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EURL ECVAM Recommendation on the Cell Transformation Assay based on the Bhas 42 cell line

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Contact information
European Reference Laboratory for Alternatives to Animal Testing
Address: Joint Research Centre, Via Enrico Fermi 2749, TP 580, 21027 Ispra (VA), Italy
E-mail: JRC-ECVAM-CONTACT@ec.europa.eu

http://ihcp.jrc.ec.europa.eu/
http://www.jrc.ec.europa.eu/

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EURL ECVAM RECOMMENDATION

on

the Cell Transformation Assay based on the Bhas 42 cell line

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This Recommendation was prepared by the EU Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM), part of the Institute for Health and Consumer Protection (IHCP), Directorate-General Joint Research Centre (DG JRC) of the European Commission.

The Recommendation was drafted on the basis of the ESAC Opinion and ESAC Working Group Report summarising the detailed scientific peer review of the Hadano-coordinated validation study of the Cell Transformation Assay based on the Bhas 42 cell line. The Recommendation further benefitted from comments and suggestions received from members of PARERE (EURL ECVAM's advisory body for Preliminary Assessment of Regulatory Relevance that brings together representatives of Member State regulatory bodies as well as EU agencies including ECHA, EFSA and EMA), and ESTAF (EURL ECVAM's Stakeholder Forum). Input was also provided by partner organisations of EURL ECVAM in the framework of the International Collaboration on Alternative Test Methods (ICATM), and via public commenting.

Coordinator of the evaluation of the test submission was Raffaella Corvi. Coordinator of the ESAC Peer Review and the EURL ECVAM Recommendation was Claudius Griesinger.

Comments or enquiries related to this EURL ECVAM Recommendation should be sent to:

EU Reference Laboratory for Alternatives to Animal Testing
European Commission DG Joint Research Centre,
Institute for Health and Consumer Protection,
Via E. Fermi 2749,
I-21027 Ispra (VA), Italy.
e-mail: JRC-ECVAM-CONTACT@ec.europa.eu

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BACKGROUND TO EURL ECVAM RECOMMENDATIONS

The aim of a EURL ECVAM Recommendation is to provide EURL ECVAM views on the validity of the test method in question, to advise on possible regulatory applicability, limitations and proper scientific use of the test method, and to suggest possible follow-up activities in view of addressing knowledge gaps.

During the development of its Recommendation, EURL ECVAM consults with its advisory body for Preliminary Assessment of Regulatory Relevance (PARERE) and the EURL ECVAM Stakeholder Forum (ESTAF). Moreover, EURL ECVAM consults with other Commission services and partner organisations of the International Cooperation on Alternative Test Methods (ICATM). Before finalising its Recommendations, EURL ECVAM also invites comments from the general public and, if applicable, from the test method submitter.

EXECUTIVE SUMMARY

In 2012, Hadano Research Institute (HRI) Food and Drug Safety Center (FDSC) finalised a validation study on the Bhas 42 Cell Transformation Assay (CTA) for carcinogenicity testing, a short-term CTA using transfected BALB/c 3T3 cells. The study on the Bhas 42 CTA was performed using two similar test method protocols, which differ only in the plate design (6-well method and 96-well method) and the methodology of foci scoring. The study was financed by the New Energy and Industrial Technology Development Organisation (NEDO) in Japan and supervised by a Validation Management Team established by the Japanese Centre for the Validation of Alternative Methods (JaCVAM). At the request of JaCVAM, EURL ECVAM charged ESAC to review the Bhas 42 CTA validation study which finalised its review in December 2012. EURL ECVAM fully endorses the ESAC Opinion (Annex 1) on the overall performance of the Bhas 42 CTA 6-well and 96-well protocols and recommends the following:

(1) Similar to previously validated *in vitro* CTAs (EC EURL ECVAM, 2012a), the Bhas 42 CTA aims at predicting the carcinogenic potential of chemicals. Based on the results of the validation study and other published data, both protocols (the 6-well and the 96-well methods) are considered sufficiently standardised, transferable, reproducible between laboratories and relevant to support the identification of potential carcinogenicity of substances. In addition, the Bhas 42 CTA may provide information on whether substances are tumour initiators or promoters.

(2) Owing to the complexity of the mechanisms of tumour development and the intrinsic limitations of in vitro CTAs (e.g. reductionist in vitro model, limited metabolic capacity), Bhas 42 CTA should not be used as a stand-alone test method, but always in combination with other complementary information sources for the purposes of hazard assessment. A CTA should be used therefore as a component in a Weight of Evidence (WoE) approach or an Integrated Testing Strategy (ITS), including chemical category formation and read-across.

(3) 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 by CTAs may be sufficient for decision-making and may thus in specific cases allow the waiving of the rodent bioassay. However, it should be noted that the use and relevance of CTA data may vary considerably from one sector to another. Therefore, like other CTAs (EC EURL ECVAM, 2012b), the Bhas 42 CTA could contribute to partial replacement and reduction of animal tests for hazard identification and risk assessment in the area of carcinogenicity, depending on other information sources available.

(4) As the Bhas 42 CTA is based on a cell line and, therefore, no experimental animals are required for the preparation of the test system, it has additional benefits from a 3Rs perspective over other validated CTAs such as the SHE CTA, which is based on primary Syrian hamster embryonic cells (EC EURL ECVAM 2012a).

(5) The 96-well format of the Bhas 42 CTA provides the possibility for the development of a high throughput version of this assay. To achieve this, automated imaging and scoring of transformed cells/foci needs to be established.

(6) Since the Bhas 42 CTA can be considered as being reliable and relevant for assessing carcinogenic potential and shows promise for inclusion within WoE and ITS approaches, EURL ECVAM

recommends that an OECD Test Guideline be developed comprising both the 6-well and 96-well plate versions of the protocol.

(7) Respecting the provision of Directive 2010/63/EU (EU, 2010) on the protection of animals used for scientific purposes, before embarking on animal experiments to identify substances with carcinogenicity potential, data from Cell Transformation Assays should be considered in combination with complementary information in order to reduce and possibly avoid animal testing. Among different possible regulatory uses of CTAs, in the context of the REACH Regulation (in agreement with the provision of Annex XI point 1.2; EU, 2006), data from non-standard testing methods such as the Bhas 42 CTA or other CTAs (e.g. SHE CTA; EC EURL ECVAM 2012a), may be used to adapt the standard information requirements in the context of weight-of-evidence judgments.

