DRAFT TEST GUIDELINE

In Vitro Carcinogenicity: Bhas 42 Cell Transformation Assay

INTRODUCTION

1. In vitro cell transformation refers to the induction of phenotypic alterations in cultured cells which are characterized as the change from non-transformed to transformed phenotype, the latter being considered typical aberrations associated with cells exhibiting neoplastic potential in vivo (1, 2). Transformed cells with the characteristics of malignant cells have the ability to induce tumors in susceptible animals (3, 4, 5); this supports the use of phenotypic alterations in vitro as criteria for predicting carcinogenic potential in vivo.

2. Various types of cell transformation assays (CTAs) have been developed for detection of carcinogenic stimuli. The Syrian hamster embryo (SHE) CTA is a primary cell system, and BALB/c 3T3, C3H10T1/2 and Bhas 42 cell CTAs are systems using established cell lines. OECD reviewed the performances of CTAs based on retrospective data (OECD Detailed Review Paper 31, DRP 31) (6). International validation studies of SHE and BALB3T3/c CTAs were performed (7) by European Centre for the Validation of Alternative Methods (ECVAM). Thereafter, an international validation study of Bhas 42 CTA was performed by the New Energy and Industrial Technology Development Organization (NEDO) in conjunction with the Japanese Center for the Validation of Alternative Methods (JaCVAM). This validation study ensured the use of a standardized Bhas 42 CTA protocol, confirmed its transferability within and between laboratories, and established its intra- and inter-laboratory reproducibility.

3. 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. Not all carcinogens, however, are known to be genotoxicants; animal carcinogenesis studies have clearly demonstrated that there exists a promotion process distinct from an initiation process (two-stage carcinogenesis) for agents that are not direct acting carcinogens (8). In addition, it has become clear that some CTAs can reproduce the two-stage carcinogenesis progression and therefore detect and distinguish between the promoting activity and the initiating activity of carcinogens (9).

4. The Bhas 42 cell line was established by the transfection of the v-Ha-ras oncogene into the BALB/c 3T3 A31-1-1 cell line and because of its resulting desirable phenotypic properties and responsiveness to chemical carcinogens was selected from among other such transfected cell lines for the CTA (10, 11). Similar to the parental BALB/c 3T3 cell line, untransformed Bhas 42 cells grow to confluence forming a contact-inhibited monolayer and such cells lack tumorigenicity upon transplantation in vivo. After exposure to carcinogenic stimuli, such cells can become morphologically altered and form independent aberrant colonies, referred to as transformed foci, capable of invading the surrounding non-transformed contact-inhibited monolayer. This focus formation is the endpoint of the Bhas 42 CTA.

5. The current protocol for the Bhas 42 CTA consists of two-assay components, the initiation assay and promotion assay for examining tumor-initiating activity and tumor-promoting activity of chemicals, respectively (12, 13). It is
acknowledged that mutation induced by chemical insult is fixed after several cell replication cycles (14, 15). Thus, in the initiation assay the cells are treated at the beginning of growth phase to allow for fixation of the induced DNA damage, and in the promotion assay the cells are repeatedly treated at stationary phase to provide a growth advantage for anomalous cells.

6. Several comprehensive studies were performed to assess the relevance and predictive reliability of the Bhas 42 CTA. These included (a) an extensive analysis of 98 chemicals (13), (b) a multi-laboratory collaborative study (16), (c) a prevalidation study (17), and (d) two international validation studies (18, 19). For the latter, the Validation Advisory Committee and the Validation Management Team were comprised of international experts from ECVAM, ICCVAM and JaCVAM. The results of all of these studies confirmed the applicability, transferability, reproducibility and reliability of the Bhas 42 CTA protocol and the assay was found to be sufficiently sensitive to predict both initiating activity and promoting activity of carcinogens.

7. 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 (20) and may reduce 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 (21).

8. This Test Guideline provides an in vitro procedure using the Bhas 42 CTA, which can be used for hazard identification of chemical carcinogens having initiating and/or promoting activity. The test method described is based upon the protocol for reported for this assay in Sakai et al. (13).

9. In this Test Guideline, the test methods using both 6-well plates and 96-well plates are described. After initial development of the Bhas 42 CTA 6-well method, the assay was adapted to a 96-well method which was designed for high-throughput analyses. Although the number of cells plated and expression of transformation frequency differ between the 6-well and 96-well methods, the overall results obtained are similar (19, 22).

