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Balb/c 3T3 Cell Transformation Assay

Prevalidation study Report

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List of Abbreviations

CRO	Contract Research Organisation
CFE	Colony Forming Efficiency
CTA	Cell Transformation Assay
CV	Crystal Violet
DMEM	Dulbecco's Modified Eagle Medium
DMEM/F12	Dulbecco's Modified Eagle Medium / Nutrient mixture F12
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
GLM	Generalised Linear Model
GLP	Good Laboratory Practice
JaCVAM	Japanese Centre for the Validation of Alternative Methods
MEM	Minimum Essential Medium
MT	Morphological Transformation
NOEL	No Observed Effect Level
OECD	Organisation for Economical Development and Co-operation
PBS	Phosphate Buffered Saline
PC	Positive Control
PE	Plating Efficiency
qRT-PCR	quantitative real time-Polymerase Chain Reaction
REACH	Registration, Evaluation, Authorisation and Restriction of CHemicals
RCG	Relative Cell Growth
rCFE	Relative Colony Forming Efficiency
SHE	Syrian Hamster Embryonic cells
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 performance of 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 the the Balb/c 3T3 A31 cell line 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 on the SHE cells were assessed: the CTAs using the protocols at pH 6.7 and 7.0, respectively.

In keeping with the objectives of this ECVAM's effort, the Validation Management Team (VMT) concluded that, on the basis of the outcome of this prevalidation study, an improved Balb/c 3T3 CTA protocol, incorporating the recommendations made by the VMT, has been developed. Further testing of this improved protocol is recommended in order to confirm its reliability.

Preface

The study presented in this report complements recent Organisation for Economic Co-operation and Development (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 (EU, 1986). 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 and pharmaceutical industry, 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 standardization 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 required 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 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 prevalidation study of select CTAs. It was determined that the SHE and Balb/c 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 on the CTA performed using the A31 subclone of the Balb/c 3T3 cell line protocol.

1.2 Intended Use

The possible use of the Balb/c 3T3 CTA is mentioned in various recent testing strategies including the supplemental data for pharmaceuticals (Jacobson-Kram and Jacobs, 2005) and the guidance on information requirements and chemical safety assessment for REACH (ECHA, 2008). For chemicals produced above 1000 tonnes per year, the REACH guidance states 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 performance of the Balb/c 3T3 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 Balb/c 3T3 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 CTA 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 Balb/c 3T3 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 Balb/c 3T3 test is mainly used in Japan 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 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 screen for non-genotoxic carcinogens (*e.g.* in agro-chemical industry), (d) to demonstrate differences and similarities across a chemical class (*e.g.* in chemical companies within REACH), (e) to screen for efficacy of chemopreventive agents (*e.g.* in pharmaceutical industry), (f) to investigate tumour promotion activity (*e.g.* in agro-chemical and chemical industries), and (g) for mechanistic studies of carcinogenicity (*e.g.* in academia and industry).

Regulatory agencies in general have been reluctant to unconditionally adopt CTA 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 recent research activities related to the CTA employing the Balb/c 3T3 cell line or Balb/c 3T3-derived cell lines, independently of the protocol used.

Recent publications have suggested that transcriptomics analysis may offer some insight toward increased understanding of the complex interactions that occur to biological systems when perturbed by carcinogens. In two recent studies, transcriptomics analysis was applied to Balb/c 3T3 CTA to identify gene signatures useful for the prediction of risk for carcinogenicity, as well as to determine the molecular mechanism of induction of Balb/c 3T3 cell transformation by specific chemicals. Using cDNA microarrays Ao *et al.* (2010) analysed the gene expression to identify chemical associated profiles induced by a variety of tumour promoting agents in cells derived from transformed foci. Characteristic clusters of genes were identified which included genes associated with oxidative stress, cell proliferation and extracellular matrix, indicating the importance of specific molecular alterations as potential biomarkers of exposure to tumour promoters (Ao *et al.*, 2010). In the other study genome wide gene expression analysis and quantitative real time-Polymerase Chain Reaction (qRT-PCR) were applied to both untransformed and transformed Balb/c 3T3 cells exposed to several chemicals used in the present prevalidation study. The data describe selective and commonly regulated carcinogenic pathways which are associated with carcinogenesis in several human cancers. This indicates that these genes are perhaps capable of predicting chemical carcinogenesis *in vivo* (Rohrbeck, in preparation).

A bottleneck in the conduct of the CTA is that classification of transformed foci relies on light microscopy scoring by a trained human expert employing prevailing conventions. Recently, Poth *et al.* (2007) have investigated the alterations in adhesion protein profiles and enzyme activities modulating cell adhesion and migration that seem to be involved in the process of focus formation. Their data indicate that these may represent promising candidates for the identification of molecular markers leading to a more objective scoring of foci and a greater acceptance of cell transformation tests in general (Poth *et al*., 2007). Another promising approach is the development of a focus classifier based on image analysis and statistical classification that is under investigation in the CTA conducted with the C3H10T1/2 cell line (Urani *et al.*, 2009). If this method is proven to be reliable it could also be

explored for the identification of Balb/c 3T3 CTA transformed foci since the foci of the two cell lines share many common features in their morphology.

An additional approach which aims at avoiding manual scoring, thus increasing the scoring objectivity and the assay throughput used the soft agar colony formation assay in the screening of anticancer compounds (Thierbach *et al*., 2009). The assay is based on the anchorage-independent growth characteristics of malignantly transformed epithelial cells held in culture (*i.e*. their ability to grow in semi-solid medium). Currently, efforts are being undertaken to automate this assay.

A short-term CTA has recently been developed, using Bhas 42 cells which were established from Balb/c 3T3 cells transfected by a v-Ha-ras gene. The Bhas 42 CTA has been reported to be capable of detecting both initiating and promoting activities of chemical carcinogens (Asada *et al.*, 2005; Ohmori *et al*., 2005; Sakai *et al*., 2009). The initiation assay involves a two-day treatment of low density cells, and the promotion assay involves treatment of near-confluent cells with a test chemical for a period of 12 days. The sensitivity of the Bhas 42 CTA was shown to be equivalent to that of the two stage Balb/c 3T3 CTA for the detection of promoting activities (Murumatsu *et al.*, 2009). Currently, JaCVAM (the Japanese Centre for the Validation of Alternative Methods) is coordinating the validation of the Bhas 42 CTA. This assay promises to be less time and labour intensive than the previous Balb/c 3T3 CTAs and it has the potential to be used in a high throughput manner.

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 ("detailed review paper") 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) 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 Balb/c 3T3 CTA for the prediction of rodent carcinogenicity was reported for 149 compounds as follows: concordance 68%, sensitivity 75%, specificity 53%, positive predictivity 77%, negative predictivity 50%, false positive 47%, false negative 25% (the prevalence, proportion of carcinogens, was 68%). 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 the data evaluated in the DRP had, in some cases, been produced employing different procedures, there was a clear need, as an ultimate goal, 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 Balb/c 3T3 CTA performed under the same conditions as those evaluated in the present study. However, two inter-laboratory studies on a variant of the Balb/c 3T3 CTA using a modified medium and a two-stage protocol are worth mentioning.

The first study has been published by Tsuchiya *et al.* (1999) and relates to the comparison of data produced in 19 laboratories testing 3-methylcholanthrene during the initiation stage and 12-O-tetradecanoylphorbol-13-acetate during the promotion stage. The initiating activity of 3-methylcholanthrene and the promoting activity of 12-O-tetradecanoylphorbol-13-acetate were 100% reproducible among laboratories (19/19) in the two-stage protocol. When 3-methylcholanthrene was tested alone, a significant positive response was obtained in 63% of the laboratories (12/19) and when 12-O-tetradecanoylphorbol-13-acetate was tested alone, a significant positive response was obtained in 47 % of laboratories (8/17).

The second study reports the comparison of results for four chemicals tested in four to five laboratories for their initiating activity, and for seven chemicals tested in three to five laboratories for their promoting activity (Tsuchiya *et al.*, 2010). In the two-stage protocol, concordance was 100% for three of the chemicals tested during the initiation phase and 50% (2/4 laboratories) for the last chemical. Results for the chemicals tested during the promotion phase were reproducible among all laboratories for six test chemicals whereas 2/3 laboratories consistently classified the last chemical. When chemicals were tested alone (one-stage protocol), the concordance was 100% for 9/11 chemicals tested overall.

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 Balb/c 3T3 assay was 68.4% (39/57 chemicals). It should be noted that these results were produced using different variants of the assay and the lower apparent reproducibility may be attributable to substantial differences in protocols (*e.g.* one-stage or two-stages, different cell culture media).

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 and the cells employed in this study are in the public domain and have not been patented.

2 Organisation of the Study

The aim of this prevalidation study was to assess the reproducibility of a standardised Balb/c 3T3 CTA protocol. In order to evaluate whether the test 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 (OECD 2007). Each *in vitro* test was conducted according to the same agreed-upon protocol in three different laboratories.

ECVAM entirely coordinated the study and sponsored two of the participating laboratories. The third laboratory was sponsored by Hatano Research Institute and a collaboration agreement was signed with ECVAM at the beginning of the study.

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 to evaluate the CTA using the Balb/c 3T3 prtocol 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 with ECVAM main goal to 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 validations (OECD 34, 2004) an independent VMT was established by ECVAM. Its role was to design the study, 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 biostatistical analysis of the *in vitro* data was the responsibility of independent biostatisticians (Sebastian Hoffmann and Andre Kleensang - ECVAM).

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

2.2 Laboratories involved

The study included three laboratories, two from Europe and one from Japan. The participating laboratories are listed below. Laboratories 1 and 3 had extensive expertise with different protocols of the assay, while Laboratory 2 had limited experience.

Laboratory 1 (Study Director: Enrico Sabbioni)
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2.3 Quality assurance schemes employed by the testing 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). Only Laboratory 2 routinely works under GLP certification and was 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 Balb/c 3T3 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 SHE CTAs, the chemical selection took into account existing results in both systems as described below. Where possible the same chemicals were selected for the evaluation of the Balb/c 3T3 and SHE 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), phenathrene (only one reference for Balb/c 3T3 and SHE pH 7.1) and o-toluidine HCl (only one reference for Balb/c 3T3 CTA and no reference for SHE pH 6.7 CTA).

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

The *in vitro* genotoxicity, *in vivo* genotoxicity and carcinogenicity characterisation of the selected compounds is reported in Annex 13.1.

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* carcinogenenicity	(References)	Suggested dose range
3-Methylcholanthrene	56-49-5	+	(Gold and Zeiger, 1997)	0.5, 1, 2, 4 µg/ml when used as positive control
3-Methylcholanthrene	56-49-5	+	(Gold and Zeiger, 1997)	0.01, 0.03, 0.1, 0.3, 1, 3, 10 µg/ml when used as coded chemical
2-Acetylaminofluorene	53-96-3	+	(Gold and Zeiger, 1997)	na
Benzo(a)pyrene	50-32-8	+	(IARC, 2009)	0.1 - 15 µg/ml
Anthracene	120-12-7	-	(IARC, 2009)	na
Phenanthrene	85-01-8	-	(IARC, 2009)	na
o-Toluidine HCl	636-21-5	+	(NTP)	20 µg/ml - 1.2 mg/ml

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.

3-Methylcholanthrene 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)

3-Methylcholanthrene 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. 3-methylcholanthrene was later used as the PC, in the subsequent phases of the study. The doses of 3-methylcholanthrene to be used were suggested by the VMT based on data from the literature.

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

Three chemicals classified as *in vivo* carcinogens (2-acetylaminofluorene, benzo(a)pyrene, o-toluidine HCl) and two chemicals classified as non-carcinogens (anthracene, phenanthrene) were selected. Dose ranges for benzo(a)pyrene, and o-toluidine HCl, were suggested by the VMT based on data from the literature. For the other two chemicals, the laboratories had to choose the dose range based on dose-range finding (DRF) tests. The VMT suggested that DMSO (dimethyl sulfoxide) should be used as the solvent 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 compounds were performed by J&JPRD and ECVAM. The ECVAM staff involved in the coding and shipment was completely independent from the staff involved in the experimental part of the validation study. The coded compounds 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.

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

Following common practice in genotoxicity studies, 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 3.6.7.

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 phase 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 in this training phase.

Since the ECVAM and the HRI laboratories had produced their historical data using two slightly different cell clones and different protocols, it was agreed that both clones and protocols, respectively, should initially be evaluated. Finally it was decided that the HRI clone and the HRI protocol version were to be used for the validation study.

Following the preliminary phase of protocol optimisation, both the transferability and the within-laboratory reproducibility were assessed by evaluating results obtained for a non-coded test chemical and a coded one. These two chemicals were the same (3-methylcholanthrene), allowing an analysis of the within-laboratory reproducibility as well as the transferability of the assay. 3-Methylcholanthrene 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 between-laboratory transfer of the test to the participating laboratories and the within-laboratory reproducibility analysis were successful, the laboratories proceeded to the experimental phase on 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 biostatistician. The spreadsheets containing the test data generated by each laboratory were returned to the biostatistician 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 lead 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	
10-11/02/2005	Kick-off meeting ECVAM, Ispra, Italy	Study Directors and members of the VMT attended the kick-off meeting and agreed on the general aspects of the protocols to be used in the optimisation phase.
03/2005-02/2006	Protocol optimisation All laboratories	Conduct of experimental work by all participating laboratories to assess best protocol conditions.
4-5/04/2005	Training week ECVAM, Ispra, Italy	Technical staff and study directors met to agree on the harmonisation of protocols and on the criteria for scoring the plates using dishes treated with transforming and non transforming chemicals.
21/08/2005	1st VMT meeting Berlin, Germany (in conjunction with the 5th World Congress on Alternative Methods)	The initial phase should assess : a) both the ECVAM clone used with the ECVAM protocol, and the HRI clone with the HRI protocol; b) the within-laboratory reproducibility and the transferability by testing 3-methylcholanthrene as positive control and as a coded chemical.
03/2006	Revision of the protocol, ECVAM	Protocol was revised based on preliminary data produced by the laboratories.
10-11/05/2006	VMT and study directors meeting ECVAM, Ispra, Italy	Evaluation and discussion of results on standardisation of the protocol, within-laboratory reproducibility and transferability
20/04/2007	*ad hoc* expert meeting on statistical analysis ECVAM, Ispra, Italy	Discussion on statistical analysis for Balb/c 3T3.
30-31/05/2007	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.
26/09/2007	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 to 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 phenomenon of morphological transformation is characterised by changes in the behaviour and cell growth of cultured cells in order for progression to the next stage in the transformation process to occur. A minimum of four phenotypic stages appears to be involved in cell transformation. These include (from primary to fully malignant cells): (a) a block in cell differentiation; (b) acquisition of immortality, characterised by unlimited lifespan, an aneuploid karyotype and decreased genetic stability; (c) acquisition of tumourigenicity, which is closely associated with the *in vitro* phenotypes of focus formation, anchorage-independent growth in semi-solid agar and autocrine growth factor production; and (d) full malignancy, including metastasis when the cells are injected in the suitable host.

The endpoints used in immortalised cells, such as Balb/c 3T3 cells, are typically the carcinogen conversion from non-tumourigenic immortality to tumourigenicity, with scoring of focus formation and anchorage-independent growth. Such effects might be caused by changes in the expression of oncogenes and/or tumour suppressor genes, block in cellular differentiation, increased genetic instability with progression *in vitro*, and autocrine growth factor production associated with altered cell signalling. The Balb/c 3T3 cell line undergoes tumourigenic conversion either spontaneously or after treatment with a carcinogen (Aarson and Todaro, 1968; Matthews *et al.,* 1993a; Matthews *et al.*, 1993b). Furthermore, the conversion to tumourigenic phenotype of the Balb/c 3T3 cells occurs after activation or inactivatation of oncogenes and tumour suppressor genes (Nakazawa *et al.*, 1990; Olson *et al.*, 1993; Silingardi *et al.*, 1994).

3.3 Biological test system: Balb/c 3T3 cell line

A CTA using an established cell line rather than early passage normal diploid cells was developed by Kakunaga (1973) who obtained a subclone, A31-714, from the Balb/c 3T3 A31 line (Aaronson and Todaro, 1968) and demonstrated its responsiveness in the induction of morphologically transformed foci with chemical carcinogens. Later, Kakunaga and Crow (1980) isolated several other variants from

the cell line that showed different susceptibility to UV-induced transformation and recommended the A31-1-1 clone as particularly suitable for the cell transformation experiment. The Balb/c 3T3 cells are contact-inhibited cells with a strong cell-to-substrate adhesion. They show a uniform morphology, a low saturation density and a high cloning efficiency (50-60%). They are characterised by a short doubling time (approximately 19 hours) and a high sensitivity to chemical transformation with a low incidence of spontaneous transformation.

3.4 Balb/c 3T3 Cell Transformation Assay

A CTA using the Balb/c 3T3 cell line was initially developed as a quantitative transformation assay (TA) for chemical carcinogens employing a subclone, A31-714, of the mouse Balb/c 3T3 A31 embryonic cell line, derived by Aaronson and Todaro (1968) mouse Balb/c 3T3 A31 embryonic cell line, the latter of which was originally established for purposes of studying viral-induced transformation. Extensive work has been carried out by many researchers using A31-714 and A31-1-1 clones (Heidelberger *et al.*, 1983). In an attempt to explore the utility, advantages, and shortcomings of different CTAs, an IARC/NCI/EPA Working Group (1985) was convened in which the Balb/c 3T3 cells and C3H/10T½ cells (Reznikoff *et al.*, 1973) systems were considered. This group prepared recommendations for practical procedures in the performance of CTAs (IARC, 1985).

Despite this and other such efforts, wherein the working group proffered recommendations regarding the Balb/c 3T3 CTA, that method and CTAs in general, have not been fully exploited for routine screening of potential carcinogens to the extent that other genotoxicity tests have been. This, in part, may have been due to ignorance regarding the underlying mechanism(s) associated with CTAs generally and the perception that such assays were technically more difficult, expensive and time-consuming than other *in vitro* genotoxicity tests that are also used for screening of potential carcinogens. Nevertheless, whereas genotoxicity assays provide indirect evidence of potential carcinogenicity, only CTAs offer the most direct *in vitro* measure of that potential. Resistance to the routine use of the Balb/c 3T3 method was also due to the fact that it had not been validated in accordance with internationally accepted validation procedures (OECD GD34, 2005). In order to overcome some of the limitations associated with the Balb/c 3T3 CTA, improvements to the basic protocol were proposed that would increase the recognition of induced cell transformation events and shorten the duration of the assay. Some such improvements included 1) enhancing the sensitivity of cells to the transforming potential of test chemicals by subsequent treatment with known tumour-promoting agents (two-stage method) (Mondal *et al.*, 1976; Sakai *et al.*, 2002), 2) amplification of the number of transformed foci by re-plating the culture after the treated cells reached confluence (Schechtman, 1985), 3) increasing the seeding cell number and treating the cells on day 2, *i.e.* one day later than the original treatment day (Matthews *et al.*, 1993), 4) using modified cell culture medium (enriched basal medium supplemented with 2% foetal bovine serum FBS) (Tsuchiya and Umeda, 1995), and 5) use of serum-reduced medium in order to increase assay sensitivity (Hayashi *et al.*, 2008) .

3.5 Protocol optimisation phase

An experimental phase was considered necessary in order to develop an optimised version of the protocol to be used in the subsequent prevalidation study.

During the kick-off meeting it became clear that the ECVAM and HRI laboratories were using the same clone of the Balb/c 3T3 cell line (clone A31-1-1), but two different cell lineages of this clone, and two different versions of the cell transformation protocol. In view of the distinctive nature of both the cell lineages and the protocols used in the two laboratories, and the fact that the historical data available from the two laboratories were likewise disparate, it was decided to initially test and compare both cell lineages with the respective protocols. Moreover, it was agreed to organise a "training week" for the participant laboratories before the initiation of the study. The aim of training week was to: 1) compare the different protocols, 2) agree on a standard protocol and provide training

on how to perform the assay, 3) compare the results derived from the ECVAM and HRI protocols and the two cell lineages used, 4) ensure that all laboratories were using the same criteria for scoring.

3.5.1 Preliminary experiments

Several preparatory experiments were performed before the training week.

For the optimisation phase of the study, two different lineages of the Balb/c 3T3 A31-1-1 clone were used (ECVAM and HRI). The ECVAM lineage was used at passage 91 and was routinely cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetalclone III serum (FCIII, Hyclone, USA), while HRI's lineage (passage 17) was cultured in Minimum Essential Medium (MEM) supplemented with 10% FBS (Moregate, Australia) and 1% penicillin 10,000 U/ml /streptomycin 10 mg/ml (PS) solution (M10F). It should be noted that the method used for determining passage number was also dissimilar in the two laboratories, thereby accounting for the dramatic numerical difference in this property.

ECVAM compared CTA in Petri dishes of different sizes (ø 60 *vs.* 90 mm), determined the best fixing and staining conditions for the Colony Forming Efficiency (CFE) test, and performed the CTA with both the ECVAM and HRI cell lineages. HRI performed the CFE test and the CTA with the HRI cell lineage and using cell culture medium supplemented with the synthetic serum employed by ECVAM. The analysis of cytotoxicity and morphological transformation (MT) at ECVAM was carried out using two inorganic compounds ($NaAsO_2$, CisPt) and one organic compound (3-methylcholanthrene).

The results from the experiments performed during the training week suggested that the cell lineages behaved differently. They differed in plating efficiencies (PEs), cytotoxicity responses, and the morphology of colonies, cell monolayers and type III foci. Interestingly, in these studies, the HRI cell lineage showed a higher number of spontaneous type III foci in the negative control compared to that routinely detected in the HRI historical controls. It was hypothesised that the HRI cell lineage used at ECVAM had undergone some transformation during cell culture, although this was not verified.

Subsequently, HRI compared the ECVAM and HRI sera using the HRI cell lineage and protocol. They concluded that the ECVAM FCIII synthetic serum is not suitable for use with the HRI protocol and cell lineage.

HRI performed some additional experiments. The results, which were reported in July 2005, were as follows:
1) The ECVAM clone was resistant to 3-methylcholanthrene induction of transformation,
2) The HRI and ECVAM clones exhibited different morphologies,
3) The different media tested yielded different cellular responses,
4) The plating efficiency (PE) of the ECVAM clone was lower than that of the HRI clone,
5) CTA and soft agar experiments with the two clones and different media showed that 3-methylcholanthrene did not induce type III foci in the ECVAM cell lineage (only type I and II foci) and no colonies were observed in soft agar.

At the SHE kick-off meeting, which was held in Berlin on the 21 August 2005, in conjunction with the 5th World Congress on the Alternatives and Animal Use in Life Sciences, the VMT also discussed some major issues regarding the Balb/c 3T3 validation study and the following decisions were taken:
1) Clones
HRI was to re-ship the Balb/c 3T3 clone to ECVAM since the clone used during the training week was considered transformed by the HRI expert. Starting from October 2005, all three laboratories were to perform the cytotoxicity experiments with both cell lineages.
2) Protocols
The HRI cell lineage was to be used with the HRI SOP. The ECVAM clone was to be used with the ECVAM SOP.

3) Dose-range finding (cytotoxicity)
CFE (ECVAM SOP) and Crystal Violet (CV) (HRI SOP) cytotoxicity assays were used to determine the appropriate test concentrations in the initial phases of the study. For the CFE assessment cells were grown for 9 days, while for CV two different time points were investigated (4 day- and 7 day-cultures). Non-coded and coded 3-methylcholanthrene were to be tested. The laboratories were not aware that these two chemicals were the same. The concentrations tested ranged from 0.01 to 10 µg/ml for non-coded 3-methylcholanthrene and from 0.01 to 25 µg/ml for coded 3-methylcholanthrene.

The data were circulated and reviewed by the VMT in February 2006.

3.5.2 Results

Figure 1 and Figure 2 show the results of the CFE and CV analyses of the HRI and ECVAM cell lineages treated with coded and non-coded 3-methylcholanthrene. Complete data sets are included in the laboratory reports of phase 1.

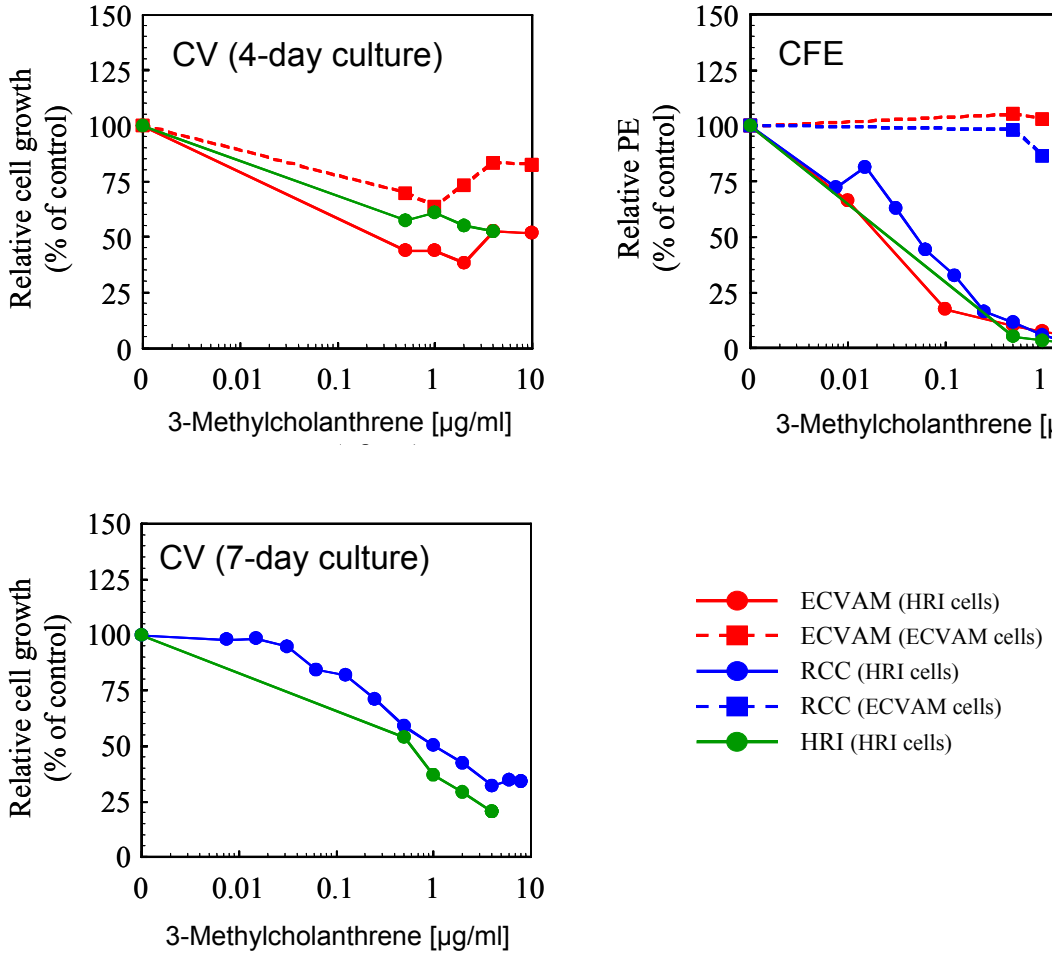

Figure 1: Crystal Violet (CV) and Colony Forming Efficiency (CFE) results of ECVAM and HRI cell lineages treated with non-coded 3-methylcholanthrene. CV was performed at 4 and 7 days of culture, whereas CFE was assessed on day 9.
PE = Plating Efficiency

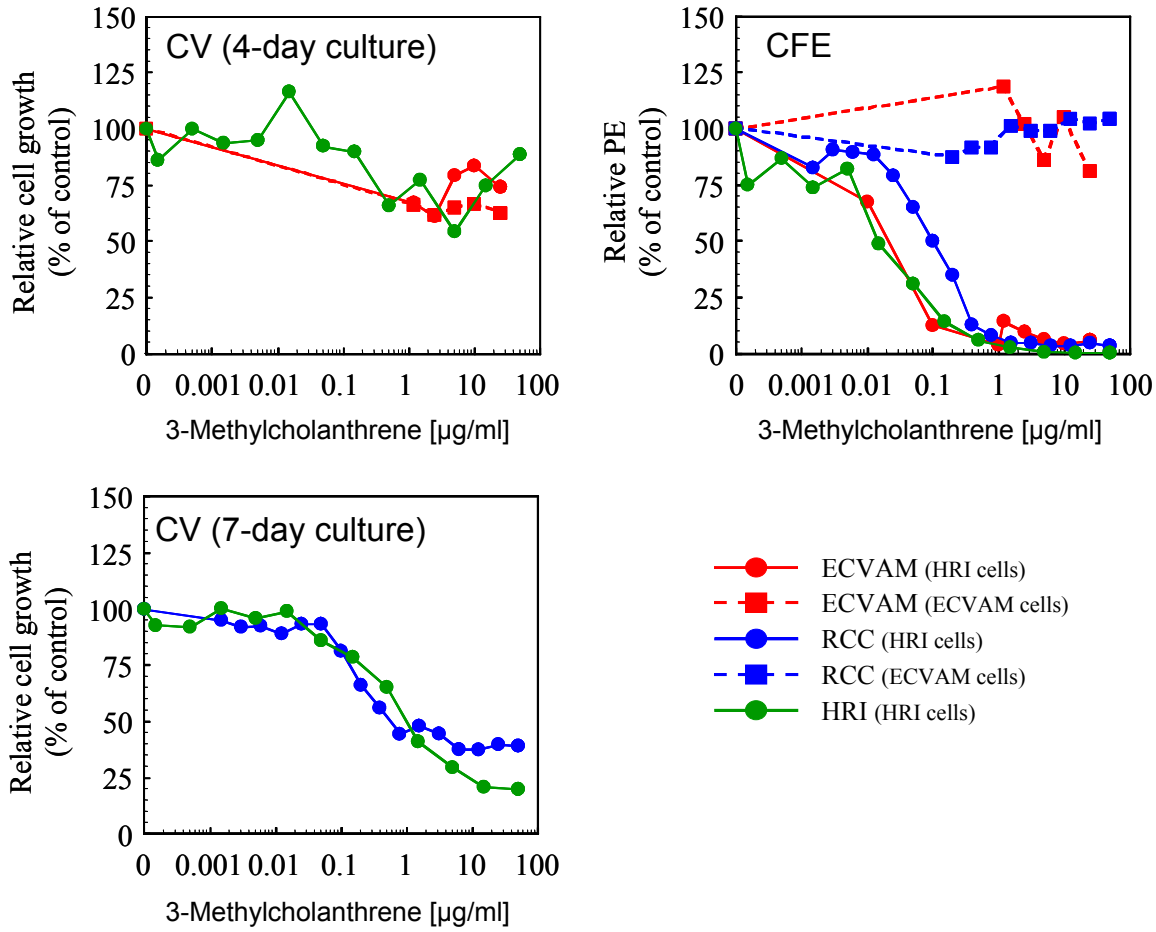

Figure 2: Crystal Violet (CV) and Colony Forming Efficiency (CFE) results of ECVAM and HRI cell lineages treated with coded 3-methylcholanthrene. CV was performed at 4 and 7 days of culture, whereas CFE was assessed on day 9.
PE = Plating Efficiency

The ECVAM cell lineage did not show any cytotoxicity to treatment with 3-methylcholanthrene, while the HRI cell lineage exhibited dose-dependent cytotoxicity when treated with the same compounds. This difference in responses of the two cell lineages was reproducible by the different laboratories.

