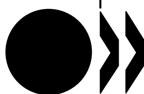


Unclassified **ENV/JM/MONO(2016)1**

Organisation de Coopération et de Développement Économiques
Organisation for Economic Co-operation and Development **08-Jan-2016**

English - Or. English

ENVIRONMENT DIRECTORATE
JOINT MEETING OF THE CHEMICALS COMMITTEE AND
THE WORKING PARTY ON CHEMICALS, PESTICIDES AND BIOTECHNOLOGY

ENV/JM/MONO(2016)1
Unclassified

GUIDANCE DOCUMENT ON THE IN VITRO BHAS 42 CELL TRANSFORMATION ASSAY

Series on Testing & Assessment
No. 231

JT03388750

Complete document available on OLIS in its original format
This document and any map included herein are without prejudice to the status of or sovereignty over any territory, to the delimitation of international frontiers and boundaries and to the name of any territory, city or area.

English - Or. English

OECD Environment, Health and Safety Publications

Series on Testing and Assessment

No. 231

GUIDANCE DOCUMENT ON THE IN VITRO BHAS 42 CELL TRANSFORMATION ASSAY

IOMC

INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS

A cooperative agreement among FAO, ILO, UNDP, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD

Environment Directorate
ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT
Paris 2016

About the OECD

The Organisation for Economic Co-operation and Development (OECD) is an intergovernmental organisation in which representatives of 34 industrialised countries in North and South America, Europe and the Asia and Pacific region, as well as the European Commission, meet to co-ordinate and harmonise policies, discuss issues of mutual concern, and work together to respond to international problems. Most of the OECD's work is carried out by more than 200 specialised committees and working groups composed of member country delegates. Observers from several countries with special status at the OECD, and from interested international organisations, attend many of the OECD's workshops and other meetings. Committees and working groups are served by the OECD Secretariat, located in Paris, France, which is organised into directorates and divisions.

The Environment, Health and Safety Division publishes free-of-charge documents in eleven different series: **Testing and Assessment; Good Laboratory Practice and Compliance Monitoring; Pesticides; Biocides; Risk Management; Harmonisation of Regulatory Oversight in Biotechnology; Safety of Novel Foods and Feeds; Chemical Accidents; Pollutant Release and Transfer Registers; Emission Scenario Documents; and Safety of Manufactured Nanomaterials.** More information about the Environment, Health and Safety Programme and EHS publications is available on the OECD's World Wide Web site (http://www.oecd.org/chemicalsafety/).

This publication was developed in the IOMC context. The contents do not necessarily reflect the views or stated policies of individual IOMC Participating Organisations.

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

This publication is available electronically, at no charge.

Also published in the Series on Testing and Assessment link

**For this and many other Environment,
Health and Safety publications, consult the OECD's
World Wide Web site (www.oecd.org/chemicalsafety/)**

or contact:

**OECD Environment Directorate,
Environment, Health and Safety Division
2 rue André-Pascal
75775 Paris Cedex 16
France**

Fax: (33-1) 44 30 61 80

E-mail: ehscont@oecd.org

© OECD 2016
Applications for permission to reproduce or translate all or part of this material should be made to: Head of Publications Service, RIGHTS@oecd.org, OECD, 2 rue André-Pascal, 75775 Paris Cedex 16, France

FOREWORD

This document presents guidance for conducting the *in vitro* Bhas 42 Cell Transformation Assay (Bhas 42 CTA).

This document, as well as a similar document on the Syrian Hamster Cells Transformation Assay (SHE CTA) , was preceded by the development of the Detailed Review Paper (DRP) 31 on “Cell Transformation Assays for Detection of Chemical Carcinogens” (OECD, 2007), pre-validation study led by ECVAM, ESAC peer review, and then from 2011 by work aimed at the development of a Test Guideline. Despite support from some countries, concerns were expressed by others regarding the CTAs, and the approval of draft TGs was considered premature.

In November 2014, the Joint Meeting discussed options for moving forward in the area of non-genotoxic carcinogenicity under the Test Guidelines Programme. The Joint Meeting advised 1) to proceed with the development of guidance documents on the SHE CTA and Bhas 42 CTA, mainly to describe the test procedures, and 2) to develop a guidance document at the OECD level outlining a conceptual framework for the identification of non-genotoxic carcinogens for priority setting (work underway).

This document has been through three WNT commenting rounds (from January to September 2015). Since all countries who commented either indicated approval or indicated that their comments did not impede approval of the document and were only of editorial nature, this document was approved by written procedure.

This document is published under the responsibility of the Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology

GUIDANCE DOCUMENT ON THE IN VITRO BHAS 42 CELL TRANSFORMATION ASSAY

PURPOSE

1. The purpose of this Guidance Document (GD) is to allow the scientific and regulatory communities to use the described method as part of a weight of evidence approach in the testing of substances for carcinogenic potential.

Background

2. Since DNA damage and mutation are known to be initiating events for carcinogenesis, several short-term *in vitro* and *in vivo* genotoxicity tests are commonly used to predict chemical carcinogenicity. However, not all carcinogens are known to be genotoxicants. Furthermore, animal carcinogenesis studies have demonstrated that carcinogenesis is itself a multi-stage process comprised of distinct initiation, promotion and progression events (1, 2). In addition, it has long been known that cell transformation assays can simulate *in vivo* initiation and promotion stages of carcinogenesis and therefore detect chemicals known to have initiating activity (tumor initiators, most of which are genotoxic carcinogens) and/or promoting activity (tumor promoters, most of which are non-genotoxic carcinogens) (3, 4).

3. *In vitro* cell transformation refers to the induction, in cultured cells, of phenotypic alterations that have long been considered alterations associated with cells exhibiting neoplastic potential *in vivo* (5, 6). Transformed cells with the characteristics of malignant cells have the ability to induce tumors in susceptible animals (7, 8, 9); this supports the practice of using specific phenotypic alterations *in vitro* as criteria for predicting carcinogenic potential *in vivo.*

4. The Bhas 42 cell line (Japanese Collection of Research Bioresources Cell Bank No.: JCRB0149) was derived as a clone formed by the stable transfection of the v-Ha-*ras* oncogene into the BALB/3T3 A31-1-1 (Japanese Collection of Research Bioresources Cell Bank No.: JCRB0601) cell line (10). Similar to the parental BALB/3T3 A31-1-1 cells, untransformed Bhas 42 cells grow to confluence forming a density-dependent contact-inhibited monolayer and subcutaneous transplantation of isolated cell populations derived from such confluent monolayers have been shown to be non-tumorigenic in nude mice. However, after exposure to carcinogenic stimuli, such cells become morphologically altered and form discrete anchorage-independent altered colonies, referred to as transformed foci, atop the confluent monolayer. Such transformed foci are capable of producing tumors *in vivo* (11). Transformation frequency can be quantified, using morphological criteria to enumerate parameters of transformed foci among Bhas 42 cells subjected to carcinogenic insult. This measure of transformation frequency, which can be extrapolated to an assessment of carcinogenic potential, constitutes the basis of the Bhas 42 cell transformation assay (CTA).

5. Unlike target cells used in other CTAs, Bhas 42 cells are considered to be initiated cells since pretreatment with a tumor initiator is not needed for subsequent induction of cell transformation by promoters in the Bhas 42 CTA (10). These data suggest that the transfected v-Ha-*ras* gene elicits the initiation process that would otherwise be induced by a chemical initiator (11). Thus, the transformation response of such initiated Bhas 42 cells may represent a late stage of the carcinogenic process, whereas the transformation responses of immortalized cells (uninitiated cells, *e.g.* BALB/3T3 A31-1-1 and C3H10T1/2 cells) and primary cells (uninitiated cells having a limited in vitro lifespan, *e.g.* Syrian hamster embryo cells) may represent prior stages (12).

6. Although the Bhas 42 cells have undergone an initial step towards transformation, they still retain the capability to respond to single treatments with tumor initiators (13) in similar ways as other CTAs. Other advantages of the Bhas 42 CTA compared to other CTAs include: a) Bhas 42 cells are especially responsive to chemical carcinogens and are readily transformed by such agents, resulting in relatively high transformation frequencies, b) the latency period of responsiveness is relatively brief, c) the number of culture vessels necessary for a given assessment of a chemical's carcinogenic potential is reduced (14,15).

7. This GD provides an *in vitro* procedure using the Bhas 42 CTA, which can be used for hazard identification of potential carcinogenicity of chemicals with initiating and/or promoting activity. The test method described is based upon the protocols of previous reports and that published by the EURL ECVAM (16, 17, 18).

8. The current protocol for the Bhas 42 CTA consists of two test components, one for examining initiating activity and one for examining promoting activity of chemicals (16, 17, 18). The initiation and promotion tests can be performed independently and the two protocols are slightly different. It is acknowledged that mutation induced by chemical insult is fixed after several cell replication cycles (19, 20). Thus, in the initiation test the cells are treated at the beginning of growth phase to allow for several cell cycles necessary for fixation of the induced DNA damage. On the other hand, it is known that cell-to-cell communication plays a key role in promotion (21, 22). Therefore, in the promotion test the cells are repeatedly treated at stationary phase to provide a growth advantage for anomalous cells.

9. In this GD, two assay formats are described, one using 6-well plates and the other using 96-well plates (16, 17, 18). After initial development of the Bhas 42 CTA 6-well format, the assay was adapted to a 96-well format that was designed for high-throughput analyses (23). Although the number of cells plated and expression of transformation frequency differ between the 6-well and 96-well formats, the overall results obtained are similar and the formats can be used interchangeably (23).

10. Several comprehensive studies were performed to assess the reliability and predictive capacity of the Bhas 42 CTA. These included (a) an extensive analysis of 98 chemicals (24), (b) a multi-laboratory collaborative study (25), (c) a prevalidation study (26) in the 6-well format, and (d) two international validation studies (16, 17). For the latter, the Validation Advisory Committee and the Validation Management Team were comprised of international experts from the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM), the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), the NTP Interagency Center for the Evaluation of Alternative Toxicological Method (NICEATM) and the Japanese Center for the Validation of Alternative Methods (JaCVAM). EURL ECVAM reviewed these studies and addressed the transferability, reproducibility and relevance of the Bhas 42 CTA protocol (27).

11. Test results derived from the Bhas 42 CTA are expected to be used as part of a testing strategy (rather than a stand-alone assay) and/or in a weight-of-evidence approach to predicting carcinogenic potential. When employed in combination with other information such as genotoxicity data, structure-activity analysis and pharmaco-/toxicokinetic information, CTAs in general and the Bhas 42 CTA specifically can contribute to the assessment of carcinogenic potential (28) and may provide an alternative to the use of *in vivo* testing. CTAs may be particularly useful for evaluating chemicals for which *in vivo* testing is not allowed (*e.g.* regulation on cosmetics in the European Union [Regulation (EC) 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products]), is limited, or is only required for chemicals identified as genotoxic (29).