INITIAL CONSIDERATIONS AND LIMITATIONS

10. The Bhas 42 cell line was established by transfection of the v-Ha-ras oncogene into the BALB/c 3T3 A31-1-1 cloned cell line, for which stable integration of the v-Ha-ras gene was demonstrated (10, 11). Like the parental BALB/c 3T3 A31-1-1 cells, the Bhas 42 cell line still maintains its non-transformed morphological properties, including that of density dependent inhibition of cell growth (contact-inhibition). It is considered to be an initiated cell line; that is, the cells are considered to have advanced beyond a “normal” condition toward a more atypical pathological state, having progressed to a certain extent along the multi-step carcinogenesis process (23). This attribute makes Bhas 42 cells highly sensitive to carcinogenic stimuli and accounts for the short latency period for expression of the transformed focus phenotype.

11. Due to their initiated state and their sensitivity to carcinogenic stimuli, Bhas 42 cells may spontaneously transform under inappropriate culture conditions. Therefore, it is important to maintain strict quality control of cells,
assay components, and test conditions, including the use low passage target cells, maintainence of a sub-confluent cell population density (≤ 70% confluence) among cell stocks to be used for treatment, and use of suitable pre-screened batches of serum (FBS). It should be noted that spontaneous transformation is a common intrinsic occurrence and is expressed at different relative frequencies among the available target cell systems used in CTAs. 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.

12. Established cell lines often do not retain the full complement of metabolic capacity with time in culture. Unless the cells are metabolically competent with respect to the substances being tested, , in vitro tests conducted with such cell lines generally require metabolic supplementation (exogenous metabolic activation) to approximate, though not entirely mimic, in vivo metabolic conditions. The fact that Bhas 42 cells respond to polycyclic aromatic hydrocarbons, 2-acetylaminofluorene, and cyclophosphamide, all of which require metabolic activation (13), suggests that Bhas 42 cells contain some level of the cytochrome P450 family of enzymes including CYP1A1 and others.

13. Initiating activity and promoting activity of carcinogens can be distinguished in the animal carcinogenicity studies using the two-stage carcinogenesis model but this distinction is not generally pursued in carcinogenicity studies in vivo. In its evaluation of the relative performance of CTAs, OECD reported on CTA responsiveness to 260 carcinogens in its DRP 31. 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/c 3T3 and C3H10T1/2 CTAs. As to the performance of the in vitro promotion assay 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 assay (13, 19, 24). These results indicate that the Bhas 42 cell promotion assay can be a valuable in vitro system for identifying potential in vivo tumor promoters. Further studies of this kind will no doubt add to the body of data demonstrating the utility of the Bhas 42 cell promotion assay.

14. Morphologically, various types of transformed foci are observed (refer to Paragraph 47 and 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 aberrant foci and in distinguishing them from non-transformed foci (see Annex 2).

**PRINCIPLE OF THE TEST METHOD**

15. 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 plates or 96-well plates. In the initiation assay, the cells are treated with a given test substance 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 assay, the treatment with the test substance 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 blind; the resulting foci are evaluated for their morphological phenotype.

16. Transformation frequency is quantified using stereomicroscopy as follows: (a) for the 6-well method transformed foci in each well are scored; (b) for the 96-well method the number of wells with transformed foci are counted. The latter counting method eliminates the imprecision that could result from attempting to score multiple foci
forming in the smaller wells of the 96-well plates. It is important, however, to be cognizant not only of those foci that form on the bottom of the wells, but also those that may adhere to the side-wall of such wells (19, 22).

17. 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/vehicle control cells. The transformation index 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/vehicle controls.

PROCEDURE

Culture media, reagents and solutions

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

Culture conditions and preparation of cell suspension

19. 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. DF5F is used for the cell growth assays and transformation assays as well as routine maintenance and subculturing of cells.

20. Bhas 42 cells are incubated at 37°C in a humidified atmosphere of 5% CO₂ 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 aberrant foci atop the confluent cell monolayer.

Preparation and cryopreservation of Bhas 42 cell stocks

21. 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).

22. 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 several 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, 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-cm² flask to a cell density not to exceed 70% confluence. They are then suspended at a cell density of 5 x 10⁵
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 cultured for 1-2 passages in M10F before cryopreservation. 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 23, which are the same as those criteria for accepting a given lot of FBS.