1. Introduction

In order to assess the performance of CTAs for carcinogenicity assessment, the Organisation for Economic Cooperation and Development (OECD) finalised in 2007 a "Detailed Review Paper on Cell Transformation Assays for Detection of Chemical Carcinogens" (DRP31) (OECD, 2007). The DRP focused on the analysis of biological relevance, predictive capacity and reliability of the Syrian Hamster Embryo (SHE) assay, the BALB/c 3T3 assay and the C3H10T1/2 assay. The DRP31 concluded that a formal assessment of the assays, in particular focusing on development of standardised protocols would be important prior to the development of OECD Test Guidelines. Following the DRP31 recommendation, EURL ECVAM performed a study on the transferability and reliability of the SHE and BALB/c 3T3 assay protocols and issued an EURL ECVAM Recommendation in March 2012 (EC EURL ECVAM, 2012a) in which the development of an OECD Test Guideline of the SHE variants was recommended. This was based on the availability of sufficiently standardised protocols (as shown in the EURL ECVAM study), a satisfactory predictive capacity, and a broad and well described applicability domain (as summarised in DRP31).

In addition to the assays covered by the DRP31, a short-term CTA using transfected BALB/c 3T3 cells (Bhas 42 CTA) has been developed and validated (Validation Study Report: Hayashi *et al.*, 2012). The Bhas 42 CTA was applied to 98 chemicals including carcinogens and non-carcinogens and it was concluded that its performance for the prediction of chemical carcinogenicity is equivalent or superior to those of conventional genotoxicity assays. Also, it was shown that the assay is capable of detecting Ames-negative and Ames-discordant carcinogens in addition to Ames-positive carcinogens (Sakai *et al.,* 2010). The study on the Bhas 42 CTA was performed using two similar test method procedures which differ only in the plate design (one protocol based on 6-well micro-plates, the other one on 96-well micro-plates) and the methodology of foci scoring (Tanaka *et al.,* 2009; Sakai *et al.,* 2011; Hayashi et al., Validation Study Report).

After completion of the study and finalisation of the validation study report in 2012 (EC EURLECVAM, 2012a), EURL ECVAM requested the EURL ECVAM Scientific Advisory Committee (ESAC) at its meeting on 6-7 November 2012 to peer-review the study (Validation Study Report: Hayashi et al.,2012) and to provide an ESAC Opinion (Annex 1), assisted through the establishment of an ESAC Working Group (WG). The ESAC WG report (EC EURL ECVAM, 2012b) and the ESAC Opinion were adopted by ESAC in December 2012.

2. Test method definition

The Bhas 42 CTA test method employs the Bhas 42 cell line as a test system. The Bhas 42 cells are available from the Japanese Collection of Research Bioresources (JCRB) Cell Bank at the National Institute of Biomedical Innovation (Osaka, Japan). Since the Bhas 42 cell line has been genetically modified, it should be noted that its use in the laboratory may be subject to country-specific authorisation provisions.

Biological and mechanistic relevance of the test method

Carcinogenesis is a complex multistage process by which normal cells are transformed into cancer cells characterised by an accumulation of changes at the cellular, genetic and epigenetic level (IARC, 2008). In vitro CTAs such as the Bhas 42 CTA have been shown to recapitulate a multistage process that closely models some stages of in vivo carcinogenesis. Cell transformation in all the systems studied to-date involves changes at each stage in order for progression to the next stage in the transformation process to occur. A minimum of four phenotypic stages appears to be involved in cell transformation. These

include (from primary to fully malignant cells): (1) a block in cellular differentiation, (2) acquisition of immortality, characterised by unlimited lifespan, an aneuploidy karyotype and a decreased genetic stability; (3) acquisition of tumorigenicity, which is closely associated with the in vitro phenotypes of foci formation, anchorage independent growth in semi-solid agar and autocrine growth factor production; and (4) full malignancy, including metastasis when the cells are injected into a suitable host, supporting the biological relevance of *in vitro* transformation to *in vivo* carcinogenicity (LeBoeuf et al., 1999). CTAs are to date the only optimised test methods that have the potential to detect both genotoxic and non-genotoxic carcinogens in vitro as shown in the large body of evidence from empirical testing of a high number of chemicals of various chemical classes (DRP31, OECD 2007).

The Bhas 42 cell line was developed from BALB/c 3T3 cells through transfection with a Harvey rat sarcoma viral mutated oncogene homolog (v-Ha-*ras*) (Sasaki *et al.*, 1988; 1990). Since Ha-*ras* is strongly related to multi-step carcinogenesis, Bhas 42 cells are more predisposed to transformation than the original cells BALB/c 3T3. The assay was first developed by Ohmori et al. (2004) to identify tumour promoters. Subsequently, Asada et al. (2005) modified the Bhas 42 CTA protocol such that it was capable of detecting tumour initiating activity, as well as tumour promoting activity of chemicals.

The current protocols consist of two assay components, the *initiation assay* and the *promotion assay* to detect the tumour-initiating and tumour-promoting activity of chemicals, respectively. In the initiation assay, the cells are inoculated at a low density and treated with test chemical in the beginning of the assay period so that the target cells can undergo cell division several times before reaching contact inhibition of growth, and thus DNA damage is fixed as mutations in the genes. In the promotion assay, the cells are seeded more densely than in the initiation assay and the treatment with a test chemical is started at sub-confluence and continued beyond confluence. A test chemical is positive (i.e. an in vitro cell transforming agent and hence considered a potential carcinogen) in the Bhas 42 CTA if the chemical is positive in one of the two assays, or in both. A negative result in both initiation and promoter assays suggest absence of transforming activity.

Transformation by initiators (genotoxic carcinogens) is caused by accumulation of genetic alterations and generally it is considered that mutation in a single gene is not sufficient to result in transformation. This may explain why Bhas 42 cells, although they have undergone an initial step towards transformation, can still be transformed by genotoxic chemicals through the accumulation of genetic alterations in their genome.

3. Overall performance of the CTA

Level of standardisation of the test method

It can be concluded from the HRI-coordinated study (Validation Study Report: Hayashi et al. 2012) that the protocols for both the 6-well and the 96-well variants of the method are transferable and reproducible between laboratories. Thus the current protocols are sufficiently well described and standardised to be recommended for routine use. EURL ECVAM will facilitate dissemination of the method protocols through its database on alternative methods (http://ecvam-dbalm.jrc.ec.europa.eu). These DB-ALM protocols will provide a comprehensive description of the method together with all the technical details needed by an end-user laboratory to implement it in a self-sufficient manner. The photo-catalogue on transformed foci that can be used as an aid in training of operators will also be included.

Reference data

The validation of alternative methods aiming to predict human health effects relies on an element of calibration of the alternative method's predictive capacity on the basis of trusted reference data. These data are typically derived from animal studies believed to be relevant for the human situation due to their phylogenetic relatedness, biological similarity and systemic integrity. However, reference data derived from animals need to be interpreted with care: studies for various endpoints where human data are available have shown that animal tests are not always fully relevant for the human situation and are not always reproducible – which complicates the determination of unambiguous reference values associated with specific validation chemicals. Such limitations have been described in particular for the current standard model system for human carcinogenicity prediction, the cancer rodent (rat, mouse) bioassay (Gottmann et al., 2001; Haseman et al., 2001; Goodman, 2001; Ennever & Lave, 2003; Alden et al., 2011). This information should be kept in mind when evaluating the performance of alternative methods that have been calibrated to a large extent against these data. In a validation study the use of chemicals classified as clear human carcinogens would be ideal, however this becomes a bottleneck especially when selecting chemicals clearly classified as non-genotoxic carcinogens and non-carcinogens in human. The present study includes, among the rodent carcinogens, some chemicals classified as human carcinogens (IARC classes 1 and 2A).