HRI and RCC laboratories reported difficulties in growing the ECVAM clone. Therefore, data for the ECVAM cell lineage were not available for all laboratories. Overall, the experiments were considered: 1) reproducible within laboratories when the results for non-coded 3-methylcholanthrene were compared with those for coded 3-methylcholanthrene in each laboratory and 2) reproducible between laboratories when the data produced by the three laboratories were compared.

Additional differences of the two cell lineages were reported by the laboratories**.** Preliminary CTA experiments showed that the morphology of the ECVAM and HRI cell lineages was different (Figure 3-Figure 4). The ECVAM cell lineage showed small colonies of different size and density, while the HRI cell lineage yielded more homogeneous colonies. Moreover, the monolayer of the HRI cell lineage showed cells with a uniform morphology, whilst cultures of the ECVAM cell lineage showed irregular growth with many clusters and islands of cells in the control culture. The PE of the ECVAM cell lineage was also lower.

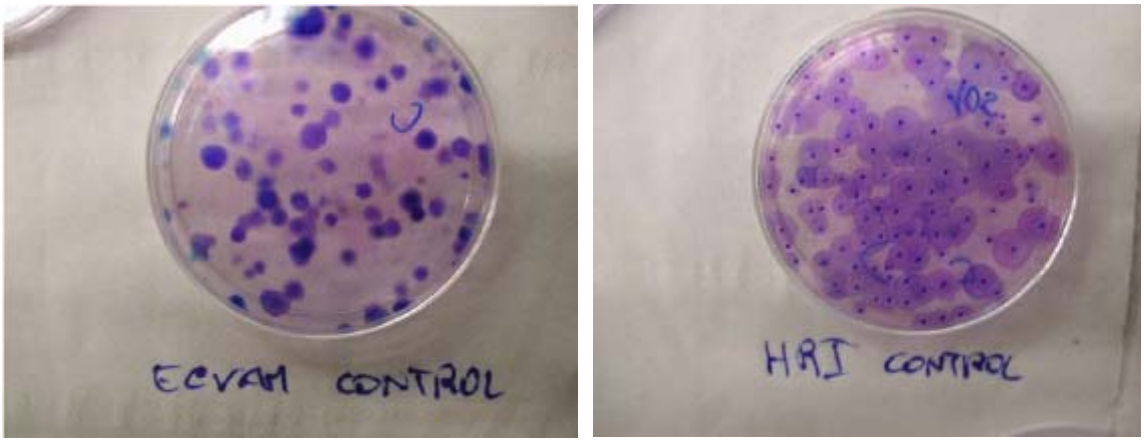

Figure 3: Colony Forming Efficiency with Balb/c 3T3 ECVAM and HRI cell lineages.

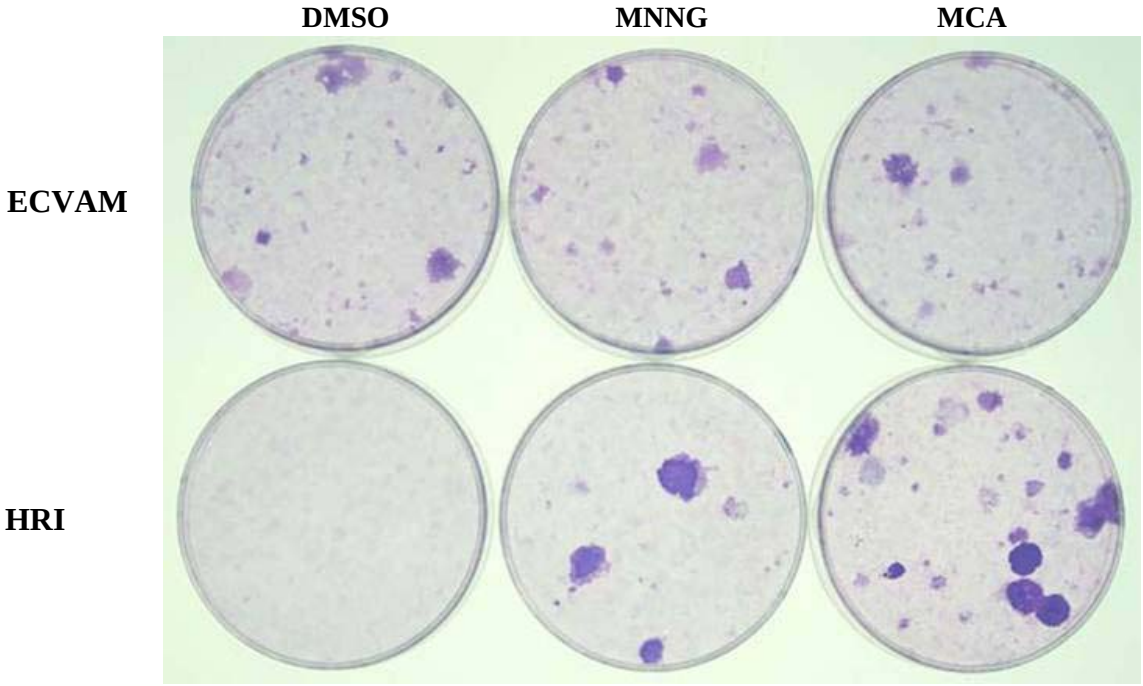

Figure 4: Morphological Transformation with Balb/c 3T3 ECVAM (top row) and HRI (bottom row) cell lineages treated with 0.5% DMSO, 1.5 µg/ml *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG), or 10 µg/ml 3-methylcholanthrene (MCA).

Although the ECVAM and HRI cell lineages seemed to had ostensibly originated from a common Balb/c 3T3 parental cell line, it is clear that some phenotypic and morphological modifications have occurred during the independent *in vitro* cultivation of the respective progeny cells over time.

The analysis of data produced with CV showed that experiments conducted with 7-day cultures performed better than those with 4-day cultures. A longer cell proliferation phase allowed a more precise discrimination between the cytotoxicity responses. The protocol was modified accordingly.

The comparison between CFE and CV was difficult due to the different sensitivities of the two methods, CFE being the most sensitive showing cytotoxicity at lower doses.

Harlan CCR and HRI reported good CTA results with the HRI cell lineage. However, no results could be provided for the ECVAM cell lineage since all the cells detached from the plastic Petri dishes after

3 weeks. ECVAM reported similar results with the ECVAM cell lineage by using Falcon Petri dishes. However, ECVAM reported good CTA results with the same clone when COSTAR dishes were used. No CTA was carried out by ECVAM with the HRI clone.

3.5.3 Conclusion of the Validation Management Team on preliminary experiments

Based on the data provided by the three laboratories and previous preliminary data, the VMT agreed that the HRI cell lineage together with the HRI SOP should be used for the completion of the validation (minutes of the teleconference of 23/02/2006 available at ECVAM).

Since the HRI cell lineage gave a higher background of type III foci the acceptance criteria in the Statement of the Work which stated "not more than one focus type III in the whole set of control dishes" was revised to read "the maximum number of Type III foci in the entire set of vehicle control dishes should not exceed five".

The data showed that the two cytotoxicity methods (CFE and CV) may have different sensitivities, CFE appears to be more sensitive in some cases. In the CV method the cell density undergoing chemical treatment is closer to that used in the MT, suggesting that the CFE might overestimate the cytotoxicity due to the different number of cells seeded. However, since CFE is the method that has been used historically to assess cytotoxicity in cell transformation experiments it was determined that its use would allow for a better comparison of data generated by this validation study with historical data. This would also be important in case a further retrospective validation study is performed in the future. Due to the difficulty of choosing the optimal cytotoxicity assay, it was agreed that the laboratories should use both cytotoxicity assays in order to allow for a more complete comparison at the conclusion of the study. However, it was agreed that the appropriate treatment doses for MT were to be determined based on the CFE data.

The protocol was revised in March 2006. The Moregate FBS necessary for the medium to be used with the HRI clone was received by ECVAM and Harlan CCR at the beginning of April 2006.

3.6 Protocol

The detailed test protocol used in this study is described in Annex 13.2.

3.6.1 Balb/c 3T3 cell line

HRI's cell lineage (passage 17) of the Balb/c 3T3 cell line was cultured in M10F. The batch of serum used was preselected by testing its cell PE (>30%), its ability to maintain a uniform contact-inhibited monolayer and to its ability to support 3-methylcholantrene-induced transformation (IARC/NCI/EPA, 1985).

The cells were distributed by HRI to the participating laboratories and used for the CTA within 3 to 4 passages. In the transformation experiments all laboratories used the same culture medium (not the same lot) from GIBCO: M10F was used during the growing stage of the cells (until day 7) and DF2I2F was used during the confluent stage of cells.

3.6.2 Serum and medium

All the laboratories used the same batches of FBS (Moregate, Australia) during the different phases of the validation study. Batch No. 2430013 serum was used during the optimisation and the within-reproducibility phases, while batch No. 94300107 serum was used for the between-laboratory reproducibility assessment. The latter was tested and selected by Harlan CCR among six serum

batches prior to use in this exercise (Report 31/10/2006, available at ECVAM), based on its ability to induce a high cloning efficiency (CFE = 52.1%), a relatively low spontaneous transformation frequency with the vehicle control VC (mean number of 0.3 transformed focus/dish under DMSO) and a high transformation frequency with a PC (mean number of 12.8 transformed foci/dish under 4 μg/ml of 3-methylcholanthrene).

The laboratories used the same media from GIBCO (MEM and Dulbecco's Modified Eagle Medium/Nutrient F12 DMEM/F12), but from different batches.

3.6.3 Controls

Positive Control: 3-methylcholanthrene (4 μg/ml dissolved in 0.5% DMSO) was used as the PC.

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

***Vehicle Control*:** The cell culture medium containing 0.5% DMSO served as the concurrent VC.

3.6.4 Test procedure

The CTA is composed of two phases:

- A preliminary dose-range finding (DRF) experiment to determine the appropriate dose-range that will be used for the transformation assay (TA),
- The TA, which represents the main experiment and which includes the assessment of morphological transformation (MT) and cytotoxicity in separate dishes.

The DRF tests are carried out by measurement of the Colony Forming Efficiency (CFE = [total number of colonies formed in the treatment dishes / total number of colonies formed in the control dishes] × 100). In parallel, the Crystal Violet (CV) cytotoxicity test (Saotome *et al.*, 1989) was also performed to calculate the Relative Cell Growth (RCG = [(Absorbance of treated well - Absorbance of medium blank well) / (Absorbance of solvent control well - Absorbance of medium blank well)] × 100).

The measurement of cytotoxicity during the TA includes both the CFE and CV cytotoxicity tests, concurrently with the MT assay. Eight doses are chosen for the TA on the basis of the DRF CFE results to cover the range from no toxicity up to 90% reduction in CFE. A PC (4 μg/ml of 3-methylcholanthrene) and a VC are included in each experiment (MT, CFE and CV). Briefly, 10 ml of cell suspension (2×10^3 cells/ml) are seeded into 100 mm-diameter dishes. Seventy-two hours after exposure (day 4), the treatment medium is removed and replaced with fresh complete medium. From day 7 to day 24 or 25 the medium is removed and replaced with DMEM/F12 medium with 2 μg/ml insulin, 2% FBS and 1% PS (DF2I2F) twice a week. One week after the last medium change, the medium is removed and the cells are washed with Phosphate Buffered Saline (PBS), fixed with methanol and then stained with 10% Giemsa solution.

For the analysis of the MT, foci consisting of more than 50 cells or more than 2 mm in diameter are evaluated using a stereomicroscope. Only type III foci are recorded, which are characterised by the following morphological criteria: deep basophilic staining of spindle-shaped cells which are morphologically different from the background monolayer cells, dense multi-layering of cells (piling up) and random orientation and invasive growth of cells at the edge of foci (criss-cross pattern) (IARC/NCI/EPA, 1985). The number of type III foci per dish is counted and this value is used for the statistical analysis.

3.6.5 Statistical Analysis of raw data

While a recommended protocol of the assay has been published by an IARC/NCI/EPA Working Group (IARC/NCI/EPA, 1985), no common statistical approach had been agreed on. Many different approaches to data evaluation have been proposed and applied for the Balb/c 3T3 CTA in toxicological literature. To clarify this issue, the VMT recommended that ECVAM would: 1) collect data from the literature on the different statistical methods that had been used to evaluate Balb/c 3T3 CTA results; and 2) organise an *ad hoc* expert group to evaluate the data and come up with a suggestion on the most suitable statistical method to use for this assay.

The experiments conducted for the assessment of the within-laboratory reproducibility were initially analysed by comparing the number of foci formed from the surviving seeded cells, *i.e.* adjusted by cytotoxicity results, in control and treated dishes using Fisher's exact test,. As this approach was considered not appropriate by the VMT, the respective experiments as well as those conducted for the assessment of the between-laboratory reproducibility were evaluated using the approaches recommended by the *ad hoc* expert group (section 3.6.5.2).

The determination of the NOEL (No Observed Effect Level) concentration was based on the ANOVA with Dunnett post test comparing each concentration with the VC with an overall α of 0.05, using Prism v5.01 (GraphPad Software Inc., La Jolla, CA, USA). The highest concentration not significantly different from the negative control was considered as NOEL concentration.

To further compare CFE and CV cytotoxicity tests, a rough comparison of the IC_{50} obtained with each method was made, based on the cytotoxicity data of the TA experiments. The IC_{50} were calculated by non-linear regression using the "Log[inhibitor] *vs.* normalised response" model with variable slope of Prism v5.01 (GraphPad Software Inc., La Jolla, CA, USA).

3.6.5.1 Report on "Analysis of statistical methods and experimental designs used in Balb/c 3T3 transformation assay"

A report on the "Analysis of statistical methods and experimental designs used in Balb/c 3T3 transformation assay" was commissioned by ECVAM. This was an extension of a former report, in which an extensive database on Balb/c 3T3 CTA results has been developed, compiling existing data on 414 chemical compounds retrieved from 250 references (ECVAM unpublished reports by M. Fischbach, available at ECVAM).

The objectives of this study were: 1) to provide a qualitative and quantitative analysis of the statistical methods used to evaluate data obtained in published Balb/c 3T3 assays, and 2) to provide an analysis of the experimental practices employed. To this end, 169 experimental references have been considered, encompassing data for 365 compounds.

A variety of statistical approaches were found in the literature. Some of them analysed the number of foci per dish quantitatively, *e.g.* by means of linear models (analysis of variance and t-test) after data transformation (Matthews, 1993), by means of the non-parametric Mann-Whitney test (Colacci *et al.*, 1996) or by assuming a modified Poisson distribution for count data. Others analysed the data as qualitative outcomes considering the proportion of dishes showing foci, *e.g.* by applying Fisher's exact test (Lubet *et al.*, 1990; Sakai, 1997). The main statistical approaches used were: Student's test (52%), modified Poisson distribution (19%), Fisher's exact test (7%), a method reported by Perocco *et al.* in 1991 (7%), dose-response trend analysis and Chi^2 analysis (2%). Seven percent of the publications evaluated were devoid of statistical analyses.

Regarding the experimental aspects of the studies, data were collected on (a) the Balb/c 3T3 derived cell clone used, (b) parallel cytotoxicity assays performed, (c) supplemental metabolic activation used, (d) a two-stage initiation/promotion procedure, and (e) a replating/amplification method (ECVAM unpublished report by M. Fischbach available at ECVAM). The most widely used cell strains were the

clone A31-1-13 used to test 92% of the chemicals (334 chemicals), followed by the cell clone A31-1-1 for 27 % of chemicals (100 chemicals). Some chemicals were examined in more than one cell strain. A concurrent cytotoxicity study was performed in 98% of the studies. Metabolic activation using an S9 fraction was performed in 24% of the studies, representing 67 tested compounds (18%). The two-stage initiation/promotion procedure is described in 26 studies (16%) for 66 compounds (18%). Finally, the replating/amplification method, which is a variation of the initial protocol, was performed in 17 studies (10%).

3.6.5.2 *Ad hoc* expert meeting on statistical analysis for Balb/c 3T3 CTA

Based on the above analysis and on the recommendation by the VMT, it was decided that a statistician *ad hoc* expert meeting should take place in order to discuss the most appropriate method for statistical analysis of the CTA in Balb/c 3T3 cells. This meeting was held at ECVAM on 20 April 2007**.**

The participants were: L. Edler (German Cancer Research Centre, Heidelberg, Germany), L. Hothorn (University of Hannover, Germany), M. Suzuki (Biosafety Research Center, Shizuoka, Japan), R. Corvi (ECVAM), S. Hoffmann (ECVAM), B.C. Thomas (ECVAM). S. Hoffmann chaired the meeting.

Reviewing the most commonly used data evaluation methods (Fischbach, unpublished data available at ECVAM), the experts concluded that none of the previously suggested methods adequately addressed the particular statistical properties of the Balb/c 3T3 CTA endpoint of number of transformed foci type III per dish. In this context, the experts discussed several statistical evaluation methods. It was agreed that the Nishiyama transformation (Nishiyama *et al.*, 2003) and an approach based on the negative binomial distribution combined with William's-type (Bretz *et al.*, 2003) protected tests were the preferred methods. They match the particular statistical properties of the Balb/c 3T3 CTA test and were recommended to be used in the present validation study.

Empirically comparing the results of the data analyses using both approaches, *i.e.* Nishiyama transformation and the approach based on negative binomial distribution, suggested that the latter was more suited for the data at hand as it better reflected the dose-concentration curve (Manuscript in preparation, minutes of the meeting available at ECVAM). Therefore, the results presented in this report have been generated with the approach based on negative binomial model (Annex 13.3). For completeness, the respective results when applying the Nishiyama transformation and the corresponding R-code are reported in Annex 13.4.
It was anticipated that for these approaches with complex multiple comparisons conventional *p*-values, such as 0.05, would not be appropriate. In this study, a *p*-value of 0.01 was considered. However, the expert group recommended to retrospectively determine an empirical threshold *p*-value for the assessment criteria based on the *p*-values obtained in the study.

3.6.5.3 Statistical method based on the negative binomial distribution

A Generalised Linear Model (GLM) with a negative binomial distribution and identity as link function was fitted. Based on that GLM downturn-protected Williams contrast tests based on unweighted combinations of concentrations were calculated and adjusted *p*-values based on the joint normal distribution of the linear function were reported.

The GLM could not be fitted in case at least one concentration and/or the VC induced the same number of foci in every dish, *i.e.* showed no variability in focus numbers. In such cases, the pragmatic solution of arbitrarily adding one focus to one of the respective dishes was applied resulting in an empirical estimation of the variability of larger than 0 (nine cases). The impact of these modifications was considered negligible.

Furthermore, the GLM could only be fitted to a balanced data set. Therefore, focus numbers of the same number of dishes for each concentration and control in a given experiment were required. In cases when less than ten dishes were available, *e.g.* due to contamination or the presence of daughter foci, the amount of dishes was harmonised either by random exclusion of dishes (one case only) or by plug-in of the median number of foci of the remaining dishes (seven cases). Impact of these modifications was considered negligible.

This approach is publicly available and implemented into R (R Development Core Team, 2009; Hothorn *et al.*, 2008; Venables and Ripley, 2002). For the specific conduct of the analysis, see Annex 13.3. The original R-codes were kindly provided by Prof. Hothorn (University of Hannover, Germany).

3.6.6 Assay acceptance criteria

The following criteria were employed in these studies, although it is important to note that modifications are described in the recommendations section (section 10) of this report based upon the outcome of the study conducted.

3.6.6.1 Concurrent cell growth assays

- Uniform cell growth on culture wells should be observed,
- At least one NOEL concentration should be used,
- Cell growth curves should cover the range between NOEL and IC_{90} (ideally six concentrations between NOEL and IC_{90}) based on CFE,
- For CFE, a minimum PE of 30% in the negative control should be achieved.

3.6.6.2 Transformation assay

- The maximum number of Type III foci in the entire set of VC dishes (10 dishes) should not exceed five,
- A minimum of six analysable concentrations is required, and they must cover the range of cytotoxicity from NOEL to IC_{90},
- A minimum of nine Petri-dishes/concentration is required.

Transformation dishes must be stored and archived in each laboratory until the end of the validation study, in the event that results need further analysis.

3.6.7 Assay assessment criteria

The following criteria were generated based on the data indicating that the negative binomial approach was the most appropriate (see Section 3.6.5). The results presented in this report were evaluated with the following criteria:

- A test chemical was considered "negative" (non-transforming) if no downturn-protected Williams contrast showed a p-value < 0.01.
- A test chemical was considered "positive" (transforming) if the downturn-protected Williams contrast with the lowest p-value:
  - (I) included at least two consecutive concentrations, and

- (II) showed a *p*-value < 0.01.

- A test chemical was considered "inconclusive" if the downturn-protected Williams contrast with the lowest *p*-value:
  - (I) included only one concentration or several non consecutive concentrations, and
  - (II) showed a *p*-value < 0.01.

4 Module 2: Within-laboratory reproducibility

This phase of the study assessed the within-laboratory reproducibility and transferability of the protocol by testing 3-methylcholanthrene as the PC and as a coded chemical. The HRI clone was used with the revised SOP (March 2006). This section provides the results of the TAs for all laboratories.

Figure 5-Figure 7 and Table 3-Table 8 show the results of the TAs. The doses for the TAs were chosen on the basis of the CFE experiments. The cytotoxicity determination was conducted in parallel by CFE and CV. Table 3-Table 8 report the cytotoxicity results of 3-methylcholanthrene as assessed by the relative Colony Forming Efficiency (rCFE) and the RCG in the CV experiment. The other columns of the tables indicate the total number of morphologically transformed foci for each concentration tested, and the mean number of foci per dish corresponding to each concentration tested.

The VC (DMSO 0.5%) gave spontaneous transformation frequencies within the expected range for the Balb/c 3T3 cells under the assay conditions employed. The mean number per plate of Type III transformed foci was 0-0.50 for ECVAM, 0.30 for Harlan CCR and 0 for HRI experiments. Further evidence of within-laboratory reproducibility is seen in the results for 3-methylcholanthrene used as the PC in the studies described in Module 4.

When the experiments did not meet the acceptance criteria, the laboratories were requested to repeat them.

4.1 Transformation Assay – non coded 3-methylcholanthrene

The non-coded 3-methylcholanthrene was initially assessed in each laboratory. The doses of 3-methylcholanthrene to be used were suggested by the VMT based on data from the literature and ranged from 0.5 to 4 µg/ml. Harlan CCR tested only the doses recommended by the VMT, while the other laboratories tested additional concentrations in order to reach NOEL. The TA results are presented in Table 3-Table 5. It should be noted that Harlan CCR and HRI used the same VC when testing non-coded and coded chemicals.

Table 3: Transformation assay results from ECVAM, testing non-coded 3-methylcholanthrene

ECVAM 3-Methylcholanthrene (µg/ml)	rCFE (%)	RCG (%) CV	Total number of foci	Mean number of foci per dish
Untreated control	na	na	1	0.10
VC (DMSO 0.5 %)	100	100	0	0
0.01	**95.6**	**82.4**	**3**	**0.30**
0.03	**94.2**	**92.6**	**6**	**0.60**
0.1	**64.8**	**83.9**	**14**	**1.40**
0.3	**62.8**	**85.5**	**10**	**1.00**
1	**51.2**	**61.5**	**12**	**1.20**
3	**83.1**	**42.2**	**47**	**4.70**
10	**4.1**	**39.2**	**94**	**9.40**

VC = Vehicle Control, rCFE = relative Colony Forming Efficiency, RCG = Relative Cell Growth, CV = Crystal Violet, na = not applicable.
Concentrations that contributed to the statistically significant result, *i.e.* which were present in the downturn-protected Williams contrast that resulted in the lowest *p*-value for a positive response (negative binomial analysis), and related values are highlighted in bold.

Table 4: Transformation assay results from Harlan CCR, testing non-coded 3-methylcholanthrene

Harlan CCR 3-Methylcholanthrene (µg/ml)	rCFE (%)	RCG (%) CV	Total number of foci	Mean number of foci per dish
Untreated control	na	na	2	0.20
VC (DMSO 0.5 %)	100	100	3	0.30
0.5	**7.2**	**314**	**16**	**1.60**
1	**3.5**	**25.8**	**44**	**4.40**
2	**2.3**	**27.4**	**39**	**3.90**
4	**1.3**	**20.0**	**67**	**6.70**

VC = Vehicle Control, rCFE = relative Colony Forming Efficiency, RCG = Relative Cell Growth, CV = Crystal Violet, na = not applicable.
Concentrations that contributed to the statistically significant result, *i.e.* which were present in the downturn-protected Williams contrast that resulted in the lowest *p*-value for a positive response (negative binomial analysis), and related values are highlighted in bold.

Table 5: Transformation assay results from HRI, testing non-coded 3-methylcholanthrene

HRI 3-Methylcholanthrene (µg/ml)	rCFE (%)	RCG (%) CV	Total number of foci	Mean number of foci per dish
Untreated control	na	na	0	0
VC (DMSO 0.5 %)	100	100	0	0
0.01	**66.6**	**95.1**	**5**	**0.50**
0.03	**54.6**	**90.4**	**7**	**0.70**
0.1	**26.6**	**83.2**	**10**	**1.00**
0.3	**18.0**	**68.6**	**15**	**1.50**
1	**10.3**	**43.7**	**34**	**3.40**
3	**6.6**	**29.7**	**119**	**11.90**
10	**5.4**	**19.7**	**224**	**22.40**

VC = Vehicle Control, rCFE = relative Colony Forming Efficiency, RCG = Relative Cell Growth, CV = Crystal Violet, na = not applicable.
Concentrations that contributed to the statistically significant result, *i.e.* which were present in the downturn-protected Williams contrast that resulted in the lowest *p*-value for a positive response (negative binomial analysis), and related values are highlighted in bold.

4.2 Transformation Assay – coded 3-methylcholanthrene

The coded 3-methylcholanthrene was assessed in each laboratory. The doses of 3-methylcholanthrene to be used had been suggested by the VMT and ranged from 0.01 to 10 µg/ml.

The TA results are presented in Table 6-Table 8.

Table 6: Transformation assay results from ECVAM, testing coded 3-methylcholanthrene

ECVAM Coded 3-methylcholanthrene (µg/ml)	rCFE (%)	RCG (%) CV	Total number of foci	Mean number of foci per dish
Untreated control	na	na	5	0.50
VC (DMSO 0.5%)	100	100	5	0.50
0.01	106.1	70.1	2	0.20
0.03	63.6	35.4	4	0.40
0.1	23.6	24.8	2	0.20
0.3	27.9	16.9	6	0.60
1	17.0	46.3	13	1.30
3	**10.9**	**13.5**	**15**	**1.50**
10	**7.3**	**15.2**	**41**	**4.10**

VC = Vehicle Control, rCFE = relative Colony Forming Efficiency, RCG = Relative Cell Growth, CV = Crystal Violet, na = not applicable.
Concentrations that contributed to the statistically significant result, *i.e.* which were present in the downturn-protected Williams contrast that resulted in the lowest *p*-value for a positive response (negative binomial analysis), and related values are highlighted in bold.

Table 7: Transformation assay results from Harlan CCR, testing coded 3-methylcholanthrene

Harlan CCR Coded 3-methylcholanthrene (µg/ml)	rCFE (%)	RCG (%) CV	Total number of foci	Mean number of foci per dish
Untreated control	na	na	2	0.20
VC (DMSO 0.5%)	100	100	3	0.30
0.01	72.0	92.0	5	0.50
0.03	53.0	69.4	2	0.20
0.1	**38.5**	**45.6**	**12**	**1.20**
0.3	**11.2**	**29.3**	**26**	**2.60**
1	**5.9**	**22.6**	**46**	**4.60**
3	**2.0**	**19.9**	**50**	**5.00**
10	**1.3**	**11.9**	**59**	**5.90**

VC = Vehicle Control, rCFE = relative Colony Forming Efficiency, RCG = Relative Cell Growth, CV = Crystal Violet, na = not applicable.
Concentrations that contributed to the statistically significant result, *i.e.* which were present in the downturn-protected Williams contrast that resulted in the lowest *p*-value for a positive response (negative binomial analysis), and related values are highlighted in bold.