23. In order to identify suitable lots of FBS for the transformation assay, several batches of FBS are checked using the cells from one master stock. The acceptance criteria for a given lot of FBS include (a) adequate plating efficiency of Bhas 42 cells (≥50%), (b) low background of spontaneous transformation, and (c) ability to facilitate Bhas 42 cell transformation by positive controls (1 µg/mL MCA and 0.05 µg/mL TPA (refer to Paragraph 27). FBS batches which fulfill the criteria in Paragraph 52 for the 6-well method and Paragraph 53 for the 96-well method are those that are selected for use in subsequent transformation assays.

24. Freshly prepared low passage cells derived from the cryopreserved cell stocks are used for each transformation assay.

25. The same cell source can be used for the cell growth assay, although cells at higher passages can also be used for dose setting.

Controls

26. The solvent/vehicle for a test substance is used as the negative control.

27. For positive controls a known tumor-initiator, MCA (final concentration of 1 µg/mL), is used in the initiation assay, and a known tumor-promoter, TPA (final concentration of 50 ng/mL), is used in the promotion assay. MCA and TPA are dissolved in dimethyl sulfoxide (DMSO), which serves as the solvent/vehicle for these two control agents. When the solvent/vehicle for the test substance 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 substance solutions

28. Test substances are dissolved or suspended in an appropriate solvent or vehicle and diluted if appropriate, prior to the treatment of the cells. Distilled water, DMSO, acetone, and ethanol can be used to dissolve test substances, and the final solvent/vehicle concentrations in the medium 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 substance and the test system, as well as their lack of transforming activity. In such cases, untreated controls devoid of the solvent of choice should also be included.

29. The maximum concentrations to be tested in cell transformation assay depend on test substance solubility and cytotoxicity. For test substances of defined composition, the highest dose level should be 0.01 M, 2 mg/mL or 2 µL/mL,
whichever is the lowest. For test substance of undefined composition, *e.g.* complex mixtures (plant extracts, tars, environmental extracts, *etc.*), the top concentration should be at least 5 mg/mL. Poorly soluble chemicals should be tested up to the first concentration producing a visible opacity (precipitation) in the final test medium observable by the unaided eye. Five to nine concentrations should be tested and these are determined according to the results of the cell growth assay. Paragraphs 34, 39–41 provide further details on the top concentration that should be tested.

**Experimental design**

30. The design of the two test methods used, *i.e.* the 6-well method and a 96-well method, are fundamentally similar in that their experimental procedures are basically the same except for the number of cells plated and the expression of transformation frequency. In the following sections, details are presented for the 6-well method and those modifications associated with the 96-well method are indicated.

31. Both methods consist of an initiation assay component and a promotion assay component. These assays can detect initiating activity and promoting activity of carcinogens, respectively. In the initiation assay, the cells are treated with chemicals in the beginning of growth phase and in the promotion assay the treatment is started at sub-confluence of cell growth. In both assays, a dose range-finding test is first conducted in which the results of a cell growth assay are used for the determination of appropriate test concentrations to be employed in the subsequent transformation assay and concurrent cell growth assay (Fig. 1).

**Figure 1: General Scheme of the Bhas 42 CTA**

**Initiation assay**

• Cell growth assay to set doses

32. The cells at ≤ 70% confluence in DF5F are suspended with trypsin and 4,000 cells are seeded into a 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 seeding, the culture medium is replaced with fresh medium containing various concentrations of a test substance, or concentrated test substance 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 methanol or 10% formalin for approximately 10 min, washed and 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.

![Day 3 0 1 4 7](Fix: Methanol or formaldehyde)

![Stain: Crystal violet](DMEM/F12 + 5% FBS (DF5F))

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<th>DMEM/F12 + 5% FBS (DF5F)</th>
<th>Chemical treatment</th>
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<td>![Cell seeding](Cell seeding)</td>
<td>![Chemical addition](Chemical addition)</td>
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<td>![Medium change](Medium change)</td>
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**Figure 2: Schematic protocol of cell growth assay in the initiation assay**

33. CV is extracted from the stained cells with 2 mL of dye extraction solution, and the OD 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 (%) = \[ \frac{(\text{Treatment} - \text{Blank})}{(\text{Control} - \text{Blank})} \times 100 \]

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

34. Five to nine concentrations 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, these concentrations that are included are: (a) at least one concentration below the no observed effect level (NOEL), (b) two concentrations between the NOEL and the 50% inhibitory concentration (IC50), and (c) two concentrations between the IC50 and the IC90. The ratio between neighboring concentrations should be less than square root of 10 (Fig. 3). Some test substances exhibit a steep concentration–response curve. With these test substances, test concentrations should be spaced at much closer intervals. In addition, it may become necessary to set up one or two more additional test concentrations below and above the expected dose range in order to allow for possible unanticipated cytotoxic fluctuations among experiments.