Reproducibility

The two Bhas 42 CTA protocols yielded results that were concordant within and between laboratories and hence reproducible for the substances tested. This supports the view that the SOP appears to be sufficiently well-developed to allow the generation of reproducible results.

Transferability

The Bhas 42 CTA test method can be transferred to laboratories with sufficient expertise in cell culture. However, to ensure successful establishment of the test method in a naïve laboratory, it is strongly recommended that the standardised protocols be strictly followed and that sufficient training of operators be provided. Training on visual scoring of foci is particularly important for the 6-well protocol which depends on the precise quantification of foci, in contrast to the 96-well plate method protocol where an assessment of the absence or presence of (any number of) foci within a well is sufficient. Moreover, it is advised to make use of the photo-catalogue of transformed foci developed during the validation study when scoring (Hayashi et al., Validation Study Report).

Predictive capacity

The Bhas 42 CTA test method has the potential to support the detection of carcinogenic chemicals, including some non-genotoxic chemicals.

For the 6-well assay (N=12 chemicals) the validation study demonstrated a 100% correlation of the predictions generated *in vitro* with the reference data generated by *in vivo* tests. In ten of the twelve cases all three labs agreed on the prediction ("call"), while in two cases two out of three labs made the correct prediction.

The 96-well assay (N=21 chemicals) revealed an 86% concordance of *in vitro* versus *in vivo* predictions (accuracy) with 83% sensitivity and 89% specificity. In twenty of the cases all labs agreed on the call.

However, in one case, only 3 of the 4 labs characterised the chemical as negative (the “correct” prediction).

Overall, these figures are in agreement with the published data on Bhas 42 performance (Sakai et al., 2010).

Although the method assay is useful for the detection of potential carcinogenicity in general, the performance characteristics of the Bhas 42 CTA test method for predicting ***tumour promoting activities of chemicals*** should be further evaluated, especially regarding the potential of the assay to discriminate between tumour initiators and promoters. While a study by Sakai et al. (2010) on 98 non-coded chemicals produced promising supporting results, the number of chemicals was considered too low to allow for definitive statements with respect to the capability of the assay to detect tumour promoters. Further assessment of the performance characteristics of the assay is desirable to understand the mechanism by which chemicals identified as carcinogens act. However, this uncertainty does not impact on the utility of the assay to predict potential carcinogenicity in vitro.

4. Applicability and limitations

The following factors should be considered when implementing the Bhas 42 CTA method:

- When using classes of chemicals different than those evaluated so far, it is recommended to verify applicability of the assay, for example by testing some chemicals of that class that have good reference data.
- CTAs may be useful to test nanomaterials (Ponti *et al.* 2009) and particulates (Ohmori *et al.*, 2013).
- CTAs may be useful to test complex mixtures and formulations (Breheny *et al.* 2005; Weisensee *et al*., 2013).
- As with many in vitro assays, the limited metabolic activity of the Bhas 42 cell line should be taken into account when interpreting results from the assay, in particular with regard to substances that are identified as negatives (i.e. non-carcinogenic). To address this in the future, the development of ITS should also include information sources that inform on potential biotransformation of the chemicals used.
- The Bhas 42 CTA requires 3 weeks per substance. Although it can be considered a long-term in vitro assay it has a significantly higher throughput compared with the BALB/c 3T3 CTA that requires 6 weeks and the cancer rodent bioassay that requires a minimum of 2 years.

5. Suggested regulatory use

The validation study showed that the Bhas 42 CTA is useful to detect carcinogenicity potential, rather than to specifically discriminate between initiators and promoters as it was initially developed for. Discrimination between genotoxic and non-genotoxic chemicals can be accomplished, however, if Bhas 42 CTA is used in parallel with genotoxicity tests.

Due to the complexity of the events leading to the final adverse effect and based on current scientific consensus, it is unlikely that a single *in vitro* method will provide sufficient information for an unequivocal assessment of the carcinogenicity potential of a substance to fully satisfy regulatory requirements. CTAs, such as the Bhas 42 CTA, 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 that can eventually contribute to the risk assessment. CTAs may thus be used for these purposes in the context of integrated approaches such as a Weight of Evidence (WoE) or Integrated Testing Strategies (ITS), including support for chemical category formation and read-across.

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 by CTAs may be sufficient for decision-making and may thus in specific cases allow waiving of the rodent bioassay (OECD, 2009). In other cases, the CTA may provide testing data that still require confirmatory testing. Therefore, it is recommended that when a CTA is conducted, this should occur before embarking on a rodent bioassay. However, the use and relevance of CTA data may vary from one sector to another (Vanparys et al., 2012). For example, the data requirements but also the information already available will differ substantially between the pharmaceutical and cosmetics sectors (Sistare et al., 2011).

Although the use of the CTA is not currently an explicit regulatory requirement in any regulatory area, its application is mentioned in different contexts in a number of guidance documents including the

guidance on information requirements and chemical safety assessment for REACH (ECHA, 2012), the Scientific Committee on Consumer Safety (SCCS)'s guidance for the testing of cosmetic ingredients (SCCS 2012), the guidance for testing cosmetics (Pfuhler *et al.,* 2010), the EFSA opinion on a strategy for genotoxicity testing (EFSA, 2011), the US FDA guidance for integration of genetic toxicology study results for pharmaceuticals (FDA, 2006) and the ICH S1B Guideline on "Testing for Carcinogenicity of Pharmaceuticals" (ICH, 2009). In the context of REACH, in agreement with the provision of Annex XI point 1.2 of the REACH Regulation (EU, 2006) data from non-standard testing methods, such as the Bhas CTA or other CTAs (e.g. SHE CTA; EC EURL ECVAM 2012a), may be used to adapt the standard information requirements in the context of weight-of-evidence judgments.

Recently, some tiered testing approaches which include the use of the CTAs have been reported (Benigni et al., 2013; Doktorova et al., 2012).

Some current uses of the CTAs include:

- to clarify the relevance of *in vitro* genotoxic positive results for prediction of carcinogenicity by weight of evidence (e.g. in chemical and cosmetics industries);
- 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);
- to screen for non-genotoxic carcinogens (e.g. in agro-chemical industry);
- for read-across purposes (e.g. chemical industry);
- in addition to the other available CTAs, the Bhas 42 CTA could be used in mechanistic studies of carcinogenicity to investigate tumour promotion activity (e.g. in the context of risk/safety assessments in agro-chemical and chemical industries, academia) and to discriminate between tumour initiators and promoters. However, some further work is required in view of assessing this potential use.

Impact on 3Rs of the suggested regulatory use

The Bhas 42 CTA has potential for regulatory use since the validation study undertaken has addressed and resolved a major regulatory concern, *i.e.* protocol standardisation.

