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ANNEX II: In vitro models - detailed description of methods and generated data WITHIN CASE STUDY ON THE USE OF INTEGRATED APPROACHES TO TESTING AND ASSESSMENT FORREAD-ACROSS BASED FILLING OF DEVELOPMENTAL TOXICITY DATA GAP FOR METHYL HEXANOIC ACID

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Annex II. In vitro models - detailed description of methods and generated data

1. ZET-1: Method description ZET test

Additional details ZET test

Every substance has been tested three times. For that, a stock solution in 100% DMSO was prepared of every compound. The test was initiated as soon as possible after egg fertilisation and terminated after 120 hours of exposure. The embryos should be immersed in the test solutions before cleavage of the blastodisc commences, at latest at the 16 cell-stage. To start the exposure with minimum delay, at least twice the number of eggs needed per treatment group are selected from the entire batch and transferred into the respective concentrations and controls (e.g., in 100 ml crystallisation dishes; eggs should be fully covered) not later than 90 minutes post fertilisation.

Viable eggs (assessed by means of stereomicroscopy [preferably ≥ 30-fold magnification], fertilised eggs undergoing cleavage and showing no obvious irregularities during cleavage [e.g., asymmetry, vesicle formation] or injuries of the chorion) are then selected and transferred into the pre-exposed 24-well plates along with 2 ml of the corresponding concentration of contaminant/control medium. Test solutions and artificial water have been replaced at 0, 24, 48, 72 and 96 h.

Before the replacement of the test solutions, the embryos were checked at 24, 48, 72, 96 and 120 hpf (hours post-fertilisation), and all effects were documented. According to OECD TG 236 (OECD, 2013), four morphological core endpoints have been identified as surrogates of death (acute lethality): (1) coagulation of the embryo, (2) non-detachment of the tail, (3) non-formation of somites, and (4) lack of a heart-beat. These are identified by direct observation in the microscope.

With respect to teratogenicity and developmental toxicity testing, any additional morphological observation may be added as additional endpoint; a non-exhaustive list would cover, e.g., scoliosis/lordosis, eye deformation, loss of pigmentation, various types of edemata and general skeletal deformations (Braunbeck *et al.*, 2005; Hollert *et al.*, 2003; Nagel, 2002).

OECD GUIDELINES FOR THE TESTING OF CHEMI CALS - Fish Embryo Acute Toxicity (FET) Test

INTRODUCTION

1. This Test Guideline (TG) 236 describes a Fish Embryo Acute Toxicity (FET) test with the zebrafish (*Danio rerio*). This test is designed to determine acute toxicity of chemicals on embryonic stages of fish. The FET-test is based on studies and validation activities performed on zebrafish (1)(2)(3)(4)(5)(6)(7)(8)(9)(10)(11)(12)(13)(14). The FET-test has been successfully applied to a wide range of substances exhibiting diverse modes of action, solubilities, volatilities, and hydrophobicities (reviewed in 15 and 16).

2. Definitions used in this Test Guideline are given in Annex 1.

PRINCIPLE OF THE TEST

3. Newly fertilised zebrafish eggs are exposed to the test chemical for a period of 96 hrs. Every 24 hrs, up to four apical observations are recorded as indicators of lethality (6): (i) coagulation of fertilised eggs, (ii) lack of somite formation, (iii) lack of detachment of the tail-bud from the yolk sac, and (iv) lack of heartbeat. At the end of the exposure period, acute toxicity is determined based on a positive outcome in any of the four apical observations recorded, and the LC50 is calculated.

INITIAL CONSIDERATIONS

4. Useful information about substance-specific properties include the structural formula, molecular weight, purity, stability in water and light, pK_a and K_{ow}, water solubility and vapour pressure as well as results of a test for ready biodegradability (OECD TG 301 (17) or TG 310 (18)). Solubility and vapour pressure can be used to calculate Henry's law constant, which will indicate whether losses due to evaporation of the test chemical may occur. A reliable analytical method for the quantification of the substance in the test solutions with known and reported accuracy and limit of detection should be available.

5. If the Test Guideline is used for the testing of a mixture, its composition should, as far as possible, be characterised, e.g., by the chemical identity of its constituents, their quantitative occurrence and their substance-specific properties (see paragraph 4). Before use of the Test Guideline for regulatory testing of a mixture, it should be considered whether it will provide acceptable results for the intended regulatory purpose.

6. Concerning substances that may be activated via metabolism, there is evidence that zebrafish embryos do have biotransformation capacities (19) (20) (21) (22). However, the metabolic capacity of embryonic fish is not always similar to that of juvenile or adult fish. For instance, the protoxicant allyl alcohol (9) has been missed in the FET. Therefore, if there are any indications that metabolites or other transformation products of relevance may be more toxic than the parent compound, it is also recommended to perform the test with these metabolites / transformation products and to also use these results when concluding on the toxicity of the test chemical, or alternatively perform another test which takes metabolism into further account.

7. For substances with a molecular weight $\geq$ 3kDa, a very bulky molecular structure, and substances causing delayed hatch which might preclude or reduce the post-hatch exposure, embryos are not expected to be sensitive because of limited bioavailability of the substance, and other toxicity tests might be more appropriate.

VALIDITY OF THE TEST

8. For the test results to be valid, the following criteria apply:

a) The overall fertilisation rate of all eggs collected should be ≥ 70% in the batch tested.

b) The water temperature should be maintained at 26 ± 1 °C in test chambers at any time during the test.

c) Overall survival of embryos in the negative (dilution-water) control, and, where relevant, in the solvent control should be ≥ 90% until the end of the 96 hrs exposure.

d) Exposure to the positive control (*e.g.*, 4.0 mg/L 3,4-dichloroaniline for zebrafish) should result in a minimum mortality of 30% at the end of the 96 hrs exposure.

e) Hatching rate in the negative control (and solvent control if appropriate) should be ≥ 80% at the end of 96 hrs exposure.

f) At the end of the 96 hrs exposure, the dissolved oxygen concentration in the negative control and highest test concentration should be ≥80% of saturation.

DESCRIPTION OF THE METHOD

9. An overview of recommended maintenance and test conditions is available in Annex 2.

Apparatus

10. The following equipment is needed:

a) Fish tanks made of chemically inert material (*e.g.*, glass) and of a suitable capacity in relation to the recommended loading (see "Maintenance of brood fish", paragraph 14);

b) Inverted microscope and/or binocular with a capacity of at least 80-fold magnification. If the room used for recording observations cannot be adjusted to 26 ± 1 °C, a temperature-controlled cross movement stage or other methods to maintain temperature are necessary;

c) Test chambers; *e.g.*, standard 24-well plates with a depth of approx. 20 mm. (see "Test chambers", paragraph 11);

d) *e.g.*, self-adhesive foil to cover the 24-well plates;

e) Incubator or air-conditioned room with controlled temperature, allowing to maintain 26 ±1 °C in wells (or test chambers);

f) pH-meter;

g) Oxygen meter;

h) Equipment for determination of hardness of water and conductivity;

i) Spawn trap: instrument trays of glass, stainless steel or other inert materials; wire mesh (grid size 2 ± 0.5 mm) of stainless steel or other inert material to protect the eggs once laid; spawning substrate (*e.g.*, plant imitates of inert material) (OECD 229, Annex 4a (23));

j) Pipettes with widened openings to collect eggs;

k) Glass vessels to prepare different test concentrations and dilution water (beakers, graduated flasks, graduated cylinders and graduated pipettes) or to collect zebrafish eggs (*e.g.*, beakers, crystallisation dishes);

l) If alternative exposure systems, such as flow-through (24) or passive dosing (25) are used for the conduct of the test, appropriate facilities and equipment are needed.

Test chambers

11. Glass or polystyrene test chambers should be used (*e.g.*, 24-well plates with a 2.5 – 5 ml filling capacity per well). In case adsorption to polystyrene is suspected (*e.g.*, for non-polar, planar compounds with high K_{OW}), inert materials (glass) should be used to reduce losses due to adsorption (26). Test chambers should be randomly positioned in the incubator.

Water and test conditions

12. Dilution of the maintenance water is recommended to achieve hardness levels typical of a wide variety of surface waters. Dilution water should be prepared from reconstituted water (27). The resulting degree of hardness should be equivalent to 100-300 mg/L $CaCO_3$ in order to prevent excessive precipitation of calcium carbonate. Other well-characterised surface or well water may be used. The reconstituted water may be adapted to maintenance water of low hardness by dilution with deionised water up to a ratio of 1:5 to a minimum hardness of 30-35 mg/L $CaCO_3$. The water is aerated to oxygen saturation prior to addition of the test chemical. Temperature should be kept at 26 ± 1 °C, in the wells, throughout the test. The pH should be in a range between pH 6.5 and 8.5, and not vary within this range by more than 1.5 units during the course of the test. If the pH is not expected to remain in this range, then pH adjustment should be done prior to initiating the test. The pH adjustment should be made in such a way that the stock solution concentration is not changed to any significant extent and that no chemical reaction or precipitation of the test chemical is caused. Use of hydrogen chloride (HCl) and sodium hydroxide (NaOH) to correct pH in the solutions containing the test chemical is recommended.

Test solutions

13. Test solutions of the selected concentrations can be prepared, *e.g.*, by dilution of a stock solution. The stock solutions should preferably be prepared by simply mixing or agitating the test chemical in the dilution water by mechanical means (*e.g.*, stirring and / or ultra-sonification). If the test chemical is difficult to dissolve in water, procedures described in the OECD Guidance Document No. 23 for handling difficult substances should be followed (28). The use of solvents should be avoided, but may be required in some cases in order to produce a suitably concentrated stock solution. Where a solvent is used to assist in stock solution preparation, its final concentration should not exceed 100 µl/L and should be the same in all test vessels. When a solvent is used, an additional solvent control is required.

Maintenance of brood fish

14. A breeding stock of unexposed, wild-type zebrafish with well-documented fertilisation rate of eggs is used for egg production. Fish should be free of macroscopically discernible symptoms of infection and disease and should not have undergone any

pharmaceutical (acute or prophylactic) treatment for 2 months before spawning. Breeding fish are maintained in aquaria with a recommended loading capacity of 1 L water per fish and a fixed 12 – 16 hour photoperiod (29)(30)(31)(32)(33). Optimal filtering rates should be adjusted; excess filtering rates causing heavy perturbation of the water should be avoided. For feeding conditions, see Annex 2. Surplus feeding should be avoided, and water quality and cleanness of the aquaria should be monitored regularly and be reset to the initial state, if necessary.

Proficiency Testing

15. As a reference substance, 3,4-dichloroaniline (used in the validation studies (1)(2)), should be tested in a full concentration-response range to check the sensitivity of the fish strain used, preferably twice a year. For any laboratory initially establishing this assay, the reference chemical should be used. A laboratory can use this chemical to demonstrate their technical competence in performing the assay prior to submitting data for regulatory purposes.

Egg production

16. Zebrafish eggs may be produced via spawning groups (in individual spawning tanks) or via mass spawning (in the maintenance tanks). In the case of spawning groups, males and females (*e.g.*, at a ratio of 2:1) in a breeding group are placed in spawning tanks a few hours before the onset of darkness on the day prior to the test. Since spawning groups of zebrafish may occasionally fail to spawn, the parallel use of at least three spawning tanks is recommended. To avoid genetic bias, eggs are collected from a minimum of three breeding groups, mixed and randomly selected.

17. For the collection of eggs, spawn traps are placed into the spawning tanks or maintenance tanks before the onset of darkness on the day prior to the test or before the onset of light on the day of the test. To prevent predation of eggs by adult zebrafish, the spawn traps are covered with inert wire mesh of appropriate mesh size (approx. 2±0.5 mm). If considered necessary, artificial plants made of inert material (*e.g.*, plastic or glass) can be fixed to the mesh as spawning stimulus (3)(4)(5)(23)(35). Weathered plastic materials which do not leach (*e.g.*, phthalates) should be used. Mating, spawning and fertilisation take place within 30 min after the onset of light and the spawn traps with the collected eggs can be carefully removed. Rinsing eggs with reconstituted water after collection from spawning traps is recommended.

Egg differentiation

18. At 26 °C, fertilised eggs undergo the first cleavage after about 15 min and the consecutive synchronous cleavages form 4, 8, 16 and 32 cell blastomers (see Annex 3)(35). At these stages, fertilised eggs can be clearly identified by the development of a blastula.

PROCEDURE

Conditions of exposure

19. Twenty embryos per concentration (one embryo per well) are exposed to the test chemical. Exposure should be such that ± 20% of the nominal chemical concentration are maintained throughout the test. If this is not possible in a static system, a manageable semi-static renewal interval should be applied (*e.g.*, renewal every 24 hrs). In these cases exposure concentrations need to be verified as a minimum in the highest and lowest test

concentrations at the beginning and the end of each exposure interval (see paragraph 36). If an exposure concentration of ± 20% of the nominal concentrations cannot be maintained, all concentrations need to be measured at the beginning and the end of each exposure interval (see paragraph 36). Upon renewal, care should be taken that embryos remain covered by a small amount of old test solutions to avoid drying. The test design can be adapted to meet the testing requirements of specific substances (*e.g.,* flow-through (24) or passive dosing systems (25) for easily degradable or highly adsorptive substances (29), or others for volatile substances (36)(37)). In any case, care should be taken to minimise any stress to the embryos. Test chambers should be conditioned at least for 24 hrs with the test solutions prior to test initiation. Test conditions are summarised in Annex 2.

Test concentrations

20. Normally, five concentrations of the test chemical spaced by a constant factor not exceeding 2.2 are required to meet statistical requirements. Justification should be provided, if fewer than five concentrations are used. The highest concentration tested should preferably result in 100% lethality, and the lowest concentration tested should preferably give no observable effect, as defined in paragraph 28. A range-finding test before the definitive test allows selection of the appropriate concentration range. The range-finding is typically performed using ten embryos per concentration. The following instructions refer to performing the test in 24-well plates. If different test chambers (*e.g.*, small Petri dishes) are used or more concentrations are tested, instructions have to be adjusted accordingly.

21. Details and visual instructions for allocation of concentrations across 24-well plates are available in paragraph 27 and Annex 4, Figure 1.

Controls

22. Dilution water controls are required both as negative control and as internal plate controls. If more than 1 dead embryo is observed in the internal plate control, the plate is rejected, thus reducing the number of concentrations used to derive the LC_{50}. If an entire plate is rejected the ability to evaluate and discern observed effects may become more difficult, especially if the rejected plate is the solvent control plate or a plate in which treated embryos are also affected. In the first case the test must be repeated. In the second one the loss of an entire treatment group(s) due to internal control mortality may limit the ability to evaluate effects and determine LC_{50} values.

23. A positive control at a fixed concentration of 4 mg/L 3,4-dichloroaniline is performed with each egg batch used for testing.

24. In case a solvent is used, an additional group of 20 embryos is exposed to the solvent on a separate 24-well plate, thus serving as a solvent control. To consider the test acceptable, the solvent should be demonstrated to have no significant effects on time to hatch, survival, nor produce any other adverse effects on the embryos (cf. paragraph 8c).

Start of exposure and duration of test

25. The test is initiated as soon as possible after fertilisation of the eggs and terminated after 96 hrs of exposure. The embryos should be immersed in the test solutions before cleavage of the blastodisc commences, or, at latest, by the 16 cell-stage. To start exposure with minimum delay, at least twice the number of eggs needed per treatment group are randomly selected and transferred into the respective concentrations and controls (*e.g.*, in

100 ml crystallisation dishes; eggs should be fully covered) not later than 90 minutes post fertilisation.

26. Viable fertilised eggs should be separated from unfertilised eggs and be transferred to 24-well plates pre-conditioned for 24 hrs and refilled with 2 ml/well freshly prepared test solutions within 180 minutes post fertilisation. By means of stereomicroscopy (preferably ≥30-fold magnification), fertilised eggs undergoing cleavage and showing no obvious irregularities during cleavage (*e.g.*, asymmetry, vesicle formation) or injuries of the chorion are selected. For egg collection and separation, see Annex 3, Fig. 1 and 3 and Annex 4, Fig. 2.

Distribution of eggs over the 24-well plates

27. Eggs are distributed to well plates in the following numbers (see also Annex 4, Fig. 1)

- 20 eggs on one plate for each test concentration;
- 20 eggs as solvent control on one plate (if necessary);
- 20 eggs as positive control on one plate;
- 4 eggs in dilution water as internal plate control on each of the above plates;
- 24 eggs in dilution water as negative control on one plate.

Observations

28. Apical observations performed on each tested embryo include: coagulation of embryos, lack of somite formation, non-detachment of the tail, and lack of heartbeat (Table 1). These observations are used for the determination of lethality: Any positive outcome in one of these observations means that the zebrafish embryo is dead. Additionally, hatching is recorded in treatment and control groups on a daily basis starting from 48 hrs. Observations are recorded every 24 hrs, until the end of the test.

Table 1. Apical observations of acute toxicity in zebrafish embryos 24 - 96 hrs post fertilisation.

	Exposure times			
	24 hrs	48 hrs	72 hrs	96 hrs
Coagulated embryos	+	+	+	+
Lack of somite formation	+	+	+	+
Non-detachment of the tail	+	+	+	+
Lack of heartbeat		+	+	+

29. *Coagulation of the embryo*: Coagulated embryos are milky white and appear dark under the microscope (see Annex 5, Fig. 1). The number of coagulated embryos is determined after 24, 48, 72 and 96 hrs.

30. *Lack of somite formation*: At 26±1°C, about 20 somites have formed after 24 hrs (see Annex 5, Figure 2) in a normally developing zebrafish embryo. A normally developed embryo shows spontaneous movements (side-to-side contractions). Spontaneous movements indicate the formation of somites. The absence of somites is recorded after 24, 48, 72 and 96 hrs. Non-formation of somites after 24 hrs might be due to a general retardation of development. At latest after 48 hrs, the formation of somites should be developed. If not, the embryos are considered dead.

31. *Non-detachment of the tail*: In a normally developing zebrafish embryo, detachment of the tail (see Annex 5, Figure 3) from the yolk is observed following posterior elongation of the embryonic body. Absence of tail detachment is recorded after 24, 48, 72 and 96 hrs.

32. *Lack of heartbeat*: In a normally developing zebrafish embryo at 26±1 °C, the heartbeat is visible after 48 hrs (see Annex 5, Figure 4). Particular care should be taken when recording this endpoint, since irregular (erratic) heartbeat should not be recorded as lethal. Moreover, visible heartbeat without circulation in aorta abdominalis is considered non-lethal. To record this endpoint, embryos showing no heartbeat should be observed under a minimum magnification of 80x for at least one minute. Absence of heartbeat is recorded after 48, 72 and 96 hrs.

33. Hatching rates of all treatment and control groups should be recorded from 48 hrs onwards and reported. Although hatching is not an endpoint used for the calculation of the LC_{50}, hatching ensures exposure of the embryo without a potential barrier function of the chorion, and as such may help data interpretation.

34. Detailed descriptions of the normal (35) and examples of abnormal development of zebrafish embryos are illustrated in Annexes 3 and 5.

Analytical measurements

35. At the beginning and at the end of the test, pH, total hardness and conductivity in the control(s) and in the highest test chemical concentration are measured. In semi-static renewal systems the pH should be measured prior to and after water renewal. The dissolved oxygen concentration is measured at the end of the test in the negative controls and highest test concentration with viable embryos, where it should be in compliance with the test validity criteria (see paragraph 7f). If there is concern that the temperature varies across the 24-well plates, temperature is measured in three randomly selected vessels. Temperature should be recorded preferably continuously during the test or, as a minimum, daily.

36. In a static system, the concentration of the test chemical should be measured, as a minimum, in the highest and lowest test concentrations, but preferably in all treatments, at the beginning and end of the test. In semi-static (renewal) tests where the concentration of the test chemical is expected to remain within ± 20% of the nominal values, it is recommended that, as a minimum, the highest and lowest test concentrations be analysed when freshly prepared and immediately prior to renewal. For tests where the concentration of the test chemical is not expected to remain within ± 20% of nominal, all test concentrations must be analysed when freshly prepared and immediately prior to renewal. In case of insufficient volume for analysis, merging of test solutions, or use of surrogate chambers being of the same material and having the same volume to surface area ratios as 24-well plates, may be useful. It is strongly recommended that results be based on measured concentrations. When the concentrations do not remain within 80-120% of the nominal concentration, the effect concentrations should be expressed relative to the geometric mean of the measured concentrations; see Chapter 5 in the OECD Guidance Document No. 23 for more details (28).

LIMIT TEST

37. Using the procedures described in this guideline, a limit test may be performed at 100 mg/L of test chemical or at its limit of solubility in the test medium (whichever is the lower) in order to demonstrate that the LC_{50} is greater than this concentration. The limit

test should be performed using 20 embryos in the treatment, the positive control and – if necessary - in the solvent control and 24 embryos in the negative control. If the percentage of lethality at the concentration tested exceeds the mortality in the negative control (or solvent control) by 10%, a full study should be conducted. Any observed effects should be recorded. If mortality exceeds 10% in the negative control (or solvent control), the test becomes invalid and should be repeated.

DATA AND REPORTING

Treatment of results

38. In this test, the individual wells are considered independent replicates for statistical analysis. The percentages of embryos for which at least one of the apical observations is positive at 48 and/or 96 hrs are plotted against test concentrations. For calculation of the slopes of the curve, LC_{50} values and the confidence limits (95%), appropriate statistical methods should be applied (38) and the OECD Guidance Document No. 54 should be consulted (39).

Test report

39. The test report should include the following information:

Test chemical:

Mono-constituent substance

- physical appearance, water solubility, and additional relevant physicochemical properties;
- chemical identification, such as IUPAC or CAS name, CAS number, SMILES or InChI code, structural formula, purity, chemical identity of impurities as appropriate and practically feasible, etc. (including the organic carbon content, if appropriate).

Multi-constituent substance, UVBCs and mixtures:

- characterised as far as possible by chemical identity (see above), quantitative occurrence and relevant physicochemical properties of the constituents.

Test organisms:

- scientific name, strain, source and method of collection of the fertilised eggs and subsequent handling.

Test conditions:

- test procedure used (*e.g.*, semi-static renewal);
- photoperiod;
- test design (*e.g.*, number of test chambers, types of controls);
- water quality characteristics in fish maintenance (*e.g.*, pH, hardness, temperature, conductivity, dissolved oxygen);
- dissolved oxygen concentration, pH, total hardness, temperature and conductivity of the test solutions at the start and after 96 hrs;

- method of preparation of stock solutions and test solutions as well as frequency of renewal;
- justification for use of solvent and justification for choice of solvent, if other than water;
- the nominal test concentrations and the result of all analyses to determine the concentration of the test chemical in the test vessels; the recovery efficiency of the method and the limit of quantification (LoQ) should also be reported;
- evidence that controls met the overall survival validity criteria;
- fertilisation rate of the eggs;
- hatching rate in treatment and control groups.

Results:

- maximum concentration causing no mortality within the duration of the test;
- minimum concentration causing 100 % mortality within the duration of the test;
- cumulative mortality for each concentration at the recommended observation times;
- the LC_{50} values at 96 hrs (and optionally at 48 hrs) for mortality with 95% confidence limits, if possible;
- graph of the concentration-mortality curve at the end of the test;
- mortality in the controls (negative controls, internal plate controls, as well as positive control and any solvent control used);
- data on the outcome of each of the four apical observations;
- incidence and description of morphological and physiological abnormalities, if any (see examples provided in Annex 5, Figure 2);
- incidents in the course of the test which might have influenced the results;
- statistical analysis and treatment of data (probit analysis, logistic regression model and geometric mean for LC_{50});
- slope and confidence limits of the regression of the (transformed) concentration-response curve.

Any deviation from the Guideline and relevant explanations.

Discussion and interpretation of results.

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ANNEX 1

DEFINITIONS

Apical endpoint: Causing effect at population level.

Blastula: The blastula is a cellular formation around the animal pole that covers a certain part of the yolk.

Epiboly: is a massive proliferation of predominantly epidermal cells in the gastrulation phase of the embryo and their movement from the dorsal to the ventral side, by which entodermal cell layers are internalised in an invagination-like process and the yolk is incorporated into the embryo.

Flow-through test is a test with continued flow of test solutions through the test system during the duration of exposure.

Internal Plate Control: Internal control consisting of 4 wells filled with dilution water per 24-well plate to identify potential contamination of the plates by the manufacturer or by the researcher during the procedure, and any plate effect possibly influencing the outcome of the test (*e.g.* temperature gradient).

IUPAC: International Union of Pure and Applied Chemistry

Maintenance water: Water in which the husbandry of the adult fish is performed.

Median Lethal Concentration (LC_{50}) is the concentration of a test substance that is estimated to be lethal to 50% of the test organisms within the test duration.

Semi-static renewal test is a test with regular renewal of the test solutions after defined periods (*e.g.*, every 24 hrs).

SMILES: Simplified Molecular Input Line Entry Specification

Somite: In the developing vertebrate embryo, somites are masses of mesoderm distributed laterally to the neural tube, which will eventually develop dermis (dermatome), skeletal muscle (myotome), and vertebrae (sclerotome).

Static test is a test in which test solutions remain unchanged throughout the duration of the test.

