DB-ALM Protocol n° 156 : Bhas 42 Cell Transformation Assay in 6- and 96-well plates

Cell transformation

The Bhas 42 cell transformation assay (CTA) is a short-term in vitro assay proposed as an alternative method for testing the carcinogenic potential of chemicals (both genotoxic and non-genotoxic). The assay is based on the change of the phenotypic features of cells undergoing the first steps of the conversion from normal cells to neoplastic-like cell foci with oncogenic properties. The protocol presented here is based on that used in the Bhas 42 CTA validation study (Hayashi *et al*., 2012).

Experimental Description

Endpoint and Endpoint Measurement:

In the Bhas 42 CTA, both cytotoxicity and morphological transformation endpoints are evaluated:

CELL NUMBER - by Crystal Violet staining, as measure of cytotoxicity

MORPHOLOGICAL TRANSFORMATION - number of transformed foci or proportion of wells having transformed foci

Endpoint Value:

RELATIVE CELL GROWTH (RCG) = {[(absorbance of treated well - absorbance of medium blank well) / (absorbance of vehicle control well - absorbance of medium blank well)] × 100)}

Morphological effects are expressed as **TRANSFORMATION FREQUENCY** measured by a number of transformed foci per well (in 6-well plates) or the proportion of wells containing transformed foci (in 96-well plates).

Experimental System(s):

Bhas 42 cells established from BALB/c 3T3 A31-1-1 cells (BALB/c mouse embryo cells) by transfection of v-Ha-ras oncogene (Sasaki *et al.*, 1990).

Discussion

Practical aspects

- Throughput: The Bhas 42 CTA requires 3 weeks per substance. It has a significantly higher throughput compared with the BALB/c 3T3 CTA that requires 5 weeks and the cancer rodent bioassay that requires a minimum 2 years.
- The CTA requires high technical skills with regard to handling of numerous cell plates simultaneously for a relatively long time period, and in particular scoring. Training and the use of the photo catalogue prepared by HRI/FDSC and appended in the end of this protocol are essential for overcoming these potential limitations.
- Bhas 42 cells may spontaneously transform under inappropriate culture conditions due to their initiated state and their sensitivity to carcinogenic stimuli. Therefore, it is important to maintain strict quality control of cells, assay components, and test conditions.
- The use of genetically modified cell lines may require official authorisation in order to comply with national legislations.

For the key principle and main applications of the assay please refer to a Method Summary **"Bhas 42 Cell Transformation Assay in 6- and 96-well plates - Summary"**, available in the DB-ALM.

Status

Participation in Validation Studies:

Pre-validation and validation studies on the 6-well method and a validation study on the 96-well method were coordinated by Hatano Research Institute (HRI) Food and Drug Safety Center (FDSC) in collaboration with the Japanese Center for the Validation of Alternative Methods (JaCVAM). They were peer reviewed by EURL ECVAM, which issued a recommendation in December 2013. An OECD draft Test Guideline for the Bhas 42 CTA is currently being developed.

Proprietary and/or Confidentiality Issues

None

Health and Safety Issues

General precautions

Even if controls are performed, biological material has always to be considered as potentially dangerous. Observe universal precautions in order to protect yourself and your colleagues. Handle the cells in a Level 2-biology safety room.

It is recommended that protective gloves and laboratory coats should be worn when handling hazardous materials.

It is recommended to work safely with a specific class of chemical or hazard (e.g. use and handle potentially carcinogenic test compounds in a fume hood).

Potentially carcinogenic waste is highly hazardous, may have mutagenic or carcinogenic properties and should be given special attention. After each experiment, chemical treated plates are closed by an adhesive film, placed in a plastic bag and appropriately disposed of, in line with local safety regulations.

Abbreviations and Definitions

3Rs: Replacement, Reduction, Refinement of animal use in experiments

CTA: Cell transformation assay

CV: Crystal violet

DF5F: Dulbecco's modified Eagle's medium/Ham's F12 supplemented with 100 units/mL of penicillin, 100 µg/mL of streptomycin and 5% FBS

DMSO: Dimethylsulfoxide

ECVAM: European Centre for the Validation of Alternative Methods. From 2011 ECVAM is established as the European Union Reference Laboratory for alternatives to animal testing (EURL ECVAM)

EDTA: Ethylenediaminetetraacetic acid

EURL ECVAM: the European Union Reference Laboratory for alternatives to animal testing

FBS: Fetal bovine serum

FDSC: Food and Drug Safety Center

HRI: Hatano Research Institute

IC50: 50% inhibitory concentration

IC90: 90% inhibitory concentration

JaCVAM: Japanese Centre for the Validation of Alternative Methods

JCRB: Japanese Collection of Research Bioresources

M10F: Eagle’s minimum essential medium supplemented with 100 units/mL of penicillin, 100 µg/mL of streptomycin and 10% fetal bovine serum

