# DB-ALM Protocol n° 137 : *In vitro* BALB/c 3T3 Cell Transformation Assay (BALB/c 3T3 CTA)

**Cell transformation**

The BALB/c 3T3 cell transformation assay (CTA) is a short-term in vitro assay recommended 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.

## Experimental Description

**Endpoint and Endpoint Measurement:**

In the BALB/c 3T3 CTA, both cytotoxicity and morphological transformation endpoints are evaluated:

**COLONY FORMATION** - the inhibition of colony formation is the basis of cytotoxicity assessment. It is expressed as: **Relative colony forming efficiency (CFE).** CFE is calculated to measure the inhibition of colony formation compared to the control group.

**CELL NUMBER** - in parallel to CFE, cell proliferation can be also measured by crystal violet method (CV), which involves cell staining, dye extraction and spectrophotometric measurement. It is expressed as **Relative cell growth (RCG)** and it is calculated to measure the decrease in cell number compared to the control group.

**MORPHOLOGICAL EFFECTS** - Carcinogenic potential assessment is based on the occurrence of morphologically transformed foci. It is expressed as **Focus count** = the total number of foci per group and the mean number of foci per dish.

**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)}

**RELATIVE COLONY FORMING EFFICIENCY (CFE)** = {[total number of colonies formed in the treatment dishes / total number of colonies formed in the control dishes] × 100}

**FOCUS COUNT** = the total number of foci per group and the mean number of foci per dish

**Experimental System(s):**

BALB/c 3T3: The mouse fibroblast cell line originating from inbred BALB/c mouse embryo cultures (Aaronson and Todaro, 1968a)

## Discussion

**Practical aspects of the assay:**

- **Cost:** the CTA is a rather costly *in vitro* test, but cheap in comparison with the rodent bioassay.
- **Throughput:** The CTA requires 5 weeks per substance, i.*e.* it has a low throughput. However, this has to be compared with the 3 years a rodent bioassay requires.
- **Complexity:** the CTA requires high 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 Sasaki *et al.* (2012b) and appended in the **Downloads** section of this protocol are essential for overcoming these potential limitations.

**Cytotoxicity assays for dose range finding test and transformation assay:**

Both the colony forming efficiency and crystal violet cytotoxicity assays were performed during the BALB/c 3T3 CTA prevalidation study. Primarily, the CFE was considered as the test that has been used historically and it would allow for a better comparison with existing data. The data showed that the two cytotoxicity methods may have different sensitivities (Tanaka *et al.*, 2012). However, the dataset generated in the prevalidation study does not allow to conclude on the equivalence or superiority of one or the other method and further studies are needed to address this important aspect. For completeness, both methods are included in the **Procedure Details** part.

**Cell clones:**

The results of the prevalidation study showed that different clones of BALB/c 3T3 cells could have a different responsiveness to morphological transformation. The cells used for this study were from the BALB/c 3T3 mouse fibroblast cell line, subclone A31-1-1 (IARC/NCI/EPA Working Group, 1985). They were provided by Hatano Research Institute (HRI) at passage 17 and used for the CTA at the lowest passage possible (*i.e.* within maximum five to six passages) to avoid the possibility of increasing spontaneous cell transformation.

In general, the cells should be obtained from a reliable and qualified source like the Health Science Research Resources Bank (HSRRB), at an early passage and should be checked for responsiveness to the transformation assay.

**Statistical approach:**

The recommended statistical methods suggested for the BALB/c 3T3 CTA are based upon the Negative Binomial Generalised Linear Model (GLM-NB) combined with a William's-type protected test or the Nishiyama transformation (NT) and described in Hoffmann *et al.* (2012). The scripts for the statistical evaluation, written in R (a freely available statistical software) are appended, in the **Downloads** section of this protocol, and include examples of output.

For the key principle and main applications of the assay please refer to a Method Summary **"Cell transformation assay with BALB/c 3T3 cells (BALB/c 3T3 CTA) - Summary"**, available in the DB-ALM.

## Status

**Participation in Validation Studies:**

EURL ECVAM coordinated, **from 2005 to 2010, a formal prevalidation study of the BALB/c 3T3 CTA** (Vanparys *et al.*, 2010; Corvi *et al.*, 2012). The BALB/c 3T3 CTA was evaluated in a multi-laboratory trial with six chemicals (Tanaka et al., 2012; EURL ECVAM, 2010).

The ESAC conclusions were summarised in the **ESAC Opinion** (EURL ECVAM, 2011 and 2012), which was followed by **EURL ECVAM Recommendation** (EURL ECVAM, 2012).

## 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 sorbonne 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 thrown into the appropriate trash.