The use of cell line based CTAs, such as the Bhas 42 CTA, has the potential to lead to partial replacement and reduction of animal tests in the regulatory and non-regulatory context. In the regulatory assessment context, the results produced by the CTAs when combined with other suitable data may be sufficient to conclude on the absence or presence of a carcinogenic chemical hazard. It is conceivable too that in certain cases high quality CTA information may allow waiving the need to conduct the cancer bioassay.

Therefore when used within integrated approaches, CTAs such as the Bhas 42 CTA have a potential 3Rs impact through partial replacement of the rodent bioassay. Moreover, the Bhas 42 CTA is more favourable than the SHE CTA in the context of 3Rs since it uses a cell line rather than primary cells.

6. Follow-up activities recommended by EURL ECVAM

(1) Preparation of a draft OECD Test Guideline (TG) including both the 6-well and 96-well standardised protocols to support the TG project already on the OECD work plan (lead country Japan). The use of the OECD TG may lead to the availability of additional high quality data to further explore the assay's applicability and predictive capacity, which may allow future revisions of the TG if necessary.

(2) Development of conceptual frameworks to define optimal combinations of CTA data with complementary information sets to address carcinogenicity assessment specific to different sectorial needs. The intrinsic metabolic competence of the test system should also be taken into account when constructing such integrated approaches.

(3) Checking the cell line stability, as part of good cell culture practice, should be continued in order to ensure the reliable and standardised use of the CTA.

(4) Further investigations whether the Bhas 42 CTA is capable of detecting tumour promoting activity, not necessarily detected by genotoxicity assays, as indicated by published literature (Ohmori et al., 2004). This capability would provide useful information on mode of action of carcinogens for risk assessment purposes.

(5) Promotion of further development in view of fully automating the 96-well plate protocol, including the scoring of cell transformation, would increase the throughput and usability of this assay.

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Annex 1 ESAC Opinion

ESAC OPINION

on

a validation study of a Cell Transformation Assay (CTA) for carcinogenicity testing based on Bhas 42 cell line coordinated by Hadano Research Institute (HRI) in collaboration with JaCVAM

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Ispra, 17.12.2012

Summary of the ESAC Opinion

The ESAC was requested to provide scientific advice to EURL ECVAM on two studies that JaCVAM had conducted in view of assessing whether the Bhas 42 CTA protocols (6-well plates and 96-well plates) have been sufficiently validated. Upon the data produced by this study, the ESAC concluded that the primary objectives of demonstrating transferability, reliability and relevance of the Bhas 42 Cell Transformation Assay (CTA) 6-well protocol and the Bhas 42 CTA 96-well protocol were achieved.

Secondary objectives of this were to assess whether the tests could identify non-genotoxic carcinogens and could discriminate between tumour initiating and tumour promoting (TP) compounds. This goal was not fully met because of difficulties in adequately defining the categories; however this difficulty is not unique to the Bhas 42 CTA . Both variations of the test did appear useful in detecting carcinogenic chemicals (including some non-genotoxic chemicals).

While the advantages and potential of these tests were recognized, a number of issues were identified that weakened the statements made by the VMT. These issues were primarily related to the study design; however, they are not expected to change the overall qualifications of the tests in terms of WLR, transferability, BLR and predictive capacity.

In spite of the differences between the applied scoring strategies, both CTA protocols were considered sufficiently standardized for assessing the cell transforming capacity of chemicals,

and these Bhas 42 protocols are suggested to be developed as the basis for an OECD Test Guideline on in vitro carcinogenicity testing.

The ESAC recommends focusing on the preparation of an OECD Test Guideline based on general ability to induce Bhas 42 cell transformation, as part of a toolbox to be used for the identification of carcinogens.

1. Mandate of the ESAC

The ESAC Working Group (EWG) was requested to conduct a scientific peer review of the Bhas 42 CTA Validation Management Team (VMT) report.
The ESAC was requested to address the following three questions (see Annex 2, ECVAM Request for ESAC advice):

1. To review whether the study of the Bhas 42 CTAs was conducted appropriately in view of the stated purpose, i.e. of assessing the CTA test method as a scientific valid method.
2. To assess whether the conclusions as presented in the Study Report are justified by the information generated during the study.
3. To express its opinion whether the CTA protocols are well standardized and could indeed be recommended to serve as a basis for an OECD Test Guideline on *in vitro* carcinogenicity testing

2. Detailed opinion of the ESAC

2.1. The study of the Bhas 42 CTAs was conducted appropriately in view of the stated purpose, i.e. assessing the CTA test method as a scientific valid method

Clarity of the definition of the study objectives.

The primary objective was clearly defined as attempting to demonstrate the transferability, reliability (reproducibility within and between laboratories) and relevance (predictive capacity) of this test system using a set of coded chemicals whose carcinogenicities are known *in vivo*.
The secondary objective is to demonstrate the utility of the test for adoption as an OECD TG that:

- can detect genotoxic and non-genotoxic carcinogens;
- is sensitive to TP;
- can discriminate tumor-initiating and tumor-promoting activity of carcinogens.

Appropriateness of the study design (e.g. chemical selection, number of chemicals used, number of laboratories, acceptance criteria).

The number of chemicals (6-well assay: 8 carcinogens (including TP), 4 non-carcinogens; 96-well assay: 12 carcinogens (including TP), 9 non-carcinogens) was acceptable for assessing the transferability and reliability of the test. While this study produced results supporting Sakai et al. (2010) (98 non-coded chemicals), the number of chemicals was considered too low to allow for strong statements with respect to predictive capacity.
The transferability of the test to inexperienced laboratories was not convincingly proven because the laboratories which were involved in the transfer phase of the study already were experienced in conducting the traditional Balb/c CTA.
The study design to demonstrate the utility of the test for adoption as an OECD TG that can:

- can detect genotoxic and non-genotoxic carcinogens;
- is sensitive to TP;
- can discriminate tumor-initiating and tumor-promoting activity of carcinogens;
- was insufficient in several ways.
- There was no selection procedure described for genotoxic and non-genotoxic carcinogens;
- none of the chemicals were unambiguously identified as genotoxic or non-genotoxic; the number of TP chemicals (N=3) was too low to demonstrate the capability of the test to discriminate tumor-initiating and tumor-promoting carcinogens.
- The number of laboratories involved at any time in the project (≥ 3) was deemed sufficient.

Appropriateness of the study execution (e.g. were there pre-defined acceptance criteria, were these respected? How were exceptions / deviations handled, e.g. retesting?)

For the 6-well assay acceptance criteria were not described in the protocol. Instead, ''low transformation in the positive control and high transformation in the negative control'' were set as criteria for repetition of the test. The acceptance criteria for the 96-well assay (including the cell growth assay) were clear and detailed.

In general the acceptance criteria were followed. However, two incomplete results were produced by the same laboratory for the 6-well assay. With respect to the 96-well assay, two incomplete results were observed. These were due to circumstances affecting compound dosing but beyond the control of the laboratories.