UVCB: substances of unknown or variable composition, complex reaction products or biological materials

ANNEX 2

MAINTENANCE, BREEDING AND TYPICAL CONDITIONS FOR ZEBRAFISH EMBRYO ACUTE TOXICITY TESTS

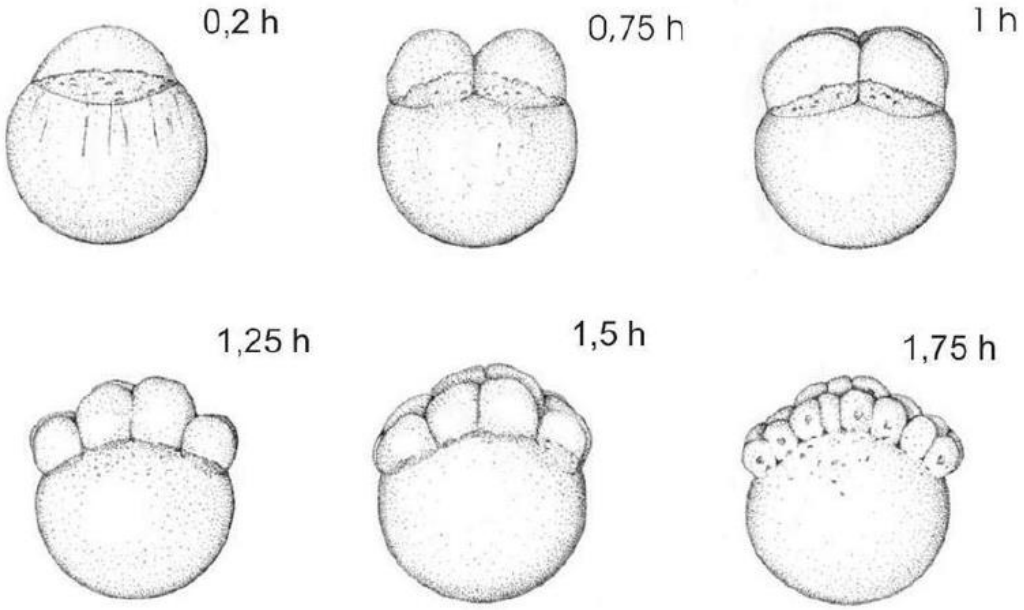
		Zebrafish (*Danio rerio*)
Origin of species		India, Burma, Malakka, Sumatra
	Sexual dimorphism	Females: protruding belly, when carrying eggs Males: more slender, orange tint between blue longitudinal stripes (particularly evident at the anal fin)
Feeding regime		Dry flake food (max. 3% fish weight per day) 3 - 5 times daily; additionally brine shrimp (*Artemia spec.*) *nauplii* and / or small daphnids of appropriate size obtained from an uncontaminated source. Feeding live food provides a source of environmental enrichment and therefore live food should be given wherever possible. To guarantee for optimal water quality, excess food and faeces should be removed approx. one hour after feeding.
Approximate weight of adult fish		Females: 0.65±0.13 g Males: 0.5±0.1 g
	Illumination	Fluorescent bulbs (wide spectrum); 10 - 20 µE/m^2/s, 540 - 1080 lux, or 50 - 100 ft-c (ambient laboratory levels); 12 - 16 hrs photoperiod
Maintenance of parental fish	Water temperature	26±1 °C
	Water quality	O_2 ≥80% saturation, hardness: e.g., ~ 30 - 300 mg/L $CaCO_3$, NO_3^-: ≤48mg/L, NH_4^+ and NO_2^-: <0.001 mg/L, residual chlorine <10 µg/L, total organic chlorine <25 ng/L, pH = 6.5 - 8.5
	Further water quality criteria	Particulate matter <20 mg/L, total organic carbon <2 mg/L, total organophosphorus pesticides <50 ng/L, total organochlorine pesticides plus polychlorinated biphenyls <50 ng/L
	Tank size for maintenance	*e.g.*, 180 L , 1 fish/L
	Water purification	Permanent (charcoal filtered); other possibilities include combinations with semi-static renewal maintenance or flow-through system with continuous water renewal
Recommended male to female ratio for breeding		2:1 (or mass spawning)
Spawning tanks		*e.g.*, 4 L tanks equipped with steel grid bottom and plant dummy as spawning stimulant; external heating mats, or mass spawning within the maintenance tanks
Egg structure and appearance		Stable chorion (i.e. highly transparent, non-sticky, diameter ~ 0.8 – 1.5 mm)
Spawning rate		A single mature female spawns at least 50 - 80 eggs per day. Depending on the strain, spawning rates may be considerably higher. The fertilisation rate should be ≥70%. For first time spawning fish, fertilisation rates of the eggs may be lower in the first few spawns.
Test type		Static, semi-static renewal, flow-through, 26±1 °C, 24 hrs conditioned test chambers (*e.g.*, 24-well plates 2.5 - 5 ml per cavity)

ANNEX 3

NORMAL ZEBRAFISH DEVELOPMENT AT 26^{O}C

Figure 1. Selected stages of early zebrafish (*Danio rerio*) development

0.2 – 1.75 hrs post-fertilisation (from Kimmel *et al.*, 1995).



The time sequence of normal development may be taken to diagnose both fertilisation and viability of eggs (see paragraph 26: Selection of fertilised eggs).

Figure 2. Selected stages of late zebrafish (*Danio rerio*) development (de-chorionated embryo to optimise visibility)

22 - 48 hrs after fertilisation (from Kimmel *et al.*, 1995 (35)).

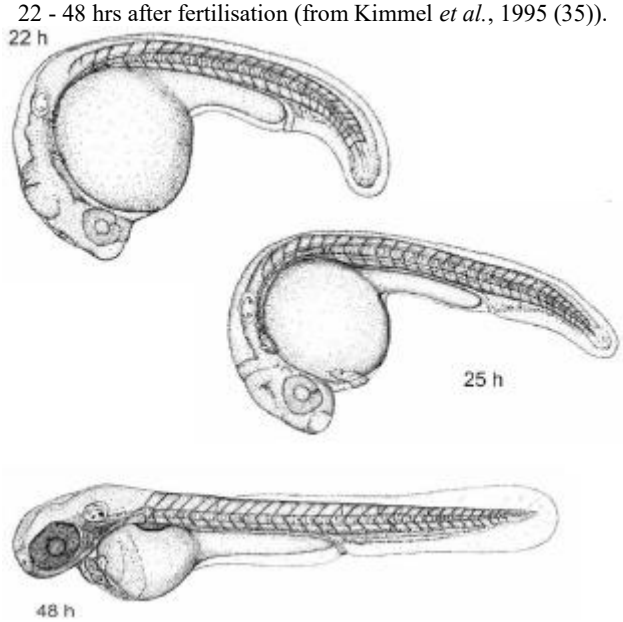
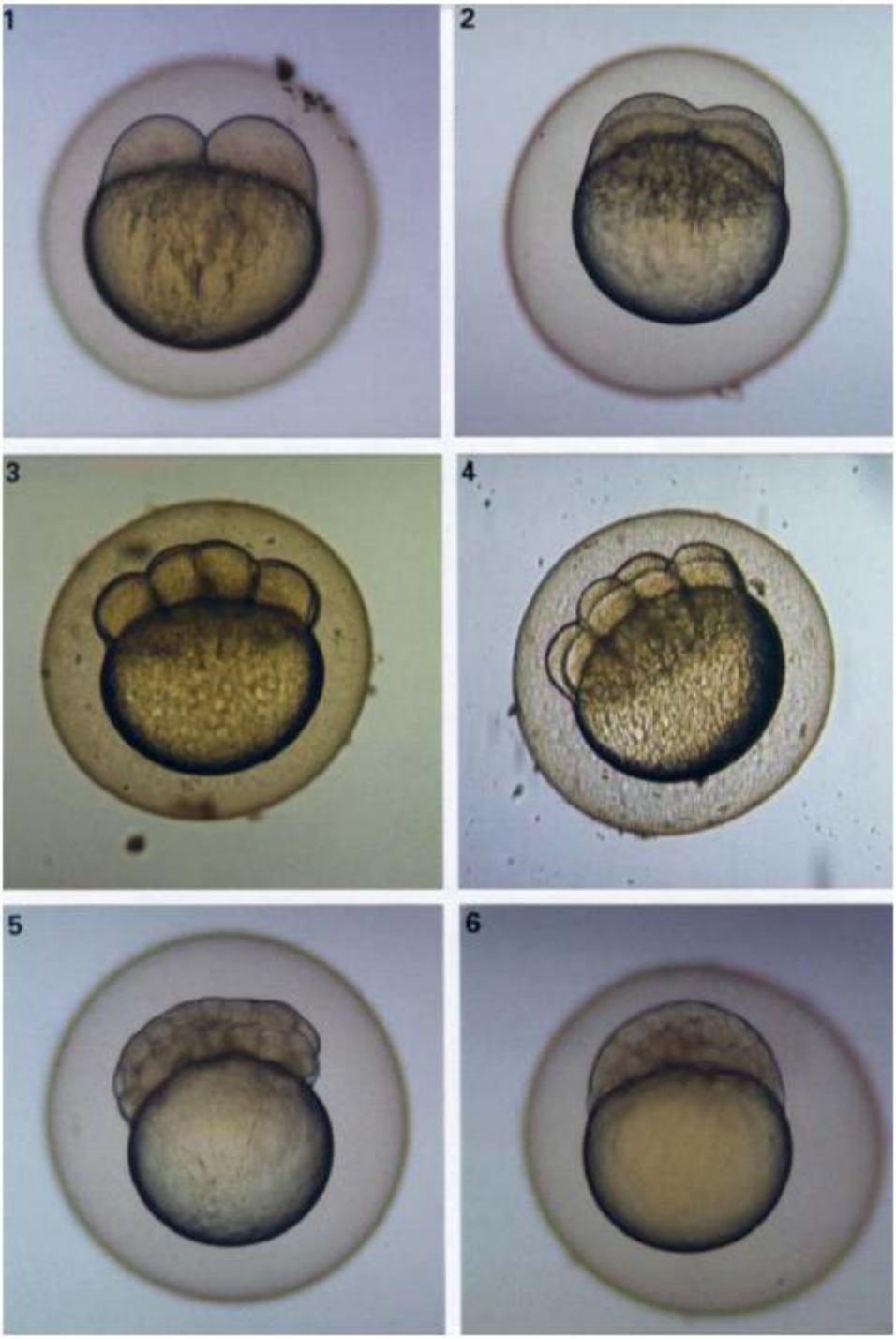


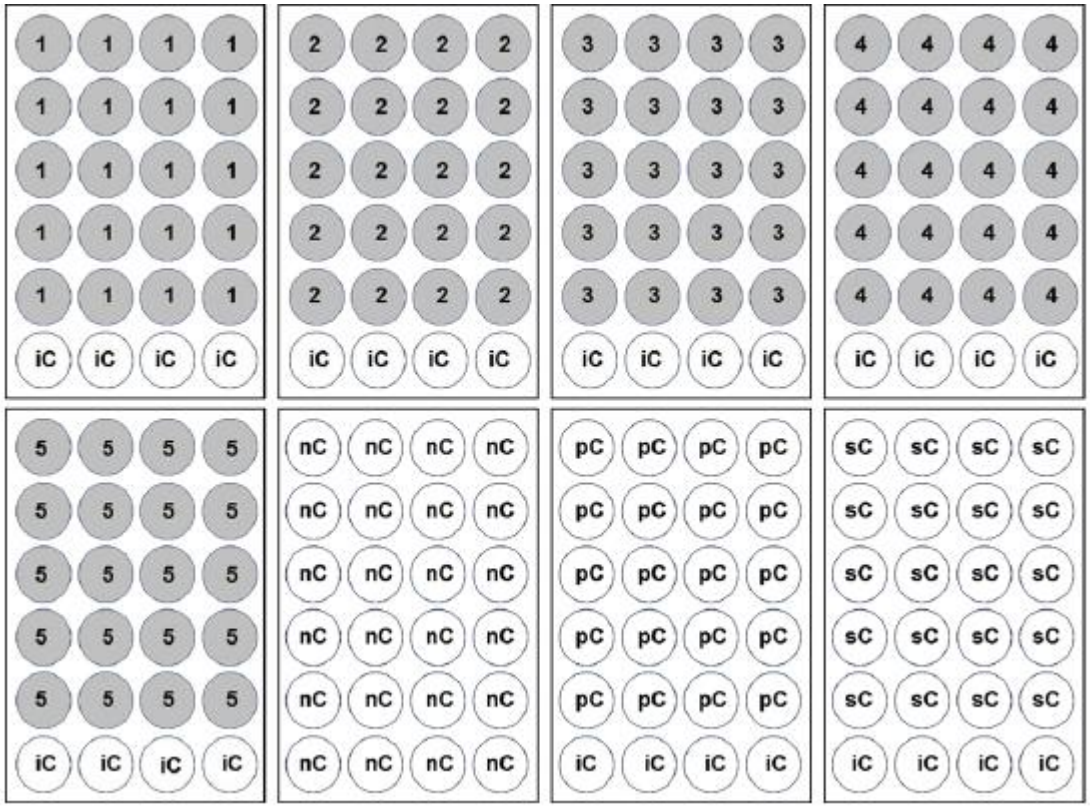
Figure 3. Normal development of zebrafish (*Danio rerio*) embryos

(1) 0.75 hrs, 2-cell stage; (2) 1 hr, 4-cell stage; (3) 1.2 hrs, 8-cell stage; (4) 1.5 hrs, 16-cell stage; (5) 4.7 hrs, beginning epiboly; (6) 5.3 hrs, approx. 50 % epiboly (from Braunbeck & Lammer 2006 (40)).



ANNEX 4

Figure 1. Layout of 24-well plates

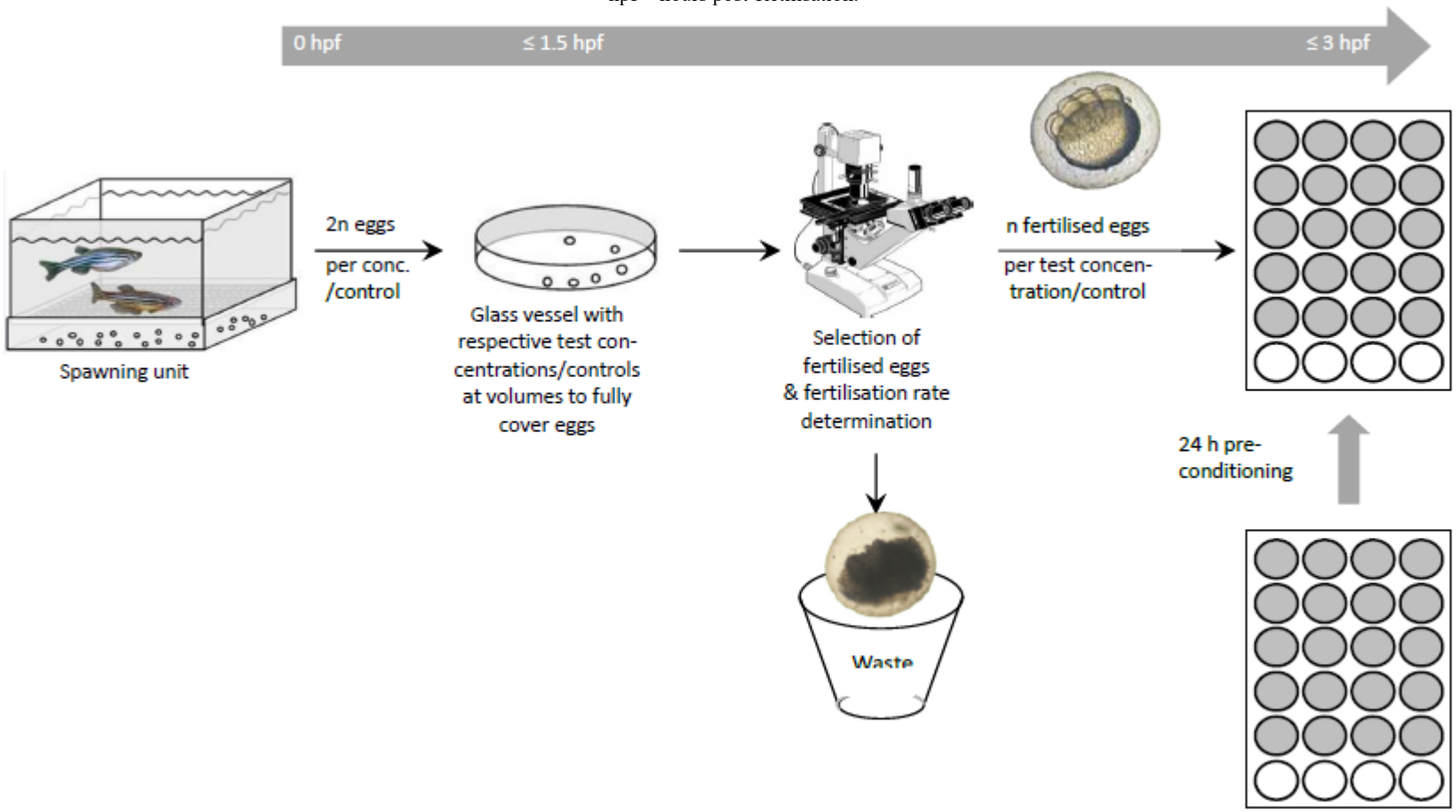


1-5 = five test concentrations / chemical; nC = negative control (dilution water); iC = internal plate control (dilution water); pC = positive control (3,4-DCA 4mg/L); sC = solvent control

Figure 2. Scheme of the zebrafish embryo acute toxicity test procedure (from left to right)

production of eggs, collection of the eggs, pre-exposure immediately after fertilisation in glass vessels, selection of fertilised eggs with an inverted microscope or binocular and distribution of fertilised eggs into 24-well plates prepared with the respective test concentrations/controls, n = number of eggs required per test concentration/control (here 20),

hpf = hours post-fertilisation.



ANNEX 5

ATLAS OF LETHAL ENDPOINTS FOR THE ZEBRAFISH EMBRYO ACUTE TOXICITY TEST

The following apical endpoints indicate acute toxicity and, consequently, death of the embryos: *coagulation of the embryo, non-detachment of the tail, lack of somite formation and lack of heartbeat*. The following micrographs have been selected to illustrate these endpoints.

Figure 1. Coagulation of the embryo

Under bright field illumination, coagulated zebrafish embryos show a variety of intransparent inclusions.

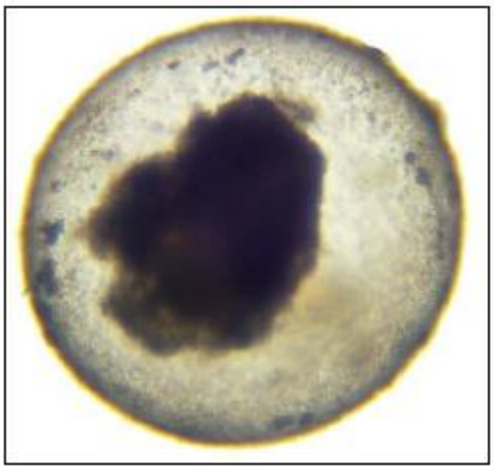


Figure 2. Lack of somite formation

Although retarded in development by approx. 10 hrs, the 24 hrs old zebrafish embryo in (a) shows well-developed somites (→), whereas the embryo in (b) does not show any sign of somite formation (→). Although showing a pronounced yolk sac oedema (*), the 48 hrs old zebrafish embryo in (c) shows distinct formation of somites (→), whereas the 96 hrs old zebrafish embryo depicted in (d) does not show any sign of somite formation (→). Note also the spinal curvature (scoliosis) and the pericardial oedema (*) in the embryo shown in (d).

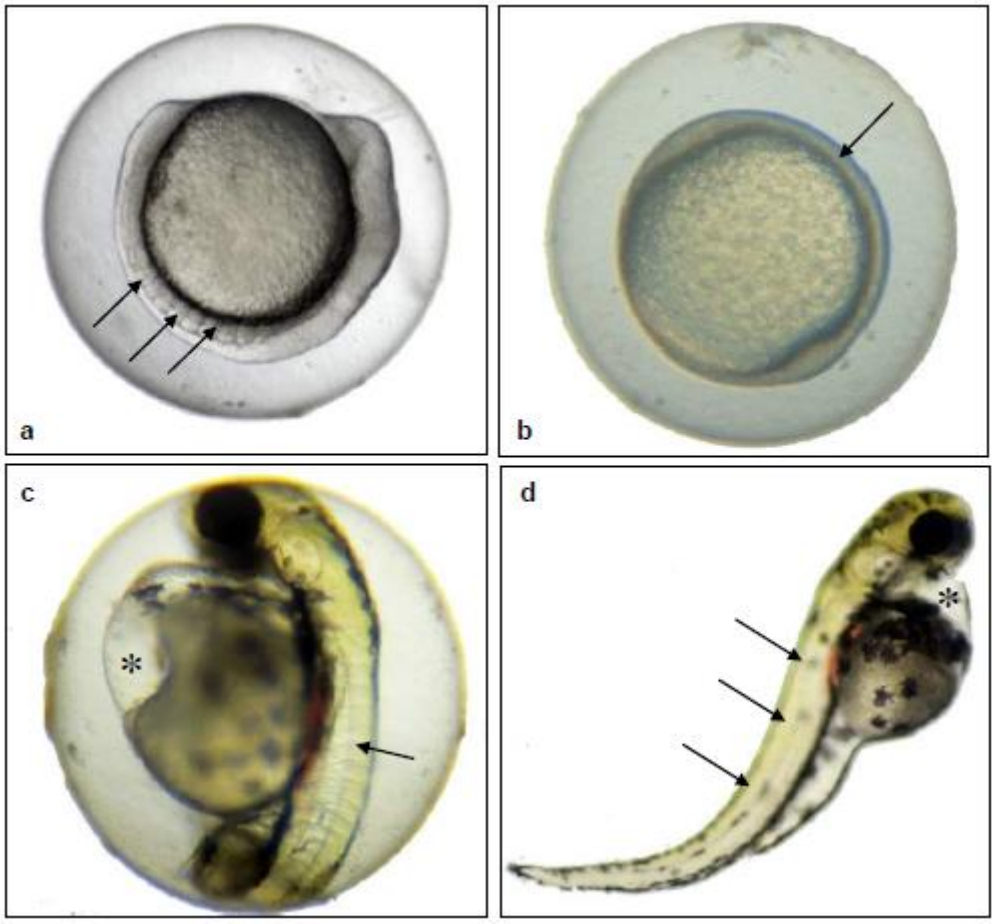


Figure 3. Non-detachment of the tail bud in lateral view (a: →; 96 hrs old zebrafish embryo). Note also the lack of the eye bud (*).

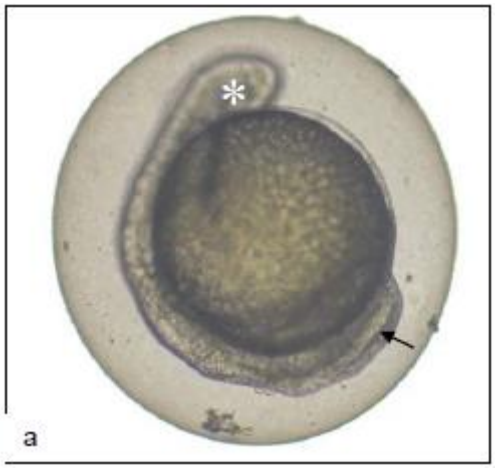
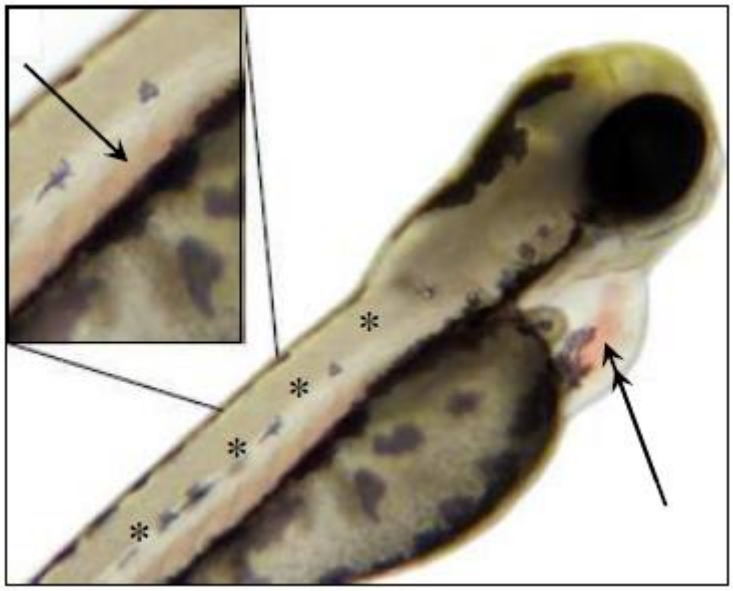


Figure 4. Lack of heartbeat is, by definition, difficult to illustrate in a micrograph.

Lack of heartbeat is indicated by non-convulsion of the heart (double arrow). Immobility of blood cells in, *e.g.*, the aorta abdominalis (→ in insert) is not an indicator for lack of heartbeat. Note also the lack of somite formation in this embryo (*, homogenous rather than segmental appearance of muscular tissues). The observation time to record an absence of heartbeat should be at least of one minute with a minimum magnification of 80×.



Fish Embryo Acute Toxicity Test with Zebrafish (ZFET) DB-ALM Protocol n° 140

Aquatic Short-Term Toxicity

The fish embryo acute toxicity test with zebrafish (ZFET) is designed to determine acute lethal effects of chemicals (including industrial chemicals, pharmaceuticals, pesticides, biocides, inorganic compounds, etc.) on embryonic stages of zebrafish (*Danio rerio*). Newly fertilised zebrafish eggs are exposed to test chemicals for 96h, lethal effects are recorded and used for calculating the LC_{50} value. Depending on the regulatory framework, this test method may be used to determine acute fish toxicity.

Objective & Application

TYPE OF TESTING	: Refinement/Replacement, Reduction, Part of an integrated testing strategy, Screening, Range-finding for higher tier testing
LEVEL OF ASSESSMENT	: Qualitative and quantitative toxic effects
PURPOSE OF TESTING	: Hazard identification, Classification and labelling

Context of Use:

The ZFET was adopted by OECD as Test Guideline No. 236: "Fish embryo acute toxicity (FET) test" (OECD, 2013), following its approval at the 25th meeting of the OECD WNT in May 2013. Depending on the regulatory framework, this test method may be used to determine acute fish toxicity as alternative to the acute fish toxicity test (TG 203; OECD, 1992), as part of a testing strategy or weight-of-evidence approach for determining acute fish toxicity. The method (also with 48h exposure) is used for research purposes, for toxicity screening of environmental samples (e.g. sediments), by industry for product development, screening and range-finding testing for higher tier fish testing (as mentioned in OECD TG 210; OECD, 2013 bis). For effluents testing, an ISO guideline is available (Standard ISO 15088:2007(E); ISO, 2007) using fewer embryos and 48h exposure.