## Abbreviations and Definitions

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

CFE: Colony forming efficiency

CRO: Contract research organisation

CTA: Cell transformation assay

CV: Crystal violet

DMEM/F12: Dulbecco's modified Eagle's medium/F12

DMSO: Dimethylsulfoxide

DRF: Dose range finding

DRP: Detailed review paper

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)

ESAC: ECVAM Scientific and Advisory Committee

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

FBS: Fetal bovine serum

GLM-NB: Negative binomial generalised linear model

HRI: Hatano Research Institute

HSRRB: Health Science Research Resources Bank

IC: Inhibitory concentration

MEM: Minimum essential medium

MTA: Morphological transformation assay

NOEL: No observable effect level

NT: Nishiyama transformation

OECD: Organisation for Economic Co-operation and Development

PBS: Phosphate-buffered saline

PE: Plating efficiency

REACH: Registration, Evaluation, Authorisation and Restriction of Chemicals

RPE: Relative plating efficiency

SCCS: Scientific Committee on Consumer Safety

SHE CTA: Syrian hamster embryo cell transformation assay

SPSF: Standard Project Submission Form

VMT: Validation Management Team

WNT: OECD Working Group of National Coordinators of the Test Guidelines Programme

*Last update:* 31 January 2013

## PROCEDURE DETAILS, 31 January 2013

***In vitro* BALB/c 3T3 Cell Transformation Assay (BALB/c 3T3 CTA)**
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The protocol represents a standard operating procedure used in the validation study BALB/c 3T3 cell transformation assay (Sasaki et al., 2012a).

Laboratories that are to implement the BALB/c 3T3 CTA should receive **proper training from experienced personnel** in order to gain the necessary level of practice to perform the assay and to correctly identify the different types of foci. In addition, it is recommended to use as a visual aid the corresponding photocatalogue, available from DB-ALM website (Sasaki et al., 2012b). Go to the section related to the Protocol No. 137 and select Related information: **Downloads**.

### Contact Details

**Dr. Kiyoshi Sasaki**
Food and Drug safety Center (FDSC)
Hatano Research Institute (HRI)
729-5 Ochiai Hadano
Kanagawa 257-8523
email: sasaki.k@fdsc.or.jp
telephone: +81-463-82-4751
fax: +81-463-82-9627

**Ph.D. Noriho Tanaka**
Food and Drug safety Center (FDSC)
Hatano Research Institute (HRI)
729-5 Ochiai Hadano
Kanagawa 257-8523
Japan
email: tanaka.n@fdsc.or.jp
telephone: +81-463-82-4751
fax: +81-463-82-9627

### Materials and Preparations

#### Cell or Test System

The BALB/c 3T3 cell line is a mouse fibroblast cell line originating from inbred BALB/c mouse embryo cultures (Aaronson and Todaro, 1968a).

The cells used for the prevalidation study were from the BALB/c 3T3 mouse fibroblast cell line, subclone A31-1-1 (Kakunaga and Crow, 1980), free from bacteria, fungi and mycoplasma. They were provided by Hatano Research Institute (HRI) at passage 17 and used for the CTA at the lowest passage possible (*i.e.* within maximum 5 to 6 passages) to avoid the possibility of increasing spontaneous cell transformation.

#### Equipment

*Fixed Equipment*

- laminar flow hood (biohazard type and restricted to cell culture assays)
- cell culture incubators (37°C; 5% $CO_2$; greater than or equal to 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*

The consumable necessary for the conduct of the assay comprises general cell culture laboratory equipment (*e.g.* glassware, filtration systems, cell culture plasticware).

**Media, Reagents, Sera, others**

***1. Culture media and serum***

Media are prepared as follows and should be stored at 4ºC for no longer than 1 month.

- **Serum:** Fetal bovine serum (FBS) was supplied by Moregate (Australia) for the prevalidation study. In general, serum batches should be tested for suitability prior to being used in the CTA (See "Checking of BALB/c 3T3 cells and FBS suitability" under the "**Routine Culture Procedure**" section).
- **M10F medium:** M10F medium is used for routine culture, cell storage, the cytotoxicity tests and the early stage of the transformation assay. After preparation, M10F medium should be stored at 4ºC. It contains:
  - **Minimum essential medium** (MEM): 500 mL. It can be obtained as liquid or powder from a commercial source and is prepared following the supplier instructions.
  - **FBS** 10% (v/v): 55.5 mL. Aliquots of 55.5 mL should be prepared aseptically and stored below -20ºC.
  - **Penicillin (10000 units/mL) - streptomycin (10 mg/mL) solution**: 5.5 mL. This solution can be obtained ready-to-use from a commercial source and should be stored at -20ºC until use.
- **DF2I2F medium:** DF2I2F medium is used for the late stage of the transformation assay (Tsuchiya and Umeda 1995; Tsuchiya *et al.,* 2010; Hayashi *et al.,* 2008). After preparation, it should be stored at 4ºC. It contains:
  - **Dulbecco's modified Eagle's medium/F12** (DMEM/F12): 500 mL. It can be obtained as liquid or powder from a commercial source and should be prepared following the supplier instructions.
  - **FBS** 2% (v/v): 10.2 mL. Aliquots of 10.2 mL should be prepared and stored below -20ºC.
  - **Penicillin (10000 units/mL) - streptomycin (10 mg/mL) solution**: 5.1 mL. This solution can be obtained ready-to-use from a commercial source and should be stored at -20ºC until use.
  - **Insulin stock solution** (2 mg/mL): 0.5 mL. Bovine pancreas insulin is dissolved in 0.1N HCl to reach the concentration of 2 mg/mL. The solution is sterilised through 0.22 µm filter. Aliquots of 0.5 mL are prepared and should be stored at -20ºC.

