



EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Institute for Health and Consumer Protection
European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM)

EURL ECVAM RECOMMENDATION

of 14th March 2012

on

three Cell Transformation Assays

using

**Syrian Hamster Embryo Cells (SHE)
and the BALB/c 3T3 Mouse Fibroblast Cell Line**

for

In Vitro Carcinogenicity Testing¹

¹ The ESAC Opinion [2011.01] was adopted on 18 February 2011. The draft EURL ECVAM Recommendation was published as a *Call for Comments* on the IHCP Internet Webpage on 7 December 2011 with a deadline for comments of 31 December 2011.

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EURL ECVAM RECOMMENDATION

of 14.03.12 on

three Cell Transformation Assays (CTA) using Syrian Hamster Embryo Cells (SHE) and the BALB/c 3T3 mouse fibroblast cell line

Executive Summary

The three CTA methods aim to predict the carcinogenic potential of chemicals.

EURL ECVAM fully endorses the ESAC Opinion (dated 18.2.2011) on the performance of these 3 cell transformation assays. In addition, EURL ECVAM recommends that:

- A draft OECD Test Guideline for the SHE CTA should be developed and, considering the limited differences between the protocols for the SHE CTA at pH 6.7 and 7.0, both CTAs should be incorporated into a single Test Guideline;
- Any use of the three CTA protocols should include the requirement of appropriate training by applying the 3 photo catalogues to ensure that scoring is as consistent as possible;
- The BALB/c 3T3 CTA protocol should be further used to expand on the reproducibility of the assay and confirm the suitability of the new statistical approach and data interpretation procedure applied. The BALB CTA should from a 3R perspective be considered more appropriate than the SHE CTA since it uses a cell line.
- The performance characteristics of the SHE CTA methods should be further evaluated by analysing existing data, complemented where necessary by generation of new data to conclude on the regulatory usability;
- The use of CTAs has the potential of partial replacement or reduction when used in a weight of evidence approach for hazard identification and risk assessment.

BACKGROUND TO THIS EURL ECVAM RECOMMENDATION

1. Introduction

- 1) The in vitro CTAs that have been in use for about 40 years model key stages of in vivo carcinogenicity. In 1998, EURL ECVAM held a workshop with the aim to "seek consensus on ways of increasing the use of mammalian CTAs, for fundamental and applied studies in carcinogenesis, and for the regulatory testing of carcinogens" (Combes et al. 1999). The workshop concluded that the CTA tests were promising but required further development, standardisation and verification prior to being proposed for regulatory use. In parallel, the OECD had in 1997 initiated work on a Detailed Review Paper (DRP) on CTAs for the detection of chemical carcinogens. The DRP was finalised in 2007 (OECD, 2007) concluding that the performance of the Syrian Hamster Embryo (SHE) and the BALB/c 3T3 CTAs were sufficiently adequate for being proposed to be developed into OECD Test Guidelines. A major criticism by regulators was the lacking standardization of protocols and objective criteria for the scoring. On the background of these two activities, and following the recommendations of an expert group which met at EURL ECVAM in 2004, EURL ECVAM coordinated a study aiming at the standardisation and subsequent evaluation of three CTA protocols in terms of transferability and reproducibility. The study was intended to complement the retrospective evaluation of the OECD DRP in view of providing prospective data on reliability but addressing predictive capacity only to a limited extent since an extensive body of existing evidence on the predictivity of CTAs was available and summarised in the OECD DRP.
- 2) The three CTA protocol variants were:
 - (a) The SHE CTA performed at pH 6.7 (SHE pH 6.7 CTA);
 - (b) the SHE CTA performed at pH 7.0 (SHE pH 7.0 CTA); and,
 - (c) the BALB/c 3T3 CTA.
- 3) After completion of the study and finalisation of the study reports in summer 2010 (EC-ECVAM, 2010a, b, c), EURL ECVAM requested the EURL ECVAM Scientific Advisory Committee (ESAC) at its meeting on 12 October 2010, to provide scientific advice on this study in the form of an ESAC opinion. ESAC established a Working Group (WG), chaired by two of its members and including internationally renowned experts on CTA, and provided the WG with the mandate to review in detail the results compiled in the three reports (EC-ECVAM, 2010d). Both the ESAC WG report (EC-ECVAM, 2011a) and the ESAC opinion (Annex) were adopted by ESAC on 18 February 2011, and made available to the OECD in time for its annual WNT meeting in mid-April 2011.
- 4) Based on the above mentioned documents (i.e. ESAC opinion, study reports and the ESAC WG report) and other relevant documents, mainly the OECD DRP (OECD, 2007), EURL ECVAM developed the present recommendation. The aim of any EURL ECVAM recommendation is to provide EURL ECVAM's views on the validity of the test method(s) in question in addition to advice on possible regulatory applicability, limitations and proper scientific use of the test methods, and to suggest possible follow-up activities.

2. Test method definition

Basis of the test method

- 5) For both SHE CTAs (at pH 6.7 and pH 7.0), the test system is based on SHE cells derived from mid-gestation embryos of Syrian golden hamsters. For the BALB/c 3T3 CTA, the test system is

based on an established mouse fibroblast cell line. The three variants of the CTA assay require visual scoring of specific parameters relating to the phenotype and growth pattern of cells. These effects can be visually detected and scored under the microscope. The parameters measured in the SHE and in the BALB/c 3T3 CTAs are the number of transformed colonies and the number of foci formed, respectively.

Biological and mechanistic relevance of the test method

- 6) In vitro CTAs have been shown to closely model some stages of the multistage process of in vivo carcinogenesis. The phenomenon of morphological transformation is characterised by changes in the behaviour and growth of cultured cells allowing for progression to the next stage in the transformation process, from a normal cell to a fully malignant cell. A minimum of four phenotypic stages appears to be involved in cell transformation (LeBoeuf et al., 1999), which includes:
 - (a) a block in cellular differentiation visualised as morphological transformation in the SHE CTA;
 - (b) the acquisition of immortality expressed by unlimited lifespan, an aneuploid karyotype and genetic instability;
 - (c) the acquisition of tumourigenicity closely associated with the in vitro phenotypes of focus formation, anchorage-independent growth in semi solid agar and autocrine factor production; and,
 - (d) full malignancy, when cells are injected into a suitable host.
- 7) Of particular interest is the fact that the CTA has the potential to detect both genotoxic and non-genotoxic carcinogens. The use of two-stage protocols can allow for the distinction between tumour initiators and tumour promoters (OECD, 2007).

3. Overall performance of the CTAs

Level of standardisation of the test method

- 8) As a result of the study, well-described standardized protocols are available for the SHE and the BALB/c 3T3 CTAs. Both of the SHE protocols appear to be transferable (at least to experienced laboratories) and reproducible between laboratories following the ESAC opinion (Annex). For the BALB/c 3T3 CTA, this protocol is due to the introduction of a new and rather specialised statistical method and the data interpretation procedure may require further attention, better definition and refinement of the acceptance and assessment criteria (see ESAC opinion). Moreover, for all three variants of the CTA assay, detailed recommendations have been made by the Validation Management Team (VMT) and the ESAC WG to support further standardisation of the protocols (see also the study reports and the ESAC WG report)(ESAC, 2011) and EURL ECVAM supports these recommendations. Importantly, during the EURL ECVAM study, photo catalogues for each variant of the assay were produced demonstrating typical effects in the three individual CTAs, and these catalogues are expected to support consistent scoring of transformed colonies and foci during training and use of the assay. The recommended protocols and photo catalogues are being published in a Special Issue of Mutation Research on Cell Transformation (Corvi and Vanparys, 2012).
- 9) EURL ECVAM concludes that the current protocols are sufficiently standardized to be recommended for routine use of the CTAs and INVITTOX protocols to which the photo-catalogues will be attached will be prepared.

Reproducibility

- 10) A formal evaluation of reproducibility on the basis of standardized protocols has been conducted in the context of the EURL ECVAM study. The ESAC agreed with the VMT that, the two SHE protocols yielded results that were concordant between laboratories and hence reproducible for the substances tested. Moreover, the reproducibility observed in the study is considered plausible with respect to existing data on the assays. This is based upon a consideration of the extensive body of existing data produced with protocols which the ESAC WG analysis indicated are appreciably similar and based upon the apparent robustness of the SHE assays (e.g. as reviewed in the OECD DRP). These conclusions are substantiated by the body of knowledge related to these assays. In particular by (i), the reproducibility evaluations of similar protocols as reported in the literature (Isfort et al., 1996) and, (ii) the overall evaluation of the data contained in the OECD DRP (OECD, 2007).
- 11) In contrast, evidence from the study supporting reproducibility of the results between laboratories for the BALB/c 3T3 protocol was considered insufficient, as suggested by the need to refine assay assessment criteria and to repeat some experiments to obtain concordant results across laboratories. Also the body of evidence in terms of available data is considerably smaller. It is recommended that the refined BALB/c 3T3 protocol is used in the future to confirm the reproducibility of the assay.
- 12) According to the ESAC, for the three CTAs evaluated, the confidence in the within-laboratory reproducibility was not sufficiently established because of the use of a single compound which was tested coded and non-coded and was further used as the positive control (Annex). Comparing data produced in different labs with the standardized protocols will help to clarify this issue, in particular if standardized data repository formats are applied. The EURL ECVAM study showed that with adequate training and by using photo catalogues of typical transformation images, a sufficient degree of consistency is reached.
- 13) EURL ECVAM concludes that data should be collected from the CTAs to enable further verification of their performance. EURL ECVAM will therefore attach standardized data reporting templates to the INVITTOX protocols that are in preparation.

Transferability

- 14) In general, the proposed test method can be performed in a laboratory that is experienced in routine cell culture techniques (Annex). Considering the nature of the readout (visual scoring), correct scoring of transformed colonies or foci is critical. In the case of the SHE protocols the successful transfer was further supported by the good between-laboratory reproducibility achieved by laboratories that had some experience with CTAs, but not necessarily with the protocol variant considered.
- 15) EURL ECVAM concludes that transferability should not be a problem for laboratories with sufficient expertise in cell culture. However, the standardized protocols should be strictly followed and sufficient training, in particular for correct scoring is essential. The photo catalogues should be used during training and subsequent routine use.

4. Suggested regulatory use of the CTA test methods

Present and past use

- 16) As part of its safety assessment process, submitters have in the past provided to the US-FDA (Food and Drug Administration) results from SHE CTA testing as part of the data submission

package. Such results were considered by FDA as supplemental information in its overall product evaluation (Jacobson-Kram and Jacobs, 2005). However, regulatory agencies in general have been reluctant to unconditionally adopt such assays in their routine safety testing schemes, especially as a full replacement for in vivo carcinogenicity testing (OECD, 2009), due, for the most part, to the lack of formal validation data of such assays. Furthermore, one of the main concerns has been the lack of objective criteria to identify and score transformed colonies and foci which could affect the reliability of the test. However, the CTAs are currently being used by academia, the chemical, agro-chemical, cosmetic, pharmaceutical and tobacco industries, and CRO's. Some current uses of the CTAs include:

- (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 (in pharmaceutical industry);
- (f) to investigate tumor promotion activity (e.g. in agro-chemical and chemical industries); and
- (g) for mechanistic studies of carcinogenicity (e.g. in academia and industry).