Applicability Domain:

The fish embryo acute toxicity test has been successfully applied to a wide range of substances exhibiting diverse modes of action, solubilities, volatilities, and hydrophobicities. During the development of OECD TG 236 and the associated validation study (OECD, 2011; OECD, 2012), 20 chemicals have been tested covering industrial chemicals, pharmaceuticals, plant protection products, inorganic compounds and biocides. Molecular weight of the chemicals ranged from 78.13 to 400,000g/mol and log K_{ow} up to 4.1.

It should be noted that many more chemicals have been tested with the (zebra) fish acute embryo toxicity test in hundreds of studies. Summarised data were published by Lammer *et al.* (2009a), Knöbel *et al.* (2012) and Belanger *et al.* (2013). In these studies, the range of compound molecular weights spans from 49 to 400,000g/mol, log K_{ow} from -4.1 to 7.9, and solubilities from 0.06mg/L to >1g/L.

Substance-specific properties (as given in OECD TG 236)

Substance-specific properties need to be available prior to the test. These include the structural formula, molecular weight, purity, stability in water and light, pK_a and K_{OW},

water solubility and vapour pressure. Special care should be taken when volatile and sorptive compounds are tested. Solubility and vapour pressure can be used to calculate Henry's law constant, which will indicate whether losses due to evaporation of the test chemical may occur.

Limitations (as given in OECD TG 236)

Regarding the testing of chemicals which may be processed via metabolism, there is evidence that zebrafish embryos do have biotransformation capacities (Weigt *et al.*, 2011; Weigt *et al.*, 2012; Incardona *et al.*, 2011, Kubota *et al.*, 2011). However, the metabolic capacity of embryonic fish is not always identical to that of juvenile or adult fish. For instance, up to the date of the publication of the OECD TG236 (July 2013), the protoxicant allyl alcohol has been the only reported substance that was missed in the FET (Knöbel *et al.*, 2012). Therefore, if there are any indications that metabolites or other transformation products of relevance may be more toxic than the parent chemical, it is recommended to perform the test with these metabolites/transformation products and to use these results when concluding on the toxicity for the test chemical, or alternatively perform another test which takes metabolism into further account. For chemicals with a molecular weight >= 3kDa, a very bulky molecular structure and chemicals causing delayed hatch which might preclude or reduce the post-hatch exposure, embryos are not expected to be sensitive because of potentially limited bioavailability of the chemical, and other toxicity tests might be more appropriate. See also Status section for more information on the use of the method in the regulatory context.

Résumé

Fish embryo tests in general allow observing effects of compounds on embryonic stages. Acute toxic effects may result in death of the embryo or retarded development and growth.

The present protocol focuses on the detection of acute lethal effects to zebrafish embryos as an indication of acute fish toxicity of the chemical tested.

Acute fish toxicity testing is a mandatory component in the environmental hazard assessment of industrial chemicals, plant protection products, biocides, veterinary pharmaceuticals, feed stuff etc. The acute fish toxicity test (OECD TG 203 / EU test method C.1; OECD, 1992; EU, 2008) is carried out with juvenile or adult fish. It is a short-term exposure test (96h) and determines the concentration that is lethal to 50% of the fish (LC_{50}). Other relevant endpoints, consistent with the OECD TG 203, can include the LC_0 and LC_{100} (0% mortality and 100% mortality). The ZFET measures LC_{50} values in zebrafish, which can be used as surrogates for fish acute toxicity data in appropriate regulatory context.

Experimental Description

Basic Procedure

Newly fertilised zebrafish eggs are exposed to at least five concentrations of the test compound and incubated for 96h at 26±1°C with an appropriate light cycle (14h light and 10h dark). Dilution water is used as negative control and 3,4-dichloroaniline (4mg/L) as positive control. Lethal effects to the embryo as coagulation, lack of somite formation, non-detachment of the tail, and lack of heartbeat are recorded on a daily basis and used for the calculation of the LC_{50}.

Data Analysis/Prediction Model

The percentage of embryos for which at least one of the lethal observations is positive at 48 and 96h are plotted against tested concentrations. For calculation of the slopes of the curve, LC_{50} values and confidence limits (95%), appropriate statistical methods should be applied. The OECD Guidance Document No. 54 (OECD, 2006) should be consulted with regard to concentration-response modelling (probit analysis, logistic regression model and geometric mean).

LC_{50} values derived with the ZFET can be used as surrogates for fish acute toxicity data. A prediction model is not required.

Test Compounds and Results Summary

The OECD validation study (carried out during the development of the TG 236) aimed to assess the transferability, intra- and interlaboratory reproducibility of the ZFET. Of the 20 chemicals (including industrial chemicals, pharmaceuticals, plant protection products and biocides), each was tested in three independent runs in at least three laboratories. The results show that the method is transferable and reproducible within and between laboratories with CVs < 30% in most laboratories and for most chemicals (OECD, 2011; OECD, 2012). Belanger *et al.* (2013) collected fish embryo acute toxicity data for 229 chemicals covering a wide range of chemical classes and functional use and compared them to acute fish toxicity data. The comparison demonstrates that fish embryo acute toxicity tests (96h exposure) predict acute fish toxicity as juvenile or adult fish would do and confirms the findings of Lammer *et al.* (2009) and Knöbel *et al.* (2012).

Modifications of the Method

The following modifications may be possible and would comply with OECD TG 236:

Limit test

According to OECD TG 236, a limit test may be performed at 100mg/L of test compound or at its limit of solubility in the test medium (whichever is the lower) in order to demonstrate that the LC_{50} is greater than this concentration. The limit test should be performed using 20 embryos in the test solution, the positive control and - if necessary - in the solvent control and 24 embryos in the negative control. If the percentage of lethality at the concentration tested exceeds the mortality in the negative control (or solvent control) by 10%, a full study should be conducted. If mortality exceeds 10% in the negative (or solvent control), the test becomes invalid and should be repeated.

Exposure schemes

Depending on the requirements of the test chemical, other exposure schemes than the semi-static exposure scheme may be more appropriate (*e.g.* flow-through; Lammer *et al*, 2009b) or passive dosing systems (Brown *et al.*, 2001) for easily degradable or highly adsorptive chemicals (Schreiber *et al.*, 2008; OECD, 2000), or others for volatile chemicals (OECD, 2004; Weil *et al.*, 2009).

Other modifications may be used but will not be in compliance with OECD TG 236:

Exposure restricted to 48h

In the context of the OECD validation study of the ZFET (OECD, 2011; OECD, 2012), LC_{50} values derived after 24, 48, 72, 96h were compared. Differences between LC_{50} 48h and LC_{50} 96h were minor for most of the compounds tested; however, toxic effects of the two high molecular weight compounds (cationic polymers) only developed after the embryos hatched, since the compounds did not pass the chorion in a sufficient quantity to cause consistent toxicity.

Use of dechorionated embryos

In specific cases, *e.g.* for chemicals which do not pass the chorion or delay the hatch, dechorionated embryos from 24h post-fertilisation might be used. Staff should be well-trained in the dechorionation technique (Henn & Braunbeck, 2011).

Acceptance Criteria and Proficiency Testing

The following acceptance criteria have to be met:

1. The overall fertilisation rate of all eggs collected should be >= 70% in the batch tested.
2. The water temperature should be maintained at 26±1°C in test chambers at any time during the test.
3. Overall survival of embryos in the negative (dilution water) control and, where relevant, in the solvent control should be >= 90% until the end of the 96h exposure.
4. Exposure to the positive control (*e.g.*, 4.0mg/L 3,4-dichloroaniline for zebrafish) should result in a minimum mortality of 30% at the end of the 96h exposure.
5. The hatching rate in the negative control (and solvent control if appropriate) should be >= 80% at the end of the 96h exposure.
6. At the end of the test, the dissolved oxygen concentration in the negative control and highest test concentration should be >= 80% of saturation.

3,4-dichloroaniline (a positive control in routine testing), should be tested in a full concentration-response range to check the sensitivity of the fish strain used, preferably twice a year. For any laboratory initially establishing this assay, a well characterised reference chemical should be used. A laboratory can use this chemical to demonstrate their technical competence in performing the assay prior to submitting data for regulatory purposes.

Discussion

Ethical issues

The use of this method will reduce the number of juvenile/adult fish used for regulatory toxicity testing and is incompliance with Council Directive 2010/63/EU on protection of animals used for scientific purposes (EU, 2010).

Special equipment needed

For ZFET, an incubator or air-conditioned room maintained at 26±1°C, equipment to ensure a light/dark cycle, and an inverted microscope and/or binocular with at minimum 30-fold magnification (80-fold for the observation of the heartbeat) and equipped with a temperature-compensated cross-movement stage, (if the room cannot be adjusted to 26±1°C) are needed. In addition, the user of the method should have immediate access to zebrafish eggs or have a zebrafish breeding facility.

Amount of training required

Staff should be trained in the observation of the lethal effects to the zebrafish embryos.

Duration of the test

At least 5 days (including pre-saturation of test vessels).

Adaptation to high-throughput testing/automation

High-throughput test format/automation might be feasible; exploration underway at the Karlsruhe Institute of Technology, Germany.

Costs

The costs vary between 500€ (routine test) and 700€ (difficult chemicals and/or adapted/modified tests). These figures do not include the costs for analytical verification of test concentrations.

Advantages and limitations

The method allows determination of toxic effects (acute and sublethal) on embryonic stages of fish. Compared to tests on juvenile and adult fish, only small quantities of test compounds/concentrations are necessary. As for any other method, special care should be taken when volatile and sorptive compounds are tested. It is not fully explored whether fish embryos possess full metabolic capacity and therefore the method might not be applicable to compounds needing metabolic activation before developing their toxicity. Some compounds may cause delayed hatch which will preclude or reduce the post-hatch exposure. In this case, other toxicity tests might be more appropriate.

Status

Known Laboratory Use:

The ZFET is used in many laboratories. The following laboratories participated in the OECD FET validation study:

University of Heidelberg, Heidelberg, Germany (Thomas Braunbeck; lead laboratory)

Procter & Gamble, Cincinnati, OH, USA (Scott Belanger)

IVM, Amsterdam, THE NETHERLANDS (Juliette Legler)

RIVM, Bilthoven, THE NETHERLANDS (Leo van der Ven)

Ipo-Pszczyna, Pszczyna, POLAND (Przemysaw Fochtman)

UBA, Berlin, GERMANY (Carola Kussatz, Christian Polleichtner)

Escuela Nacional de Ciencias Biológicas, IPN, México City, MEXICO (Fernando Martínez-Jerónimo)

BASF, Ludwigshafen, GERMANY (Edward Salinas)

Merck KGaA, Darmstadt, GERMANY (Nicole Hübler)

UFZ, Leipzig, GERMANY (Stefan Scholz)

VITO, Mol, BELGIUM (Hilda Witters)

Participation in Validation Studies:

The transferability, intra- and inter-laboratory reproducibility of the ZFET was assessed within the OECD FET validation study (coordinated by EURL ECVAM). This study was linked to the OECD project 2.7 - "New test guideline Fish Embryo Toxicity [FET] Test".

The reports (including trial plans and SOP) are available on the OECD website (OECD 2011, 2012). The validation management group concluded that the ZFET was successfully transferred from the lead laboratory to the participating laboratories and that, for the vast majority of the chemicals, it has a good intra- and interlaboratory reproducibility with coefficients of variation (CV) below 30% regardless of the chemical or the laboratory.

In addition, Belanger *et al.* (2013) performed a retrospective analysis of acute fish embryo toxicity data and acute fish toxicity data in order to assess the predictive capacity of the (zebra)fish embryo acute toxicity test for acute fish toxicity testing.

EURL ECVAM has asked its Scientific Advisory Committee (ESAC) to perform a peer review of the scientific validity of the ZFET. The ESAC peer review was launched in October 2012 and finalised in March 2013. The EURL ECVAM recommendation is pending.

Regulatory Acceptance:

After finalisation of the validation study, OECD continued with the development of the new test guideline. The fish embryo acute toxicity test with zebrafish was adopted by OECD on 26th July 2013 as **Test Guideline No. 236: "Fish embryo acute toxicity (FET) test"** (OECD, 2013), following its approval in May 2013 at the 25t h meeting of the OECD WNT.

Abbreviations and Definitions

3,4-DCA	3,4-Dichloroaniline
CV	Coefficient of variance
ESAC	Scientific Advisory Committee to EURL ECVAM
EU	European Union
EURL ECVAM	European Union Reference Laboratory for Alternatives to Animal Testing
FET	Fish embryo toxicity test
ISO	International Organization for Standardization
Kow	Partition coefficent octanol/water, indicator of the capacity of test compound to penetrate cell membranes and bind to plastics

LC50	Concentration lethal to 50% of the test species
MSDS	Material Safety Data Sheet
OECD	Organisation for Economic Co-operation and Development
SOP	Standard Operating Procedure
TG (OECD)	Test Guideline
ZFET	Zebrafish embryo toxicity test

Last update: 30 May 2013

PROCEDURE DETAILS, 30 May 2013

Fish Embryo Acute Toxicity Test with Zebrafish (ZFET) DB-ALM Protocol n° 140

The protocol was used in the EURL ECVAM coordinated OECD validation study assessing the transferability, intra- and interlaboratory reproducibility of the zebrafish embryo acute toxicity test (ZFET; OECD 2011; OECD 2012) and complies with the OECD Test Guideline No. 236.: **Fish Embryo Acute Aquatic Toxicity (FET) Test**.

Annexes to this protocol are available from DB-ALM as downloadable content and can be found in the section related to the Protocol No. 140, under Related information: Downloads box.

For technical questions please contact **Prof. Dr Thomas Braunbeck** (details below). For regulatory questions please contact **Susanne Walter-Rohde** (details below)

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Health and Safety Issues

General Precautions

There are no specific health and safety issues associated with this protocol; however, health and safety issues might be associated with the test compounds and respective MSDS should be consulted.

MSDS Information

The MSDS information listed here refers to the compounds needed for preparation of test reagents, e.g. dilution water, controls.

Compounds	CAS	MSDS (example)	Use
Calcium chloride dehydrate [$CaCl_2$ x $2H_2O$]	10035-04-8	102382 Merck	Dilution water
Magnesium sulfate heptahydrate [$MgSO_4$ x $7H_2O$]	10034-99-8	105886 Merck	Dilution water
Sodium carbonate [$NaHCO_3$]	144-55-8	106329 Merck	Dilution water
Potassium chloride [KCl]	7447-40-7	104936 Merck	Dilution water
Hydrochloric acid [HCl]	7647-01-0	109063 Merck	pH adjustment
Sodium hydroxyde [NaOH]	1310-73-2	109136 Merck	pH adjustment
3,4-Dichloroaniline [$Cl_2C_6H_3NH_2$]	95-76-1	35827 Fluka	Positive control

Materials and Preparations

Cell or Test System

Newly fertilised zebrafish eggs deriving from a breeding stock of unexposed and healthy mature zebrafish (*Danio rerio*), wild-type strain, with an age between 4 and 18 months are used.

Equipment

Fixed Equipment

1. Equipment for maintenance of zebrafish breeding stock
    - Fish tanks made of chemically inert material (*e.g.* glass) and of a suitable capacity in relation to the recommended loading.
2. Equipment for the production of zebrafish eggs
    - Spawn trap:
        - instrument trays of glass, stainless steel or other inert material;
        - wire mesh of stainless steel or other inert material (*e.g.* grid size 2±0.5mm) to protect the eggs once laid;
        - spawning substrate (*e.g.*, plant imitates of inert material).
3. Equipment for the FET test
    - Inverted microscope and/or binocular with at minimum 80-fold magnification. If the room used for the recording of the observations cannot be adjusted to 26±1°C, a temperature-compensated cross movement stage is necessary (*e.g.* Minitüb HT 200, Tiefenbach, Germany);

- Incubator or air-conditioned room maintained at 26±1°C; equipped with an automatic light control;
- pH-meter;
- Oxygen meter;
- Equipment for determination of hardness of water and conductivity;
- In case a flow-through system is used, facilities and equipment necessary to maintain flow-through exposure conditions.

Consumables

- Pipettes with widened openings to collect newly fertilised zebrafish eggs;
- Glass vessels to collect newly fertilised zebrafish eggs (*e.g.* beakers, crystallisation dish);
- Glass vessels to prepare dilution water, stock solutions, and test chemical solutions (*e.g.* beakers, graduated flasks, graduated cylinders, crystallisation dish);
- Graduated glass pipettes;
- Test chambers; *e.g.*, standard 24-well plates with a depth of approx. 20mm (*e.g.* Nunc multidish Nunclon 144530; Renner TPP 92424);
- Self-adhesive foil to cover the 24-well plates (*e.g.* Nunc Sealing Tape SH, no. 236269).

Media, Reagents, Sera, others

1. Chemicals for preparing the dilution water according to ISO 7346-3 (ISO, 1996) or ISO 15088 (ISO, 2007).

Name [formula]	CAS number	Purity	Supplier	Catalogue number
Calcium chloride dehydrate [$CaCl_2$ x 2 H_2O]	10035-04-8	p.a.	e.g., Merck	102382
Magnesium sulfate heptahydrate [$MgSO_4$ x 7 H_2O]	10034-99-8	p.a.	e.g., Merck	105886
Sodium carbonate [$NaHCO_3$]	144-55-8	p.a.	e.g., Merck	106329
Potassium chloride [KCl]	7447-40-7	p.a.	e.g., Merck	104936

2. Chemicals for adjusting the pH

Name [formula]	CAS number	Purity	Supplier	Catalogue number
Hydrochloric acid [HCl]	7647-01-0	p.a.	e.g., Merck	109063
Sodium hydroxyde [NaOH]	1310-73-2	p.a.	e.g., Merck	109136

3. Positive control

Name [formula]	CAS number	Purity	Supplier	Catalogue number
3,4-Dichloroaniline [$Cl_2C_6H_3NH_2$]	95-76-1	99%	Sigma Aldrich (Fluka)	35827

Preparations

Media and Endpoint Assay Solutions

Dilution water

Dilution water is used for preparation of the stock solution of the chemicals, the test concentrations, and controls. It is prepared from reconstituted water according to ISO 7346-3 (ISO, 1996) or ISO 15088 (ISO, 2007).

100-fold stocks solutions of the four chemicals are prepared as follows:

Chemical	g	Distilled or deionised water (mL)
$CaCl_2$ x 2 H_2O	147	500
$MgSO_4$ x 7 H_2O	6.165	500
$NaHCO_3$	31.5	500
KCl	2.75	500

For having 1L of dilution water, 10ml of each of the above stock solutions are added to 960ml distilled or deionised water. The conductivity of the distilled or deionised water used for preparing the dilution water should not exceed 10µS/cm.

The final salt concentration in the dilution water is:

- 294.0 mg/L $CaCl_2$ x 2 H_2O;
- 123.3 mg/L $MgSO_4$ x 7 H_2O;
- 63 mg/L $NaHCO_3$;
- 5.5 mg/L KCl

The resulting degree of total hardness should be equivalent to 100-300 mg/L $CaCO_3$ to prevent excessive precipitation of calcium carbonate.

NOTE: Other well-characterised surface or well water may be used. The reconstituted water may be adapted to maintenance water of low hardness by dilution with deionised water up to a ratio of 1:5 to a minimum hardness of 30-35 mg/L $CaCO_3$).

The water is aerated to oxygen saturation prior to the addition of the test chemical and its temperature should be 26.0±1.0°C when used for preparation of test chemical concentrations/controls and throughout the test.

The pH should be adjusted to a range between pH 6.5 and 8.5 (use of HCL and NaOH recommended), and not vary within this range by more than 1.5 units during the course of the test.

If the pH does not remain in this range, a second test could be carried out, adjusting the pH of the stock solution to that of the dilution water before addition of the test chemical. The pH adjustment should be made in such a way that the stock solution concentration is not changed to any significant extent and that no chemical reaction or precipitation of the test chemical is caused.

Test Compounds

- Test chemical solutions of the selected concentrations can be prepared, e.g., by dilution of a stock solution. The stock solutions should preferably be prepared

by simply mixing or agitating the test chemical in the dilution water by mechanical means (e.g., stirring or ultrasonification).

- Use of HCl and NaOH solutions to correct the pH in the solutions containing test chemicals to the pH of the dilution water (6.8 - 8.5) is recommended.
- If the test chemical is difficult to dissolve in water, procedures described in the OECD Guidance Document No. 23 for handling difficult chemicals should be followed (OECD, 2000). The use of solvents should be avoided but may be required in some cases in order to produce a suitably concentrated stock solution.
- In case a solvent is required to assist in stock solution preparation, its final concentration should not exceed 100µl/L and should be the same in all test vessels.
- The examples of suitable solvents are given in OECD Guidance Document No. 23 (OECD, 2000).
- When a solvent is used, a solvent-only control is required.

Positive Control(s)

3,4-dichloroaniline (3,4-DCA) is used as positive control at a concentration of 4mg/L.

The final 3,4-DCA solution is freshly prepared (= on the same day) with dilution water. The temperature of the dilution water should be 26±1°C.

Preparation of 3,4-DCA stock solution:

- Dissolve 50 mg 3,4-DCA in 500mL dilution water
- Stir in a closed, light-proof vessel for 24h at room temperature
- Adjust pH to the pH of the dilution (±0.5) water using NaOH or HCl solutions
- Stock solution can be kept dark in fridge (1-8°C) for up to 2 months maximum
- Before use of the stock solution, stir at room temperature for at least 30min to ensure a uniform concentration of 3,4-DCA

Negative Control(s)

Pure dilution water is used as negative control and internal plate control.

If a solvent is used for the preparation of the test chemical concentrations, a solvent control is needed at the final concentration used.

Method

Test System Procurement

Maintenance of zebrafish breeding stock (according to OECD TG 236)

A breeding stock of unexposed, wild-type zebrafish strain with well-documented fertilisation rate of eggs is used for egg production. Fish should be free of macroscopically discernible symptoms of infection or disease and should not have been treated with any pharmaceuticals (acute or prophylactic) for 2 months before spawning. Breeding fish are

maintained in aquaria with a recommended loading capacity of 1L water per fish and a fixed 12-16h light photoperiod (Laale, 1977; Westerfield, 2000; CCAC, 2005; EC, 2007; EU, 2010). Optimal filtering rates should be adjusted; excess filtering rates causing heavy perturbation of the water should be avoided. Surplus feeding should be avoided, and water quality and cleanness of the aquaria should be monitored regularly and reset to the initial state, if necessary. More information on the maintenance and breeding of zebrafish as well as specific conditions for the ZFET is available in Annex 2 of OECD TG No. 236.

Routine Culture Procedure

Annex 1 provides an overview of the workflow of the FET (available in Downloads)

NOTE: A single mature female spawns at least 50-80 eggs per day. Depending on the strain, spawning rates may be considerably higher. The fertilisation rate should >= 70%. In case first time spawning fish are used, fertilisation rates may be lower in the first few spawns.

1. Production of eggs

Eggs may be produced via spawning groups in individual spawning tanks or via mass spawning in maintenance tanks.

In case of spawning groups, males and females (e.g. in ratio 2:1) are placed in spawning tanks a few hours before the onset of darkness on the day prior to the test. Since spawning groups of zebrafish may occasionally fail to spawn, the parallel use of at least three spawning tanks is strongly recommended. To avoid genetic bias, eggs should be collected from a minimum of three breeding groups.

2. Collection of eggs

For the collection of the eggs, spawn traps are placed into the spawning or maintenance tanks before the onset of darkness on the day prior to the test or before the onset of light on the day of the test. To prevent predation of the eggs by the adult zebrafish, the spawn traps are covered with inert wire mesh of appropriate grid size (2±0.5mm). If considered necessary, artificial plants made of green inert material (e.g. plastic or glass) can be fixed to the mesh as spawning stimulus (Braunbeck *et al*, 2005; ISO, 2007; Nagel, 2002; Laale, 1977; Nagel, 1986). To avoid any contamination via leaching of chemicals from plastic, weathered plastic material should be used. Mating, spawning and fertilisation take place within 30min after the onset of light and the spawn traps with the collected eggs can be carefully removed.

3. Differentiation of eggs

At 26°C, fertilised eggs undergo the first cleavage after about 15min and the consecutive synchronous cleavages form 4, 8, 16 and 32 cell blastomers (see *Annex 2* in Downloads section). At these stages, fertilised eggs can be clearly identified by the development of a blastula.

Test Material Exposure Procedures

1. Test chemical concentrations

Normally, at least five concentrations of the test chemical spaced by a constant factor not exceeding 2.2 are needed to meet the statistical requirements. Preferably, the highest concentration tested should result in 100% mortality, and the lowest concentration tested should give no observable effect. A range-finding test before the definitive test allows

selection of the appropriate concentration range and it is typically performed using ten embryos per concentration.

2. Conditioning of test chambers (24-well plates)

24-well plates should be pre-saturated with the respective test chemical concentrations/controls at least 24h before starting the test. They are filled with the required quantity of freshly prepared test chemical concentrations (i.e. prepared on the same day) and respective controls.

3. Preparation of glass vessels for selection of eggs and 24-well plates for incubation

On the day of the test, glass vessels (an appropriate volume to fully cover the eggs during the selection) and 24-well plates (2ml/well) are filled with the respective freshly prepared test chemical concentrations/controls.

4. Selection of fertilised eggs & start of exposure

NOTE: The embryos should be immersed in the test solutions before cleavage of the blastodisc commences, or, at latest, by the 16 cell-stage.

In order to start the exposure with a minimum delay, at least twice of the number of eggs needed per test chemical concentration/control group are randomly selected and transferred into the prepared glass vessels (see ***Point 3***. above) not later than 1h post fertilisation (past the onset of light).