Current knowledge and understanding about mechanisms involved in cell transformation

12. The concept that carcinogenesis is a multistage process is widely accepted (see Paragraph 4). Molecular mechanisms of the multistage process were determined through *in vitro* studies, mainly by gene transfection techniques in cell transformation assays. Transformed cells were induced by the following combinations including active *ras* genes in specific cell types: a) human c-Ha-*ras* plus *myc* genes (30) and human c-Ha-*ras* plus polyoma virus middle-T genes (31) in primary cells, b) c-Ha-*ras* gene alone in established cells but not in primary cells (32), c) c-Ha-*ras* gene in primary cells followed by treatment with carcinogens (32), and d) *ras* genes plus 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in primary and established cells (33, 34, 35). These results suggest that activation of *ras* genes is an important event in cell transformation and that other (*e.g.* chemicals) stimuli can serve to induce expression of such genes thereby resulting in the altered transformation phenotype.

13. The *ras* genes were found to be activated by mutations in chemically induced tumors *in vivo* (36). On the other hand, the c-*myc* gene was expressed following treatment with the tumor promoter TPA (37). These studies together with others in which cell transformation resulted in cells with active c-Ha-*ras* plus *myc* genes (30) suggested that cells containing integrated v-Ha-*ras* gene and which could be transformed by TPA should be able to detect suspected chemical carcinogens. It was on that basis that the Bhas 42 cells were isolated and cloned because of their potential utility in identifying chemical carcinogens (10).

14. There is a close correlation between *in vitro* transformed Bhas 42 cells, identified as such by their morphologically altered phenotype, and *in vivo* tumorigenicity. When chemically transformed Bhas 42 cells were inoculated subcutaneously into nude mice, 100% (four out of four) transformed clones formed tumors (11). In contrast, non-transformed Bhas 42 cells did not induce tumors. Almost all of the transformed Bhas 42 clones (11/12) expressed v-Ha-*ras* genes 2- to 14-fold higher than the non-transformed cells (38). Furthermore, transformed Bhas 42 cells induced by transfection with MTG8 (leukemia-related gene) (39) or HP-MP1 genes (tumor necrosis factor alpha-inducing membrane protein in Helicobacter pylori) (40) were also tumorigenic *in vivo*. In contrast, these genes did not induce transformation in progenitor BALB/3T3 A31-1-1 cells. These data suggest that not only carcinogens but also potential oncogenic genes work together with v-Ha-*ras* genes to induce transformation in Bhas 42 cells.

15. Not all mechanisms leading to cell transformation involve mutations of *ras* genes. Other genetic and epigenetic alterations are also associated with cell transformation (41, 42). Such alterations are also found in immortalized cells (12). In fact, alterations of p53 tumor suppressor gene have been found to have occurred in the parental BALB/3T3 A31-1-1 cells from which Bhas 42 cells were originally derived (43). Involvement of p53 and *ras* mutations suggest that similar to *in vivo* carcinogenesis, *in vitro* cell transformation is manifest through a multistage process, which may help explain why Bhas 42 cells are so responsive to chemical insult.

16. A common mechanism of tumor promotion is still not clear because of the chemical diversity of such agents. Nevertheless, cell proliferation, inflammation, dedifferentiation and inhibition of apoptosis appear to correlate with tumor promotion. On the other hand, the principal mechanism that has been associated with tumor initiation is that of genetic alterations. Furthermore, ligand binding to receptors, gene expression, signal transduction, DNA methylation, histone tail modification and microRNA alteration have been analyzed at molecular level (44, 45). Taken together, these studies suggest that multiple signaling pathways are activated, resulting in continuous cell proliferation and subsequent tumor promotion. *Ras* signaling pathway is one of the major signal transduction cascades and constitutive activation of *ras* is an

important factor in malignant growth (46). Moreover, DNA synthesis of Bhas 42 cells was found to persist longer than that of BALB/3T3 A31-1-1 cells following treatment with TPA (10), which might be due to the integration of v-Ha-*ras* gene.

17. Inhibition of gap junctional cell-to-cell communication has been known as one of early events in tumor promotion (22). When Bhas 42 cells were co-cultured with BALB/3T3 A31-1-1 cells and treated with TPA, the transformation frequency decreased depending on the numbers of BALB/3T3 A31-1-1 cells present (11). In addition, an increase of transformation frequency correlated with inhibition of cell-to-cell communication by TPA in BALB/3T3 A31-1-1 cells (47). These data suggest that the suppression and induction of Bhas 42 transformed foci are regulated by enhancement and inhibition of cell-to-cell communication, respectively.

INITIAL CONSIDERATIONS AND LIMITATIONS

18. The chromosome number of Bhas 42 cells, which are hyper-triploid, ranges from 58 to 72 and the modal chromosome number is 60 (42%). Bhas 42 cells also have marker chromosomes similar to parental BALB/3T3 A31-1-1 cells. The results of fluorescence *in situ* hybridization (FISH) indicated that transfected v-Ha-*ras* genes were present in all nuclei of Bhas 42 cells examined, with an average of 2.4 copies/nucleus and that the v-Ha-*ras* genes were located on chromosome numbers 17 and 19 (11). In addition, v-Ha-*ras* genes that had become integrated into the genome of Bhas 42 cells have maintained the sequence of the original plasmid. The studies from which these data were obtained employed Bhas 42 cells that had undergone more than 18 passages (sub-cultivations) indicating that v-Ha-*ras* genes were stably transferred to daughter cells and that the integrated v-Ha-*ras* genes were not lost even after repeated sub-cultivation (11).

19. Due to their initiated state and their sensitivity to carcinogenic stimuli, Bhas 42 cells may spontaneously transform under sub-optimal culture conditions. Therefore, it is important to maintain strict quality control of cells, assay components, and test conditions, including the use of low passage target cells, maintenance of a sub-confluent cell population density (≤ 70% confluence) among cell stocks to be used for treatment, and use of suitable pre-screened lots of foetal bovine serum (FBS) (16, 17, 48). It should be noted that spontaneous transformation is a common intrinsic occurrence among the various CTAs and is expressed at different relative frequencies among the available target cell systems used in CTAs (6). Irrespective of the CTA system, those spontaneous transformation rates are moderated by adhering to the strict quality control measures described above. In this way, the spontaneous and chemically induced transformation frequencies are readily distinguishable.

20. Initiating and promoting activities of carcinogens can be distinguished in the *in vivo* carcinogenicity studies using the two-stage carcinogenesis model but this distinction is not generally pursued. In its evaluation of the relative performance of CTAs, OECD, in its DRP 31 (49), reported on CTA responsiveness to 260 carcinogens. Only 9 *in vivo* tumor promoters (3.5%) were included in the review, and all of them showed positive results in all or either of the SHE, BALB/3T3 A31-1-1 and C3H10T1/2 CTAs. As to the performance of the *in vitro* promotion test using the Bhas 42 CTA, 14 *in vivo* tumor promoters were investigated, 13 (92.9%) of which were positive in the Bhas 42 cell promotion test (17, 24, 50). These results indicate that the Bhas 42 cell promotion test can be a valuable *in vitro* system for identifying potential *in vivo* tumor promoters.

21. Eight mouse Cyp isoforms (Cyp1a1, Cyp1a2, Cyp2b10, Cyp2c29, Cyp2c65, Cyp2d22, Cyp2e1 and Cyp3a11) were not detected in Bhas 42 cells by quantitative real-time PCR. However, when Bhas 42 cells were treated with 3-methylcholanthrene (3MC, predominantly metabolized by Cyp1a1 to active

carcinogenic forms), only Cyp1a1 was found to be markedly induced from Day 1 and reached a plateau on Day 2-3 (51). These results suggest that although Cyp1a1 is not expressed in normal culture conditions, some chemicals induce Cyp1a1 and they are converted to active metabolites.

22. In the Bhas 42 CTA, 8 out of 10 (80%) carcinogenic polycyclic aromatic hydrocarbons (predominantly metabolized by Cyp1a1) examined were positive (11). These data suggest that the Bhas 42 CTA can efficiently detect carcinogens that require metabolic activation by Cyp1a1. In addition, the Cyp1a1 expression following chemical treatment for three days indicates that the three-day treatment in the initiation test (16, 17, 18) is a suitable protocol to detect carcinogens requiring metabolic activation.

23. Cyp isoforms except Cyp1a1 were not expressed, however, 2-acetylaminofluorene (2AAF, predominantly metabolized by Cyp1a2) and cyclophosphamide (CPD, predominantly metabolized by Cyp2b10) induce transformation in Bhas 42 cells. The eight mouse Cyp isoforms were selected based upon human data, *i.e.* the human CYP homologs of the mouse Cyp isoforms are mainly involved in drug metabolism *in vivo* (52, 53). Currently, about 80 mouse Cyp isoforms have been identified and almost all chemicals are metabolized by multiple enzymes. 2AAF and CPD may induce transformation due to their metabolic activation catalyzed by Cyp isoforms other than the eight that have chosen for measurement.

24. Carcinogens (including cytokines) that bind to specific receptors are thought to act through receptor-mediated pathways because tumors are not induced in knockout mice which lack the gene responsible for coding such receptors (54, 55). Some of these receptors have been identified in BALB/3T3 A31-1-1 cells where transformation has been induced (56, 57). Although similar receptors have yet to be identified in Bhas 42 cells, it is possible, given the derivation of Bhas 42 cells, that they might also be transformed through receptors similar to those of BALB/3T3 A31-1-1 cells.

25. Morphologically, various types of transformed foci are observed (48, Paragraph 60, Annex 2). For this reason, adequate training of laboratory personnel engaged in the identification and scoring of transformed foci is essential. A photo catalog of various examples of untransformed and transformed foci has been found to be a valuable tool with which to assist in the recognition of such transformed foci and in distinguishing them from non-transformed foci (Annex 2).

PRINCIPLE OF THE TEST METHOD

26. Bhas 42 cells proliferate exponentially and when they reach confluence, they form a contact-inhibited monolayer. Appropriate numbers of Bhas 42 cells are plated into each well of 6-well or 96-well plates. In the-initiation test, the cells are treated with a given test chemical at a low cell density for three days (from Day 1 to Day 4), allowed to replicate and then fixed and stained on Day 21 after plating. In the promotion test, the treatment with the test chemical is commenced at sub-confluence and continued for 10 days (from Day 4 to Day 14). The cells are then fixed and stained on Day 21 after plating. Plates are coded and scored; the resulting foci are evaluated for their morphological phenotype.

27. Transformation frequency is quantified using stereomicroscopy as follows: (a) for the 6-well format, transformed foci in each well are scored; (b) for the 96-well format, the number of wells with transformed foci are counted. The second method overcomes the difficulty in scoring multiple foci in 96-well plates. The scoring should include the foci at the bottom of the wells as well as the foci growing on the walls of each well (17, 23).