17) The CTAs are also used to evaluate certain classes of chemicals that have a low predictive capacity in the traditional in vitro genotoxicity tests (e.g. in chemical and cosmetic industries), like the use of the SHE pH 6.7 CTA for testing aromatic amines.

Possible regulatory use

- 18) Due to the complexity of the events leading to the final adverse effect and based on current opinion, no single in vitro method can provide sufficient information for an unequivocal assessment of the carcinogenicity potential of a substance to satisfy regulatory requirements fully. The CTAs may however provide useful information about possible genotoxic and non-genotoxic carcinogenicity potential for use in conjunction with other data to generate supporting information for hazard identification and risk assessment. The assay may thus be used for these purposes in the context of a weight of evidence approach. Depending on the regulatory context and the extent of other information available from non-testing and testing approaches, it is conceivable that information on the transforming potential of chemicals generated with the CTA may be sufficient for decision-making and may thus in specific cases allow waiving the use of the rodent bioassay. In other cases, the CTA may provide testing data that still require confirmatory testing.
- 19) The possible use of the SHE and BALB/c 3T3 CTAs for regulatory purposes is mentioned in various recent testing strategies including the FDA guidance for integration of genetic toxicology study results for pharmaceuticals (FDA, 2006) and the guidance on information requirements and chemical safety assessment for REACH (ECHA, 2008). Further possible uses of the SHE CTAs are mentioned in the Scientific Committee on Consumer Products (SCCP)'s notes of guidance for

testing For Testing of Cosmetic Ingredients (SCCP 2010), and in the guidance for testing cosmetics (Pfuhrer et al., 2010).

- 20) However, for use of other classes of chemicals than those so far evaluated, it is recommended to verify that the assay is suitable for that specific application (e.g. testing some reference chemicals of interest). Data from the OECD DRP (OECD, 2007) refer to pure chemicals and show that CTAs can be applied to organic and inorganic chemicals and that they can be used to identify genotoxic and non-genotoxic rodent carcinogens. It is plausible that CTAs can be applied to nanoparticles (Ponti et al., 2009). While there is no sufficient evidence on the performance of the assays using mixtures and formulations, there are no scientific reasons to exclude a priori that the CTAs are not suitable assays to test chemical mixtures and formulations (Breheny et al., 2005).
- 21) EURL ECVAM concludes that the CTAs have a promising potential for regulatory use, as the study has addressed two major regulatory concerns, i.e. scoring and protocol standardization. However, before concrete regulatory use beyond weight of evidence approach can be recommended, quality controlled historical data shall be used together with new data generated with the standardised protocols for further establishing the capacity of the CTAs to predict the outcome of the rodent bioassay.

Impact on the three Rs

- 22) The use of the CTAs has the potential to lead to partial replacement and reduction of animal tests (mainly life-time cancer bioassays, OECD 2009) in the regulatory and non-regulatory context. In research, CTAs are, and can be used for research targeting the biological mechanism underlying carcinogenicity. In the regulatory and risk assessment context, the results produced by the CTAs, when considered in conjunction with other available data, may allow concluding on the absence or presence of a carcinogenic chemical hazard. Therefore, high quality CTA information may allow waiving the need to conduct the cancer bioassay. For the SHE CTAs, the use of primary cells from Syrian hamster embryos using pregnant female hamsters may be considered sensitive and appropriate methods of humane killing need to be applied (as outlined in the protocols).
- 23) EURL ECVAM concludes that the CTAs have a potentially significant 3R impact, as partial replacement to the rodent bioassay, however, it should be noted that from a 3R perspective the BALB CTA is considerably more appropriate since it uses a cell line and not primary embryonic hamster cells, as is the case for the SHE CTA.

5. Limitations

- 24) There are no known apparent limitations related to specific classes of chemicals that can be tested with the CTAs (OECD, 2007). Implementation and routine use of the CTAs can be limited by the following factors:

Applicability domain:

- The CTA works for pure chemicals falling into the range of the chemicals used in the EURL ECVAM study and included in the OECD DRP. Outside of this range it is recommended to run some suitable reference chemicals to ensure that the CTA can be used.

- There are no available data for the use of mixtures but it is plausible that the CTAs may be applicable for this use as well. In any case, it is essential to ensure that the test chemical reaches the cells.

Practical aspects:

- Cost: the CTA is a rather costly in vitro test (12-35 k€ per substance), but cheap in comparison with the rodent bioassay (1-1.5 M € per substance).
- Throughput: The CTA requires 2-7 weeks per substance, i.e. it has a low throughput. However, this has to be compared with the 3 years a rodent bioassay requires.
- Complexity: the CTA requires high skills with regard to handling of numerous cell plates simultaneously for a relatively long time period, and in particular scoring. Training and the use of the photo catalogues are essential for overcoming these potential limitations.
- X-ray: for the SHE CTAs, the need for X-ray exposed feeder cells to support the growth of target cells requires the access of an irradiation facility.
- The statistical method proposed for the BALB/c 3T3 CTA is not widely used and requires a certain level of expertise and appropriate IT tools.

6. Follow-up activities recommended by EURL ECVAM

- Prepare and publish INVITTOX protocols with the photo catalogues and the data repository template attached, EURL ECVAM has already embarked on this.
- Collection of high quality historical data for retrospective validation, whenever possible supported by new high-quality data generated with the standardised protocols, suggested to be followed-up by the OECD CTA Expert Group.
- Preparations of a combined draft OECD Test Guideline for the two SHE protocols.
- Research and development should be promoted for: (i) human cell based CTAs; (ii), elucidating tumour promoting mechanisms as manifest in CTAs; and, (iii), increasing throughput and reliability of CTAs, e.g. by automation of the visual scoring, if possible together with cost-reduction.

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ANNEX

ESAC OPINION

**based on the ESAC Peer Review
of a EURL ECVAM-coordinated validation study**

of

**three Cell Transformation Assay (CTA) protocols
for in vitro carcinogenicity testing**

ESAC Opinion Nr.	2011-01
Relevant ESAC request Nr.	2010-02
Date of opinion	18.2.2011

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EXECUTIVE SUMMARY

The potential carcinogenicity of chemicals, pharmaceuticals and food additives is a toxicity effect of great concern. To date, the standard approach to assess carcinogenicity for regulatory purposes is the two year bioassay in rodents (OECD TG 451). Several in vitro alternative methods have been developed. While in vitro genotoxicity tests address only induction of genetic damage as a mechanisms leading to carcinogenicity, in vitro Cell Transformation Assays (CTAs) have been shown to recapitulate stages of in vivo carcinogenesis. Exposure of cultured cells to carcinogenic substances in the CTA can lead to cell transformation involving changes in cell behaviour/phenotype (e.g. proliferation control, altered cell morphology, changed colony growth patterns, anchorage independent growth). Transformed cells can lead to tumour formation in vivo when injected in a suitable host, underlining the biological relevance of the CTAs for carcinogenicity testing.

Continuing previous evaluations of the potential utility of the CTAs for standardised applications including regulatory testing (Combes et al., 1997), EURL ECVAM conducted, from 2005 to 2010, a prevalidation study on three protocol variants of the CTA. Two of the three protocols were based on cells from Syrrian Hamster Embryos (the 'SHE' variant of the CTA), the "SHE pH 6.7" and "SHE pH 7.0" assays. One protocol was based on the BALB/c 3T3 cell line, the "BALB/c assay". The study addressed the three aspects of prevalidation (EC- ECVAM, 1995): protocol refinement, transfer and preliminary assessment of, within the limits of a small scale study, protocol reproducibility within and between laboratories.

Following a request from EURL ECVAM to ESAC in October 2010 (EC-ECVAM 2010d; c.f. Annex 2) for scientific advice on this study, the ESAC set up a Working Group (ESAC WG) charged with the detailed scientific peer review of this prevalidation study.

After careful peer review of the study reports (EC-ECVAM 2010 a-c) and considering the detailed ESAC WG peer review consensus report (EC-ECVAM 2011), the ESAC concludes, in agreement with the Validation Management Team (VMT), that the reliability of the BALB/c protocol was not adequately addressed in the present study.

In contrast, in case of the SHE pH 6.7 and SHE pH7.0, the study data indicate that sufficiently standardised protocols have been produced which appear transferable. While the data relating to the assessment of within-laboratory reproducibility were considered insufficient, the data indicated acceptable between-laboratory reproducibility for the compounds tested and when considering the type of study (i.e. prevalidation).

In view of the possible standardised use of the SHE protocols including for regulatory purposes, the development of a common SHE protocol is recommended describing both pH variants (i.e. pH6.7 and pH7.0). In a next step, test performance needs to be characterised on the basis of a larger set of chemicals covering a broad range of chemical classes and mechanisms of action. This should in particular include the evaluation of more data for non-carcinogens.

However, when planning future activities, the extent to which existing testing information on such chemicals could be used to describe SHE protocol performance for a specific purpose should be considered carefully. Such information may either be published or reside with relevant stakeholders. In the opinion of the ESAC it is conceivable and plausible, considering the extensive body of information available, that historical SHE testing data could be used to arrive at a robust characterisation of the SHE test method performance to support possible standardised use, including for regulatory purposes.

This view is based, firstly, on the apparent robustness of the SHE assays as demonstrated by an analysis of published data in the OECD DRP: the predictions compiled in this report were obtained using non-standardised protocols and showed nevertheless a high degree of concordance. Secondly, an analysis carried out by the ESAC WG (cf. Annex 2 of ESAC WG Report) indicated appreciable

similarity of the historical SHE protocols and the standardised protocols in this study, supporting the possible integration of prospective with existing testing data.

Finally, the ESAC recommends, that future activities towards the possible use of these assays should start with the definition of the intended purpose which is expected to facilitate a detailed and targeted characterisation of test performance (e.g. predictive capacity, limitations) on the basis of new or existing information.

1. Mandate of the ESAC

On its meeting on 12 October 2010, the ESAC was requested by EURL ECVAM (see Annex 2) to conduct a scientific review of an EURL ECVAM-coordinated prevalidation study on three protocols of the Cell Transformation Assay (CTA) for carcinogenicity testing ("CTA prevalidation study"). Two of the three protocols were based on cells from Syrian Hamster Embryos (the 'SHE' variant of the CTA). The two protocols differed mainly with respect to the pH of the medium in which the cells are kept (either pH 6.7 or pH 7.0) and the protocols are hereunder referred to as "SHE pH 6.7" and "SHE pH 7.0" assays. One protocol was based on the BALB/c 3T3 cell line and is referred to hereunder as "BALB/c assay". The study addressed the three aspects of prevalidation (ECVAM 1995): protocol refinement, transfer and preliminary assessment, on the basis of a small scale study, of protocol reproducibility within and between laboratories.