Viable fertilised eggs should be separated from unfertilised eggs and be transferred to 24-well plates (see ***Point 3***. above) within 3h post fertilisation. The glass vessels containing the eggs are placed under an inverted microscope or a binocular with a minimum magnification of 30x to identify fertilised eggs and determine the fertility rate. Fertilised eggs can easily be identified by their transparency, at best by putting the glass vessels on a black pad and using flexible swan neck lights or transverse light under the binocular.

In the following, details on the appearance of developmental stages critical for the identification of fertilised eggs are given (see *Annex 2*; Kimmel *et al* 1995):

- Freshly spawned eggs are characterised by a fully transparent perivitelline space surrounded by the egg membrane and containing the yolk, and the germinal disc, which has already formed at the animal pole.
- After fertilisation, the first cell division is initiated at 26°C after about 15min.
- From the 4-cell stage onwards, fertilised eggs can unambiguously be distinguished by their transparency from non-fertilised eggs.
- Eggs with obvious anomalies (asymmetries, formation of vesicles) or damaged chorion should be discarded.
- Non-fertilised eggs can be identified by a lack of blastomer formation and, at later stages, by their non-transparency.

NOTE: Only fertilised eggs between the 4- and 128-cell stages should be used.

5. Distribution of fertilised eggs over 24-well plates

One egg is used per well containing 2ml of the respective test chemical concentration/control. The following distribution scheme is recommended (see *Annex 3* in Downloads section):

- 20 eggs on one plate for each of five test chemical concentrations;
- 20 eggs as solvent control on one plate (if necessary);
- 20 eggs as positive control on one plate;
- 4 eggs in dilution water as internal plate control on each of the above plates; and
- 24 eggs in dilution water as negative control on one plate

6. Incubation and exposure duration

The 24-well plates are covered with self-adhesive foil and incubated at 26±1°C for 96h. Control of the light cycle (12–16h) is achieved by keeping the plates in either an incubator or separate room equipped with an automatic light control.

7. Semi-static exposure with semi-static renewal technique of the test chemical concentrations/controls after 24, 48, 72h

The following steps are carried out after the daily recording of the lethal effects:

- Test chemical concentrations / controls are freshly prepared from the stock solution
- Solutions are removed by using an appropriate pipette or vacuum suction (cell culture-fitted vacuum pump plus suction bottle).
- For the removal of each test chemical concentration, separate pipette tips must be used.

  NOTE: In any case, contact with the embryo must be avoided and care should be taken that the embryos remain covered by a small amount of the old test solutions to avoid drying.

- At least 90% of the volume of each well must be removed and immediately replaced with the corresponding volume of freshly prepared test chemical solutions/controls.

Endpoint Measurement

Recording of lethal effects / observations

Dead embryos are recorded on a daily basis. For this purpose, the 24-well plates are controlled using an inverted microscope or binocular.

Any of the following observations (see Annex 4) indicate a lethal effect:

- *Coagulation of the embryo (Annex 4, Figure 1)*

  Coagulated embryos are milky white and appear dark under the microscope. The number of coagulated embryos is determined after 24, 48, 72 and 96h.

- *Lack of somite formation (Annex 4, Figure 2)*

  At 26±1°C, about 20 somites have formed after 24h in a normally developing zebrafish embryo. A normally developed embryo shows spontaneous movements (side-to-side contractions) indicating the formation of somites. The absence of somites is recorded after 24, 48, 72 and 96h. Non-formation of

somites after 24h might be due to a general retardation of development. At latest after 48h, the formation of somites should be visible. If not, the zebrafish embryos are considered dead.

- *Non-detachment of the tail (Annex 4, Figure 3)*

  In a normally developing zebrafish embryo, detachment of the tail from the yolk is observed following posterior elongation of the embryonic body. Absence of tail detachment is recorded after 24, 48, 72 and 96h.

- *Lack of heartbeat (Annex 4, Figure 4)*

  In a normal developing zebrafish embryo at 26±1°C, the heartbeat is visible after 48h. Particular care should be taken when recording this endpoint, since irregular (erratic) heartbeat should *not* be recorded as lethal. Moreover, visible heartbeat without circulation in aorta abdominalis is considered *non-lethal*. To record this effect, embryos should be observed under a minimum magnification of 80× for at least one minute. Lack of heartbeat is recorded after 48, 72 and 96h.

In addition, hatching rates should be recorded in the treatment and control groups from 48h onwards. Hatching is not an endpoint used for the calculation of the LC_{50}, however it ensures that the zebrafish embryos are exposed to the test chemical without a potential barrier function of the chorion and as such may help data interpretation.

Analytical measurements

- *Test conditions*

  At the beginning and at the end of the test, pH, total hardness and conductivity in the control(s) and in the highest test chemical concentration are measured. In semi-static renewal systems the pH should be measured prior to and after water renewal. The dissolved oxygen concentration is measured at the end of the test in the negative controls and highest test chemical concentration with viable embryos, where it should be in compliance with the test acceptance criteria (see below). The temperature is measured in three randomly selected vessels and it should be recorded preferably continuously during the test or, as a minimum, daily.

- *Measurement of test compound concentrations*

  (to be considered when test results are used for regulatory purposes)

  OECD TG 236 recommends the following approach for semi-static tests:

  As a minimum, the concentration of the test chemical should be measured in the highest and lowest test concentrations, and preferably in all treatments, at the beginning and end of the test in a static system.

  In semi-static (renewal) tests where the concentration of the test chemical is expected to remain within ±20% of the nominal values, it is recommended that, as a minimum, the highest and lowest test concentrations be analysed when freshly prepared and immediately prior to renewal.

- For tests where the concentration of the test chemical is not expected to remain within ±20% of nominal, all test concentrations must be analysed following the same regime as for more stable chemicals. In case of insufficient volume for

analysis, merging of test solutions or use of surrogate chambers being of the same material and having the same volume to surface area ratios as 24-well plates, may be useful.

It is strongly recommended that result calculation is based on measured concentrations. When the concentrations do not remain within 80-120% of the nominal concentration, the effect concentrations should be determined and expressed relative to the geometric mean of the measured concentrations for semi-static tests; see Chapter 5 in the OECD Guidance Document No. 23 for more details (OECD, 2000).

Acceptance Criteria

The following acceptance criteria have to be met:

1. The overall fertilisation rate of all eggs collected should be >= 70% in the batch tested.
2. The water temperature should be maintained at 26±1°C in test chambers at any time during the test.
3. Overall survival of embryos in the negative (dilution water) control and, where relevant, in the solvent control should be >= 90% until the end of the 96h exposure.
4. Exposure to the positive control (*e.g.*, 4.0mg/L 3,4-dichloroaniline for zebrafish) should result in a minimum mortality of 30% at the end of the 96h exposure.
5. The hatching rate in the negative control (and solvent control if appropriate) should be >= 80% at the end of the 96h exposure.
6. At the end of the test, the dissolved oxygen concentration in the negative control and highest test concentration should be >= 80% of saturation.

NOTE: The mortality in the internal plate control should not exceed one embryo per plate. If more than 1 dead embryo is observed in the internal plate control, it could be considered to only discard the plate concerned and not the complete test. In this case, fewer concentrations will be available to derive the LC_{50}. The test should be repeated when increased mortality is observed in the internal plate control of the solvent control plate.

Data Analysis

LC_{50} calculation

The individual wells are considered independent replicates for statistical analysis. The percentages of embryos for which at least one of the lethal observations is positive at (48h and) 96h are plotted against test chemical concentrations. For calculation of the slopes of the curve, LC_{50} values and confidence limits (95%), appropriate statistical methods should be applied (ISO, 2006) and the OECD Guidance Document No. 54 (OECD, 2006) should be consulted.

Prediction Model

The LC_{50} values derived with the zebrafish embryo acute toxicity can be directly used as surrogates for the LC_{50} values of fish acute toxicity tests. A prediction model is not needed.

Annexes

Annexes 1- 4 are available in the **Downloads** section of this protocol in the DB-ALM (http://ecvam-dbalm.jrc.ec.europa.eu/)

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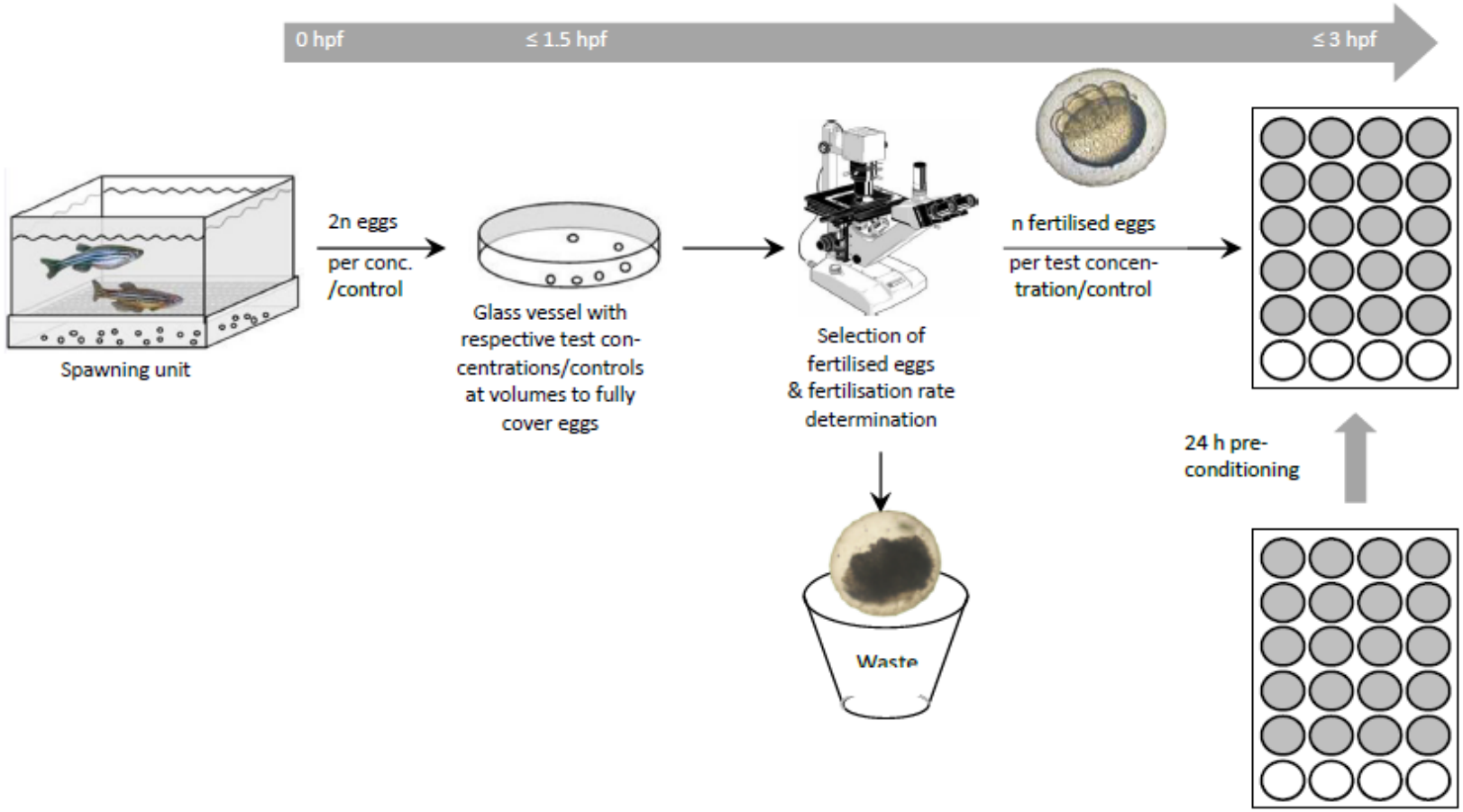
Annex 1. Scheme of fish embryo acute toxicity test with zebrafish

From left to right: production & collection of eggs, immediately after fertilisation pre-exposure of eggs in glass vessels, selection of fertilised eggs with an inverted microscope or binocular and distribution of fertilised eggs into 24-well plates prepared with the respective test concentrations/controls, n = number of eggs required per test concentration/control (here 20/24 eggs); hpf = hours post-fertilisation (modified according to OECD TG 236).


0 hpf
≤ 1.5 hpf
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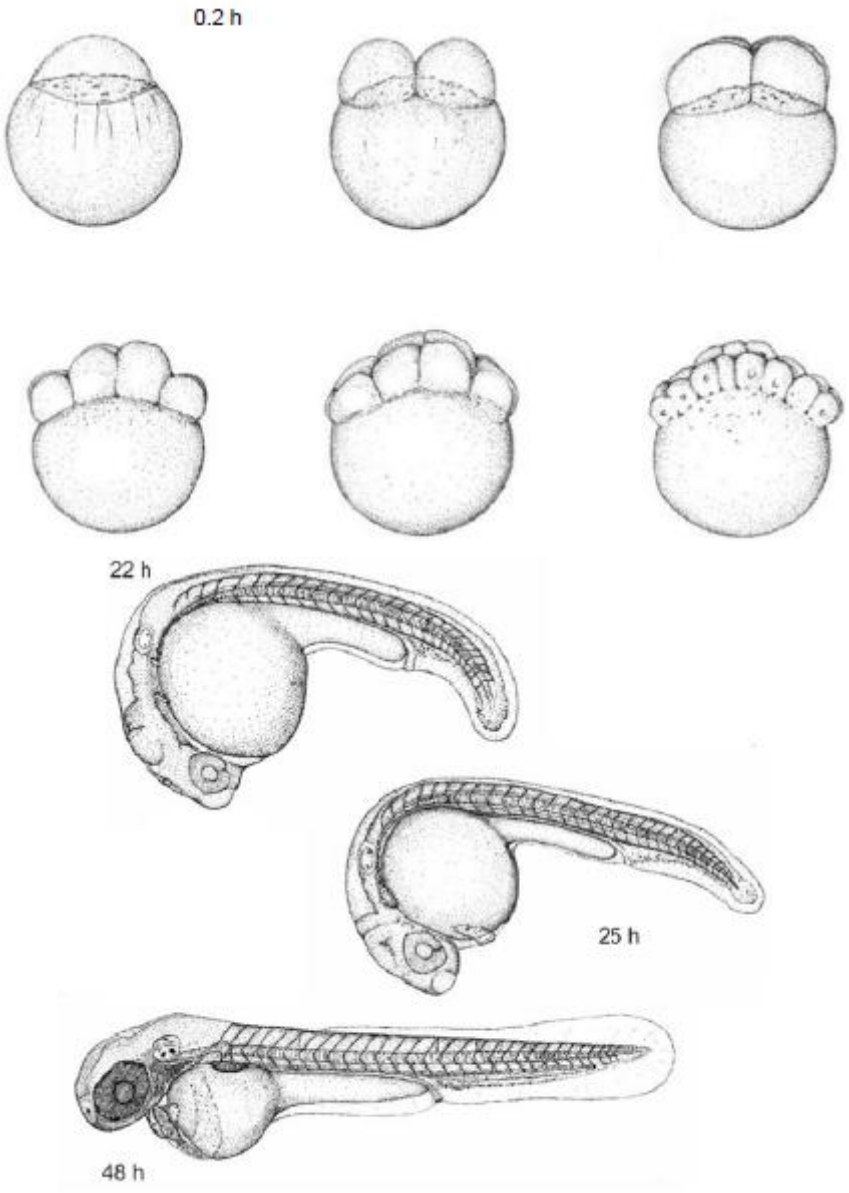
Spawning unit
2n eggs
per conc. /control
Glass vessel with respective test con-centrations/controls at volumes to fully cover eggs
Selection of fertilised eggs & fertilisation rate determination
Waste
n fertilised eggs
per test concen-tration/control
24 h pre-conditioning

Annex 2. Normal zebrafish development at 26°C al (as given in OECD TG 236)

Figure 1. Selected stages of early zebrafish (*Danio rerio*) development

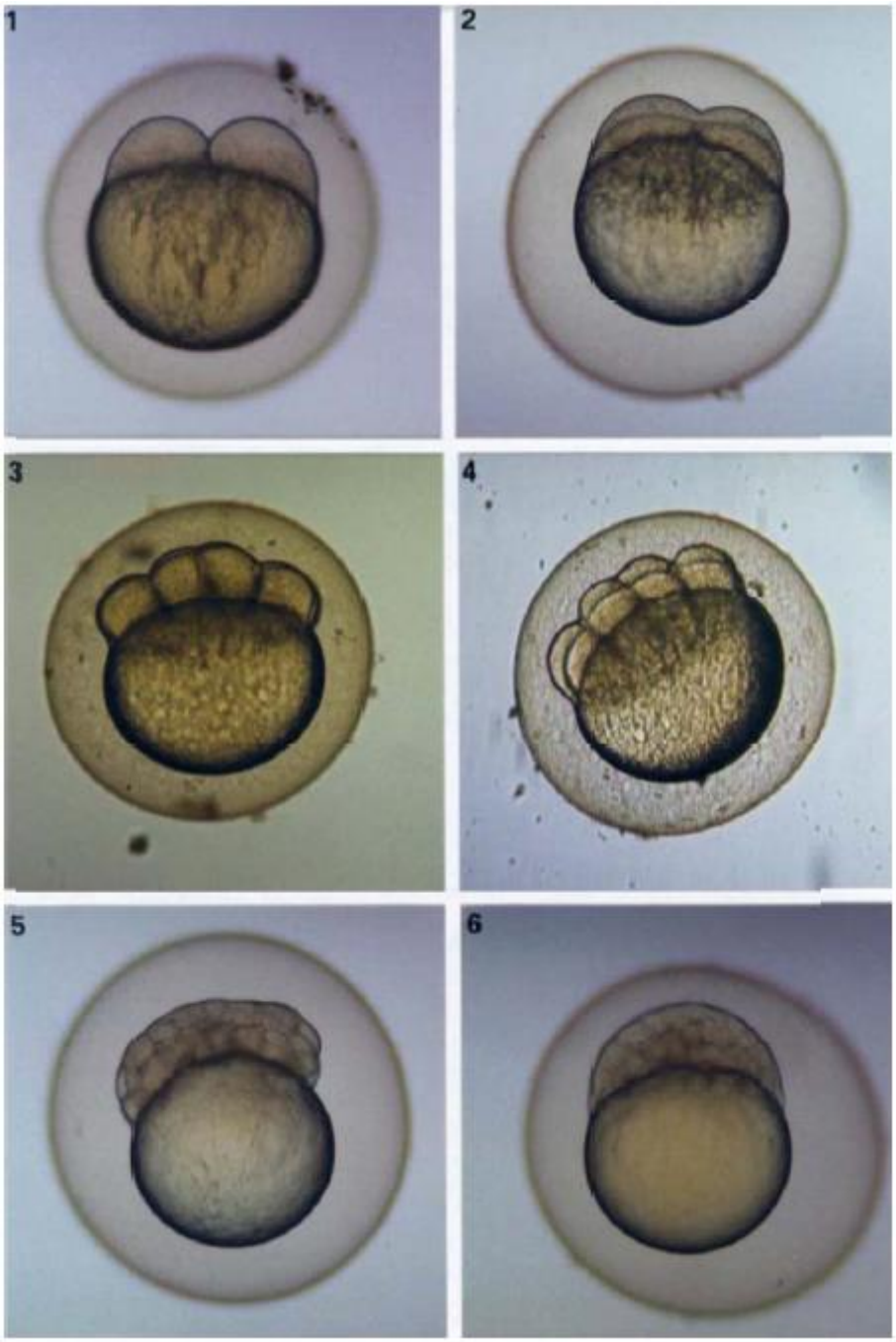
0.2 – 1.75 hrs post-fertilisation (from Kimmel *et al.*, 1995*). The time sequence of normal development may be taken to diagnose both fertilisation and viability of eggs (see paragraph 26: Selection of fertilised eggs).



*Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B. and Schilling, T.F. (1995) Stages of embryonic development of the zebrafish. Developmental Dynamics, 203, 253-310, **Wiley**

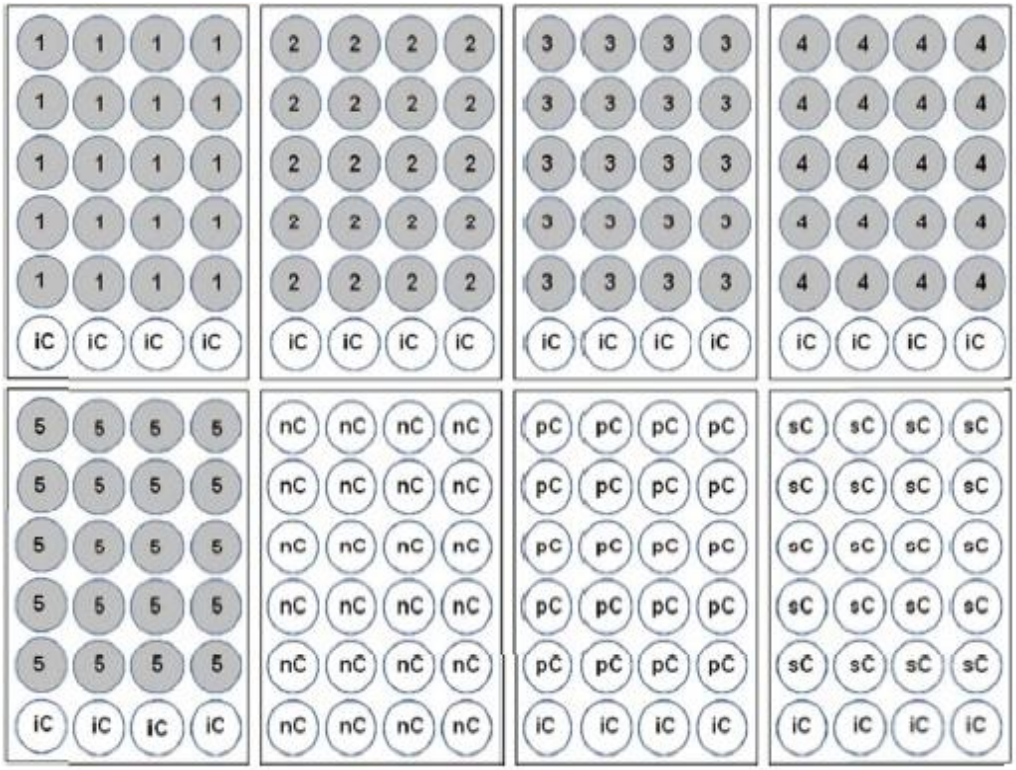
Figure 3. Normal development of zebrafish (*Danio rerio*) embryos

(1) 0.75 hrs, 2-cell stage; (2) 1 hr, 4-cell stage; (3) 1.2 hrs, 8-cell stage; (4) 1.5 hrs, 16-cell stage; (5) 4.7 hrs, beginning epiboly; (6) 5.3 hrs, approx. 50 % epiboly (from Braunbeck & Lammer 2006: *Detailed review paper "Fish embryo toxicity assays". UBA report under contract no. 20385422 German Federal Environment Agency, Berlin. 298 pp*).



Annex 3. 24-well plate layout (as given in OECD TG 236)

1-5 = five test concentrations / chemical; nC = negative control (dilution water); iC = internal plate control (dilution water); pC = positive control (3,4-DCA 4mg/L); sC = solvent control



Annex 4. Lethal effects on zebrafish embryos (as given in OECD TG 236)

The following apical endpoints indicate acute toxicity and, consequently, death of the embryos: *coagulation of the embryo, non-detachment of the tail, lack of somite formation and lack of heartbeat*. The following micrographs have been selected to illustrate these endpoints.

Figure 1. Coagulation of the embryo

Under bright field illumination, coagulated zebrafish embryos show a variety of intransparent inclusions.

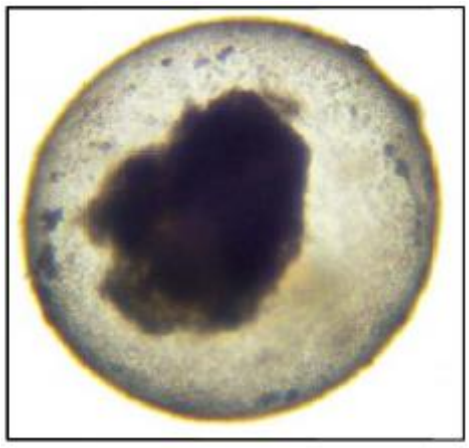


Figure 2. Lack of somite formation

Although retarded in development by approx. 10 hrs, the 24 hrs old zebrafish embryo in (a) shows well-developed somites (→), whereas the embryo in (b) does not show any sign of somite formation (→). Although showing a pronounced yolk sac oedema (*), the 48 hrs old zebrafish embryo in (c) shows distinct formation of somites (→), whereas the 96 hrs old zebrafish embryo depicted in (d) does not show any sign of somite formation (→). Note also the spinal curvature (scoliosis) and the pericardial oedema (*) in the embryo shown in (d).