***2. Reagents***

- **Cell freezing solution:** This solution consists of M10F medium containing 10% (v/v) of DMSO. It should be prepared fresh every time.
- **EDTA-2Na solution:** This 2% (w/v) solution is prepared by dissolving 2 g of EDTA-2Na in 100 mL of distilled or MilliQ water. The solution should be sterilised by autoclaving (121ºC, 15-20 min). It can be stored at room temperature.
- **Washing solution:** This solution consists of $Ca^{2+}$/$Mg^{2+}$-free Phosphate-buffered saline (PBS) containing 0.02% (w/v) of EDTA-2Na. It is prepared by adding 5 mL of 2% EDTA-2Na solution to 500 mL of $Ca^{2+}$/$Mg^{2+}$-free PBS. It can be stored at room temperature.
- **Trypsin solution:** The 0.25% (w/v) trypsin solution is obtained from a commercial source and is stored at -20°C. Aliquots can be prepared and should be stored at -20°C. When an aliquot is thawed and used, the remaining trypsin solution can be stored at 4°C for further use, but care should be taken to avoid leaving it at room temperature for a long period.

### Preparations

*Media and Endpoint Assay Solutions*

- **Fixing solutions:**
  - **Methanol** is used for cell fixation in the CFE test and in the transformation assay.
  - **10% (v/v) formalin (*i.e.* 3.7% (w/v) solution of formaldehyde)** is used for cell fixation in the crystal violet cell growth assay and is prepared by a 10-fold dilution in distilled or MilliQ water of 100% (v/v) formalin (*i.e.* 37% (w/v) solution of formaldehyde).
- **Staining solutions:**
  - **A 0.04% (w/v) Giemsa solution** is used for cell staining in the CFE test and the transformation assay. It is obtained *e.g.* by a 10-fold dilution (10%, v/v) of a 0.4% (w/v) Giemsa solution from Sigma-Aldrich (Catalogue number GS500) or by a 20-fold dilution (5%, v/v) of a 0.7% (w/v) Giemsa solution from Merck (Catalogue number 1.09204) in distilled or MilliQ water.
  - **0.1% (w/v) crystal violet (CV) solution** is used for cell staining in the CV cell growth assay. One gram of CV is first dissolved in 50 mL of ethanol and volume is then adjusted to 100 mL by the addition of 50 mL of distilled or MilliQ water. The 1% (w/v) CV solution obtained is then diluted 10-fold in distilled or MilliQ water to reach a final concentration of 0.1% (w/v).
- **Crystal violet extraction solution:** This solution is used for extracting CV from stained cells prior to spectrophotometry and consists of a 50% (v/v) ethanol solution containing 0.02 mol/L of HCl. It is prepared by adding 50 volumes of ethanol and 2 volumes of 1 mol/L HCl to 48 volumes of distilled or MilliQ water.

*Test Compounds*

Test substances are dissolved or suspended in an appropriate vehicle and diluted with M10F complete medium. The preferred vehicle is DMSO. In general, the vehicle should not interact with the test substances nor affect cell survival or focus formation.

The final vehicle concentration in the medium should be less than 10% (v/v) when the vehicle is distilled water or saline, and less than 0.1% (v/v) when the vehicle is DMSO, acetone or ethanol (however, a maximum concentration of 0.5% (v/v) of DMSO and acetone is acceptable in the medium when test substances do not dissolve at lower concentrations of these two organic vehicles).

*Positive Control(s)*

**Positive control:** 3-methylcholanthrene (4 µg/mL dissolved in 0.5% (v/v) DMSO)
The positive control is not necessary for the dose-range finding test but mandatory for the transformation assay.