1.1 General objective of the advice to be given by ESAC

Given the background made available in Section 3 of the associated request of EURL ECVAM to ESAC (EC-ECVAM 2010d; Annex 2) and all documentation made available to the ESAC (EC-ECVAM 2010d; Annex 2), the opinion of the ESAC should provide expert advice to EURL ECVAM on a prevalidation study that EURL ECVAM conducted in view of assessing whether three protocols of the Cell Transformation Assay (the variants were the SHE pH6.7, the SHE pH7.0 and BALB/c protocols) have been sufficiently standardised to be transferable to other laboratories and reproducible between different laboratories and may therefore be fit for future use.

In providing this advice, ESAC is requested to take account of the existing information, in particular the OECD DRP (OECD, 2007) and address also the suitability of the three CTA assays/protocols in question to be used as a basis for the development of OECD test guidelines as foreseen by the OECD in the context of the OECD DRP which led to the present study.

1.2 Questions that should be addressed by the ESAC and its Working Group

The specific questions related to this mandate are listed in section 4.2 of Annex 2 (EC-ECVAM 2010d).

1.3 Background to the ESAC Mandate

1.3.1 Background to the study

It is important to note that this EURL ECVAM study, performed from 2005 to 2010, was planned on the background of past EURL ECVAM activities towards the possible use of CTAs for carcinogenicity testing (Combes et al., 1997) but also on the background of the, at the time ongoing, OECD project towards the drafting of a Detailed Review Paper (DRP) on historical CTA data (the "OECD DRP") which took place from 1997 to 2007.

Both projects therefore temporally overlapped to some extent and information on progress in both projects was mutually taken into consideration: while the EURL ECVAM studies relied to a great

extent on reference data compiled in the OECD DRP, the recommendations made in the OECD DRP took already into account possible results of the prevalidation study conducted by EURL ECVAM at that time and aiming at the refinement of selected CTA protocols.

According to the recommendations of the OECD DRP (published in 2007 while the EURL ECVAM study was still ongoing), the present studies (if successful) should contribute to decisions regarding the incorporation of the CTA assays into an OECD test guideline / guidelines. However, the specific purpose of the tests within the framework of an OECD test guideline was not defined in the OECD DRP.

Since the OECD DRP was to provide detailed information on the predictive capacity of the CTAS, the EURL ECVAM study was planned as a complement to this DRP. Consequently, the study focused on the development and evaluation (i.e. transfer/reproducibility) of standardised and well documented protocols that could serve as a possible basis for an OECD test guideline. In contrast, predictive capacity, which typically is addressed to a preliminary extent during prevalidation studies, was not defined as a study objective and there is hence no description of the assays' accuracy (sensitivity/specificity) in the study reports.

However, since test items with reference data of the rodent bioassay (and in part of IARC) were used to assess reproducibility (see figure 1 of ESAC WG report), the predictive capacity of the standardised protocols could be calculated on the basis of the test items assessed (see Annex 1 of the ESAC WG report).

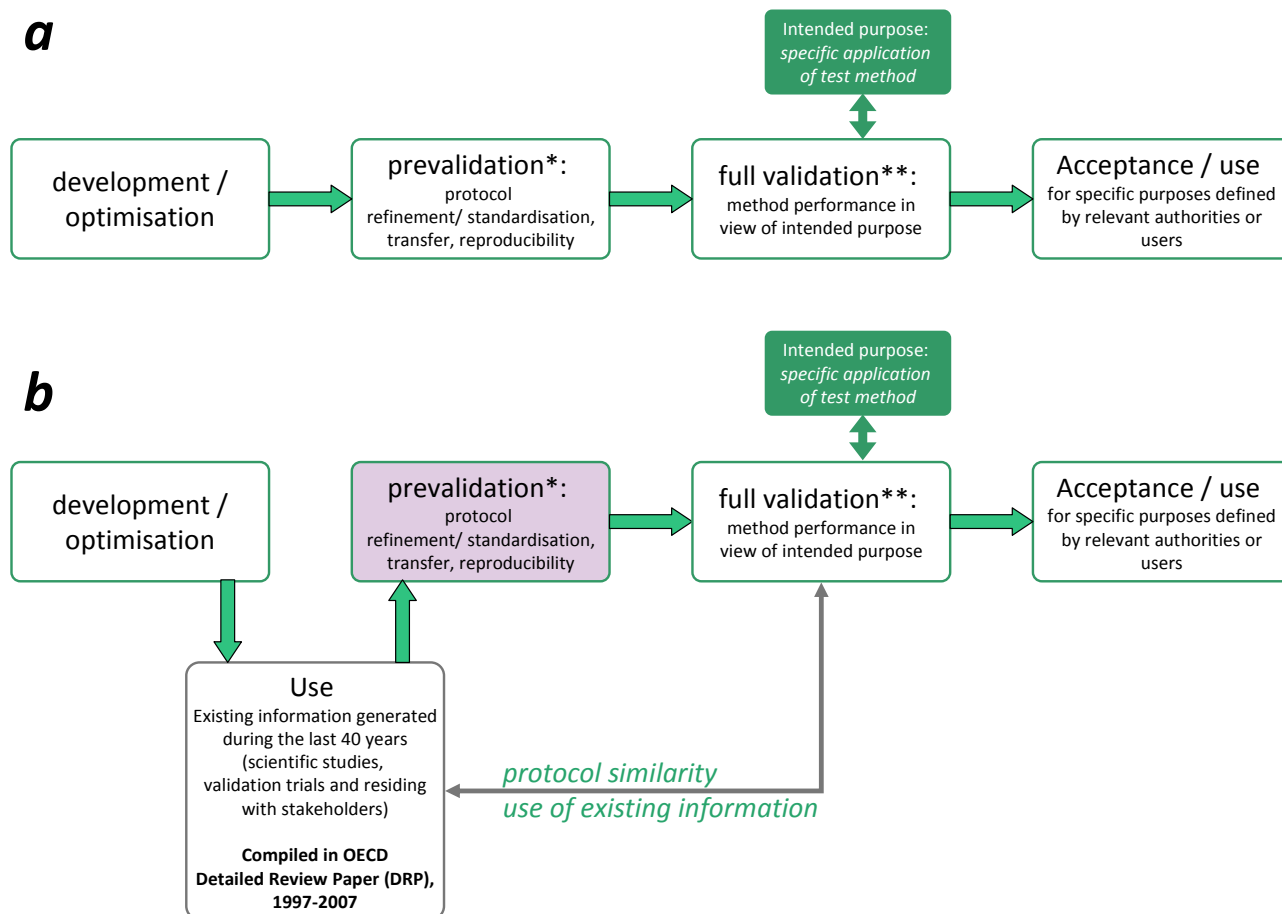
1.3.2 Specifics of this study

From the background to the study outlined in 1.3.1, it is obvious that the current prevalidation study represents a specific case (see figure 1).

Typically, a prevalidation study follows the development / optimisation phase of a new test method (figure 1a). Prevalidation studies are designed to further refine and standardise the test method's protocol (Standard Operating Procedure, SOP) and transfer the SOP from a lead laboratory to other laboratories for an assessment of between-laboratory reproducibility on the basis of a limited set of test items. This is done in order to determine whether the new method and in particular its associated protocol (SOP) is mature and robust enough to merit progressing into a costly and time-consuming full validation study which aims at a performance characterisation of the assay in view of a specific purpose.

In contrast, the present prevalidation study was planned on the background of an extensive body of data, compiled in the OECD DRP (figure 1b). The aim of the prevalidation study was, as for all prevalidation studies, the standardisation of protocols, their transfer to other laboratories and subsequent assessment of protocol reproducibility. However, in view of full test performance characterisation, the existing information and the extent to which it can be used, is an important issue and has been touched on in the recommendations (section 3.15) of this document. An important aspect in this context is the similarity of historical protocols versus protocols as generated during this study. This issue has been addressed, in a preliminary manner, by this review and is presented in more detail in Annex 2 of the ESAC WG report.

Figure 1: Validation flow. (a) Typical flow from development over prevalidation, validation to acceptance and use. **(b)** The situation for the CTAs. The assays have been in use for considerable time and a large body of data has been produced on the basis of the CTAs. The extent to which existing information can be used for validation and performance characterisation needs to be carefully considered.



*) Upon completion, information requirements (modular information) only partly fulfilled.

***) Upon completion, all information requirements are satisfactorily fulfilled in view of purpose

2. Summary of the ESAC Opinion

Taking into account (a) the detailed review of the ESAC WG including the WG's analysis concerning the similarity of existing protocols and those generated during the prevalidation study (EC-ECVAM 2011), (b) the information made available to ESAC by EURL ECVAM including the Validation Study Reports (EC-ECVAM 2010 a-c), (c) the EURL ECVAM request for ESAC advice outlining the ESAC's mandate (EC-ECVAM 2010d; c.f. see Annex 2) the ESAC has the following opinion:

(1) The reliability of the BALB/c protocol was not adequately addressed in the present study. Major concerns are, inter alia, that repeat testing was executed without blinding and also that the 'assay assessment criteria' (allowing translating the measurements into predictions on the transforming potency of substances) require further refinement as already suggested by the VMT coordinating the study. The ESAC appreciates why further refinements were made to the BALB/c protocol as a consequence of testing during the study, but believes that the final protocol having undergone these modifications should be tested in future trials.

(2) Despite shortcomings in study design and execution (see point 6), the study data indicate acceptable reliability for both the SHE pH 6.7 and SHE pH 7.0 protocols for the compounds tested and when considering the type of study (i.e. prevalidation). Reproducibility was assessed by analysing

the concordance of predictions made in the participating laboratories and when comparing the predictions to in vivo carcinogenicity reference data from respected sources reviewed in the OECD Detailed Review Paper (OECD, 2007) including from IARC (IARC 2009), the National Toxicology Program (NTP database available online) and Gold and Zeiger (Gold & Zeiger 1997). Moreover, successful transfer can be concluded from the data on between-laboratory reproducibility.

(3) Although the number of test items used and consequently the chemical domain occupied is rather limited due to a shortcoming of study design (see paragraph 6) and the nature of the study (prevalidation), the ESAC is nevertheless of the opinion that it is plausible that this level of reproducibility would extend to other chemical domains as well. This notion is supported by (a) the apparent similarity of the historical versus the current protocols (c.f. analysis of protocol similarity performed by the ESAC WG, EC-ECVAM 2011) as well as (b) the apparent robustness of the CTA assays in general (see point 6 for more details). Therefore, the SHE assay protocols as standardised during this study are at least sufficiently reproducible, for those chemicals tested, to be considered for use in a regulatory setting.