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<th>NOEL</th>
<th>IC50</th>
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<td>At least one dose</td>
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<td>Two doses</td>
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**Figure 3: Dose setting for the initiation assay component of the transformation assay**

35. In the 96-well method, the cell growth assay is carried out in the same manner as the 6-well method except the following conditions.
- Into each well, 200 cells are seeded 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 substance or solvent/vehicle alone at two times the final desired concentrations, so that the final volume of the medium becomes 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**

36. The frozen working stock cells are rapidly thawed, suspended in M10F and cultured in 100-mm culture plates at a volume of 10 mL medium. When the cells reach approximately 70% confluence, they are trypsinized, suspended in DF5F at an appropriate density (7,000 to 10,000 cells/mL is suggested) and cultured in 100-mm culture plates (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 seeded into each well 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 in the same way as the cell growth assay (Day 1-4). The medium is changed with fresh DF5F on Day 4, 7, 10 (or 11) and 14. On Day 21, the cells are fixed with methanol and stained with 5% Giemsa solution for approximately 15 min (Fig. 4). The positive control (1 µg/mL MCA) and the negative (solvent/vehicle) control(s) are included in the transformation assay for each test substance. When the solvent/vehicle used for the test substance is not DMSO, DMSO is still necessary as the negative control for MCA and thus, two solvent controls (one for the test article and one for the positive control) are required. In addition, an untreated control devoid of the solvent used for the test article should also be included in cases when the solvent/vehicle used is one other than those commonly employed (see Paragraph 28). 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).

![Figure 4: Schematic protocol for the initiation assay component of the transformation assay](image)

37. In the 96-well method, the transformation assay is carried out in the same manner as in the transformation assay for the 6-well method except that 200 cells are seeded/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 substance on Day 1. One 96-well plate (96 wells) is prepared for the transformation assay and eight wells are prepared for the concurrent cell growth assay, respectively.
**Promotion assay**

- **Cell growth assay to set doses.**

38. The experimental procedure is basically the same as the initiation assay, 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 substance solution (Fig. 5). Three wells are prepared for each group.

![Diagram of cell growth assay](image)

**Figure 5:** Schematic protocol of cell growth assay in the promotion assay

39. There are two different types of chemicals that exhibit promoting activity. One group includes those chemicals that markedly enhance cell growth. With these test substances, 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 NOEL, three concentrations in the range of growth enhancement and one concentration in the range of weak growth inhibition are assessed (Fig. 6).

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<td>One dose</td>
<td>Three doses</td>
<td>One dose</td>
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**Figure 6:** Dose setting for the promotion assay component of the transformation assay for chemicals that exhibit marked growth enhancement

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

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<td>At least two doses</td>
<td>Two doses</td>
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**Figure 7:** Dose setting for the promotion assay component of the transformation assay for chemicals that inhibit growth
41. It is noteworthy that there are chemicals that induce more pronounced growth inhibition in the transformation assay than would otherwise be observed in the concurrent cell growth assay. 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 3 days for the cell growth assay (26).

42. When test substances exhibit a steep concentration–response curve, considerations similar to those described in the initiation assay (refer to Paragraph 34) may need to be taken into account, e.g. inclusion of additional test concentrations and test concentration intervals, thereby ensuring an acceptable assay outcome.

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

- **Transformation assay**

44. The transformation assay is carried out in the same manner as the initiation assay 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) 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 substance for 10 days, from Day 4 to Day 14.

- The chemical treatment is carried out on Day 4, 7 and 10 (or 11) by exchanging existing medium with fresh medium containing the chemical solution or solvent/vehicle alone.

- On Day 14, the medium is changed with the fresh medium containing neither solvent/vehicle nor test substance.

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

![Figure 8: Schematic protocol for the promotion assay component of the transformation assay](image-url)
45. In the 96-well method, the transformation assay is carried out in the same manner as that for the 6-well method except for the plating of 400 cells in 0.1 mL of DF5F (Day 0). One 96-well plate (96 wells) is prepared for the transformation assay and eight wells are employed for the concurrent cell growth assay.