28. Cytotoxicity is evaluated colorimetrically by estimating the amount of dye (crystal violet) extracted from the treated cells (25). For this purpose, the relative optical density (OD) is obtained by calculating the ratio of the OD determined for the treated cells to the OD of solvent control cells. The transformation frequency is statistically determined from the relative increase in the number of morphologically transformed foci observed in the treated group compared to the number of such foci appearing in the solvent controls.

PROCEDURE

Culture media, reagents and solutions

29. The culture media, reagents and solutions are described in Annex 1.

Culture conditions and preparation of cell suspension

30. Minimum Essential Medium supplemented with 10% FBS (M10F) is used for population expansion of cells so as to generate master cell stocks and working cell stocks, all of which are stored frozen in a liquid nitrogen tank. Cell cultures used for cytotoxicity and transformation assays are derived from those frozen cell stocks. Dulbecco's Modified Eagle's Medium/F12 supplemented with 5% FBS (DF5F) is used for the cell growth assays and transformation assays as well as routine maintenance and subculturing of cells.

31. Bhas 42 cells are incubated at 37ºC in a humidified atmosphere of 5% CO_2 and air. It is important that all cell stocks and working cultures be maintained at a sub-confluent density at all times prior to use in transformation assays, such that they do not exceed 70% confluence and thereby retain their property of density- dependent inhibition of cell growth. This ensures that loss of cell-to-cell contact inhibition is the result of treatment with chemical carcinogens and not a function of failure to maintain the necessary pre-assay cell culture conditions. The necessity of this becomes clear when it is realized that those cells that are no longer contact-inhibited and exhibit unrestricted growth are those that are transformed and preferentially form altered foci atop the confluent cell monolayer.

Preparation and cryopreservation of Bhas 42 cell stocks

32. Bhas 42 cells should be obtained from a reliable source, specifically, JCRB Cell Bank, National Institute of Biomedical Innovation (NIBIO, Osaka, Japan) [http://cellbank.nibio.go.jp/english/] and shown to be free of adventitious contaminating agents (*e.g.* mycoplasma).

33. If the Bhas 42 cells are cultured with sufficient care using acceptable pre-screened lots of FBS and proper attention paid to maintenance of sub-confluent cell density, the cells can be passaged 2-3 times without losing the properties that make them suitable for use as a CTA target cell system. The most practical solution to ensure the uninterrupted availability of such suitable cell populations is to have available a large stock of frozen early passage cells. For this purpose, the procedure is as follows (48);

- Initial master cell stocks are generated and cryopreserved in a liquid nitrogen tank in aliquots that will eventually serve to generate working cell stocks.

- Cells are cultured with M10F in a 100- or 150-mm dish or in a 75- or 150-cm^2 flask to a cell density not to exceed 70% confluence.

- They are then suspended at a cell density of 5×10^5 cells/mL in cold fresh M10F containing a suitable cryoprotective agent (*e.g.* 5% dimethyl sulfoxide) to make a master cell stock from which 0.5 mL aliquots are cryopreserved and stored in liquid nitrogen.

- Cells from one master stock are thawed and cultured for 1-2 passages in M10F before a second cryopreservation step.

- From this cell population, approximately 100 aliquots are prepared and cryopreserved so as to provide sufficient working cell stocks.

- The quality of those cells is then confirmed for their ability to fulfill the acceptance criteria described in Paragraph 34.

- It is noteworthy that the same criteria need to be met to assess cell quality as those used for accepting a given lot of FBS.

34. In order to identify suitable lots of FBS for the transformation assay, several lots of FBS are checked using the cells from one master stock. The acceptance criteria for a given lot of FBS include (a) ability to support adequate plating efficiency (PE) of Bhas 42 cells (≥50%, calculated with the formula below, generally 100 cells are plated per 60-mm dish or well of 6-well plate and cultured for 6-10 days), (b) low background of spontaneous transformation, and (c) ability to facilitate Bhas 42 cell transformation by positive controls (1 µg/mL MCA and 50 ng/mL TPA, refer to Paragraph 38). FBS lots that fulfill the criteria in Paragraphs 66-68 are those that are selected for use in subsequent transformation assays.

PE (%) = [(total number of colonies per dish)/(total number of cells plated per dish)] x 100

35. Freshly prepared 2-3 passage cells derived from the cryopreserved cell stocks are used for each transformation assay.

36. The cells at higher passages (within 10 passages) can be used for dose setting.

Controls

37. The solvent for a test chemical is used as the negative control. The solvent should be chosen to optimize the solubility of the test chemical without adversely affecting the conduct or outcome of the assay, *e.g.* cell growth and morphology, cell-to-cell interaction, integrity of the test material, augment or diminish the effects of the test chemical on the target cells, reaction with culture vessels.

38. For positive controls, a known tumor-initiator, 3-methylcholanthrene (MCA, final concentration of 1 µg/mL), is used in the initiation test, and a known tumor-promoter, 12-*O*-tetradecanoylphorbol-13-acetate (TPA, final concentration of 50 ng/mL), is used in the promotion test. MCA and TPA are dissolved in dimethyl sulfoxide (DMSO), which serves as the solvent for these two control agents. When the solvent for the test chemical is not DMSO, DMSO is still necessary as the negative control for MCA or TPA. The stock solutions of MCA and TPA in DMSO can be stored in frozen aliquots at -20°C for at least two years.

Preparation of test chemical solutions

39. Test chemicals are dissolved or suspended in an appropriate solvent and diluted, if appropriate, prior to the treatment of the cells. Distilled water, DMSO, acetone, and ethanol can be used to dissolve test chemicals, and the final solvent concentrations in the medium for each of these vehicles should not exceed 5%, 0.5%, 0.5% and 0.1%, respectively. Although the concentration of DMSO can be as high as 0.5%, 0.1% is recommended when possible. If solvents other than the above well-established ones are employed, their use should be supported by data indicating their compatibility with the test chemical and the test system, as well as their lack of inherent transforming activity. In such cases, untreated controls devoid of the solvent of choice should also be included against which to compare the possible transforming activity associated with the solvent. Gaseous or volatile chemicals should be tested by appropriate methods, determined on a case-by-case basis. Fresh preparations of the test chemical should be used unless stability data demonstrate that storage of test chemical is acceptable. Solutions at different concentrations of the test

chemical should be prepared under UV filtered lights or protected from exposure to ambient light for photo-sensitive chemicals.

40. Several concentrations for the transformation assay (at least four concentrations, if the test chemical is not toxic) should be tested and these are determined according to the results of the cell growth assay. Paragraphs 47, 52-55 provide further details on the top concentration that should be tested.

41. For poorly soluble test chemicals that are not cytotoxic at concentrations lower than the lowest insoluble concentration, the highest concentration analysed should produce turbidity or a precipitate visible by eye or with the aid of an inverted microscope at the end of the treatment with the test chemical. Even if cytotoxicity occurs above the lowest insoluble concentration, it is advisable to test at only one concentration producing turbidity or with a visible precipitate because artifactual effects may result from the precipitate. At the concentration producing a precipitate, care should be taken to assure that the precipitate does not interfere with the conduct of the test (*e.g.* staining or scoring). The determination of solubility in the culture medium prior to the experiment may be useful.

42. If no precipitate or limiting cytotoxicity is observed, the highest test concentration should correspond to 10 mM, 2 mg/mL or 2 µl/mL, whichever is the lowest. When the test chemical is not of defined composition *e.g.* substance of unknown or variable composition, complex reaction products or biological materials (*i.e.* UVCBs), environmental extracts, *etc*., the top concentration may need to be higher (*e.g.* 5 mg/ml) in the absence of sufficient cytotoxicity, to increase the concentration of each of the components.

Experimental design

43. The 6-well and the 96-well formats are quite similar since the experimental procedures differ only in the plated cells and the calculation of the transformation frequency. In the following sections, experimental details are provided for the 6-well format, including modifications associated with the 96-well format.

44. Both formats consist of an initiation test and a promotion test components. These test components can detect initiating activity and promoting activity of carcinogens, respectively. In the initiation test, the cells are treated with chemicals in the beginning of the growth phase and in the promotion test the treatment is started at sub-confluence of cell growth. Both tests consist of two steps. In the first step, a dose range-finding assay is performed in which test chemical concentrations are selected (preliminary cell growth assay) using a broad dose range. In the second step, the transformation assay itself and a concurrent cell growth assay are performed, the latter serving to verify that the selected doses meet the acceptation criteria for the tests (Fig. 1).

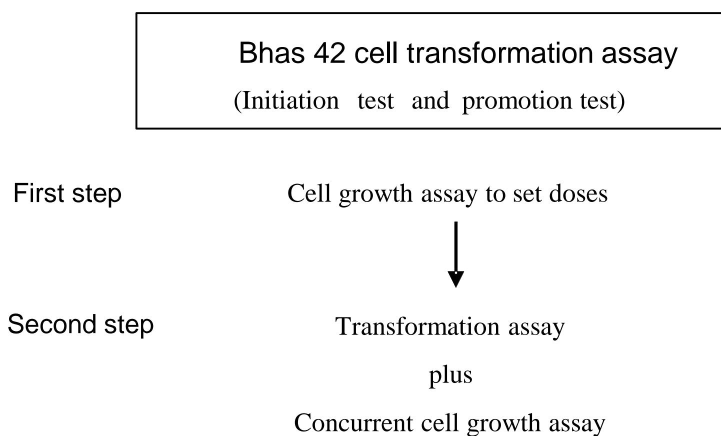


Figure 1: General scheme of the Bhas 42 CTA

Initiation test

Cell growth assay to set doses

45. The cells, at a density of ≤ 70% confluence in DF5F, are trypsinized and 4,000 cells are plated into each well with 2 mL of DF5F (Day 0). Wells containing medium alone are also prepared for the blank control in the colorimetric analysis (the blank control can be shared among different assays performed simultaneously). At 20-24 hours (Day 1) after cell plating, the culture medium is replaced with fresh medium containing various concentrations of a given test chemical, or concentrated test chemical solutions are added to each well without medium replacement. The medium is changed with fresh medium on Day 4. On Day 7, the cultures are fixed with ethanol or methanol for approximately 10 min, washed and air-dried. The cells are stained with crystal violet (CV) solution for approximately 15 min, rinsed well with water and dried (Fig. 2). Three wells are prepared in each group.