MCA: 3-Methylcholanthrene

NOEL: No observable effect level

OD: Optical density

OECD: Organisation for Economic Co-operation and Development

PBS: Phosphate Buffered Saline

SHE CTA: Syrian hamster embryo cell transformation assay

TPA: 12-O-tetradecanoylphorbol-13-acetate

v-Ha-ras: Harvey rat sarcoma viral oncogene homolog

Last update: 9 January 2014

PROCEDURE DETAILS, 9 January 2014

Bhas 42 Cell Transformation Assay in 6- and 96-well plates
DB-ALM Protocol n° 156

The protocol represents a standard operating procedure used in the validation study of Bhas 42 cell transformation assay in 6- and 96-well plates.

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Materials and Preparations

Cell or Test System

Bhas 42 cells established from BALB/c 3T3 A31-1-1 cells (BALB/c mouse embryo cells) by transfection of v-Ha-*ras* oncogene (Sasaki *et al.*, 1998). Japanese Collection of Research Bioresources (JCRB) Cell Bank at the National Institute of Biomedical Innovation (Osaka, Japan) [**http://cellbank.nibio.go.jp/english/**] supplies frozen Bhas 42 cells.

Equipment

Fixed Equipment

- laminar flow hood (biohazard type and restricted to cell culture assays)
- cell culture incubators (37°C; 5% CO_2; >=85% humidity)
- low-speed centrifuge
- water bath (37°C)
- inverse phase microscope
- micropipettors
- computer
- refrigerator (4°C)
- freezers (-20°C and -80°C)
- containers for storage in liquid nitrogen
- autoclave (for instruments and for bio-hazardous waste materials)
- balance
- pH meter
- osmometer
- cell counting (Bürker-Türk chamber or Coulter counter)
- spectrophotometer with a 540 nm filter (alternatively any filter between 540 nm and 570 nm can be used)

Consumables

Micro-plates with 6 wells and 96 wells are used for the 6-well method and 96-well method, respectively. Multichannel micropipettes are used for the 96-well method. In addition, general cell culture equipment

is necessary.

Media, Reagents, Sera, others

- For cell culturing:
  - Dulbecco's modified Eagle's medium,
  - Penicillin G sodium,
  - Streptomycin sulphate,
  - Fetal bovine serum (FBS).
- For cell passage:
  - 0.25% trypsin,
  - 0.02% EDTA-PBS(-).
- For cell cryo-preservation and as solvent for test chemicals:
  - Dimethylsulfoxide (DMSO).
- For fixing cells:
  - Formalin (37% formaldehyde),
  - Methanol.
- For staining cells in cell growth assays:
  - Crystal violet (CV) solution: in 50 mL of ethanol 1 g of crystal violet is dissolved, and the total volume is adjusted to 1 L with distilled water;
  - Dye extraction solution (0.02 mol/L HCl and 50% ethanol). Mix 480 mL distilled water + 500 mL ethanol + 20 mL 1 M HCl.
- For staining cells in transformation assays:
  - 5% Giemsa solution.

Preparations

Media and Endpoint Assay Solutions

> The assay itself and all the preliminary steps (i.e. cell stock preparation, reagent and treatment solution preparations) should be performed under sterile conditions.

Culture media

- Eagle's minimal essential medium (with 2.2 g/L $NaHCO_3$ and 0.292 g/L L-glutamine) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 units/mL penicillin G sodium and 100 µg/mL streptomycin sulfate) (M10F) is used for the proliferation of Bhas 42 cells, cell cryo-preservation and the first culture after cell thawing.
- Dulbecco's modified Eagle's medium/F12 (with 1.2 g/L $NaHCO_3$) supplemented with 5% FBS and antibiotics (100 units/mL penicillin G sodium and 100 µg/mL streptomycin sulfate) (DF5F) is used for second culture after cell thawing, cell growth assays and transformation assays. (Other antibiotics could be used after examination for applicability.)

Selection of FBS

Although most batches of FBS are generally usable for the transformation assay, it is recommended that at least 3 batches be screened for their ability to support plating efficiencies of at least 50% as well as chemically-induced transformation. The batch of FBS which results in lowest transformation frequency in the negative control groups and highest transformation frequency in positive control groups in the **6-well** method and/or **96-well** method of Bhas 42 cell transformation assay should be selected. The FBS batches should fulfil all of the acceptance criteria described below (*Section 4.3*) in the **6-well** method and/or **96-well** method of the Bhas 42 cell transformation assay using the negative control DMSO and

the positive controls, 1 µg/mL MCA and 50 ng/mL TPA.

