Embryonic Stem Cell Test (EST)
DB-ALM Protocol n° 113

Developmental toxicity

The potential embryotoxicity of xenobiotics is assessed by its interference with the cell differentiation of permanent murine embryonic stem cell lines, compared to cytotoxic effects in these cells and 3T3 mouse fibroblasts.

Objective & Application

Disturbances of ES cell differentiation following treatment of xenobiotics indicate a potential of embryotoxicity for early pre-implantation stages of mammalian development. The Embryonic Stem cells Test (EST) has been proposed as a screening assay for potentially embryotoxic substances and for their classification into three different classes of *in vivo* embryotoxicity (strong, weak and not embryotoxic) (Genschow *et al*., 2002; Spielmann *et al*., 1997).

The Embryonic Stem Cells Test (EST) is a validated assay for potential embryotoxicants (Genschow *et al*. 2002, Spielmann *et al*. 1997; Marx-Stoelting *et al*., 2009). However, according to the recent studies of Augustine-Rauch *et al*. (2010), Bremer *et al*. (2005), Marx-Stoelting *et al*. (2009), Ozolins (2009), Spielmann *et al*. (2006) or Stummann and Bremer (2008), the EST on its own is not yet suitable for regulatory purposes, but may be used in an integrated test strategy.

Currently chemicals are tested for potential toxic effects on developmental toxicity with *in vivo* studies according to OECD Testing Guidelines (414, 415, 416, 421 and 422; OECD, 1983, 2001a,b, 1995 and 1996), EU test methods (B.31, B.34, B.35; EU, 2008) and segment 2 and 3 studies (teratogenicity and embryotoxicity; pre- and postnatal toxicity) according to the ICH guidelines (ICH, 2005).

Résumé

Embryotoxicity testing is either performed *in vivo* using pregnant animals or *in vitro* on cultured embryos or embryonic tissues and cells from pregnant animals. Both for *in vivo* and for *in vitro* testing pregnant animals have to be sacrificed (Doetschmann *et al.*, 1985). Taking advantage of the potential of embryonic stem cells to differentiate in culture, a new *in vitro* embryotoxicity test with permanent cell lines from the mouse was proposed, the embryonic stem cell test (Spielmann *et al.*, 1995). This test is based on the determination of the most important embryotoxic parameters. The inhibition of differentiation is combined with the study of differences in sensitivity between embryonic and adult tissues to cytotoxic damage. Two permanent mouse cell lines are used, ES cells (D3), to represent embryonic tissue, and fibroblasts (3T3 cells), to represent adult tissue. The test has been developed only after it was found that ES cells can be maintained in the undifferentiated stage in the presence of the cytokine leukemia inhibiting factor (LIF). When released from the undifferentiated stage, ES cells will form embryo bodies (EBs) and differentiate under appropriate conditions into the major embryonic tissues. Cytotoxicity data show that ES cells are more sensitive to toxic agents than adult cells (Laschinski *et al.*, 1991). The inhibition of differentiation of ES cells and the inhibition of growth of ES cells and 3T3 cells are the three selected endpoints in the EST for predicting the embryotoxic potential of chemicals.

A comprehensive bibliographic review document "Embryonic Stem Cell Test" is available as "Method Summary" in DB-ALM.

Experimental Description

Biological Endpoint and Measurement:

CELL DIFFERENTIATION: Inhibition of ES cell differentiation into cardiac myoblasts, measured by light microscopy

CELL PROLIFERATION: Inhibition of 3T3 and ES cell proliferation

CELL VIABILITY: Inhibition of 3T3 and ES cell viability determined by the MTT assay

Endpoint Value:

IC_{50}: 50% inhibition of growth and viability of the cells

ID_{50}: 50% inhibition of differentiation of ES cells into cardiac myoblasts

Experimental System(s):

3T3 FIBROBLASTS (MOUSE): permanent mouse cell line BALB/c 3T3 cells, clone A31

D3 EMBRYONIC STEM CELLS (mouse)

Basic Procedure

Differentiation of ES cells.
The mouse ES cell line D3 is cultured permanently in the presence of LIF, a differentiation inhibition factor. In the absence of LIF, ES cells start to differentiate spontaneously. Several concentrations of the test chemical are added to a stem cell suspension. Drops of ES cell suspension in supplemented DMEM (Dulbecco's Modified Eagle's Medium) are placed on the lids of 10 cm petri dishes ("hanging drop" culture according to Wobus *et al.*, 1991). After cultivation for 3 days the aggregates are transferred into bacterial (non tissue culture treated) petri dishes. 2 days later EBs are placed into 24-well plates (tissue culture treated) where further development of EBs proceeds into different embryonic tissues (Spielmann *et al.*, 1995; Heuer *et al.*, 1994a and b). Differentiation into contracting myocardial cells is determined by light microscopy after another 5 days of culture.

Cytotoxicity measurement with ES D3 cells and 3T3 cells in the MTT assay.
Exponentially growing 3T3 cells and ES cells in the absence of LIF are inoculated into 96-well microtiter plates. 2 hrs after cell seeding 8 concentrations of the test chemical, dissolved in assay medium or appropiate solvent, are added to each well. After 10 days of culture the MTT assay is performed. The absorbance is read on an ELISA reader at 570 nm and using a reference wavelength of 630 nm.

Data Analysis/Prediction Model

The original Prediction Model (Spielmann *et al.*, 1997) was refined in the ECVAM prevalidation study (Scholz *et al.*, 1999), then evaluated and applied during the formal validation study (Genschow *et al.*, 2002).

The three experimental endpoints determined in two different cell lines (50% inhibition of differentiation of ES cells into cardiac myoblasts ID50, and 50% inhibition of cell growth in ES and 3T3 cells in the MTT assay, respectively IC50 D3and IC50 3T3) are used to derive a classification model based upon three variables, which were found to correlate significantly with embryotoxic potential. The chemicals are classified into three classes of the *in vivo* embryotoxicity (*not embryotoxic, weak*and*strong embryotoxic*).

To compare the *in vitro* with the *in vivo* classifications, contingency statistics were obtained assessing the data accuracy, the method predictivity and precision for all three toxicity classes of the test chemicals. (Genschow *et al.*, 2002; Spielmann *et al*., 1997)

For further details see section 3 **"Evaluation: the prediction of embryotoxic potential"** of the attached Procedure Details.