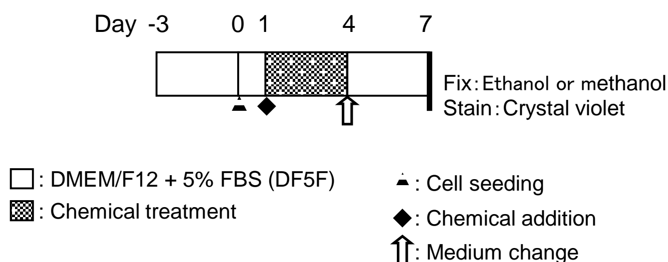


Figure 2: Time line of the cell growth assay component of the initiation test

46. The CV is extracted from the stained cells with 2 mL of dye extraction solution (see Annex 1), and the OD (optical density) is measured at a wavelength between 540-570 nm. The relative cell growth of cultures treated with a chemical is calculated as follows:

Relative cell growth (%) = [(Treatment – Blank)/(Control – Blank)] x 100

“Treatment”, “Control” and “Blank” refer to the absorbance of the CV extracts of each treatment group, the solvent control group and the medium only group, respectively.

47. Five to nine concentrations of test chemical are set up based on the results of the cell growth assay. These concentrations cover a range from little or no toxicity to the highest acceptable level of toxicity (less than 20% survival compared to the negative control). Ideally, those concentrations include: (a) at least one concentration below the non-toxic level (around 80-120% of cell growth), (b) two concentrations between the non-toxic level and the 50% inhibitory concentration (IC50), and (c) two concentrations between the IC50 and the IC90. Subsequent concentrations should have a ratio of square root of 10 or less (Fig. 3). Some test chemicals exhibit a steep concentration–response curve. With these test chemicals, test concentrations should be spaced at much closer intervals. In addition, it may become necessary to include one or two additional test concentrations below and above the expected dose range in order to allow for possible unanticipated cytotoxic fluctuations among experiments.

-------------------- Non-toxic level -----------------------------IC50-------------------------IC90

At least one dose	Two doses	Two doses

<u>Figure 3</u>: Dose setting for the initiation test component of the transformation assay

48. In the 96-well format, the cell growth assay is carried out in the same manner as the 6-well format except for the following conditions.

- Into each well, 200 cells are plated with 0.05 mL of DF5F (Day 0).

- The cultures in 0.05 mL of medium are treated by the addition of another 0.05 mL of medium containing a test chemical or solvent alone at two times the final desired concentrations, so that the final volume of the medium is 0.1 mL (Day 1).

- The volumes of CV solution and dye extraction solution are 0.1 mL/well.

- For each group, eight wells are prepared.

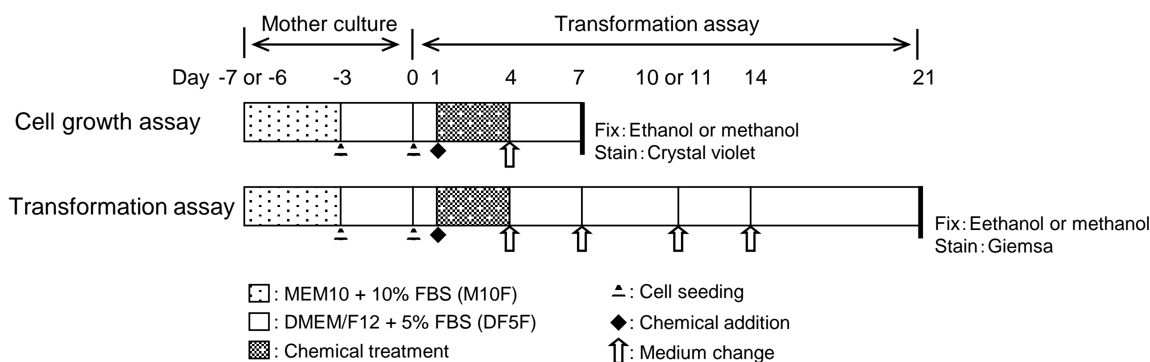
Transformation assay

49. The transformation assay is carried out as follows (Fig. 4):

- The frozen working cell stocks are rapidly thawed, suspended in M10F and cultured in 100-mm dishes in a volume of 10 mL medium. When the cells reach approximately 70% confluence, they are trypsinized, plated in DF5F at an appropriate density (70,000 to 100,000 cells) per 100-mm dish (Day -3).

- When these cells reach approximately 70% confluence, they are again trypsinized and suspended in DF5F at 2,000 cells/mL. The cell suspension is plated into each well of a 6-well plate at a volume of 2 mL (4,000 cells/well) for the transformation assay and the concurrent cell growth assay (Day 0). Twenty to 24 hours (Day 1) after seeding, the cells are treated for three days (Day 1-4) in the same way as the cell growth assay. The medium is changed with fresh DF5F on Day 4, 7, 10 (or 11) and 14.

- On Day 7, the cultures for the concurrent cell growth assay are fixed with ethanol or methanol for approximately 10 min, washed and dried. The cells are stained with CV solution for approximately 15 min, rinsed well with water and dried (Fig. 2). On Day 21, the cells for transformation assay are fixed with ethanol or methanol and stained with 5% Giemsa solution for approximately 15 min (Fig. 4). The positive control (1 µg/mL MCA) and the negative (solvent) control(s) are included in the transformation assay for each test chemical. Nine wells are prepared for each group (one plate of six wells for the transformation assay and three wells for the concurrent cell growth assay).



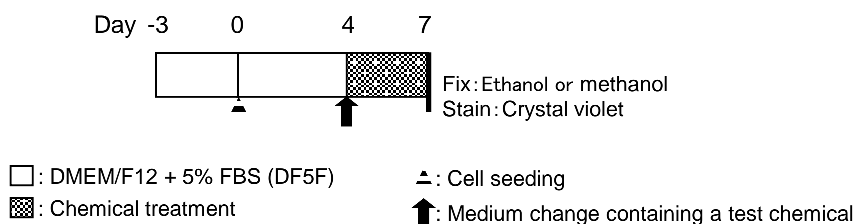
<u>Figure 4</u>: Time line for the initiation test component of the transformation assay

50. The transformation assay using the 96-well format is carried out in the same manner as the transformation assay using the 6-well format except that 200 cells are plated/well in 0.05mL of medium on Day 0, to which is added 0.05 mL medium containing twice the desired final concentration of test chemical on Day 1. One 96-well plate (96 wells) for each group is prepared for the transformation assay and eight wells are prepared for the concurrent cell growth assay.

Promotion test

Cell growth assay to set doses

51. The experimental procedure is basically the same as the initiation test, except for the number of cells plated and timing of chemical treatment. Cells are plated at 14,000 cells/well in 2 mL of DF5F on Day 0, and chemical treatment is started on Day 4 by exchanging existing medium with fresh medium containing the test chemical solution (Fig. 5). Three wells are prepared for each group.



<u>Figure 5</u>: Time line of the cell growth assay component of the promotion test

52. There are two different types of chemicals that exhibit promoting activity. One group includes those chemicals that enhance cell growth more than 20% of that of the control. With these test chemicals, concentrations are selected to cover the range from little or no growth enhancement effect to concentrations that enhance cell growth. In practice, one concentration below the non-toxic level, three concentrations in the range of growth enhancement and one concentration in the range of weak growth inhibition are assessed (Fig. 6).

---------- Non-toxic level ----- Growth enhancement-------- Growth inhibition

One dose	Three doses	One dose

<u>Figure 6</u>: Dose setting for the promotion test component of the transformation assay for chemicals that induce growth enhancement

53. The second chemical group that exhibits promoting activity is that which inhibits cell growth. For these test chemicals, concentrations are selected to cover the range from the non-toxic level to a level below the IC50. Ideally, at least two concentrations below the non-toxic level, two concentrations between the non-toxic level and the IC50 and one concentration between IC50 and IC90 are evaluated (Fig. 7).

----------------------- Non-toxic level --------------------------IC50---------------- IC90

At least two doses	Two doses	One dose

<u>Figure 7</u>: Dose setting for the promotion test component of the transformation assay for chemicals that inhibit growth

54. It is noteworthy that there are chemicals that cause more pronounced growth inhibition in the transformation assay than would otherwise be observed in the concurrent cell growth assay (See paragraph 59). This phenomenon can be attributed to the difference in the duration of the treatment periods for each, *i.e.* 10 days for the transformation assay versus three days for the cell growth assay (58).

55. When test chemicals exhibit a steep concentration–response curve, considerations similar to those described in the initiation test (refer to Paragraph 47) may need to be taken into account, *e.g.* inclusion of additional test concentrations and test concentration intervals, thereby ensuring an acceptable test outcome.

56. In the 96-well format, the cell growth assay is carried out in the same manner as the 6-well format except for the plating of 400 cells in 0.1 mL of DF5F (Day 0). Eight wells are prepared for each group.

Transformation assay

57. The transformation assay is carried out in the same manner as the initiation test except for the following (Fig. 8):

- The cells are suspended in DF5F at 7,000 cells/mL, of which a volume of 2 mL is plated into each well (14,000 cells/well) of a 6-well plate on Day 0. Nine wells are prepared for each group (one plate of six wells for the transformation assay and three wells for the concurrent cell growth assay).

- The cells are exposed to the test chemical for 10 days, from Day 4 to Day 14.

- Following the initial exposure of cells to test chemical on Day 4, cells are re-exposed on days 7 and 10 (or 11) by exchanging existing medium with fresh medium containing the chemical solution or solvent alone.

- On Day 14, the treatment medium is replaced with fresh medium containing neither solvent nor test chemical.

- TPA (50 ng/mL) is used for the positive control.

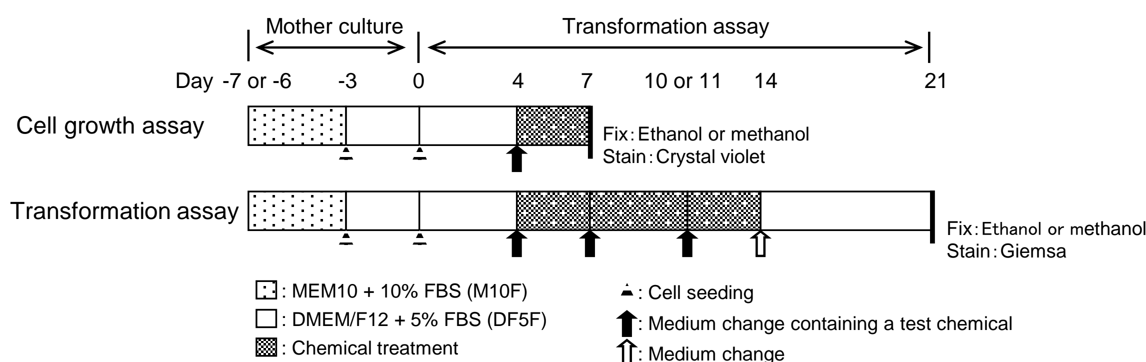


Figure 8: Time line for the promotion test component of the transformation assay

58. In the 96-well format, the transformation assay is carried out in the same manner as that for the 6-well format except for the plating of 400 cells in 0.1 mL of DF5F (Day 0). One 96-well plate (96 wells) for each group is prepared for the transformation assay and eight wells for each group are employed for the concurrent cell growth assay.