Test Compounds

Distilled water, DMSO, acetone, and ethanol can be used to dissolve chemicals, 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%, it is recommended that it does not exceed 0.1%. The absence of cytotoxicity should dictate the highest allowable concentration of solvent/vehicle. Test chemicals are dissolved or suspended in an appropriate solvent or vehicle and diluted with the solvent/vehicle to each individual concentration before being added to cell cultures or culture media so that all chemical treatment media contain an equal concentration of the solvent/vehicle.

In initiation assay, in **6-well** method, a test chemical solution at various concentrations or solvent/vehicle alone is added to each well without replacement of medium. In case of**96-well** method, the cultures are treated by the addition of 0.05 mL of medium containing a chemical solution or solvent/vehicle alone at two times the final concentration added to each well containing already 0.05 mL of medium (the final volume of medium is 0.1 mL/well). Care should be taken to immediately mix the medium. This is especially important in the case of DMSO, which has a higher density than water and therefore sinks to the bottom of the dish where it could kill the cells. It is therefore important that DMSO solutions are added whilst evenly moving the dishes.

In promotion assay, in both **6- and 96-well plate** methods the medium is removed and it is replaced with fresh medium containing a test chemical solution or solvent/vehicle alone.

Test-chemical solutions are prepared before use as a general rule. To reduce labor, stock and/or working solutions may be preserved in aliquots at -20°C for less than 10 days and thawed before use, but must not be re-frozen. The suspensions of test chemicals must be prepared before use and cannot be stored for the future use.

Test Chemical concentrations

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 cell growth assay.

Positive Control(s)

A known tumour-initiator, 3-methylcholanthrene (MCA, final concentration of 1 µg/mL), and a known tumour-promoter, 12-O-tetradecanoylphorbol-13-acetate (TPA, final concentration of 50 ng/mL), are used for the positive controls in the initiation assay and promotion assay, respectively. MCA and TPA are dissolved in DMSO.

The stock solutions of MCA and TPA in DMSO can be stored in aliquots at -20°C for at least two years, provided they are not thawed

Negative Control(s)

The solvent/vehicle for a test chemical is used as the negative control. MCA and TPA, which are used for the positive controls in the initiation assay and promotion assay are dissolved in DMSO. When the solvent/vehicle for the test chemical is not DMSO, DMSO is still necessary as the negative control for MCA or TPA.

Method

The studies on the Bhas 42 CTA were performed using two test method procedures which differ only in the plate design: one protocol based on**6-well micro-plates** (6-well method), the other one on **96-well microplates** (96-well method) and in the way how the scoring of foci is conducted: in the 6-well method the total number of foci is counted whereas in the 96-well method, the end value is the percentage of wells containing one or more foci, regardless of how many foci are contained within a single well. (Tanaka et al., 2009; Sakai et al., 2011; Hayashi et al., 2012). Furthermore, similar results are obtained with both methods.

The Bhas 42 CTA consists of an initiation assay and a promotion assay (Asada et al., 2005). These assays can detect genotoxic and non-genotoxic carcinogens, respectively (Ohmori et al., 2004, 2005; Sakai, 2008; Sakai et al., 2010, 2011). 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 subconfluence of cell growth. A test chemical is positive (i.e. an in vitro cell transforming agent and hence considered a potential carcinogen) in the Bhas 42 CTA if the chemical is positive in one of the two assays, or in both. A negative result in both initiation and promoter assays suggest absence of transforming activity.

Cell growth assay is carried out prior to the transformation assay for the determination of appropriate test concentrations, and concurrently with the transformation assay to confirm the toxic effects of chemical treatment (**Figure 1**).

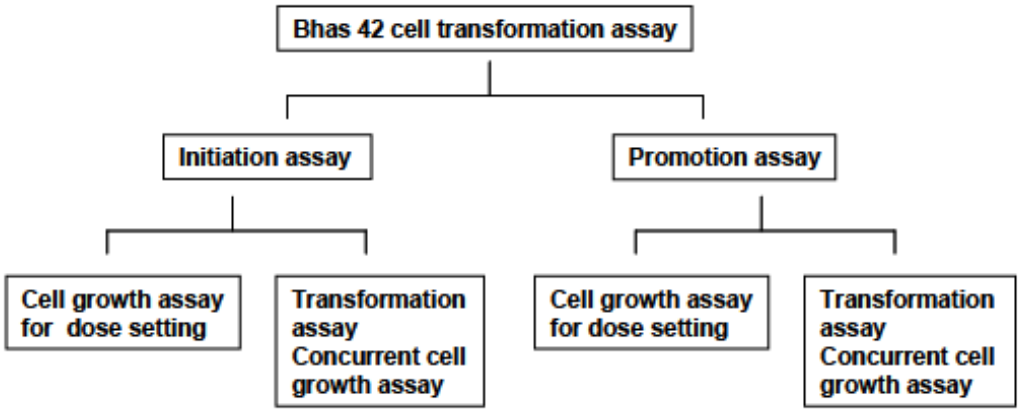

Figure 1: Schematic of the Bhas 42 cell transformation assay

Test System Procurement

Stock cells

The cells should be obtained from a reliable source, e.g. Japanese Collection of Research Bioresources (JCRB) Cell Bank at the National Institute of Biomedical Innovation (Osaka, Japan).