*Negative Control(s)*

- a **Vehicle control**: medium added with the appropriate vehicle (*e.g.* 0.5% (v/v) DMSO)
- an **Untreated control**: medium only
- In addition for the CV test, a **Blank** for spectrophotometric measurement is included: medium without cells

## Method

### Test System Procurement

- **Cell procurement**
  In general, BALB/c 3T3 cells should be obtained from a reliable and qualified source like the Health Science Research Resources Bank (HSRRB), at an early passage and should be checked for responsiveness to the transformation assay (See “Checking of BALB/c 3T3 cells and FBS suitability” under the **“Routine Culture Procedure”** section).
- **Cell stock preparation and freezing**
  **Cell master stock** and **working stock** are prepared in the same way and should be stored in liquid nitrogen at an early passage.

> ***Note:***
> *The following suggested procedure is adapted for large-scale amplification, but it can be scaled down when needed.*

1. A frozen tube containing BALB/c 3T3 cells is thawed in 37ºC water.
2. Cells are immediately suspended in 20 mL of M10F complete medium and seeded in two 100-mm dishes.
3. Cells are grown at 37ºC in a humidified 5% $CO_2$ incubator until subconfluence (approximately 70% of confluence).
4. When subconfluent, cells are trypsinised and plated in 16 dishes (100 mm) containing each 10 mL of M10F medium.
5. Once cells have reached 70% confluence, they are trypsinised, counted and suspended in M10F medium at 5 to $10\times10^5$ cells/mL.
6. The cell suspension is then aliquoted in freezing tubes (0.5 mL/tube), before addition of 0.5 mL of freezing solution/tube (final concentration of DMSO is 5% (v/v)).
7. Finally the tubes are frozen in a deep freezer at -70 to -80ºC for one day, before transfer into liquid nitrogen.

**Routine Culture Procedure**

- **Checking of BALB/c 3T3 cells and FBS suitability**
  In general, serum batches and cell batches should be tested for suitability prior to being used in the CTA. A batch should be selected based on its ability:
  - to maintain the contact-inhibited cell monolayer,
  - to induce a high cloning efficiency (colony forming efficiency (CFE) should be at least 30% in the vehicle control dishes), and
  - to support a relatively low spontaneous transformation frequency with the vehicle control as well as a high transformation frequency with a positive control (IARC/NCI/EPA Working Group, 1985). See also the **"Acceptance Criteria"** section.
- **Cell thawing and culturing**
  One working stock tube should be used for one transformation assay within two passages. For the cell growth assay, cells can be used several times within 10 passages.
  1. One tube of frozen cells is thawed at 37ºC in warm water.
  2. Cells are immediately suspended in 10-20 mL of M10F complete medium and seeded in 1-2 100-mm dishes.
  3. Cells are then cultured at 37ºC in a humidified 5% $CO_2$ incubator until subconfluence (approximately 70% of confluence).
- **Cell passage and maintenance**
  1. When the cultures have reached about 70% of confluence, the medium is removed and dishes are washed with washing solution.
  2. Cells are treated with 0.25% trypsin solution until they detach from the dish surface.
  3. Trypsin is then inactivated by the addition of M10F complete medium.
  4. After pipetting several times to get a single-cell suspension, cells are counted.
  5. Cell concentration is adjusted with M10F complete medium so as to obtain the required cell density.
  6. The cells are seeded in 100-mm dish(es) and grown at 37ºC in a humidified 5% $CO_2$ incubator.
  7. The cells are transferred or harvested when they have reached approximately 70% of confluence.

***Note** :*

- *A homogeneous cell monolayer can be obtained by gently moving the newly seeded dishes back and forth, and right and left a few times before placing them in the incubator.*
- *It is critical to passage the cells before they reach 80% confluence to ensure proper cell maintenance, because the BALB/c 3T3 cells tend to induce spontaneously transformed cells when kept under the culture condition of cell-to-cell contact.*
- *The following passage schedules are suggested as examples to ensure passage at about 70% confluence: 4-5 × $10^4$ cells seeded per 100-mm dish and cultured for three days before trypsinisation or 1-2 × $10^4$ cells seeded per dish and cultured for four days.*

- **Cell counting**

1. Cells are counted using a Bürker-Türk chamber (or other device - please see note below) after dilution of the cell suspension in trypan blue solution.
2. Two dilutions can be prepared depending on cell density/concentration: 1/10 (10 µl of cell suspension + 90 µl of trypan blue) or 1/2 (50 µl of cell suspension + 50 µl of trypan blue).
3. Living cells remain unstained with a clear cell membrane.
4. The number of cells is calculated using the formula **N = (a / b) x $10^4$ x DF** , where

    - **"N"** is the number of cells/mL,
    - **"a"** is the total number of cells counted in a minimum of 3 squares,
    - **"b"** = number of squares considered (minimum 3),
    - **$10^4$** is the conversion factor for chamber volume, and
    - **"DF"** is the Dilution Factor (usually equal to 2 or 10).