(4) Despite the small number of items tested (see paragraph 6), these nevertheless reflect a certain range of the possible combinations concerning carcinogenicity and genotoxicity profiles². Briefly, in case of the SHE assays, 4/6 substances tested are carcinogens (benzo(a)pyrene, 2,4-diaminotoluene, o-toluidine, 3-Methylcholantrene), while 2/6 of the substances are non-carcinogens (anthracene and phthalic anhydride). 2/4 of the carcinogenic substances are clearly genotoxic in vivo and in vitro assays (benzo(a)pyrene and 2,4-diaminotoluene), while for one the overall evidence suggests that it is a genotoxic carcinogen despite some inconclusive in vivo genotoxicity data (3-methylcholanthrene)³. The remaining substance (o-toluidine HCL) has equivocal data from in vivo and in vitro genotoxicity tests and could be regarded as a non-genotoxic carcinogen.

(5) While reproducibility was promising for the SHE assays, note should be taken of the fact that robust conclusions on reproducibility cannot be drawn from this prevalidation study alone. The dataset generated during this study is too small to allow sufficient characterisation of key items of test method performance. These include: reproducibility on the basis of a larger and different set of chemicals (including weakly transforming agents), predictive capacity, applicability and possible limitations.

(6) The ESAC noted that there were some shortcomings with regard to study design and execution. Although these did not critically influence the outcome of the study, they nevertheless may constitute deviations from what may be seen as good practice in validation according to EURL ECVAM's approach and International guidance (OECD 2005). These issues include the planning and execution of the selection of reference data (see 3.4), the low number of test items (n=6), even when considering that this is a prevalidation study, the within-laboratory phase (see 3.6), the transferability phase (see 3.7) and the lack of clear provisions for retesting (e.g. number of admissible retests in case of data not fulfilling test acceptance criteria).

(7) In view of possible recommendations concerning future activities towards application of these assays for standardised testing purposes, the ESAC has the following opinion: the next step following on from this prevalidation exercise normally would be a full prospective validation study to characterise the performance of the test methods in view of standardised use including possible regulatory use. Such a study would comprise a set of test substances covering a wide range of chemical classes / possible mechanisms of action (e.g. genotoxic/non-genotoxic) and which is large enough for a statistical evaluation of predictions into two (dichotomous) classes: transforming or

² A description of these mechanism-related issues relating specifically to chemical selection can be found in Vinken et al (2008).

³ Evidence for the genotoxicity of 3-Methylcholantrene is provided for instance in: Moorthy et al., 2007; Xu et al, 2005; Rihn et al., 2000; Moorthy et al., 1993; Bryla & Wyand, 1992; for bibliographic references see section 15.

non-transforming agents. In line with the OECD DRP it is noted by the ESAC that also pharmaceuticals should be included in future analyses of test performance. The ESAC recommends extending this to food additives (e.g. flavours, fragrances and other food supplements).

(8) However, when planning future activities, it should be carefully considered to which extent existing information can be used. In the opinion of the ESAC it is conceivable and plausible that historical SHE testing data could be used to arrive at a robust characterisation of the SHE test method performance to support possible regulatory use. This view is based, firstly, on the apparent robustness of the SHE assays as demonstrated by an analysis of published data in the OECD DRP: the predictions compiled in this report were obtained using non-standardised protocols and showed nevertheless a high degree of concordance. Secondly, an analysis carried out by the ESAC WG (EC-ECVAM 2011: Annex 2) indicated appreciable similarity of the historical SHE protocols and the standardised protocols in this study supporting the possible integration of prospective with existing test data. About 500 coded and un-coded compounds have up to now been tested using the SHE assay by many laboratories. Careful use and reanalysis of these historical data, which include validation studies, may be able to supplement or substitute for a new full prospective validation study of the SHE assay. The ESAC WG draws attention to two publications that highlight the good predictive capacity of the SHE assays (Isfort et al, 1996; Mauthe et al, 2001).

(9) The ESAC is of the opinion, that any further activities towards assay performance characterisation should pay attention to the chemical selection and, if using existing information, to an appropriate description of the toxicity potency of substances, their physicochemical properties, chemical class and mechanism of action in order to define applicability and, in particular, possible limitations of the assays. The ESAC has two concerns with respect to the information compiled in the OECD DRP (OECD, 2007): (a) the lack of explicitness regarding the completeness of the existing data presented and whether data selection criteria based on study quality were defined and had been applied and (b) the lack of a description of the transforming potency of the chemicals analysed. Reproducibility may have been overestimated if most data are based on transforming / non-transforming agents that have generally shown unequivocal results in the past (i.e. no record of discordant results between laboratories). Thus, future activities using existing information should address these issues before new prospective studies are planned and/or before drawing conclusions on test performance on the basis of existing data. Finally, the ESAC recommends, that future activities towards the possible use of these assays should start with the definition of the intended purpose which is expected to facilitate a detailed and targeted characterisation of test performance (e.g. predictive capacity, limitations) on the basis of new or existing information.

3. Detailed opinion of the ESAC

The following paragraphs follow largely the same structure as used in the ESAC WG report.

3.1 Data collection

3.1.1 Reference Data

All reference data used in the prevalidation study are derived from the Detailed Review Paper on Cell Transformation Assays for Detection of Chemical Carcinogens published by the OECD in 2007 (hereunder abbreviated as "OECD DRP").

The current prevalidation studies make use of data from six of the chemicals reported in the OECD DRP as reference data to assess reproducibility as measured through the **concordance of predictions within and between laboratories** and in reference to in vivo carcinogenicity classifications as

published in the OECD DRP, which in turn – in case of these chemicals – refers to classifications by the International Agency for Research on Cancer (IARC), Gold and Zeiger (1997) and the U.S. National Toxicology Program (NTP) database.

Briefly, the predictions generated by the Cell Transformation Assay (CTA) protocols in this study allow classification of the test chemical either as a "transforming agent" or as a "non-transforming agent" (for SHE, based on calculation of "Morphological Transformation Frequency", MTF; for BALB/c 3T3, based on measurement of the number of type III foci). Predictions (transforming / non-transforming agent) obtained in different laboratories were assessed for consistency (concordance) between the laboratories and were compared with in vivo carcinogenicity data as reported in the OECD DRP as the "reference standard".

Moreover, relevant EURL ECVAM workshop reports and recent research papers related to (a) the scoring of observed effects, (b) the mechanistic understanding and (c) between-laboratory reproducibility are discussed in the prevalidation study reports (sections 1.4 to 1.7 pp.10-13 in all three reports), but have not been used as reference data.

For additional details, see section 1.1 of ESAC WG report

3.1.2 Search strategy to retrieve reference data associated with the test items

The ESAC working group (ESAC WG) noted that there was apparently no detailed search strategy established for identifying suitable reference data. However, taking into account that this was a small scale study which did not attempt to define the predictive capacity or the applicability domain of the three CTAs studied, but focused on protocol refinement and reproducibility, this fact was not considered relevant in this context.

For additional details, see section 1.2 of ESAC WG report

3.1.3 Selection criteria for reference data

The ESAC WG noted that there was apparently no detailed set of selection criteria established to reject/accept retrieved data. The OECD DRP was taken as reliable source although it is not clearly described in the OECD DRP how the quality of the data had been controlled.

For additional details, see section 1.3 of ESAC WG report

3.2 Study objective

3.2.1 Clarity of the study objective

The objective of the studies was considered clear and comprehensive: standardisation of CTA protocols and subsequent assessment of these protocols for reproducibility and transferability.

For additional details, see section 2.1 of ESAC WG report

3.2.2 Intended scientific rationale

The intended scientific rationale was explained as far as our current understanding of the cellular mechanisms involved in carcinogenesis (primarily in rodent cells) allows. The reported prevalidation study does not contribute to this scientific understanding, but builds upon evidence (provided primarily by the OECD DRP) that genotoxic as well as non-genotoxic carcinogens induce cell transformation in SHE and BALB/c 3T3 cells while non-carcinogenic substances do not.

For additional details, see section 2.2 of ESAC WG report.

3.2.3 Regulatory rationale

The regulatory rationale remains somewhat open although it is acknowledged by the ESAC WG that even screening data and supportive data within a Weight of Evidence framework can be used for regulatory purposes and may thus constitute a "regulatory rationale". However, recommendations for a more precise definition of the regulatory usability of these tests should have been made in the reports since such use is mentioned as one of the motives for the study (see also Section 15. Recommendations). For additional details, see section 2.3 of ESAC WG report

3.2.4 Appropriateness of study design

Overall, the study design was considered appropriate for assessing the reproducibility and transferability of the standardised protocols, despite shortcomings relating to the design/planning of (1) the test item selection (even when considering that this is a prevalidation study, there are concerns regarding the number representativeness of test chemicals with regard to chemical class and mechanism of action), (2) the within-laboratory variability phase and (3) the transferability phase.

For additional details, see section 2.4 of ESAC WG report

3.2.5 Appropriateness of statistical evaluation

The statistical evaluation of the test data generated during the study appears appropriate. However, the methods of statistical analysis used in the test method procedures (SOP) and the assay assessment criteria need a critical revision.

For additional details, see section 2.5 of ESAC WG report

3.3 Test definition

Overall the tests were adequately defined considering the objective of this study. The overall purpose of the study (development of OECD guidelines) was clear, but the specific purpose of the tests was neither defined by OECD nor by the VMT. Validation is the assessment of the satisfactory performance of a system designed for a specific purpose. Considering this, the absence of a clearer definition of the purpose or possible use of the tests may have influenced the study design, e.g. with respect to the test chemical selection. It is therefore recommended that the intended purpose is sufficiently considered when planning a validation study including a prevalidation exercise. The SOPs were found acceptable provided some minor revisions, including the ones recommended by the VMT.

For additional details, see section 3. of ESAC WG report

3.4 Data quality

3.4.1 Quality of the evaluated data

In general, the data quality was good. Acceptance criteria are broad enough to anticipate different outcomes of the assays when applied properly. Discrepancies were explained.

3.4.2 Sufficiency of the evaluated data in view of the study objective

The data generated and evaluated did not allow for either a proper assessment of within-laboratory reproducibility or the success of the test transfer within the context of a dedicated study phase (transferability) for all three assays.

In contrast, the data produced for assessing between-laboratory reproducibility were considered sufficient for the SHE assays and may moreover be used to infer the success of transfer. The lack of appropriate testing for both modules - within-laboratory reproducibility and transferability - is, however, not considered compliant with standard practice in validation.

3.4.3 Quality of the reference data

The quality of the reference data was assumed sufficient for assessing reproducibility, being based on the OECD DRP and well-regarded sources (i.e. IARC, Gold & Zeiger and NTP database). However, it was noted that there were apparently no provision for assessing the quality of data reported in the OECD DRP and consequently in the present study.