The cells are expanded by growing them up to 50-70% confluence in culture plates with M10F so as to avoid the increase of transformed variants. It is important to maintain a sub-confluent state in all the area of the cultures since the transformed cells, which lose the characteristic of cell-to-cell contact inhibition, preferentially multiply at confluence.

The cells collected from cell populations that do not exceed 50-70% confluence are washed once with 0.02% EDTA-PBS(-), trypsinized using 0.25% trypsin and subcultured.

After two passages, the expanded cells are suspended at a cell density of 5 x 10^5 cells/mL in cold fresh M10F containing 5% DMSO and cryo-preserved in 0.5 mL aliquots to make a master stock in liquid nitrogen.

Using one tube of the master stock, working cell stock for transformation assays are prepared using the same procedures and stored in liquid nitrogen until required for experiments. Generally, large numbers of tubes of the master and working stock cells are prepared so as not to be depleted prematurely.

The transformation assay must be started using the cryo-preserved stock cells. Conventionally, the cells at higher passages can be used for the cell growth assay for dose setting, but should not be used for the transformation assays themselves in order to avoid excessive spontaneous transformation.

The cultures are maintained in a humidified incubator with a 5% CO_2 atmosphere at 37ºC.

Checking of the Bhas 42 cells

Bhas 42 cells may spontaneously transform under inappropriate culture conditions due to their initiated state and their sensitivity to carcinogenic stimuli. Therefore, it is important to maintain strict quality control of cells, assay components, and test conditions, including the use low passage target cells, maintenance of a sub-confluent cell population density (less than or equal to 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.

In order to determine the utility of the cryo-preserved cells, every batch of working stock cells must be confirmed for:

- adequate plating efficiency (greater than or equal to 50%),
- low background of spontaneous transformation, and
- their ability to be transformed by archetypical chemical carcinogens.

To be considered suitable for the transformation assay, the fulfilment of acceptance criteria described in *Section 4.3* must be achieved in the 6-well method and/or 96-well method of the Bhas 42 cell transformation assay using the negative control DMSO and the positive controls, 1 µg/mL MCA and 50 ng/mL TPA.

Test Material Exposure Procedures

Procedure was evaluated during validation studies and below protocol is now recommended for Bhas 42 cell transformation assay in **6-** and **96-well plates**.

1. ***Medium change***

   For the **6-well plates** the final volume of medium is 2 mL per well. The medium is removed by pipette.

   For the **96-well plates** the final volume of medium is 0.1 mL per well. The medium is removed by inverting the plates and shaking out the medium over paper towels or disposable diapers three times to drain off the medium completely. The medium is replaced by gently dispensing it down the walls of the wells over the cells using micro-tips attached to a multichannel micropipette.

2. ***Initiation assay***

   2.1. ***Initiation cell growth assay***

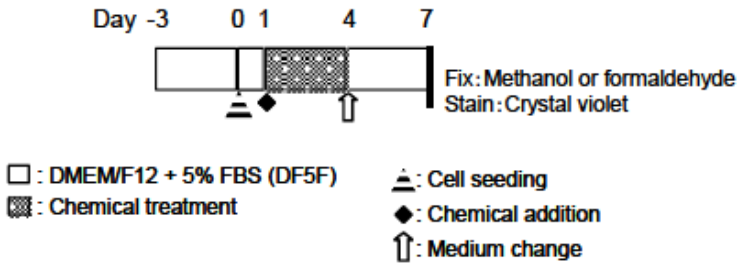

Figure 2: Schematic protocol of cell growth assay in the initiaiton assay

The cells at less than or equal to 70% confluence in DF5F are trypsinized.

In 6-well plates 4000 cells are seeded into a well with 2 mL of DF5F (Day 0). Three wells are prepared for each treatment condition.

In case of **96-well plates**, 200 cells are seeded into each well with 0.05 mL of DF5F (Day 0).

For each treatment condition, 8 wells are prepared.

Wells containing medium alone are also prepared for the blank control in photometry (The blank control can be shared among different assays performed on different chemicals provided they are tested simultaneously). At 20-24 hours after seeding, a test chemical solution at various concentrations or solvent/vehicle alone is added to each well without replacement of medium, which is the same practice used in the initiation transformation assay (*Section 2.3*).