Evaluation of the results

Determination of transformation frequency

59. Transformed foci are scored using a stereomicroscope. If the cells in the transformation assay do not reach confluence at a given concentration of test chemical because of cytotoxicity, that concentration is considered unacceptable for transformation assessment and is excluded from focus-counting. In such situations, "toxicity" is recorded in the data sheet.

60. Transformed foci are characterized by the following morphological properties: (a) more than 100 cells, (b) spindle-shaped cells differing in appearance from the contact-inhibited monolayer cells, (c) deep basophilic staining, (d) random orientation of cells, especially visible at the edge of foci (criss-cross misalignment of individual cells), (e) dense multi-layering of cells (piling up), and (f) invasive growth into the surrounding confluent monolayer of contact-inhibited cells. It should be noted that all transformed foci do not necessarily need to exhibit all of these morphological characteristics, but a few of these characteristics should show clear-cut morphological alterations (see Annex 2). For quantification, the number of transformed foci in each well is recorded for each group.

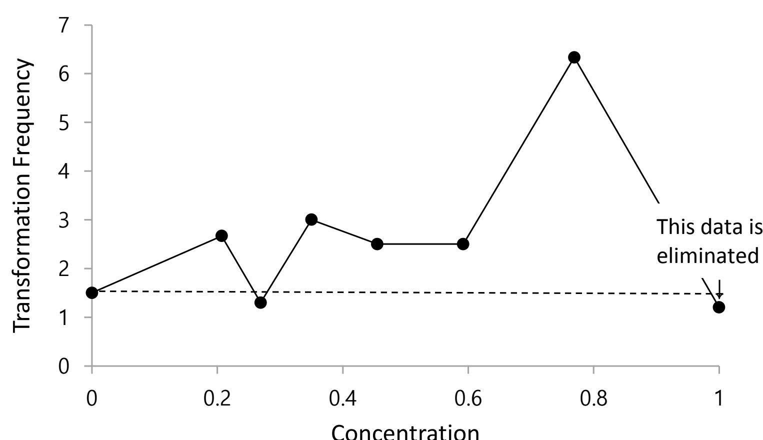
61. In the 96-well format, transformed foci are judged using the same criteria as in the 6-well format. For assay scoring, the number of wells having transformed foci relative to the number of wells observed is recorded for every group. Thus, a well having one focus is counted as one and a well having two or more foci is likewise counted as one.

Statistical analysis

62. For statistical purposes, the quantifiable unit in the 6-well format is the number of transformed foci per well. Test chemical-induced transformation frequency is statistically analyzed by multiple comparison using the one-sided Dunnett's test ($p<0.05$, upper-sided). For the positive controls, the statistical significance is evaluated by the one-sided Student's t-test or Aspin-Welch test ($p<0.05$, upper-sided) depending on the results of the F-test for homoscedasticity (homogeneity of variance).

63. For statistical purposes, the quantifiable unit in the 96-well format is the number of wells with one or more transformed foci. Test chemical-induced transformation frequency is statistically analyzed using the Holm's test ($p<0.05$, upper-sided). For the positive controls, the statistical significance is evaluated by the one-sided chi-square test ($p<0.05$, upper-sided).

64. If statistical significance is obtained at only one concentration, dose dependency is analyzed by the Jonckheere test ($p<0.05$, upper-side) for the 6-well format and by the Cochran-Armitage test for the 96-well format ($p<0.05$, upper-side). If a bell-shaped pattern is observed, eliminating the value(s) that is lower than that of the corresponding negative control in the dose ranges showing a decrease trend is recommended (See Figure 9).



<u>Figure 9</u>: An example of bell-shaped response in the transformation assay

Assay acceptance criteria

65. The following criteria (Paragraphs 66-70) must be fulfilled for a given assay to be considered valid. When considered invalid, the initiation or promotion test is repeated independently, as needed, to satisfy the assay acceptance criteria.

66. The following criteria in the negative control must be fulfilled for a given assay to be considered valid:

- In the 6-well format, the number of (spontaneous) transformed foci must be ≤10 per well in the initiation test and ≤12 per well in the promotion test. The reason why the acceptability criteria for spontaneous transformation is different in the initiation and promotion assays is that a higher density of plated cells (as is used in the promotion test) increases the spontaneous transformation frequency.

- In the 96-well format, the number of wells in the negative control plates having (spontaneous) transformed foci must be ≤15 wells/plate in the initiation test; if damaged wells are present, the number of scorable wells with transformed foci must be ≤16%. In the promotion test, the number of wells in the negative control plates having (spontaneous) transformed foci must be ≤20 wells/plate; if damaged wells are present, the number of undamaged wells with transformed foci must be ≤21%.

67. The following criteria in the positive control must be fulfilled for a given assay to be considered valid:

- There must be a biologically relevant and statistically significant increase in the transformation incidence compared to the corresponding negative control.

68. When contamination or technical problems are observed, the following requirement must be fulfilled for a given assay to be considered valid:

- In the 6-well format, a minimum of two undamaged wells per group is necessary in the concurrent cell growth assay and a minimum of five undamaged wells per group is necessary in the transformation assay.

- In the 96-well format, a minimum of four undamaged wells per group is necessary in the concurrent cell growth assay and a minimum of 90 undamaged wells per group is necessary in the transformation assay.

- In cases where such technical difficulties are encountered, "contamination", "accident", "technical error", *etc.* are recorded in the data sheet.

69. In the 6-well and 96-well formats, the following criteria must be fulfilled for a given assay to be considered valid:

A transformation assay is considered acceptable if at least four test chemical concentrations are available to be evaluated and the following conditions are satisfied.

- In the initiation test, the results of the concurrent cell growth assay include at least one concentration near the non-toxic level and three concentrations in the range between the non-toxic level and the IC90.

-In the promotion test showing growth enhancement, the results of the concurrent cell growth assay include at least one concentration near the non-toxic level and two concentrations in the range of growth enhancement.

- In the promotion test showing growth inhibition, the results of the concurrent cell growth assay include at least two concentrations below the non-toxic level and two concentrations between the non-toxic level and the IC50.

When test chemical properties bring about atypical test conditions such as precipitation and/or a steep concentration–response curve, and the above assay acceptance conditions cannot be satisfied, a detailed explanation along with the rationale for accepting vs. rejecting the test should be provided.

70. In the initiation and promotion tests, when cytotoxicity from chemical treatment results in an inhibition of confluence at the end of transformation assay such that at least four test chemical concentrations are not available to be evaluated, the following criteria can be invoked in deciding whether or not to repeat such an experiment:

- If a minimum of two sequential doses induces statistically significant increases in transformation frequency, then a repeat experiment is not necessary and the result is judged as positive.

- Other outcomes, *e.g.* one test chemical concentration induces a statistically significant increase in transformation frequency and dose dependency is not observed, would necessitate a repeat experiment at lower concentrations (*e.g.* those that would not inhibit confluence).

- Other experimental results in which an insufficient number of test chemical concentrations remains available for scoring should be evaluated on a case-by-case basis to determine the design for a repeat study.

Data interpretation criteria

71. The interpretation of test results relies on both statistical significance and biological relevance of data, although the latter is considered to be of requisite importance. The bell-shaped pattern observed frequently in Bhas 42 CTA have often suggested the results to be not significant in the trend tests (Jonckheere and Cochran -Armitage), although transformation frequencies were clearly positive. Therefore, use of the trend tests in the results showing the significant difference in multiple doses is not recommended. The concentration(s) of test chemical that increase the transformation frequency is carefully considered, taking into account the range of cytotoxic/non-cytotoxic concentrations.

72. The results of initiation and promotion tests in the 6-well and 96-well formats are evaluated independently and judged as follows:

- The result~~s~~ are judged positive when there are two or more sequential doses that induce statistically significant increase in the transformation frequencies compared with the solvent control and at least one of these doses exceeds the distribution range of the historical negative control data (e.g. 95% confidence interval).

- The result~~s~~ are judged positive when a statistically significant increase in the transformation frequencies compared with the solvent control is observed at only one dose and that one dose exceeds the distribution range of the historical negative control data (e.g. 95% confidence interval), and further statistical analysis is suggestive of a dose-related response.

- The results are judged negative when there is no dose showing a statistically significant increase in the transformation frequencies.

- When the statistically significant increase occurs at only one dose and the response is not dose dependent, the test result is regarded as equivocal, in which case the initiation and/or promotion test(s) should be repeated. The repeat test should employ modified experimental conditions in which test chemical concentrations are adjusted so as to employ a broader or narrower range, as appropriate, in order to preclude the probability of such equivocal results.

- When results are positive in the initiation and/or promotion test(s) based upon the above criteria, the test chemical is considered to have transforming activity in this test system. Positive results in either the initiation or promotion tests may be considered as indicative of *in vivo* carcinogenic potential.

- Negative results in both tests are taken to mean that the test chemical does not possess potential carcinogenic activity.

Laboratory Proficiency

73. In order to assure the proficiency of a given laboratory, the laboratory should perform tests using two negative chemicals and four positive chemicals, each of the latter acting via different mechanisms of action in both the initiation and promotion test(s). Those chemicals recommended for this purpose are listed in Table 2. All such tests should also include the appropriate negative (untreated and solvent) controls as described in Paragraph 39, above. When negative and positive results are obtained within the ranges in the validation reports (16, 17), those results are considered as acceptable. During the course of these tests, the laboratory should establish:

- A historical response range and distribution for both untreated controls and solvent controls.

- A historical response range and distribution for positive controls.

Even if laboratory competency has already been established as described, if major changes to experimental conditions are introduced into the assay (*e.g.* modifications in methodology, use of assay materials other than those described, use of automated instead of manual scoring techniques), re-evaluation of laboratory proficiency is recommended. Additionally, changes in laboratory personnel responsible for the conduct of the assay may warrant re-evaluation of adeptness.

Before utilizing this GD for regulatory assessment purposes, it is recommended that personnel be trained in a qualified laboratory with acknowledged experience in this assay. Furthermore, the laboratory expecting to generate data for such purposes should be able to demonstrate prowess in conducting the assay.

Table 2: Chemicals for Assessing Laboratory Proficiency (see reference 16)

Category	Chemical	CAS number
1. Positive chemicals in initiation test of the Bhas42 CTA (genotoxic carcinogens)		
	N-Methyl-*N′*-nitro-*N*-nitrosoguanidine (MNNG)	70-25-7
	3-Methylcholanthrene (MCA)	56-49-5
2. Positive chemicals in promotion test of the Bhas 42 CTA (non-genotoxic carcinogens)		
	12-*O*-tetradecanoylphorbol-13-acetate (TPA)	16561-29-8
	Mezerein	34807-41-5
3. Negative chemicals in both tests of the Bhas 42 CTA (non-carcinogens)		
	Caffeine (CFN)	58-08-2
	Mannitol (MAN)	69-65-8

REPORTING

Test report

74. The test report should include the following information:

Test chemical

Mono-constituent substance:

- physical appearance, water solubility, and additional relevant physicochemical properties
- chemical identification, such as IUPAC or CAS name, CAS number, SMILES or InChI code, structural formula, purity, chemical identity of impurities as appropriate and practically feasible, *etc.*

Multi-constituent substance and mixtures:

- characterized to the extent possible by chemical identity (see above), quantitative occurrence and relevant physicochemical properties of the constituents.