For additional details on data quality, see section 4. of ESAC WG report

3.5 Test items

3.5.1 Sufficiency of the number of evaluated test items in view of the study objective

The number of chemicals (n=6) is judged to be sufficient, with respect to statistical requirements, to assess reproducibility (the main study objective).

However, although it is acknowledged that this is not a full validation study, the number of substances tested is low. More specifically, the number appears low to adequately cover, also for the purposes of a prevalidation study, the range of possible types of chemicals in view of the most prominent underlying mechanism of action (i.e. genotoxic / non-genotoxic) for an endpoint as complex as carcinogenicity.

Thus, the reproducibility assessment is restricted in this case to substances that belong to the same chemical classes (i.e. organic substances; inorganic compounds have not been tested) and which have the same mechanism of action as the ones tested in the prevalidation study. Considering the more advanced SHE assays, 3/6 of the substances were clear genotoxic carcinogens, only 1/6 of the substances was a possible non-genotoxic carcinogen (more details in 3.5.2).

For additional details, see section 5.1 of ESAC WG report

3.5.2 Representativeness of the test items with respect to the applicability domain

It is not the objective of this study to assess the limitations of the tests. However, based on the ESAC WG's analysis (cf. section 12.1; Annex 2) showing the apparent similarity of the historical protocols compared with the protocols from this prevalidation study, it is conceivable that historical data as reported in the OECD DRP could help describe the applicability domain of the test methods in the future.

When considering the test items of this study, it appears that mainly clear positives (transforming agents) and clear negatives (non-transforming agents) have been tested, but no "equivocal" substances (known to be able to lead to discordant results within and between laboratories) which may have challenged the reproducibility of the protocols more adequately.

For the two SHE protocols, a few materials, such as reserpine, cinnamyl anthranilate, or ethylene thiourea, which have given discordant results in previous interlaboratory studies (Tu et al. 1986; Jones et al. 1988) might have helped better define the transferability and reproducibility of these newly standardised protocols.

When considering the complexity of the endpoint (i.e. regarding decisions on "genotoxic/non-genotoxic" and "carcinogenic/non-carcinogenic") as well as considering the small number of test items (n=6), the test items covered a range of the possible combinations of (non)genotoxic and

(non)carcinogenic (cf. ESAC WG report section 5.2, Box 1 'categorisation tree' and Box 2 reproducing table 35, p68 in the SHE pH6.7 assay).

Briefly, in case of the SHE assays, 4/6 substances tested are carcinogens. These are benzo(a)pyrene, 2,4-diaminotoluene, o-toluidine and 3-methylcholanthrene. 2/6 are non-carcinogens when considering reference data from the rodent bioassay (anthracene and phthalic anhydride). 1 of these non-carcinogens (anthracene) is currently not classifiable according to IARC.

Furthermore, 2/4 carcinogenic substances studied are clearly genotoxic in vivo and in vitro assays (benzo(a)pyrene and 2,4-diaminotoluene), while for one the overall evidence suggests that it is a genotoxic carcinogen despite some inconclusive in vivo genotoxicity data (3-methylcholanthrene). The remaining substance (o-toluidine HCL) has equivocal data from in vivo and in vitro genotoxicity tests and could be regarded as a non-genotoxic substance.

As the concept of non-genotoxic carcinogens has only been accepted rather recently and many substances found to be carcinogens have been tested repeatedly for genotoxicity, it is possible that such substances with equivocal genotoxicity data could be regarded as non-genotoxic carcinogens (e.g. o-toluidine).

For additional details, see section 5.2 of ESAC WG report

3.6 Within laboratory reproducibility

The ESAC WG feels that within-laboratory reproducibility was not clearly established due to inadequate study design: only one chemical was tested. Moreover, this substance was the positive control (benzo(a)pyrene for the SHE CTAs, 3-methylcholanthrene for the BALB/c 3T3 CTA). These may, due to their strong transforming potency, lead to an overestimation of reproducibility. While, within laboratory reproducibility for this single substance was, not surprisingly, high in the SHE and BALB/c assays and also between-laboratory reproducibility was good, one chemical only (in addition the PC) cannot be regarded as a sufficient dataset to conclude on this module in compliance with good validation practice.

For additional details, see section 6. of ESAC WG report

3.7 Transfer phase / Transferability

The transfer phase was adequately described and appropriately executed so allowing proper test method conduct in the other laboratories for the subsequent analysis of between laboratory reproducibility. However, how the success of the transfer was assessed and what criteria were used to judge the transfer successful was not clearly described. Moreover, ease of transferability was not assessed through the testing of test items. While this is not a prerequisite for prevalidation studies, the current study nevertheless did not fully address one of its objectives (i.e. assessment of the transferability module).

The success of the transfer programme was not demonstrated in separate experiments. All participating laboratories had some experience with CTAs. Thus, the ease of transferability to a laboratory without any CTA experience was not demonstrated. However, this is in any case not a formal requirement for a prevalidation study (OECD guidance document Nr. 34), and in this case it is noted that successful transfer may be inferred from the good between-laboratory reproducibility.

For additional details, see section 7. of ESAC WG report

3.8 Between-laboratory reproducibility

The final outcome following the implementation of the assessment criteria was considered reproducible for the SHE assays. For the BALB/c 3T3 assay, refinement of the assessment criteria is required.

Between-laboratory reproducibility was assessed through analysis of the concordance of predictions for the six test substances obtained by the involved laboratories. The predictions concerned the classification of test substances as potential transforming agents / non-transforming agents in the CTA assays. The CTA predictions were compared with the reference data associated with the test chemicals. These data are *in vivo* carcinogenicity predictions taken from the OECD DRP report which, for the test chemicals, are based on IARC classifications, the Gold & Zeiger and the NTP databases.

Based on the data generated and reported, the ESAC WG agrees with the VMT that the two SHE protocols yield results which are concordant between laboratories and hence reproducible for the substances tested.

In contrast, evidence supporting reproducibility of the results between laboratories for the BALB/c 3T3 protocol was considered insufficient, as suggested by the need to refine assay assessment criteria and to repeat some experiments to obtain concordant results across the laboratories.

For additional details, see section 8. of ESAC WG report

3.9 Predictive capacity

Although predictive capacity was outside the scope of the study objective, it is noteworthy that the predictions made by the SHE assays for the six chemicals were in most cases correct (6/6 corrections were correct in the SHE pH7.0, while 5/6 were correct in the SHE pH6.7). While the chemicals selected may have a bias towards reproducible results (clear negatives and strong positives), the results are nevertheless reassuring and add to the database of CTA testing data. However, based on the ESAC WG's analysis (cf. section 12.1; Annex 2) showing apparent similarity of the historical protocols with the protocols from the prevalidation study, it is conceivable that historical data as reported in the OECD DRP could help describe the predictive capacity of the SHE test methods in the future.

3.10 Applicability and limitations of the test methods

Since this study is not a full validation study, the assessment of the applicability domain is rather limited. However, based on the ESAC WG's preliminary analysis (cf. Annex 2 of ESAC WG report) showing apparent similarity of the historical protocols with the protocols from the prevalidation study, it is conceivable that historical data as reported in the OECD DRP could help describe the applicability and limitations of the SHE test methods in the future.

3.11 Performance Standards

Not applicable to this study.

3.12 Readiness for standardised use

3.12.1 Readiness for regulatory use

The data generated during this prevalidation study, when viewed on their own, are insufficient to draw conclusions on readiness for regulatory use of the SHE assay protocols, although these showed acceptable reproducibility.

However, an analysis by the ESAC WG identified considerable similarity in the historical SHE protocols and the standardised protocols in this prevalidation study. This supports the view that a substantial amount of the existing testing information from the SHE assays could be used for future considerations on their performance (e.g. predictive capacity, applicability / limitations) required to define their regulatory utility.

This view is further supported by the apparent robustness of the SHE assays as demonstrated in the OECD DRP: the data show considerable concordance with regard to the predictions made even though these older protocols may have differed to some extent and clearly no standardised test procedures had been used. These predictions are moreover relevant when compared with in vivo carcinogenicity data derived from respected sources (e.g. IARC, NTP database).

The ESAC WG, therefore, believes that future activities aimed at more precise definition of test method performance of the SHE assay and possible regulatory utility of the associated SHE protocols can be based on both, prospective testing but also on the analysis of existing historical information (e.g. a meta-analysis using defined search and data selection criteria based on study quality).

The ESAC WG notes, based upon current opinion, that no single method can provide sufficient information for an unequivocal assessment of the carcinogenicity potential of a substance to satisfy regulatory requirements fully. The SHE assays may provide information about possible genotoxic and non-genotoxic carcinogens for use in conjunction with other data (e.g. in the context of a "weight-of-evidence" approach). Some recommendations on possible approaches towards the expansion of the performance characterisation of these methods are made in section 3.15, notwithstanding the fact that the specific regulatory use needs to be defined by the relevant authorities for the purpose in mind.

The study results show that, in contrast to the SHE data, the BALB/c 3T3 protocol still requires optimisation (concerning for example the assessment criteria for the assay) and is at present neither ready to enter full validation nor consideration for regulatory use based on existing information.

For additional details, see section 12.1 of ESAC WG report

3.12.2. Assessment of the readiness for other uses

The ESAC considers the CTAs useful for testing compounds belonging to the same class of chemicals as those used in the reported prevalidation studies (screening purposes) and to generate supporting information for hazard identification and risk assessment (weight of evidence). Moreover, the CTAs will continue to be useful also for mechanistic studies of the transformation process.

For additional details, see section 12.2 of ESAC WG report

3.12.3 Critical aspects impacting on standardised use

The performance characteristics of the SHE methods need to be carefully analysed through prospective testing and/or analysis of existing information (protocol similarity supports the use of historical data) before the SHE protocols can be used in standardised applications (regulatory or non-regulatory). This analysis should include a careful examination of the chemical classes tested. Moreover, some improvement of the SHE protocols such as the development of common protocol for the two pH variants and a better description of some of the protocol steps (cell preparation) should be performed before standardised use is considered.

Concerning the BALB/c 3T3 CTA, further optimisation of the protocol is needed. These modifications, including those suggested by the VMT, should be tested in further trials before standardised use is considered.

For additional details, see section 12.3 of ESAC WG report

3.13 Other considerations

Detailed suggestions on prevalidation conduct and test method SOPs and their use have been made in the ESAC WG report (section 15.).

3.14 Conclusions on the study

As a scientific piece of work the study is impressive and succeeded in generating, in case of the SHE assays, standardised protocols including associated photo catalogues to support consistent scoring. Sufficient between-laboratory reproducibility was demonstrated for these standardised protocols. Moreover, the predictions yielded were in most cases relevant when compared to reference data (rodent bioassay and IARC class, where available).