***Note** :*
*Cell counting can be done without trypan-blue staining, where refractile cells with clear cell membrane are counted. For cell counting, other appropriate types of cell counting chambers or electronic particle counters can be used.*

**Test Material Exposure Procedures**

**The CTA is composed of two phases:**

- **A preliminary dose-range finding (DRF)** cytotoxicity test to determine the treatment doses that will be used for the transformation assay
- **The transformation assay (TA)**, which represents the main experiment and which includes in parallel: the scoring of morphologically transformed foci in the morphological transformation assay (MTA) and the evaluation of cytotoxicity in separate dishes.

The DRF tests and the assessment of cytotoxicity during the transformation assay are carried out by the measurement of both the CFE and Crystal Violet tests (Saotome *et al.*, 1989) .

**The testing procedure of the transformation assay can be summarised as follows:**

- 8 doses are chosen for the transformation assay on the basis of the DRF CFE and/or CV results to cover the range from no toxicity up to 90% reduction in cell viability.
- 2 × $10^4$ cells are seeded per 100 mm-diameter dishes.
- 24h after seeding (day 1), treatment media containing the different test concentrations of the substance are prepared. The culture medium is removed from the dishes and 10 mL of the treatment medium are added to each of the corresponding dishes.
- 72h after exposure (day 4), the treatment medium is removed and replaced with fresh complete

medium.

- From day 7 to day 24 or 25 the medium is removed and replaced with DMEM/F12 medium with 2 µg/mL insulin, 2% FBS and 1% PS (DF2I2F) twice a week.
- 1 week after the last medium change (day 31 or 32), the medium is removed and cells are washed with phosphate-buffered saline (PBS), fixed with methanol and then stained with 10% Giemsa solution.
- For the analysis of the MTA, foci consisting of more than 50 cells or more than 2 mm in diameter are evaluated using a stereomicroscope. Only type III foci are recorded, which are characterised by the following morphological criteria: deep basophilic staining of spindle-shaped cells which are morphologically different from the background monolayer cells, dense multi-layering of cells (piling up) and random orientation and invasive growth of cells at the edge of foci (criss-cross pattern) (IARC/NCI/EPA Working Group, 1985).
- The number of type III foci per dish is counted and this value is used for the statistical analysis.

***1. Cell growth assays for the dose range finding***

Doses for the morphological transformation experiment are selected based on the results of the preliminary cytotoxicity tests: CFE and/or CV experiments.

The data showed that the two cytotoxicity methods may have different sensitivities (Tanaka *et al.*, 2012). However, the limited dataset generated in this study doesn't allow us to conclude on the equivalence or superiority of one or the other method and further studies are needed to address this important aspect. For completeness, both methods are described hereafter.

***1.1 Colony Forming Efficiency (CFE) method***

Only M10F medium is used in the CFE test. It is conducted as follows:

- **Day 0: Cell seeding** :

  1. Cells at approximately 70% of confluence are trypsinised and suspended in M10F complete medium.
  2. Cells are then seeded in 60-mm dishes at a density of 200 cells/dish, in 4 mL of M10F complete medium:

     - 4 dishes/dose
     - 4 dishes for each of the controls: untreated control (medium alone) and vehicle control (medium added with vehicle).

> ***Note***:
> *In order to inoculate 200 cells/dish a cell suspension of 50 cells/mL can be prepared and 4 mL are seeded in each dish. Alternatively, a 2 × 10 $^{3}$ cells/mL cell suspension can be prepared and 0.1 mL is seeded in each dish already containing 4 mL of M10F medium.*

- **Day 1: Cell treatment** (24h after seeding). Culture medium is replaced by 4 mL of treatment medium.

> ***Note***:
> - *24h after seeding is the time necessary for the cells to exit the stationary phase of cell growth.*
> - *Treatment media containing the various concentrations of test substances are prepared and used for medium change. Alternatively, concentrated test substance solutions can be added directly into the culture medium without changing the medium.*
> - *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.*

- **Day 4**: **End of treatment** (72h after exposure). Treatment medium is replaced by 4 mL of fresh complete medium.
- **Day 9**: **Cell fixation and staining** :

1. After culture medium has been removed, cells are washed with saline and fixed with methanol (about 3 mL/dish) for 10 min.
2. Then methanol is removed and cells are stained with a Giemsa solution for 30 min.
3. After the staining solution is removed, the dishes are air-dried.

- **Colony count**: The colonies that have been grown are counted under a stereomicroscope.