**Evaluation of the results**

- **Determination of transformation frequency**

46. Transformed foci are scored using a stereomicroscope. If a given concentration results in a failure of the target cells to reach confluence because of cytotoxicity, that concentration is considered not acceptable for transformation assessment and is excluded from focus-counting. For such situations, “toxicity” is recorded in the data sheet.

47. 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 need not necessarily exhibit all of these morphological characteristics to be regarded as transformed, but that observation of clear-cut morphological aberrations such as these is generally sufficient to classify transformed foci as such (see Annex 2). For quantification, the number of transformed foci in each well are recorded for each group.

48. In the 96-well method, transformed foci are judged using the same criteria as in the 6-well method. 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**

49. Test substance-induced transformation frequency (number of transformed foci/well) in the 6-well method is statistically analyzed by multiple comparison using the one-sided Dunnett test (p<0.05, upper-sided). For the positive controls, the statistical significance is evaluated by the one-sided t-test or Aspin-Welch test (p<0.05, upper-sided) depending on the results of F-test for homoscedasticity (homogeneity of variance).

50. Test substance-induced transformation frequency (proportion of wells with one or more transformed foci) in the 96-well method is statistically analyzed using the chi-square test with Bonferroni adjustment (p-value<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).

- **Assay acceptance criteria**

51. The following criteria (Paragraphs 52 and 53) must be fulfilled for a given assay to be considered valid. When considered invalid, the initiation or promotion assay is repeated independently, as needed, to satisfy the assay acceptance criteria.
52. In the 6-well method, the following criteria must be fulfilled for a given assay to be considered valid:

- When contamination or technical problems are observed in wells, 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 cases where such technical difficulties are encountered, “contamination”, “accident”, “technical error”, etc. are recorded in the data sheet.

- In the negative control, the number of (spontaneous) transformed foci must be 10 or less per well in the initiation assay and 12 or less per well in the promotion assay.

- In the positive control, there must be a statistically significant increase in the number of transformed foci per well compared to the corresponding negative control.

- A transformation assay is considered acceptable if four test chemical concentrations persist and the following conditions are satisfied.

  - In the initiation assay, it is recommended that the results of the concurrent cell growth assay include at least one concentration near the NOEL and three concentrations in the range between the NOEL and the IC90.

- In the promotion assay showing growth enhancement, it is recommended that the results of the concurrent cell growth assay include at least one concentration near the NOEL and two concentrations in the range of growth enhancement.

- In the promotion assay showing growth inhibition, it is recommended that the results of the concurrent cell growth assay include at least two concentrations below the NOEL and two concentrations between the NOEL and the IC50.

53. In the 96-well method, the following criteria must be fulfilled for a given assay to be considered valid:

- When contamination or technical problems are observed, 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.

- In the initiation assay, the number of wells in the negative control plates having (spontaneous) transformed foci must be 15 wells/plate or less; if damaged wells are present, the number of undamaged wells with transformed foci must be \(\leq 15.625\%\). In the promotion assay, the number of wells in the negative control plates having (spontaneous) transformed foci must be 20 wells/plate or less; if damaged wells are present, the number of undamaged wells with transformed foci must be \(\leq 20.833\%\).

- In the positive control, there must be a statistically significant increase in the proportion of wells having transformed foci.
- A transformation assay is considered acceptable if four test chemical concentrations persist and the following conditions are satisfied.

  - In the initiation assay, it is recommended that the results of the concurrent cell growth assay include at least one concentration near the NOEL and three concentrations in the range between the NOEL and the IC90.

  - In the promotion assay showing growth enhancement, it is recommended that the results of the concurrent cell growth assay include at least one concentration near the NOEL and two concentrations in the range of growth enhancement.

  - In the promotion assay showing growth inhibition, it is recommended that the results of the concurrent cell growth assay include at least two concentrations below the NOEL and two concentrations between the NOEL and the IC50.

54. In the initiation and promotion assays, 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 induce 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 two concentrations do not, would necessitate a repeat experiment at lower concentrations (i.e. 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

55. The assay results in the 6-well method and 96-well method are judged as follows:

  - The results in the initiation and promotion assays are judged positive if there are two or more sequential doses that induce statistically significant increases in the number of transformed foci per well relative to the corresponding vehicle control.

  - The results in the initiation and promotion assays are judged negative if there is no dose showing a statistically significant increase in the number of transformed foci per well.

  - If the statistically significant increase occurs at only one or non-sequential doses, the assay result is regarded as equivocal, in which case the initiation or promotion assay should be repeated. Modification of experimental conditions
in which a broader or narrower range of test chemical concentrations, as appropriate, should be considered in such follow-up experiments to eliminate such equivocality.