Despite some shortcomings in study design (mainly with respect to the number of items tested, but also the design of the within-laboratory reproducibility requirement), the study succeeded with respect to its stated goals. The extent to which the various information requirements of this prevalidation study were addressed and fulfilled in view of the objective of the study is summarised in table 1.

For additional details, see section 14 of ESAC WG report

Table 1: Extent to which information requirements were addressed and fulfilled in view of the objective of the prevalidation study

	Protocol standardisation	Within-laboratory reproducibility	Transferability	Between-laboratory reproducibility
SHE pH 6.7	Achieved. Single reporting format would have been beneficial. Development of the photo catalogue is considered a major merit of the study.	Not sufficiently addressed Only one substance tested. In some cases this was the PC (intrinsic propensity to generate reproducible results). Study design of WLR phase was very variable.	Successfully transferred to experienced laboratories. Success of transfer was not tested empirically but can be deduced from information on BLR.	Satisfactorily demonstrated Satisfactory for the substances tested*
SHE pH 7.0	Achieved Single reporting format would have been beneficial. Development of the photo catalogue is considered a major merit of the study.	Not sufficiently addressed Only one substance tested. In some cases this was the PC (intrinsic propensity to generate reproducible results). Study design of WLR phase was very variable.	Successfully transferred to experienced laboratories. Success of transfer was not tested empirically but can be deduced from information on BLR.	Satisfactorily demonstrated Satisfactory for the substances tested*
BALB/c 3T3	Not finalised Assessment criteria were insufficient at outset of study. Further definition suggested by VMT and ESAC WG. These improvements need now to be assessed by testing.	Not sufficiently addressed Only one substance tested. In some cases this was the PC (intrinsic propensity to generate reproducible results). Study design of WLR phase was very variable.	Successfully transferred to experienced laboratories. Success of transfer was not tested empirically but can be deduced from information on BLR.	Promising but insufficient Further refinement of the test method required.

3.15 Recommendations for future work in view of standardised use of the test methods

As briefly outlined in section 1.3, the specific purpose of the CTAs within the framework of, for example, an OECD test guideline was neither defined in the OECD DRP nor by the Validation Management Team (VMT) when planning the current study. This has implications on the future strategy regarding test performance characterisation and possible (regulatory) use of the assays.

Test performance characteristics of a test method (e.g. applicability, limitations, predictive capacity) are to some extent dependent on the specific intended purpose of a test method. Thus, in the absence of a clear intended purpose (e.g. 'use within a test strategy for industrial chemicals to identify positives to waive confirmatory in vivo testing'), the characterisation of test performance remains difficult or on a general level which does not reflect the needs and constraints of possible applications.

Vice versa, the intended regulatory use of test method is easier to define if a precise description of test performance is available. This mutual interdependency of the test method performance characterisation and test method purpose description may, if not addressed in a forward-looking manner, hamper implementation of test methods that appear to be promising for standardised applications including regulatory testing.

In order to avoid such a situation the ESAC recommends that future activities aiming at the potential use of the SHE assays (e.g. OECD test guideline development) should commence with a definition of the intended use of the assays based on the information available (e.g. the current study and the OECD DRP). This will allow a targeted and in-depth description of test performance for the purpose in mind. A strategy towards test method characterisation has been outlined below, using existing information to the extent possible.

3.15.1. Recommendations for the SHE assays

Although the present study succeeded in generating standardised protocols which appear reproducible, the SHE assays are at present not yet ready for regulatory use.

In any case, a revision of the protocols with the aim of incorporating the two SHE cell protocols into one single protocol describing both pH variants (pH6.7 and pH7.0) should be considered. As a minimum, the two protocols should be harmonized as much as possible. Moreover, considering the nature of the readout (visual scoring), it is recommended that the SOPs contain a specific subsection on training and transfer of the assays to naïve laboratories⁴. The definition of proficiency chemicals would support such transfer and help laboratories to assess whether they are capable of conducting the assay.

More importantly, the assays require still a complete description of their performance on the basis of a considerably larger set of chemicals including, if necessary for the envisaged purpose, pharmaceuticals and food additives. Future test substances should include substances that challenge the transferability and reproducibility (i.e. substances with discordant results between laboratories) as well as substances representing a range of possible mechanisms of action. Such performance characterisation should include information on (a) predictive capacity, (b) applicability and, more importantly, limitations of the assays, (c) reproducibility, as well as (d) ease of transferability.

When planning future steps of performance characterisation, the extent to which historical data (including earlier validation studies) can be taken into account, should be carefully considered, as these data could supplement or even substitute for a full new validation study of the SHE assays.

⁴ The word 'naïve laboratories' in the context of validation refers to laboratories that are inexperienced with regard to the use of a specific test method, i.e. they have not conducted this method or variants of it before.

Moreover, the extent to which prospective testing is required to fully characterise the SHE assays will depend on (a) the intended purpose of the assays including a more precise concept concerning their possible regulatory use and (b) the information that may have – in the meantime – become available in the literature.

The following strategy is recommended in order to gain more robust information towards a complete test performance characterisation of the SHE cell assays especially for regulatory purposes:

STEP 1 – Analysis of existing information:

Any future activity towards the standardised / regulatory use of the SHE method should start with a critical analysis of the considerable body of existing testing information (either published or residing with stakeholders).

It is conceivable that, after analysis of the historical datasets (e.g. with respect to chemical class, mechanism of action, carcinogenic potency) test performance can be satisfactorily described through retrospective validation and meta-analysis of data alone, without further need for prospective testing.

Importantly, such an analysis should also go back to original data and not only rely on processed data such as contained in the OECD DRP. Moreover, an evidence-based approach should be employed using a predefined search strategy for retrieving all relevant information and minimum acceptance criteria for data quality.

Should this analysis show that there are gaps in the existing data sets (e.g. with regard to chemical classes, transforming potency), STEP 2 or STEP 3 should be considered.

STEP 2 – Targeted prospective testing of gap substances:

Should the retrospective evaluation of existing information performed in STEP 1 not suffice for a satisfactory description of test performance in view of the intended purpose, a small and targeted prospective study should be conducted providing information on assay performance for those "gap substances" identified in STEP 1. The testing information generated during STEP 2 may then supplement the existing information compiled in STEP 1.

STEP 3 – Full prospective validation:

Should the information generated during STEP 1 and/or STEP2 not suffice for the intended purpose, a full prospective validation study should be conducted using the SHE protocol(s) produced during this prevalidation study but taking into account the improvements of the SOPs as suggested by the EURL ECVAM/ESAC.

3.15.2. Recommendations for the BALB/c 3T3 assay

The BALB/c 3T3 assay is at present and following this prevalidation study not yet ready for regulatory use requires further optimisation, including refinement of the acceptance and assessment criteria.

However, considering the specificities of the BALB/c 3T3 assay (e.g. use of a well established cell line, no feeder cells needed so no irradiation facility required) compared to the SHE assays, further use of the refined protocol is encouraged to expand the data on assay reproducibility and the appropriateness of the assay assessment criteria (including statistical methodology used) for generating relevant predictions. These steps should precede a more complete test performance characterisation which may follow the same strategy as outlined for the SHE assays (see 3.15.1).

For additional details, e.g. concerning suggestions for improvement of the SOPs of the SHE and BALB/c CTAs, see section 15. of ESAC WG report.

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- EC-ECVAM (2010b) Report of the Validation Management Team on the ECVAM prevalidation study concerning the SHE pH7.0 CTA.
- EC-ECVAM (2010c) Report of the Validation Management Team on the ECVAM prevalidation study concerning the BALB/c 3T3 CTA.
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Annex 1 of the ESAC Opinion

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ESAC Secretariat

Dr. Claudius GRIESINGER
Dr. Pascal PHRAKONKHAM (specific support)

Annex 2 of the ESAC Opinion

EURL ECVAM request to ESAC for scientific advice concerning the EURL ECVAM-coordinated CTA prevalidation study

ESAC Request 2010-02

EURL ECVAM Scientific Advisory Committee (ESAC)

EURL ECVAM REQUEST FOR ESAC ADVICE

on an EURL ECVAM-coordinated prevalidation study concerning the protocols of three Cell Transformation Assays (CTA) for carcinogenicity testing

Title page information	
Abbreviated title of ESAC request	ESAC peer review of and ESAC opinion on the EURL ECVAM-led prevalidation study of three cell transformation assays for carcinogenicity testing: 1) SHE pH 6.7 assay 2) SHE pH 7.0 assay 3) Balb/c 3T3 assay.
ESAC REQUEST Nr.	2010-02
Filename	ESAC REQUEST_2010 02_CTA+ESAC-WG-Mandate-approved.doc
Template used for preparing request	EP 2.01
Date of finalising request	2010-09-14
Date of submitting request to ESAC	2010-10-02
Request discussed through	Plenary at ESAC33 at 2010/10/12
Opinion expected at (date)	Through written procedure in January 2011 (before OECD WNT in March)

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1. TYPE OF REQUEST

Request Type	Identify request ("YES")
R1 ESAC Peer Review of a Prevalidation Study or Validation Study	YES
If R1)applies please specify further:	
▶ Prevalidation Study	<p>YES</p> <p>The study is a complement to the recommendations of the OECD Detailed Review Paper on Cell Transformation Assays. The study addressed protocol <u>standardisation</u>, <u>transferability</u> and <u>reproducibility</u> (but not performance) of three protocols of cell transformation assays in view of establishing standardised protocols for future consistent use, e.g. through the development of OECD test guidelines for in vitro carcinogenicity testing.</p>
▶ Prospective Validation Study	
▶ Retrospective Validation Study	
▶ Validation Study based on Performance Standards	
R2 Scientific Advice on a test method submitted to EURL ECVAM for validation (e.g. the test method's biological relevance etc.)	
R3 Other Scientific Advice (e.g. on test methods, their use; on technical issues such as cell culturing, stem cells etc.)	

2. TITLE OF STUDY OR PROJECT FOR WHICH SCIENTIFIC ADVICE OF THE ESAC IS REQUESTED

Prevalidation of three cell transformation assays for carcinogenicity testing:

- 1) SHE pH 6.7 assay
- 2) SHE pH 7.0 assay
- 3) Balb/c 3T3 assay

3. BRIEF DESCRIPTION OF THE STUDY OR PROJECT

1) Background to carcinogenicity testing and available alternative methods

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 (OECD TG 451; Ref. 1).