Test Compounds and Results Summary

Pharmaceuticals, agricultural and industrial chemicals, food additives and contaminants (Brown, 2002).

Discussion

Embryonic stem cells of mouse will differentiate into different embryonic tissues depending on culture conditions. Therefore, in the past several groups have used ES cells of the mouse to establish an *in vitro* embryotoxicity assay. Laschinsky *et al.* (1991) compared cytotoxicity in ES cells and in mouse fibroblasts to assess the embryotoxic potential of teratogenic agents. Heuer *et al.* (1994a, b) compared cytotoxicity and inhibition of differentiation of ES cells for the same purpose. Newall and Beedles (1994) measured cytotoxicity and colony forming potential of ES cells after 7 days of culture in the presence of teratogenic agents, which has the advantage for routine testing, because one endpoint, colony forming, is determined automatically. In all of these assays, only a few embryotoxic agents could be classified correctly. This is probably due to the fact that in the ES assays only two of the three essential endpoints were selected for biostatistical evaluation, which seems to be insufficient.

To overcome the limitations of the previously mentioned ES cell tests, Spielmann *et al*. (1997) determined three different experimental endpoint values (ID50, IC50 D3 and IC50 3T3). From these endpoints, three variables are derived, which are the basis for the classification of the test compounds into three *in vivo* embryotoxicity classes (see Prediction Model). This approach has the advantage to provide also information on the different sensitivity between embryonic and adult tissue concerning

cytotoxicity damage of chemical compounds.
In fact, using discriminant analysis, 16 test chemicals have already correctly been assigned to the proposed 3 classes of *in vivo* embryotoxicity (Genschow *et al.*, 2002; Spielmann *et al.*, 1997).

So far, no other *in vitro* test has performed as well. Moreover, this is the first *in vitro* embryotoxicity test in which no pregnant animals have to be sacrificed to obtain embryonic tissue for the *in vitro* culture. The development of the standard operating procedure (SOP), presented in this Protocol, for the current Validation Study, overcomes the problem concerning the maintenance of ES in laboratories, which usually tend to differentiate spontaneously. Furthermore, the ECVAM validation study has shown that the correlation between the *in vitro* data and *in vivo* data was good (accuracy 78%), according to the performance criteria defined. The predictivity (100%) for *strongly embryotoxic* chemicals was reported to be excellent, and the precision (81%) was considered good. The predictivity for *non-* (72%) and *weak* (70%) *embryotoxicants* and the precision for *non-embryotoxicants* (70%) were reported to be sufficiently high (>/=65%) (Anon., 2002).

However, a special problem has occurred with the misclassification of methylmercury chloride, a strongly embryotoxic chemical, that was predicted to be non-embryotoxic instead of strongly embryotoxic in four of eight experiments. This misclassification probably occurred because the training set used for developing the PM did not include chemicals of a similar cytotoxicity pattern (Genschow *et al*., 2002). A low rate of correct predictivity was also observed with other metals in the EST: correct predictions were only obtained for Li, CrIII and CrVI, whereas Cd, MeHg, AsIII and AsV and the organic metabolites of arsenic were misclassified. Reasons for this finding may be a comparable cytotoxicity towards D3 and 3T3 cells and/or or the absence of an inhibition of cardiac differentiation via specific mechanisms (Stummann *et al*., 2007; 2008).

In contrast to the promising results of EST, a limitation of the test is the lack of a biotransformation system. Therefore, the combination of the EST with a suitable *in vitro* metabolic system was intended to be evaluated during ReProTect Project (2004-2009). The pluripotency of the embryonic stem cells offers to study effects on the development of additional target tissues, namely neural and skeletal derivatives, and this was the scope of ReProTect. Interspecies variations represent a major problem in drug testing, therefore it was the aim to adapt the murine system to human embryonic stem cells. This would allow the prediction of developmental toxic effects in humans more precisely (Hareng *et al*., 2005).

Within the ReProTect project, an extension of the EST data base with 13 selected chemicals was performed in 2 independent laboratories: the EST yielded no satisfactory results, with only minor differences between the two laboratories. Only 2 of the 13 substances were classified correctly (Marx-Stoelting *et al*., 2009). Furthermore, the attempts to develop a metabolising system within the project have also not been successful. New SOPs have been established for differentiation into neuronal, cartilage and bone cells as well as for additional endpoints, and first steps were achieved in establishing a hEST. However, the results are unpublished to date (Adler *et al*., 2010; Marx-Stoelting *et al*., 2009; Spielmann, 2009).

A feasibility study was performed by Schenk *et al*. (2010) within the ReProTect project. They tested 10 chemicals with a test battery consisting of 14 *in vitro* tests. Developmental toxicity *in vitro* was examined by a combination of three test systems, including the EST. The results from the EST led to a correct prediction of the occurrence of *in vivo* observed developmental effects for 7 chemicals (5 correctly positive, 2 correctly negative) and to a false prediction for 2 chemicals (1 false positive, 1 false negative). One chemical gave ambiguous results *in vivo* and *in vitro* (Schenk *et al*., 2010).

Status

Known Laboratory Use:

Centre de Recerca en Toxicologia (CERETOX) Barcelona (**link to a demonstration movie)**

Participation in Evaluation Studies:

The validated Embryonic Stem cell test (EST) has been included in the **“ReProTect” project** (**www.reprotect.eu**, last accessed on 17.12.2012), a **5-years (July 2004-2009) integrated project funded by the European Commission under the EU 6th Framework Programme for Research, Technological Development and Demonstration** (Hareng *et al.*, 2005). The overall aim of the project was to develop a testing strategy to cover the entire mammalian reproductive cycle in the area of reproductive toxicity (Hareng *et al*., 2005). The further development and evaluation of the EST followed the modular approach presented by ECVAM in 2004 (Hartung *et al*., 2004).

Within the frame of the ReProTect project the aim was to conclude module 2 (assessment of

reproducibility of experimental data in same laboratory) for the human EST, module 3 (assessment of reproducibility of experimental data in a second laboratory) for the test system involving skeletal differentiation, module 4 (assessment of reproducibility of experimental data in 2-4 laboratories) for the test system involving neuronal differentiation and for the test including a biotransformation system and module 5 (assessment of predictive capacity of the prediction model associated with the test system) for the original EST system.