Table 8: Transformation assay results from HRI, testing coded 3-methylcholanthrene

HRI Coded 3-methylcholanthrene (µg/ml)	rCFE (%)	RCG (%) CV	Total number of foci	Mean number of foci per dish
Untreated control	na	na	0	0
VC (DMSO 0.5%)	100	100	0	0
0.01	**76.9**	**86. 7**	**2**	**0.20**
0.03	**48.6**	**82.0**	**3**	**0.30**
0.1	**31.7**	**72.3**	**10**	**1.00**
0.3	**16.3**	**60.1**	**9**	**0.90**
1	**10.6**	**39.3**	**39**	**3.90**
3	**6.3**	**24.8**	**89**	**8.90**
10	**6.0**	**19.4**	**211**	**21.10**

VC = Vehicle Control, rCFE = relative Colony Forming Efficiency, RCG = Relative Cell Growth, CV = Crystal Violet, na = not applicable.
Concentrations that contributed to the statistically significant result, *i.e.* which were present in the downturn-protected Williams contrast that resulted in the lowest *p*-value for a positive response (negative binomial analysis), and related values are highlighted in bold.

4.2.1 Concurrent cytotoxicity: Crystal Violet

Cytotoxicity of 3-methylcholanthrene was evaluated by the CV method in all laboratories (Figure 5).

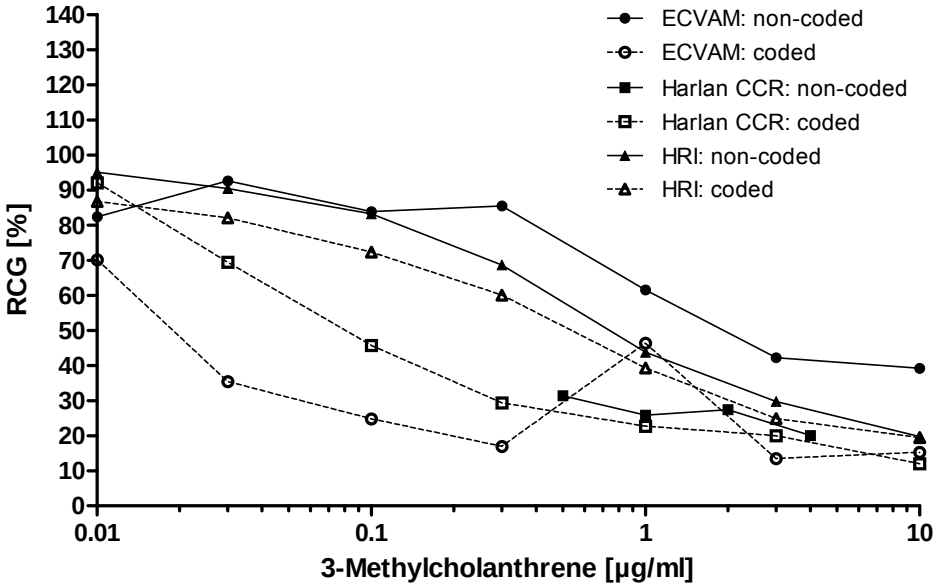

Figure 5: Relative cell growth (RCG) measured with the crystal violet assay compared to vehicle control in all laboratories testing non-coded and coded 3-methylcholanthrene

4.2.2 Concurrent cytotoxicity: Colony Forming Efficiency

Cytotoxicity of 3-methylcholanthrene was evaluated by CFE assessment in all laboratories (Figure 6). The results show a dose-dependent cytotoxicity with non-coded and coded chemicals in all laboratories. The cytotoxicity curves are similar with the exception of the non-coded 3-methylcholanthrene tested by ECVAM, which showed in general a lower level of cytotoxicity and an unexpected result at the dose of 3 µg/ml, suggesting that a mistake in the chemical dilution for this concentration had been made.

In general CFE seemed to be more sensitive than CV, except for ECVAM.

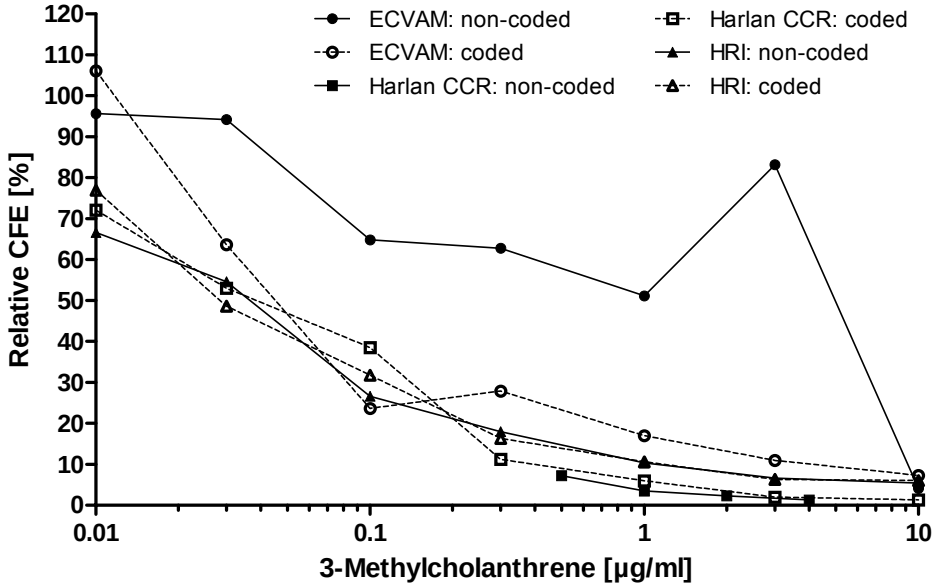

Figure 6: Relative Colony Forming Efficiency (CFE) compared to vehicle control in all laboratories testing non-coded and coded 3-methylcholanthrene

4.2.3 Morphological transformation

MT results with non-coded and coded 3-methylcholanthrene are shown in Figure 7. Although the dose-range tested by Harlan CCR for the non-coded 3-methylcholanthrene was limited, the trend in the formation of transformed foci by non-coded and coded 3-methylcholanthrene was similar in all laboratories. In HRI laboratory the overall response was higher than in other laboratories.

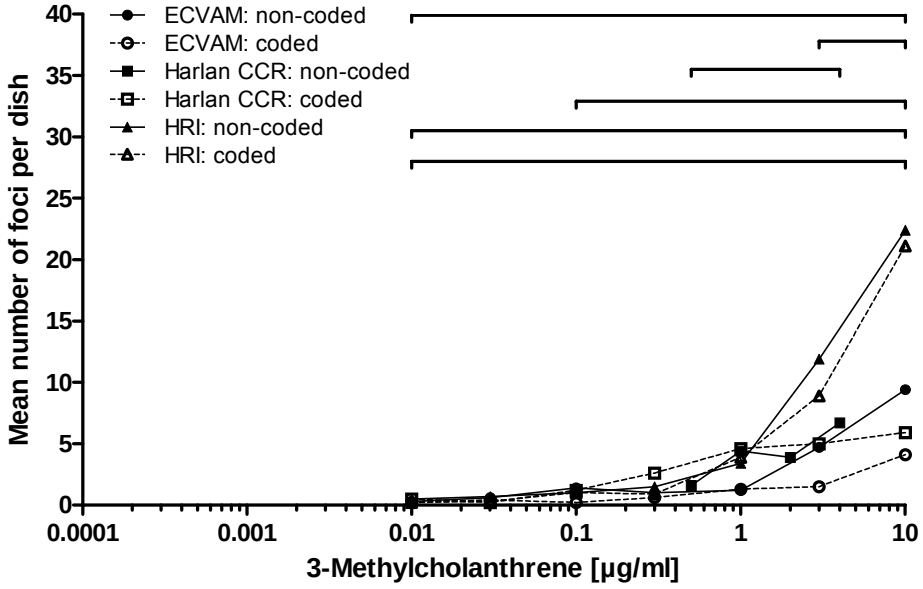

Figure 7: Number of transformed foci for all laboratories testing non-coded and coded 3-methylcholanthrene.

Results of the negative binomial statistical analysis are shown: concentrations that contributed to the statistically significant result, *i.e.* which were present in the downturn-protected Williams contrast that resulted in the lowest *p*-value for a positive response, are indicated by a horizontal line next to the corresponding laboratory name in the legend. The assessment criteria are described in section 3.6.7.

4.2.4 Acceptance criteria

Most of the acceptance criteria were met in the three laboratories (Table 9), except for the PE criteria of coded 3-methylcholanthrene in ECVAM laboratory, which did not reach 30%. However this experiment was considered acceptable by the VMT since a clear positive response in morphological transformation could be observed. In this phase of the validation study, the criteria that one NOEL concentration and at least six analysable concentrations should be tested were not taken into consideration, since the doses to be tested were fixed and had been previously recommended by the VMT.

Table 9 : Acceptance criteria and assessment of non-coded and coded 3-methylcholanthrene results

3-Methylcholanthrene							
Criteria		**Laboratory**					
		ECVAM non-coded	**ECVAM coded**	**Harlan non-coded**	**Harlan coded**	**HRI non-coded**	**HRI coded**
CFE	Concentration range (rCFE%)	96-4%	106-7%	7-1%	72-1%	67-5%	77-6%
	PE of untreated control	41.4	22.8	32.6	32.6	41.6	41.6
	PE of vehicle control >30%	**yes** (43.0)	**no** (20.6)	**yes** (38.0)	**yes** (38.0)	**yes** (43.8)	**yes** (43.8)
MT	Vehicle used	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO
	Number of foci of vehicle control <6	**yes** (0)	**yes** (5)	**yes** (3)	**yes** (3)	**yes** (0)	**yes** (0)
	Number of analysable concentrations	7	7	4	7	7	7
Fulfillment of all assay acceptance criteria		**YES**	**NO#**	**YES**	**YES**	**YES**	**YES**
Assessment (Fisher's exact test)	Call	**+**	**+**	**+**	**+**	**+**	**+**
Assessment (negative binomial)	Concentrations in the contrast [µg/ml]*	0.01-10 (7 conc.)	3-10 (2 conc.)	0.5-4 (4 conc.)	0.1-10 (5 conc.)	0.01-10 (7 conc.)	0.01-10 (7 conc.)
	Call	**+**	**+**	**+**	**+**	**+**	**+**

CFE = Colony Forming Efficiency, rCFE = relative Colony Plating Efficiency, MT = Morphological Transformation, PE = Plating Efficiency.

considered acceptable for overall study evaluation.

* for positive assessment, the concentrations that contributed to the statistically significant result, *i.e.* which were present in the downturn-protected Williams contrast that resulted in the lowest *p*-value, are indicated. The assessment criteria are described in section 3.6.7.

4.3 Statistical analysis

The statistical analysis of these data was performed using the Fisher's exact test, by comparing number of foci formed in the surviving seeded cells, *i.e.* adjusted by cytotoxicity results, of control and treated dishes. This analysis is based on the assumption that the relative cell survival in the CFE test is the same as in the MT component of the assay. However, this is not completely correct since the survival in CFE may depend, in part, on the density of the cells seeded on the dish, which is different in CFE and in MT, since the experiments are carried out independently. Moreover the cell survival is measured at a different time point than the MT. Though other statistical approaches had been used in the past, no agreement was reached on the most appropriate statistical method for this analysis.

Consequently the VMT (at the meeting of May 2006) recommended that ECVAM would organise an *ad hoc* expert group to evaluate the data and come up with a suggestion on the most suitable statistical method to use for this assay (see section 3.6.5). Accordingly, the above data were re-analysed a posteriori using the negative binomial approach and showed a statistically significant increase in transformed foci in all experiment. Note that for the data generated with 3-methylcholanthrene, both the Fischer's exact test and the negative binomial approach gave comparable results and led to the same conclusions.

4.4 Conclusions of the Validation Management Team on Module 2

The data generated showed satisfactory within-laboratory reproducibility between the non-coded and the coded 3-methylcholanthrene. Moreover the results demonstrated that the method had been successfully transferred to all laboratories and provided an initial indication of between-laboratory reproducibility for 3-methylcholanthrene.

The curves of cytotoxicity tests for non-coded and coded 3-methylcholanthrene were similar for Harlan and HRI, whereas they varied for ECVAM. Overall, the CFE test was more sensitive than the CV test, although the ranges of their separation differed among the laboratories.

Since all laboratories reproducibly induced a positive response in morphological transformation with non-coded and coded 3-methylcholanthrene, even though there was some variance in response rates, the VMT agreed that the laboratories were ready to proceed to the between-laboratory reproducibility phase of the validation study. These results agree with published data (Heidelberger *et al*., 1983; Kajiwara Y. and Ajimi S., 2003)

5 Module 3: Transferability

5.1 General Aspects

In general, the Balb/c 3T3 CTA 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 and adequately trained to identify morphological transformation events 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.

Scoring of transformed foci 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 QC-tissue culture laboratories. The operator should, in particular, be trained in the scoring of transformed foci. 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 ECVAM in April 2005. Representatives from all laboratories involved in the study participated in the training. Agreement was reached on criteria for scoring the plates, using dishes treated with both the vehicle and reference chemicals. In addition, a comparison was conducted between two methods of cytotoxicity, the CFE and CV. Moreover, historical data produced by the ECVAM and HRI laboratories were also evaluated. The experimental team agreed on the scoring of morphologically transformed foci as follows: 1) only type III foci would need to be scored; 2) a detailed description of the morphology of type III foci would be produced; 3) foci would to be scored using a stereomicroscope. Overall, the training was extremely useful for harmonising the procedures among the laboratories to be used in the prevalidation experiments. A report of the training week is available at ECVAM.

As part of this validation exercise a photo catalogue was produced by the HRI laboratory, in collaboration with the other participating laboratories, with the aim of standardising the scoring. Although the classification of foci into distinct morphological categories can be difficult since their development is a continuous process, three types of foci have been reported and defined. Although this categorisation was originally defined for the C3H/10T½ CTA (Reznikoff *et al*., 1973), it has since been applied to the Balb/c 3T3 CTA. The catalogue includes pictures of both non malignantly transformed foci (type I, type II) and malignantly transformed foci (type III). Examples of clearly scorable foci, foci with questionable or mixed morphology, as well as examples of altered foci that should not be scored as transformed were included in the catalogue to obtain an overview of the different types of foci that can be encountered during the Balb/c 3T3 CTA experiment.

5.3 Conclusions 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 photograph catalogue of foci was found to be very useful in establishing consistency in assessing focus 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.

6 Module 4: Between-laboratory reproducibility

The between-laboratory reproducibility experiments were conducted using the "SOP_rev_June_06" version (Annex 13.2), which is the resulting version that was previously revised after the within-laboratory and transferability phases of the validation study had been completed. The following coded chemicals were tested (Table 1): 2-acetylaminofluorene, benzo(a)pyrene, anthracene, phenanthrene, o-toluidine HCl. The Safety Officers identified by the laboratories received the instructions to dissolve all coded chemicals in DMSO. HRI laboratory did not follow these instructions and used instead water, therefore the laboratory was requested to repeat some of the experiments using DMSO as the solvent. HRI laboratory stated that they had not received this information. Most probably the Safety Officer had not passed the information to the laboratory staff.

The results of the between-laboratory reproducibility studies are presented in this section and are summarised by compound and laboratory. The data are shown in tables for each individual laboratory. Each table includes information on: the measures of cytotoxicity used shown by the rCFE (%), and the RCG (%) in the CV experiment; the total number of morphologically transformed foci, and the mean number of foci per dish corresponding to each concentration of chemical tested, to vehicle and positive controls.

An initial DRF test using the CFE was performed by all laboratories to determine the experimental doses to be used in the TA, for each chemical (Figure 8, Figure 13, Figure 18, Figure 23 and Figure 28). Subsequently, the complete TA including concurrent cytotoxicity tests (Figure 9, Figure 10, Figure 14, Figure 15, Figure 19, Figure 20, Figure 24, Figure 25, Figure 29 and Figure 30) and MT assays were performed (Figure 11, Figure 16, Figure 21, Figure 26 and Figure 31). The concurrent cytotoxicity tests were performed in parallel by CFE and CV in order to allow a comparison between the two methods.

PE of vehicle and negative controls were similar in all the experiments performed.

VCs gave levels of transformed foci within the acceptable range dictated by the acceptance criteria established for this Balb/c 3T3 CTA, except for four experiments where the level of transformed foci was higher than five. The PC chemical 3-methylcholanthrene induced an increase in morphologically transformed foci in all experiments. The average number of transformed foci per dish in each experiment was always higher than 10 foci per dish (section 6.6).

All results were sent to the statistician as soon as they became available. A VMT meeting was held on the 30 May 2007 to discuss the between-laboratory results.

Due to costs and resources needed to perform such an assay it was necessary to minimise the repetition of experiments. Therefore, some experiments were still considered by the VMT although they did not fulfill all acceptance criteria. Justifications are given in these cases.

6.1 2-Acetylaminofluorene

6.1.1 Dose-range finding test

Figure 8 shows the results of the DRF tests with 2-acetylaminofluorene. The results were reproducible in all laboratories and showed a narrow cytotoxic concentration range. ECVAM and HRI laboratories performed the DRF twice.

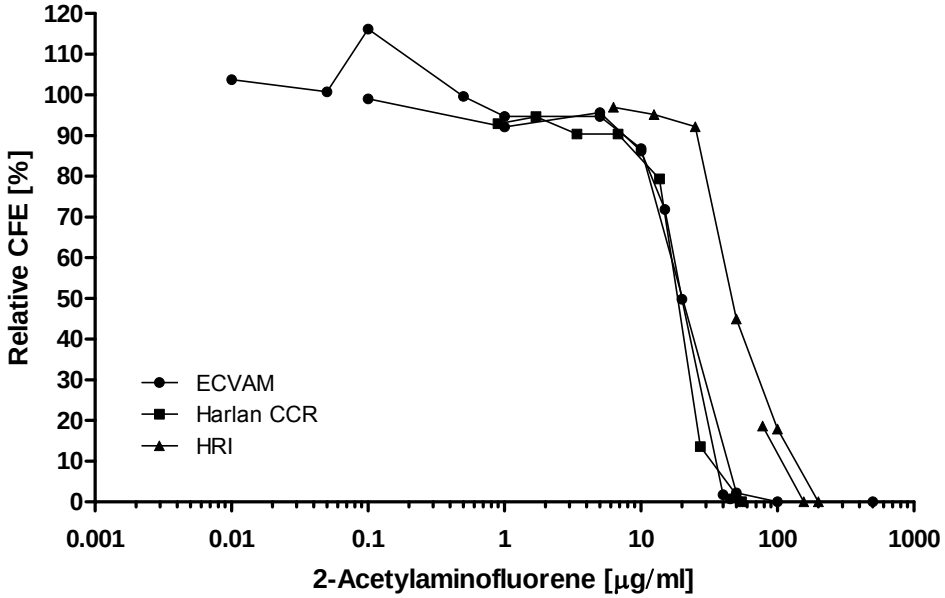

Figure 8: Relative Colony Forming Efficiency (CFE) compared to vehicle control in all laboratories testing coded 2-acetylaminofluorene, for the Dose-Range Finding. ECVAM and HRI repeated the experiment.

6.1.2 Transformation assay

The following tables and figures show the MT and concurrent cytotoxicity test results upon treatment with 2-acetylaminofluorene at the test concentrations which were selected based on solubility and DRF tests.

6.1.2.1 ECVAM

2-Acetylaminofluorene was dissolved in DMSO. The concentrations were selected on the basis of the DRF results and evaluated by ECVAM (Table 10). This chemical induced a statistically significant positive response in morphological transformation.

Table 10: Transformation assay results from ECVAM, testing coded 2-acetylaminofluorene

ECVAM 2-Acetylaminofluorene (µg/ml)	rCFE (%)	RCG (%) CV	Total number of foci	Mean number of foci per dish
Untreated control	na	na	4	0.44
VC (DMSO 0.5%)	100	100	12	1.20
0.05	nd	nd	6	0.60
0.1	104.5	108.9	7	0.78
10	nd	nd	3	0.33
15	54.2	95.4	5	0.50
20	**26.3**	**37.0**	**12**	**1.20**
25	**17.4**	**21.5**	**36**	**4.00**
30	**10. 8**	**10.6**	**123**	**12.30**
35	**4.5**	**9.5**	**114**	**12.67**
PC	1.8	15.9	182	20.22

VC = Vehicle Control, PC = Positive Control, rCFE = relative Colony Forming Efficiency, RCG = Relative Cell Growth, CV = Crystal Violet, na = not applicable, nd = not determined.

Concentrations that contributed to the statistically significant result, *i.e.* which were present in the downturn-protected Williams contrast that resulted in the lowest *p-value* for a positive response (negative binomial analysis), and related values are highlighted in bold.

6.1.2.2 Harlan CCR

2-Acetylaminofluorene was dissolved in DMSO. The concentrations were selected on the basis of the DRF results and evaluated by Harlan CCR (Table 11). This chemical induced a statistically significant positive response in morphological transformation at a single dose. As such, the result was considered inconclusive.
Harlan CCR was requested to repeat the assay since it gave an inconclusive call, however this was not done due to lack of resources.

Table 11: Transformation assay results from Harlan CCR, testing coded 2-acetylaminofluorene

Harlan CCR 2-Acetylaminofluorene (µg/ml)	rCFE (%)	RCG (%) CV	Total number of foci	Mean number of foci per dish
Untreated control	na	na	3	0.30
VC (DMSO 0.5%)	100	100	3	0.30
1	91.0	96.3	2	0.20
2.5	82.7	100.7	4	0.40
5	90.4	94.5	5	0.50
10	77.8	87.8	3	0.30
15	44.4	79.8	3	0.30
20	12.3	31.1	3	0.30
25	5.9	22.5	7	0.78
30	**1.2**	**4.8**	**24**	**2.40**
PC	1.5	22.0	113	11.30

VC = Vehicle Control, PC = Positive Control, rCFE = relative Colony Forming Efficiency, RCG = Relative Cell Growth, CV = Crystal Violet, na = not applicable.
Concentration that contributed to the statistically significant result, *i.e.* which was present in the downturn-protected Williams contrast that resulted in the lowest *p*-value for a positive response (negative binomial analysis), and related values are highlighted in bold**.**

6.1.2.3 HRI

2-Acetylaminofluorene was dissolved in ultrapure water by HRI. The concentrations were selected on the basis of the DRF results and evaluated by HRI (Table 12). This chemical induced a statistically significant positive response in morphological transformation.
Although this chemical was tested in water, HRI was not asked to repeat the experiment in DMSO because 1) HRI results on cytotoxicity are similar to cytotoxicity curves from the other laboratories, 2) 2-acetylaminofluorene produces a statistically significant positive response related to multiple doses and, 3) to avoid a waste of resources.

Table 12: Transformation assay results from HRI, testing coded 2-acetylaminofluorene

HRI 2-Acetylaminofluorene (µg/ml)	rCFE (%)	RCG (%) CV	Total number of foci	Mean number of foci per dish
Untreated control	na	na	5	0.50
VC (water)	100	100	2	0.20
10	98.6	104.6	1	0.10
25	**67.2**	**47.3**	**32**	**3.20**
50	**34.7**	**6.6**	**16**	**1.60**
75	**21.4**	**4.1**	**10**	**1.00**
100	**11.8**	**5.0**	**17**	**1.70**
150	**2.1**	**6.6**	**32**	**3.20**
PC	11.0	-3.6	161	16.10

VC = Vehicle Control, PC = Positive Control, rCFE = relative Colony Forming Efficiency, RCG = Relative Cell Growth, CV = Crystal Violet, na = not applicable.
Concentrations that contributed to the statistically significant result, *i.e.* which were present in the downturn-protected Williams contrast that resulted in the lowest *p*-value for a positive response (negative binomial analysis), and related values are highlighted in bold.

6.1.2.4 Concurrent cytotoxicity: Colony Forming Efficiency

Cytotoxicity of 2-acetylaminofluorene was evaluated by CFE assessment in all laboratories (Figure 9). All laboratories showed cytotoxicity within a narrow concentration range: ECVAM (10-30 µg/ml), Harlan CCR (6.8-27.3 µg/ml) and HRI (25-100 µg/ml).

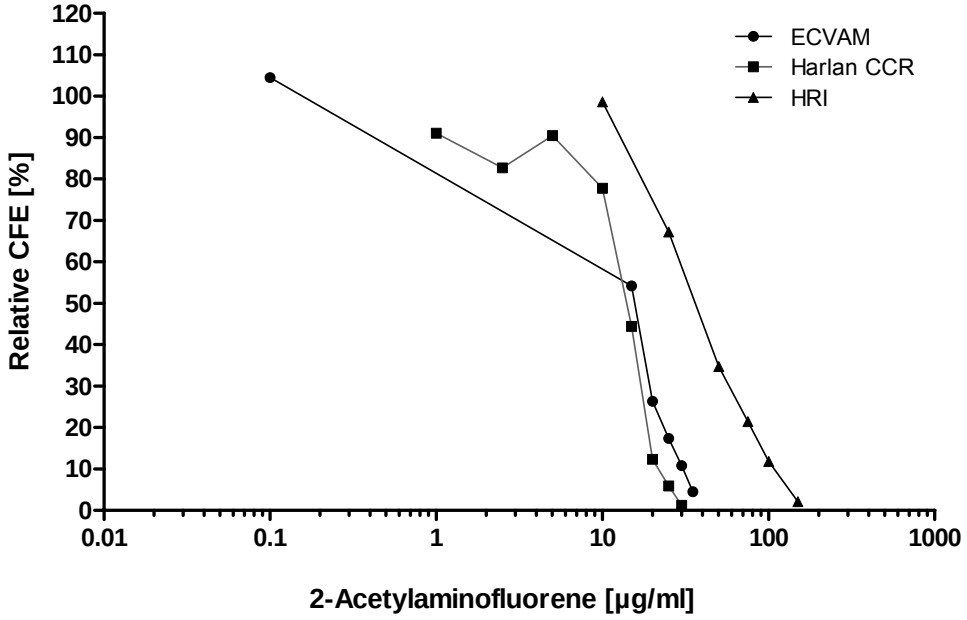

Figure 9: Relative Colony Forming Efficiency (CFE) compared to vehicle control in all laboratories testing coded 2-acetylaminofluorene

6.1.2.5 Concurrent cytotoxicity: Crystal Violet

Cytotoxicity of 2-acetylaminofluorene was evaluated by the CV method in all laboratories (Figure 10). All laboratories showed cytotoxicity within a narrow concentration range. For this chemical no clear differences were noted between the cytotoxicity curves of the CFE and CV tests.

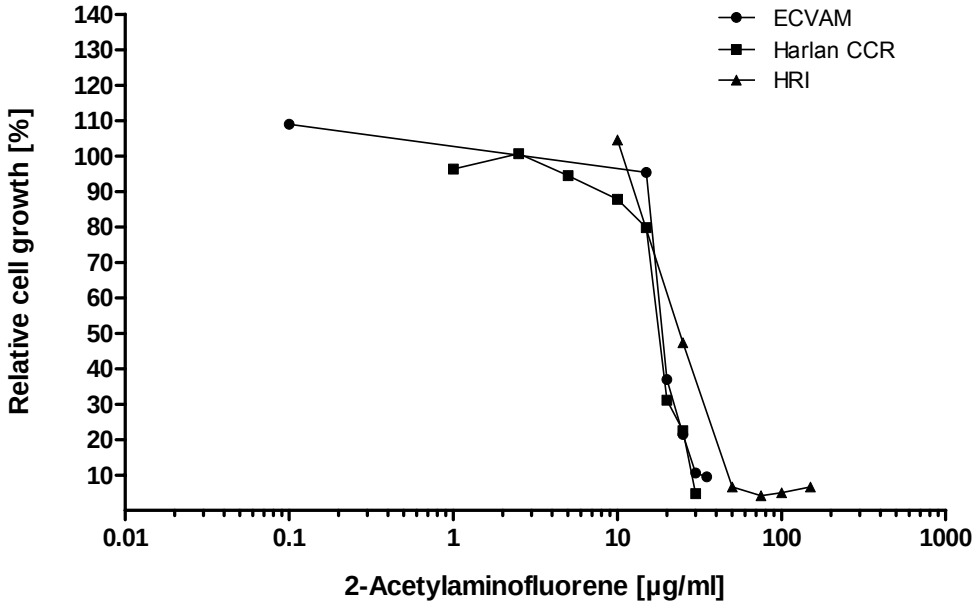

Figure 10: Relative cell growth compared to vehicle control in all laboratories testing coded 2-acetylaminofluorene

6.1.2.6 Morphological transformation

MT results with 2-acetylaminofluorene are shown in Figure 11. Both HRI and ECVAM showed a statistically significant increase in the number of transformed foci, while Harlan CCR showed a statistically significant increase of transformed foci related to the highest dose tested only. The transformed foci appeared at doses corresponding to IC_{80} and more.