Several in vitro alternatives have been developed for predicting carcinogenicity. Of these, the in vitro genotoxicity tests address only one mechanism involved in carcinogenicity, the 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: CTAs can detect phenotypic changes of cultured cells as a result of exposure to test materials (i.e. chemicals, products etc.). These phenotypic/morphological changes are a result of the transformation of cultured cells which involves changes in cell behaviour and proliferation control (e.g. altered cell morphology, changed colony growth patterns and anchorage –independent growth). Moreover, transformed cells can evolve to be tumorigenic when injected in a suitable host. Importantly, CTAs are to date the only optimised tests that allow the detection of both genotoxic and non-genotoxic carcinogens. CTAs have been in use for about 40 years and are currently being performed by academia, the chemical, agro-chemical, cosmetic and pharmaceutical industries. CTAs are conducted in-house as well as at contract research organisations to screen for potential carcinogenicity as well as investigate mechanisms of carcinogenicity. While CTAs are currently not used routinely for regulatory testing, they are frequently used for internal (in-house) safety assessment of chemicals, drugs, etc. and are considered to provide additional useful information to the prevailing tests that are used for assessing carcinogenic potential.

2) The OECD Detailed Review Paper as the basis for this prevalidation study

In order to systematically assess the performance of the CTAs, the Organisation for Economic Co-operation and Development (OECD) finalised in 2007 a "Detailed Review Paper on Cell Transformation Assays For Detection of Chemical Carcinogens" (OECD DRP). The OECD DRP focused on the analysis of the predictive capacity (relevance) of three CTAs and addressed also some elements of reliability: (1) the Syrian hamster embryo (SHE) assay, (2) the BALB/c 3T3 assay and (3) the C3H10T1/2 assay. A substantial body of existing and published data was evaluated (SHE n=264 chemicals; BALB/c 3T3 n=184; C3H10T1/2 n=141). The OECD DRP concluded that the performances of two of the assays, the SHE assay and Balb/c 3T3 assay, were sufficiently adequate and should be developed into formal OECD test guidelines (OECD DRP, Ref. 2). Further, the same OECD DRP recommended that although considerable data on the performance of the assays were available, a formal assessment of the assays, in particular focusing on development of a standardised transferable and reproducible protocol, would be important for preparation of such OECD test guidelines.

3) Study objectives and design

Based on the OECD DRP and several EURL ECVAM expert meetings (Combes et al., 1999, Ref. 3), EURL ECVAM initiated a study on the two CTAs found most relevant by the OECD DRP on the basis of the available information, the SHE and the BALB/c 3T3 assays. The study constitutes a complement to the extensive OECD DRP and its conclusions. In agreement with the conclusions of the OECD DRP, EURL ECVAM focused on the development and evaluation of standardised, well-documented protocols that could serve as a basis for an OECD test guideline. In summary, the study was organised and designed taking into account:

- the **objective of the study** to address protocol standardisation and an assessment of transferability and reproducibility of the standardised CTA protocols but not their predictive capacity (which is addressed by the OECD DRP) and
- the **high costs and considerable time** required to perform the assays as well as the limited funding and resources which could be made available by EURL ECVAM.

The study addressed the three classical aspects of Prevalidation: I) protocol refinement/standardisation; II) protocol transfer and III) protocol performance (ECVAM 1995, Ref.4; OECD guidance document on validation, 2005, Ref. 5). With respect to the modular approach of validation (Hartung et al., 2004, Ref. 6), the study assessed information concerning module 1) test

definition, module 2) within-laboratory reproducibility, module 3) transferability, module 4) between-laboratory reproducibility.

The study addressed three variants of CTA protocols: two SHE protocol variants (cells at pH 6.7 and at pH 7.0, respectively) and the CTA based on the Balb/c 3T3 A31 cell line. Each protocol was assessed using six chemicals. In contrast to the Balb/c 3T3 protocol which required more substantial refinement, both SHE protocols were already available in the literature and results of these have been reported in the OECD DRP. Between-laboratory reproducibility was examined in three laboratories except for the SHE 7.0 protocol, where four laboratories were involved.

4) Results and Conclusions

The Validation Management Team (VMT) concluded that, for the SHE pH 6.7 and the SHE pH 7.0 CTAs, the study had demonstrated that standardised protocols were available which could be the basis for future use. These protocols and the assay system itself have been shown to be transferable between laboratories, and are reproducible within- and between-laboratories. For the Balb/c 3T3 method, an improved protocol has been developed, which allowed obtaining reproducible results. However, further testing of the improved Balb/c 3T3 protocol is recommended (see Validation Study Reports, Ref. 7-9). Moreover, the VMT concluded that the appropriate training and the use of the photo catalogues (see Photo Catalogues, Ref. 10-12) developed during the protocol refinement phase, led to a consistent scoring of transformed colonies and foci.

Overall, these results in combination with the extensive database summarized in the OECD DRP support the utility of in vitro CTAs for the assessment of carcinogenicity potential.

References

- 1 OECD TG 451 on rodent long term carcinogenicity testing
- 2 OECD. Detailed Review Paper on Cell Transformation Assays for Detection of Chemical Carcinogens, OECD Environment, Health and Safety Publications, Series on Testing and Assessment, No. 31 (2007).
- 3 Combes R., Balls M., Curren R., Fischbach M., Fusenig N., Kirkland D., Lasne A., Landolph J., LeBoeuf R., Marquardt H., McCormick J., Mueller L., Rivedal E., Sabbioni E., Tanaka N., Vasseur P. and Yamasaki H. Cell transformation assay as predictors of human carcinogenicity. *Alter. Lab. Anim.*, 27 (1999) 745-67.
- 4 ECVAM Prevalidation Task Force Report 1: The role of prevalidation in the development, validation and acceptance of alternative methods. *ATLA* 23, 211-217 (1995)
- 5 OECD Series on Testing and Assessment Number 3: Guidance document on the validation and international acceptance of new or updated test methods for hazard assessment. OECD, Paris, 2005.
- 6 Hartung T., Bremer S., Casati S., Coecke S., Corvi R., Fortaner S., Gribaldo L., Halder M., Hoffmann S., Roi A.J., Prieto P., Sabbioni E., Scott L., Worth A. and Zuang V. A modular approach to the ECVAM principles on test validity. *Alter. Lab. Anim.*, 32 (2004) 467-72.
- 7 VMT-study report SHE pH 6.7
- 8 VMT-study report SHE pH 7.0
- 9 VMT-study report Balb/c 3T3
- 10 CTA SHE pH 6.7 photo catalogue
- 11 CTA SHE pH 7.0 photo catalogue

4. OBJECTIVES, QUESTIONS, TIMELINES

4.1 OBJECTIVE

<p>Objective</p> <p>Why does EURL ECVAM require advice on the current issue?</p>	<p>Given the background in Section 3, the opinion of the ESAC should provide expert advice to EURL ECVAM on three studies that EURL ECVAM conducted in view of assessing whether the three CTA protocols (SHE 6.7; SHE 7.0 and BALB/c) have been sufficiently standardised to be transferable to other laboratories and reproducible between different laboratories and may therefore be fit for future use.</p> <p>In providing this advice, ESAC is requested to take account of the existing information (in particular the OECD DRP) and address also the suitability of the three CTA assays/protocols in question to be used as a basis for the development of OECD test guidelines as foreseen by the OECD in the context of the OECD DRP which led to the present study.</p>
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4.2 QUESTION(S) TO BE ADDRESSED

<p>Questions</p> <p>What are the questions and issues that should be addressed in view of achieving the objective of the advice?</p>	<p>The ESAC is requested to address the following three questions:</p> <p>1) to review whether the study of the three CTAs was conducted appropriately in view of the stated purpose, i.e. of assessing whether the CTA protocols are sufficiently standardised to be transferable and reproducible.</p> <p>In particular the following issues should be addressed:</p> <ol style="list-style-type: none"> 1. Clarity of the definition of the study objective. 2. Appropriateness of the study design (e.g. chemical selection, number of chemicals used, number of laboratories, acceptance criteria). 3. Appropriateness of the study execution (e.g. were there pre-defined acceptance criteria, were these respected? How were exceptions / deviations handled, e.g. retesting?). 4. Appropriateness of the statistical analysis as used in the protocols and for analysing reproducibility. <p>2) to assess whether the conclusions as presented in the Study Reports by the Validation Management Team are justified by the information generated during the study and whether they are plausible with respect to existing information and current views (e.g. literature), in particular the OECD DRP on CTAs.</p> <p>In particular the following issues should be addressed:</p> <ol style="list-style-type: none"> a) Provide a qualitative discussion of the study results/deliverables achieved within the limits of this prevalidation study: <ul style="list-style-type: none"> • Clarity and completeness of the standardised protocol. • Within laboratory reproducibility • Transferability (critical issues and how they were
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	<p>handled)</p> <ul style="list-style-type: none"> • Between laboratory reproducibility <p>b) Provide a clear presentation of the conclusions presented in the study reports</p> <p>c) Evaluate to which extent the conclusions are justified by the study results alone</p> <p>d) Discuss the plausibility of the conclusion in the light of the study results AND existing historical information as available to the EWG (in particular the OECD DRP which led to this study).</p> <p>3) to express its opinion with regard to the question whether the CTA protocols standardised and evaluated during the study could indeed be recommended to serve as a basis for an OECD test guideline on in vitro carcinogenicity testing.</p> <p>In particular the following issues should be addressed:</p> <p>a) Similarity of the standardised protocols with respect to the historical protocols (provide to the extent possible a direct comparison and discuss the relative importance of any difference identified).</p> <p>b) Other critical issues and gap analysis (what further work may be useful/required). Please provide a rationale for your proposed position.</p>
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4.3 TIMELINES

Timelines concerning this request	Timeline	Indication
When does EURL ECVAM require the advice?	Finalised ESAC Opinion required by:	The ESAC opinion should be available latest during the second half of February 2011 (e.g. 20.1.2011). An attempt will be made to finalise the opinion by written procedure.
	Request to be presented to ESAC by written procedure (e.g. <u>due to urgency</u>) prior to the next ESAC	NO
	Request to be presented to ESAC at ESAC plenary meeting	October 2010

5. EURL ECVAM PROPOSALS ON HOW TO ADDRESS THE REQUEST WITHIN ESAC

5.1 EURL ECVAM PROPOSAL REGARDING REQUEST-RELATED STRUCTURES REQUIRED

Specific structures required within ESAC to address the request	Structure(s) required	Required according to EURL ECVAM?
Does the advice require an ESAC working group, an ESAC rapporteur etc.?	S1 ESAC Rapporteur	NO
	S2 ESAC Working Group	YES
	S3 Invited Experts	NO
	Ad S3: If yes – list names and affiliations of suggested experts to be invited and specify whether these are member of the EEP	NO
	If other than above (S1-S3):	NO

5.2 DELIVERABLES AS PROPOSED BY EURL ECVAM

Deliverables	Title of deliverable other than ESAC opinion	Required?
What deliverables (other than the ESAC opinion) are required for addressing the request?	D1 ESAC Rapporteur Report and draft opinion	NO
	D2 ESAC Peer Review Report and draft opinion	YES (EURL ECVAM proposal)
	If other than above (D1-D2):	NO