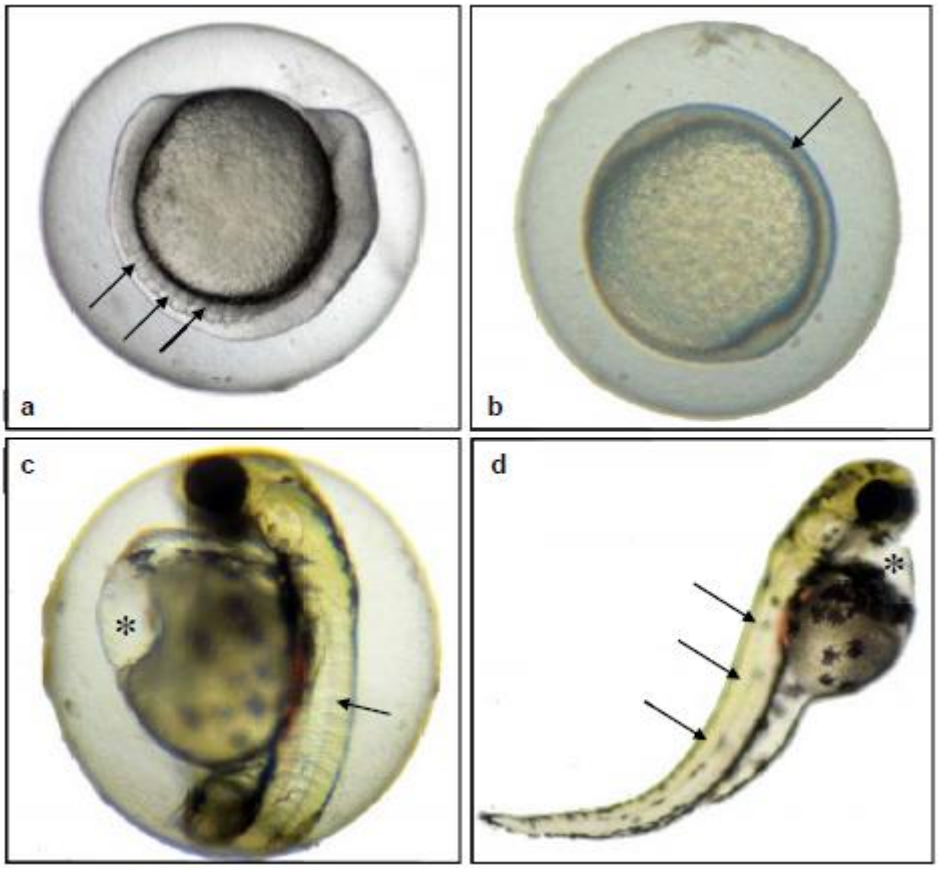


Figure 3. Non-detachment of the tail bud in lateral view (a: →; 96 hrs old zebrafish embryo). Note also the lack of the eye bud (*).

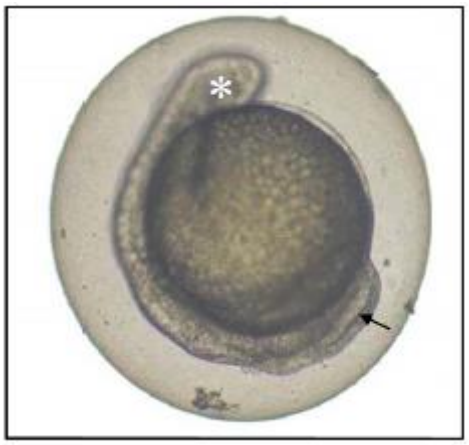
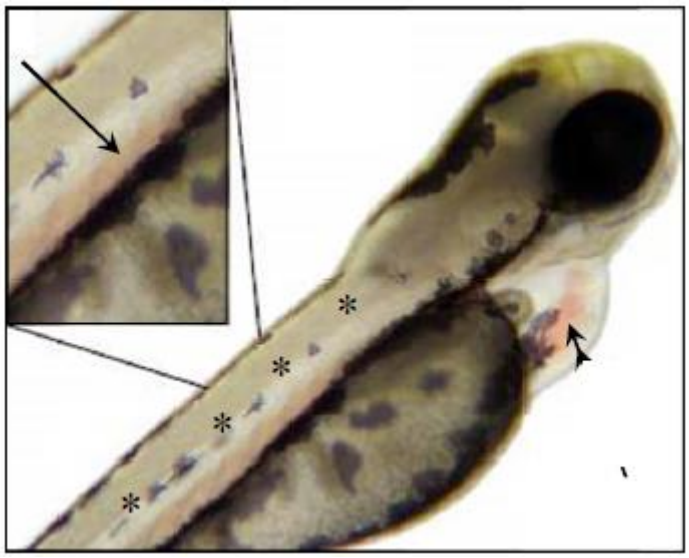


Figure 4. Lack of heartbeat is, by definition, difficult to illustrate in a micrograph.

Lack of heartbeat is indicated by non-convulsion of the heart (double arrow). Immobility of blood cells in, *e.g.*, the aorta abdominalis (→ in insert) is not an indicator for lack of heartbeat. Note also the lack of somite formation in this embryo (*, homogenous rather than segmental appearance of muscular tissues). The observation time to record an absence of heartbeat should be at least of one minute with a minimum magnification of 80×.

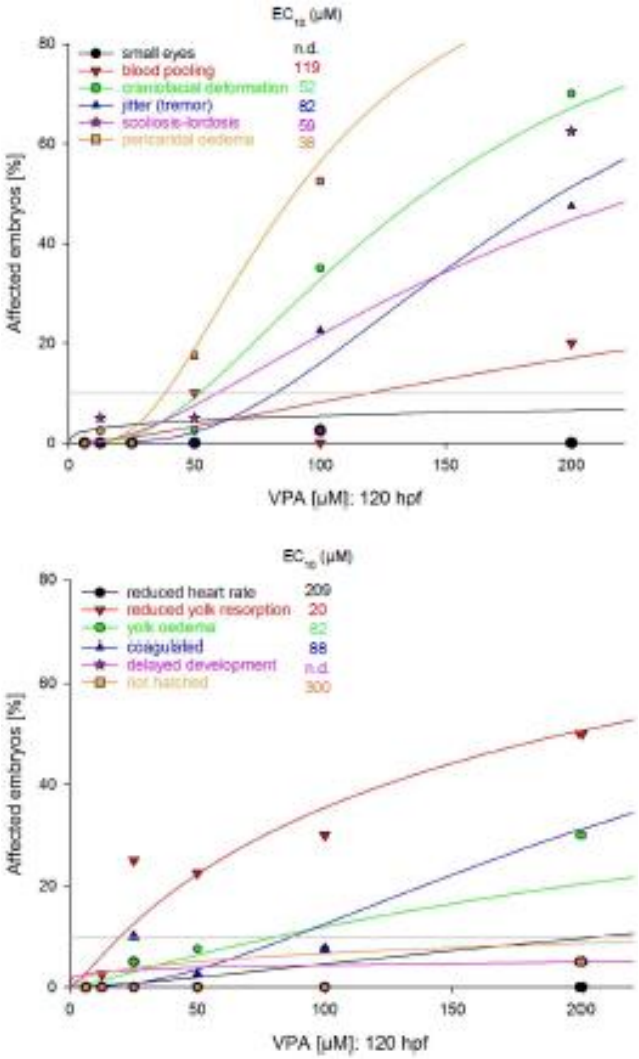


2. ZET-2: Individual effect curves and EC_{10} for all analogues

A. Separate effect graphs of every compound including tables with the individual EC_{10}, LOEC and NOEC (nominal concentration)

Note: n.d.: not detectable

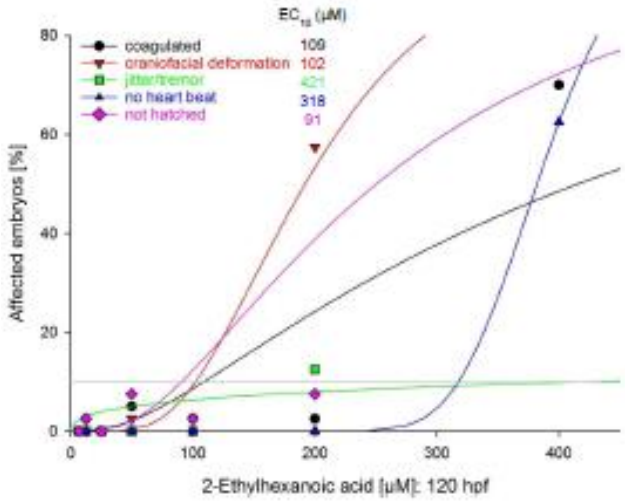
A1. Valproic acid

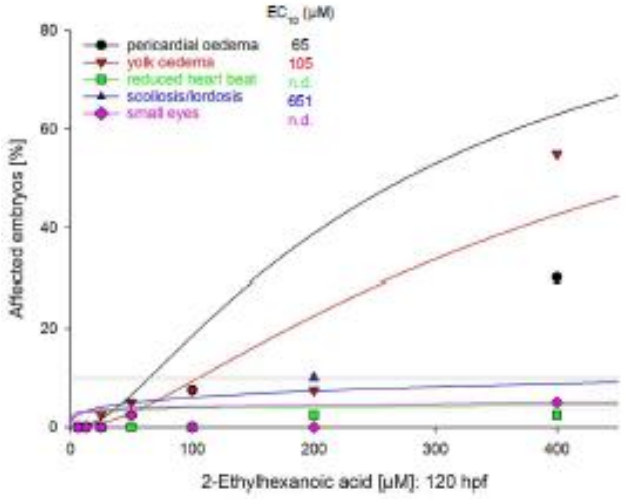


Effect	EC_{10} (μM)	LOEC (μM)	NOEC (μM)
Small eyes	n.d.	400	200
Blood Pooling	119	200	100
Craniofacial deformation	52	100	50
Jitter/tremor	82	100	50
Scoliosis/lordosis	59	50	6.25
Pericardial oedema	38	50	25
Reduced heart beat	209	400	200
Reduced yolk resorption	20	25	12.5
Yolk oedema	82	25	12.5
Coagulated	88	400	12.5
Delayed development	n.d.	400	200
No hatched	300	400	200

Most sensitive effects: pericardial oedemata, reduced yolk resorption
Most prominent effects: craniofacial deformation, small eyes (& pericardial oedemata)
Please note: pericardial and yolk oedemata appeared with all tested substances (even at the lowest test concentrations). It can be the first sign of the embryo feeling unwell. This effect can also be reversible.

A2. 2-Ethyl hexanoic acid

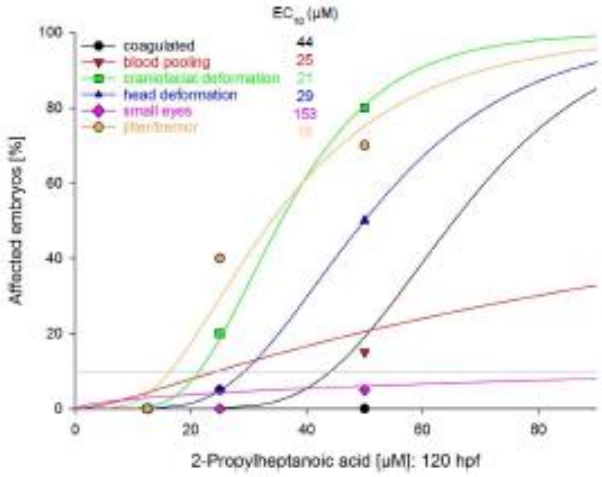


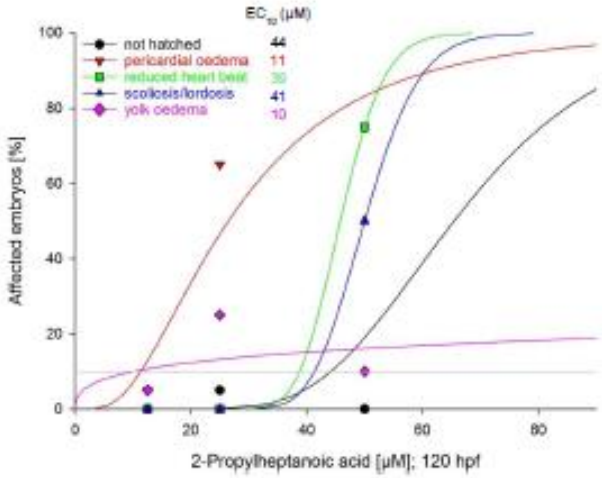


Effect	EC_{10} (µM)	**LOEC** (µM)	**NOEC** (µM)
Coagulated	109	50	6.25
Craniofacial deformation	102	200	100
Jitter/tremor	421	200	100
No heart beat	318	400	200
No hatched	91	400	50
Pericardial oedema	65	50	25
Yolk oedema	105	100	50
Reduced yolk resorption	n.d.	200	100
Scoliosis/lordosis	651	200	12.5
Small eyes	n.d.	400	25

Most sensitive effects: pericardial oedemata
Most prominent effects: craniofacial deformation, pericardial oedemata
Please note: pericardial and yolk oedemata appeared in all tested substances (even at the lowest test concentrations). It can be the first sign of the embryo feeling unwell. This effect can also be reversible.

A3. 2-Popyl heptanoic acid

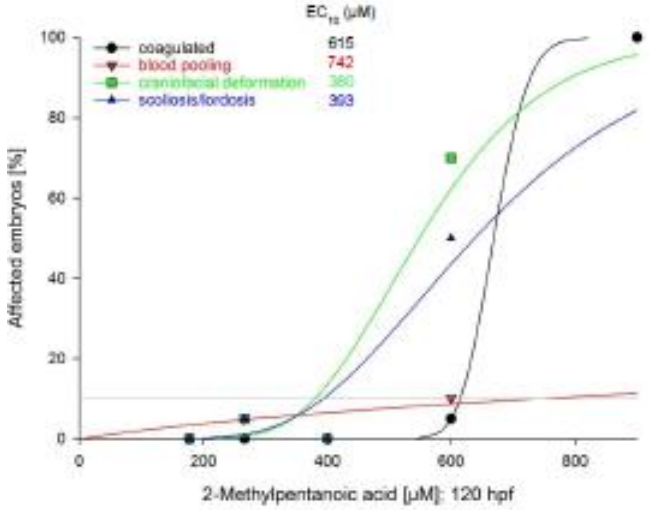


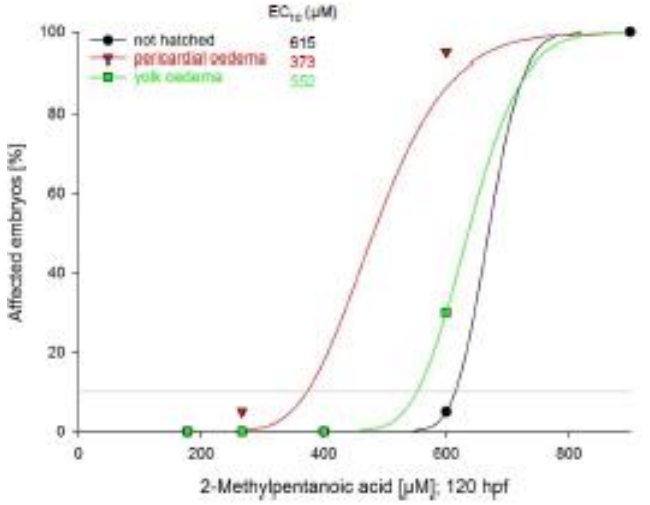


Effect	EC_{10} (µM)	LOEC (µM)	NOEC (µM)
Coagulated	44	25	12.5
Blood pooling	25	50	12.5
Craniofacial deformation	21	50	12.5
Head deformation	29	50	25
Small eyes	153	50	25
Jitter/tremor	16	50	<16
No hatched	44	25	12.5
Pericardial oedema	11	12.5	<12.5
Reduced heart beat	39	50	25
Scoliosis/lordosis	41	50	25
Yolk oedema	10	50	<10

Most sensitive effects: pericardial oedemata, reduced heart rate, small eyes
Most prominent effects: small eyes, craniofacial deformation
Please note: pericardial and yolk oedemata appeared in all tested substances (even at the lowest test concentrations). It can be the first sign of the embryo feeling unwell. This effect can be also reversible.

A4. 2-Methyl pentanoic acid

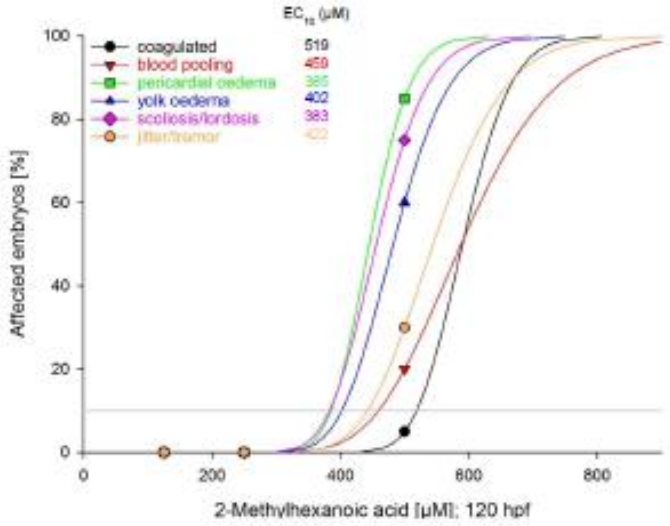




Effect	EC_{10} (µM)	**LOEC** (µM)	**NOEC** (µM)
Coagulated	615	900	600
Blood pooling	742	600	<177.7
Craniofacial deformation	380	266.6	<177.7
Scoliosis/lordosis	393	600	266.6
No hatched	615	900	600
Pericardial oedema	373	600	266.6
Yolk oedema	552	600	400

Most sensitive effects: blood pooling, pericardial oedemata
Most prominent effects: scoliosis/lordosis, craniofacial deformation
Please note: pericardial and yolk oedemata appeared in all tested substances (even at the lowest test concentrations). It can be the first sign of the embryo feeling unwell. This effect can be also reversible.

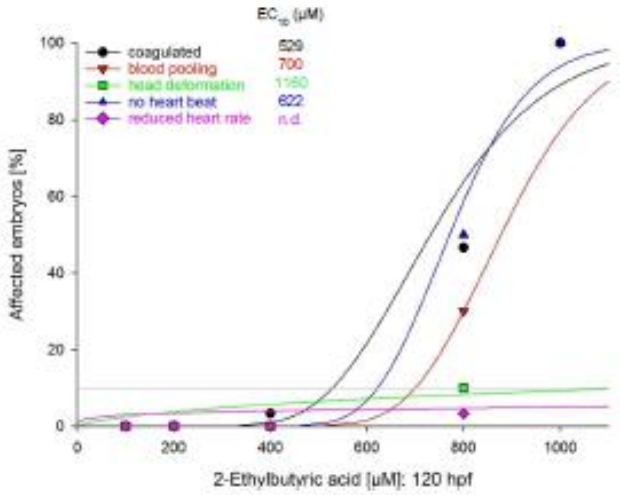
A5. 2-Methyl hexanoic acid

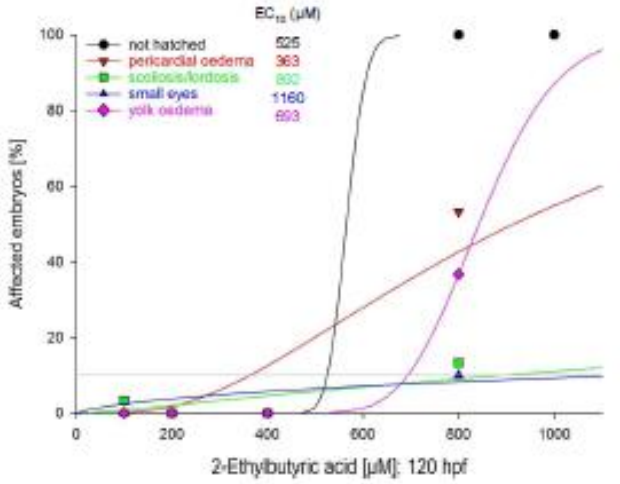


Effect	EC_{10} (µM)	**LOEC** (µM)	**NOEC** (µM)
Coagulated	519	1000	500
Blood pooling	459	500	250
Pericardial oedema	385	500	250
Yolk oedema	402	500	250
Scoliosis/lordosis	383	500	250
Jitter/tremor	442	500	<442

Most sensitive effects: scoliosis/lordosis
Most prominent effects: pericardial oedemata, craniofacial deformation
Please note: pericardial and yolk oedemata appeared in all tested substances (even at the lowest test concentrations). It can be the first sign of the embryo feeling unwell. This effect can be also reversible.

A6. 2-Ethyl butyric acid

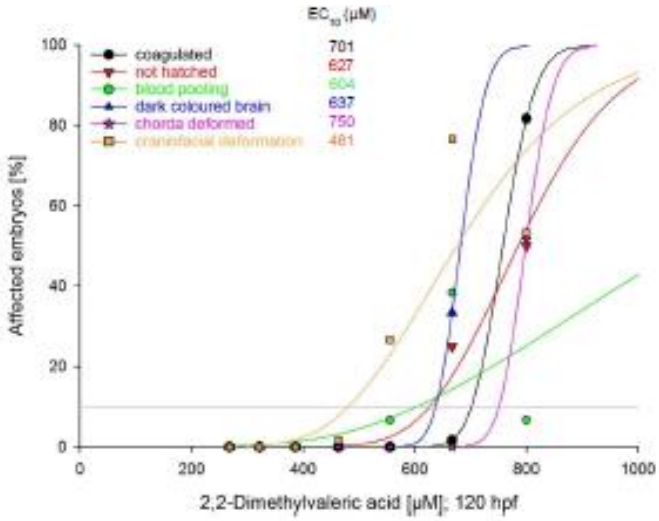


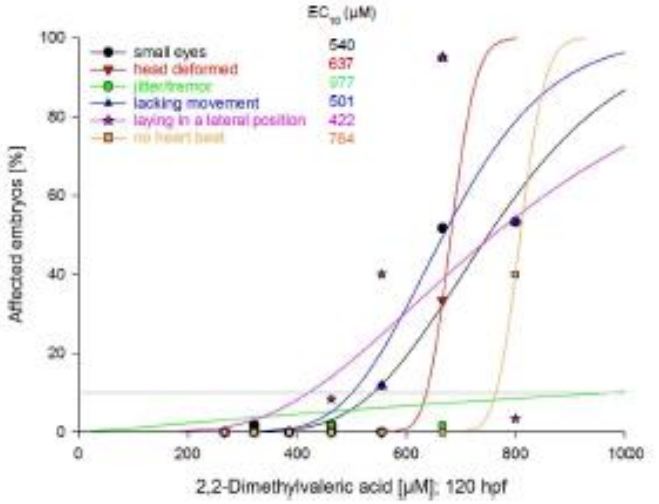


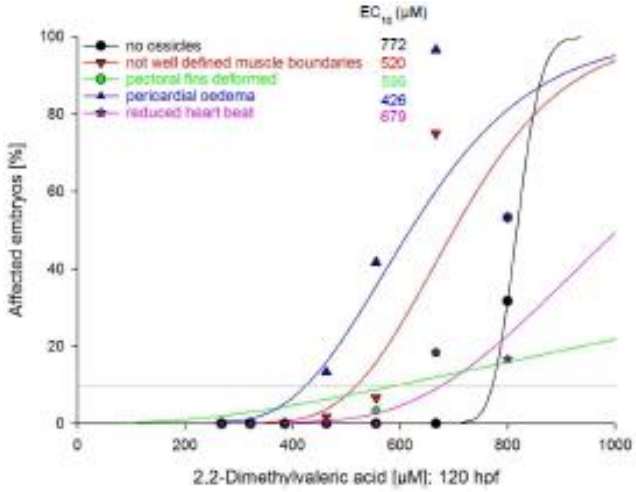
Effect	EC_{10} (µM)	**LOEC** (µM)	**NOEC** (µM)
Coagulated	529	800	400
Blood pooling	700	800	400
Head deformation	1160	800	≤800
No heart beat	622	800	400
Reduced heart beat	n.d.	800	≤800
No hatched	525	800	400
Pericardial oedema	363	400	200
Scoliosis/lordosis	892	>800	800
Small eyes	1160	800	400
Yolk oedema	693	800	400

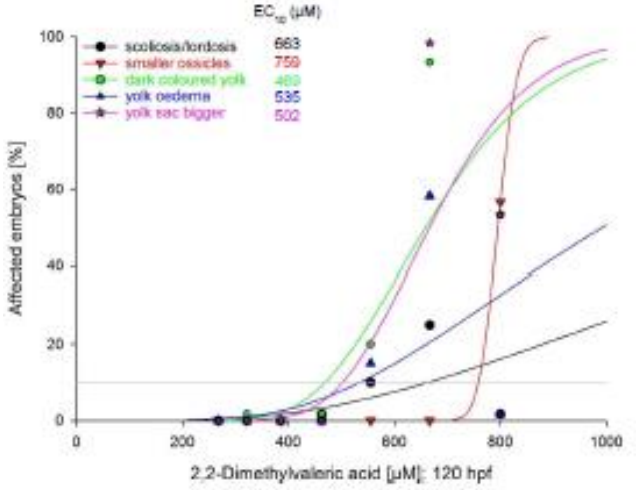
Most sensitive effects: no ossicles (bone formation), pericardial oedemata
Most prominent effect: head deformed, not hatched
Please note: pericardial and yolk oedemata appeared in all tested substances (even at the lowest test concentrations). It can be the first sign of the embryo feeling unwell. This effect can be also reversible.