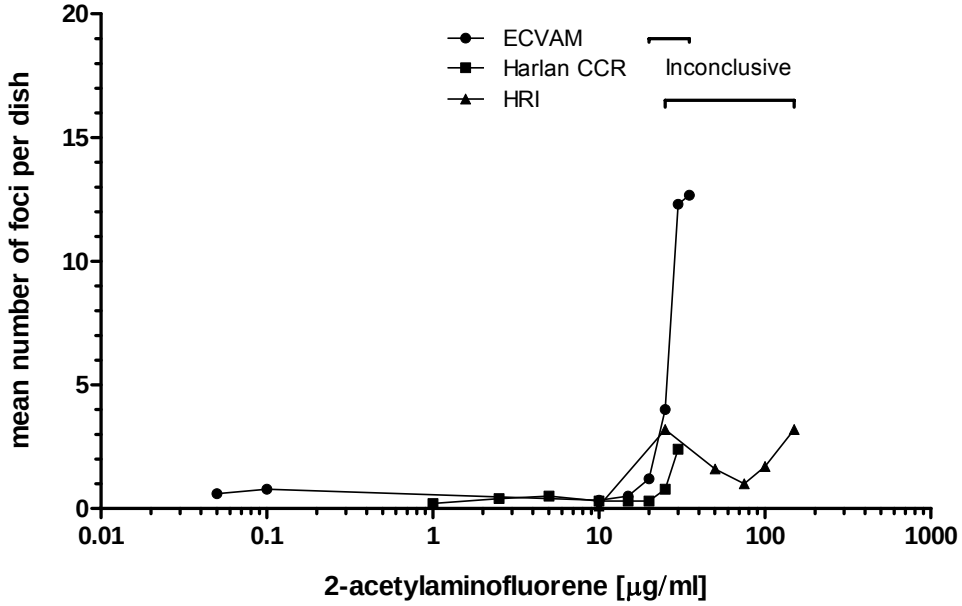

Figure 11: Mean number of foci per dish for all laboratories testing coded 2-acetylaminofluorene.

Results of the negative binomial statistical analysis are shown. For positive assessment the concentrations that contributed to the statistically significant result, *i.e.* which were present in the downturn-protected Williams contrast that resulted in the lowest *p*-value, are indicated by a horizontal line next to the corresponding laboratory name in the legend. Assessment for Harlan CCR was reported inconclusive since the downturn-protected Williams contrast with the lowest *p*-value showed a *p*-value < 0.01 but included only one concentration (30 µg/ml). The assessment criteria are described in section 3.6.7.

MT results for the different control treatments are summarised in Figure 12. The untreated, vehicle and positive controls were reproducible across the laboratories.

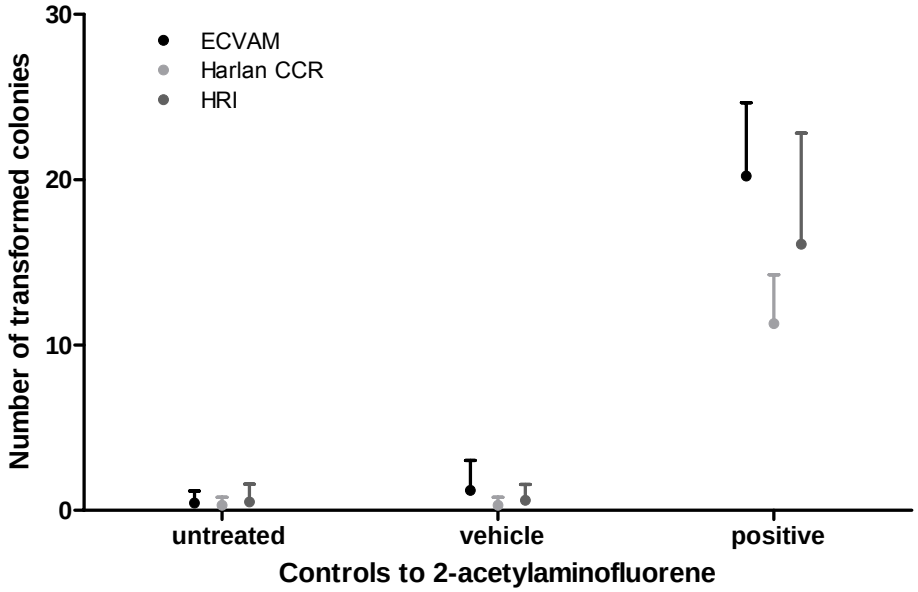

Figure 12: Number of transformed colonies (mean + standard deviation) for untreated, vehicle and positive controls in all laboratories testing coded 2-acetylaminofluorene

6.1.2.7 Acceptance and assessment criteria

All acceptance criteria were fulfilled upon treatment with 2-acetylaminofluorene, except for the number of transformed foci in the VC in the ECVAM laboratory (Table 13).

Although the ECVAM experiment did not fulfil all acceptance criteria, it was considered acceptable for the overall study evaluation since a clear dose-response transforming effect was observed.

Table 13: Acceptance criteria and assessment of 2-acetylaminofluorene results

2-Acetylaminofluorene				
Criteria		**Laboratory**		
		ECVAM	**Harlan CCR**	**HRI**
CFE	1 NOEL [µg/ml]	**yes** (0.1)	**yes** (5)	**yes** (10)
	PE of untreated control	44.8	38.3	62.9
	PE of vehicle control >30%	**yes** (41.7)	**yes** (40.5)	**yes** (64.8)
MT	Vehicle used	DMSO	DMSO	water
	Number of foci of vehicle control <6	**no** (12)	**yes** (3)	**yes** (2)
	Number of analysable concentrations ≥6	**yes** (8)	**yes** (8)	**yes** (6)
Fulfilment of all assay acceptance criteria		**NO**[#]	**YES**	**YES**
Assessment	Concentrations in the contrast [µg/ml]*	20 - 35 (4 conc.)	30 (1 conc.)	25 - 150 (5 conc.)
	Call	**+**	**i****	**+**

CFE = Colony Forming Efficiency, MT = Morphological Transformation, NOEL = No Observed Effect Level, PE = Plating Efficiency, i = inconclusive.
[#] considered acceptable for overall study evaluation.
* for positive assessment, the concentrations that contributed to the statistically significant result, *i.e.* which were present in the downturn-protected Williams contrast that resulted in the lowest *p*-value, are indicated.
** for Harlan CCR assessment was reported inconclusive since the downturn-protected Williams contrast with the lowest *p*-value showed a *p*-value < 0.01 but included only one concentration (30 µg/ml).
The assessment criteria are described in section 3.6.7.

6.1.3 Conclusion

Treatment with 2-acetylaminofluorene produced a statistically significant positive response in morphologically transformed foci in ECVAM and HRI laboratories, and a clear increase in the number of foci at the highest concentration only in Harlan laboratory. Since the assessment criteria for a positive call for MT required at least two concentrations to be in the downturn-protected Williams contrast with the lowest *p*-value, the resulting call for Harlan CCR was inconclusive and should have been repeated. Due to a lack of resources and time reasons Harlan CCR could not repeat this experiment.

It can be concluded that, according to the assessment criteria established for this study, the experiments were reproducible in two laboratories, and that 2-acetylaminofluorene was considered to be a positive transforming agent in the Balb/c 3T3 CTA. These results agree with published data (Hedelberger *et al.,* 1983; Matthews *et al.*, 1993c).

6.2 Benzo(a)pyrene

6.2.1 Dose-range finding test

Figure 13 shows the results of the DRF tests with benzo(a)pyrene. Harlan CCR and HRI laboratories observed a dose-dependent cytoxicity induced by benzo(a)pyrene at the concentrations tested. HRI repeated the experiment twice.

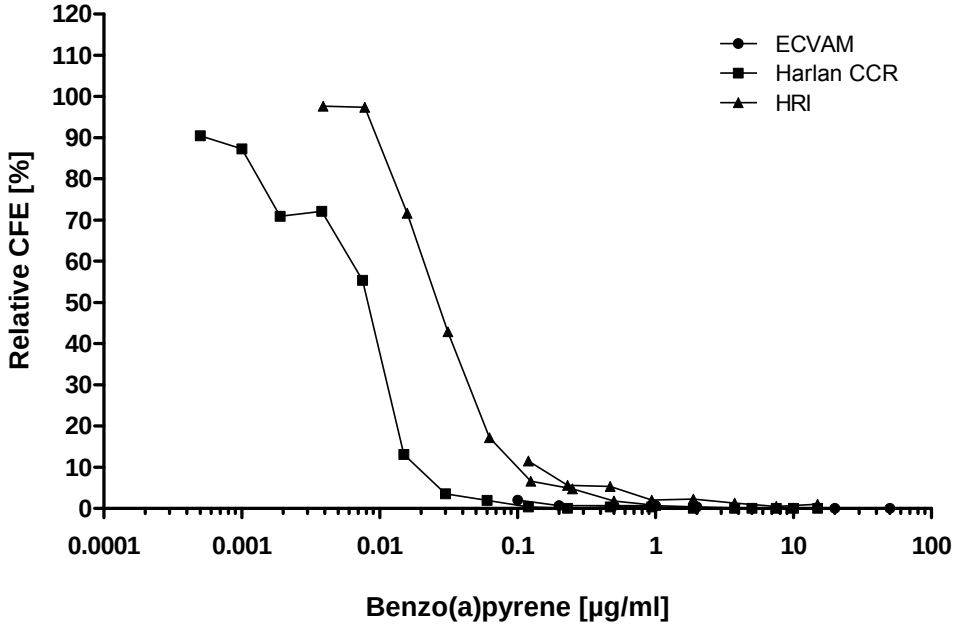

Figure 13: Relative Colony Forming Efficiency (CFE) compared to vehicle control in all laboratories testing coded benzo(a)pyrene, for the Dose-Range Finding.

6.2.2 Transformation Assay

The following tables and figures show the MT and concurrent cytotoxicity test results upon treatment with benzo(a)pyrene at the test concentrations which were selected based on solubility and DRF tests.

6.2.2.1 ECVAM

Benzo(a)pyrene was dissolved in DMSO. The concentrations were selected on the basis of the DRF results and evaluated by ECVAM (Table 14). This chemical induced a statistically significant positive response in morphological transformation.

Table 14: Transformation assay results from ECVAM, testing coded benzo(a)pyrene

ECVAM Benzo(a)pyrene (µg/ml)	CFE (%)	RCG (%) CV	Total number of foci	Mean number of foci per dish
Untreated control	na	na	5	0.50
VC (DMSO 0.5%)	100	100	4	0.40
0.0005	**85.8**	**83.7**	**6**	**0.60**
0.001	**nd**	**nd**	**2**	**0.20**
0.005	**62.1**	**89.0**	**15**	**1.50**
0.05	**0.0**	**49.3**	**69**	**6.90**
0.125	**0.8**	**10.4**	**91**	**9.10**
0.625	**0.4**	**3.2**	**110**	**11.00**
3.125	**nd**	**nd**	**75**	**7.50**
15	**0.0**	**2.6**	**13**	**1.30**
PC	7.1	25.0	131	13.10

VC = Vehicle Control, PC = Positive Control, rCFE = relative Colony Forming Efficiency, RCG = Relative Cell Growth, CV = Crystal Violet, na = not applicable, nd = not determined

Concentrations that contributed to the statistically significant result, *i.e.* which were present in the downturn-protected Williams contrast that resulted in the lowest *p*-value for a positive response (negative binomial analysis), and related values are highlighted in bold.

6.2.2.2 Harlan CCR

Benzo(a)pyrene was dissolved in DMSO. The concentrations were selected on the basis of the DRF results and evaluated by Harlan CCR (Table 15). This chemical induced a statistically significant positive response in morphological transformation.

Table 15: Transformation assay results from Harlan CCR, testing coded benzo(a)pyrene

Harlan CCR Benzo(a)pyrene (µg/ml)	rCFE (%)	RCG (%) CV	Total number of foci	Mean number of foci per dish
Untreated control	na	na	4	0.40
VC (DMSO 0.5%)	100	100	0	0
0.01	**65.3**	**76.3**	**5**	**0.50**
0.015	**49.6**	**79.4**	**7**	**0.70**
0.02	**37.5**	**70.4**	**19**	**1.90**
0.03	**21.5**	**89.8**	**21**	**2.10**
0.04	**4.5**	**73.0**	**21**	**2.10**
0.06	**1.8**	**58.2**	**33**	**3.30**
0.08	**1.3**	**34.3**	**39**	**3.90**
0.1	**0.8**	**27.1**	**45**	**4.50**
0.12	**0.8**	**23.5**	**48**	**4.80**
0.2	**0.5**	**9.2**	**72**	**7.20**
PC	6.3	25.9	135	15.00

VC = Vehicle Control, PC = Positive Control, rCFE = relative Colony Forming Efficiency, RCG = Relative Cell Growth, CV = Crystal Violet, na = not applicable.
Concentrations that contributed to the statistically significant result, *i.e.* which were present in the downturn-protected Williams contrast that resulted in the lowest *p*-value for a positive response (negative binomial analysis), and related values are highlighted in bold.

6.2.2.3 HRI

Benzo(a)pyrene was dissolved in DMSO. The concentrations were selected on the basis of the DRF results and evaluated by HRI (Table 16). This chemical induced a statistically significant positive response in morphological transformation.

Table 16: Transformation assay results from HRI, testing coded benzo(a)pyrene

HRI Benzo(a)pyrene (µg/ml)	rCFE (%)	RCG (%) CV	Total number of foci	Mean number of foci per dish
Untreated control	na	na	2	0.22
VC (DMSO 0.5%)	100	100	5	0.50
0.003	90.2	96.4	6	0.67
0.010	70.1	83.3	7	1.00
0.030	**15.5**	**23.6**	**20**	**2.22**
0.100	**3.5**	**6.1**	**17**	**1.70**
0.300	**1.4**	**4.4**	**27**	**3.38**
1.000	**0.4**	**4.4**	**65**	**6.50**
PC	8.8	9.2	250	25.00

VC = Vehicle Control, PC = Positive Control, rCFE = relative Colony Forming Efficiency, RCG = Relative Cell Growth, CV = Crystal Violet, na = not applicable.
Concentrations that contributed to the statistically significant result, *i.e.* which were present in the downturn-protected Williams contrast that resulted in the lowest *p*-value for a positive response (negative binomial analysis), and related values are highlighted in bold.

6.2.2.4 Concurrent cytotoxicity: Colony Forming Efficiency

Cytotoxicity of benzo(a)pyrene was evaluated by measuring CFE in all laboratories (Figure 14). Benzo(a)pyrene was shown to be cytotoxic in all laboratories as a function of concentration. Cytotoxicity was similar in all laboratories.

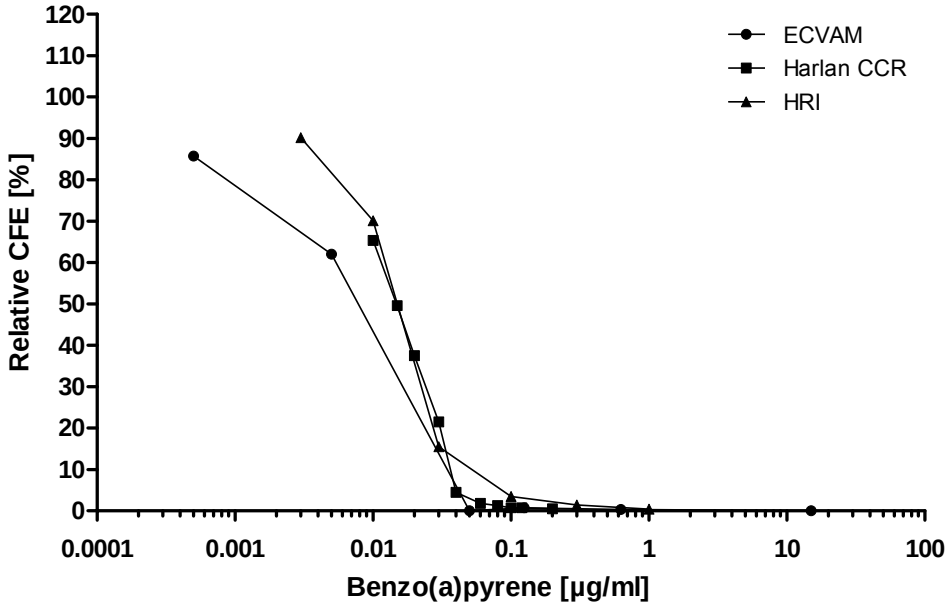

Figure 14: Relative Colony Forming Efficiency (CFE) compared to vehicle control in all laboratories testing coded benzo(a)pyrene

6.2.2.5 Concurrent cytotoxicity: Crystal Violet

Cytotoxicity of benzo(a)pyrene was evaluated by the CV method in all laboratories (Figure 15).
The results suggest that CV may be less sensitive than CFE in the assessment of cytotoxicity of benzo(a)pyrene.

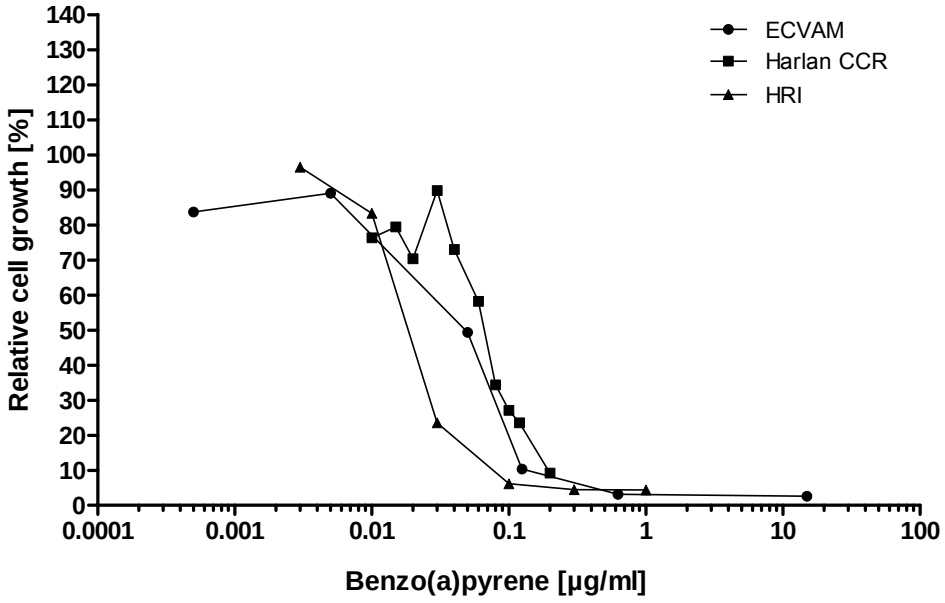

Figure 15: Relative cell growth compared to vehicle control in all laboratories testing coded benzo(a)pyrene

6.2.2.6 Morphological Transformation

MT results with benzo(a)pyrene are shown in Figure 16. All three laboratories clearly demonstrated that benzo(a)pyrene induced a positive response in a relatively broad concentration range: from 0.005 µg/ml to 15 µg/ml for ECVAM, from 0.02 µg/ml for Harlan, and from 0.03 µg/ml to the highest tested concentration of 1.0 µg/ml for HRI. The decrease in the number of foci at the two highest concentrations for ECVAM can be explained by the high cytotoxicity induced at these doses.

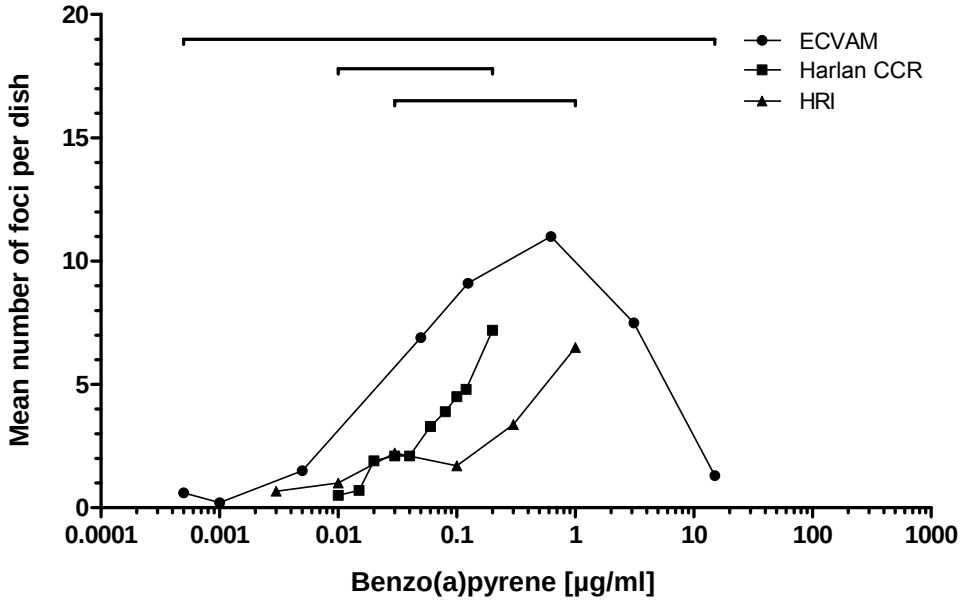

Figure 16: Mean number of foci per dish for all laboratories testing coded benzo(a)pyrene.
Results of the negative binomial statistical analysis are shown: for positive assessment, the concentrations that contributed to the statistically significant result, *i.e.* which were present in the downturn-protected Williams contrast that resulted in the lowest *p*-value, are indicated by a horizontal line, next to the corresponding laboratory name in the legend. The assessment criteria are described in section 3.6.7.

MT results for the different control treatments are summarised in Figure 17. The untreated, vehicle and positive controls were reproducible across the laboratories. HRI shows a higher value for the PC.

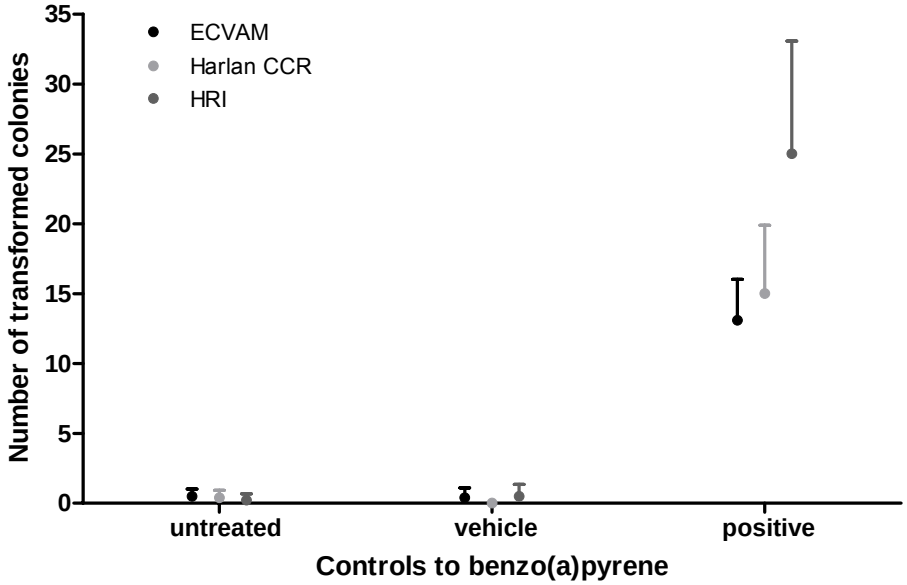

Figure 17: Number of transformed colonies (mean + standard deviation) for untreated, vehicle and positive controls in all laboratories testing coded benzo(a)pyrene

6.2.2.7 Acceptance and assessment criteria

ECVAM met all the assay acceptance criteria (Table 17). Although one of the assay acceptance criteria was not met in Harlan (absence of NOEL) and HRI (number of analysable concentrations), benzo(a)pyrene induced a clearly positive response in transformed foci in all laboratories. Therefore despite these limitations, these experiments were considered acceptable for the overall study evaluation.

Table 17: Acceptance criteria and assessment of benzo(a)pyrene results

Benzo(a)pyrene				
Criteria		**Laboratory**		
		ECVAM	**Harlan CCR**	**HRI**
CFE	1 NOEL [µg/ml]	**yes** (0.0005)	**no**	**yes** (0.003)
	PE of untreated control	32.5	49.9	62.6
	PE of vehicle control >30%	**yes** (31.6)	**yes** (47.6)	**yes** (61.1)
MT	Vehicle used	DMSO	DMSO	DMSO
	Number of foci of vehicle control <6	**yes** (4)	**yes** (0)	**yes** (5)
	Number of analysable concentrations ≥6	**yes** (8)	**yes** (10)	**no** (4*****)
Fulfilment of all assay acceptance criteria		**YES**	**NO**[#]	**NO**[#]
Assessment	Concentrations in the contrast [µg/ml]**	0.0005 - 15 (All conc.)	0.01 - 0.2 (All conc.)	0.03 - 1 (4 conc. *)
	Call	**+**	**+**	**+**

CFE = Colony Forming Efficiency, MT = Morphological Transformation, NOEL = No Observed Effect Level, PE = Plating Efficiency.

[#] considered acceptable for overall study evaluation.

* only concentrations with at least 9 dishes available were used for the assessment (*i.e.* 0.003, 0.03, 0.1 and 1 µg/ml).

** for positive assessment, the concentrations that contributed to the statistically significant result, *i.e.* which were present in the downturn-protected Williams contrast that resulted in the lowest *p*-value (negative binomial analysis), are indicated. The assessment criteria are described in section 3.6.7.

6.2.3 Conclusion

Treatment with benzo(a)pyrene produced a positive response in morphologically transformed foci in all laboratories.

It can be concluded that, according to the assessment criteria established for this study, the experiments were reproducible in all laboratories, and that benzo(a)pyrene was considered to be a positive transforming agent in the Balb/c 3T3 CTA. These results agree with published data (Heidelberger *et al.*, 1983, Sheu *et al.*, 1994).

6.3 Anthracene

6.3.1 Dose-range finding test

Figure 18 shows the results of the DRF tests with anthracene. This chemical does not show clear signs of toxicity up to the limits of solubility. ECVAM performed the DRF twice. HRI tested a different concentration range since they used water as a solvent.

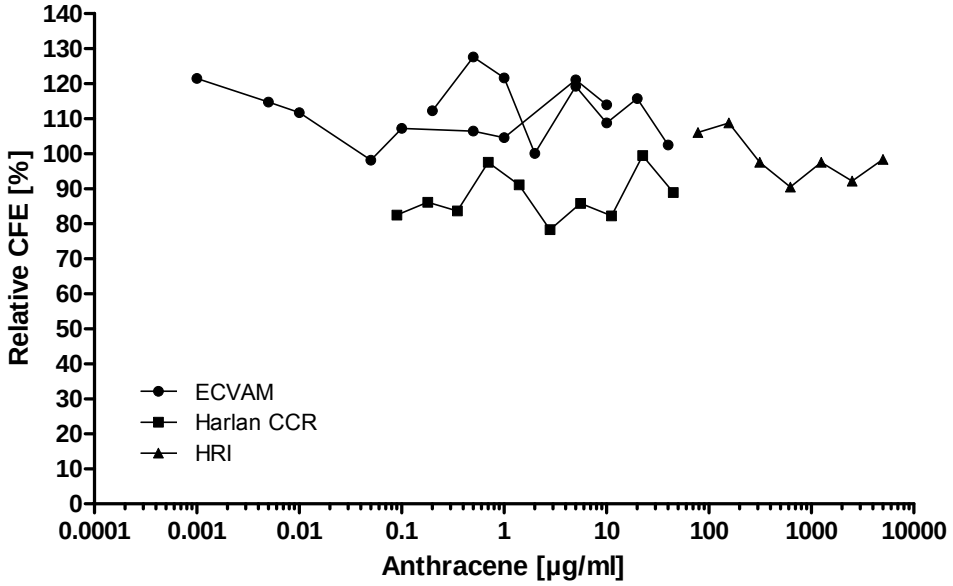

Figure 18: Relative Colony Forming Efficiency (CFE) compared to vehicle control in all laboratories testing coded anthracene, for the Dose-Range Finding. ECVAM repeated the experiment.

6.3.2 Transformation assay

The following tables and figures show the MT and concurrent cytotoxicity test results upon treatment with anthracene at the test concentrations which were selected based on solubility and DRF tests.

6.3.2.1 ECVAM

Anthracene was dissolved in DMSO by ECVAM. The concentrations were selected on the basis of the DRF results and evaluated by ECVAM (Table 18). The chemical was tested up to precipitating doses. No positive response in morphological transformation was observed upon treatment with this chemical.

Table 18: Transformation assay results from ECVAM, testing coded anthracene

ECVAM Anthracene (µg/ml)	rCFE (%)	RCG (%) CV	Total number of foci	Mean number of foci per dish
Untreated control	na	na	4	0.40
VC (DMSO 0.5%)	100	100	5	0.50
0.1	104.5	96.5	3	0.30
0.2	nd	nd	4	0.40
0.4	94.9	84.1	15	1.50
1	nd	nd	8	0.80
2	108.6	90.5	7	0.70
10	98.8	83.5	16	1.60
20	100.8	74.1	14	1.40
40	91.9	86.6	13	1.30
PC	9.8	38.7	156	15.60

VC = Vehicle Control, PC = Positive Control, rCFE = relative Colony Forming Efficiency, RCG = Relative Cell Growth, CV = Crystal Violet, na = not applicable, nd = not determined

6.3.2.2 Harlan CCR

Anthracene was dissolved in DMSO by Harlan CCR. The concentrations were selected on the basis of the DRF results and evaluated by Harlan CCR (Table 19). The chemical was tested up to precipitating doses. No positive response in morphological transformation was observed upon treatment with this chemical.