However, the attempts within the ReProTect project to broaden the data basis of the validation study (Genschow *et al*., 2002) with 13 selected chemicals in the EST yielded no satisfactory results: the previously established prediction model (PM) was not applicable to the new data (Marx-Stoelting *et al*., 2009). Furthermore, the attempts to develop a metabolising system within the project have also not been successful. A feasibility study was performed by Schenk *et al*. (2010) within the ReProTect project. The results from the EST lead to a correct prediction of the occurrence of *in vivo* observed developmental effects for 7 out of 9 chemicals (Schenk *et al*., 2010).

Participation in Validation Studies:

Following prevalidation (March-December 1997) (Anon., 1998b), the SOP presented in this Protocol has participated in the international (ECVAM) embryotoxicity validation project: **"*In vitro* tests for embryotoxicity: Prevalidation and validation of assays employing micromass cultures, rat embryo cultures and embryonic stem cells"** that started in December 1996 and has successfully been concluded in January 2000.

Based on the successful outcome of the study, in October 2001 **the ECVAM Scientific Advisory Committee (ESAC)** unanimously endorsed the statement that the results obtained in the definitive phase of the study with the Embryonic Stem Cell test were highly reproducible, the correlation between *in vitro* and *in vivo* data was good, and the test proved applicable to testing a diverse group of chemicals of different embryotoxic potentials. **The ESAC therefore agreed with the conclusion that the EST is a scientifically validated test which is ready to be considered for regulatory purposes** (Anon., 2002; Genschow *et al*., 2002). However, even if the three embryotoxicity methods evaluated do not represent replacements for current animal tests for reproductive toxicity as a whole, ESAC underlined that they could provide suitable means for reducing and/or refining the use of animal procedures in the context of testing strategies (Anon., 2002).

Regulatory Acceptance:

According to Augustine-Rauch *et al*. (2010), Bremer *et al*. (2005), Marx-Stoelting *et al*. (2009), Ozolins (2009), Spielmann *et al*. (2006) or Stummann and Bremer (2008), the EST on its own is not yet suitable for regulatory purposes, but may be used in an integrated test strategy.

The protocol introduction has been updated in 2010 within the Thematic Review Project of European Partnership for Alternative Approaches to Animal Testing (EPAA).

Last update: December 2010

PROCEDURE DETAILS, 28 January 1999*

Embryonic Stem Cell Test (EST)
DB-ALM Protocol n° 113

The protocol presents the standard operating procedure used in the validation study: "*In Vitro* tests for embryotoxicity: prevalidation and validation of assays employing micromass cultures, rat embryo cultures and embryonic stem cells".

This SOP, scientifically validated by the ECVAM Scientific Advisory Committee (ESAC) in October 2001 was included in the EU Integrated project "ReProTect" (**www.reprotect.eu**) for further development and introduction of a metabolic system.

**The accuracy of the SOP has been confirmed by the responsible laboratory in May 2000. The DB-ALM is contacting again the person responsible for the method to update or confirm the herewith included SOP. As soon as new information will become available this version will be updated.*

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1. Materials

1.1. CELL LINES

Balb/c 3T3 cells, clone A31, obtained from ICN-Flow, Eschewege, Germany (Cat. No. 03-465-83,) or American Type Culture Collection (ATCC; Cat. No. CCL-163).
Embryonic stem cells, D3, obtained from Prof. Rolf Kemler (Max Planck Institute, Freiburg, Germany) or American Type Culture Collection (ATCC; Cat. No. CRL-1934).

1.2. TECHNICAL EQUIPMENT

- Incubator (37°C ± 1°C), humidified, 5% ± 1% CO2/air
- Laminar flow clean bench (standard: "biological hazard")
- Water bath (37°C ± 1°C)
- Phase contrast microscope
- Laboratory burner
- Laboratory balance
- 96-well plate photometer (Immuno reader)
- Shaker for microtiter plates
- Cell counter or haemocytometer
- Dilution blocks: Greiner, Cat No. 975 502

- Dilution tubes: Greiner, Cat. No. 102 201

- safe lock tubes ambra: Eppendorf, Cat. No. 0030 120.191

- Pipetting aid

- Pipettes, 8-channel-pipettes

- Cryotubes (2 ml)

- Tissue culture vials:

  - for routine culture:
    T-flasks 25 cm^2: Corning, Cat. No. 430168;
    T-flasks 75 cm^2: Corning, Cat. No. 25110;
    petri dishes 60 x 15 mm Ø: Corning, Cat. No. 25010 or Falcon, Cat. No. 3004;
    petri dishes 100 x 20 mm Ø: Corning, Cat. No. 25010, Falcon, Cat. No.3003;
  - for hanging drop culture: petri dishes 100 x 20 mm Ø: Falcon, Cat. No. 3003, Corning, Cat. No. 25010
  - for preparation of cell/EB suspensions: bacterial petri dishes 60x15mm Ø, Greiner, Cat. No. 628102
  - 96-well flat-bottomed tissue culture microtiter plates: Falcon, Cat. No. 3072
  - 24-well tissue culture plates: Falcon, Cat. No. 3047
  - Plate sealers: Dynatech, Cat No. M 30 or equivalent

1.3. CHEMICALS, MEDIA, SERA

- Dulbecco's Modified Eagle's Medium (DMEM) with L-glutamine, Glucose and NaHCO3: Gibco, Cat. No. 41965-039 [Europe] or Gibco, Cat. No. 11965-092 [USA]
- L-Glutamine: Gibco, Cat. No. 25030-024
- Foetal Calf Serum (FCS; has to be tested): HyClone Cat. No. A-1111-L; Boehringer Mannheim Cat. No. 210471
- Trypsin/EDTA solution, Gibco, Cat. No. 45300-019 or Clonetics, Cat. No. cc-5012
- Penicillin/Streptomycin solution: Gibco, Cat. No. 15140-114
- Dimethylsulphoxide (DMSO): Sigma, Cat. No. D2650
- Non essential amino acids (NAA): Gibco, Cat. No. 11140-035
- b-Mercaptoethanol: Sigma, Cat. No. M7522
- m LIF: Gibco, Cat. No. 13275-011 or -029
- MTT: Sigma, Cat. No. M5655
- Propan-2-ol: Merck, Cat. No. 995 and Sigma Cat. No. IO398
- SDS (sodium dodecylsulphate): Fluka, Cat. No. 71727
- Phosphate buffered saline (PBS) without Ca++ and Mg ++ Biochrom, Cat. No. L1825; or Gibco, Cat. No. 14190-144
- 5-Fluorouracil: Sigma, CAS No. 51-21-8, Cat. No.F-6627
- Penicillin G: Sigma, CAS No. 69-57-8, Cat. No.PEN-NA
- BSA, tissue culture tested: Sigma, Cat. no. A2058 or equivalent
- Trypan Blue
- Ethanol (analytical grade)
- Aqua Bidest