Appropriateness of the statistical analysis as used in the protocols and for analysing reproducibility and predictive capacity.
The statistical analysis is based upon a conservative approach that minimizes the number of false positive results. This approach is acceptable for identification of clear positive and negative compounds, but may be problematic for less unambiguous compounds. The approach is dependent on the number of doses used in the test, with increasing number of doses reducing the statistical significance of the differences between control and test doses.
A minor criticism on the design of the study and one which could possibly affect the outcome of the statistical analysis is that controls are isolated on separate plates, increasing the risk that differences between treatments (representing individual doses and controls) are due to the conditions affecting each specific plate and not to the concentration of the compound.

2.2. The overall conclusions as presented in the Study Report are generally justified by the information generated during the study although issues were identified that weaken the strength of the conclusions.

Qualitative discussion of the study results/deliverables achieved within the limits of this study:

Clarity and completeness of the protocol(s).

(See 2.b)

Within laboratory reproducibility (WLR)

Overall the results were concordant. A different statistical method was used depending on the protocol (6-well or 96-well) which may affect the identification of the weakly positive chemicals. In general the acceptance criteria were followed. However, incomplete results were produced by one laboratory for the 6-well assay. Two incomplete results observed for the 96-well assay were due to circumstances affecting compound dosing but beyond the control of the laboratories.

Transferability

Details of the training program for the 6-well assay were not provided in the report. The transferability of the test to a laboratory not previously experienced in CTA was not demonstrated. The WG considers the test to be transferable to laboratories with experience in cell culturing techniques. For the 96-well assay post-training data were presented, and these data were satisfactory.

Between-laboratory reproducibility (BLR)

The BLR of the 6-well assay was assessed on 12 chemicals (coded) by 3 laboratories. In 9/12 (75%), concordant results (number of foci) were obtained. Caffeine gave incomplete data in one laboratory due to not compliance with the protocol. The two remaining laboratories produced concordant results. It is unclear why results for anthracene and o-toluidine were not reproducible. The results for the positive controls MCA and TPA range between 10 and 50 foci per well. It is not possible to determine from the information given the reason(s) for this spread. The BLR of the 96-well assay was assessed by comparing laboratory results within the validation study. Twenty-two chemicals were tested by 4-6 labs. Results for initiation, promotion and transformation were acceptable (>90% for each endpoint).

Predictive capacity

The predictive capacity of the assays was assessed by comparing the “transformation” results for each of the tested chemicals and comparing that to the reported carcinogenicity *in vivo*. In the vast majority of the cases, all the laboratories agreed on the “transformation” call, but in a few instances, noted below, the judgement of the majority of the laboratory calls was accepted for the comparison.

For the 6-well assay (N=12 chemicals) this comparison resulted in 100% correlation. In 10 of the 12 cases all three labs agreed on the call. However, for anthracene only2 of the 3 labs characterized it as negative (the “correct” call), and for o-toluidine only 2 of the 3 labs characterized it as positive (the “correct” call).

The 96-well assay (N=21 chemicals) revealed an 86% concordance, 83% sensitivity and 89% specificity. In twenty of the cases all labs (n = 2–8) agreed on the call. However, for phenanthrene, only 3 of the 4 labs characterized it as negative (the “correct” call). For common chemicals between the 96-well assay and the 6-well assay, sodium arsenite and o-toluidine were negative in the 96-well assay, with transformed foci on the side of the wells not scored as prescribed by the scoring criteria. Thus the 96-well method seems to need more investigation of scoring before the protocol is finalized.

The conclusions presented in the study report are endorsed by the EWG with minor modifications.

Clarity and completeness of the protocol(s)

The protocol for the 6-well assay lacks sufficient technical detail, while the protocol for the 96-well assay was more detailed. It is the ESAC WGs opinion that a lack of sufficient detail provided

in the scoring atlas may have contributed to the observed differences in the foci scored between experiments and laboratories.

Within laboratory reproducibility (WLR)

As concluded by the VMT, the within-laboratory reproducibility was shown to be satisfactory in all laboratories for the vehicle controls, the positive controls and for the test chemicals.

Transferability

The report did not include a detailed training program, the data produced during training or the quality of these data. Post-training data were only presented for the 96-well assay, and these data were satisfactory.

The transferability of the test to a laboratory not previously experienced in CTA was not demonstrated. The WG considers the test to be transferable to laboratories with experience in cell culturing techniques, but it requires more than one day of training. Considerable training in scoring of foci (and the use of the catalogue) might be required, especially for the 6-well assay. The value of the catalogue could be enhanced with description phrases.

Between-laboratory reproducibility (BLR)

The BLR of the 6-well assay was assessed on 12 chemicals (coded) by 3 laboratories. In 9/12 (75%), concordant results were obtained. Caffeine gave incomplete data in one laboratory due to a lack of compliance with the protocol. The two remaining laboratories produced concordant results. It is unclear why results for anthracene and o-toluidine were not reproducible, although an answer could likely be determined if a re-evaluation of the foci was conducted. The values for MCA and TPA in the participating laboratories ranged from 10 to 50 foci per well which was considered a wide variation. It is not possible to determine from the information provided the reason(s) for this spread. The BLR (20 coded chemicals tested by 4-6 labs) of the 96-well assay was assessed by comparing laboratory results within the validation study. Results for both initiation and promotion were acceptable (95% for initiation and 85% promotion).

A high level of between-laboratory reproducibility was demonstrated for both assays. However, significant quantitative differences (number of foci counted) between laboratories were observed for the positive control of the 6-well assay. It is unclear how these differences would affect the detection of chemicals of low potency.

Predictive capacity

The predictive capacity of the assays was assessed by comparing the “transformation” results for each of the tested chemicals and comparing that to the reported carcinogenicity in vivo. In the vast majority of the cases, all the laboratories agreed on the “transformation” call, but in a few instances, noted below, the judgement of the majority of the laboratory calls was accepted for the comparison. For the 6-well assay (N=12 chemicals) this comparison resulted in 100% correlation. In ten of the twelve cases all three labs agreed on the call. However, for anthracene, only 2 of the 3 labs characterized it as negative (the “correct” call), and for o-toluidine only 2 of the 3 labs characterized it as positive (the “correct” call). The 96-well assay (N=21 chemicals) revealed a 86% concordance, 83% sensitivity and 89% specificity. In twenty of the cases all labs (n = 2 – 8) agreed on the call. However, for phenanthrene, only 3 of the 4 labs characterized it as negative (the “correct” call).For common chemicals between the 96-well assay and the 6-well assay, sodium arsenite and o-toluidine were negative in the 96-well assay, with transformed foci on the side of the wells not scored as prescribed by the scoring criteria. Thus the 96-well method seems to need more investigation of scoring before the protocol is finalized. While this study produced promising results supporting Sakai et al. (2010) (98 non-coded chemicals), the number of chemicals was considered too low to allow for strong statements with respect to its predictive capacity.