Table 19: Transformation assay results from Harlan CCR, testing coded anthracene

Harlan CCR Anthracene (µg/ml)	rCFE (%)	RCG (%) CV	Total number of foci	Mean number of foci per dish
Untreated control	na	na	4	0.40
VC (DMSO 0.5%)	100	100	4	0.40
0.7	83.3	94.8	1	0.11
1.4	95.8	100.0	5	0.50
2.8	96.1	94.9	5	0.50
5.6	79.4	92.1	1	0.10
11.2	101.1	92.7	5	0.50
22.5	88.9	90.6	2	0.20
45	88.6	92.9	0	0
PC	0	23.9	137	13.70

VC = Vehicle Control, PC = Positive Control, rCFE = relative Colony Forming Efficiency, RCG = Relative Cell Growth, CV = Crystal Violet, na = not applicable.

6.3.2.3 HRI

A suspension of anthracene in water was tested by HRI. The concentrations were selected on the basis of the DRF results and evaluated by HRI (Table 20). No positive response in morphological transformation was observed upon treatment with this chemical.

Although this chemical was tested in water, HRI was not asked to repeat the experiment in DMSO, the solvent requested by the VMT, because 1) anthracene was clearly non cytotoxic in DMSO and water and 2) anthracene was clearly negative in DMSO and water.

Table 20: Transformation assay results from HRI, testing coded anthracene

HRI Anthracene (µg/ml)	rCFE (%)	RCG (%) CV	Total number of foci	Mean number of foci per dish
Untreated control	na	na	9	0.90
VC (water)	100	100	6	0.60
100	98.6	85.3	5	0.50
300	104.0	123.4	2	0.20
500	100.2	77.2	3	0.30
1000	98.0	94.0	3	0.30
3000	98.6	105.4	2	0.20
5000	101.2	140.2	0	0
PC	15.0	0.3	207	20.70

VC = Vehicle Control, PC = Positive Control, rCFE = relative Colony Forming Efficiency, RCG = Relative Cell Growth, CV = Crystal Violet, na = not applicable.

6.3.2.4 Concurrent cytotoxicity: Colony Forming Efficiency

Cytotoxicity of anthracene was evaluated by CFE assessment in all laboratories (Figure 19) and was non cytotoxic at the concentrations tested (up to the limits of solubility). HRI curve is shifted to the right because water instead of DMSO has been used as solvent. For the DMSO the upper limit was 40-45 µg/ml for ECVAM and Harlan CCR due to precipitation of the compounds into the medium, while HRI tested up to 5000 µg/ml. In none of the conditions cytotoxicity was reached.

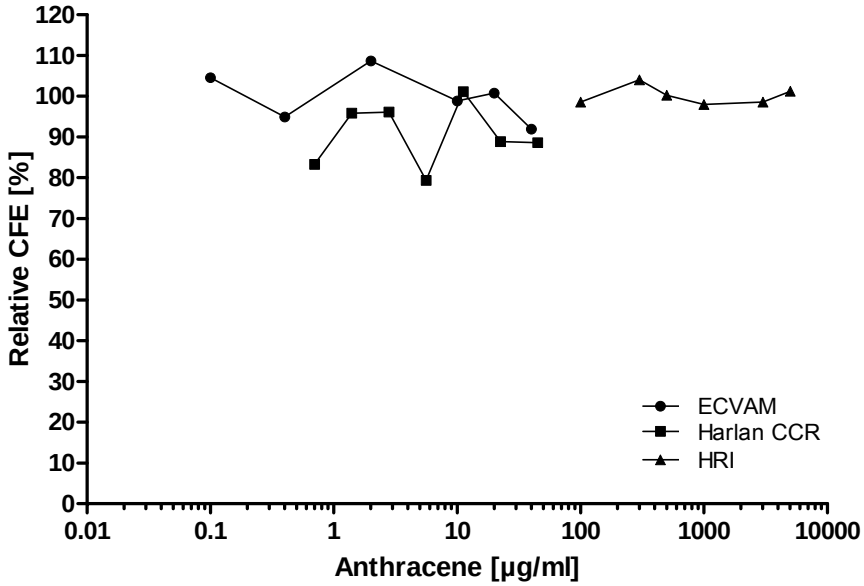

Figure 19: Relative Colony Forming Efficiency (CFE) compared to vehicle control in all laboratories testing coded anthracene

6.3.2.5 Concurrent cytotoxicity: Crystal Violet

Cytotoxicity of anthracene was evaluated by CV in all laboratories (Figure 20). HRI curve is shifted to the right because water instead of DMSO has been used as solvent.

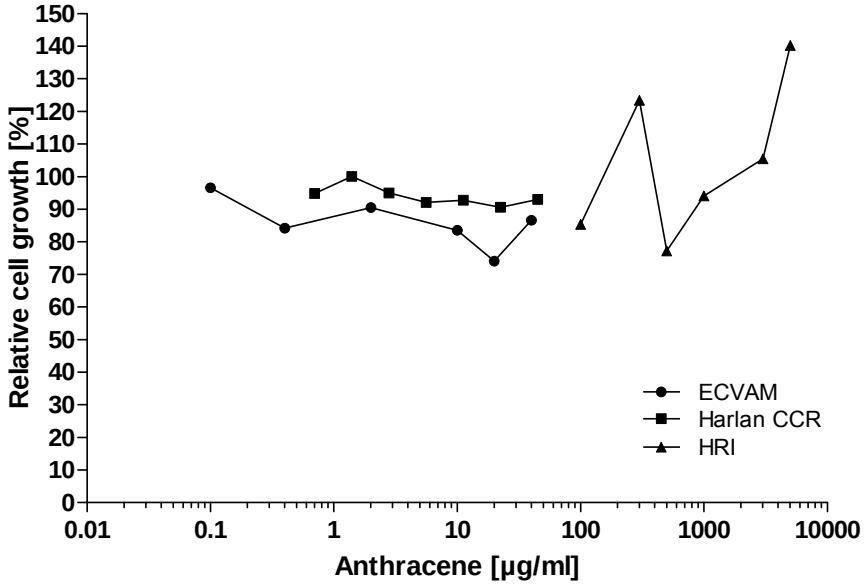

Figure 20: Relative cell growth compared to vehicle control in all laboratories testing coded anthracene

6.3.2.6 Morphological transformation

MT results with anthracene are shown in Figure 21. A negative response was observed in all laboratories. HRI curve is shifted to the right because water instead of DMSO has been used as solvent.

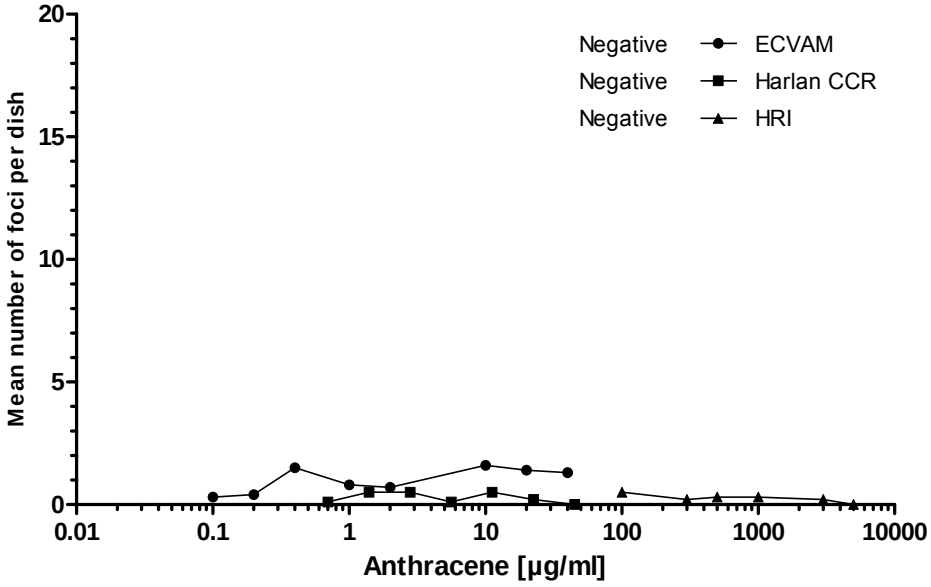

Figure 21: Mean number of foci per dish for all laboratories testing coded anthracene.
Results of the negative binomial statistical analysis are shown. Negative = no downturn-protected Williams contrast resulted in a statistically significant *p*-value for any laboratory. The assessment criteria are described in section 3.6.7.

MT results for the different control treatments are summarised in Figure 22. The untreated, vehicle and positive controls were reproducible across the laboratories.

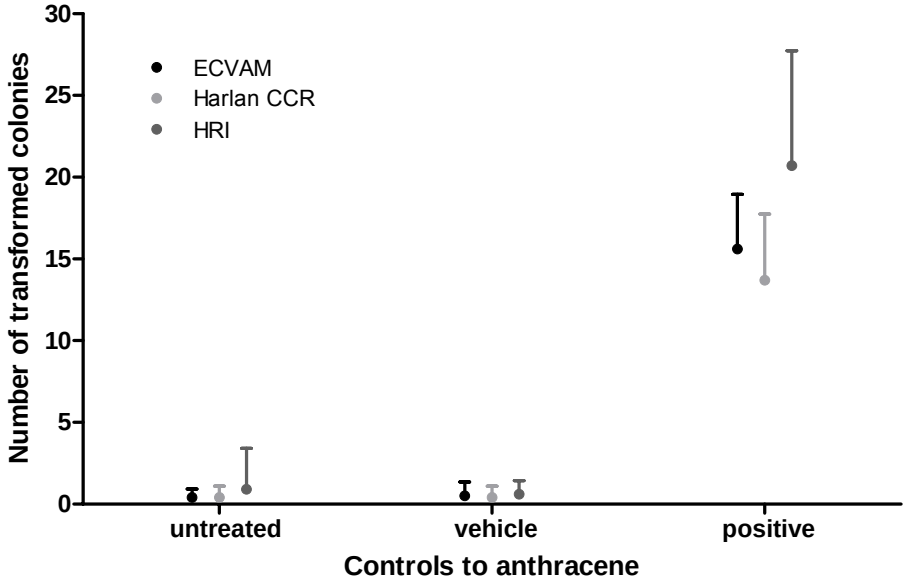

Figure 22: Number of transformed colonies (mean + standard deviation) for untreated, vehicle and positive controls in all laboratories testing coded anthracene

6.3.2.7 Acceptance and assessment criteria

For ECVAM and Harlan CCR, all acceptance criteria were fulfilled. For HRI, all acceptance criteria were met except that the number of transformed foci in the VC was slightly above the limit: six foci were observed instead of five as stated by the acceptability criteria (Table 21). Since the number of foci in the treatment groups was within the VC range, the data were accepted and a negative call was assigned.

Table 21: Acceptance criteria and assessment of anthracene results

Anthracene				
Criteria		**Laboratory**		
		ECVAM	**Harlan CCR**	**HRI**
CFE	1 NOEL [µg/ml]	**yes** (40 = max. conc.)	**yes** (45 = max. conc.)	**yes** (5000 = max. conc.)
	PE of untreated control	63.4	38.1	62.4
	PE of vehicle control >30%	**yes** (63.6)	**yes** (44.9)	**yes** (62.0)
MT	Vehicle used	DMSO	DMSO	water
	Number of foci of vehicle control <6	**yes** (5)	**yes** (4)	**no** (6)
	Number of analysable concentrations ≥6	**yes** (8)	**yes** (7)	**yes** (6)
Fulfilment of all assay acceptance criteria		**YES**	**YES**	**NO**[#]
Assessment	Concentrations in the contrast [µg/ml]*	na	na	na
	Call	-	-	-

CFE = Colony Forming Efficiency, MT = Morphological Transformation, NOEL = No Observed Effect Level, PE = Plating Efficiency, na = not applicable.

[#] considered acceptable for overall study evaluation

* The assessment criteria are described in section 3.6.7.

6.3.3 Conclusion

Treatment with anthracene produced negative response in morphologically transformed foci in all laboratories.

It can be concluded that, according to the assessment criteria established for this study, the experiments were reproducible in all laboratories, and that anthracene was considered to be a non transforming agent in the Balb/c 3T3 CTA. These results agree with published data (Heidelberger *et al.,* 1983).

6.4 Phenanthrene

6.4.1 Dose-range finding test

Figure 23 shows the results of the DRF tests with phenanthrene. The DRF experiments show that phenanthrene induced cytotoxicity in a very narrow concentration range, in all laboratories. ECVAM laboratory performed the DRF twice.

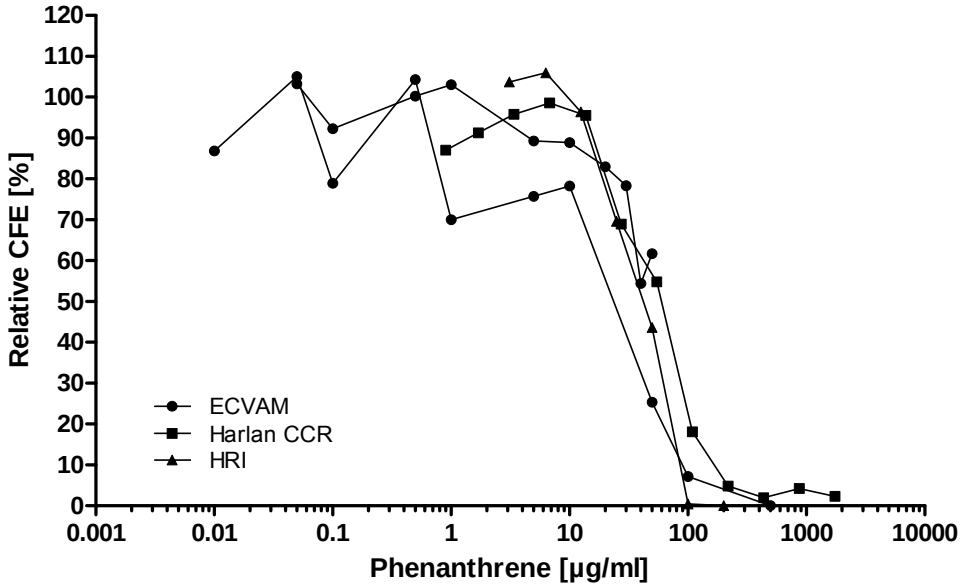

Figure 23: Relative Colony Forming Efficiency (CFE) compared to vehicle control in all laboratories testing coded phenanthrene, for the Dose-Range Finding. ECVAM repeated the experiment.

6.4.2 Transformation assay

The following tables and figures show the MT and concurrent cytotoxicity test results upon treatment with phenanthrene at the test concentrations which were selected based on solubility and DRF tests.

6.4.2.1 ECVAM

Phenanthrene was dissolved in DMSO. The concentrations were selected on the basis of the DRF results and evaluated by ECVAM (Table 22).

Table 22: Transformation assay results from ECVAM (1st experiment), testing coded phenanthrene

ECVAM Phenanthrene (µg/ml) TA1	rCFE (%)	RCG (%) CV	Total number of foci	Mean number of foci per dish
Untreated control	na	na	4	0.40
VC (DMSO 0.5%)	100	100	4	0.40
0.01	nd	nd	0	0
0.1	106.5	103.0	5	0.50
1	nd	nd	7	0.70
10	85.7	91.3	4	0.40
20	76.3	84.8	1	0.10
30	59.1	64.5	5	0.50
50	19.2	9.9	22	2.20
75	4.2	1.3	0	0
PC	2.9	14.9	114	11.40

TA1 = Transformation Assay 1, VC = Vehicle Control, PC = Positive Control, rCFE = relative Colony Forming Efficiency, RCG = Relative Cell Growth, CV = Crystal Violet, na = not applicable, nd = not determined

According to the statistical analysis the assessment of phenanthrene for the ECVAM laboratory was negative. However, an increase in the number of transformed foci was observed at the highest testable dose (50 µg/ml), suggesting that phenanthrene could have a transforming potential. To verify this suspicion the VMT requested the laboratory to retest the same chemical (TA2) focusing on more closely spaced concentrations (Table 23). A retest with this chemical showed a statistically significant positive response in morphological transformation.

Table 23: Transformation assay results from ECVAM (2nd experiment), testing coded phenanthrene

ECVAM Phenanthrene (µg/ml) TA2	rCFE (%)	RCG (%) CV	Total number of foci	Mean number of foci per dish
Untreated control	na	na	4	0.40
VC (DMSO 0.5%)	100	100	6	0.60
10	85.1	86.7	2	0.20
20	nd	nd	4	0.40
30	62.2	90.5	7	0.70
40	42.3	75.1	16	1.60
45	17.7	49.2	4	0.40
50	14.1	32.2	16	1.60
60	**nd**	**nd**	**32**	**3.20**
75	**0.0**	**11.7**	**80**	**8.00**
PC	25.7	41.8	161	16.10

TA2 = Transformation Assay 2, VC = Vehicle Control, PC = Positive Control, rCFE = relative Colony Forming Efficiency, RCG = Relative Cell Growth, CV = Crystal Violet, na = not applicable, nd = not determined
Concentrations that contributed to the statistically significant result, *i.e.* which were present in the downturn-protected Williams contrast that resulted in the lowest *p*-value for a positive response (negative binomial analysis), and related values are highlighted in bold.

6.4.2.2 Harlan CCR

Phenanthrene was dissolved in DMSO. The concentrations were selected and evaluated by Harlan CCR on the basis of the DRF results (Table 24).

Table 24: Transformation assay results from Harlan CCR (1st experiment), testing coded phenanthrene

Harlan CCR Phenanthrene (µg/ml) TA1	rCFE (%)	RCG (%) CV	Total number of foci	Mean number of foci per dish
Untreated control	na	na	2	0.20
VC (DMSO 0.5%)	100	100	3	0.30
1	102.0	105.4	1	0.10
2.5	88.2	105.9	1	0.10
5	89.7	106.8	2	0.20
7.5	118.1	99.1	4	0.40
10	86.8	95.8	2	0.20
30	52.9	74.8	5	0.50
60	**22.5**	**38.7**	**28**	**2.80**
100	**1.0**	**15.0**	**74**	**7.40**
PC	2.9	18.3	115	11.50

TA1 = Transformation Assay 1, VC = Vehicle Control, PC = Positive Control, rCFE = relative Colony Forming Efficiency, RCG = Relative Cell Growth, CV = Crystal Violet, na = not applicable.
Concentrations that contributed to the statistically significant result, *i.e.* which were present in the downturn-protected Williams contrast that resulted in the lowest p-value for a positive response (negative binomial analysis), and related values are highlighted in bold.

Although, the first experiment did not fulfil one of the assay acceptance criteria (PE <30%), the data showed a statistically significant positive response at the two highest dose levels. As such, the VMT considered the test to be acceptable. The VMT requested a repeat study to confirm these results (Table 25). A retest with phenanthrene, in which all of the assay acceptance criteria were fulfilled, confirmed this result, showing a statistically significant positive response in morphological transformation.

Table 25 : Transformation assay results from Harlan CCR (2nd experiment), testing coded phenanthrene

Harlan CCR Phenanthrene (µg/ml) TA2	rCFE (%)	RCG (%) CV	Total number of foci	Mean number of foci per dish
Untreated control	na	na	3	0.30
VC (DMSO 0.5%)	100	100	1	0.10
10	**86.2**	**101.2**	**3**	**0.30**
30	**77.1**	**79.5**	**2**	**0.20**
40	**47.1**	**53.6**	**14**	**1.56**
50	**30.3**	**30.0**	**15**	**1.50**
60	**12.8**	**20.7**	**17**	**1.70**
70	**3.4**	**12.5**	**28**	**2.80**
80	**1.7**	**14.8**	**39**	**3.90**
90	**1.0**	**15.29**	**48**	**4.80**
100	**0.7**	**16.4**	**51**	**5.10**
PC	0.7	15.8	136	13.60

TA2 = Transformation Assay 2, VC = Vehicle Control, PC = Positive Control, rCFE = relative Colony Forming Efficiency, RCG = Relative Cell Growth, CV = Crystal Violet, na = not applicable.
Concentrations that contributed to the statistically significant result, *i.e.* which were present in the downturn-protected Williams contrast that resulted in the lowest *p*-value for a positive response (negative binomial analysis), and related values are highlighted in bold.

6.4.2.3 HRI

Phenanthrene was initially dissolved in water (TA1) and according to the statistical analysis the assessment of phenanthrene was negative (see Annex 13.5.1). The VMT asked the laboratory to retest using DMSO which was the solvent originally requested (TA2). The concentrations were selected and evaluated by HRI on the basis of the DRF results (Table 26). A statistically significant positive response in morphological transformation was observed at two concentrations.

Daughter foci were observed in a single dish of the PC, but were not considered during scoring.

Table 26: Transformation assay results from HRI (2nd experiment), testing coded phenanthrene

HRI Phenanthrene (µg/ml) TA2	rCFE (%)	RCG (%) CV	Total number of foci	Mean number of foci per dish
Untreated control	na	na	0	0
VC (DMSO 0.5%)	100	100	0	0
10	108.2	114.9	0	0
15	99.8	105.6	0	0
22	83.5	104.0	0	0
32	68.0	84.1	1	0.10
45	50.6	58.5	1	0.10
70	26.3	38.5	2	0.20
100	**6.8**	**34. 8**	**32**	**3.20**
150	**0.9**	**16.0**	**16**	**1.60**
PC	21.4	36.4	93	10.33

VC = Vehicle Control, PC = Positive Control, rCFE = relative Colony Forming Efficiency, RCG = Relative Cell Growth, CV = Crystal Violet, na = not applicable.
Concentrations that contributed to the statistically significant result, *i.e.* which were present in the downturn-protected Williams contrast that resulted in the lowest *p*-value for a positive response (negative binomial analysis), and related values are highlighted in bold.

6.4.2.4 Concurrent cytoxicity: Colony Forming Efficiency

Cytotoxicity of phenanthrene was evaluated by CFE assessment in all laboratories (Figure 24). Cytotoxicity was induced in very narrow concentration range in all laboratories.

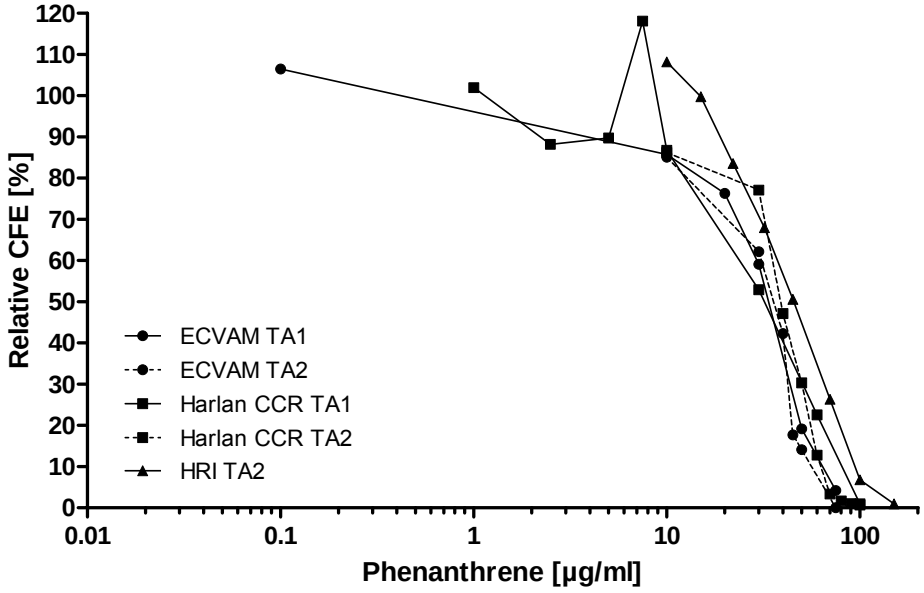

Figure 24: Relative Colony Forming Efficiency (CFE) compared to vehicle control in all laboratories testing coded phenanthrene. ECVAM and Harlan CCR repeated the experiment.

6.4.2.5 Concurrent cytotoxicity: Crystal Violet

Cytotoxicity of phenanthrene was evaluated by the CV method in all laboratories (Figure 25).

The cytotoxicity of phenanthrene measured by the CV method gave similar results to that measured by the CFE method.

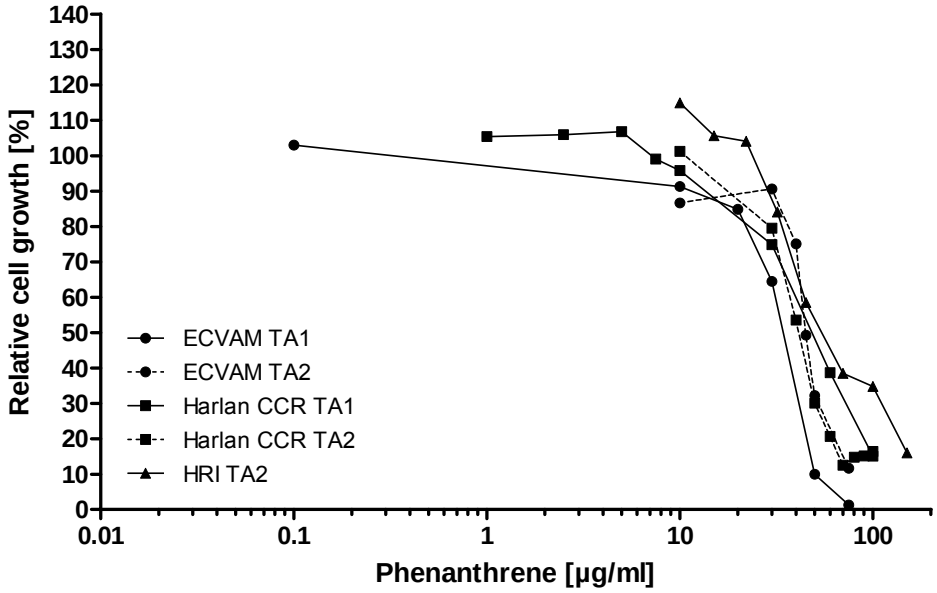

Figure 25: Relative cell growth compared to vehicle control in all laboratories testing coded phenanthrene. ECVAM and Harlan CCR repeated the experiment.

6.4.2.6 Morphological transformation

MT results with phenanthrene are shown in Figure 26.

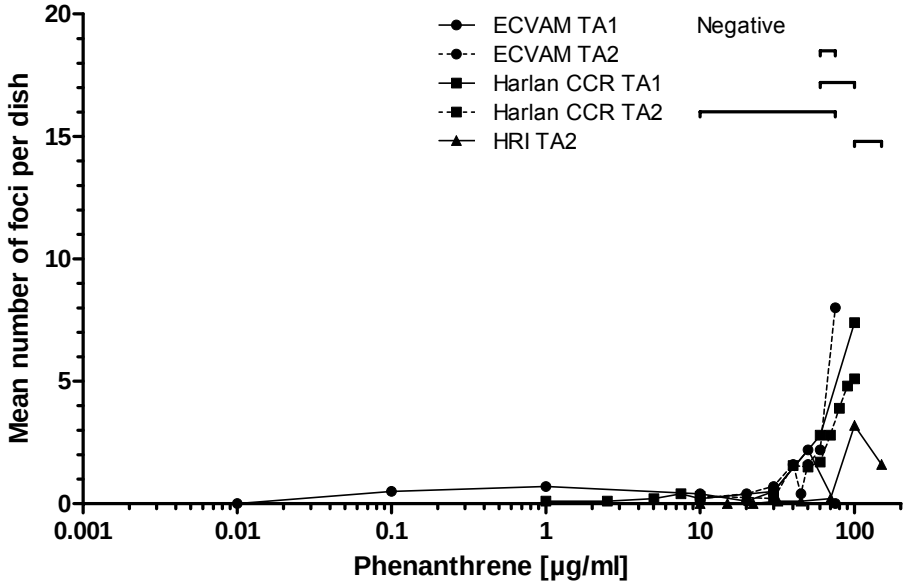

Figure 26: Mean number of foci per dish for all laboratories testing coded phenanthrene. ECVAM and Harlan CCR repeated the experiment.

Results of the negative binomial statistical analysis are shown: for positive assessment, the concentrations that contributed to the statistically significant result, *i.e.* which were present in the downturn-protected Williams contrast that resulted in the lowest *p*-value, are indicated by a horizontal line, next to the corresponding laboratory name in the legend. The assessment criteria are described in section 3.6.7.

MT results for the different control treatments are summarised in Figure 27. The untreated, vehicle and positive controls were reproducible across the laboratories.

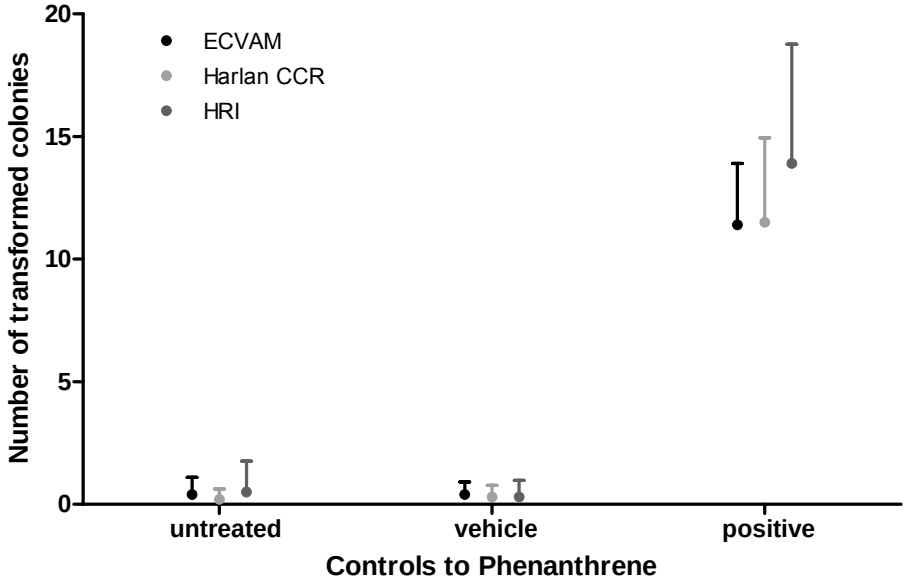

Figure 27: Number of transformed colonies (mean + standard deviation) for untreated, vehicle and positive controls in all laboratories testing coded phenanthrene

6.4.2.7 Acceptance and assessement criteria

Two experiments did not fulfil all the acceptance criteria for phenanthrene TA results (Table 27), however in both cases a clear transforming response was observed and the data were considered acceptable for the overall study evaluation.