1.4. PREPARATIONS

A) m LIF
LIF is provided as solution by the manufacturer and added directly to the culture plates/T-flasks during routine passaging of ES cells. LIF solution at a concentration of 10^6 U/ml (Cat. no. 13275-011) is stored in aliquots at -20°C. Once thawed, aliquots are stored at 4°C (stable for up to 1 year). If LIF at 10^7 U/ml (Cat. no. 13275-029) is used, prepare a 1:10 dilution in PBS (containing 1% BSA as carrier) or in cell culture medium and store as above according to the manufacturers instructions.
BSA, tissue culture tested, from Sigma, Cat. No. A2058 or similar grade should be used.

B) Fetal Calf Serum
FCS is heat inactivated after thawing at 56°C for 30 min.

C) b-ME
A 10 mM working solution of b-ME can be prepared in PBS and can be used for up to 1 week (store at 4°C).

D) Culture medium
Complete media (routine culture or assay media) are prepared without LIF and used no longer than one week (stored at 4°C).

All supplements for media are provided as solutions. They are stored in aliquots at 4°C or -20°C according to the manufacturers instructions.

Complete media contain the following supplements in DMEM (Dulbecco's Modified Eagle's Medium; final concentrations of compounds are quoted):

3T3 cells	**ES cells**
(A I) for Routine culture	**(A II) for Routine culture**
10 % FCS 4 mM Glutamine 50 U/ml Penicillin 50 µg/ml Streptomycin	20 % FCS 2 mM Glutamine 50 U/ml Penicillin 50 µg/ml Streptomycin 1 % NAA 0.1 mM b-Mercapto-ethanol 1000 U/ml m LIF (added directly to the plates)
(B I) for Assay	**(B II) for Assay**
(see (AI)	20 % FCS 2 mM Glutamine 50 U/ml Penicillin 50 µg/ml Streptomycin 1 % NAA 0.1 mM b-Mercapto-ethanol
(C I) for Freezing	**(C II) for Freezing**
20 % FCS 4 mM Glutamine 50 U/ml Penicillin 50 µg/ml Streptomycin 10 % DMSO	40 % FCS 2 mM Glutamine 50 U/ml Penicillin 50 µg/ml Streptomycin 1 % NAA 0.1 mM b-Mercapto-ethanol 10% DMSO

E) MTT Solution

5 mg MTT/ml PBS
Prepare stock solution, filter (0.2 µm) and store aliquots at -200°C.

F) MTT Desorb

3.49 ml	20% SDS stock solution (final conc. = 0.7%)
	(20g SDS dissolved in 100 ml aqua bidest, store at RT)
+96.51 ml/100.00 ml	2-propanol

Prepare freshly prior to use (warm to 37°C if precipitates occur).

2. Methods

2.1. ES CELL DIFFERENTIATION ASSAY

2.1.1. Concentration of test chemicals

- Dissolve test chemical in appropriate solvent. The recommended maximum final solvent concentrations are indicated in the table below, the maximum test concentration of any chemical is 1000µg/ml.

Solvent	max. concentration
DMEM (not supplemented) PBS Aqua bidest	1%
DMSO	0.25%
Ethanol	0.5%

Do not use complete (supplemented) media for preparation of stock solutions since serum proteins, test chemicals or other components may precipitate upon repeated freezing and thawing.

- Chemical solutions have to be weigthed and dissolved prior to each experiment, including 5-Fluorouracil (5-FU) for the positive control experiment. For use on days 3 and 5 (change of medium), the stock preparation made prior to the beginning of the experiment can be used, if stored in aliquots at -20°C. The positive and negative control chemical Penicill G may be used from frozen stocks of 100 mg/ml in PBS as well as 5-FU for the fixed concentration concurrent positive control concentration.
- The final solvent concentration should be kept constant, not be cytotoxic and should not have any other effects on the cell differentiation at this concentration.
  A range finder experiment is not necessary in the differentiation assay for solvents described above.
  For a strategy for pretesting of solubility of test chemicals see ANNEX H.
- Since strong acids and bases may influence the buffer capacity of medium, check the pH of the highest test concentration of a given chemical after dilution in medium by optical inspection. If the medium turns violet or brightly yellow (pH >8 or <6.5), the stock solution of the test chemical should be neutralised with 0.1N NaOH or 0.1N HCl.

Note: Prepare highest concentration of the chemical in ~ 80% of the solvent volume, measure pH, neutralise, add solvent to the final volume.

- Avoid prolonged exposure to light (e.g. under the microscope) when testing chemicals sensitive to light. Check cells under the microscope before changing of medium. Use light tight tubes (Eppendorf safe lock ambra) or wrap tubes containing chemical solutions in aluminium foil. Main experiment: Make up 6-8 concentrations with a 1.2-3 fold dilution factor covering the relevant range of "dose response" according to the cytotoxicity range finder experiment. For the design of concentration series see ANNEX G.

2.1.2. Assay procedure (see also ANNEX A)

Day 0

Prepare a concentration range of test chemical in assay medium (= test solutions) and the solvent with ES cells (3.75 x 10^4 cells/ml). ES cells are trypsinised and added last, after preparation of test chemicals in medium to avoid prolonged storage outside the incubator. The ES cell suspension is prepared in 60 mm Ø bacterial petri dishes to prevent adherence of ES cells. Keep the cells in suspension by frequent gentle agitation during the following steps and leave at room temperature only for the shortest time period necessary (viability of the cells can be checked by staining an aliquot of the cell suspension with trypan blue. A viability of >=90% is acceptable).