Solvent (if appropriate)

- identification
- justification for choice of solvent
- concentrations tested and preparation of the dosing solutions
- signs of precipitation (presence or absence)

Cells and media

- source of cells
- number of cell subcultures (passage number): cryopreserved cell stocks, working cell stocks
- maintenance of cell cultures
- absence of cell culture contamination, especially mycoplasma
- identification of media and serum (provider and lot number) used for cell culture cryopreservation, maintenance, and assays

Test conditions

- rationale for selection of test chemical concentrations, including cytotoxicity data and solubility limitations
- composition of media
- serum concentration, origin, quality, selection criteria
- concentrations of test chemicals
- volume of solvent and test chemical added
- duration of treatment
- incubation temperature
- incubation atmosphere: percent CO_2 and air
- number of cells plated for cell growth assays, concurrent cytotoxicity tests and transformation assays
- positive and negative controls: identification, CAS numbers, concentrations
- criteria for scoring morphologically altered foci

Results

- results of the dose range finding test
- results of the concurrent cell growth assay
- solubility of test chemical, pH and signs of precipitation in medium at the beginning and the end of the treatment
- number of total valid (quantifiable) wells, number of wells lost and the reason(s) for the loss
- number of total foci (6-well format)
- transformation frequency: transformed foci/well (6-well format), the number of wells with transformed foci/total number of wells (96-well format)
- dose-response relationship, if any
- statistical analyses: statistical test(s) employed, statistical analysis of results
- concurrent negative (solvent) control data, untreated control data where appropriate, and positive control data
- historical negative (solvent) and positive control data, with ranges, means and standard deviations
- consistency of concurrent negative (solvent and/or untreated) and positive control data relative to historical control data

Data should be presented in tabular form (see Annex 3). The following values should be presented for each group (treated group, solvent and positive control groups):

i. results of the concurrent cell growth assay
ii number of total valid (quantifiable) wells
iii number of total foci (6-well format) or the number of wells with transformed foci (96-well format)
iv transformation frequency
v statistical test(s) employed, statistical analysis of results

Discussion of results

Conclusion

REFERENCES

1. Foulds, L. (1969), *Neoplastic Development*, Academic Press, New York, NY.
2. Berenblum, I. (1975), Sequential aspects of chemical carcinogenesis: Skin, In: Becker, F.F. ed. *Cancer: A Comprehensive Treatise, Vol. 1*, 323-344, Plenum Press, New York, NY.
3. Lasne, C., Gentil, A. and Chouroulinkov, I. (1974), Two-stage malignant transformation of rat fibroblasts in tissue culture. *Nature*, 247, 490-491.
4. Tsuchiya, T., Umeda, M., Tanaka, N., Sakai, A., Nishiyama, H., Yoshimura, I., Ajimi, S., Asada, S., Asakura, M., Baba, H., Dewa, Y., Ebe, Y., Fushiwaki, Y., Hagiwara, Y., Hamada, S., Hamamura, T, Iwase, Y, Kajiwara, Y, Kasahara, Y, Kato, Y, Kawabata, M, Kitada, E, Kaneko, K, Kizaki, Y., Kubo, K., Miura, D., Mashiko, K., Mizuhashi, F., Muramatsu, D., Nakajima, M., Nakamura, T., Oishi, H., Sasaki, T., Shimada, S., Takahashi, C., Takeda, Y., Wakuri, S., Yajima, N., Yajima, S. and Yatsushiro, T. (2010), Application of the improved BALB/c 3T3 cell transformation assay to the examination of the initiating and promoting activities of chemicals: the second interlaboratory collaborative study by the non-genotoxic carcinogen study group of Japan, *Altern. Lab. Anim.*, 38, 11-27.
5. Barrett, J.C. and Ts'o, P.O.P. (1978), Evidence for the progressive nature of neoplastic transformation in vitro, *Proc. Natl. Acad. Sci. USA*, 75, 3761-3765.
6. Kakunaga, T. and Yamasaki, H. eds. (1985), *Transformation Assay of Established Cell Lines: Mechanisms and Application, IARC Scientific Publications No. 67*, International Agency for Research on Cancer, Lyon, France.
7. Berwald, Y. and Sachs, L. (1963), In vitro cell transformation with chemical carcinogens, *Nature*, 200, 1182-1184.
8. Newbold, R.F., Overell, R.W. and Connell, J.R. (1982), Induction of immortality is an early event in malignant transformation of mammalian cells by carcinogens, *Nature*, 299, 633-635.
9. Elias, Z., Poirot, O., Pezerat, H., Suquet, H., Schneider, O., Danière, M.C., Terzetti, F., Baruthio, F., Fournier, M. and Cavelier, C. (1989), Cytotoxic and neoplastic effects of industrial hexavalent chromium pigments in Syrian hamster embryo cells, *Carcinogenesis*, 10, 2043-2052.
10. Sasaki, K., Mizusawa, H. and Ishidate, M. (1988), Isolation and characterization of ras-transfected BALB/3T3 clone showing morphological transformation by 12-O-Tetradecanoylphorbol-13-acetate, *Jpn. J. Cancer Res.*, 79, 921-930.
11. Sasaki, K., Umeda, M., Sakai, A., Yamazaki, S. and Tanaka, N. (2015), Transformation assay in Bhas 42 cells: a model using initiated cells to study mechanisms of carcinogenesis and predict carcinogenic potential of chemicals. *J. Environ. Sci. Health. C Environ. Carcinog. Ecotoxicol. Rev.*, 33, 1-35.
12. Stepanenko, A.A. and Kavsan, V.M. Immortalization and malignant transformation of eukaryotic cells, (2012), *Cytol. Genet.*, 46, 36-75.
13. Asada, S., Sasaki, S., Tanaka, N., Takeda. K., Hayashi. M. and Umeda, M. (2005), Detection of initiating as well as promoting activity of chemicals by a novel cell transformation assay using v-Ha-ras-transfected BALB/c 3T3 cells (Bhas 42 cells), *Mutat. Res*., 588, 7-21.
14. Ohmori, K., Sasaki, K., Asada, S., Tanaka, N. and Umeda, M. (2004), An assay method for the prediction of tumor promoting potential of chemicals by the use of Bhas 42 cells, *Mutat. Res*., 557, 191-202.
15. Muramatsu, D., Sasaki, K., Kuroda, S., Hayashi, K., Tanaka, N. and Sakai, A. (2009), Comparison of sensitivity to arsenic compounds between a Bhas 42 cell transformation assay and a BALB/c 3T3 cell transformation assay, *Mutat. Res*., 675, 66–70.
16. Sakai, A., Sasaki, K., Hayashi, K., Muramatsu, D., Arai, S., Endou, N., Kuroda, S., Poth, A., Bohnenberger, S., Kunkelmann, T., Asakura, M., Hirose, H., Ishii, N., Mizuhashi, F., Kasamoto, S., Nagai, M., Pant, K., Bruce, S.W., Sly, J.E., Yamazaki, S, Umeda, M, and Tanaka, N. (2011), An international validation study of a Bhas 42 cell transformation assay for the prediction of chemical carcinogenicity, *Mutat. Res.*, 725, 57-77.

17. Hayashi, M., Kojima, H., Corvi, R., Stokes, W., Jacobs, A., Morita, T., Schechtman, L. and Suzuki, M. (2012), Bhas 42 cell transformation assay validation study report, *EURL ECVAM Recommendations*, Ispra, Italy.
18. EURL ECVAM DataBase service on Alternative Methods to Animal Experimentation (DB-ALM) protocol No.156 on in vitro Bhas 42 cell transformation assay (http://ecvam-dbalm.jrc.ec.europa.eu/)
19. Chu, E.H. and Malling, H.V. (1968), Mammalian cell genetics. II. Chemical induction of specific locus mutations in Chinese hamster cells in vitro, *Proc. Natl. Acad. Sci. USA.*, 61, 1306-1312.
20. Duncan, M.E. and Brookes, P. (1973), The induction of azaguanine-resistant mutants in cultured Chinese hamster cells by reactive derivatives of carcinogenic hydrocarbons, *Mutat. Res.*. 21, 107-118.
21. Mondal, S., Brankow, D.W. and Heidelberger, C. (1976), Two-stage chemical oncogenesis in cultures of C3H/10T1/2 cells, *Cancer Res.*, 36, 2254-2260.
22. Yamasaki, H., Ashby, J., Bignami, M., Jongen, W., Linnainmaa, K., Newbold, R.F., Nguyen-Ba, G., Parodi, S., Rivedal, E., Schiffmann, D., Simons, J.W. and Vasseur, P. (1996), Nongenotoxic carcinogens: development of detection methods based on mechanisms: a European project, *Mutat. Res.*, 353, 47-63.
23. Arai, S., Sakai, A., Hayashi, K., Sasaki, K., Muramatsu, D., Endou, N., Umeda, M. and Tanaka, N. (2013), A high-throughput cell transformation assay applicable to automation for detecting potential chemical carcinogens using Bhas 42 cells, (2013), *AATEX.*, 18, 1-18.
24. Sakai, A., Sasaki, K., Muramatsu, D., Arai, S., Endou, N., Kuroda, S., Hayashi, K., Lim, Y.M., Yamazaki, S., Umeda, M. and Tanaka, N. (2010), A Bhas 42 cell transformation assay on 98 chemicals: the characteristics and performance for the prediction of chemical carcinogenicity, *Mutat. Res.*, 702, 100-122.
25. Ohmori, K., Umeda, M., Tanaka, N., Takagi, H., Yoshimura, I., Sasaki, K., Asasda, S., Sakai, A., Araki, H., Asakura, M., Baba, H., Fushiwaki, Y., Hamada, S., Kitou, N., Nakamura, T., Nakamura, Y., Oishi, H., Sasaki, S., Shimada, S., Tsuchiya, T., Uno, Y., Washizuka, M., Yajima, S., Yamamoto, Y., Yamamura, E. and Yatsushiro, T., Non-Genotoxic Carcinogen Study Group in the Environmental Mutagen Society of Japan (2005), An inter-laboratory collaborative study by the Non-Genotoxic Carcinogen Study Group in Japan, on a cell transformation assay for tumour promoters using Bhas 42 cells, *Altern. Lab. Anim.*, 33, 619-639.
26. Tanaka, N., Sasaki, K., Hayashi, K., Sakai, A., Asada, S., Muramatsu, D, Kuroda, S, Mizuhashi, F., Nagai, M., Suzuki, H., Imamura, T., Asakura, M., Satoh, H., Sakamoto, A., Nakao, R., Hirose, H., Ishii, N. and Umeda, M. (2009), An international collaborative study on a cell transformation assay using Bhas 42 cells, *AATEX.*, 14, 831-848.
27. EURL ECVAM Recommendation on the Cell Transformation Assay based on the Bhas 42 cell line 1. EURL ECVAM Recommendation, ESAC Opinion and ESAC WG Report, https://eurl-ecvam.jrc.ec.europa.eu/eurl-ecvam-recommendations/files-bhas/EURL_ECVAM_Recommendation_Bhas-CTA_2013.pdf.
28. Creton, S., Aardema, M., Carmichael, P.L., Harvey, J.S., Martin, F.L., Newbold, R.F., O'Donovan, M.R., Pant, K., Poth, A., Sakai, A., Sasaki, K., Scott, A.D., Schechtman, L.M., Shen, R.R., Tanaka, N. and Yasaei, H. (2012), Cell transformation assays for prediction of carcinogenic potential: state of the science and future research needs, *Mutagenesis*, 27, 93–101.
29. Vanparys, P., Corvi, R., Aardema, M.J., Gribaldo, L., Hayashi, M., Hoffmann, S. and Schechtman, L. (2012), Application of in vitro cell transformation assays in regulatory toxicology for pharmaceuticals, chemicals, food products and cosmetics, *Mutat. Res.*, 744, 111-116.
30. Land, H., Parada, L.F. and Weinberg, R.A. (1983), Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes, *Nature*, 304, 596-602.
31. Ruley, H.E. (1983) Adenovirus early region 1A enables viral and cellular transforming genes to transform primary cells in culture, *Nature*, 304, 602-606.
32. Newbold, R.F. and Overell, R.W. (1983), Fibroblast immortality is a prerequisite for transformation by EJ c-Ha-ras oncogene, *Nature*, 304, 648-651.