A7. 2,2-Dimethyl valeric acid





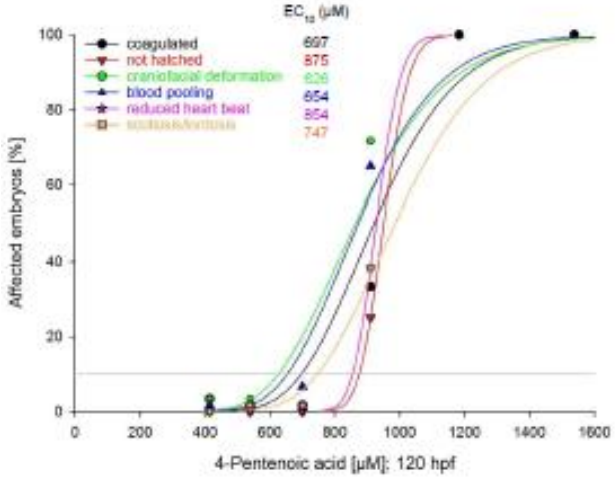

EC10 (µM)
no ossicles 772
not well defined muscle boundaries 520
pectoral fins deformed 590
pericardial oedema 426
reduced heart beat 679
Affected embryos [%]
2,2-Dimethylvaleric acid [µM]; 120 hpf

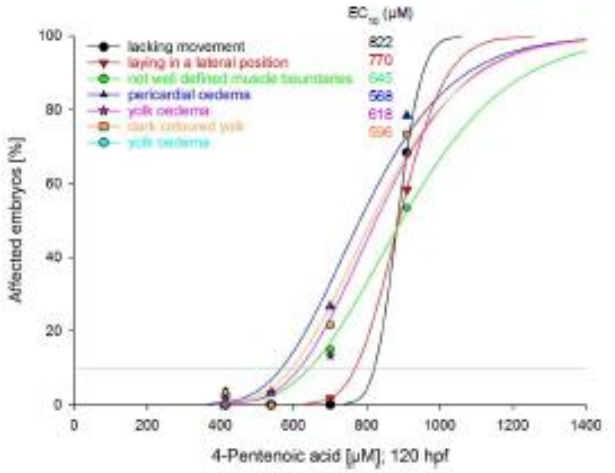

EC10 (µM)
scoliosis/lordosis 663
smaller ossicles 759
dark coloured yolk 460
yolk oedema 535
yolk sac bigger 502
Affected embryos [%]
2,2-Dimethylvaleric acid [µM]; 120 hpf

Effect	EC_{10} (µM)	**LOEC** (µM)	**NOEC** (µM)
Coagulated	701	800	666.6
No hatched	627	666.6	555.5
Blood pooling	604	555.5	462.9
Dark coloured brain	637	666.6	555.5
Chorda deformed	750	800	666.6
Craniofacial deformation	481	555.5	462.9
Eyes too small	540	666.6	462.9
Head deformed	637	666.6	555.5
Jitter/tremor	977	666.6	≤666.6
Lacking movement	501	555.5	462.9
Laying in a lateral position	422	800	385.8
No heart beat	764	800	666.6
No ossicles	772	800	666.6
No well defined muscle boundaries	520	462.9	385.8
Pectoral fins deformed	599	666.6	555.5
Pericardial oedema	426	462.9	385.8
Reduced heart beat	679	666.6	555.5
Scoliosis/lordosis	663	462.9	385.8
Smalles ossicles	759	800	666.6
Dark coloured yolk	469	555.5	462.9
Yolk oedema	535	800	462.9
Yolk sac bigger (compared to NK)	502	555.5	462.9

Most sensitive effects: no ossicles (bone formation), pericardial oedemata, blood pooling
Most prominent effect: craniofacial deformation, not well defined muscle boundaries, lacking movement
Please note: pericardial and yolk oedemata appeared in all tested substances (even at the lowest test concentrations). It can be the first sign of the embryo feeling unwell. This effect can be also reversible.

A8. 4-Pentenoic acid

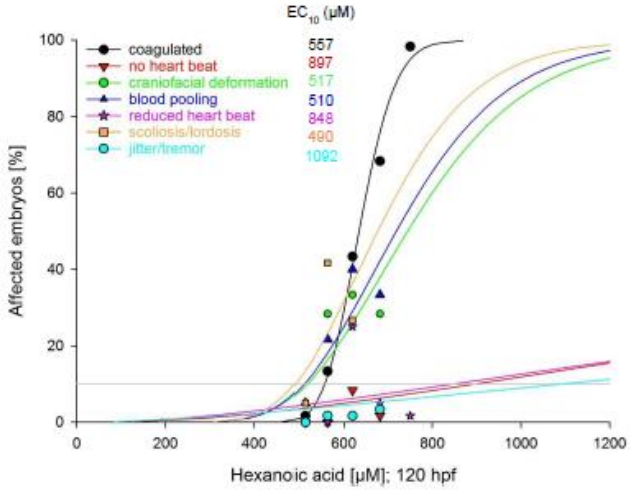


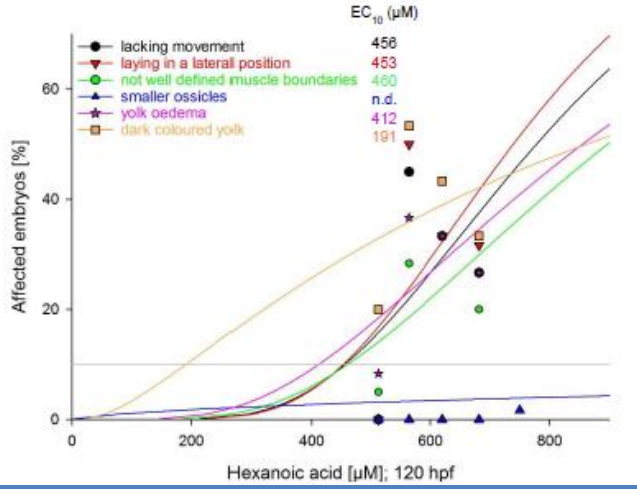


Effect	EC_{10} (µM)	**LOEC** (µM)	**NOEC** (µM)
Coagulated	697	910.33	538.66
No hatched	875	910.33	700.26
Craniofacial deformation	626	414.35	<414.35
Blood pooling	654	414.35	<414.35
Reduced heart beat	854	910.33	700.26
Scoliosis/lordosis	747	910.33	700.26
Lacking movement	822	910.33	700.26
Laying in a lateral position	770	910.33	700.26
Not well defined muscle boundaries	645	700.26	538.66
Pericardial oedema	568	700.26	538.66
Yolk oedema	618	700.26	538.66
Dark coloured yolk	596	700.26	538.66

Most sensitive effects: yolk oedemata, pericardial oedemata, blood pooling
Most prominent effect: craniofacial deformation
Please note: pericardial and yolk oedemata appeared in all tested substances (even at the lowest test concentrations). It can be the first sign of the embryo feeling unwell. This effect can be also reversible.

A9. Hexanoic acid

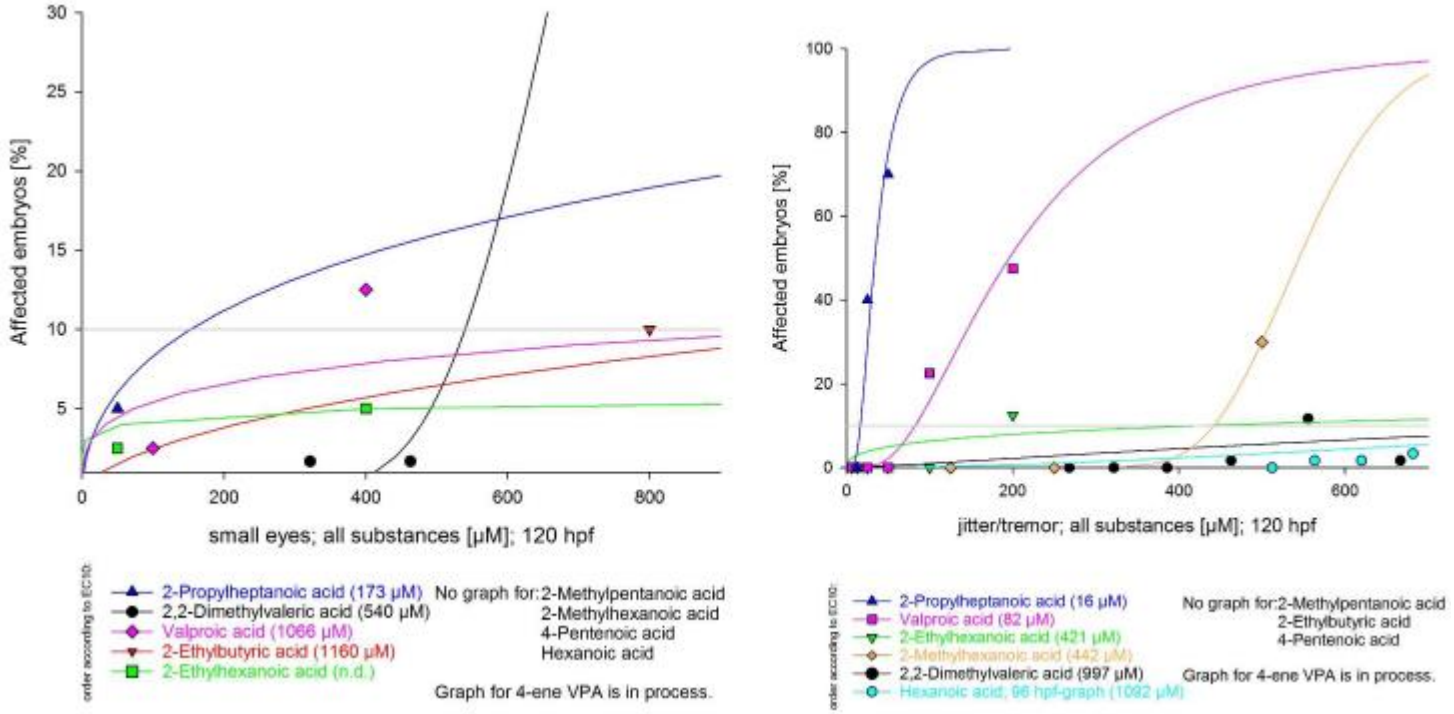


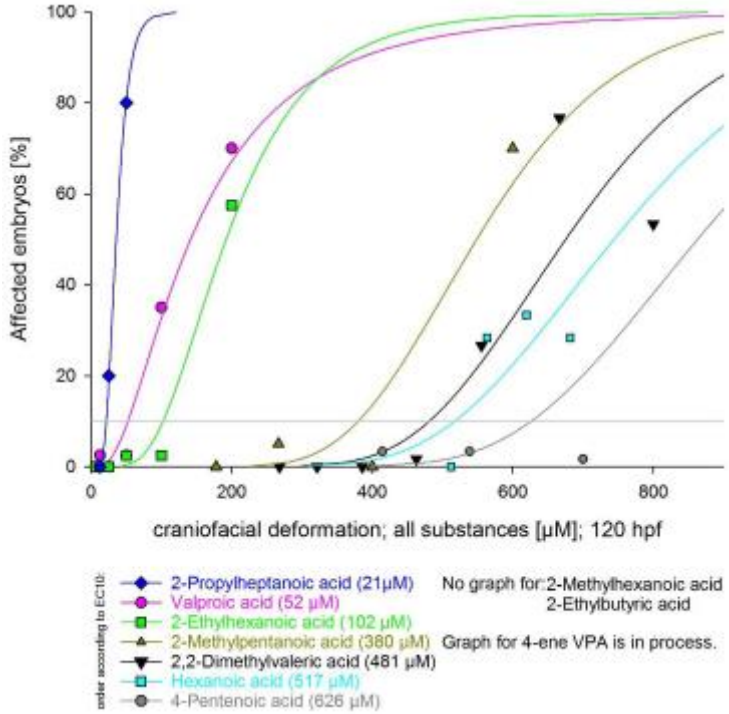


Effect	EC_{10} (µM)	LOEC (µM)	NOEC (µM)
Coagulated	557	563.49	512.26
Jitter/tremor	1092 (96 hpf)	681.81	<681.81
No heart beat	897	681.81	619.83
Craniofacial deformation	517	563.49	512.26
Blood pooling	510	563.49	512.26
Reduced heart beat	848	750	563.49
Scoliosis/lordosis	490	563.49	<490
Lacking movement	456	563.49	<456
Laying in a lateral position	453	563.49	<453
Not well defined muscle boundaries	460	750	>460
Smaller ossicles	n.d.	750	681.81
Yolk oedema	412	512.26	<412
Dark coloured yolk	191	512.26	<191

Most sensitive effects: blood pooling, pericardial oedemata, yolk oedemata
Most prominent effect: craniofacial deformation, laying in a lateral position (but still moving)
Please note: pericardial and yolk oedemata appeared in all tested substances (even at the lowest test concentrations). It can be the first sign of the embryo feeling unwell. This effect can be also reversible

B1. Effects in zebrafish embryo relating to NTD in human; comparison of all substances for three effects (nominal concentration; small eyes, jitter/tremor, craniofacial deformation).




Affected embryos [%]
100
80
60
40
20
0
0
200
400
600
800
craniofacial deformation; all substances [µM]; 120 hpf
order according to EC10:
2-Propylheptanoic acid (21µM)
Valproic acid (52 µM)
2-Ethylhexanoic acid (102 µM)
2-Methylpentanoic acid (380 µM)
2,2-Dimethylvaleric acid (481 µM)
Hexanoic acid (517 µM)
4-Pentenoic acid (626 µM)
No graph for:2-Methylhexanoic acid
2-Ethylbutyric acid
Graph for 4-ene VPA is in process.

3. ZET-3: Method description ZET Reporter CHBA

Zebrafish ceratohyal angle assay description

Background:

Most bony features of the face are formed by endochondral ossification of pre-existing cartilage structures. As an assay for craniofacial developmental and reproductive toxicity (DART) effects, the ceratohyal angle assay assesses the morphological appearance of the ceratohyal as a representation of overall facial cartilage development. The ceratohyal is a characteristic V-shaped facial cartilage structure, which is clearly visible in ventral views of the rostral part of zebrafish embryos of the fluorescent collagen reporter line TG (col2:mCherry) at 5 days post fertilisation (DPF) (figure 1). As the angle of the ceratohyal increases along with general exacerbation of cartilage deterioration, it serves as a directly quantifiable proxy for craniofacial DART effects of chemical substances see (figure 2).

Assay description:

The exposure regime is a repeat-dose exposure, with exposure medium changes every 24 hours for 5 days, adapted from the OECD 236 test guideline for zebrafish embryonic toxicity. Embryos are positioned for ventral view image acquisition in a semiautomatic manner, using the Vertebrate Automated Screening Technology (VAST) bioimager (Union Biometrica, USA) (figure 3), and fluorescence images are acquired at 40 times magnification, using a standard fluorescent microscope. CHA values are measured using imageJ.

Data handling:

CHA measurements are transformed into a percentage response based on normalisation to control measurements made in age matched control siblings, which defines 0% response, and 180 (the ceratohyal forming a straight line) which is defined as 100% response (figure 4).

After normalisation CHA datapoints are plotted against log-transformed exposure values, and curves are fitted by non-linear regression (figure 5). EC_{10} values are derived using Graphpad Prism 7 (Graphpad Software, USA).

Acceptance criteria:

The following acceptance criteria should be applied for the control of embryonic batch quality and validity of CHA measurements:

- Overall fertilisation rate of all eggs collected should be ≥ 80% per batch.

- Overall survival of embryos in the negative (dilution-water) control, and in the solvent control should be ≥ 80% until the end of the 120 hours of exposure.
- Hatching rate should be ≥ 80% at the end of 120 hours of exposure.

Uncertainties:

There are two principal elements of uncertainty associated with the interpretation of data generated from this assay, which relates to the interpretation of positive and negative results, these are discussed in turn:

I) One very general source of uncertainty concerns compounds for which a dose-response curve can be fitted, and is related to the accuracy of the curve and derived parameters such as EC_{10}. This assay is very specific to craniofacial cartilage structures and relies on accurate orientation of the larvae. In some cases, notably with compounds exhibiting very low potencies in the CHA assay, this leads to difficulties in capturing data points approaching 100% effect, due to apparently aspecific toxicological effects and overall morphological distortions of the larvae. This uncertainty is reflected directly in the accuracy of the subsequent curve fitting to the doseresponse data points, and thus can be addressed directly through the R^2 value. Based on our observations we consider R^2 values above 0,9 as low uncertainty, R^2 values between 0,7 and 0,9 as medium uncertainty and R^2 values below 0,7 ad high uncertainty. EC_{10} values derived from dose-response curves with R^2 values below 0,7 should be considered ambiguous results and should be used with caution. In this data set hexanoic acid provides a good example. As it only begins to exhibit clear CHA effects at concentrations when serious morphological distortions and lethality become apparent. The VAST bioimager offers the possibility to store brightfield images of embryos as they are positioned and rotated for fluorescent microscopy and image acquisition. It is recommended that the fluorescent image output is supplemented by representative brightfield images, to provide an estimate of overall morphological distortion of the embryos at increasing compound concentration, in support of uncertainty assessment (see figure 6).

II) Another type of uncertainty concerns the interpretation of data derived from compounds for which no doseresponse curve could be fitted. This will be the case when the compound fails to exert any measurable effect on the CHA, and the uncertainty relates to the interpretation of the reason for this lack of measurable response. Since the endpoint is recorded at 5 DPF, compounds exhibiting potent lethal effects at earlier timepoints may result in the inability of the assay to return a dose-response curve. Therefore, it may be similarly difficult to ascribe an EC_{10} value to a potent but aspecifically toxic compound as to a compound for which a no effect can be observed at all, specific or not. In this data set, 2-methyl hexanoic acid and pentenoic acid, both registering N/A in EC_{10}, are representatives of the former and the latter example respectively (see figure 7).

To overcome such difficulties, it is recommended that notes detailing observations regarding toxicity manifestations at time points leading up to the 5 DPF endpoint are recorded, e.g. during range finding experiments preceding the detailed CHA assessment.

Figure 1. The ceratohyal angle (CHA) in ventral views of 5 DPF zebrafish embryos

Ventral view of the rostral part of a 5 DPF *col2a:mCherry* embryo. The ceratohyal is visible as a V-shaped, highly fluorescent collagen expressing structure. The CHA is measured directly in ventral-view images like the one depicted here.

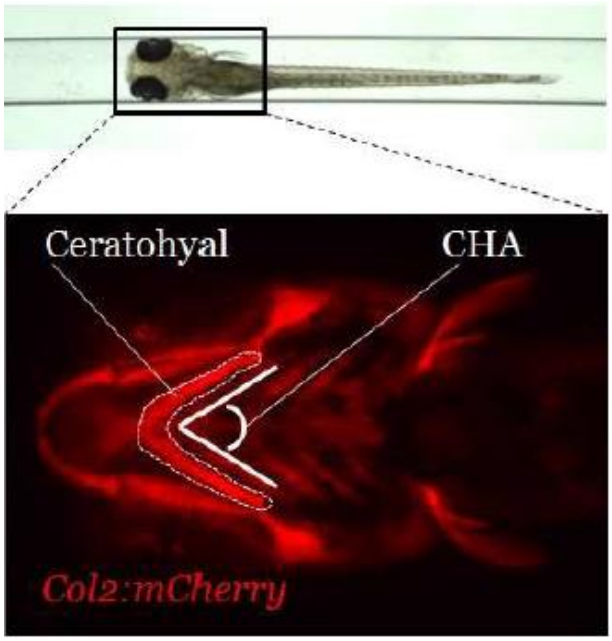


Figure 2. Example of dose-dependent CHA expansion

The well-known DART causing compound Valproic acid causes morphological distortions to all facial cartilage structures in a dose-dependent manner which is quantified through the increase in CHA.

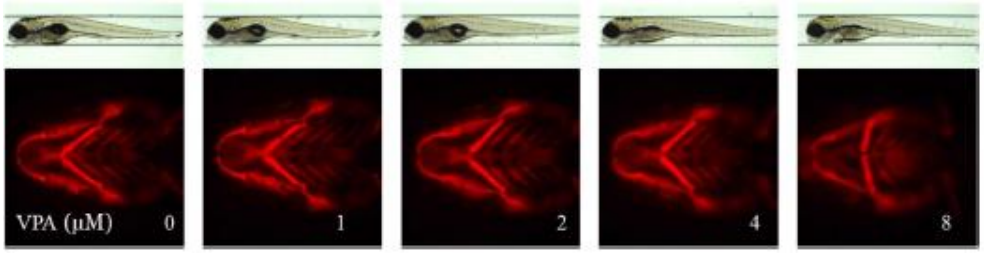


Figure 3. Vertebrate Automated Screening Technology (VAST) bioimager

The VAST bioimager is a semiautomated system facilitating rapid and uniform image acquisition in any orientation along the body axis of test subjects. The test subjects are pumped through tubing to a glass capillary (blow-up in the top left corner). By rotating the capillary it is possible to capture ventral views in a uniform manner suitable for standardised image analysis. (Reproduced with permission from Union Biometrica, USA).

Figure 4. Data normalisation.

CHA data is normalised based on the mean of the control group, which is defined as 0% response. For relativisation purposes maximum response is defined as 180° when the ceratohyal forms a straight line.

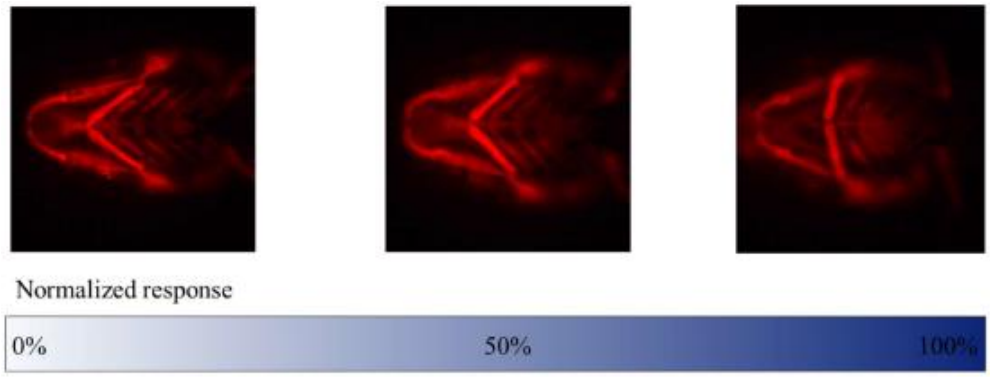


Figure 5. Dose-response curves

After normalisation CHA response data points are plotted against logtransformed nominal concentration values and a dose response curve is fitted to the data points by nonlinear regression. R^2 values indicate the accuracy of curve fitting.

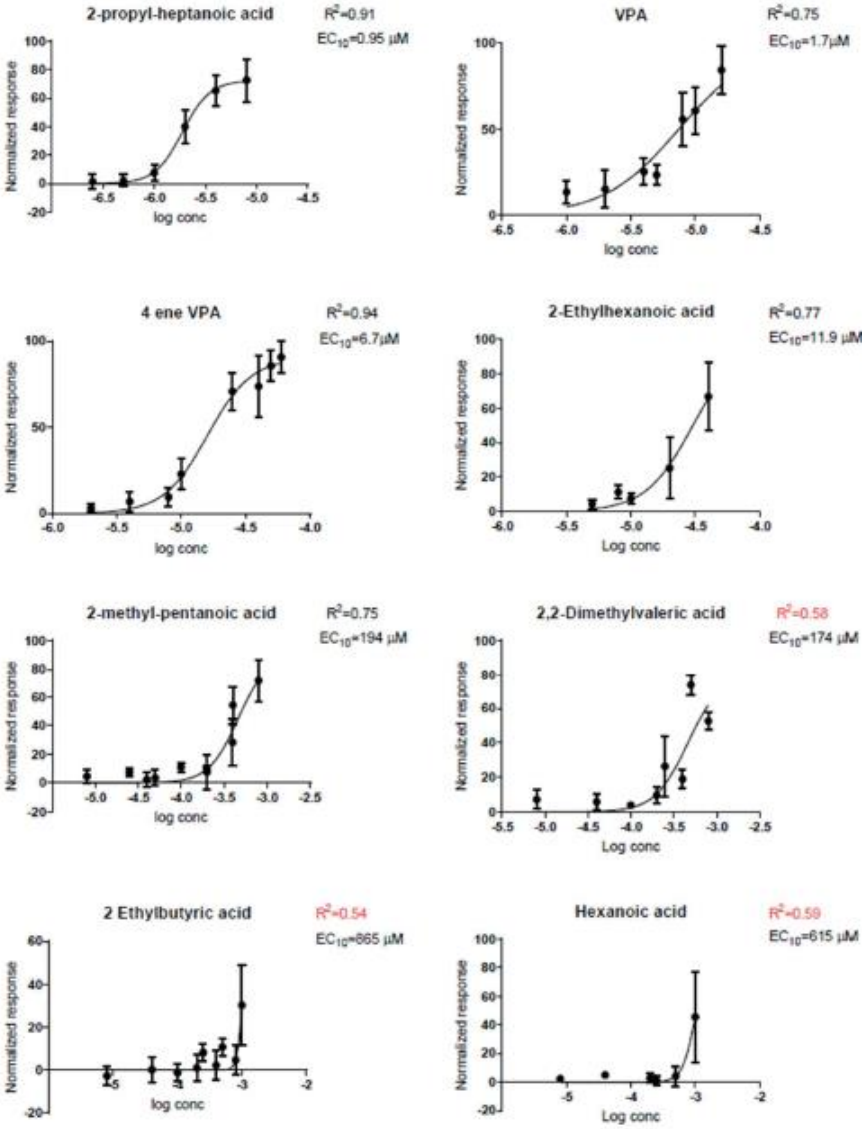


Figure 6. Uncertainty in interpreting positive results, curve fitting

One type of uncertainty of data interpretation concerns all those compounds for which a dose dependent response could be recorded, the majority of compounds tested in this assay. The most critical aspect influencing this uncertainty is the level of overlap between specific CHA effects and aspecific morphological manifestations. Aspecific toxic effects which are usually visible as distortions to embryonic morphology may affect the accuracy of the CHA assessment and may lead to difficulties in acquiring high quality data points in the high effect range. One example of this difficulty is hexanoic acid, which displays strong overlapping morphological manifestations of aspecific toxicity at compound concentrations when CHA measurements begin to become apparent, as it is visible in the brightfiels images depicted above the fluorescent images. This uncertainty is accurately captured by the R^2 estimate of dose-response curvefit.