Take care not to exceed the highest solvent concentration allowed and to keep the solvent at a constant concentration with each concentration of test chemical.

Using a pipette, dispense 20µl of cell suspension containing the apppropriate test chemicals (º750 cells) on the inner side of a 100 mm Ø tissue culture petri dish lid. 50-80 drops are pipetted per lid. Use one petri dish per concentration of the test chemical as well as for the untreated control (= assay medium) and the solvent control.

Turn lid carefully into its regular position and put on top of the petri dish filled with 5 ml PBS.

Incubate the "hanging drops" for 3 days in a humidified atmosphere with 5% CO2 at 37°C.

Day 3

Prepare the same test solutions as on day 0.
Use one petri dish per concentration of the test chemical also for the untreated control (= assay medium) and solvent control.
Pipette 5ml of medium containing the appropriate concentration of test chemical or solvent into the lid of the "hanging drop" culture dish. Hold the lid at approximately a 45° angle to rinse the Embryoid Bodies (EBs) down to the bottom. Using a sterile 5ml pipette (to avoid damage to the EBs), gently transfer the total suspension to a 60 mm Æ bacterial petri dish.
Take care that chemical concentration of the "hanging drops" and the petri dish are identical.
Cultivate this EB suspension culture for 2 days in a humidified atmosphere with 5% CO2 at 37°C.

Day 5

Prepare the same test solutions as on day 0.
Use one 24-well plate per concentration of the test chemical as well as for the untreated control (= assay medium) and solvent control.
Pipette 1 ml of test solution into each well of a 24-well tissue culture plate.
Add one EB (in a small volume (£40µl) with blue tip or cut yellow tip) per well. Take care that EBs originated from a certain test solution are transferred to a test solution with an identical concentration.

Incubate 24-well plates for 5 days in a humidified atmosphere with 5% CO_2 at 37°C.

2.1.3. Assay endpoint

On day 10 of the assay differentiation into contracting myocardial cells is determined under the light microscope. Each well of the plate will be checked and the number of wells containing spontaneously contracting cells will be recorded. Use the spreadsheet (ANNEX F) for data recording.

2.1.4. Independent runs

- Repeat the experiment at least once (2 valid experiments).
- Prepare the medium and the test solution prior to starting the experiment.
- Use independent preparations of the reagents in a second experiment.

2.1.5. Quality check of cells

At the end of the differentiation period (day 10) check the solvent control plate(s). The assay is acceptable, if at least 21 out of the 24 EBs have differentiated into spontaneously contracting myocardial cells. According to historical data a 100 % differentiation (= 24 wells with contracting cells) is obtained in ~ 50% of the assays, whereas the acceptable range of 21-24 differentiated EBs covers ~ 95% of all assays. Compare the data of the negative control plate (assay medium) to the data of the solvent control plate to be sure that the solvent has no effect on ES cell differentiation.

If the highest allowed solvent concentration is used, both a solvent control and a medium control are made.

2.1.6. Quality check of the assay (positive control)

After thawing a new batch of frozen cells and before testing chemicals of interest, the quality of the assay is checked using 5-fluorouracil (Sigma Cas. No. 51-21-8; Cat. No.F-6627) as a positive reference chemical. ID 50 values are determined with ES cells (according to section 3 **"Evaluation: Prediction of embryotoxic potential"**). Final concentrations of 0.07, 0.06, 0.05, 0.04, 0.03 µg/ml 5-FU (0,02µg/ml optional) are tested

(prepared from a 2 mg/ml stock solution in PBS).
The ID 50 for 5-FU shall be within the range of 0.048-0.06 µg/ml (preliminary calculation).

2.1.7. Quality check of foetal calf serum

FCS batches of interest are tested in differentiation assays without chemicals first, which should result in at least 21 out of 24 wells containing contracting myocard in at least two independent runs (according to section 2.1.5 **"Quality check of cells"**). This should be done with a batch of cells known to be of good quality. Following this, 5-FU should be tested as positive control with concentrations according to section 2.1.6 **"Quality check of the assay"** at least twice.

2.2. CYTOTOXICITY ASSAY WITH ES CELLS AND 3T3 CELLS

2.2.1. Concentration of test chemicals

- Dissolve test chemical in DMEM or appropriate solvent (see also section 2.1.1. and ANNEX H). If solvent is used, the final solvent concentration should not be cytotoxic and kept at a constant concentration.

  The dilution series is best prepared using the scheme below (example for a 2 fold dilution series using dilution blocks):

- Avoid prolonged exposure to light (e.g. under the microscope) when testing chemicals sensitive to light. Check cells under the microscope before changing medium. Use light tight tubes or wrap tubes containing chemical solutions in aluminium foil.
- **Range finder:** Use the highest soluble concentration of test chemical and non cytotoxic concentration of solvent as highest test concentration. Make a dilution series of 8 dilutions each with a factor of 1:10.
- **Main experiment**: Make up 7 concentrations with a smaller dilution factor covering the relevant range of "dose response" determined in the range finder experiment (see ANNEX G) and 1 concentration of the positive control chemical (see section 2.2.6. **"Quality check of the assay (positive control)"**). The minimum practical dilution factor is 1.5 fold.
- The maximum test concentration of any chemical is 1000µg/ml
- Since volatile chemicals tend to evaporate under the conditions of testing, plates shall be sealed with CO2 permeable plastic film (Dynatech, Cat No. M 30) that is impermeable to volatile chemicals thus decreasing evaporation.
- Before starting an assay with an unknown chemical, exclude a chemical reaction between MTT, the test chemical and the medium by measuring the OD value at 550-570 nm (add 20µl MTT solution to 200µl of medium containing the highest test concentration of chemical). After 2h of incubation at 37°C the OD value should be £ 0.05. If the OD exceeds this value and if the respective concentration is within the range of the expected IC50, medium of all wells of the plate (except blanks) is replaced by assay medium (without test chemical) before addition of MTT on day 10 of the assay.