3. The CTA protocols were sufficiently standardized in the proposed protocol and could be recommended to serve as a basis for an OECD Test Guideline on *in vitro* carcinogenicity testing

a). In spite of the differences between the applied scoring strategies, both CTA protocols were considered sufficiently standardized for assessing the cell transforming capacity of chemicals, and could be recommended to serve as a basis for an OECD Test Guideline on *in vitro* carcinogenicity testing, in line with the Balb/c 3T3 and SHE CTAs.

It is however recommended to focus on the preparation of an OECD guideline based on general ability to induce Bhas 42 cell transformation, as part of a tool box to be used for cancer risk assessment.

b). Other Issues

Critical issues:

1. There was no clear definition provided in the report for genotoxic and non-genotoxic carcinogens.
2. Presentation of the assays for identification of genotoxic and non-genotoxic carcinogens should be reconsidered until a more systematic review of the genotoxicity and non-genotoxicity of tested compounds is conducted.
3. Compounds with TP activity were few and not well defined.
4. Scoring of foci is critical and the scoring atlas could be improved with textual descriptions of the reasons foci are described as transformed or not.
5. The transferability of the test to a laboratory not previously experienced in CTA is not known.

Gap analysis:

The extent to which discordant results (e.g. o-toluidine) are due to scoring issues in the study is still not known. This should be clarified by a systematic re-evaluation of the existing plates.
Lack of compounds with clear 'initiator' and 'promoter' activity makes it impossible to evaluate the capacity of the tests to discriminate between both sets of chemicals.

3. Informative background to the Mandate and Opinion

The carcinogenic potential of compounds is a crucial aspect in human hazard and risk assessment of chemicals. To date, the standard approach to assess carcinogenicity at a regulatory level is the 2-year bioassay in rodents (OECD TG 451, TG 453).

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.

The Bhas 42 CTA is a sensitive short-term system that has reduced associated cost and labor compared with those associated with the conventional BALB/c 3T3 CTA. The Bhas 42 cells were developed from the BALB/c 3T3 cells through the transfection with v-Ha-*ras* [Sasaki *et al.*, 1988]. Using these cells Asada *et al*. [2005] generated a modified Bhas 42 CTA capable of detecting tumor-initiating activity as well as tumor-promoting activity of chemicals.

This current protocol consists of two assay components, the initiation assay and the promotion assay, proposed to detect the tumor-initiating activity and the tumor-promoting activity of chemicals, respectively. A test chemical is positive in the Bhas 42 CTA if the chemical is positive in one of the two assays. Because of the increased sensitivity of cells to transformation, the assay duration has been reduced to 3 weeks (instead of 6 weeks in the BALB/c 3T3 CTA), and the assay scales such as the dish size, the dish number, the medium volume, the serum concentration in the cultures have been reduced.

The Hatano Research Institute (HRI) undertook a research project financed by the New Energy and Industrial Technology Development Organization (NEDO, Japan) to characterize, evaluate,

refine and validate the Bhas 42 CTA. Under the project, the Bhas 42 CTA has been applied to 98 chemicals including carcinogens and non-carcinogens, and it has been confirmed that its performance for the prediction of chemical carcinogenicity is superior or equivalent to those of conventional genotoxicity assays and the assay is capable of detecting Ames-negative and Ames-discordant carcinogens in addition to Ames-positive carcinogens [Sakai *et al.*, 2010]. In the meantime, JaCVAM has submitted an SPSF for the development of a Test Guideline based on the Bhas 42 CTA to the OECD, which was added to the rolling work-plan in 2007.
The above mentioned studies on the Bhas 42 CTA, development, improvement and application to 98 chemicals, were performed using 6-well plates (6-well method). Meanwhile, the Bhas 42 CTA using 96-well microplates (96-well method) has been developed to be utilized for high throughput automated applications. The assay procedures are fundamentally the same between the 6-well and 96-well methods, except for the statistical methods used.
Two validation studies on the Bhas 42 CTA, a validation study on the 6-well method [Sakai *et al*., 2011] and a validation study on the 96-well method, were carried out under the NEDO project. EURL ECVAM requests ESAC advice on the latter two validation studies which were the JaCVAM-initiative.
Given this background, the opinion of the ESAC should provide expert advice to EURL ECVAM on two studies that JaCVAM conducted in view of assessing whether the Bhas 42 CTA protocols (6-well plates and 96-well plates) have been sufficiently validated.
In providing this advice, ESAC is requested to take account of the information presented in the validation report and address also the suitability of the CTA assays/protocols in question to be used as a basis for the development of an OECD Test Guideline as foreseen by the OECD work plan.

4. References

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Annex 2 EURL ECVAM request for ESAC advice

EURL ECVAM request for scientific advice on validation study of the Cell Transformation Assay (CTA) for carcinogenicity testing based on Bhas 42 cell line, coordinated by Hadano Research Institute in collaboration with JaCVAM

ESAC Request 2012-02

Title page information	
Abbreviated title of ESAC request	Bhas 42 CTA
ESAC REQUEST Nr.	2012-02
Template used for preparing request	EP 2.01
Date of finalising request	2012-08-20
Date of submitting request to ESAC	2012-08-22
Request discussed through	Written procedure
ESAC WG report / opinion expected at (date)	ESAC37, 6-7/11-12

1. TYPE OF REQUEST

Request Type		**Identify request ("YES")**
1. ESAC Peer Review of a Pre-validation Study or Validation Study		**YES**
If 1) applies please specify further:		
Prevalidation Study		
Prospective Validation Study	YES The study addressed protocol standardisation, transferability, reproducibility and predictivity of the Bhas 42 CTA in view of establishing standardized protocols for future consistent use, e.g. through the development of OECD Test Guidelines for *in vitro* carcinogenicity testing.	
Retrospective Validation Study		
Validation Study based on Performance Standards		
2. Scientific Advice on a test method submitted to ECVAM for validation (e.g. the test method's biological relevance etc.)		
3. Other Scientific Advice (e.g. on test methods, me-too tests, performance standards, 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

Validation study of Bhas 42 cell transformation assays for evaluation of carcinogenicity potential

3. BRIEF DESCRIPTION OF THE STUDY OR PROJECT

1) Background to carcinogenicity testing and available alternative methods

The carcinogenic potential of compounds is a crucial aspect in human hazard and risk assessment of chemicals. To date, the standard approach to assess carcinogenicity at a regulatory level is the 2-year bioassay in rodents (OECD TG 451, TG 453).