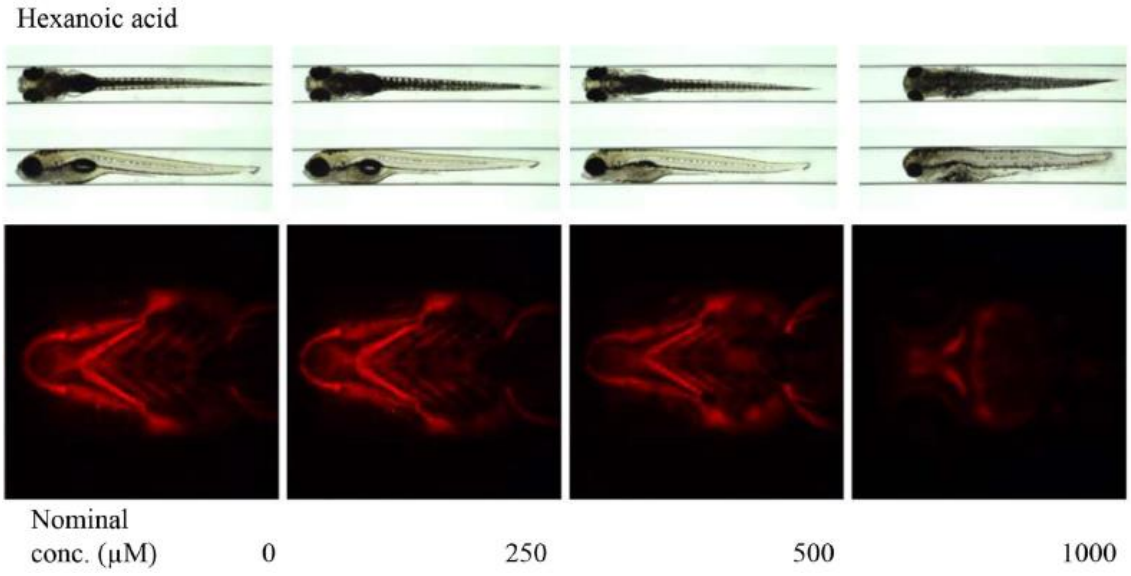
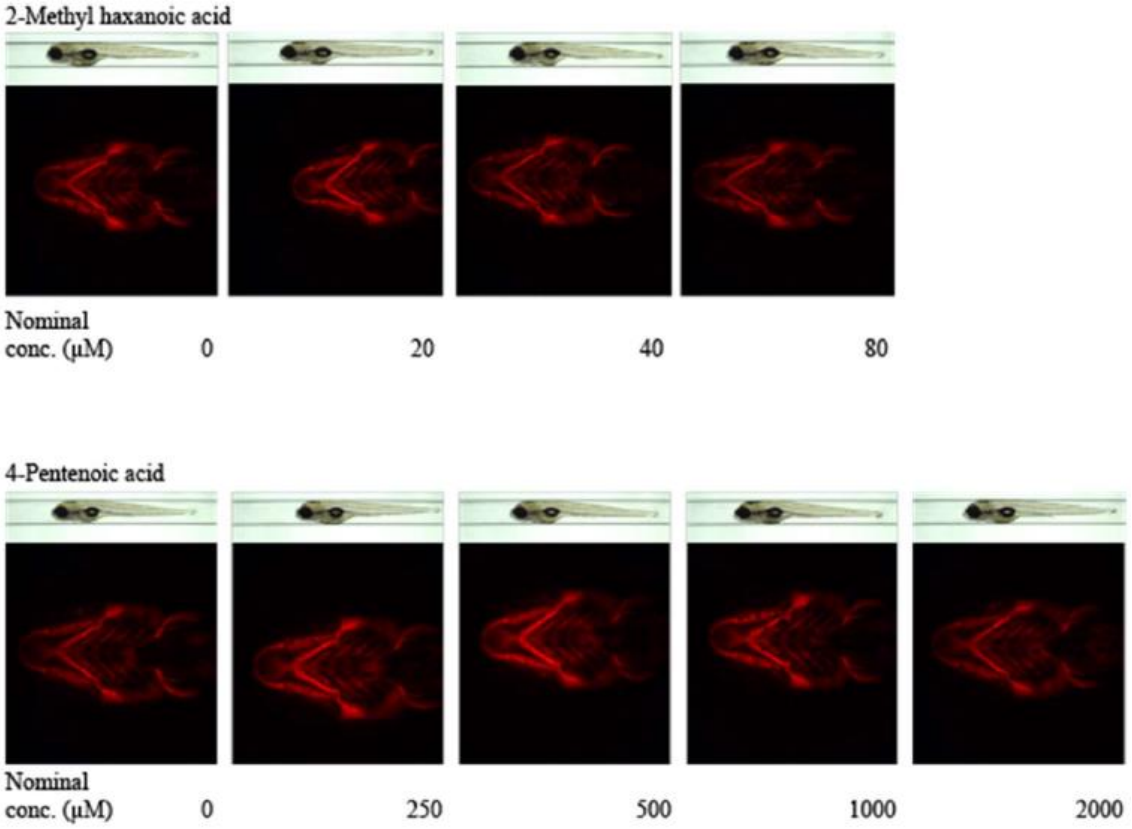


Figure 7. uncertainty in interpreting negative results

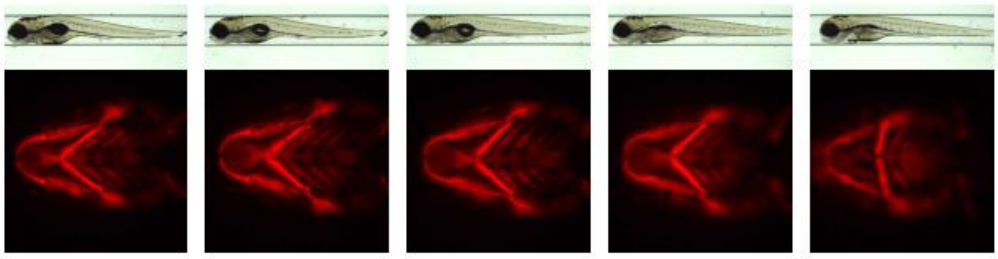
Another type of uncertainty concerns compounds for which no response could be recorded. There are two scenarios in which a compound can fail to show an effect: For some compounds lethal toxicity may manifest itself before 5 DPF or at concentrations below those which cause measureable CHA effects, in which case the assay will not provide the data to fit a dose-response curve to. In this data set, 2-methyl hexanoic acid is an example of such a compound. There are no measurable effects of exposure up to 80 µM nominal concentration dose, but at 160 µM the test subjects die before 5 DPF. Other compounds, like 4-pentenoic acid, test negative even at the highest tested concentrations. In which case the assay will also not be able to provide an estimate of effective concentrations.



4. ZET-4: Individual effect curves and EC_{10} for all analogues

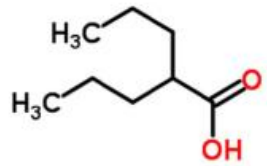
CHA effects of valproic acid analogues

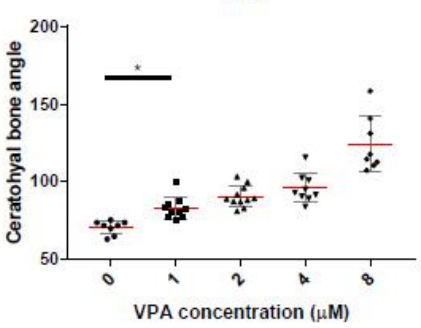
Valproic acid (VPA)



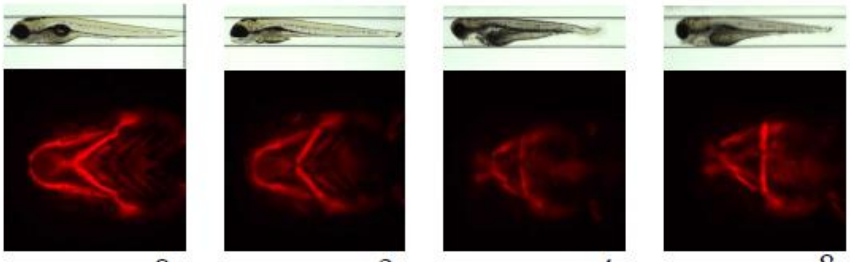
Compound Conc. (µM) 0 1 2 4 8

Positive



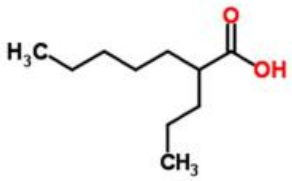


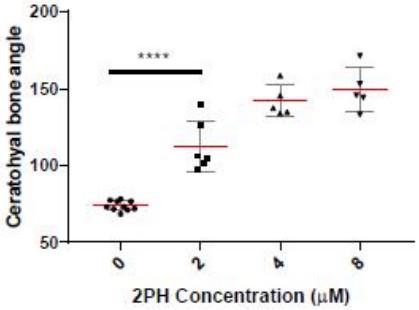
2-propyl-heptanoic acid (2PH)



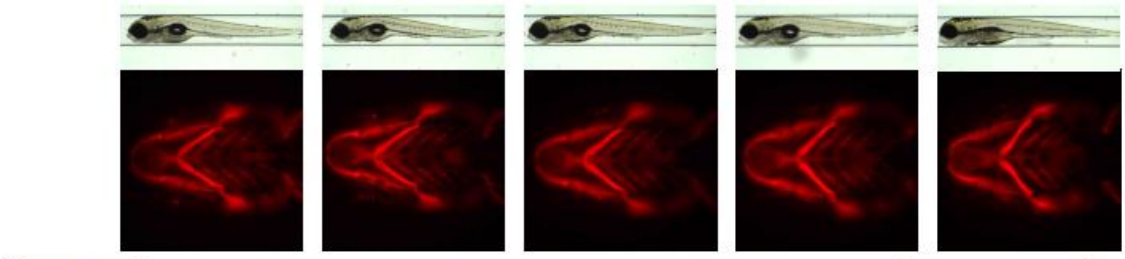
Compound Conc. (µM) 0 (0,1% DMSO) 2 4 8

Positive



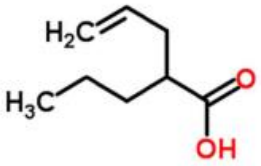


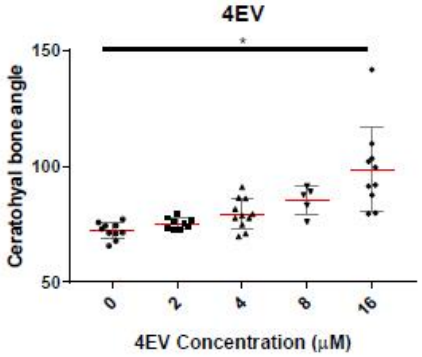
4-ene-VPA (4EV)



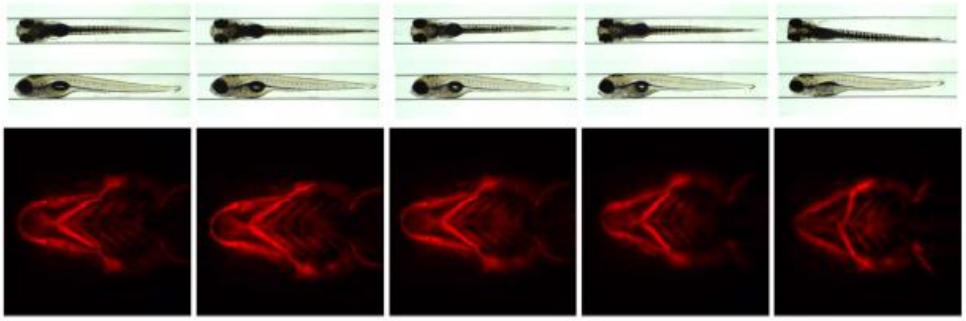
Compound Conc. (µM) 0 2 4 8 16

Positive



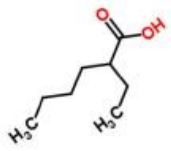


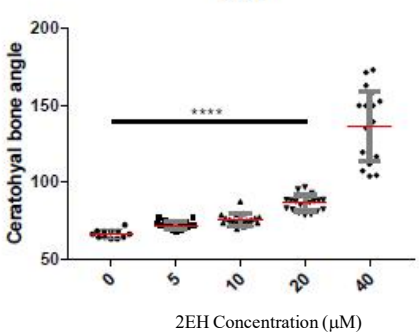
2-Ethylhexanoic acid (2EH)



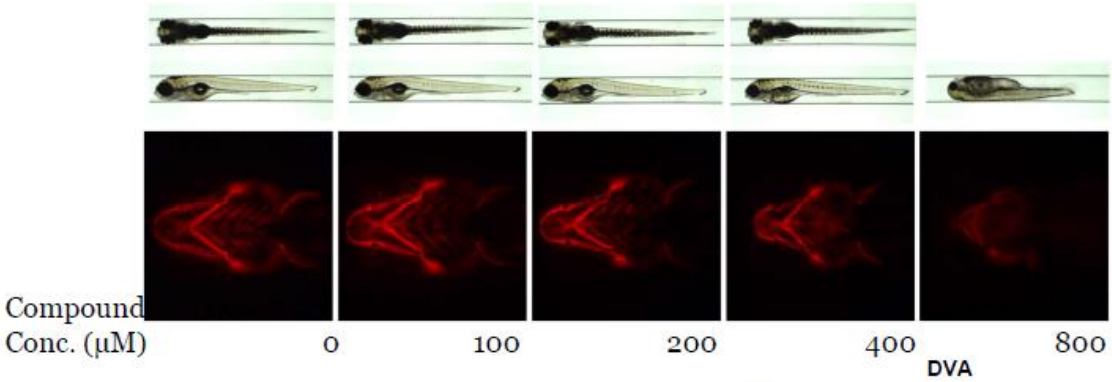
Compound Conc. (µM) 0 5 10 20 40

Positive

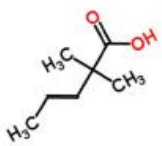


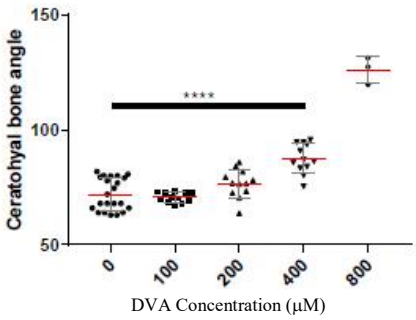


2,2-Dimethylvaleric acid (DVA)

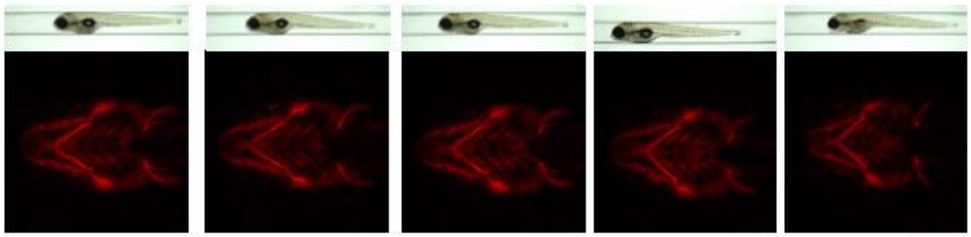


Positive, reaching statistical significance only at high concentrations.

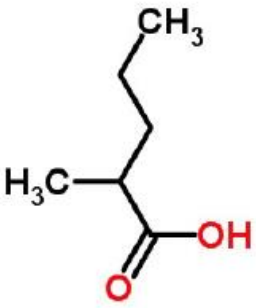


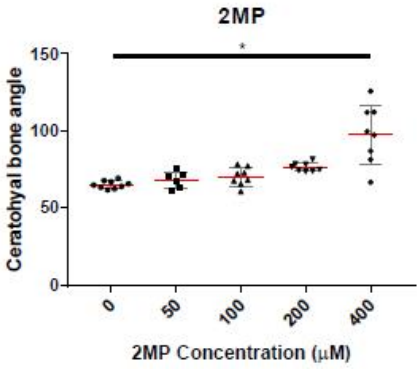


2-Methylpentanoic acid (2MP)

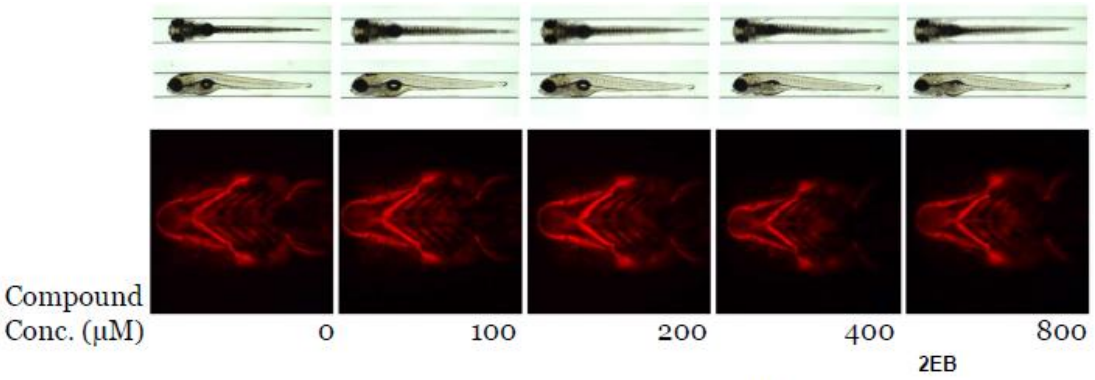


Positive, reaching statistical significance only at high concentrations.

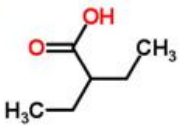


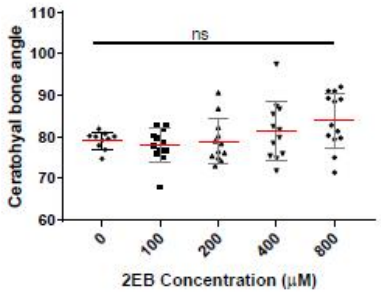


2-Ethylbutyric acid (2EB)

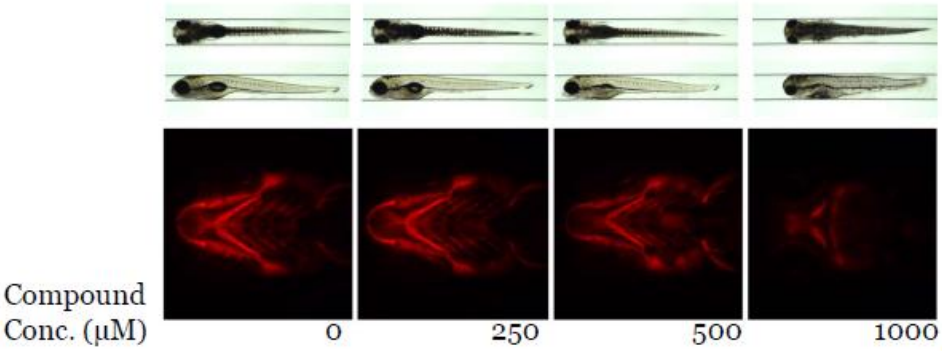


Negative at concentrations tested. Substantial mortality was observed at 1 mM in range-finding experiment

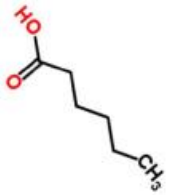


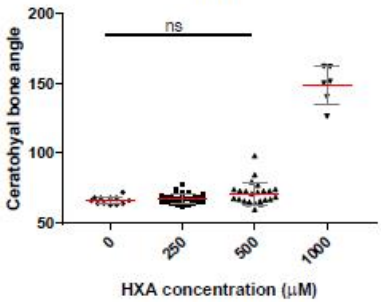


Hexanoic acid (HXA)

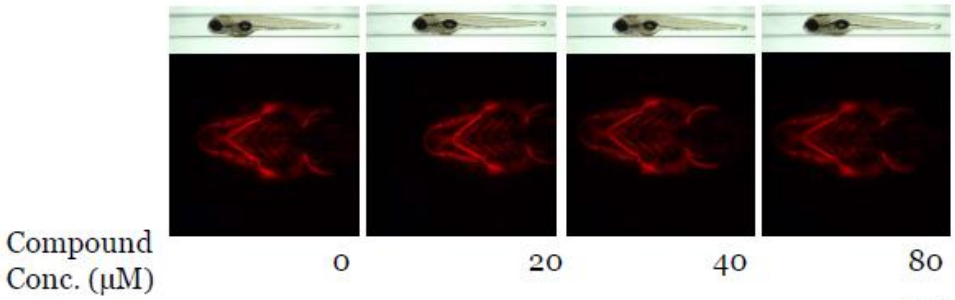


Negative at the concentrations tested. Several dead and unhatched embryos at 1 mM.

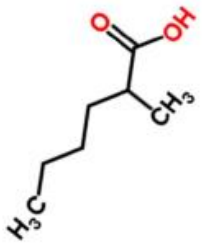


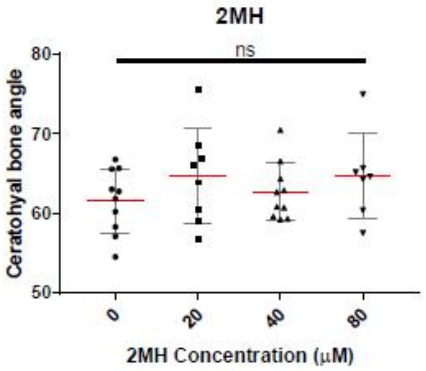


2-Methylhexanoic acid

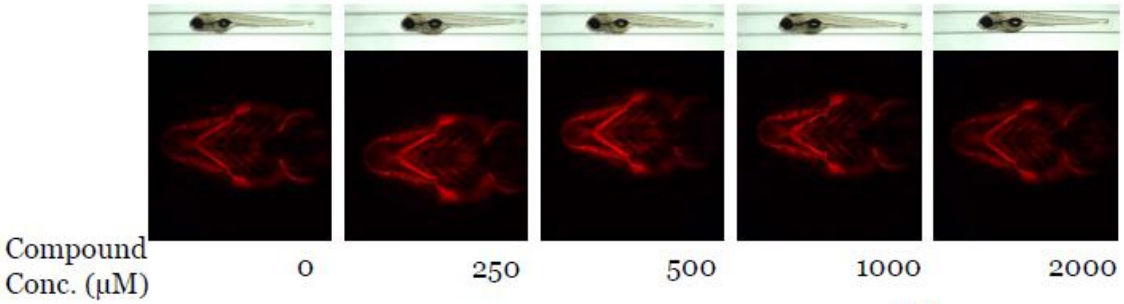


Negative, substantial mortality at 160 µM

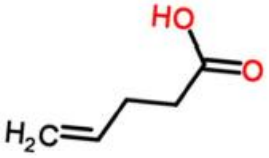


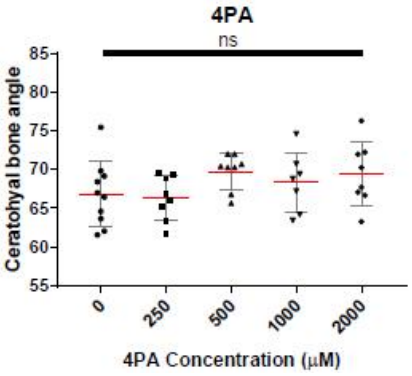


4-Pentenoic acid (4PA)



Negative





Summary of DART experiments

Compounds tested	Result:
- VPA	+++
- 2-propyl-heptanoic acid	+++
- 4-ene VPA	++
- 2-Ethylhexanoic acid	++
- 2,2-Dimethylvaleric acid	+
- 2-Methylpentanoic acid	+
- 2-Ethylbutyric acid	-
- Hexanoic acid	-
- 2-Methylhexanoic acid	-
- 4-Pentenoic acid	-

Key:
+++ Significant below 10 µM
++ Significant below 100 µM
+ Significant below 1000 µM
- Not significant

5. EST-1: Method description EST (DB-ALM)

EST_Data Content Last Update: September 2013 (v.9.4)

The present template is based on the Content Criteria for Protocols, which are designed for the provision of technical details that enable the documented alternative method to be transferred to other laboratories without the need of additional information.

The Content Criteria, on which the presented template is based, are generic and based on the analysis of common descriptors from hundreds of different non-animal experimental methods and techniques. However, not all parameters or sections indicated are applicable to all protocols. It is the responsibility of the author to provide content where relevant and as appropriate related to a precise protocol. The compilation is to be performed by completing the individual sections with the information related to your method. A review for consistency, completeness in relation to the technique described and compliance with the Content Criteria in place is always performed by the JRC staff. Furthermore, before any protocol is published via the DB-ALM, the final draft is reviewed and approved by designated contact person(s) (method's owner and/or experienced user).

A summary description of the main method features bringing it in a context regarding its intended purpose(s) and application(s), as well as the scientific rationale is always to be provided with each protocol. The content criteria for the method summary will be sent in due course.

The DB-ALM is operated by the European Reference Laboratory for Alternatives to Animal Testing of the Joint Research Centre

Part A. Protocol Introduction

Protocol Name: Embryonic Stem Cell Test

Abstract: The principle of the Embryonic Stem Cell Test for Embryotoxicity/ Teratogenicity is based on the unique capacity of embryonic stem cells (ES cells) to differentiate into several cell types.

Résumé

The *in vitro* mouse Embryonic Stem Cell Test (mEST) assay is an implemented routine assay at Roche. The original EST was developed by Horst Spielmann and his group in 1997 as an *in vitro* model for the screening of embryotoxicity, based on a blastocyst-derived permanent embryonic mouse ESC (mESC) D3 cell line derived from mouse 129 strains and was validated by European Centre for the Validation of Alternative Methods (ECVAM). We further optimised and modified the approach allowing for the application of the assay to pharmaceutical compounds.

Experimental Description

Biological Endpoint and Endpoint Measurement:

The cytotoxicity (inhibition of growth) of 3T3 fibroblasts, which represents differentiated cells and the cytotoxicity of undifferentiated embryonic stem cells (D3) after 10 days of substance treatment serve as two assay endpoints. This is determined by the use of dehydrogenase enzymes, which are present in the intact mitochondria of living cells to convert yellow soluble substrate 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) into a dark blue insoluble formazan product, which gets sequestered within the cells and is detected quantitatively using an absorbance reader (570nm) after solubilising the cell membrane.

The third endpoint is the inhibition of differentiation of ES cells into myocards which are cardiac muscle cells after 10 days of treatment. The beating of this cells is evaluated by microscopy.

Endpoint Value:

Viability: IC_{50} 3T3

Viability: IC_{50} D3

Inhibition of differentiation: ID_{50} D3

Experimental System:

Embryonic Stem cell test is performed with two permanent mouse cell lines, 3T3 fibroblasts and embryonic stem (ES) cells of the D3 line.