### *1.2. Crystal Violet (CV) method*

Only M10F medium is used in the CV test. It is conducted as follows:

- **Day 0: Cell seeding**

1. Cells at approximately 70% of confluence are trypsinised and suspended in M10F complete medium.
2. Cells are then seeded in 6-well plates at a density of $3\times10^3$ cells per well, in 1.5 mL of M10F complete medium:

   - At least 3 wells / concentration
   - 3 wells for each control: untreated control (medium alone), vehicle control (medium added with vehicle), blank (medium without cells).

***Note***:

- *In order to inoculate $3x10^3$ cells/well a cell suspension of $2x10^3$ cells/mL can be prepared and 1.5 mL are seeded in each well of the 6-well plates .*
- *In the prevalidation study 24-well plates were employed. Further experience with this assay suggests the use of 6-well plates rather than 24-well plates in order to avoid inhomogeneous cell growth on the smaller culture surface of the 24-well plates. Further, with this change, the cell density inoculated into the wells of 6-well plates (about 330 cells/$cm^2$ ) becomes much closer to that of 100-mm dish (about 350 cells/$cm^2$ ), whilst the cell density in the wells of 24-well plates is about 500 cells/$cm^2$.*

- **Day 1: Cell treatment** (24h after seeding). Treatment media containing various concentrations of test substances are prepared, and used for medium change. Cells are exposed to the treatment medium for 72h (See the ***Note*** for Day 1 in section 1.1).
- **Day 4: End of treatment**. Medium is replaced with fresh complete medium. Cell growth is checked under a microscope.
- **Day 7: Cell fixation and staining**

1. Medium is removed and cells are fixed with 10% formalin for 30 min.
2. Cells are washed with water, air-dried, and then stained with 0.1% CV solution for 15 min.
3. After rinsing with water, the plates are air-dried.

- **Spectrophotometric measurement of the stained dye:**

1. The stained dye in each well is extracted with 1.5 mL of extraction solution/well for 10

min under gentle shaking.

2. The optical density of each well is measured at 540 nm (alternatively any filter between 540 nm and 570 nm can be used).

### *1.3. Dose determination for the morphological transformation assay*

For the selection of doses to be used in the MTA the results of the DRF tests should contain at least:

- one dose between the NOEL (no observable effect level) and IC50
- another one between IC50 and IC90, as presented in **Figure 1**.
- From the DRF cytotoxicity curves, the MTA treatment doses should be determined as presented in **Figure 2**.

> ***Note**:*
> *Some substances with steep cytotoxicity curves induce transformed foci within almost two times the concentration range above IC50. With such chemicals transformed foci are sometimes induced at concentrations above IC90. Moreover, during the prevalidation study, a slight deviation was observed between the cytotoxicity curves for repeated experiments. For such substances it is sometimes necessary to adopt one or two more doses over IC90 and to test within a narrow concentration range, which can be achieved by setting a dilution increment such as 1/12 log or smaller. Using a 1/12 log increment allows to test three concentrations within the 2.2-fold range.*

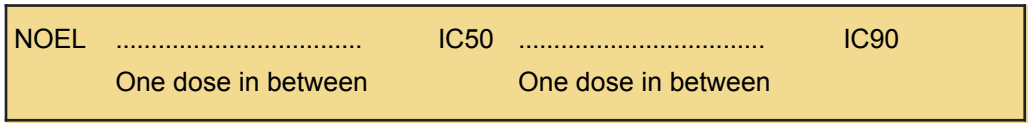


**Figure 1:** Doses required in the preliminary dose range finding tests for selecting the doses to be used in the morphological transformation assay of the BALB/c 3T3 CTA.

| ..... NOEL ..... IC50 ..... IC90 | | |
|---|---|---|
| One dose below | Two doses in between | Two to three doses in between |

**Figure 2:** Dose selection for the morphological transformation assay, based on the results from the dose range finding experiment of the BALB/c 3T3 CTA.

***2. Morphological transformation assay and concurrent cell growth assays***

The MTA is conducted with doses selected on the basis of the data from the cytotoxicity tests.

Three controls, *i.e.* medium control (no addition), vehicle control (medium added with the vehicle) and positive control (4 µg/mL of 3-methylcholanthrene dissolved in DMSO), are included in every experiment.

Cytotoxicity tests are performed in parallel to every MTA according to the above-mentioned protocols (Section 1) in order to confirm the appropriate dosing of the corresponding experiment.