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 a large amount of data was available. Taking advantage of this, the Organisation for Economic Cooperation and Development (OECD) finalised in 2007 a "Detailed Review Paper on Cell Transformation Assays for Detection of Chemical Carcinogens" [2007, Ref. 1] that focused on the capability of three CTAs (the Syrian hamster embryo cells (SHE), the BALB/c 3T3 and the C3H10T1/2 assays) to predict rodent carcinogenicity. Recently, EURL-ECVAM conducted a prevalidation study of three CTAs using SHE cells at pH 6.7 and pH 7.0 and the BALB/c 3T3 mouse fibroblast cell line with the aim of assessing protocol standardisation, transferability, within- and between-laboratory reproducibility. This study was submitted to ESAC for Peer Review and EURL ECVAM subsequently issued a recommendation on the three CTAs [EURL-ECVAM recommendation and ESAC Opinion; Ref. 2]. Based on these activities, in April 2012 the OECD WNT agreed to proceed with the drafting of a Test Guideline for the SHE CTA.

3) Bhas 42 CTA: Development, characteristics, two assay methods and validation

The Bhas 42 CTA is a sensitive short-term system that has reduced associated cost and labor compared with those associated with the conventional BALB/c 3T3 CTA. The Bhas 42 cells were developed from the BALB/c 3T3 cells through the transfection with v-Ha-*ras* [Sasaki *et al.*, 1988, Ref. 3]. The Bhas 42 cells are transformed by known tumor promoters, including 12-*O*-tetradecanoylphorbol-13-acetate (TPA), okadaic acid and lithocholic acid, without initiating treatment with a known tumor initiator such as 3-methylcholanthrene (MCA) [Omori *et al*., 2004, Ref. 4], and are presumed to be initiated toward transformation by the introduced *ras* sequence [Sasaki *et al.*, 1990, Ref. 5]. Asada *et al*. generated a modified Bhas 42 CTA capable of detecting tumor-initiating activity as well as tumor-promoting activity of chemicals [2005, Ref. 6]. This current protocol consists of two assay components, the initiation assay and the promotion assay, to detect the tumor-initiating activity and the tumor-promoting activity of chemicals, respectively. A test chemical is positive in the Bhas 42 CTA if the chemical is positive in one of the two assays. Because of the increased sensitivity of cells to transformation, the assay duration has been reduced to 3 weeks (instead of 6 weeks in the BALB/c 3T3 CTA), and the assay scales such as the dish size, the dish number, the medium volume, the serum concentration in the cultures have been reduced.

The Hatano Research Institute (HRI) undertook a research project financed by the New Energy and Industrial Technology Development Organization (NEDO, Japan) to characterize, evaluate, refine and validate the Bhas 42 CTA. Under the project, the Bhas 42 CTA has been applied to 98 chemicals including carcinogens and non-carcinogens, and it has been confirmed that its performance for the prediction of chemical carcinogenicity is superior or equivalent to those of conventional genotoxicity assays and the assay is capable of detecting Ames-negative and Ames-discordant carcinogens in addition to Ames-positive carcinogens [Sakai *et al.*, 2010, Ref. 7]. In the meantime, JaCVAM has submitted an SPSF for the development of a Test Guideline based on the Bhas 42 CTA to the OECD,

which was added to the rolling work-plan in 2007.
The above mentioned studies on the Bhas 42 CTA, development, improvement and application to 98 chemicals, were performed using 6-well plates (6-well method). Meanwhile, the Bhas 42 CTA using 96-well microplates (96-well method) has been developed to be utilized for high throughput automated applications. The assay procedures are fundamentally the same between the 6-well and 96-well methods, except for the statistical methods used.
Two validation studies on the Bhas 42 CTA, a validation study on the 6-well method [Sakai *et al*., 2011, Ref. 8] and a validation study on the 96-well method, were carried out under the NEDO project. EURL ECVAM requests ESAC advice on the latter two validation studies which were the JaCVAM- initiative.

4) Study objectives and design

The study objective is to validate the Bhas 42 CTA in formal inter-laboratory studies in order to assess its transferability, the within- and between-laboratory reproducibility and the predictive capacity. The ultimate goal is to demonstrate the utility of the assay for adoption as an OECD Test Guideline and for use as a component of a test battery for the prediction of chemical carcinogenicity.
With respect to the modular approach of validation [Hartung *et al.*, 2004, Ref. 9], the study assessed information concerning module 1) test definition, module 2) within-laboratory reproducibility, module 3) transferability, module 4) between-laboratory reproducibility and 5) predictive capacity.
The study addressed two protocols of Bhas 42 CTA, the 6-well method and the 96-well method. In the validation of the 6-well method, six laboratories joined in the study, twelve chemicals were examined, and each chemical was tested by three laboratories. In the validation of the 96-well method, four laboratories participated, the study was forwarded stepwise, and a total of 25 chemicals were tested including duplicate chemicals between phases. In the assessment of predictivity, in-house data which were obtained by HRI in Japan and had been published [Sakai *et al*., 2010, Ref. 7] were referred to in addition to the results of the validation study.

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4. OBJECTIVES, QUESTIONS, TIMELINES

4.1 OBJECTIVE

Objective *Why does EURL ECVAM require advice on the current issue?*	Given the background in Section 3, the opinion of the ESAC should provide expert advice to EURL ECVAM on two studies that JaCVAM conducted in view of assessing whether the Bhas 42 CTA protocols (6-well plates and 96-well plates) have been sufficiently validated. In providing this advice, ESAC is requested to take account of the information presented in the validation report and address also the suitability of the CTA assays/protocols in question to be used as a basis for the development of an OECD Test Guideline as foreseen by the OECD work plan.

4.2 QUESTION(S) TO BE ADDRESSED

Questions *What are the questions and issues that should be addressed by the ESAC WG?*	The ESAC is requested to address the following three questions: 1) to review whether the study of the Bhas 42 CTAs was conducted appropriately in view of the stated purpose, i.e. of assessing the CTA test method as a scientific valid method. In particular the following issues should be addressed: Clarity of the definition of the study objective. Appropriateness of the study design (e.g. chemical selection, number of chemicals used, number of laboratories, acceptance criteria). Appropriateness of the study execution (e.g. were there pre-defined acceptance criteria, were these respected? How were exceptions / deviations handled, e.g. retesting?). Appropriateness of the statistical analysis as used in the protocols and for analysing reproducibility and predictive capacity. 2) to assess whether the conclusions as presented in the Study Report are justified by the information generated during the study. In particular the following issues should be addressed: Provide a qualitative discussion of the study results/deliverables achieved within the limits of this study: Clarity and completeness of the protocol(s). Within laboratory reproducibility Transferability (critical issues and how they were handled) Between-laboratory reproducibility Predictive capacity Evaluate to which extent the conclusions presented in the study report are justified by the study results and present the working group conclusions. 3) to express its opinion whether the CTA protocols are well standardized and could indeed be recommended to serve as a basis for an OECD Test Guideline on *in vitro* carcinogenicity testing, in addition to: The similarity of the 6 and 96-well protocols. Please provide a recommendation whether they could be merged into one integrated protocol. Other critical issues and gap analysis; if necessary, what further work may be useful/required?