Table 27: Acceptance criteria and assessment of phenanthrene results

Phenanthrene						
Criteria		**Laboratory**				
		ECVAM TA1	**ECVAM TA2**	**Harlan CCR TA1**	**Harlan CCR TA2**	**HRI TA2**
CFE	1 NOEL [µg/ml]	**yes** (0.1)	**yes** (10)	**yes** (10)	**yes** (10)	**yes** (15)
	PE of untreated control	36.1	47.9	25.8	38.4	55.8
	PE of vehicle control >30%	**yes** (38.5)	**yes** (45.3)	**no** (25.5)	**yes** (37.1)	**yes** (53.1)
MT	Vehicle used	DMSO	DMSO	DMSO	DMSO	DMSO
	Number of foci in vehicle control < 6	**yes** (4)	**no** (6)	**yes** (3)	**yes** (1)	**yes** (0)
	Number of analysable concentrations ≥6	**yes** (8)	**yes** (8)	**yes** (8)	**yes** (9)	**yes** (8)
Fulfilment of all assay acceptance criteria		**YES**	**NO**[#]	**NO**[#]	**YES**	**YES**
Assessment	Concentrations in the contrast [µg/ml]*	na	60 - 75 (2 conc.)	60 - 100 (2 conc.)	10 - 100 (All conc.)	100 - 150 (2 conc.)
	Call	**-**	**+**	**+**	**+**	**+**

CFE = Colony Forming Efficiency, MT = Morphological Transformation, NOEL = No Observed Effect Level, PE = Plating Efficiency, na = not applicable.

[#] considered acceptable for overall study evaluation.

* for positive assessment, the concentrations that contributed to the statistically significant result, *i.e.* which were present in the downturn-protected Williams contrast that resulted in the lowest *p*-value (negative binomial analysis), are indicated. The assessment criteria are described in section 3.6.7.

6.4.3 Conclusion

Treatment with phenanthrene in the repeated tests performed by ECVAM and in all other studies produced a positive response in morphologically transformed foci. As such, phenanthrene was considered to be a positive transforming agent in the Balb/c 3T3 CTA.

Surprisingly, the reproducible positive call for phenanthrene is in contrast with previously published results (Heidelberger *et al.,* 1983). Although phenanthrene has been classified by IARC as in Group 3 (not classifiable as to its carcinogenicity to humans), there is some evidence that phenanthrene is mutagenic in the Ames test and in human cells in culture when S9 fraction was prepared by Aroclor 1254 induction (Oesch *et al.*, 1981, IARC, 1983) and that may produce papillomas when applied at high doses to mouse skin (Scribner and Suss, 1978).

6.5 o-Toluidine HCl

6.5.1 Dose-range finding test

Figure 28 shows the results of the DRF tests with o-toluidine HCl.

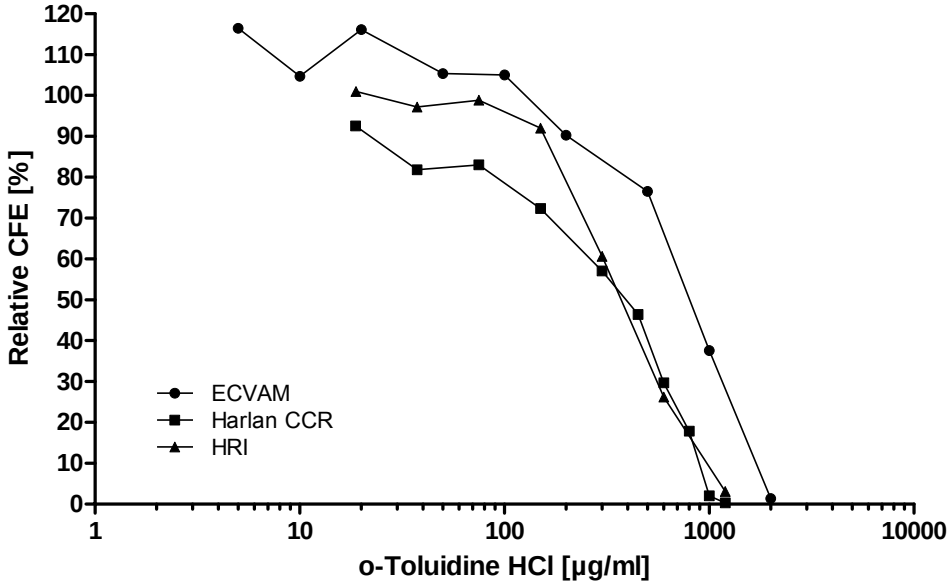

Figure 28: Relative Colony Forming Efficiency (CFE) compared to vehicle control in all laboratories testing coded o-toluidine HCl, for the Dose-Range Finding

6.5.2 Transformation assay

The following tables and figures show the MT and concurrent cytotoxicity test results upon treatment with o-toluidine HCl at the test concentrations which were selected based on solubility and DRF tests.

6.5.2.1 ECVAM

o-Toluidine HCl was dissolved in DMSO. The concentrations were selected on the basis of the DRF experiments and evaluated by ECVAM (Table 28). This chemical induced a statistically significant positive response in morphological transformation.

Table 28: Transformation assay results from ECVAM, testing coded o-toluidine HCl

ECVAM o-Toluidine HCl (µg/ml)	rCFE (%)	RCG (%) CV	Total number of foci	Mean number of foci per dish
Untreated control	na	na	3	0.30
VC (DMSO 0.5%)	100	100	8	0.80
20	nd	nd	5	0.50
100	87.9	111.1	3	0.30
200	nd	nd	4	0.40
500	52.0	103.3	8	0.80
800	22.3	105.1	15	1.50
1000	**10.9**	**57.0**	**35**	**3.50**
1200	**7.1**	**37.6**	**37**	**3.70**
1750	1.9	22.7	26	2.60
PC	11.3	45.0	119	11.90

VC = Vehicle Control, PC = Positive Control, rCFE = relative Colony Forming Efficiency, RCG = Relative Cell Growth, CV = Crystal Violet, na = not applicable, nd = not determined.

Concentrations that contributed to the statistically significant result, *i.e.* which were present in the downturn-protected Williams contrast that resulted in the lowest *p*-value for a positive response (negative binomial analysis), and related values are highlighted in bold.

6.5.2.2 Harlan CCR

o-Toluidine HCl was dissolved in DMSO. The concentrations were selected on the basis of the DRF experiments and evaluated by Harlan CCR (Table 29). This chemical induced a statistically significant positive response in morphological transformation.

Table 29: Transformation assay results from Harlan CCR, testing coded o-toluidine HCl

Harlan CCR o-Toluidine HCl (µg/ml)	rCFE (%)	RCG (%) CV	Total number of foci	Mean number of foci per dish
Untreated control	na	na	5	0.50
VC (DMSO 0.5%)	100	100	3	0.30
5	105.9	99.2	3	0.30
15	101.7	98.6	8	0.80
75	102.5	103.2	4	0.40
150	83.1	105.2	0	0
300	60.1	104.1	3	0.30
450	28.4	78.0	8	0.80
600	**17.7**	**52.5**	**12**	**1.20**
800	**5.6**	**23.1**	**32**	**3.20**
1000	**1.4**	**15.3**	**48**	**4.80**
1200	**2.0**	**12.6**	**52**	**5.20**
PC	11.5	39.9	109	10.90

VC = Vehicle Control, PC = Positive Control, rCFE = relative Colony Forming Efficiency, RCG = Relative Cell Growth, CV = Crystal Violet, na = not applicable.
Concentrations that contributed to the statistically significant result, *i.e.* which were present in the downturn-protected Williams contrast that resulted in the lowest *p*-value for a positive response (negative binomial analysis), and related values are highlighted in bold.

6.5.2.3 HRI

o-Toluidine HCl was initially dissolved in water (TA1) and according to the statistical analysis the assessment of o-toluidine was negative (Annex 13.5.2). The experiment was repeated using DMSO, which was the solvent suggested by the VMT (TA2). The concentrations were selected on the basis of the DRF experiments and evaluated by HRI (Table 30).

Daughter foci were observed in two dishes of the PC, one dish at 500 µg/ml, and one dish at 800 µg/ml, but were not considered during scoring (see section 3.6.5.3 for analysis of the data).

Table 30: Transformation assay results from HRI (2nd experiment), testing coded o-toluidine HCl

HRI o-Toluidine HCl (µg/ml) TA2	rCFE (%)	RCG (%) CV	Total number of foci	Mean number of foci per dish
Untreated control	na	na	1	0.10
VC (DMSO 0.5%)	100	100	0	0
63	106.0	115.5	1	0.10
125	98.1	109.8	0	0
250	91.0	99.5	0	0
500	71.5	82.0	1	0.11
630	48.8	63.9	1	0.10
800	27.1	45.5	2	0.22
1000	17.4	43.6	0	0
1200	4.9	11.2	17	1.70
PC	22.0	37.5	90	11.25

TA1 = Transformation Assay 1, VC = Vehicle Control, PC = Positive Control, rCFE = relative Colony Forming Efficiency, RCG = Relative Cell Growth, CV = Crystal Violet, na = not applicable.

According to the statistical analysis the assessment of o-toluidine HCl for the HRI laboratory was negative. However, an increase in the number of transformed foci was observed at the highest dose tested (1200 µg/ml), suggesting that o-toluidine HCl could have a transforming potential. To verify this suspicion the VMT requested the laboratory to retest the same chemical (TA3) focusing on more closely spaced concentrations (Table 31). A retest with o-toluidine confirmed this result, showing a statistically significant positive response in morphological transformation.

Table 31: Transformation assay results from HRI (3rd experiment), testing coded o-toluidine HCl

HRI o-Toluidine HCl (µg/ml) TA3	rCFE (%)	RCG (%) CV	Total number of foci	Mean number of foci per dish
Untreated control	na	na	3	0.30
VC (DMSO 0.5%)	100	100	0	0
100	95.5	95.7	1	0.10
200	93.4	82.6	0	0
400	58.5	42.8	1	0.10
600	36.2	20.7	2	0.20
800	**24.4**	**15.3**	**15**	**1.50**
900	**18.0**	**15.7**	**27**	**2.70**
1000	**14.3**	**11.4**	**27**	**2.70**
1100	**12.2**	**9.8**	**29**	**2.90**
1200	**7.9**	**4.1**	**20**	**2.00**
PC	30.0	9.8	161	16.10

TA2 = Transformation Assay 2, VC = Vehicle Control, PC = Positive Control, CFE = Colony Forming Efficiency, RCG = Relative Cell Growth, CV = Crystal Violet, na = not applicable.
Concentrations that contributed to the statistically significant result, *i.e.* which were present in the downturn-protected Williams contrast that resulted in the lowest *p*-value for a positive response (negative binomial analysis), and related values are highlighted in bold.

6.5.2.4 Concurrent cytotoxicity: Colony Forming Efficiency

Cytotoxicity of o-toluidine HCl was evaluated by CFE assessment in all laboratories (Figure 29).

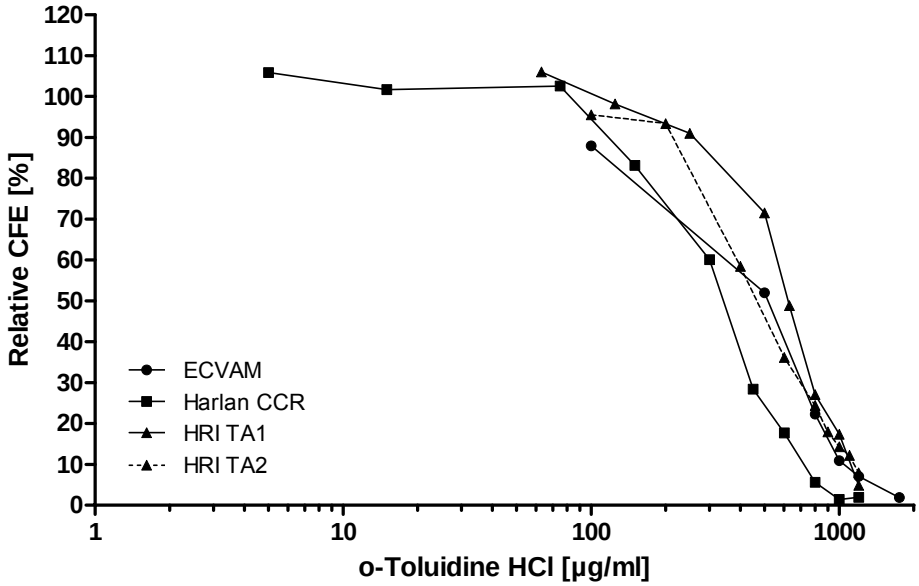

Figure 29: Relative Colony Forming Efficiency (CFE) compared to vehicle control in all laboratories testing coded o-toluidine HCl. HRI repeated the experiment.

6.5.2.5 Concurrent cytotoxicity: Crystal Violet

Cytotoxicity of o-toluidine HCl was evaluated by the CV method in all laboratories (Figure 30).
Due to the quantitative variability of the responses in the above experiments, it was difficult to clearly compare CFE with CV results.

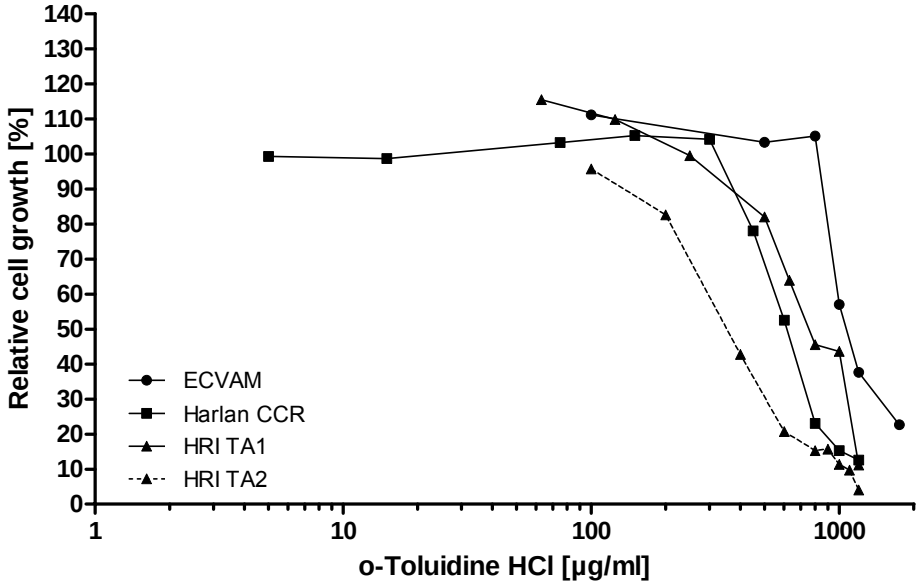

Figure 30: Relative cell growth compared to vehicle control in all laboratories testing coded o-toluidine HCl. HRI repeated the experiment.

6.5.2.6 Morphological transformation

MT results with o-toluidine HCl are shown in Figure 31. The results showed that o-toluidine induced an increase in the number of transformed foci in a narrow concentration range in all laboratories.

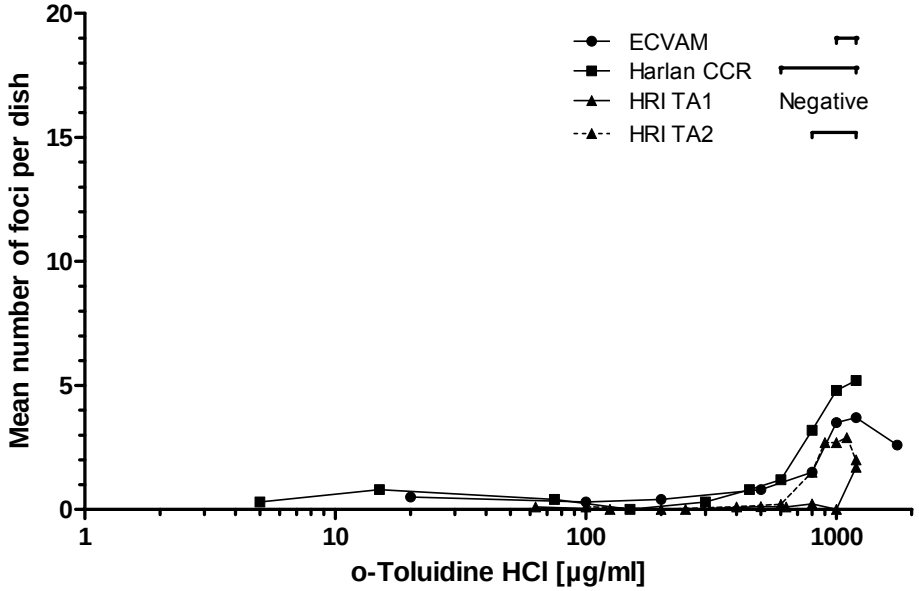

Figure 31: Mean number of foci per dish for all laboratories testing coded o-toluidine HCl. HRI repeated the experiment.
Results of the negative binomial statistical analysis are shown. For positive assessment, the concentrations that contributed to the statistically significant result, *i.e.* which were present in the downturn-protected Williams contrast that resulted in the lowest *p*-value, are indicated by a horizontal line, next to the corresponding laboratory name in the legend. Negative = No downturn-protected Williams contrast resulted in a statistically significant *p*-value in the corresponding laboratory. The assessment criteria are described in section 3.6.7.

MT results for the different control treatments are summarised in Figure 32. The untreated, vehicle and positive controls were reproducible across the laboratories.

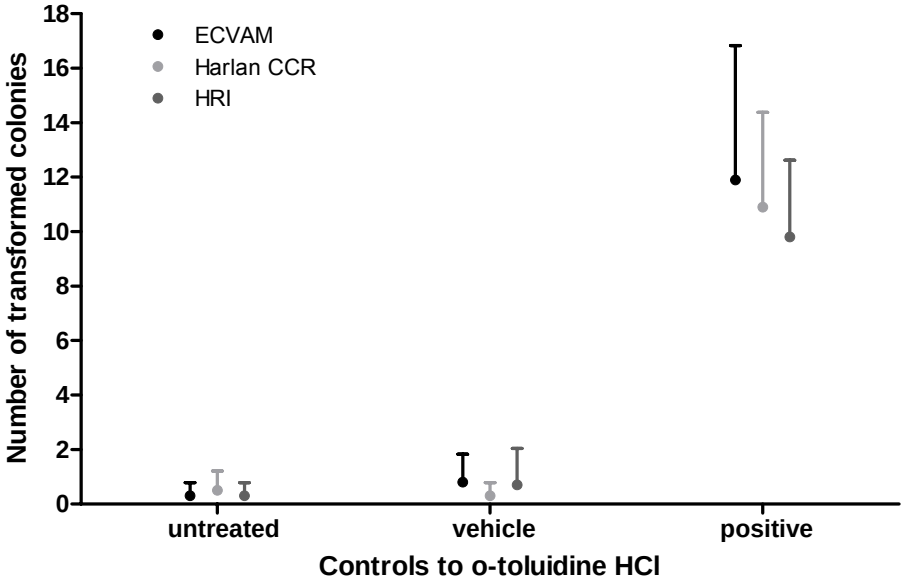

Figure 32: Number of transformed colonies (mean + standard deviation) for untreated, vehicle and positive controls in all laboratories testing coded o-toluidine HCl

6.5.2.7 Acceptance and assessment criteria

Harlan CCR and HRI experiments fulfilled all the assay acceptance criteria for o-toluidine HCl TA results, while the ECVAM experiment did not fulfil the criterion for the number of foci in the VC (Table 32). The ECVAM experiment was still considered acceptable by the VMT for the overall evaluation since a clear positive response was observed.

Table 32: Acceptance criteria and assessment of o-toluidine HCl results

o-Toluidine HCl					
Criteria		**Laboratory**			
		ECVAM	**Harlan CCR**	**HRI TA2**	**HRI TA3**
CFE	1 NOEL [µg/ml]	**yes** (100)	**yes** (75)	**yes** (250)	**yes** (200)
	PE of untreated control	67.0	44.8	54.9	58.6
	PE of vehicle control >30%	**yes** (65.1)	**yes** (44.5)	**yes** (54.0)	**yes** (58.4)
MT	Vehicle used	DMSO	DMSO	DMSO	DMSO
	Number of foci of vehicle control < 6	**no** (8)	**yes** (3)	**yes** (0)	**yes** (0)
	Number of analysable concentrations ≥6	**yes** (8)	**yes** (10)	**yes** (8)	**yes** (9)
Fulfilment of all assay acceptance criteria		**NO**[#]	**YES**	**YES**	**YES**
Assessment	Concentrations in the contrast [µg/ml]*	1000-1200 (2 conc.)	600-1200 (4 conc.)	na	800-1200 (5 conc.)
	Call	**+**	**+**	**-**	**+**

CFE = Colony Forming Efficiency, MT = Morphological Transformation, NOEL = No Observed Effect Level, PE = Plating Efficiency, na = not applicable.
[#] considered acceptable for overall study evaluation.
* for positive assessment, the concentrations that contributed to the statistically significant result, *i.e.* which were present in the downturn-protected Williams contrast that resulted in the lowest *p*-value (negative binomial analysis), are indicated. The assessment criteria are described in section 3.6.7.

6.5.3 Conclusion

Treatment with o-toluidine HCl in the repeated tests of HRI and in the other studies conducted by the other participating laboratories produced a positive response in morphologically transformed foci. As such, o-toluidine HCl was considered to be a positive transforming agent in the Balb/c 3T3 CTA. These results agree with published data (Matthews *et al.*, 1993c).

6.6 Distributions of all experimental controls

The negative and vehicle controls gave in general levels of transformed foci within the acceptable range expected for Balb/c 3T3 cells. The average number of foci was 0.3 ± 0.2 in the negative controls and 0.4 ± 0.3 in the VCs. The average number of transformed foci per experiment was less than 1.0 per dish, except in one experiment where it was 1.2 ± 1.8. However, in four experiments the number of transformed foci in the VC exceeded five, which was the acceptance criterion. A revision of this acceptance criterion for future work is recommended.

The PC chemical 3-methylcholanthrene (at 4 µg/ml) always induced a positive response. The average focus number was 14.7 ± 4.0. The average number of transformed foci in each experiment was always higher than 10 per dish. At the beginning of this study no acceptance criteria for a PC were established. Based on the data collected in this study, additional criteria for the acceptability of the PC might be recommended for future application of the Balb/c 3T3 CTA.

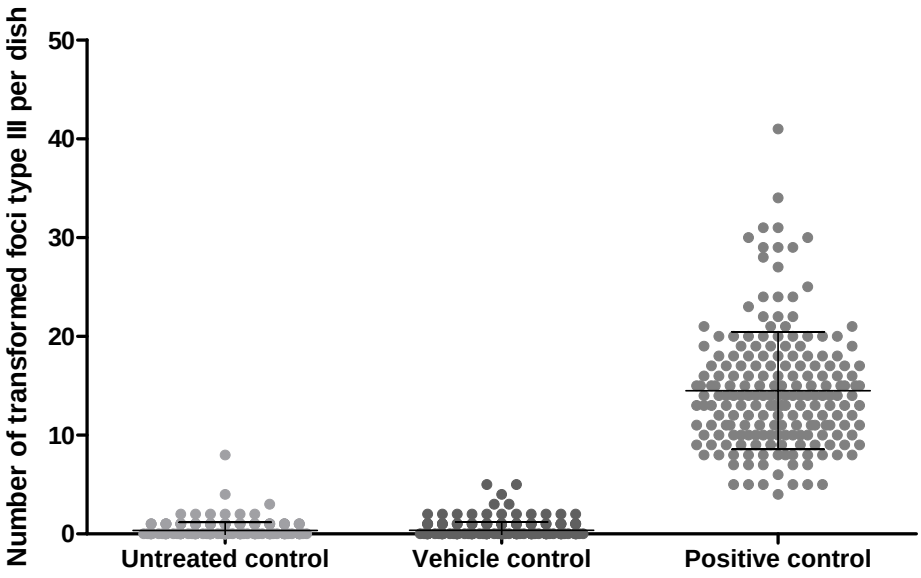

Figure 33: Untreated, vehicle and positive controls for all experiments in all laboratories for cell transformation

6.7 Comparison of CFE and CV methods for cytotoxicity assessment

In this study, the appropriate dose-range for the TA was based on the data produced by the CFE, which is the principal cytotoxicity method that has been used in published studies. In order to investigate whether the CV cytotoxicity method was also suitable to identify the appropriate doses for the TA, the CV was run in parallel with the CFE during the TA and the IC_{50} obtained with both methods were compared. The CV method is less laborious than the CFE method and uses a more similar cell density (350 cells/cm^2) to the TA (500 cells/cm^2). The CFE test uses a much lower cell density than the TA (approximately 10 cells/cm^2) and it was thought this may influence the measure of cytotoxicity.

When the results from the two methods were compared, a larger difference between the two cytotoxicity measures was observed for 3-methylcholanthrene and benzo(a)pyrene (Table 33). Although the CFE test has a tendency to be more sensitive than the CV test (based on the IC_{50}), this was not always the case.

Moreover, we compared the different cytotoxicity measures assessed with CFE and CV in the TA experiments. This qualitative analysis suggests that if the doses would have been chosen based on the CV method alone, the range of concentrations tested according to the protocol would have included the concentrations that contributed to the positive results in the present study.

No clear conclusions on the difference between the two methods can be drawn from the data produced in this study and further studies in this area are needed to determine whether CV is an appropriate alternative to CFE.

Table 33: Summary table of IC_{50} values measured by CFE and CV

Chemical	Laboratory	CFE IC_{50} (µg/ml)	CV IC_{50} (µg/ml)
3-Methylcholanthrene	ECVAM	0.063	0.016
	Harlan CCR	0.037	0.11
	HRI	0.036	0.52
2-Acetylaminofluorene	ECVAM	16	20
	Harlan CCR	14	14
	HRI	37	25
Benzo(a)pyrene	ECVAM	0.0066	0.047
	Harlan CCR	0.015	0.060
	HRI	0.014	0.019
Anthracene	ECVAM	na	na
	Harlan CCR	na	na
	HRI	na	na
Phenanthrene	ECVAM	31	33
	ECVAM (TA2)	34	45
	Harlan CCR (TA1)	32	49
	Harlan CCR (TA2)	39	42
	HRI	44	63
o-Toluidine HCl	ECVAM	506	1126
	Harlan CCR	329	622
	HRI (TA1)	624	785
	HRI (TA2)	480	366

na = not applicable because compound was not toxic.

6.8 Conclusion of the Validation Management Team on between-laboratory reproducibility results

The between-laboratory reproducibility was shown to be satisfactory when the repeated experiments were taken into consideration. Two of the experiments required repetition because a biological effect was observed although the statistical analysis was negative.

Overall, according to the assessment criteria established for this study and considering the repeated experiments, all chemicals tested (except 2-acetylaminofluorene that produced an inconclusive result in one laboratory and could not be repeated) as well as vehicle and positive controls gave reproducible results in the three laboratories.

7 Summary of results

An optimised Balb/c 3T3 CTA protocol was produced and agreed upon by the different laboratories during the initial phase of the 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 foci morphology and in scoring experimental results. Using the improved protocol and providing that repeated tests were considered, test reproducibility was shown to be satisfactory within as well as between laboratories. Table 34 summarises the results obtained with each chemical.

Table 34: **Summary table of the reproducibility results.** The assessment was made using the negative binomial statistical approach, $p < 0.01$.

Chemical	Expected result[#]	Laboratory						
		ECVAM		Harlan CCR		HRI		
		TA1	TA2	TA1	TA2	TA1	TA2	TA3
3-Methylcholanthrene	+	+ [$]	+	+ [$]	+	+ [$]	+	
2-Acetylaminofluorene	+	+		i *		+		
Benzo(a)pyrene	+	+		+		+		
Anthracene	-	-		-		-		
Phenanthrene	-	- **	+	+	+	nc	+	
o-Toluidine HCl	+	+		+		nc	- **	+

[#] Based on previous results from the litereature (see sections 2.4.1.1 and 2.4.1.2)
i = inconclusive call; + = positive call; - = negative call; nc = not considered.
[$] Chemical tested non coded in module 2
* Assessment was reported inconclusive since the downturn-protected Williams contrast with the lowest *p*-value showed a *p*-value < 0.01 but included only one concentration (30 µg/ml). This study should have been repeated. See section 6.1.2.2 for explanation.
** An increase in the number of foci was observed at the highest dose tested, but the result was considered negative according to the assessment criteria.

7.1 3-Methylcholanthrene

The results of 3-methylcholanthrene, tested as a coded and a non-coded chemical were reproducible within and between the laboratories. Moreover, the within- and between-laboratory reproducibility of 3-methylcholanthrene was demonstrated when this chemical was used as PC in the different experiments.