> ***Note*** *:*
> *When DMSO or water is used as vehicle, medium control can be omitted as these substances are well known to have little effect on cell growth under these experimental conditions.*

***2.1. Morphological transformation assay (MTA)***

The MTA is conducted as follows (**Figure 3**):

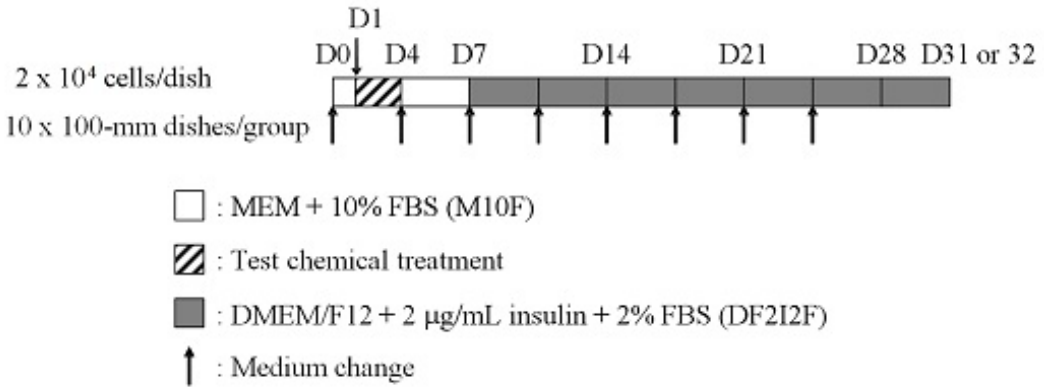


**Figure 3:** Schematic outline of the BALB/c 3T3 CTA protocol.

- **Day 0: Cell seeding**

  1. Cells at approximately 70% of confluence are trypsinised and suspended in M10F complete medium.
  2. Cells are then seeded in each 100-mm dish at a density of $2\times10^{4}$ cells/dish. 10 dishes are plated per dose and per control.

***Note**:*

- *In order to inoculate 2 × $10^4$ cells/dish a cell suspension of 2 × $10^3$ cells/mL can be prepared and 10 mL are seeded in each Petri dish.*
- *In this protocol, 10 100-mm dishes are used per group (i.e. for each treatment dose and control group) in order to check the variability of focus formation. The IARC/NCI/EPA Working Group protocol previously recommended the use of 10 60-mm dishes per group (Tsuchiya and Umeda 1995; Tsuchiya et al. 2010; Hayashi et al., 2008). Hayashi et al. (2008), conducting a similar assay, demonstrated that clear dose response curves can be obtained with carcinogens using only four 100-mm dishes per group. Thus, in order to increase practicality of the present assay, the use of five 100-mm dishes or of 10 60-mm dishes may be considered.*

- **Day 1: Cell treatment** (24h after seeding):

  1. Treatment media containing various concentrations of the substance are prepared.
  2. The culture medium is removed from the dishes and 10 mL of the treatment medium are added to each of the corresponding dishes.
  3. Cells are exposed to the treatment medium for 72h (See the note for Day 1 in section 1.1).

- **Day 4: End of treatment**

  Treatment medium is replaced with 10 mL of fresh M10F complete medium.

- **Day 7 through Day 24 or 25: Medium change**

  1. M10F medium is replaced with 10 mL of fresh DF2I2F complete medium at Day 7.
  2. Thereafter DF2I2F medium is changed twice a week.

- **Day 31 or 32: Cell fixation and staining**

  1. 1 week after the last medium change cells are fixed with methanol for 10 min.
  2. Then cells are stained with a Giemsa solution for 30 min.
  3. After the staining solution is removed, dishes are air-dried.

- **Focus count:** The foci that have been grown are counted under a stereomicroscope and classified.

### *2.2. Concurrent cell growth assays*

**CFE** and **CV** cytotoxicity tests are run in parallel to the MTA, following the procedures described in section 1.1 and 1.2, respectively.

Identical treatment doses and controls must be tested in all three assays.

## Endpoint Measurement

In the BALB/c 3T3 CTA, both cytotoxicity and morphological transformation endpoints are evaluated. Both parameters are determined for each concentration and control, after observation under a stereomicroscope and classification of cell foci as normal or morphologically transformed.

### *Cytotoxicity*

Cytotoxicity assessment is based on the inhibition of colony formation, as reflected by the PE and the RPE in the CFE test and by the RCG in the CV test.

- **In the CFE test**, only foci consisting of more than about 50 cells or being more than about 2 mm in diameter are counted. Relative CFE and plating efficiency (PE) are

recorded, and the results are expressed as relative mean CFE (%) ± SD:

- **Relative CFE (%) =** [total number of colonies formed in the treatment dishes / total number of colonies formed in the control dishes] × 100.
- **PE (%)** = number of colonies formed in the control dishes × 100 / 200, where 200 is the total number of cells seeded in the CFE dishes.
- **In the CV test**, growth rates relative to the control culture are calculated from the absorbance using the following formula:
- **Relative cell growth (% of control)** = (absorbance of treated well – absorbance of medium blank well) / (absorbance of vehicle control well – absorbance of medium blank well) × 100

***Morphological Transformation***

Carcinogenic potential assessment is based on the occurrence of morphologically transformed foci, as reflected by the number of Type III foci per dish.