33. Hsiao, W.L., Gattoni-Celli, S. and Weinstein, I.B. (1984), Oncogene-induced transformation of C3H 10T1/2 cells is enhanced by tumor promoters, *Science*, 226, 552-555.
34. Dotto, G.P., Parada, L.F. and Weinberg, R.A. (1985), Specific growth response of ras-transformed embryo fibroblasts to tumour promoters, *Nature*, 318, 472-475.
35. Hsiao, W.L., Wu, T. and Weinstein, I.B. (1986), Oncogene-induced transformation of a rat embryo fibroblast cell line is enhanced by tumor promoters, *Mol. Cell. Biol.*, 6, 1943-1950.
36. Zarbl, H., Sukumar, S., Arthur, A.V., Martin-Zanca, D. and Barbacid, M. (1985), Direct mutagenesis of Ha-ras-1 oncogenes by N-nitroso-N-methylurea during initiation of mammary carcinogenesis in rats, *Nature*, 315, 382-385.
37. Müller, R., Bravov R., Burckhardt, J. and Curran, T. (1984), Induction of c-fos gene and protein by growth factors precedes activation of c-myc, *Nature*, 312, 716-720.
38. Lim, Y.M., Sasaki, K., Muramatsu, D., Arai, S., Endou, N., Sakai, A., S., Umeda, M. and Tanaka, N. (in preparation), Enhancement of expression of v-Ha-ras gene in chemically induced transformed Bhas 42 cells. .
39. Sueoka, E., Sueoka, N., Okabe, S., Komori, A., Suganuma, M., Kozu, T. and Fujiki, H. (1998), Tumourigenicity of MTG8, a leukaemia-related gene, in concert with v-Ha-ras gene in BALB/3T3 cells, *Br. J. Haematol.*, 101, 737-742.
40. Suganuma, M., Kurusu, M., Okabe, S., Sueoka, N., Yoshida, M., Wakatsuki, Y. and Fujiki, H. (2001), Helicobacter pylori membrane protein 1: a new carcinogenic factor of Helicobacter pylori, *Cancer Res.*, 61, 6356-6359.
41. Hahn, W.C. and Weinberg, R.A. (2002), Rules for making human tumor cells, *N. Engl. J. Med.*, 347, 1593-1603.
42. Futscher, B.W. (2013), Epigenetic changes during cell transformation, *Adv. Exp. Med. Biol.*, 754, 179-194.
43. Harvey, D.M. and Levine, A.J. p53 alteration is a common event in the spontaneous immortalization of primary BALB/c murine embryo fibroblasts, (1991), *Genes Dev.*, 5, 2375-2385.
44. Rundhaug, J.E. and Fischer, S.M. (2010), Molecular mechanisms of mouse skin tumor promotion, *Cancers*, 2, 436-482.
45. Bustaffa, E., Stoccoro, A., Bianchi, F. and Migliore, L. (2014), Genotoxic and epigenetic mechanisms in arsenic carcinogenicity, *Arch. Toxicol.*, 88, 1043-1067.
46. Zenonos, K. and Kyprianou, K. (2013), RAS signaling pathways, mutations and their role in colorectal cancer, *World J. Gastrointest. Oncol.*, 5, 97-101.
47. Enomoto, T. and Yamasaki, H. (1985), Phorbol ester-mediated inhibition of intercellular communication in BALB/c 3T3 cells: relationship to enhancement of cell transformation, *Cancer Res.*, 45, 2681-2688.
48. Sasaki, K., Huk, A., El Yamani N., Tanaka, N. and Dusinska, M. (2014), Bhas 42 cell transformation assay for genotoxic and non-genotoxic carcinogens, In: Sierra, L.M. and Gaivão, I. eds. *Genotoxicity and DNA Repair -A Practical Approach-, Methods in Pharmacology and Toxicology*, 343-362, Springer, New York, NY.
49. OECD (2007), 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 ENV/JM/MONO(2007)18, http://www.oecd.org/chemicalsafety/testing/37863750.pdf
50. Sakai, A., Iwase, Y., Nakamura, Y., Sasaki, K., Tanaka, N. and Umeda, M. (2002), Use of a cell transformation assay with established cell lines, and a metabolic cooperation assay with V79 cells for the detection of tumour promoters: a review, *Altern. Lab. Anim.*, 30, 33-59.
51. Sasaki, K., Wakuri, S., Gondoh, M., Endou, N., Tanaka, N. and Yamakage, K. (in preparation), Effects of carcinogens on cytochrome P450 isoenzymes induction in v-Ha-ras-transfected BALB/c 3T3 cells (Bhas 42 cells).
52. Kashuba, A.D.M. and Bertino, J.S. Jr. (2001), Mechanisms of drug interactions I. In: Piscitelli, S.C. and Rodvold, K.A. eds. *Drug Interactions in Infectious Diseases, Second Edition*, Humana Press Inc., 13-39, Totowa, NJ.

53. Paine, M.F., Hart, H.L., Ludington, S.S., Haining, R.L., Rettie, A.E. and Zeldin, D.C. (2006), The human intestinal cytochrome P450 "pie", *Drug. Metab. Dispos.*, 34, 880-886.
54. Dickson, R.B. and Stancel, G.M. (2000), Estrogen receptor-mediated processes in normal and cancer cells, *J. Natl. Cancer Inst. Monogr.*, 27, 135-145.
55. Shimada, T. and Fujii-Kuriyama, Y. (2004), Metabolic activation of polycyclic aromatic hydrocarbons to carcinogens by cytochromes P450 1A1 and 1B1, *Cancer Sci.*, 95, 1-6.
56. Chida, K., Hashiba, H., Sasaki, K. and Kuroki T. (1986), Activation of protein kinase C and specific phosphorylation of a Mr 90,000 membrane protein of promotable BALB/3T3 and C3H/10T1/2 cells by tumor promoters, *Cancer Res.*, 46, 1055-1062.
57. Zhang, L., Zhao, R., Ye, S.Q., Zhou, L., Wu, Y.N. and Zeng, Y. (2013), Synergistic effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin and N-nitrosodiethylamine on cell malignant transformation, *Biomed. Environ. Sci.*, 26, 323-330.
58. Hayashi. K., Sasaki, K., Asada, S., Tsuchiya, T., Hayashi, M., Yoshimura, I., Tanaka, N. and Umeda, M. (2008), Technical modification of the Balb/c 3T3 cell transformation assay: the use of serum-reduced medium to optimise the practicability of the protocol, *Altern. Lab. Anim.*, 36, 653-665.
59. Arai, S., Tanaka, N., Sasaki, K. and Sakai, A. (2010), A study on the dose setting of test chemicals for the promotion assay in Bhas 42 cell transformation assay, *AATEX.*, 15, 6-13.

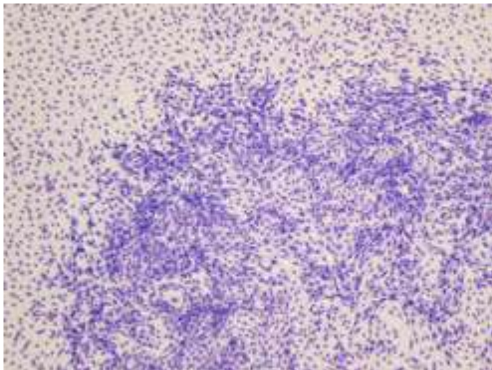
Annex 1: Culture media, reagents and solutions

Media and supplements:
- MEM: Minimum essential medium with 2.2 g/L $NaHCO_3$ and 0.292 g/L L-glutamine.
- DMEM/F12: Dulbecco's modified Eagle's medium/F12 with 1.2 g/L $NaHCO_3$.
- FBS: Fetal bovine serum, selected based upon its ability to support a low frequency of spontaneous transformed focus formation and its ability to induce a high frequency of transformed focus formation in the positive control.
- PS: Penicillin G sodium (10,000 units/mL) and streptomycin sulfate (10 mg/mL).
- M10F: MEM + 10% FBS + 1% PS (500 mL MEM + 56 mL FBS + 5 mL PS): used for cell population expansion, cell storage, and the first culture after thawing.
- DF5F: DMEM/F12 + 5% FBS + 1% PS (500 mL DMEM/F12 + 26.5 mL FBS + 5 mL PS): used for routine subculturing of cells, cell growth assays and transformation assays.