In **6-well plates**, in the case of water as solvent, 100µl/well is added (to obtain final concentration 5%). In the case of DMSO as solvent, 2µl/well is added (to obtain final concentration 0.1%).

In **96-well plates**, the cultures are treated by 0.05 mL of medium containing a test chemical solution or solvent/vehicle alone at two times the final concentration added to each well so that the final volume of medium is 0.1 mL.

The medium is changed with fresh medium on Day 4.

On Day 7, the cultures are fixed with methanol or 3.7% formaldehyde for 30 min, washed and dried. The cells are stained with 1.5 mL/well or 0.1 mL/well of crystal violet (CV) solution for 15 min in **6-** or **96-well plates** respectively, rinsed well with water and dried (**Figure 2**).

CV is extracted from cells with 2 mL/well of dye extraction solution in **6-well plates** and 0.1 mL/well in **96-well plates**, and the optical density (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 = [(At –Ab)/(Ac –Ab)] x 100

where **At** is the absorbance of CV extract from a well with the chemically-treated cells, **Ac** is the absorbance of CV extract from a well with the solvent/vehicle-treated cells and **Ab** is the absorbance of CV extract from a well with the medium only.

*2.2. **Dose setting for the initiation transformation assay (6- and 96-well plates)***

Five to nine concentrations are set up based on the results of cell growth assays. These concentrations cover a range from the highest cytotoxicity (less than 20% survival compared to the negative control) to little or no toxicity. Ideally, one concentration below the no observed effect level (NOEL), two concentrations between the NOEL and the 50% inhibitory concentration (IC_{50}) and two concentrations between the IC_{50} and the IC_{90} are selected. The ratio between neighbouring concentrations should be less than square root 10 (**Figure 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.

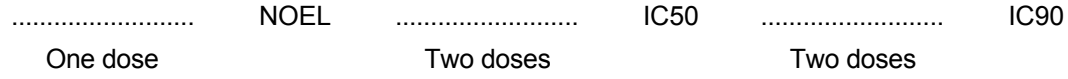

Figure 3: Dose setting for the transformation assay in the initiation assay.

2.3. *Initiation transformation assay*

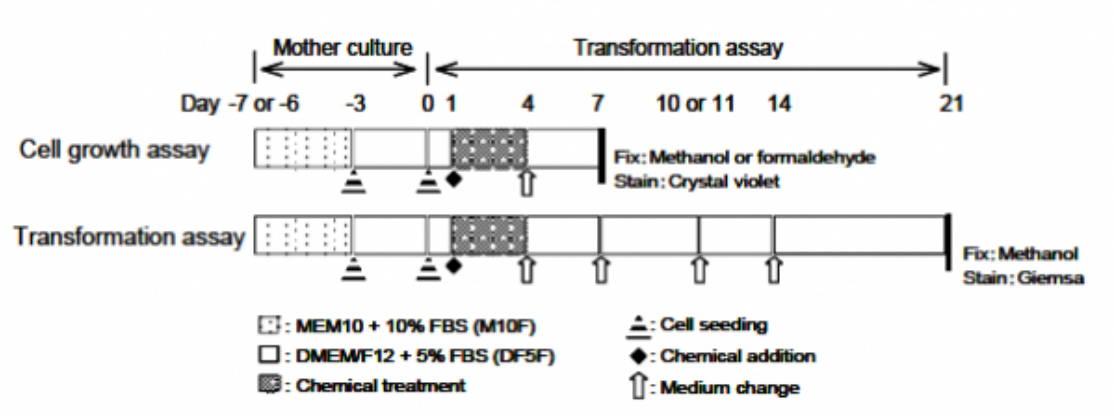

Figure 4: Schematic protocol of transformation assay in the initiation assay.

The frozen stock cells are rapidly thawed, suspended in M10F and cultured in Φ100-mm culture plates at a volume of 10 mL medium. When the cells reached about 70% confluence, they are trypsinized, suspended in DF5F at an appropriate density (7,000 to 10,000 cells /mL is suggested) and cultured in Φ100-mm culture plates (Day -3). The cells at about 70% confluence are trypsinized, and suspended in DF5F at appropriate concentrations.

In **6-well plates**, 4,000 cells are seeded into each well with a volume of 2 mL of DF5F for the transformation assay and the concurrent cell growth assay (Day 0). Nine wells are prepared for each dose (one plate of 6 wells for the transformation assay and 3 wells for the concurrent cell growth assay). 20-24 hours after seeding, a chemical solution is added to each well without replacement of medium. In the case of water as solvent, 0.1 mL/well is added (to obtain final concentration 5%). In the case of DMSO as solvent, 2 µL/well is added (to obtain final concentration 0.1%).