- **In the MTA, focus count is performed according to the following:**
- Foci consisting of more than about 50 cells or being more than about 2 mm in diameter are evaluated using a stereomicroscope.
- Only type III foci are recorded, which are characterised by the following morphological criteria: deep basophilic staining of spindle-shaped cells which are morphologically different from the background monolayer cells, dense multi-layering of cells (piling up), random orientation and invasive growth of cells at the edge of foci (criss-cross pattern) (IARC/NCI/EPA Working Group, 1985)
- Since there is a certain degree of subjectivity associated with the identification of transformed foci in the BALB/c 3T3 CTA and a correct scoring is critical, training is necessary to ensure a scoring that is as consistent and objective as possible. A photo catalogue was produced during the BALB/c 3T3 CTA prevalidation study and it has proven to be a valuable aid in establishing consistency in assessing morphology of the foci and for scoring of the experiments (Sasaki *et al.,* 2012b)
- The number of type III foci per dish is counted and this value is used for the statistical analysis.

> ***Note:***
> *In some cases, daughter foci were observed during the prevalidation study, which represent secondary foci developing from cells that break away from the "parent" focus. These foci are scattered on the dish having the same cell morphology and a tendency to detach at the centre when they grow larger. Although this is a rare phenomenon, dishes exhibiting such secondary foci should not be considered for the statistical analysis. To limit cell detachment and to avoid the occurrence of this phenomenon, care should be taken when changing medium.*

**Acceptance Criteria**

The following criteria must be fulfilled for the validity of the assay:

- ***Concurrent cell growth assays***
  - Uniform cell growth in culture wells should be observed.
  - At least one NOEL-concentration should be tested.
  - Cell growth curves should cover the range between NOEL and IC90 (more than 2 and ideally 6 concentrations between NOEL and IC90).
  - For CFE, a minimum plating efficiency of 30% in the vehicle control should be achieved.
- ***Morphological transformation assay***
  - Each laboratory needs to develop its own database and criteria for an acceptable number of foci in the vehicle control, which should be as low as possible. For instance, in the BALB/c 3T3 CTA prevalidation study, the maximum number of Type III foci in the entire set of vehicle control dishes (10 dishes) should not have exceeded 5.
  - An acceptance criterion for the positive control should be added to the protocol. For data with similar properties to those observed in our study, especially regarding the number

of foci in the vehicle and positive controls, the average number of Type III foci induced by the positive control should always exceed 10 per dish. However, the criterion should be adjusted on the basis of the historical data of the laboratory. Alternatively, either an approach based on the definition of an absolute number of foci above the vehicle control or a statistical approach may be developed for the positive control.

- A minimum of 6 analysable concentrations is required, and they must cover the range of cytotoxicity from NOEL to IC90.
- A minimum of 9 scorable dishes out of the 10 dishes/concentration is required for the statistical analysis.

## Data Analysis

The negative binomial distribution combined with William's-type protected tests is a recommended method for the statistical analysis. It matches the particular statistical properties of the BALB/c 3T3 CTA test and it was developed and used in the prevalidation study. Details on this method are described in a dedicated paper by Hoffmann *et al.* (2012) .

The scripts of the statistical evaluation programs written in R language (a freely available statistical analysis software) are appended in the **Downloads** section of the Protocol and include an example of a possible output.

## Prediction Model

The following criteria were generated based on the use of the negative binomial approach for statistical evaluation of the BALB/c 3T3 CTA prevalidation study (Hoffmann *et al.,* 2012).

- A test substance is considered **"negative" (non-transforming)** if no downturn-protected Williams contrast shows a *p*-value < 0.01.

  If, in the case of a negative call, one or more acceptance criteria are not fulfilled, the experiment should be repeated.

- A test substance is considered **"positive"** (transforming) if the downturn-protected Williams contrast with the lowest *p*-value:

  i. includes at least two consecutive concentrations, and
  ii. shows a p-value < 0.01.

  An experiment need not be repeated if a clear positive result is obtained even if one of the acceptance criteria is not fulfilled.

- A test substance is considered "inconclusive" if the downturn-protected Williams contrast with the lowest*p*-value:

  i. includes only one concentration or several non consecutive concentrations, and
  ii. shows a *p*-value < 0.01.

  An inconclusive experiment should be repeated.

- An experiment that does not produce a statistically significant positive response, but shows a marked increase in foci at a single dose needs to be repeated.

## Annexes

- The use of the photo catalogues for the BALB/c 3T3 CTA (Sasaki *et al.*, 2012b) is recommended with the protocol to support consistency in focus scoring and assay results. A copy is available in the **Downloads** section of the Protocol.
- The reporting templates used during the ECVAM prevalidation study are available in the **Downloads** section of the Protocol.

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