2.2.2. Seeding of monolayers and assay procedure (see also ANNEX B)

Day 0

Prepare a cell suspension of $1x10^4$cells/ml in routine culture medium. Using a multi-channel pipette, dispense 50 µl medium only into the peripheral wells of a

96-well tissue culture microtiter plate (blank).
In the remaining wells dispense 50 µl volumes of the cell suspension of $1x10^4$ cells/ml (= 500 cells/well). Viability of the cells can be checked by staining an aliquot of the cell suspension with trypan blue. A viability of ³90% is acceptable.

Incubate the cells for 2hrs in a humidified atmosphere with 5% CO2 at 37°C. This incubation period allows adherence of cells.

After 2 hrs incubation, add 150 µl assay medium containing the appropriate concentration of test chemical (test solution; for the pipetting scheme see also ANNEX C; note that the 150µl vol has to contain 1.333x the final chemical concentration).

Into the peripheral wells (blanks) pipette 150µl of assay medium without chemical. Incubate cell cultures at 5% CO_2 and 37°C for 3 days.

Day 3

Remove test solution using a Pasteur-pipette attached to a pump or a multichannel pipette (except peripheral wells). Take care not to destroy the cell layer on the bottom of the wells. Add 200 µl freshly prepared test solution (final concentration/well as on day 0). Incubate cell cultures at 5% CO_2 / 37°C for 2 days.

Day 5

Remove test solution using a Pasteur-pipette attached to a pump or a multichannel pipette and add again 200µl new test solution (final concentrations/well as on day 0). Incubate cell cultures at 5% CO_2 / 37°C for 5 days.

Determination of cell growth inhibition will be performed at day 10 of the assay (see below).

A) Microscopic Evaluation

- Examine cells under a phase contrast microscope.
- Record changes in morphology due to cytotoxic effects of the test chemical. This check is performed to exclude experimental errors. Microscopical analysis of cytotoxicity is not used as an endpoint of the assay.

B) Measurement of MTT (4)

- Add 20 µl MTT (5 mg/ml) to all wells of the plate and incubate at 37°C in a humidified atmosphere of 5% CO_2, for 2 hrs.
- After 2 hrs incubation, decant the MTT solution carefully or remove test solution using a Pasteur-pipette attached to a pump. Place plate upside down on a blotting paper for 1 minute.
- Add exactly 130 µl MTT desorb solution (prewarmed to 37°C) to each well.
- Shake microtiter plate thoroughly on a microtiter plate shaker for 15 min to dissolve blue formazan until the solution is cleared and no more clumps are visible. If aggregates still exist after this incubation, precipitates can be resuspended by pipetting up and down with an octapipette before measuring the absorption.
- Measure the absorption of the resulting coloured solution at 550-570 nm in a microtiter plate reader using 630 nm as a reference wavelength.

Reference filters may have a tolerance of ± 5%, so that reference measurement may still be in the absorption curve of blue formazan (see spectrum below). This can significantly reduce the signal. In this case readings should be performed without reference filter.

2.2.3. Independent runs

- Repeat the experiments at least once.
- Prepare medium and test solution prior to use.
- Use independent cell cultures.

2.2.4. Quality check of cells

Normal growing behaviour of cells is a prerequisite in all cytotoxicity assays based upon determination of growth inhibition. Therefore, on day 10 after the MTT assay has been performed, check the absolute optical density ($OD_{550-570}$) of solvent control wells (columns 2 and 11 of the 96-well plate, see also Annex C). According to historical data the following confidence ranges (preliminary calculation) have to be met:

CELL LINE	SOURCE	95% CONFIDENCE INTERVAL ($OD_{550-570}$)
D3 Kemler	ATCC	0.15-0.8 0.90-1.6
3T3	ICN-Flow ATCC	0.50-1.3 0.15-0.6

2.2.5. Quality check of the assay (positive control)

After thawing a new batch of frozen cells and before testing chemicals of interest, the quality of the assay is checked using 5-fluorouracil (Sigma Cas. No. 51-21-8; Cat. No.F-6627) as a positive control and Penicillin G (Sigma Cas. 69-57-8, Cat. No. PEN-NA) as a negative control. Highest test concentrations of 1 µg/ml 5-FU for ES and 3T3 cells (prepared from a 2 mg/ml stock in PBS) are used and diluted in a 2 fold dilution series. IC50 values for 5-FU are determined with both ES cells and with 3T3 cells.
The IC50 values for 5-fluorouracil shall be in the range of 0.048 - 0.086 µg/ml with ES cells and 0.12 - 0.5 µg/ml with 3T3 cells (preliminary calculation).

One concentration of Penicillin G (1000µg/ml) is concurrently run in 1 column of the plate (column 3). The Pen G concentration should not have an effect on viability of the cells (to be confirmed).
In addition one fixed dilution of the positive control chemical 5-fluorouracil is included in each cytotoxicity assay concurrently with 7 dilutions of the test chemical. The concentration of 5-fluorouracil is derived from historical mean values of IC50 for ES and 3T3 cells. The concentrations of 5-fluorouracil to be included as the positive control are: 0.29 µg/ml for 3T3 cells and 0.06 µg/ml for ES cells. With these concentrations inhibition should be in the range of 20-80%.

3. Evaluation: prediction of embryotoxic potential

3.1. GENERAL REMARKS:

- Evaluation of results is based upon 3 experimental endpoints determined in two cell lines: ID50, IC50 D3 and IC50 3T3 . ID50 is reflecting 50% inhibition of ES cell differentiation and IC50 50% inhibition of cell growth with ES cells (IC50 D3) and 3T3 cells (IC50 3T3). It shall always be backed by a graded concentration response curve. It is, therefore, not sufficient to calculate a 50% inhibiting concentration by interpolation between an "all or nothing" effect, unless the concentration-response curve is extremely steep and a graded response is not obtainable even with testing narrow concentration

steps.

- To calculate ID50, IC50 D3 and IC50 3T3 from experimental data several methods are adequate and may be used:
  The most simple way is a graphical determination using probability paper with x=log and y=probit scales, where the test concentrations are assigned to the x-axis and the % effects are assigned to the y-axis. Biostatistical methods modeling the concentration-response curves reveal a more precise calculation of the ID50, IC50 and, in addition, allow to calculate the confidence intervals for these values. For calculation of the ID50, the method of Litchfield & Wilcoxon (1949) or probit analysis according to Finney (1971) is recommended. For calculation of IC50 the method of Holzhütter & Quedenau (1995) is recommended.