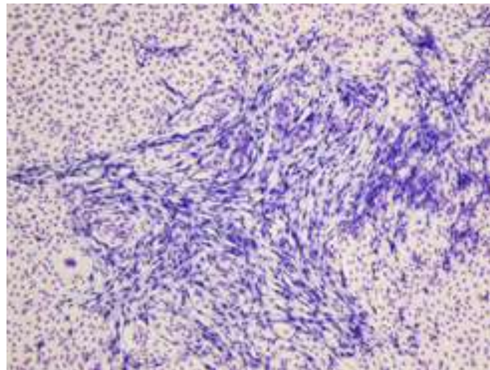
Fixatives and staining solutions:
- Formalin (37% formaldehyde): used for fixing cells.
- Ethanol: used for fixing cells.
- Methanol: used for fixing cells.
- 0.1% crystal violet (CV) solution: used for staining cells in cell growth assays. CV, 1 g, is dissolved in 50 mL of ethanol, and the total volume is adjusted to 1 L with distilled water/ultra-pure water.
- Extraction solution: 0.02 mol/L HCl and 50% ethanol (480 mL distilled water/ultra-pure water + 500 mL ethanol + 20 mL 1 M HCl) used for extracting CV in cell growth assays.
- 5% Giemsa solution: used for staining cells in transformation assays.

Annex 2: Photo catalog of foci in Bhas 42 CTA (see * below for definitions of abbreviations used)

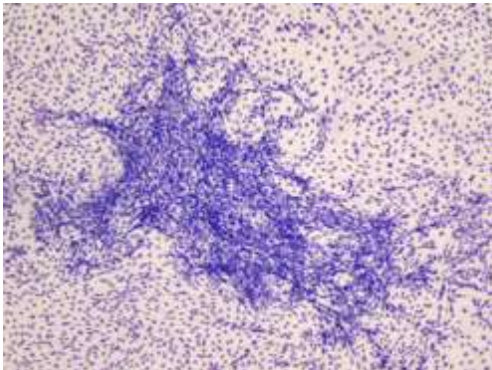
<Negative (Non-transformed) Foci>



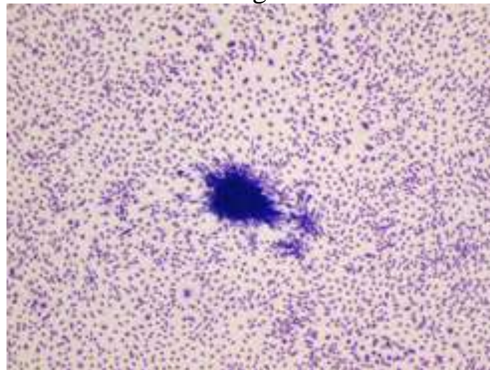
B- S- M- R- I-
The cells simply gather together (cluster).



B+/- S+ M- R- I-
The morphology of the cells changes to spindle-shaped. However, all other characteristics are negative.

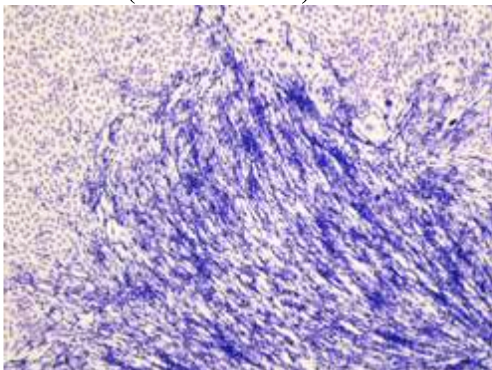


B+/- S+ M+/- R- I+
Piling up is scarcely observed and other positive characteristics are barely discernible.

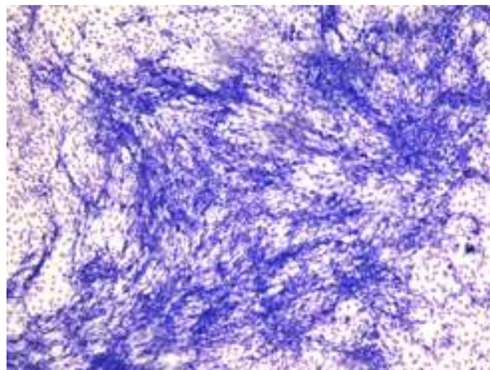


B+ S+ M+ R+ I+
Morphological characteristics resembling transformation are observed but the focus is exceedingly small.

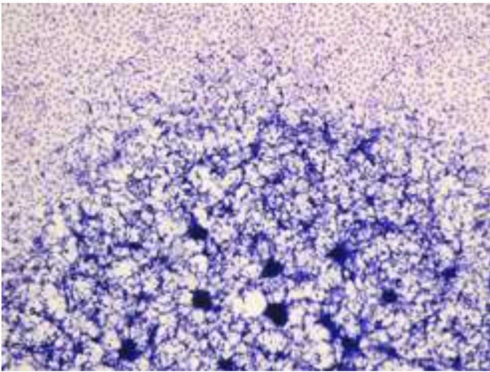
<Positive (Transformed) Foci>



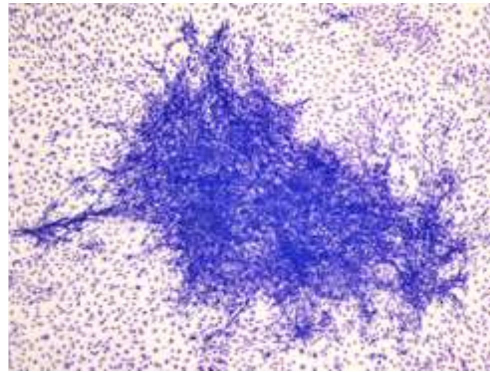
B+ S++ M+/- R+ I+
Piling up is limited. The cells comprising the Focus are markedly spindle-shaped (tapered and elongated), displaying a swirling parallel arrangement.



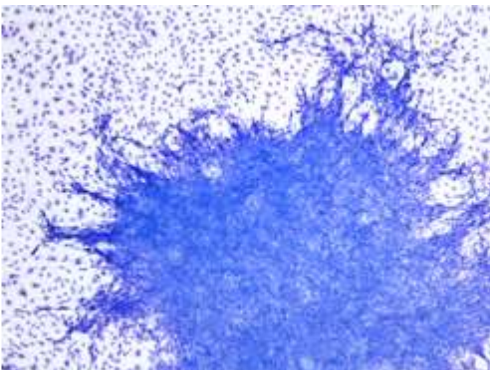
B+ S++ M+ R+ I+
Some areas of piling up are observed within the focus. Foci consist of markedly spindle-shaped (tapered and elongated) cells generally aligned relative to each other.



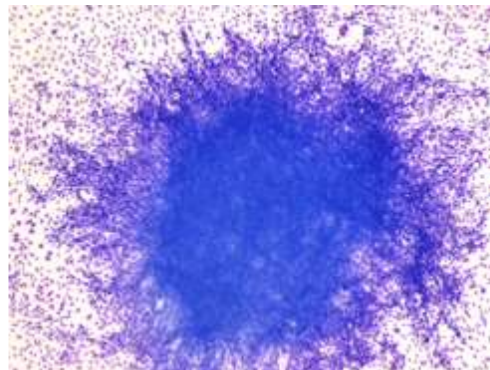
B+ S++ M+/- R++ I+
Scattered areas of piling up and knotting (dense clustering) of cells are observed. Cells are exceedingly spindle-shaped (tapered and elongated) and randomly orientated.



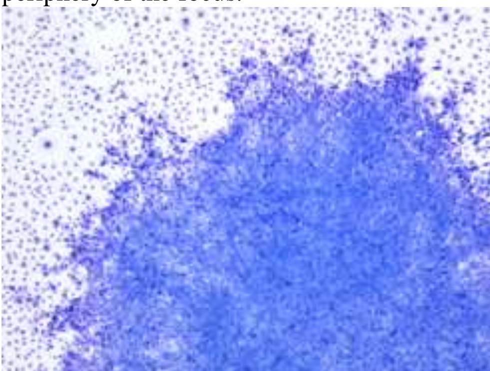
B+ S+ M+ R+ I+
All properties that are characteristic of the transformed phenotype are moderately expressed.



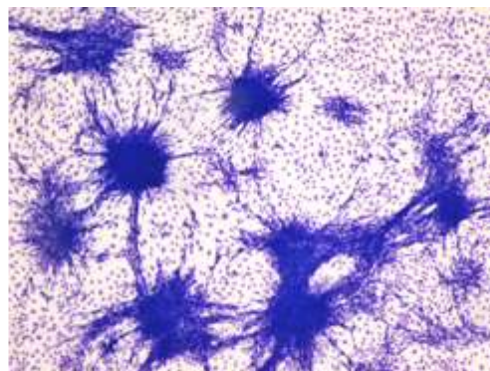
B+ S++ M++ R++ I+
All aberrant phenotypic characteristics are clearly visible. With the exception of multilayered areas, which are too dense to discern individual cell morphology, the atypical properties of the transformed focus are readily observed at the periphery of the focus.



B+ S+ M++ R+ I+
The cells comprising the periphery of the focus are less densely packed and their spindle-shape and random orientation, although apparent, are less striking. The interlaced cells at the edge of the focus invade the surrounding monolayer.



B+ S- M++ R- I+
The multi-layering and density of cells are apparent throughout the focus. The cells comprising the periphery of the focus are not obviously spindle-shaped or randomly orientated but do invade the contact-inhibited monolayer.



B+ S++ M++ R++ I+
Daughter (secondary) foci originating from a single parent focus and exhibiting typical and uniform transformed morphology. These are found in close proximity, are often connected by cellular appendages, and are scored as one transformed focus.

*Abbreviations: B, basophioic; S, spindle-shaped; M, multilayer; R, random orientated; I, invasive.

Annex 3: Data collection forms for the Bhas 42 CTA (Example)

Data Sheet for Cell Growth Assay for 6-well Format

Cell growth assay	Well No.	Blank	Solvent (%)	Concentrations (mM)								
OD	1											
	2											
	3											
	Average											
	SD											
	Average - Blank											
Relative cell growth (%)												

Data Sheet for Cell Transformation Assay for 6-well Format

Cell growth assay	Well No.	Blank	Solvent (%)	Concentrations (mM)									Negative control	Positive control
													0.1% DMSO	MCA (1 ug/mL)
OD	1													
	2													
	3													
	Average													
	SD													
	Average - Blank													
Relative cell growth (%)														

TTransformation assay	Well No.		Solvent (%)	Concentrations (mM)									Negative control	Positive control
													0.1% DMSO	MCA (1 ug/mL)
Foci/Well	1													
	2													
	3													
	4													
	5													
	6													
	Average													
	SD													
Transformation frequency (foci/well)														

Data Sheet for Cell Growth Assay for 96-well Format

Cell growth assay	Well No.	Blank	Solvent (%)	Concentrations (mM)								
OD	1											
	2											
	3											
	4											
	5											
	6											
	7											
	8											
	Average											
	SD											
	Average - Blank											
Relative cell growth (%)												

Data Sheet for Cell Transformation Assay for 96-well Format

Cell growth assay	Well No.	Blank	Solvent (%)	Concentrations (mM)									Negative control	Positive control
													0.1% DMSO	MCA (1 ug/mL)
OD	1													
	2													
	3													
	4													
	5													
	6													
	7													
	8													
	Average													
	SD													
	Average - Blank													
Relative cell growth (%)														