- When results in the initiation assay are determined to be positive based upon the above criteria, the test substance is considered to have carcinogenic initiating activity.

- When results in the promotion assay are determined to be positive based upon the above criteria, the test substance is considered to have carcinogenic promoting activity.

- A positive result in either the initiation assay or the promotion assay is taken to mean that the test chemical possesses potential carcinogenic activity, irrespective of which endpoint is positive.

**Laboratory Proficiency**

56. In order to assure the proficiency of a given laboratory, the laboratory should perform tests using four positive chemicals, each acting via different mechanisms of action in both the initiation assay and the promotion assay, and two negative chemicals. Those chemicals recommended are listed in Table 2. During the course of these tests, the laboratory should establish:

- A historical negative (untreated, solvent) control range and distribution.

- A historical positive control range and distribution.

Re-evaluation of laboratory proficiency is recommended if major changes to experimental conditions are introduced in the assay (e.g. use of automated instead of manual scoring techniques). Additionally, changes in laboratory personnel responsible for the conduct of the assay may warrant re-evaluation of adeptness.

Before using this Test Guideline, it is recommended that personnel be trained in a laboratory experienced in this assay.

**Table 2: Chemicals for Assessing Laboratory Proficiency**

<table>
<thead>
<tr>
<th>Category</th>
<th>Chemical</th>
<th>CASRN</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Positive chemicals for initiation assay</td>
<td>N-Methyl-N'-nitro-N-nitrosoguanidine (MNNG)</td>
<td>70-25-7</td>
</tr>
<tr>
<td></td>
<td>3-Methylcholanthrene (MCA)</td>
<td>56-49-5</td>
</tr>
<tr>
<td>2. Positive chemicals for promotion assay</td>
<td>12-O-tetradecanoylphorbol-13-acetate (TPA)</td>
<td>16561-29-8</td>
</tr>
<tr>
<td></td>
<td>Methapyrilene HCl</td>
<td>135-23-9</td>
</tr>
<tr>
<td>3. Negative chemicals for both assays</td>
<td>Caffeine (CFN)</td>
<td>58-08-2</td>
</tr>
<tr>
<td></td>
<td>Mannitol</td>
<td>69-65-8</td>
</tr>
</tbody>
</table>
REPORTING

Test report

57. The test report should include the following information:

Test substance
- identification and CAS number. (if known)
- physical nature and purity
- physical properties relevant for conducting the assay
- stability of the test substance (if known)

Solvent (if appropriate)
- identification
- justification for choice of solvent/vehicle
- concentrations tested and preparation of the dosing solutions
- signs of precipitation (if appropriate)

Cells and media
- source of cells
- number of cell subcultures (passage number)
- maintenance of cell cultures
- absence of cell culture contamination, especially mycoplasma
- identification of media and serum (provider and batch 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 substances
- volume of solvent and test substance added
- duration of treatment
- incubation temperature
- incubation atmosphere: percent CO₂ 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, signs of precipitation in medium
- number of total valid (quantifiable) wells, number of wells lost and the reason(s) for the loss
Discussion of results

Conclusion

REFERENCES


http://www.oecd.org/chemicalsafety/testing/37863750.pdf

7. ECVAM (2012), Recommendation concerning the cell transformation assays (CTA) using Syrian Hamster Embryo cells (SHE) and the BALB/c 3T3 mouse fibroblast cell line for in vitro carcinogenicity testing, including the ESAC opinion (Annex 1) based on the ESAC peer review of an EURL ECVAM-coordinated validation study of three CTA protocols for in vitro carcinogenicity testing


Annex 1: Culture media, reagents and solutions

Media and supplements:
- MEM: Minimum essential medium with 2.2 g/L NaHCO₃ and 0.292 g/L L-glutamine.
- DMEM/F12: Dulbecco’s modified Eagle’s medium/F12 with 1.2 g/L NaHCO₃.
- FBS: Fetal bovine serum, selected based upon a low frequency of spontaneous transformed focus formation and a high induced 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.
- 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

<Negative (Non-transformed) Foci>

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

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

<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.
Scattered areas of piling up and knotting (dense clustering) of cells is observed. Cells are exceedingly spindle-shaped (tapered and elongated) and randomly orientated.

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.

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.

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, basophilic; S, spindle-shaped; M, multilayer; R, random orientated; I, invasive.