In case of **96-well plates**, 200 cells are seeded into each well with 0.05 mL of DF5F (Day 0). For each treatment condition, one 96-well plate is prepared for the transformation assay and 8 wells are prepared for the cell growth assay, respectively. 20-24 hours after seeding, the cultures are treated by the addition of 0.05 mL of medium containing a chemical solution or solvent/vehicle alone at two times the final concentration added to each well so that the final volume of medium is 0.1 mL.

Irrespective of the plate type, the cells are exposed to the chemical for three days (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 (**Figure 4**).

The positive control (1 µg/mL MCA) and the negative control are included in the transformation assay for each chemical (when the solvent/vehicle of test chemical is not DMSO, DMSO is still necessary as the negative control for MCA).

The cell growth assay is carried out in parallel with the transformation assay.

3. ***Promotion assay***

3.1. ***Promotion cell growth assay***

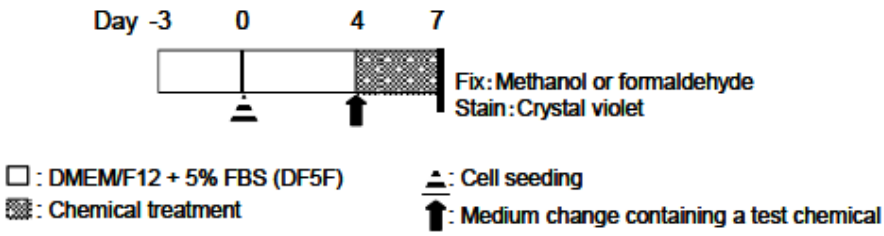

Figure 5: Schematic protocol of cell growth assay in the promotion assay.

The cells at less than or equal to 70% confluence in DF5F are trypsinized.

In case of **6-well plates** , 14000 cells are seeded into a well in 2 mL of medium (Day 0). Three wells are prepared for each treatment condition.

In **96-well plates** , 400 cells are seeded into each well with 0.1 mL of DF5F (Day 0). For each treatment condition, 8 wells are prepared.

Wells containing medium alone are also prepared for the blank control in photometry (the blank control can be shared with the cell growth assay related to the initiation assay which is performed simultaneously).

On Day 4, the medium is removed and it is replaced with fresh medium containing a test chemical solution or solvent/vehicle alone.

On Day 7, the cultures are fixed with methanol or 3.7% formaldehyde for 30 min, washed and dried. The cells are stained with 1.5 mL/well or 0.1 mL/well of crystal violet solution for 15 min in **6-** or **96-well plate** respectively, rinsed well with water and dried (**Figure 5**).

The following procedures are the same as those in the cell growth assay in the initiation assay (*Section 2.1*).

3.2. ***Dose setting for the promotion transformation assay (6- and 96-well plates)***

The possible outcomes of the cell growth assay are that the test concentration has either no effect, inhibits or stimulates the growth of the treated cells.

For the chemicals that exhibit marked growth enhancement, concentrations are selected to cover the range from little effect to enhancement on cell growth. Ideally, 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 (**Figure 6**).

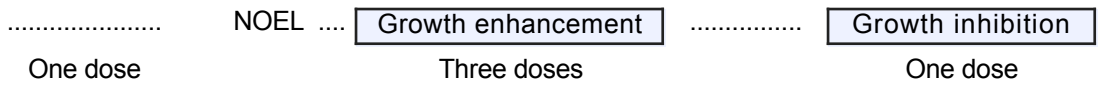

Figure 6: Dose setting for transformation assay in the promotion assay of chemicals that exhibit marked growth enhancement.

For the chemicals that inhibit growth, concentrations are selected to cover the range from the NOEL to the level below 50% inhibition on cell growth. Ideally, two concentrations below the NOEL, three concentrations between the NOEL and the IC_{50} and one concentration above the IC_{50} are evaluated (**Figure 7**).

..... NOEL ..... IC50 .....

Two doses Three doses One dose

Figure 7: Dose setting for transformation assay in the promotion assay of chemicals that inhibit growth.

Whether a chemical shows growth enhancement or inhibition, other experimental conditions, such as the number of concentrations set up and the ratio between neighbouring concentrations, are the same as described in the initiation assay (see *Section 2.2*).

For substances which exhibit a steep concentration–response curve, 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.