6. LIST OF DOCUMENTS TO BE MADE AVAILABLE TO THE ESAC

Count	Description of document	Available (YES/NO)	File name
1	VMT-study report SHE pH 6.7	YES	1)ER2010-02_SHE6.7.pdf
2	VMT-study report SHE pH 7.0	YES	2)ER2010-02_SHE7.0.pdf
3	VMT-study report Balb/c 3T3	YES	3)ER2010-02_Balb.pdf

4	CTA SHE pH 6.7 photo catalogue	YES	4)ER2010-02_SHE6.7-photo.pdf
5	CTA SHE pH 7.0 photo catalogue	YES	5)ER2010-02_SHE7.0-photo.pdf
6	CTA Balb/c 3T3 photo catalogue	YES	6)ER2010-02_Balb-photo.pdf
7	OECD. Detailed Review Paper on Cell Transformation Assays for Detection of Chemical Carcinogens, OECD Environment, Health and Safety Publications, Series on Testing and Assessment, No. 31 (2007).	YES	7)ER2010-02_OECD-DRPonCTAs.pdf
8	Combes R., Balls M., Curren R., Fischbach M., Fusenig N., Kirkland D., Lasne A., Landolph J., LeBoeuf R., Marquardt H., McCormick J., Mueller L., Rivedal E., Sabbioni E., Tanaka N., Vasseur P. and Yamasaki H. Cell transformation assay as predictors of human carcinogenicity. <i>Alter. Lab. Anim.</i> , 27 (1999) 745-67.	YES	8)ER2010-02_ECVAM-WS-Report-on-CTAs.pdf
9	OECD TG 451 on rodent long term carcinogenicity testing	YES	9)ER2010-02_OECD-TG-451.pdf
10	ECVAM Prevalidation Task Force Report 1: The role of prevalidation in the development, validation and acceptance of alternative methods. <i>ATLA</i> 23, 211-217 (1995)	YES	10)ER2010-02_ECVAM-prevalidation.pdf
11	OECD Series on Testing and Assessment Number 3: Guidance document on the validation and international acceptance of new or updated test methods for hazard assessment. OECD, Paris, 2005.	YES	11)ER2010-02_OECD-GuidanceDocument.pdf
12	Hartung T., Bremer S., Casati S., Coecke S., Corvi R., Fortaner S., Gribaldo L., Halder M., Hoffmann S., Roi A.J., Prieto P., Sabbioni E., Scott L., Worth A. and Zuang V. A modular approach to the ECVAM principles on test validity. <i>Alter. Lab. Anim.</i> , 32 (2004) 467-72.	YES	12)ER2010-02_ECVAM-modular-approach.pdf

7. TERMS OF REFERENCE OF THE ESAC WORKING GROUP

7.1 ESTABLISHMENT OF THE ESAC WORKING GROUP

During its 33rd meeting on 12 October 2010 the ESAC plenary unanimously decided to establish an ESAC Working Group charged with the detailed scientific review of a study on three Cell Transformation (CTA) protocols.

7.2 TITLE OF THE ESAC WORKING GROUP

Full title:

"ESAC Working Group on the scientific review of 3 Cell Transformation Assay (CTA) prevalidation studies (SHE 6.7, SHE 7.0, BALB)".

Abbreviated title:

7.3 MANDATE OF THE ESAC WG

The EWG is requested to conduct a scientific review of the EURL ECVAM study concerning three protocols of the Cell Transformation Assay (CTA). The review needs to address the questions put forward to ESAC by EURL ECVAM.

The review should focus on the appropriateness of design and conduct of the study in view of the study objective and should provide an appraisal to which extent the conclusions of the Validation Management Team (VMT) are substantiated by the information generated during the study and how the information generated relates to the scientific background available.

7.4 DELIVERABLE OF THE ESAC WG

The ESAC WG is requested to deliver to the chair of the ESAC and the ESAC Secretariat a detailed **ESAC Working Group Report** outlining its analyses and conclusions. A reporting template has been appended (Appendix 1) intended to facilitate the drafting of the report.

The conclusions drawn in the report should be based preferably on consensus. If no consensus can be achieved, the report should clearly outline the differences in the appraisals and provide appropriate scientific justifications.

7.5 PROPOSED TIMELINES OF THE ESAC WG

The Secretariat has proposed timelines which should be agreed upon during the first Teleconference (Item 1 in the table):

Item	Proposed date/time	Action	Deliverable
1	Teleconference 5 November 2010, 14:00 CET	Kick-off teleconference to <ul style="list-style-type: none"> • discuss the mandate, deliverables, timelines, study background • agree on timelines and meeting dates/times (see item2) • distribute (if appropriate) work and agree on further communication (e.g. TCs if required) 	<ul style="list-style-type: none"> • Agreed timelines • Agreed work plan and distribution
2	First ESAC WG meeting in Ispra <ul style="list-style-type: none"> • Option 1 (preferred): 30.11. – 2.12.2010 (3 days) • Option 2: 6.12 - 7.12.2010 (2 days) • Option 3 (least preferred): 14.12. – 16.12.2010 (3 days) 	<ul style="list-style-type: none"> • Discussions of the relevant material and preliminary analysis and possible conclusion. • Identification of unresolved issues and disagreements • Identification of process to resolve possible disagreements • Further work distribution and communication means (e.g. TCs) 	Possibly preliminary versions of <ul style="list-style-type: none"> • ESAC WG Report

		<ul style="list-style-type: none"> Beginning of drafting process of report 	
3	Teleconference 10. January 2011, 14:00 CET	Refresher teleconference (if required) to revisit the status of the work, plan what remains to be done before the second meeting.	
4	Second (last) ESAC WG meeting in Ispra <ul style="list-style-type: none"> Option 1: 12.1. – 14.1.2011 (3 days) Option 2: 19.1. – 21.1.2011 (3 days) 	Finalisation of ESAC WG Report	Final versions of <ul style="list-style-type: none"> ESAC WG Report
5	Tuesday 25.1.2011	Handover of report to ESAC chair and Secretariat	Final edited versions (ready for distribution to ESAC): <ul style="list-style-type: none"> ESAC WG Report

7.6 QUESTIONS WHICH SHOULD BE ADDRESSED BY THE ESAC WG

The ESAC WG is requested to address the three questions posed to the ESAC which have been broken down further in more specific questions by the ESAC chair, the chair of the ESAC WG and the Secretariat (see section 4.2).

When preparing the final ESAC WG report to address these questions, the ESAC WG is requested to use a pre-defined reporting template. This template (see appendix 1) follows EURL ECVAM's modular approach and addresses to which extent the standard information requirements have been addressed by the study. The template allows moreover for addressing the issues specific studies outlined in section 4.2. The Secretariat will provide guidance if necessary.

APPENDIX 1

REPORTING TEMPLATE FOR THE ESAC WG REPORT

The following suggested template follows the EURL ECVAM modular approach and allows at the same time for the description of the analysis and conclusions concerning more specific questions. The template can be used for various types of validation studies (e.g. prospective full studies, retrospective studies, performance-based studies and prevalidation studies). Depending on the study type and the objective of the study, not all sections may be applicable. However, for reasons of consistency and to clearly identify which information requirements have not been sufficiently addressed by a specific study, this template is uniformly used for the evaluation of validation studies.

Text in red is explanatory, not intended to be part of the title.

One section is clearly not applicable to the present CTA study (identified).

1. Data collection

- 1.1 Information / data sources used (e.g. reference data)
- 1.2 Search strategy
- 1.3 Selection criteria applied to the available information

2. Study objective and design

- 2.1 Clarity of the definition of the study objective
- 2.2 Analysis of the scientific rationale provided
- 2.3 Analysis of the regulatory rationale provided
- 2.4 Appropriateness of the study design
(selection of test items, number of test items, number of laboratories, retesting in case of unqualified tests etc.)
- 2.5 Appropriateness of the statistical evaluation
(independence of statisticians, statistical method)

3. Test definition (Module 1)

- 3.1 Quality and completeness of the overall test definition
(test system, protocol, test acceptance criteria etc.)
- 3.2 Quality of the background provided concerning the purpose of the test method
- 3.3 Quality of the documentation and completeness of (a) standardised protocols (SOPs) and (b) prediction models

4. Data quality

- 4.1 Overall quality of the evaluated data
- 4.2 Sufficiency of the evaluated data in view of the study objective
- 4.3 Quality of the reference data for evaluating reliability and relevance⁵

5. Test materials

- 5.1 Sufficiency of the number of evaluated test items in view of the study objective
- 5.2 Representativeness of the test items with respect to the applicability domain

6. Within-laboratory reproducibility (Module 2)

- 6.1 Assessment of repeatability and reproducibility in the same laboratory
- 6.2 Conclusion on within-laboratory reproducibility as assessed by the study

⁵ OECD guidance document Nr. 34 on validation defines relevance as follows: "Description of relationship of the test to the effect of interest and whether it is meaningful and useful for a particular purpose. It is the extent to which the test correctly measures or predicts the biological effect of interest. Relevance incorporates consideration of accuracy (concordance) of a test method."

7. Transferability (Module 3)

- 7.1 Quality of design and analysis of the transfer phase
- 7.2 Conclusion on transferability to a second laboratory as assessed by the study
In particular: where critical issues that may impact on transferability identified or addressed?

8. Between-laboratory reproducibility (Module 4)

- 8.1 Assessment of reproducibility in different laboratories
- 8.2 Conclusion on reproducibility as assessed by the study

9. Predictive capacity (Module 5) N.B. Predictive capacity was outside the scope of the study

- 9.1 Adequacy of the assessment of the predictive capacity in view of the purpose
- 9.2 Overall relevance (biological relevance and accuracy) of the test method in view of the purpose

10. Applicability domain (Module 6) N.B. Since this study is not a full validation study, the assessment of the applicability domain is rather limited

- 10.1 Appropriateness of study design to conclude on applicability domain, limitations and exclusions
- 10.2 Quality of the description of applicability domain, limitations, exclusions

11. Performance standards (Module 7) N.B. Not applicable to the current study.

- 11.1 Adequacy of the proposed Essential Test Method Components
- 11.2 Adequacy of the Reference Chemicals
- 11.3. Adequacy of the defined Accuracy Values

12. Readiness for standardised use

- 12.1 Assessment of the readiness for regulatory purposes
- 12.2. Assessment of the readiness for other uses (in house screening etc.)
- 12.3 Critical aspects impacting on standardised use
- 12.4 Gap analysis
Identify, if appropriate, gaps in the study design and/or execution that impact on the stated study objective or the conclusions drawn.

13. Other considerations

Please address any other consideration you might have in relation to the proposed approach under this section.

14. Conclusions and recommendation

- 14.1 Summary of the study results and conclusions
- 14.2 Extent to which conclusions are justified by the study results alone
- 14.3 Extent to which conclusions are plausible in the context of existing information
- 14.4 Recommendations