3.2. CALCULATION OF ENDPOINTS

3.2.1. ES cell differentiation assay

- On day 10 determine the number of wells with contracting myocardial cells in the 24-well solvent control plate.
- Set this number = 100%.
- Determine the number of wells with contracting areas for each of the plates treated with a given concentration of the test chemical.
- Calculate the inhibition of differentiation as % of the solvent control plate.
- Use the spread sheet (ANNEX F) for data recording.
- Use these values for calculating the ID50 value according to section 3.1., 2nd paragraph.

3.2.2. Cytotoxicity assay in ES cells and 3T3 cells

- Determine the mean OD 550-570 of the blank wells and substract this value from all OD values of the 96 well plate (this corrects all values for adherence of the dye to the plastic material of the plates).
- Determine the mean OD 550-570 of the untreated solvent controls (columns: 2 B-G and 11 B-G, see also ANNEX C). Set this value to a cell viability of 100%.
- Determine the mean OD 550-570 for each of the columns 4 to 10, each representing a concentration of the test chemical. Express this value as cell viability (% of untreated controls).

3.2.3. Data recording

Data files of optical densities (OD 570) generated by a microplate reader are directly transferred/copied into the EXCEL spreadsheet (ANNEX F) this spreadsheet saves data in a standard format and allows biostatistic evaluation of data generated by different labs. Mean OD values, standard deviations and viabilities are calculated automatically. IC50 values can be calculated graphically from the spreadsheet or by the ELISA reader software used in each laboratory. All fields of the template have to be filled out.

3.3. CLASSIFICATION

To predict embryotoxic potential of a test chemical a prediction model (PM) originally proposed for the EST (Spielmann *et al*., 1997) was refined using data obtained at the ZEBET laboratory during the prevalidation study (Scholz *et al.*, 1999).

Basically, for the improved Prediction Model (iPM), the endpoints were not changed,

except that IC50 and ID50 concentrations above 1000µg/ml are not calculated any more. If an IC50 or ID50 value exceeds this concentration, it is set to 1000µg/ml by definition, which implies that for future testing, the maximum test concentration will be 1000µg/ml. In the iPM, the combination of endpoints, which were applied as variants in the previous linear discriminant analysis, have been modified:

improved Prediction Model

Endpoints:	IC50 3T3	**Variables:**	lg(IC50 3T3)
	IC50 D3		lg(IC50 D3)
	ID50		(IC503T3-ID50)/IC503T3

Linear discriminant functions I, II and III:

I: 5.916 lg(IC50 3T3) + 3.500 lg (IC50 D3) - 5.307 [(IC503T3-ID50) / IC503T3] -15.27

II: 3.651 lg (IC503T3) + 2.394 lg (IC50D3) - 2.033 [(IC503T3-ID50) / IC503T3] - 6.85

III: -0.125 lg (IC503T3) - 1.917 lg (IC50D3) +1.500 [(IC503T3-ID50) / IC503T3] - 2.67

Classification criteria:

Class 1 **Not embryotoxic**	If I>II and I>III
Class 2 **Weak embryotoxic**	If II>I and II>III
Class 3 **Strong embryotoxic**	If III>I and III>II

The iPM has proved to be more appropriate than the original PM for data sets from different laboratories as well as for different sets of chemicals.

A classification table containing the formula for prediction of embryotoxic potential was provided to each test laboratory as an EXCEL file (version 5.0).

Annexes

ANNEX A: EXPERIMENTAL STEPS OF THE ES CELL DIFFERENTIATION ASSAY

ANNEX B: EXPERIMENTAL STEPS OF THE CYTOTOXICITY ASSAY (ES- AND 3T3 CELLS)

ANNEX C: PIPETTING SCHEME FOR THE CYTOTOXICITY ASSAY

The pipetting scheme shown below shall be used for one test chemical per plate (the positive control is placed next to the lowest test concentration).

	1	2	3	4	5	6	7	8	9	10	11	12
A	b	b	b	b	b	b	b	b	b	b	b	b
B	b	CO	P		TEST	SO	LU	TION			CO	b
C	b	CO	P								CO	b
D	b	CO	P								CO	b
E	b	CO	P	LOW	CON	CEN	TRA	TION	->	HIGH	CO	b
F	b	CO	P								CO	b
G	b	CO	P								CO	b
H	b	b	b	b	b	b	b	b	b	b	b	b

CO *= SOLVENT CONTROL*
b *= BLANKS (assay medium)*
P *= POSITIVE CONTROL (see section 2.2.6.*
"Quality check of the assay (positive control)" *;*
negative control in the positive control experiment=1000µg/ml PenG)

ANNEX D: CELL MAINTENANCE AND CULTURE PROCEDURES

Routine culture of ES cells and Balb/c 3T3 cells

ES cells and Balb/c 3T3 cells are routinely grown as a monolayer in petri dishes or culture flasks at 37°C in a humidified atmosphere of 5% CO2. The cells should be examined on a daily basis under a phase contrast microscope. Any changes in morphology or their adhesive properties should be noted.

Prior to use solutions should be prewarmed to 37°C in a water bath or incubator.

When the cells approach 80 % confluence they should be removed from the petri dish or culture flask by trypsinisation as follows:

- Decant the medium and rinse the cultures with PBS twice without Ca ++ and Mg ++.
- Wash cells by gentle agitation to remove any culture medium additives, which might inhibit trypsin activity.
- Discard the washing solution.

A) For trypsin treatment of ES cells:

- Add 1 - 2 ml of prewarmed Trypsin/EDTA solution to the monolayer for a few seconds.
- Add 6 ml culture medium to the Trypsin-EDTA solution.
- Resuspend the rest of the cells from the bottom of the flask.
- Centrifuge the cell suspension at 800 U/min for 10 minutes.

B) For trypsin treatment of Balb/c 3T3 cells:

- Add 1 - 2 ml Trypsin-EDTA solution to the monolayer for a few seconds.
- Remove excess Trypsin-EDTA solution and incubate the cells at 37°C.
- After 2 - 3 min, lightly tap the culture flask or petri dish to detach the cells into a single cell suspension.

Cell Counting

- After centrifuging and discarding the supernatant or detaching the cells, add 0.1 - 0.2 ml of routine culture medium/cm².