3.3. ***Promotion transformation assay***

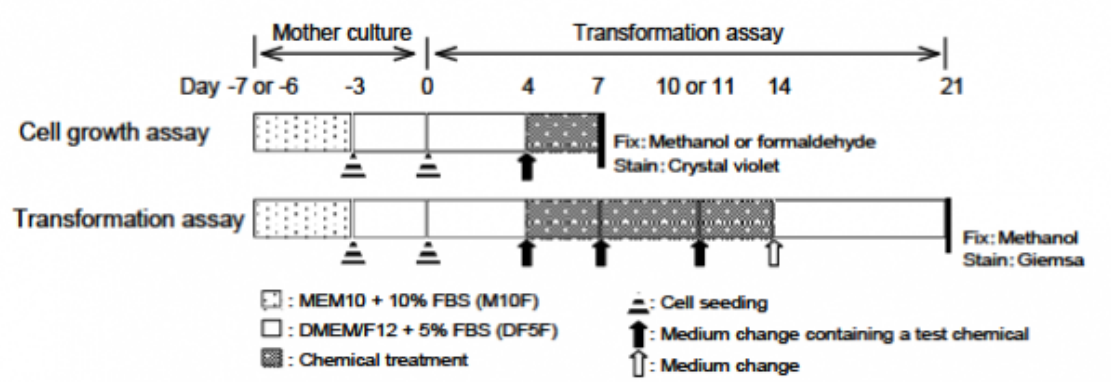

Figure 8: Schematic protocol of transformation assay in the promotion assay.

The transformation assay is carried out in the same manner as in the initiation assay except for the following (**Figure 8**):

- The cells are suspended in medium at appropriate concentration, and 14,000 cells with 2 mL (**6-well plates**) or 400 cells with 0.1 mL of DF5F (**96-well plates**) are plated into each well (Day 0).
- In case of **96-well plates**, for each treatment condition, one plate is prepared for the transformation assay and 8 wells are prepared for the cell growth assay, respectively.
- The cells are exposed to a test chemical for 10 days from Day 4 to Day 14.
- The chemical treatment is carried out by exchanging existing medium with fresh medium containing a chemical solution or solvent/vehicle alone on Day 4, 7 and 10 (or 11).
- On day 14 the medium is changed with the normal medium containing neither solvent/vehicle nor test chemical.
- TPA (50 ng/mL) is used for the positive control instead of MCA.

4. ***Evaluation of the results***

4.1. ***Record of transformation frequency***

Transformed foci are scored using a stereomicroscope. Transformed foci are characterized by the following morphological properties:

- more than 100 cells,
- spindle-shaped cells differing in appearance from the contact-inhibited monolayer cells,

- deep basophilic staining,
- random orientation of cells at the edge of foci (criss-cross),
- dense multilayering of cells (piling up),
- invasive growth into the surrounding monolayer of contact-inhibited cells.

It should be noted that there are transformed foci that do not exhibit all these characteristics, but observation of some of the above clear-cut morphological properties is generally sufficient to classify transformed foci.

In **6-well plates method**, the number of transformed foci in each well are recorded for every concentration.

In **96-well plates method**, the number of wells having transformed foci versus the number of wells observed is recorded for every concentration: a well having one focus is counted as one and a well having two or more foci is also counted as one.

*4.2. **Statistical analysis***

In **6-well plates method**, for the increases in the number of the transformed foci produced by a test chemical, a statistical analysis is performed by multiple comparison using the one-sided Dunnett test ($p<0.05$).

For the positive controls, the statistical significance is evaluated by one-sided t-test or Aspin-Welch test ($p<0.05$) depending on the result of F-test for homoscedasticity (homogeneity of variance).

In **96-well plates method**, for the increases in the proportion of wells with transformed foci in the plate treated with a test chemical, a statistical analysis is performed by chi-square test with Bonferroni adjustment (p-value<0.05, upper-sided) (Snedecor and Cochran, 1989). For multiplicity, the number of concentrations (at least 4) that satisfy the assay acceptance criteria is considered by this methodology.

For the positive controls, the statistical significance is evaluated by one-sided chi-square test ($p<0.05$, upper-sided).

*4.3. **Assay acceptance criteria***

The following criteria must be fulfilled for a given assay to be considered valid. The initiation or promotion assay is repeated independently, as needed, to satisfy the assay acceptance criteria.

Concurrent cell growth assay

- When contamination or technical problems are observed, two (in **6-well plates**) or four (in **96-well plates**) undamaged wells are necessary at the minimum for each concentration for cell growth assessment.