7.2 2-Acetylaminofluorene

According to the assessment criteria established for this validation study, the results were reproducible in two laboratories and an experiment was found inconclusive in one laboratory. 2-Acetylaminofluorene was considered to be a positive transforming agent in the Balb/c 3T3 CTA, which was the expected result.

Since the assessment criteria for a positive call required two concentrations to be in the downturn-protected Williams contrast with the lowest *p*-value, the resulting call for Harlan CCR was inconclusive and should have been repeated using a narrower concentration range around the positive concentration. However, Harlan CCR could not repeat the experiment due to time restrictions and other commitments.

7.3 Benzo(a)pyrene

The final results of benzo(a)pyrene were reproducible between the laboratories and benzo(a)pyrene was shown to be a positive transforming agent in the Balb/c 3T3 CTA, which was the expected result.

7.4 Anthracene

The final results of anthracene were reproducible between the laboratories and anthracene was shown to be negative in the Balb/c 3T3 CTA, which was the expected result.

7.5 Phenanthrene

The results of phenanthrene were reproducible in all laboratories when repeated tests were considered. All laboratories showed a reproducible dose-dependent increase in the number of transformed foci and the statistical analysis showed phenanthrene to be positive in all laboratories. Surprisingly, the positive call for phenanthrene is in contrast with previously published results.

The IARC classification of phenanthrene is Group 3, not classifiable as to its carcinogenicity to humans (IARC, 1983). In spite of this, there is some evidence that phenanthrene is mutagenic in the Ames test and in human cells in culture when S9 fraction was prepared by Aroclor 1254 induction (Oesch *et al.*, 1981, IARC, 1983). It also induced sister chromatid exchange in Chinese hamster bone-marrow cells *in vivo* (IARC, 1983). Moreover, Scribner and Suss (1978) described that papillomas were obtained when applying phenantrene at high doses to mouse skin. In the present study all three laboratories produced positive calls at doses above 50 µg/ml (but still within acceptable cytotoxicity ranges). The data reported in the literature resulted from using lower concentrations, which may explain the CTA results reported herein.

7.6 o-Toluidine HCl

The experiments were reproducible in all laboratories and statistically significant MT was seen in all laboratories above 800 µg/ml of o-toluidine HCl when the repeated tests were considered. It can be concluded that, o-toluidine HCl was shown to be a positive transforming agent in the Balb/c 3T3 CTA, which was the expected result.

8 Discussion

In the present study some experiments did not fulfil all acceptance criteria, ideally triggering their repetition. However for reason of costs, resources and time needed for the conduct of the CTA, the repetition of experiments was kept at a minimum. Justifications for considering the experiments that did not fulfil all acceptance criteria were given and were based on biological relevance, such as the presence of a clear transforming dose-response.

For uniformity in testing, all the laboratories were requested by the VMT to dissolve their compounds in DMSO. This recommendation was not initially adopted in all cases and in those situations the compounds had been dissolved in water. In some of those cases, a different outcome was obtained. It is possible that the bioavailability of compounds dissolved in DMSO may be increased, explaining the different results obtained. Water causes a shift in cytotoxicity influencing the choice of the dose range used in the CTA. This variance in outcome depending on the solvent used suggests that further care should be taken that the same solvent is used when comparing data from different studies. It also emphasizes the fact that the choice of solvent is critical to the outcome of a given assay.

The Balb/c 3T3 prevalidation results clearly demonstrated that it is critical to test the correct dose range in order to produce the accurate results. This is especially important for chemicals that show a cytotoxic response in a very narrow concentration range.

The training of laboratory personnel is important to ensure consistency in assessing the morphology of foci both within a given laboratory and between laboratories. Moreover, the photo catalogue proved to be an invaluable aid during the scoring. Overall, the results produced in this study demonstrated that if the laboratories are well trained, the manual scoring of foci and the potential subjectivity in identifying transformed foci are not problematic issues.

The CFE test has a tendency to be more sensitive than the CV test in some cases and a larger difference between the two cytotoxicity measures (based on IC_{50} values) was observed for 3-methylcholanthrene and benzo(a)pyrene. The results presented here and historical data from the literature were based on the use of CFE for dose selection. It can be noted that in the present study the concentrations that contributed to the statistically significant results for the positive chemicals would also have been tested if the DRF had been based on the cytotoxicity values obtained with CV instead of CFE. However, the limited dataset generated in the present study doesn't allow a conclusion to be made on the equivalence or superiority of one or the other method and further studies are needed to address this important aspect.

The data produced during this study suggest that the acceptance criteria initially established were not always appropriate for the conduct of this protocol. In some experiments the following criteria were not fulfilled: the number of foci in the VC (exceeded five foci in four experiments), the use of a NOEL concentration (one experiment), the PE (less than 30% in two experiments), the number of analysable concentrations (one experiment). From the data collected, it is clear that under these experimental conditions the acceptable number of foci in the VC needs to be increased, but there are insufficient data to define what the limit should be. In most cases the effect of a slight increase in the Type III foci in the VC will be only marginal and does not affect the overall call when there is a large response with a test article. Moreover, it should be noted that in the case of a clear positive call with a statistically significant dose-dependent increase of foci, experiments were not repeated in the present study. In fact, seven experiments which did not fulfil one of the assay acceptance criteria each showed a clear positive response and as such the chemicals were considered to be positive transforming agents.

Various statistical approaches had been used in the past to evaluate the Balb/c 3T3 CTA data, while their appropriateness has never been assessed in detail. Consequently the VMT (at the meeting of May 2006) recommended that ECVAM organise an *ad hoc* expert group to evaluate the CTA data and the various related statistical methods previously employed and to come up with a recommendation on the

most suitable statistical method to use for analysing data typically generated with this assay (see section 3.6.5). The experts concluded that none of the statistical methods used in the past were appropriate for this kind of analysis and proposed using an analysis based on the so-called Nishiyama transformation and an approach based on the negative binomial distribution to evaluate the data produced in this study. From a statistical point of view, the approach based on the negative binomial distribution seemed more appropriate. However due to the limited amount of data produced in this study it is premature to draw conclusions on whether the assessment criteria based on this approach provide the properties required for the analysis of the CTA. Further use of this statistical approach will allow for the generation of more data with which to evaluate its relevance and applicability.

When a single concentration induced an increase in transformed foci but the statistical analysis indicated the result was negative, repetition of the experiment with additional concentrations that bracketed the concentration yielding the increase in foci resulted in a clear positive result. This occurred in two experiments where an increase in transformed foci was observed at the highest concentration only, although it was not significant as demonstrated by the statistical approach used. In both cases the repetition of the experiments proved that the chemicals tested had indeed a transforming effect. Based on this observation, it is recommended that when an increase in foci is observed at a single concentration, a repeat study is conducted with more doses covering the concentration range where the increase was observed.

As for any other statistical approach, the choice of the *p*-value defines the statistical significance of the results. As the significance is directly linked to the interpretation (significant = positive; not significant = negative), the *p*-value impacts on the sensitivity and specificity of the method. Since it was the first time that the proposed statistical method was used for the given assay, the data generated were used to start to empirically derive a suitable *p*-value. Based on the very limited set of chemicals from this prevalidation study there are too few carcinogens and non-carcinogens to define an appropriate *p*-value for an optimised balance between sensitivity and specificity. Therefore, it is recommended that further formal evaluation of the performance of the new proposed statistical method, including an optimisation of the *p*-value that should be used as a decision criterion, be performed.

At the beginning of this prevalidation study no acceptance criteria for a PC had not been established due to the lack of historical data produced with the same optimised and agreed upon protocol used in this study. Since the formal definition of an acceptable range of transformed foci for the PC is an essential part of every assay, based on the data collected in this study, it is recommended that such a criterion for the PC be defined and incorporated into the protocol for future Balb/c 3T3 CTA studies.

During the conduct of the study some concerns were raised regarding the formation of “daughter” foci, which represent secondary foci developing from cells that break away from the “parent” focus. Although this is a rare phenomenon, it was suggested that dishes exhibiting such secondary foci not be counted for the statistical analysis.

9 Overall conclusion by the Validation Management Team

The aim of the study was to prevalidate the Balb/c 3T3 CTA, in a formal inter-laboratory study, following the modular approach (Hartung *et al*., 2004) and concentrating on modules 1-4: test definition, within-laboratory reproducibility, transferability and between-laboratory reproducibility. Table 35 summarises the conclusion by the VMT on the assessment of the Balb/c 3T3 CTA.

Table 35: 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 - Protocol available with improvement in the interpretation of data and clearer definition of a valid study (*e.g*. some acceptance and assessment criteria have been refined) - A new statistical approach was applied but needs to be further evaluated	**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**	- Test method is transferable 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, when considering repeated tests **, # Confirmation needed with further testing	**yes** &

* One experiment did not fulfil one of the acceptance criteria but was considered valid by the VMT for the overall evaluation.
** Seven experiments did not fulfil one of the acceptance criteria but were considered valid by the VMT for the overall evaluation since they all showed a statistically significant dose-response effect.
# Between-laboratory reproducibility was initially not considered satisfactory, leading to the revision of the criteria for repetition of a test to achieve reliable results. Two experiments were repeated because a biological effect was observed although the statistical analysis was negative.
& According to revised protocol.

On the basis of the outcome of this prevalidation study, an improved protocol, incorporating the recommendations made by the VMT has been developed. The recommended changes to the protocol relate only to data interpretation (refinement of acceptance and assessment criteria). More experimental data are required to allow for further refinement and evaluation of the statistical method

which may impact future study design (number of plates, concentrations tested, requirements for repeat studies, etc).

If the repeated experiments and the modifications to the data interpretation of the improved protocol are taken into consideration, including the importance of considering biological relevance, it can be concluded that the assay is transferable between laboratories and, reproducible within and between laboratories. Moreover, this study demonstrated that with the appropriate training and the use of the photo catalogue, the scoring of foci was not problematic despite the concerns raised in the past. It is recommended that this improved protocol be used in the future in order to confirm its utility.

Furthermore, although limited, these prevalidation data add to the fifth module *i.e.* predictive capacity, which has been addressed by the OECD DRP evaluation.

10 Recommendations

Taking into account clarifications and modifications introduced into the protocol by the VMT and the participating laboratories, an improved protocol has been generated and it is recommended that this protocol be used in the future to produce new data in order to expand the body of evidence on assay reproducibility, and to address remaining issues described below such as appropriate statistical analysis. Based upon the experience gained from this effort, points that should be taken into consideration in the future conduct of the assay include the following:

- Check that the cell lineage and the culture conditions are suitable to be used in the CTA. This should be done by establishing that the PC induces a clear and reproducible increase and the VC exhibits a low and reproducible response.

- Carefully assess the correct dose range to be used for chemicals that show a cytotoxic response in a very narrow concentration range. In such cases, more closely spaced doses may be required.

- Since there is a certain degree of subjectivity associated with the identification of transformed foci in the Balb/c 3T3 CTA and correct scoring is critical, training is necessary to ensure scoring which is as consistent and objective as possible. The photo catalogue produced during this study has proven to be an invaluable aid in establishing consistency in assessing morphology of the foci 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 protocol be made available to laboratories conducting the Balb/c 3T3 CTA. It is intended that such a photo catalogue will be published by ECVAM in the near future for that purpose.

- An acceptable background of spontaneous foci in the VC could not be defined. Each laboratory needs to develop its own database and criteria for an acceptable number of foci in VC, which should be as low as possible.

- An acceptance criterion for the PC should be added to the protocol. For data that have similar properties to those observed in our study, especially regarding the number of foci in VC and PC, the average number of Type III foci induced by the PC chemical should always exceed 10 per dish. However, the criterion should be adjusted in relation to the historical data of the laboratory. Alternatively, either an approach based on the definition of an absolute number of foci above the VC or a statistical approach may be developed for the PC.

- If in the case of a negative call, one or more acceptance criteria are not fulfilled, the experiment should be repeated. This *proviso* should be added in the recommended protocol.

- An experiment need not be repeated if a clear positive result is obtained even if one of the acceptance criteria is not fulfilled.

- An experiment that does not produce a statistically significant positive response, but shows a marked increase in foci at a single dose needs to be repeated.

- It is recommended that further formal evaluation of the performance of the new proposed method, including an optimisation of the *p*-value that should be used as a decision criterion, be performed.

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13 Annexes

13.1 Chemicals selected for the prevalidation of Balb/c 3T3 CTA

Chemicals were selected based on the genotoxicity and carcinogenicity data compiled by the OECD DRP31 (2007) and Kirkland *et al.* (2005). The *in vitro* genotoxicity, *in vivo* genotoxicity and carcinogenicity characterisation of the selected compounds is reported in Table 36.

Table 36: Genotoxicity and carcinogenicity data on the chemicals selected for the Balb/c 3T3 CTA prevalidation study

Chemical	CAS number	Genotoxic profile *in vitro*				Genotoxic *in vivo*	IARC class	*in vivo* carcinogenicity
		Ames	MLA	MNT	CA			
3-Methylcholanthrene	56-49-5	+	+	+	+	i	nd	+*
2-Acetylaminofluorene	53-96-3	+	+	+	+	+ (gene mutation, MN)	nd	+*
Benzo(a)pyrene	50-32-8	+	+	+	+	+ (gene mutation, MN)	1	+
Anthracene	120-12-7	+/-	+	nd	-	i	3	-
Phenanthrene	85-01-8	+/-	nd	nd	nd	i	3	-
o-Toluidine HCl	636-21-5	+/-	+/-	+/-	+/-	+/-	2A	+

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

13.2 Amended protocol for Phase 2

Pre-validation study on Balb/c 3T3 cell transformation assay to detect carcinogenic potential of chemicals

Phase II

The following Standard Operating Procedures (SOPs) describe the protocols used for the pre- validation of the Balb/c 3T3. SOPs are provided for dose-range findings (cytotoxocity) and for morphological transformation assay induced by chemicals in mouse fibroblast Balb/c 3T3 clone A31-1-1 cell line provided by Hatano Research Laboratory (Japan).
Materials and reagents used and their catalogue numbers are listed in Appendix 1.

This document amends the previous ones.

Following the recommendations of the Validation Management Team during the meeting of the 10^{th}-11^{th} May 2006 in Ispra, minor modifications were made to the original protocols before proceeding to phase 2.

Media, Reagents and Supplements

(Appendix 1 contains all the information regarding the suppliers)

Cell line. Balb/c 3T3 clone A31-1-1 cells (1,2): Mouse fibroblasts, free from bacteria, fungi and mycoplasma, distributed by HRI Cell Bank at the passage number 12.
Culture media: prepared as follows and stored at 4ºC for no longer than 1 month.
The following media, reagents and supplements will be prepared.
All the media and reagents will be labeled with the date of the preparation.
M10F: MEM (Minimum essential medium) + 10% FBS (Fetal Bovine Serum).
M10F is used for the routine passage, the cell storage and the early stage of transformation experiments.
- MEM (500 ml): Minimum essential medium (GIBCO, CAT No.: 11095-080 (Japan), 31095-029 (Europe), liquid, with low glucose (1000 mg/l), L-glutamine (292 mg/l) and sodium bicarbonate (2200 mg/l) is stored at 4ºC.
- FBS (55.5 ml): Fetal bovine serum (Moregate Lot N. 94300107), stored below -20ºC upon arrival, is thawed and added to MEM to reach 10% final concentration of FBS.
- PS (5.5 ml): Penicillin (10000 units/ml) - Streptomycin (10 mg/ml) solution from GIBCO (CAT No.: 15140-122 (Japan and Europe) is stored at -20ºC until needed. 5.5 ml of the solution are added to the culture medium to reach a final concentration of 1%.

DF2I2F: DMEM/F12 (Dulbecco's modified Eagle's medium/F12) + 2 µg/ml insulin + 2% FBS.
DF2I2F is used for the later stage of transformation experiments (3,4).
- DMEM/F12 (500 ml): Dulbecco's modified Eagle's medium/F12 (GIBCO, CAT No.: 11330-032 (Japan), 31330-038 (Europe)), liquid, with high glucose (3151 mg/l), L-glutamine (365 mg/l) and sodium bicarbonate (1200 mg/l) is stored at 4ºC.
- FBS (10.2 ml): Fetal Bovine Serum (Moregate Lot N. 94300107), stored below -20ºC upon arrival, is thawed and added to DMEM/F12 to reach a final concentration of 2%.
- PS (5.1 ml): Penicillin (10000 units/ml) - Streptomycin (10 mg/ml) solution from GIBCO (CAT No.: 15140-122) is stored at -20ºC until needed. 5.1 ml of the solution are added to the medium to reach a final concentration of 1%.
- 0.5 ml of insulin (2 mg/ml) is added to reach a final concentration of 2 µg/ml.

Serum: FBS is stored at -20ºC upon arrival. To prepare aliquots, 500 ml bottle is thawed at 4ºC in a refrigerator, and aliquots of 55.5 ml or 10.2 ml are prepared aseptically and stored at -20ºC. One aliquot of 55.5 ml is added to 500 ml of MEM bottle to reach a final concentration of 10% FBS, and one aliquot of 10.2 ml is added to a 500 ml bottle of DMEM/F12 to reach a final concentration of 2% FBS.

Insulin stock solution (2 mg/ml): Insulin (SIGMA I5500) is dissolved in 0.1N HCl to reach the concentration of 2 mg/ml. The solution is filtered with 0.22 µm filter. Aliquots of 5 ml are prepared and stored at -20 ºC. Once thawed, the aliquot is used within one month.
0.5 ml of the solution is added to DMEM/F12 to a reach final concentration of 2 µg/ml.

Freezing solution (M10F containing 10% DMSO): Freezing solution is prepared freshly every time needed by the addition of 10% DMSO to the M10F medium.

2% EDTA-2Na solution: 2 g of EDTA-2Na is dissolved in 100 ml of distilled water, and the solution was sterilized by autoclaving (121ºC, 10 min). It is used under sterile conditions and discarded after 6 months from the day of the preparation.

Washing solution (PBS containing 0.02% EDTA-2Na): 5 ml of 2% EDTA-2Na solution were added to 500 ml bottle of PBS (GIBCO, CAT NO.: 14190-144 (Japan and Europe). It is used under sterile conditions, stored at room temperature and discarded after 6 months from the day of the preparation.

Fixing solutions: (stored at room temperature and discarded after 6 months from the day of the preparation)

- Pure methanol is used for cell fixation in the transformation assay and the Colony Forming Efficiency (CFE) assessment.
- 10% formalin (3.7% formaldehyde): 100% formalin (37% formaldehyde solution) is diluted with distilled or MilliQ water to make a concentration of 10%. This is used for fixation of cell growth assay.

Staining solutions: (stored at room temperature and discarded after 6 months from the day of the preparation)

- 0.04% Giemsa solution: One volume of Giemsa solution contained 0.4% Giemsa is diluted with 9 volumes of distilled or MilliQ water. This is used for staining in the transformation assay and the CFE assessment. (***Note:*** Giemsa solution from Merck (Cat. No.: 1.09204) is 0.4% Giemsa).
- 0.1% crystal violet (CV) solution: Crystal Violet is dissolved in MilliQ water to make a 1% solution. The 1% solution is diluted in distilled or MilliQ water to a final concentration of 0.1%. This is used for cell staining in the CV cell growth assay.

Extraction solution (for crystal violet): 50 volumes of ethanol and 1 volume of 1 mol/l HCl are added to 49 volumes of distilled water. Extraction solution consists of 50% ethanol containing 0.02 mol/l HCl (final concentration 0.01 mol/l HCl) and is used for extracting CV from stained cells.

Trypsin: Trypsin (GIBCO, Cat No. 15050-065) is stored at -20°C upon arrival. To prepare aliquots, trypsin is thawed and aliquots of 10 ml are prepared aseptically and re-stored at -20°C. When needed, aliquots are thawed and used. After use, always in sterile conditions, trypsin can be stored at 4°C up to one month from the date of thawing. Special care should be taken to avoid leaving trypsin at room temperature for a long time.

Test article solution: Test chemicals are dissolved or suspended in an appropriate solvent or vehicle and diluted with M10F complete medium. The solvent/vehicle should not interact with test chemicals and affect survival and focus formation of the cells. Final concentration of solvent/vehicle in medium is < 10 vol% with distilled water or saline, and < 0.1 vol% with DMSO (maximum dose of 0.5 vol% is permissible when test chemicals do not dissolve).

Methods for cell culture

Cell freezing:
Preparation of **MASTER STOCK** and **WORKING STOCK** is performed the same way. The cells should be stocked in liquid nitrogen at an early passage generation. One of the frozen vial is thawed in warm water (approximately 37ºC). The thawed tube must be immediately put in ice cold water. The thawed cells are suspended in 20 ml of M10F complete medium and seeded onto 2 culture dishes and cultured in a humidified 5% CO_2 incubator at 37ºC until subconfluence (60 to 70% confluence). Then, the cells are dislodged, and plated in 16 petri dishes (Ø90 mm) with 10 ml of fresh complete medium. After the cultures reach 70% confluence, the cells are dislodged, and cell number is adjusted to 1×10^6 cells/ml with M10F. 0.5 ml of cell suspension is transferred into freezing vials, then 0.5 ml of freezing solution is added and the vials are frozen in a deep freezer at -70 to -80ºC for 1 day, and then transferred into liquid nitrogen.
One working stock tube should be used for one transformation assay within 2 passages. For the cell growth assay, cells can be used several times within 9 passages.

Note: When cells are seeded in the petri dish, cell suspension should be mixed in the appropriate amount of medium, and should not be distributed before adding medium into the dishes. This is to allow the cells to attach homogeneously to the petri dish.

Cell thawing and culturing: One vial of frozen cells is thawed in warm water (approximately 37ºC). The thawed tube must be immediately put in ice cold water. The thawed cells are suspended in 20 ml M10F complete medium and seeded onto 2 culture dishes and cultured in a humidified 5% CO_2 incubator at 37ºC until subconfluence (70% confluence).

Cell passage and maintenance: When the cultures reach about 70% confluence, medium is removed and petri dishes are washed with washing solution, then treated with 0.25% trypsin solution until the cells dislodge from the culture surface. Trypsin is inactivated by the addition of M10F complete medium. The number of cells in the cell suspension is counted. The cells are adjusted to make a required cell density with M10F complete medium and seeded onto Ø90 mm dish(es). The cells are cultured in M10F complete medium in a humidified 5% CO_2 incubator at 37ºC. The cells should be harvested when they have reached 60 to 70% confluence.

Cell count
Only cells with whole cell membrane are counted using Bürker chamber and trypan blue solution (two dilutions can be prepared depending on the cell density/concentration: 10 µl cells + 90 µl trypan blue or 50 µl cells + 50 µl trypan blue).
Calculate the number of cells with the formula $\mathbf{N = (a / b) \times 10^4 \times DF}$
where **"N"** is the number of cells/ml; **"a"** is the number of cells counted in minimum 3 squares; **"b"** = number of squares considered (minimum 3); $\mathbf{10^4}$ is the conversion factor of chamber volume and **"DF"** is the Dilution Factor (usually equal to 2 or 10).
Record the cell count and passage number in the ad hoc working sheet.

Experimental procedures

Cell growth assays for the determination of test concentrations

CV method

Day 0: Cells at 60 to 70% confluence are trypsinized and suspended in M10F complete medium at 2 x 10^3 cells/ml. 0.5 ml of the cell suspension is transferred to each well of 24-well plates (1 x 10^3 cells/well). At least 3 wells of 24-well plates are used for each concentration. Three wells for medium alone (medium control), 3 wells for medium plus solvent controls (solvent control), and 3 wells for blank (medium without cells) must be included.
In order to obtain homogeneously growing cells in 24-well plates, it is necessary to keep plates without moving for 10 to 15 minutes after cell inoculation. Meanwhile, cells sediment to the plate surface and don't move thereafter under carrying the plates.
Day 1: Media containing various concentrations of test chemical are prepared, and used for medium change. Cells are exposed to the treatment medium for 72 hours.
Day 4: Medium is replaced with fresh complete medium. Check cell growth under microscope.
Day 7: Medium is removed. Cells are fixed with 10% formalin for 30 min, washed with water, air dried, and then stained with 0.1% CV solution for 15 min. After rinsing with water, the plates are air dried.

The staining dye in each well is extracted with 0.5 ml/well of extraction solution for 10 minutes (under gentle shaking) and the optical density of each well is measured at 540-570 nm (depending on the photometer filters available). Growth rates relative to the control culture are calculated from the absorbance with the formula:

Relative cell growth (% of control) = (Absorbance of treated well – Absorbance of medium blank well) / (Absorbance of **solvent** control well – Absorbance of medium blank well) x 100

Colony Forming Efficiency (CFE) method

1) SEEDING

Cells at 60 to 70% confluence are trypsinized and resuspended in M10F complete medium (as already mentioned before). Cells are seeded at the density of 200 cells/dish in 4 ml of M10F complete medium (60x15 mm petri dish, 4 dishes/dose).

Note: Cell suspension should be mixed in the appropriate amount of medium, and seeded into petri dish where the medium has already been placed. This will allow the cells to attach homogeneously to the petri dish.

2) TREATMENT

Day 0: 200 cells/dish (60x15 mm petri dish) are seeded, 4 dishes/dose

Day 1: Replace culture medium with 4 ml treatment medium (72 hours of exposure)

Day 4: (72 hours after exposure): Remove treatment medium without washing with PBS and replace with 4 ml of complete fresh medium

Day 7: Replace culture medium with fresh complete medium

Day 9: Remove medium and fix petri dishes with methanol (1 ml/ dish) for 3 minutes. Stain petri dish with filtered Giemsa 0.04% solution for 20 minutes. Then remove the solution and air dry the dishes.

The colonies that have been grown are counted under stereomicroscope. Only colonies with a well defined centre and more than 50 cells/colony are scored (5)

3) DATA COLLECTION

Relative CFE and plating efficiency (PE) are recorded.
Results are expressed as Relative CFE (%) mean ± SD.

Relative CFE (%) = total number of colonies formed in the treatment dishes x 100 / total number of colonies formed in the control dishes

For **PE (%) =** number of colonies formed in the control x 100 / 200,
Where 200 is the total number of cells seeded in the CFE dishes.

Suggested templates to be used for data collection are presented in Appendix 2.

Transformation assay and concurrent cell growth assays (CV and CFE)

For the second part of Phase 1, CV and CFE are performed in parallel to the morphological transformation according to the above-mentioned protocols.

Day 0: Cells at 60 to 70% confluence are trypsinized and harvested in M10F complete medium at 2 x 10^3 cells/ml. Ten ml (10 ml) of the cell suspension are plated into each Ø90 mm petri dish (2 x 10^4 cells/dish, 10 dishes/dose).
Note: Homogeneously growing cells can be obtained if dishes are gently moved back and forth and right and left a few times, and then placed in the incubator.
Day 1: Medium containing various concentrations of test chemical is prepared. 10 ml of treatment medium is replaced in each dish. Cells are exposed to the treatment solution for 72 hours.
Day 4: Treatment medium of Ø90 mm dishes is replaced with 10 ml of fresh M10F complete medium.
Day 5 through Day 24 or 25: Medium is changed with 10 ml of fresh DF2I2F complete medium twice a week.
Day 31 or 32: One-week after the last medium change, cells are fixed with methanol for 10 min and stained with 10% Giemsa solution for 30 min. Then staining solution is removed and Petri dishes are air dried.
Transformed foci type III are colonies with the following characteristics: deep basophilic staining, dense multilayered, cells randomly orientated at focus edge, cells spindle-shaped different from the background monolayer cells, multi-layering (piling up) cells (5). Foci consisting of more than 50 cells are scored. Only foci type III are considered tumourigenic because it has been shown that they are the only ones inducing neoplastic transformation in nude mice with a frequency of 85% (5, 8).
Number of foci in each dish is scored. Average number and standard deviation in each group are calculated and recorded. (To be further clarified with the photo catalogue)

Transformation dishes must be stored and archived in each laboratory until the end of the pre-validation, in case that a not clear result needs clarification.

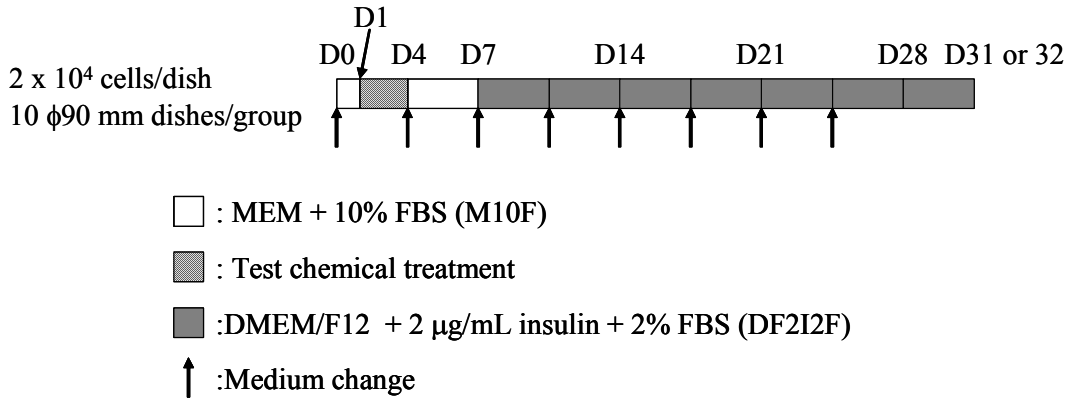

Fig. BALB/c 3T3 Cell Transformation Assay Protocols

General experimental design

Note: Following the suggestions of the Management Team after the teleconference of the 23rd February 2006, the concentrations for the morphological transformation and the concurrent CV and CFE assays (MCA and Compound X) have been chosen:
0.01 μg/ml, 0.03 μg/ml, 0.1 μg/ml, 0.3 μg/ml,1 μg/ml, 3 μg/ml, 10 μg/ml
This has been applied only to the second part of Phase 1.
For Phase 2, the general experimental design is specified below.