  Note: if you want to start a differentiation assay, the resulting density of the D3 cells should not be lower than 1x10 ^{6} cells/ml at this step.

- Disperse the monolayer by gentle trituration. It is important to obtain a single cell suspension for exact counting.
- Count a sample of the cell suspension obtained using a haemocytometer or cell counter.

Subculture

After determination of cell number, the culture can be either subcultured or used in an assay. ES cells and Balb/c 3T3 cells are routinely passaged at a cell density of approximately 5 x 10 ^{4} cells/ml (»1 x 10 ^{4} cells/cm ^{2}) every 2 to 3 days. For ES cells 25 cm ^{2} t-flasks or dishes (60 mm Ø) are used, for 3T3 cells 75 cm ^{2} t-flasks or dishes (100 mm Ø).

Freezing

Stocks of ES cells and Balb/c 3T3 cells can be stored in sterile, freezing tubes in liquid nitrogen. DMSO is used as a cryoprotective agent.

- Centrifuge trypsinised cells at 200 xg for 10 minutes.
- Discard the supernatant and resuspend the cells in freezing medium (see section 1.4 **"Preparations"**

) at a concentration of 1 to 5 x 10^6 cells/ml and fill 1 ml cell suspension per freezing tube.

- Freeze cells at a freezing rate of 1°C / min until -70 to -80°C are reached. This may be achieved using different techniques.
- Place the frozen tubes into liquid nitrogen for storage.

Thawing

- Thaw cells by putting ampoules into a water bath at 37°C. Leave for as brief a time as possible.
- Resuspend the cells in routine culture medium and centrifuge to remove DMSO. Decant supernatant and resuspend the cell pellet in routine culture medium.

  Attachment of ES cells to the plate after thawing is highly improved by using gelatine treated petri dishes. For routine culture (after the first passage) non gelatine treated dishes are used.

- Incubate at 37°C in a humidified 5 % CO_2 atmosphere.
- Passage two to three times before using the cells in a cytotoxicity test.
- Cells should be maintained no longer than 25 passages after thawing (which corresponds to 2 months with 3 passages per week).

ANNEX E: HISTORICAL DATA OBTAINED WITH THE EST

The following 16 chemicals tested at ZEBET/BgVV served as a data base for developing the prediction model. All chemicals were classified correctly (Spielmann *et al.* , 1995). The table gives summarised information, which may help to establish the assay in other laboratories.

Test chemical	**CAS No**	**Sigma** Order No	**MTT** mean IC50 (µg/ml) 3T3-cells	ES-cells	**Differentiation** Mean ID50 (µg/ml) ES-cells
Group 1: non teratogen					
Ascorbic acid	134-03-2	A7631	25.5	138	408
Isoniazid	54-85-3	I3377	375	750	360
Penicillin G	69-57-8	PEN-NA	1586	2950	3450
Saccharin	82385-42-0	S1002	3000	3498	2000
Group 2: weak / moderate teratogen					
Aspirin	50-78-2	A5376	230	220	248
Caffein	58-08-2	C0750	155	165	185
Dexamethasone	50-02-2	D1756	26	23	18.3
Diphenhydramine	147-24-0	D3630	30	29.5	6.7
Diphenylhydantoin	630-93-3	D4505	35	27.3	20
Indomethacin	53-86-1	I7378	27	29	66
Methotrexate	59-05-2	A6770	0.015	0.074	0.020
Group 3: strong teratogen					
Busulphan	55-98-1	B2635	4.8	2.1	4.6
Cytosine arabinoside	69-74-9	C6645	0.033	0.024	0.029
5-Fluorouracil	51-21-8	F6627	0.17	0.103	0.0289
Hydroxyurea	127-07-1	H8627	7.2	2.0	1.7
Retinoic acid	302-79-4	R2625	1.0	0.005	0.000105

ANNEX F: EXCEL SPREADSHEET FOR DATA RECORDING:

ANNEX G: DECIMAL GEOMETRIC CONCENTRATION SERIES

In general **dose-response relationships are non linear** , but can be linearised to some extend by logarithmic transformation of the x-axis. Usually this has to be done when IC50 values are calculated either by regression analysis or by graphical estimation.

If dose series (in cell culture: **concentration** series) are done with arithmetic steps, transformation of the x-axis will result in an unequal distribution of measuring points. Therefore, a geometric concentration series (= constant dilution factor) is recommended. The most simple geometric series are dual geometric ones, like e.g. factor 2. These series have the disadvantage of permanently changing chains within the series (2, 4, 8, 16, 32, 64, 128,...).

The **decimal geometric series** , first described by **Hackenberg & Bartling (1959)** for the use in toxicological and pharmacological studies has the advantage, that independent experiments with wide and with narrow dose factors can be easily compared, and, furthermore can be calculated together:

10						**31.6**						**100**
10				**21.5**				**46.6**				**100**
10		**14.7**		**21.5**		**31.6**		**46.6**		**68.1**		**100**
10	**12.1**	**14.7**	**17.8**	**21.5**	**26.1**	**31.6**	**38.2**	**46.6**	**56.1**	**68.1**	**82.2**	**100**

The dose factor of **3.16** (= $\sqrt[2]{10}$) divides a decade into 2 equal chains, the dose factor of **2.15** (= $\sqrt[3]{10}$) divides a decade into 3 equal chains, the dose factor of **1.47** (= $\sqrt[6]{10}$) divides a decade into 6 equal chains, and the dose factor of **1.21** (= $\sqrt[12]{10}$) divides the decade into 12 equal chains.

Therefore, for reasons of an easier biometrical evaluation of the data it is recommended to use decimal geometric concentration series rather than dual geometric series.

The production of decimal geometric concentration series is very easy, e.g. factor 1.47: dilute 1 volume of highest dose by adding 0.47 volumes of diluent. Then dilute 1 volume of this solution with 0.47 volumes of diluent...(and so on).

Due to the limited number of concentrations it may be useful to have concentration series which incorporate larger dilution steps at the ends of the scale (e.g. 3.16 or 2.15) and narrower steps near the expected IC50 (e.g. 1.47 or 1.21).

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ANNEX H: PRETESTING OF SOLUBILITY OF TEST CHEMICALS

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