Transformation assay

a. ***Initiation assay and promotion assay***

- If a given chemical concentration results in excessive cell death and/or cells fail to reach confluence at the end of transformation assay because of chemical toxicity, the concentration is not valid for transformation assessment and is excluded from focus-counting, statistical analysis and judgment, and “toxicity” should be recorded in the data sheet.
- When contamination or technical problems are observed, if, for a given concentration, the number of damaged wells is greater than or equal to 2 in case of **6-well plates** or greater than or equal to 7 in case of **96-well plates**, the concentration is not considered valid for transformation assessment and is excluded from focus-counting, statistical analysis and judgment. In such cases, “contamination”, “accident”, “technical error”, etc. should be recorded in the data sheet.
- In the positive control, in **6-well plate** method, there must be a statistically

significant increase in the number of transformed foci per well compared to the corresponding negative control (one-sided t-test or Aspin-Welch test, $p<0.05$). In case of **96-well method**, there must be a statistically significant increase in the proportion of wells having transformed foci compared to the corresponding negative control (one-sided chi-square test, $p<0.05$, upper-sided).

b. ***Initiation assay***

- In the negative control, in **6-well plate** method, the number of transformed foci must be ten or less per well, in case of **96-well method**, the number of wells having transformed foci must be 15 wells/plate or less (if there exist damaged wells, less than or equal to 15.625% of undamaged wells).
- If there is no statistically significant increase in the number of transformed foci (in **6-well plates**) or in the proportion of wells having transformed foci (in **96-well plates**) at any dose, four valid test chemical concentrations are necessary, at a minimum, to accept the transformation assay for evaluating a chemical as negative. Those concentrations should include at least one concentration near the NOEL and three concentrations in the range between the NOEL and the IC_{90} in the concurrent cell growth assay.

c. ***Promotion assay***

- In the negative control, in **6-well plate method**, the number of transformed foci must be twelve or less per well. In case of **96-well plate method**, the number of wells having transformed foci must be 20 wells/plate or less (if there exist damaged wells, less than or equal to 20.833% of undamaged wells).
- If there is no statistically significant increase in the number of transformed foci (in **6-well plates**) or in the proportion of wells having transformed foci (in **96-well plates**) at any dose (in case of negative results), four valid test chemical concentrations are necessary, at a minimum, to accept the transformation assay for evaluating a chemical. Those concentrations should include at least one concentration near the NOEL and two concentrations in the range of growth enhancement when the chemical enhances cell growth (increases cell density in the cell growth assay).
- When the chemical does not induce growth enhancement but induces cytotoxicity, the cytotoxicity observed in the concurrent cell growth assay may not be similar to that observed in the transformation assay, since the durations of chemical exposure to the cells are different between the cell growth assay (3 days) and the transformation assay (10 days). Consequently, chemical toxicity is sometimes accumulated over the 10 day duration of the transformation promotion assay and the valid plates may be lost because of chemical toxicity. In such cases, four valid plates are also necessary in the concentration range where cells are not killed and are confluent at the end of the transformation assay. If excessive toxicity is encountered, it may be necessary to repeat the assay in a lower concentration range.
- 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 IC_{50}.

Data Analysis

Experimental records and data analysis should take the following information into account:

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: per cent 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, signs of precipitation in medium,
- number of total valid (quantifiable) wells, number of wells lost and the reason(s) for the loss,
- number of total foci,
- transformation frequency: transformed foci/well (6-well method), the number of wells with transformed foci/ total number of wells (96-well method),
- dose-response relationship, where one exists,
- statistical analyses: statistical test(s) employed, analytical 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.

Discussion of results

Conclusion

Prediction Model

The assay results are judged as follows:

- The results in the initiation and promotion assays are judged **positive** if there exist two or more sequential doses that induce statistically significant increases in the number of transformed foci per well relative to the corresponding vehicle control (multiple comparison using one-sided Dunnett test, $p<0.05$) in case of **6-well plate method** or in the proportion of wells having transformed foci (number of wells having transformed foci/number of wells observed) using chi-square test with Bonferroni adjustment, $p<0.05$, upper-sided in **96-well plate method**.
- 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 (**6-well plates**) or in the proportion of wells having transformed foci (**96-well plate)**.
- If the statistically significant increase is at only one or non-sequential doses, the assay result is regarded as **equivocal**, and then the initiation or promotion assay should be repeated together with the concomitant cell growth assay, including the dose that caused the statistically significant increase of transformation frequency in the first assay. Broader or narrower range of test chemical concentrations should be considered in such follow-up experiments to eliminate such equivocality. The chemical is then judged to be positive if a statistically significant increase in the number of transformed foci per well (**6-well plates**) or in the proportion of wells having transformed foci (**96-well plate)** is induced at one or more concentrations in the second assay; otherwise, the chemical is judged to be negative.
- A test chemical is positive in the Bhas 42 CTA, if it is positive in one of the assays (initiation or promotion assay), or in both.

Annexes

- The use of the photo catalogues for the Bhas 42 CTA (Hayashi *et al.*, 2012) is recommended with the protocol to support consistency in focus scoring and assay results and available in the **Downloads** section of the Protocol.
- The reporting templates used during the validation study are available in the **Downloads** section of the Protocol.

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