Crystal Violet method

MCA has been chosen as a positive control.

- Fixed dose of MCA to be tested for the transformation test is 4.0 μg/ml- 3 wells/dose. A concurrent growth assessment must be done.
- Test chemicals are dissolved or suspended in an appropriate solvent or vehicle and diluted with the medium. The solvent/vehicle should not interact with test chemicals and affect survival and focus formation of the cells. Final concentration of solvent/vehicle in medium is < 10 vol% with distilled water or saline, and < 0.1vol% with DMSO (maximum dose of 0.5% is permissible when test chemicals do not dissolve).
- The highest concentration is 5 mg/ml or 10 mM, whichever is the lowest (6,7), or is determined based on historical studies.
- Five or more concentration levels are set by an appropriate serial dilution factor such as 2 or square root 10.
- Each transformation experiment (for each compound) in Phase I will be performed with untreated and solvent controls.
- It is desirable that cell growth curve covers the range between NOEL and >IC_{90}.

Colony Forming Efficiency (CFE)

MCA has been chosen as positive control.

- Fixed doses of MCA to be tested for the transformation test is 4.0 μg/ml - 4 dishes/dose. A cell growth assessment must be done.
- The second compound will be coded and a dose-curve for cell growth assay will be performed before transformation assay.
- Test chemicals are dissolved or suspended in an appropriate solvent or vehicle and diluted with medium. The solvent/vehicle should not interact with test chemical and affect survival and focus formation of the cells. Final concentration of solvent/vehicle in medium is < 10 vol% with distilled water or saline, < 0.1vol% with DMSO (maximum dose of 0.5% is

permissible when test chemicals do not dissolve).
- Each transformation experiment (for each compound) in Phase II will be performed with untreated and solvent controls.
- The doses that should be assessed for the selected compounds (ideally) are:

NOEL……IC50 ……IC_{90}

One dose in between	Two doses in between

Morphological transformation assay

- MCA will be used as a positive control and tested at 4.0 µg/ml.
- Test concentrations of the chemicals to be tested after a dose-curve for cell growth will be (ideally) as follows;

……NOEL……IC50 ……..……IC_{90}

One dose	Two doses in	Two doses in

- Ten (Ø90 mm) Petri dishes (20000 cells/dish) are used for each concentration.

Acceptance Criteria

Concurrent cell growth assays
- Homogeneous cell growth on culture wells should be observed.
- At least one NOEL-concentration.
- Cell growth curves cover the range between NOEL and IC_{90} (ideally six concentrations between NOEL and IC_{90}).
- For CFE, minimum plating efficiency 30% in the negative control.

Transformation assay
- The maximum number of Type III foci in the entire set of solvent control dishes should not exceed 5.
- If, while performing experiment, one entire set-concentration is lost, the experiment is still OK. If 2 concentrations are lost, this might still be acceptable if the remaining concentrations still cover the range of toxicity.
- If one Petri-dish is lost (out of the 10 dishes/concentration) by accident or contamination, the experiment is still acceptable.

References

(1) T. Kakunaga, A quantitative system for assay of malignant transformation by chemical carcinogens using a clone derived from BALB-3T3, *Int. J. Cancer*, **12** (1973) 463-473.
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(3) T. Tsuchiya, M. Umeda, Improvement in the efficiency of the in vitro transformation assay method using BALB/3T3 A31-1-1 cells, *Carcinogenesis*, **16** (1995) 1887-1894.
(4) T. Tsuchiya, M. Umeda, H. Nishiyama, I. Yoshimura, S. Ajimi, M. Asakura, H. Baba, Y. Dewa, Y. Ebe, Y. Fushiwaki, S. Hamada, T. Hamamura, M. Hayashi, Y. Iwase, Y. Kajiwara, Y. Kasahara, M. Kawabata, E. Kitada, K. Kudo, K. Mashiko, D. Miura, F. Mizuhashi, F. Mizuno, M. Nakajima, Y. Nakamura, N. Nobe, T. Oishi, E. Ota, A. Sakai, M. Sato, S. Shimada, T. Sugiyama, C. Takahashi, Y. Takeda, N. Tanaka, C. Toyoizumi, T. Tsutsui, S. Wakuri, S. Yajima, N. Yajima, An interlaboratory validation study of the improved transformation assay employing BALB/c 3T3 cell: Results of a collaborative study on the two-stage cell transformation assay by the non-genotoxic carcinogen study group, *ATRA*, **27** (1999) 685-702.
(5) IARC/NCI/EPA Working Group, Cellular and molecular mechanisms of cell transformation and standardization of transformation assays of established cell lines for prediction of carcinogenic chemicals: Overview and recommended protocols, *Cancer Res*., **45** (1985) 2395-2399.
(6) OECD Guidelines for the Testing of Chemicals. Test No. 473: *In vitro* Mammalian Chromosome Aberration Test.
(7) OECD Guidelines for the Testing of Chemicals. Test No. 476: *In vitro* Mammalian Cell Gene Mutation Test.
(8) U. Saffiotti, M. Bignami, F. Bertolero, E. Cortesi, C. Ficorella, M.E. Kaighn, Studies on chemically induced neoplastic transformation and mutation in the BALB/3T3 Cl A31-1-1 cell line in relation to the quantitative evaluation of carcinogens, *Toxicol. Pathol.*, **12** (1984) 383-390.

Appendix 1

	Supplier	Cat. No.
Culture reagents		
Minimum Essential Medium	GIBCO	11095-080 (Japan) 31095-029 (Europe)
Fetal bovine serum Phase 1	Moregate	Lot No. 24300133
Fetal Bovine serum Phase 2	Moregate	Lot No. 94300107
Dulbecco's modified Eagle's medium/F12	GIBCO	11330-032 (Japan) 31330-038 (Europe)
Penicillin (10000 U/ml) - Streptomycin (10 mg/ml)	GIBCO	15140-122
Insulin	Sigma	I5500
Other reagents		
Phosphate buffered saline	GIBCO	14190-144
EDTA	SIGMA	E5134
Trypsin	GIBCO	15050-065
DMSO	SIGMA	D8418
Fixation and staining solutions		
Methanol	SIGMA	15490-3
Giemsa solution *	MERK	1.09204
Giemsa solution (0.4%)	Sigma	GS 500
Formalin (37% formaldehyde)	SIGMA	F8775
Crystal violet	SIGMA	C3886
Ethanol		
HCl		
Culture vessels **		
60 x 15 mm dishes	Falcon or Costar can be used	
100 x 20 mm dishes	Falcon or Costar can be used	
24-well microplates	Falcon or Costar can be used	

* Giemsa solution purchased from Merck is 0.4%
** for phase 1 FALCON vessels were used

Appendix 2

Colony forming efficiency with COMPOUND XX

COLONY FORMING EFFICIENCY (CFE)									
Coded compound	Conc. (µg/mL)	Number of colonies 1. dish	2. dish	3. dish	4. dish	Mean	SD	Relative PE (%)	Relative CFE (%)
Untreated control[1]	-								-
Solvent control[2]	-								
Positive control[3]	4.00								

[P] Precipitation occurred at the end of treatment
[1] Complete culture medium
[2] DMSO 0.5 % (v/v)
[3] MCA

Crystal violet determination with COMPOUND XX

CRYSTAL VIOLET (CV)								
Coded compound	Conc. (µg/mL)	Number of colonies 1. well	2. well	3. well	4. well	Mean	SD	Relative growth (%)
Untreated control[1]	-							-
Solvent control[2]	-							
Positive control[3]	4.00							

[P] Precipitation occurred at the end of treatment
[1] Complete culture medium
[2] DMSO 0.5 % (v/v)
[3] MCA

Morphological transformation with COMPOUND XX

Coded compound	Conc. (µg/mL)	Number of Type III foci										Mean	SD	Sum
		1. dish	2. dish	3. dish	4. dish	5. dish	6. dish	7.dish	8.dish	9.dish	10. dish			
Untreated control[1]	-													
Solvent control[2]	-													
Positive control[3]	4.00													

[P] Precipitation occurred at the end of treatment
[1] Complete culture medium
[2] DMSO 0.5 % (v/v)
[3] MCA

13.3 Example of statistical analysis of Balb/c 3T3 CTA results, using the Generalised Linear Model approach with a negative binomial distribution

Following the conclusions of the ad hoc expert group meeting held at ECVAM in April 2007 to discuss the most suitable statistical method to use for the Balb/c 3T3 CTA, a Generalised Linear Model (GLM) with a negative binomial distribution and identity as link function was designed.
That GLM is applied for the calculation of downturn-protected Williams contrast tests based on unweighted combinations of concentrations and for the calculation of adjusted *p*-values based on the joint normal distribution of the linear function.

This approach is publicly available and implemented into R (http://www.R-project.org). The original R-codes were kindly provided by Pr. Hothorn (University of Hannover, Germany).

Reported below is one example of the analysis of morphological transformation results (number of foci per dish), using the statistical method developed for this validation study.

1/ Data File

Filename: BALB TB Hatano 3nd_exp.txt

Original data

NC	0	0	0	0	0	0	0	0	0	0
SC	0	0	0	0	0	0	0	0	0	0
PC	17	20	13	18	18	11	15	18	14	17
c100	0	1	0	0	0	0	0	0	0	0
c200	0	0	0	0	0	0	0	0	0	0
c400	1	0	0	0	0	0	0	0	0	0
c600	0	1	0	0	0	0	0	0	0	1
c800	0	2	1	0	1	6	1	3	1	0
c900	8	2	1	3	3	3	2	2	2	1
c1000	6	5	1	3	1	3	2	3	1	2
c1100	6	5	2	6	4	2	2	0	1	1
c1200	2	0	1	2	3	3	2	3	2	2

Modified data (used for the prediction)

NC	0	0	0	0	0	0	0	0	0	0
SC	0	**1**	0	0	0	0	0	0	0	0
PC	17	20	13	18	18	11	15	18	14	17
c100	0	1	0	0	0	0	0	0	0	0
c200	0	0	0	0	0	0	**1**	0	0	0
c400	1	0	0	0	0	0	0	0	0	0
c600	0	1	0	0	0	0	0	0	0	1
c800	0	2	1	0	1	6	1	3	1	0
c900	8	2	1	3	3	3	2	2	2	1
c1000	6	5	1	3	1	3	2	3	1	2
c1100	6	5	2	6	4	2	2	0	1	1

c1200	2	0	1	2	3	3	2	3	2	2

columns → dishes (ten in total)
rows → NC negative control, SC solvent control, PC positive control and test concentrations

Remark
Because of mathematical reasons (at least one event per row is necessary → probability > 0), "zero" values for solvent control (dish 2) and concentration 200 µg/ml (dish 7) were changed into one (negative control values were not modified although all "zero" since they are not used in the analysis)

2/ R-Code for the Generalised Linear Model with a negative binomial distribution, including comments

```
#####################################################################
# BALB Phase II                                                     #
#                                                                   #
# Andre Kleensang                                                   #
# European Commission, JRC, IHCP, Systems Toxicology Unit           #
# Code developed by Prof. Dr. Hothorn                               #
# University of Hannover                                            #
# 07 November 2008                                                  #
# Filename: BALBanalysis TB Hatano 3nd.R                            #
# Remark: If applied to new data the code needs to be adapted       #
#         based on the number of test concentrations and            #
#         their values as given in the comments!                    #
#####################################################################

# Libraries
library(multcomp)
library(MASS)

# Function for defining the contrasts for the downturn-protected Williams tests
protwill <- function(n){
  k <- length(n)
  CM <- c()
  if (!is.null(names(n))) varnames <- names(n) else varnames <- 1:length(n)
  for (j in 1:(k-1)) {
    for (i in 1:(k - j)) {
      helper <- c(-1, rep(0, k - i - j), n[(k - i + 1):k]/sum(n[(k - i + 1):k]), rep(0, j-1))
      CM <- rbind(CM, helper)
    }
  }
  rownames(CM) <- paste("C", 1:nrow(CM))
  colnames(CM) <- varnames
  CM
}

# Read file, second line needs to be adapted based on the number of test concentrations and their values
```

```
CTA1 <- read.table(file("BALB TB Hatano 3nd_exp.txt", encoding="latin1"))
compound <- c("control","solvent","positive","100","200","400","600","800","900","1000","1100","1200")

# Transforming the matrix into a flat-file format:
CTA1stk <- stack(CTA1[,2:ncol(CTA1)])
compound <- rep(compound, ncol(CTA1)-1)
CTA <- data.frame(foci = CTA1stk[,1], dish = CTA1stk[,2], compound)

# Arranging factor levels
# First line needs to be adapted based on the number of test concentrations and their values
lcmpd <- c("control","solvent","100","200","400","600","800","900","1000","1100","1200","positive")
CTA$compound <- factor(CTA$compound, levels=lcmpd)

# Omitting P and C by sub-setting the dataset and redefinition of factor levels
# First line needs to be adapted based on the number of test concentrations and their values
lcmpdsub <- c("solvent","100","200","400","600","800","900","1000","1100","1200")
CTAs <- subset(CTA, compound != "control" & compound != "positive")
CTAs$compound <- factor(CTAs$compound, levels=lcmpdsub)

# Defining the contrasts for the downturn-protected Williams tests
n <- tapply(CTAs$foci, CTAs$compound, length)
K <- protwill(n)
K

# Fitting GLM with negative binomial distribution and identity link function
nb.CTAs <- glm.nb(foci ~ compound, data=CTAs, link = identity)

# Calculation of p-values for the Williams downturn-protected contrasts tests
# adjusted for multiple testing based on the joint normal distribution of the linear function
MCP.CTAs <- glht(nb.CTAs, linfct = mcp(compound = K), alternative="greater")
pval <- summary(MCP.CTAs)$test$pvalues
pval
```

3/ Results

pval (Variable with *p*-values)

```
 [1] 8.867174e-04 1.307175e-08 1.296629e-12 5.551115e-16 0.000000e+00
 [6] 1.110223e-16 1.110223e-15 1.443290e-14 1.533218e-13 3.663518e-05
[11] 1.180222e-09 8.404388e-14 2.442491e-15 1.409983e-14 1.115774e-13
[16] 1.249667e-12 1.994904e-11 6.550699e-05 1.781550e-09 1.818046e-11
[21] 8.806822e-11 9.012645e-09 5.546341e-09 6.336666e-08 5.676274e-05
[26] 2.016288e-07 2.119999e-06 1.537223e-05 3.443053e-05 1.465107e-04
[31] 7.091067e-03 1.258352e-02 3.490272e-02 7.941265e-02 1.467802e-01
[36] 8.926942e-01 9.403999e-01 9.575313e-01 9.656967e-01 9.858827e-01
[41] 9.858841e-01 9.858818e-01 9.858830e-01 9.858835e-01 9.858843e-01
attr(,"error")
[1] 0.0007979342
```

K (Variable with downturn-protected Williams contrasts)

	solvent	100	200	400	600	800	900	1000	1100	1200
C 1	-1	0.0000000	0.0000000	0.0000000	0.0000000	0.0000000	0.0000000	0.0000000	0.0000000	1.0000000
C 2	-1	0.0000000	0.0000000	0.0000000	0.0000000	0.0000000	0.0000000	0.0000000	0.5000000	0.5000000
C 3	-1	0.0000000	0.0000000	0.0000000	0.0000000	0.0000000	0.0000000	0.3333333	0.3333333	0.3333333
C 4	-1	0.0000000	0.0000000	0.0000000	0.0000000	0.0000000	0.2500000	0.2500000	0.2500000	0.2500000
C 5	**-1**	**0.0000000**	**0.0000000**	**0.0000000**	**0.0000000**	**0.2000000**	**0.2000000**	**0.2000000**	**0.2000000**	**0.2000000**
C 6	-1	0.0000000	0.0000000	0.0000000	0.1666667	0.1666667	0.1666667	0.1666667	0.1666667	0.1666667
C 7	-1	0.0000000	0.0000000	0.1428571	0.1428571	0.1428571	0.1428571	0.1428571	0.1428571	0.1428571
C 8	-1	0.0000000	0.1250000	0.1250000	0.1250000	0.1250000	0.1250000	0.1250000	0.1250000	0.1250000
C 9	-1	0.1111111	0.1111111	0.1111111	0.1111111	0.1111111	0.1111111	0.1111111	0.1111111	0.1111111
C 10	-1	0.0000000	0.0000000	0.0000000	0.0000000	0.0000000	0.0000000	0.0000000	1.0000000	0.0000000
C 11	-1	0.0000000	0.0000000	0.0000000	0.0000000	0.0000000	0.0000000	0.5000000	0.5000000	0.0000000
C 12	-1	0.0000000	0.0000000	0.0000000	0.0000000	0.0000000	0.3333333	0.3333333	0.3333333	0.0000000
C 13	-1	0.0000000	0.0000000	0.0000000	0.0000000	0.2500000	0.2500000	0.2500000	0.2500000	0.0000000
C 14	-1	0.0000000	0.0000000	0.0000000	0.2000000	0.2000000	0.2000000	0.2000000	0.2000000	0.0000000
C 15	-1	0.0000000	0.0000000	0.1666667	0.1666667	0.1666667	0.1666667	0.1666667	0.1666667	0.0000000
C 16	-1	0.0000000	0.1428571	0.1428571	0.1428571	0.1428571	0.1428571	0.1428571	0.1428571	0.0000000

```
C 17      -1 0.1250000 0.1250000 0.1250000 0.1250000 0.1250000 0.1250000 0.1250000 0.1250000 0.0000000
C 18      -1 0.0000000 0.0000000 0.0000000 0.0000000 0.0000000 0.0000000 1.0000000 0.0000000 0.0000000
C 19      -1 0.0000000 0.0000000 0.0000000 0.0000000 0.0000000 0.5000000 0.5000000 0.0000000 0.0000000
C 20      -1 0.0000000 0.0000000 0.0000000 0.0000000 0.3333333 0.3333333 0.3333333 0.0000000 0.0000000
C 21      -1 0.0000000 0.0000000 0.0000000 0.2500000 0.2500000 0.2500000 0.2500000 0.0000000 0.0000000
C 22      -1 0.0000000 0.0000000 0.2000000 0.2000000 0.2000000 0.2000000 0.2000000 0.0000000 0.0000000
C 23      -1 0.0000000 0.1666667 0.1666667 0.1666667 0.1666667 0.1666667 0.1666667 0.0000000 0.0000000
C 24      -1 0.1428571 0.1428571 0.1428571 0.1428571 0.1428571 0.1428571 0.1428571 0.0000000 0.0000000
C 25      -1 0.0000000 0.0000000 0.0000000 0.0000000 0.0000000 1.0000000 0.0000000 0.0000000 0.0000000
C 26      -1 0.0000000 0.0000000 0.0000000 0.0000000 0.5000000 0.5000000 0.0000000 0.0000000 0.0000000
C 27      -1 0.0000000 0.0000000 0.0000000 0.3333333 0.3333333 0.3333333 0.0000000 0.0000000 0.0000000
C 28      -1 0.0000000 0.0000000 0.2500000 0.2500000 0.2500000 0.2500000 0.0000000 0.0000000 0.0000000
C 29      -1 0.0000000 0.2000000 0.2000000 0.2000000 0.2000000 0.2000000 0.0000000 0.0000000 0.0000000
C 30      -1 0.1666667 0.1666667 0.1666667 0.1666667 0.1666667 0.1666667 0.0000000 0.0000000 0.0000000
C 31      -1 0.0000000 0.0000000 0.0000000 0.0000000 1.0000000 0.0000000 0.0000000 0.0000000 0.0000000
C 32      -1 0.0000000 0.0000000 0.0000000 0.5000000 0.5000000 0.0000000 0.0000000 0.0000000 0.0000000
C 33      -1 0.0000000 0.0000000 0.3333333 0.3333333 0.3333333 0.0000000 0.0000000 0.0000000 0.0000000
C 34      -1 0.0000000 0.2500000 0.2500000 0.2500000 0.2500000 0.0000000 0.0000000 0.0000000 0.0000000
C 35      -1 0.2000000 0.2000000 0.2000000 0.2000000 0.2000000 0.0000000 0.0000000 0.0000000 0.0000000
C 36      -1 0.0000000 0.0000000 0.0000000 1.0000000 0.0000000 0.0000000 0.0000000 0.0000000 0.0000000
C 37      -1 0.0000000 0.0000000 0.5000000 0.5000000 0.0000000 0.0000000 0.0000000 0.0000000 0.0000000
C 38      -1 0.0000000 0.3333333 0.3333333 0.3333333 0.0000000 0.0000000 0.0000000 0.0000000 0.0000000
C 39      -1 0.2500000 0.2500000 0.2500000 0.2500000 0.0000000 0.0000000 0.0000000 0.0000000 0.0000000
C 40      -1 0.0000000 0.0000000 1.0000000 0.0000000 0.0000000 0.0000000 0.0000000 0.0000000 0.0000000
C 41      -1 0.0000000 0.5000000 0.5000000 0.0000000 0.0000000 0.0000000 0.0000000 0.0000000 0.0000000
C 42      -1 0.3333333 0.3333333 0.3333333 0.0000000 0.0000000 0.0000000 0.0000000 0.0000000 0.0000000
C 43      -1 0.0000000 1.0000000 0.0000000 0.0000000 0.0000000 0.0000000 0.0000000 0.0000000 0.0000000
C 44      -1 0.5000000 0.5000000 0.0000000 0.0000000 0.0000000 0.0000000 0.0000000 0.0000000 0.0000000
C 45      -1 1.0000000 0.0000000 0.0000000 0.0000000 0.0000000 0.0000000 0.0000000 0.0000000 0.0000000
```

4/ Prediction

The downturn-protected Williams contrast with the lowest *p*-value (the 5th contrast) **(I)** includes at least two consecutive concentrations (concentrations 800 – 1200; in total 5), and **(II)** showed a *p*-value of < 0.01 (*p*-value <1e-16) → **positive prediction**

13.4 Nishiyama transformation analysis

The *ad hoc* expert group discussed several statistical evaluation methods for the analysis of the Balb/c 3T3 CTA results. The Nishiyama transformation (Nishiyama *et al*., 2003) and an approach based on the negative binomial distribution combined with William's-type (Bretz *et al*., 2003) protected tests were recommended for use in this validation study since they both match the particular statistical properties of the Balb/c 3T3 CTA test. The latter method was suggested to be more suited for the data at hand as it better reflected the dose-concentration curve (Manuscript in preparation, minutes of the meeting available at ECVAM).

Nevertheless, for completeness, the results obtained when applying the Nishiyama transformation and the corresponding R-code are reported below.

13.4.1 Summary of results

The predictions based on Nishiyama transformation and ordinary least squares linear model in combination with downturn-protected Williams contrasts are presented for each chemical in Table 37.

The results were evaluated with the following criteria:

- A test chemical was considered "negative" (non-transforming) if no downturn-protected Williams contrast showed a *p*-value < 0.01.
- A test chemical was considered "positive" (transforming) if the downturn-protected Williams contrast with the lowest *p*-value:
  - (I) included at least two consecutive concentrations, and
  - (II) showed a *p*-value < 0.01.
- A test chemical was considered "inconclusive" if the downturn-protected Williams contrast with the lowest *p*-value:
  - (I) included only one concentration or several non consecutive concentrations, and
  - (II) showed a *p*-value < 0.01.

Table 37: **Summary table of the predictions based on Nishiyama transformation and ordinary least squares linear model in combination with downturn-protected Williams contrasts (*p* < 0.01).**

Chemical	Expected result[#]	Laboratory						
		ECVAM		Harlan CCR		HRI		
		TA1	TA2	TA1	TA2	TA1	TA2	TA3
3-Methylcholanthrene	+	+[$]	i	+[$]	+	+[$]	+	
2-Acetylaminofluorene	+	+		i		i		
Benzo(a)pyrene	+	+		+		i		
Anthracene	-	-		-		-		
Phenanthrene	-	i	i	i	+	nc	i	
o-Toluidine HCl	+	+		+		nc	i	+

[#] Based on previous results from the litereature (see sections 2.4.1.1 and 2.4.1.2)
i = inconclusive call; + = positive call; - = negative call; nc = not considered.
[$] Chemical tested non coded in module 2

All inconclusive assessments reported were as such because the downturn-protected Williams contrast with the lowest *p*-value showed a *p*-value < 0.01 but included only one concentration.

13.4.2 R-Code for the Nishiyama transformation, including comments

```
# Libraries
library(multcomp)
library(MASS)

# Function for defining the contrasts for the downturn-protected Williams tests
protwill <- function(n){
  k <- length(n)
  CM <- c()
  if (!is.null(names(n))) varnames <- names(n) else varnames <- 1:length(n)
  for (j in 1:(k-1)) {
    for (i in 1:(k - j)) {
      helper <- c(-1, rep(0, k - i - j), n[(k - i + 1):k]/sum(n[(k - i + 1):k]), rep(0, j-1))
      CM <- rbind(CM, helper)
    }
  }
  rownames(CM) <- paste("C", 1:nrow(CM))
  colnames(CM) <- varnames
  CM
}

# Read file, second line needs to be adapted based on the number of test concentrations and their values
CTA1 <- read.table(file("BALB TB Hatano 3nd_exp.txt", encoding="latin1"))
compound <- c("control","solvent","positive","100","200","400","600","800","900","1000","1100","1200")

# Transforming the matrix into a flat-file format:
CTA1stk <- stack(CTA1[,2:ncol(CTA1)])
compound <- rep(compound, ncol(CTA1)-1)
CTA <- data.frame(foci = CTA1stk[,1], dish = CTA1stk[,2], compound)

# Arranging factor levels
# First line needs to be adapted based on the number of test concentrations and their values
lcmpd <- c("control","solvent","100","200","400","600","800","900","1000","1100","1200","positive")
CTA$compound <- factor(CTA$compound, levels=lcmpd)

# Omitting P and C by sub-setting the dataset and redefinition of factor levels
# First line needs to be adapted based on the number of test concentrations and their values
```

```
lcmpdsub <- c("solvent","100","200","400","600","800","900","1000","1100","1200")
CTAs <- subset(CTA, compound != "control" & compound != "positive")
CTAs$compound <- factor(CTAs$compound, levels=lcmpdsub)

# Defining the contrasts for the downturn-protected Williams tests
n <- tapply(CTAs$foci, CTAs$compound, length)
K <- protwill(n)
K

# Square root transformation of Nishiyama et al. 2003
CTAt <- CTAs
CTAt$foci <- sqrt(CTAt$foci) + sqrt(CTAt$foci + 1)

# Fitting ordinary least squares linear model
fit.CTAt <- lm(foci ~ compound, data=CTAt)

# Calculation of p-values for the Williams downturn-protected contrasts tests
# adjusted for multiple testing based on the joint normal distribution of the linear function
n <- tapply(CTAt$foci, CTAt$compound, length)
K <- protwill(n)
K
MCP.CTAt <- glht(fit.CTAt, linfct = mcp(compound = K), alternative="greater")
pval <- summary(MCP.CTAt)$test$pvalues
pval
```

13.5 Repeated experiments

13.5.1 HRI – Phenanthrene

In the first experiment by HRI (TA1), phenanthrene was dissolved in water. Table 38 shows the test concentrations which were evaluated. The VMT asked the laboratory to retest using DMSO which was the solvent originally requested (see section 6.4.2.3).

Table 38: Transformation assay results (1st experiment) from HRI, testing coded phenanthrene

HRI Phenanthrene (µg/ml) TA1	rCFE (%)	RCG (%) CV	Total number of foci	Mean number of foci per dish
Untreated control	na	na	5	0.50
VC (water)	100	100	3	0.30
250	76.1	10.7	7	0.70
500	78.0	9.0	6	0.60
750	23.7	9.9	15	1.50
1000	9.3	6.5	12	1.20
1500	14.4	5.8	8	0.80
2000	5.1	5.3	8	0.80
PC	12.4	8.0	139	13.9

VC = Vehicle Control, PC = Positive Control, rCFE = relative Colony Forming Efficiency, RCG = Relative Cell Growth, CV = Crystal Violet, na = not applicable.

13.5.2 HRI – o-toluidine HCl

In the first experiment by HRI (TA1), o-Toluidine HCl was dissolved in water (TA1). Table 39 shows the test concentrations which were evaluated. The VMT asked the laboratory to retest using DMSO which was the solvent originally requested (see section 6.5.2.3).

Table 39: Transformation assay results (1st experiment) from HRI, testing coded o-toluidine HCl

HRI o-Toluidine HCl (µg/ml) TA1	rCFE (%)	RCG (%) CV	Total number of foci	Mean number of foci per dish
Untreated control	na	na	3	0.30
VC (water)	100	100	7	0.70
50	96.3	62.1	4	0.40
100	99.0	62.0	0	0
250	87.4	64.0	2	0.20
500	76.6	60.9	3	0.30
750	59.5	43.8	2	0.20
1000	28.3	44.7	9	0.90
PC	11.8	8.6	98	9.80

TA1 = Transformation Assay 1, VC = Vehicle Control, PC = Positive Control, rCFE = relative Colony Forming Efficiency, RCG = Relative Cell Growth, CV = Crystal Violet, na = not applicable.