4.3 TIMELINES

Timelines concerning this request	**Timeline**	**Indication**
	Finalised ESAC Opinion required by:	ESAC37, 6-7/11-12
	Request to be presented to ESAC by written procedure (e.g. due to	Yes

When does EURL ECVAM require the advice?	**urgency) prior to the next ESAC**	
	Request to be presented to ESAC at ESAC plenary meeting	

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 *Does the advice require an ESAC working group, an ESAC rapporteur etc.?*	**Structure(s) required**	**Required according to EURL ECVAM? (YES/NO)**
	ESAC Rapporteur	Yes
	ESAC Working Group	Yes, Dr. Rodger Curren and Dr. Erwin Roggen
	Invited Experts	Dr. David Lovell Dr. Edgar Rivedal Dr. Takeki Tsutsui
	If yes – list names and affiliations of suggested experts to be invited and specify whether these are member of the EEP	
	If other than above :	

5.2 DELIVERABLES AS PROPOSED BY EURL ECVAM

Deliverables *What deliverables (other than the ESAC opinion) are required for addressing the request?*	**Title of deliverable other than ESAC opinion**	**Required? No**
	ESAC Rapporteur Report and draft opinion	No
	ESAC Peer Review Report and draft opinion	Yes
	If other than above :	

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

Count	**Description of document**	**Already available? (YES/NO)**	**File name**
1	VMT study report on Bhas 42 CTA	YES	1. Bhas_Validation_Study_Report.pdf
2	Supplier, catalogue number and lot number of test chemicals in the 6-well method validation study.	YES	Annex_1_Ver _3_6-Well_Chemicals_Supplier.pdf
3	Protocol of Bhas42 CTA 6-well method (version 2)		Annex_2_6-Well_Protocol.pdf
4	Photo Catalog for the Judgment of		Annex_3_Bhas_Foci_Catalog.pdf

	Foci in the Bhas 42 CTA		
5	Results (raw data) Submitted from Laboratories in the Validation Study of 6-Well Method		Annex_4_Raw_Data_6-Well[1].pdf
6	Supplier, catalogue number and lot number of test chemicals in the 96-well method validation study		Annex_5_Ver _3_96-Well_Chemicals_Supplier.pdf
7	Protocol of Bhas42 CTA 96-well method (version 2) – prevalidation study.		Annex_6_96-Well_Pre-Val_Protocol.pdf
8	Protocol of Bhas42 CTA 96-well method (version 3) – phase 1		Annex_7_96-Well_Phase_I_Protocol-3.pdf
9	Protocol of Bhas42 CTA 96-well method (version 4) – phase 2		Annex_8_96-Well_Phase_II_Protocol-3.pdf
10	Results (raw data) Submitted from Laboratories In the Pre-validation Phase of Validation Study of 96-Well Method		Annex_9_Raw_Data_96-Well_Pre-Val[1].pdf
11	Results (raw data) Submitted from Laboratories In the Phase I of Validation Study Of 96-Well Method		Annex_10_Raw_Data_96-Well_Phase_I[1].pdf
12	Results (raw data) Submitted from Laboratories In the Phase II of Validation Study of 96-Well Method		Annex_11_Raw_Data_96-Well_Phase_II[1].pdf
13	Recommended Protocol for the Bhas 42 Cell Transformation Assay (2012.7.27)		Annex_12_Bhas_CTA_Recommended_Protocol.pdf
14	Transformation frequency of negative and positive controls		Annex_13_Transformation_Frequency_Of_Controls.pdf
15	Chemical properties and classes of 12 coded test chemicals and positive controls for the 6-well method validation study		Annex_14_6-Well_Chemical_Properties.pdf
16	Chemical properties and classes of test chemicals for the pre-validation phase, phase I and phase II of 96-well method validation study		Annex_15_96-Well_Chemical_Properties.pdf
17	Sakai, A. et al (2010) A Bhas 42 cell transformation assay on 98 chemicals: The characteristics and performance for the prediction of chemical carcinogenicity. Mutation Research 702 (2010) 100–122.		Annex_16_Article_On_98_Chemicals.pdf

Note: Other documents that may be useful for conducting the peer review (e.g. manuscripts) are available at EURL ECVAM upon request.

7. TERMS OF REFERENCE OF THE ESAC WORKING GROUP

7.1 ESTABLISHMENT OF THE ESAC WORKING GROUP

During its 36th meeting on 21 March 2012 the ESAC plenary unanimously decided to establish an ESAC Working Group charged with the detailed scientific review of a study on the Bhas 42 CTA.

7.2 TITLE OF THE STUDY OR PROJECT

Full title:
Validation study of BHAS 42 cell transformation assays for evaluation of carcinogenicity potential

Abbreviated title:
Bhas 42 CTA

7.3 MANDATE OF THE ESAC WG

The EWG is requested to conduct a scientific peer review of the Bhas 42 CTA test method. The peer review needs to address the questions in Section 4.2 of this request to ESAC by EURL ECVAM. A general template for reporting is attached as Annex 1.

7.4 REQUESTED DELIVERABLES OF THE ESAC WG

The ESAC WG is requested to deliver to the chair of the ESAC the following two documents:
Draft ESAC WG Report detailing its analyses and conclusions
Draft ESAC Opinion outlining the key findings and recommendations
The conclusions drawn in the report should be based preferably on consensus. If no consensus can be achieved, the draft Report and Opinion 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	1-2 October 2012	Meeting of the EWG	Develop the EWG Draft Report and Opinion
2	6-7 November 2012	ESAC37	Deliver the final EWG Report and the Draft Opinion

END OF EURL ECVAM RECOMMENDATION

European Commission
EUR 26374– Joint Research Centre – Institute for Health and Consumer Protection

Title: EUR 26374 - EURL ECVAM Recommendation on the Cell Transformation Assay based on the Bhas 42 cell line

Luxembourg: Publications Office of the European Union

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Abstract

The carcinogenic potential of compounds is a crucial aspect in human hazard and risk assessment of substances. Among the various alternatives developed for carcinogenicity prediction, the cell transformation assays (CTAs) have been shown to closely model some key stages of the in vivo carcinogenesis process. Similar to previously validated in vitro CTAs, the CTA in Bhas 42 cells aims at predicting carcinogenic potential. Based on the results of a validation study coordinated by Hadano Research Institute (HRI) Food and Drug Safety Center (FDSC) and other published data, the Bhas 42 CTA protocol (including the 6-well and 96-well plate versions) was considered to be sufficiently standardised, transferable, reproducible between laboratories and relevant to support the identification of potential carcinogenicity of substances. Following independent scientific peer review by the EURL ECVAM's Scientific Advisory Committee (ESAC) and having considered the input from regulators, stakeholders, international partners and the general public, EURL ECVAM concluded that the CTA in Bhas 42 cells shows promise for inclusion within weight of evidence or integrated testing strategy approaches to assess carcinogenic potential or to support chemical category formation and read-across. Thus EURL ECVAM recommends that an OECD test Guideline be developed. In addition, further investigations on the capability of the assay to detect tumour promoters would provide useful information on mode of action of carcinogens for risk assessment purposes.

LB-NA-26374-EN-N

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