Discussion

The mEST does not use embryonic tissue from pregnant animals but only two permanent mouse cell lines.

The mEST-Assay test duration is 10 days with several medium change intervals.

The test should be performed at least under Biosafety level 1 and done in a sterile bench with a vacuum pump system to collect all liquid waste.

A special training is needed to know the method especially the evaluation of contracting cardiomyocytes by microscopy and for data interpretation.

A critical attention point is the addition of fetal bovine serum which is necessary to allow the spontaneous differentiation. The serum ingredients are batch dependent, therefore several preliminary tests such as a differentiation-test have to be run to achieve the an appropriate cell differentiation.

Status

In Development:

Fully established as an alternative test method

Known Laboratory Use:

n/a

Participation in Evaluation Study:

The mEST Assay has shown increased robustness and concordance to *in vivo* adverse developmental effects, whilst maintaining a low false positive rate.

Participation in Validation Study:

n/a

Regulatory Accepted:

The mEST is not a regulatory requirement only 'voluntary' testing of early pharma pipeline.

Proprietary and/or Confidentiality Issues

See actual agreement with F. Hoffman-La Roche AG.

Health and Safety Issues

General precautions:

The Test should be performed under Biosafety level 1. All solid and liquid waste should be cleared accordingly to biosafety level 1.

MSDS Information:

mESCells: https://www.atcc.org/Products/All/CRL-1934.aspx#documentation

mouse Fibroblasts: https://www.atcc.org/Products/All/CCL-163.aspx#documentation

DMEM: https://www.thermofisher.com/order/catalog/product/41966029

5-Flurouracil:
https://www.sigmaaldrich.com/catalog/product/sigma/f6627?lang=de&region=CH

SDS: http://shop.biosolve-chemicals.eu/detail.php?id=2330

mLIF: https://www.sigmaaldrich.com/catalog/product/sigma/l5158?lang=de&region=CH

β-Mercapthoethanol:
https://www.sigmaaldrich.com/catalog/product/aldrich/m6250?lang=de&region=CH

MTT: https://www.tocris.com/products/mtt_5224

N, N-DMF:
https://www.sigmaaldrich.com/catalog/product/sial/40250?lang=de&region=CH

DMSO:
https://www.sigmaaldrich.com/catalog/product/sigma/d2650?lang=de&region=CH

L-Glutamin: https://www.thermofisher.com/order/catalog/product/25030024?SID=srch-hj-25030024

Penicillin/Streptomycin:
https://www.thermofisher.com/order/catalog/product/15140122?SID=srch-hj-15140-122

0.05% Trypsin/EDTA:
https://www.thermofisher.com/order/catalog/product/25300054?SID=srch-hj-25300-054

NAA (100x): https://www.thermofisher.com/order/catalog/product/11140035?SID=srch-hj-11140-035

PBS (-$CaCl_2$/-$MgCl_2$):
https://www.thermofisher.com/order/catalog/product/14190094?SID=srch-hj-14190-094

Distilled Water Ultra-Pure:
https://www.thermofisher.com/search/results?query=10977-035&focusarea=Search%20All

Trypan Blue (0.4%) für Cell Counter:
https://www.thermofisher.com/order/catalog/product/T10282?SID=srch-hj-T10282

FCS:
https://cdn.gelifesciences.com/dmm3bwsv3/AssetStream.aspx?mediaformatid=10061&destinationid=10016&assetid=18092

Abbreviations and Definitions

mEST	Mouse embryonic stem cell test
ES Cells	Embryonic stem cells
3T3	Mouse Fibroblasts
D3	ES Cells
5 - FU	5 - Fluorouracil
SDS	Sodium Dodecyl Sulfate
FCS	Foetal calf serum
N,N-DMF	Dimethylformamid
DMSO	Dimethylsulfoxid
PBS	Phosphat Buffered Saline
DMEM	**D**ulbecco's **M**odified **E**agle's **M**edium
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium Bromid
β-ME	β-Mercapthoethanol
mLIF	Mouse Leukemia Inhibitory Factor
NAA	**N**on **E**ssential **A**mino**a**cids

Last Update: 28.06.2018

Part B. Technical Description

Procedure Details, Latest Version: 14.12.17

Protocol Name: Embryonic Stem Cell Test

Final modified protocol

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Materials and Preparations

CELL OR EXPERIMENTAL SYSTEM

Balb/c 3T3 cell clone A31, American Type Culture Collection (ATCC) Cat No CCL-163
ES-D3 (D3), American Type Culture Collection (ATCC) Cat No CRL-1934

EQUIPMENT

Fixed Equipment

Laminar flow hood, Incubator, Water bath, Automated Cell Counter, Microscope, MTPShaker, Absorbance reader, centrifuge, Fridge, Freezer, Ultrasonic, autoclave, analytical balance, Vortex, N2-Tank, magnetic stirrer, pipettes (multichannel and/or one channel) 10µl, 100µl, 200µl, 1000µl and 1200µl

Consumables

Plastic ware (T-Flasks, 25 cm^2 and 75 cm^2, Petri dishes Ǿ100mm and Ǿ60mm, Falcon tubes 5ml, 14ml, 15ml and 25ml, microtiter plates 96well and 24well, Millipore filter 250ml and 500ml, 96er deep-well plate 500µ, cryotubes 2ml)

Disposable materials (countess chamber slides, serological pipettes 2ml, 5ml, 10ml, 25ml and 50ml, Erlenmeyer flasks 250ml and 125 ml, Socorex reservoir 12 channel, LLGdisposable reservoir, Eppendorf tubes 1.5 ml)

MEDIA, REAGENTS, SERA, OTHERS

FCS	Hyclone, Cat No SH30070.03
DMEM with Glucose, Glutamin, $NaHCO_3$	Gibco, Cat No 41966-029
L-Glutamine (100x)	Gibco, Cat No 25030-024
Penicillin/Streptomycin	Gibco, Cat No 15140-122

B-Mercaptoethanol	Sigma, Cat No M6256
m-LIF	Sigma, Cat No L5158-5UG
0.05% Trypsin/EDTA	Gibco, Cat No 25300-054
NEAA (100x)	Gibco, Cat No 11140-035
Trypan blue 0.04%	Gibco, Cat No T10282
5-Fluorouracil	Sigma, Cat No F-6627-5G
PBS (-$CaCl_2$/-$MgCl_2$)	Gibco, Cat No 14190-094
Distilled Water, Ultra-Pure	Gibco, Cat No 10977-035
MTT Tocris	Bioscience, Cat No 5224/500
DMSO	Fluka, Cat No D2650

PREPARATIONS

Media and Endpoint Assay Solutions

Culture Media:

3T3	D3
10% FCS	20% FCS
4 mM Glutamin	2 mM Glutamin
50 U/ml Penicillin	50 U/ml Penicillin
50 µg/ml Streptomycin	50 µg/ml Streptomycin
	1% NAA
	0.1 mM β-ME
	1000 U/ml m-Lif (only added separately to subculture)

Assay Media:

3T3	D3
10% FCS	20% FCS
4 mM Glutamin	2 mM Glutamin
50 U/ml Penicillin	50 U/ml Penicillin
50 µg/ml Streptomycin	50 µg/ml Streptomycin
	1% NAA
	0.1 mM β-ME

Media for Freezing Cells:

3T3	D3
20% FCS	40% FCS
4 mM Glutamin	2 mM Glutamin
50 U/ml Penicillin	50 U/ml Penicillin
50 µg/ml Streptomycin	50 µg/ml Streptomycin
	1% NAA
	0.1 mM β-ME
7 % DMSO	7 % DMSO

β-Mercapthoethanol (β-ME) (10 mM)

17.5µl β-ME added to 25 ml of PBS

Store at 4°C for max. 1 week

FCS

Thaw FCS once up by water bath (37°C) and make aliquots of 100ml, 50 ml and 25ml.

Avoid multiple thawing

Store at -20°C, Expiry date: see Date on bottle

MTT-Solution

5mg MTT/ml PBS

Use a sterile Filter from Milipore and make aliquots of 8ml and 4 ml

Store at -20°C, Expiry date: see Date on bottle

MTT-Desorb-Solution

20% SDS solved in water/DMF, 1:1, adjust the pH to 4.5 with acetic acid

Test Compounds

Stock solution: 200 mM

Solvent: 100% DMSO

1. Compound Stock Preparation (Concentration A)
    - Weigh powder accordingly and dissolve in 100% DMSO to achieve a concentration of 200 mM (stock solution A).
    - This stock solution is re-used for each medium change of 10-day assay incubation but is made up fresh for a new assay run (e.g. confirmation run).
    - Note down any visible precipitation, cloudiness or colour changes.
2. Intermediate DMSO stocks: compound dilutions for concentrations B to G
    - Dilute an aliquot of the 200 mM stock 1:5 with 100% DMSO
    - Example: for concentration B, dissolve 20 µl of 200mM stock into 100 µl of 100% DMSO
    - Mix well by up and down pipetting, then take aliquot of concentration B, and dilute 1:5 in 100% DMSO
    - Prepare other concentrations accordingly.
    - Note down any visible precipitation, cloudiness or colour changes.
3. Final compound dilutions in assay
    - Final concentration of DMSO in assay: 0.25 % (for all concentrations)
    - Dilution factor: 1:400
    - Dilute each concentration 1:400 in pre-warmed culture medium (for example: 5 µl in 2 ml prepared for each concentration separately) for sufficient volume to give 3 replicates in a 96 well plate.

- For the dose-range finder, the final assay concentrations are 500, 100, 20, 4, 0.8, 0.16 and 0.032 µM.
- For the cytotoxicity assay, all 7 concentrations are used (plus a solvent control = 0.25% DMSO).
- For the differentiation assay, 6 concentrations (500 to 0.16 µM) are used (plus solvent control).
- Note down any visible precipitation, cloudiness or colour changes (also during media changes).

Positive Control(s)

5-Fluoruracil: (stocksolutions are prepared with DMSO)

-conc for mESC: 2mg/ml

-conc for 3T3: 2.5 mg/ml

Dilute with factor 400 direct into medium

Negative Control(s)

Solvent control: Add solvent (usually DMSO) in the appropriate conc to the media.

Method

EXPERIMENTAL SYSTEM PROCUREMENT

N/A

ROUTINE PROCEDURES

1) Cell passage with trypsin 0.05% EDTA for D3 and 3T3 cells

- check Falcon flat flask with microscope
- pre-incubate trypsin and media at 37°C
- remove media from flask with pipette / vacuum
- add 5 ml PBS, wash, remove supernatant
- add 1 ml trypsin / EDTA, incubate 40 sec at 37°C / 5% CO_2
- shake and hit the flask to separate cells from walls
- add 8 ml media
- transfer into a 15 ml Falcon tube
- centrifuge at 1000 x g for 5 min (Mulifuge 3SR Heraeus)
- discard supernatant (pour out)
- dilute cells with 2 – 6 ml media (depends on the size of the pellet)

2) Assembly of the cell suspension

- mix dilution of 10µL trypan blue solution and 10µL cell suspension, mix thoroughly and count cells in Hemocytometer, calculate appropriate cell number:

EScells	Subcultivation is performed every 2 – 3 days. Add cell suspension in a T25 cm^2 cell culture flask each with $0.6x10^6$ cells (Monday & Wednesday) and 1.5 x 10^5 cells to 10 ml medium in total. Add 1 µl m-LIF to 1 ml of fresh culture medium.
3T3-cells	Subcultivation is performed every 3 – 4 days. Add cell suspension of 4 x 10^5 cells/ml to 15 ml medium into a T75 cm^2 culture flask (every Monday and Friday).

TEST MATERIAL EXPOSURE PROCEDURES

DIFFERENTIATION

DAY 0

1) Cell passage with trypsin 0.05% EDTA for D3 cells

Cell Count: 2.5 x 10^4 cells/ ml

- check Falcon flat flask with microscope
- pre-incubate trypsin and media at 37°C
- remove media from flask with pipette / vacuum pump
- add 5 ml PBS, wash, remove supernatant
- add 1 ml trypsin / EDTA, incubate 40.sec. at 37 °C / 5% CO_2
- shake and hit the flask to separate cells from walls
- add 10 ml media
- transfer into a 15 ml Falcon tube
- centrifuge at 1000 x g for 5 min
- discard supernatant (pour out)
- dilute cells with 2 – 6 ml media (dependent on the size of the pellet)

2) Assembly of the cell suspension

- count cells in Hemocytometer by adding 10 µL trypan blue solution to 10 µL of cell suspension
- dilute cells to 2.5 x 10^4 / ml with 18 ml media (for each test) in 50 ml Falcon tubes

3) Prepare the petridishes (PD)

- add 5-10 ml sterile Dulbecco's PBS (Gibco) into each dish bottom, distribute over the whole dish

4) Dilution series of compound in 5 ml PP tubes

- 6 tubes for the concentrations; fill with 2 ml cell suspension
- 1 tube for DMSO (solvent control) fill with 2 ml cell suspension

5) Dilution series of test compound in Eppendorf tubes

- stock solution: preparation see "Test compound"

- 6 concentrations (A-F). Dilution factor 1:5 (for the first run, after that depends on the results, according to the study plan)
- add 5 µl of compound (1:400 dilution) and 5µl control solution (DMSO) to 2 ml cell suspension, vortex

6) Preparation of hanging drops in petridishes

- vortex tube, aspirate suspension with automatic pipette and multi-dispense 20 µl drops onto the lid of the petridish, add 2 ml in total in a concentric circle of drops (~ 100 drops)
- quickly but smoothly turn the cover and put on PD
- incubate for 3 days at 37°C / 5% CO_2

DAY 3

1) Dilution series of compound in 14 ml PP tubes

- 6 tubes for the concentrations; fill with 5 ml Media
- 1 tube for DMSO (solvent control) fill with 5 ml Media

2) Dilution series of compound in Eppendorf tubes

- 6 concentrations (A-F). Dilution factor 1:5 (for the first run, after that depends on the results, according to the study plan)
- add 12.5 µl of compound (1:400 dilution) and 12.5 µl control solution, vortex

3) Transfer of embryoid bodies in a bacterial Petri dish

- Carefully turn PD lid, check the drops for fungus contamination
- Rinse several times the drops down with 5 ml of the prepared solution
- Transfer in a bacterial PD
- incubate for 3 days at 37 °C / 5% CO_2

DAY 5

1) Dilution series of compound in 50 ml tubes

- 6 tubes for the concentrations; fill with 25 ml media
- 1 tube for DMSO (solvent control) fill with 25 ml media

2) Dilution series of compound in 1.5 ml tubes

- stock solution: preparation see “Test compound”
- 6 concentrations (A-F). Dilution factor 1:5 (for the first run, after that depends on the results, according to the study plan)
- add 62.5 µl of compound (1:400 dilution) and control solution (DMSO), vortex

3) Preparation of 96 well plates

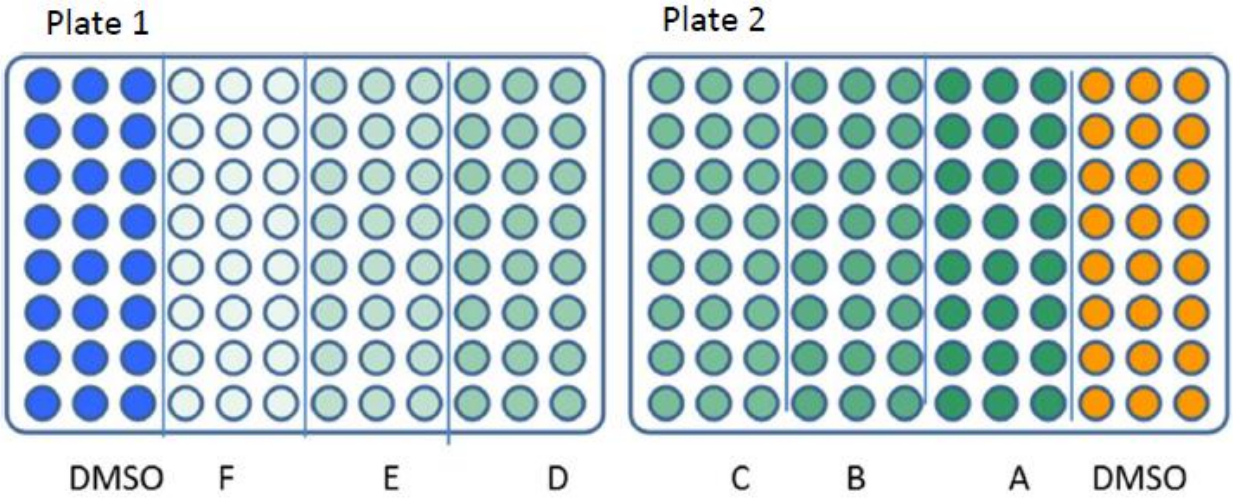
- 2 plates for each compound, see Compound-plate-Layout
- add 220 µl media/compound/solvent mix in all 96 wells

- start with low concentration

4) Pipetting of embryoid bodies

- visually control the EB's in the PD
- with a 25 µl tip, pipette one EB in each well
- check visually the plate to ensure that at least one EB is present in each well
- incubate for 3 days at 37°C / 5% CO_2

Compound-plate-layout



DAY 10

- visualise each well with microscope for beating myocard cells
- Media and DMSO controls should show at least 80% of beating cardiomyocyte cells.

CYTOTOXICITY

Stock solution with a concentration of 0.2 mol/L is created for all substances. Test substances are diluted in DMSO solution.

DAY 0

Create cell suspension for D3 and 3T3 cell lines

$2.5x10^4$ cells/ml for 3T3, $1.5x10^4$ cells/ml for D3cells

Determine cell concentration with hematocytometer and create at least 5 ml

Suspension per 96-well plate

Pipetting of 200 µL medium in the outer wells of a 96-well multi well plate (blanks) Add 50 µL cell suspension into to the remaining inner wells of the 96-well multi well plate (samples)

Incubate for 2 h at 37°C/ 5% CO_2 to let the cell adhere

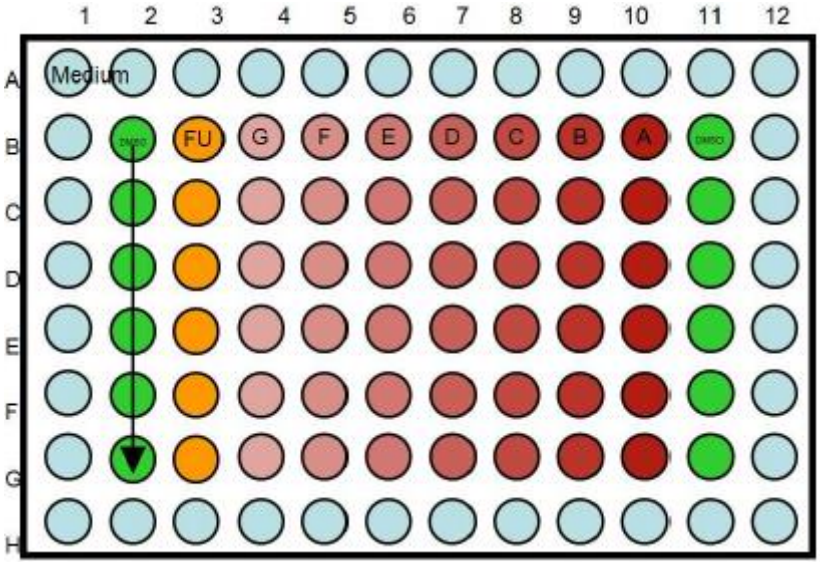
Pipetting of the test substances or DMSO controls (see pipetting scheme of the cytotoxicity assay)

Create concentrations of 2 ml medium and 6.67 µL test substance in a 5 ml tube

Add 150 µL/well of the solution into the sample wells (200 µL/well in total)

Incubate for 3 days at 37°C/ 5% CO_2

Pipetting Layout of cytotoxicity assay:



DAY 3, 5 AND 7

Dilute 2 ml of the medium (3T3 or D3 cell medium) with adding 5 µL of test substance (or DMSO control) (1:400) in a ml tube

Remove the medium with a vacuum pump without damaging of the cell layer on the bottom

Add 200 µL of the diluted test substances (and DMSO controls) into to the appropriate sample wells

Incubation:

Day 3: 2 days at 37°C/ 5% CO_2

Day 5: 2 days at 37°C/ 5% CO_2

Day 7: 3 days at 37°C/ 5% CO_2

DAY 10

Preliminary observe cellular changes, substance precipitation or any other effects visually under the light microscope

MTT-Measurements:

Create final MTT solution by adding 4 ml MTT into 40 ml DMEM and warm up to 37°C

Remove medium out of the 96-well plates by discarding the medium carefully

Add 200 µL of MTT solution to each well with a multiwall pipet

Incubate the plates for 3h at 37°C/ 5% CO_2

Warm up the MTT-Desorb solution to 37°C

Remove the MTT-solution carefully

Add 130 µL of MTT-Desorb solution into each well and incubate the plates for 30 min at 37°C in the incubator, then put the plates for at least 2-3 hours on a plate shaker

Measure the absorption on a plate reader at 570 nm

ACCEPTANCE CRITERIA

Differentiation Endpoint: at least 80% of beating cardiomyocytes in a total assay needed for acceptance of a valid assay

Cytotoxicity Endpoints:

Acceptable ranges of DMSO control and POS control and the determination of OD values of D3 (about 1.8 - 2.2) and 3T3 (0.8 - 1.0) should be in their appropriate ranges

Data Analysis

Differentiation Endpoint:

Determination of the total number of beating cardiomyocytes (at least one beating cardiomyocyte per well = one positive count, no beating cardiomyocytes per well = negative count), normalisation to the positive DMSO controls

Cytotoxicity Endpoints:

Determination of the mean values of the OD_{570} of the blanks (value indicates the adhesion of the dye to plastic material and residual amount of medium). Subtract this value from sample values and continue to calculate with the corrected values. Determination of the mean values of the OD_{570} of the treated sample wells. Determination of the mean values of the OD_{570} of the solvent control wells are set as 100%. The Viability is calculated in % normalised to the DMSO solvent control.

Prediction Model

Data files of optical densities (OD_{570}) generated by a microplate reader were copied into an EXCEL spreadsheet. Mean OD values, standard deviations and viabilities were calculated automatically. The following endpoints from the assays could be calculated graphically from the concentration-response curve in the spreadsheet:

IC_{50}D3 - the concentration of test substance at which 50% of D3 cells have died

IC_{50}3T3 - the concentration of test substance at which 50% of the 3T3 cells have died.

ID_{50}D3 - the concentration of test substance at which there is a 50% reduction in the differentiation of D3 cells into contracting cardiomyocytes.

The IC_{50} values of the D3 and 3T3 cells from the cytotoxicity assay and the ID_{50} of the D3 differentiation assay were entered into the statistical evaluation developed from the modified prediction model used by Scholz *et al.* 1999a:

$$\text{D12\_3} = \frac{lg\ IC_{50}\ D3 + lg\ IC_{50}\ 3T3}{2} - lg\ ID_{50}$$

D12_3 < 0.5 → negative

D12_3> 0.6 → positive

Predictive scores between 0.5 and 0.6 are labelled borderline results.

Inconclusive results are also possible, for example, if solubility limits the dose ranges tested to an extent that no IC_{50} or ID_{50} values can be determined for one or more dose response curves (Withlow *et al.* 2007)

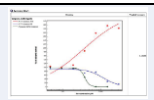
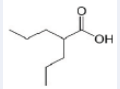
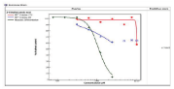
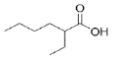
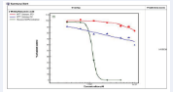
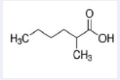
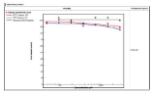
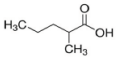
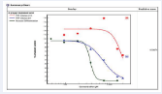
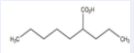
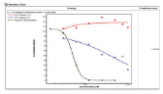
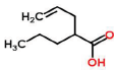
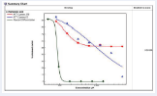
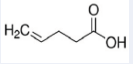
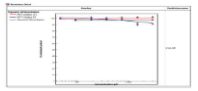
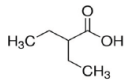
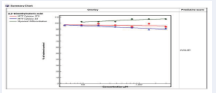
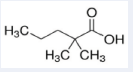
Annexes

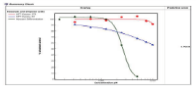
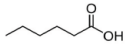
	Definition
Confluency	Possible density arrangement of adherent cells as monolayer in culture
Passage	The transfer of cells from one culture vessel to another, wherein usually a dilution of the cells takes place. The number of passages increases by +1.
Teratogenicity	Property of a substance to cause damage to the embryo during pregnancy (embryonic development).
Toxicity	Change of common physiological functions of an organism or cells, which may be caused by various external influences. In cell culture also known as cytotoxicity. The toxic effects, eg. changes in morphology, attachment conditions or growth must be recorded.
embryotoxic	Damaging of the embryo. A harmful or deadly effect u. a. chemical substances, ionising radiation and infections.
pluripotent	In terms of stem cells, the ability of a single stem cell to differentiate into all cell types for the development of an organism.
ECVAM	European Centre for the Validation of Alternative Methods
Monolayer	Single layer, mostly formed by adherent cells that depend on cell-cell contacts

Bibliography

- Genschow, Elke & Spielmann, Horst & Scholz, Gabriele & Seiler, Andrea & Brown, Nigel & Piersma, Aldert & Brady, Madeleine & Clemann, Nicole & Huuskonen, Hannele & Paillard, Francoise & Bremer, Susanne & Becker, Klaus. (2001). The ECVAM international validation study on *in vitro* embryotoxicity tests: Results of the definitive phase and evaluation of prediction models. European Centre for the Validation of Alternative Methods.
- Genschow, Elke & Spielmann, Horst & Scholz, Gabriele & Pohl, Ingeborg & Seiler, Andrea & Clemann, Nicole & Bremer, Susanne & Becker, Klaus. (2004). Validation of the embryonic stem cell test in the International ECVAM Validation Study on three *in vitro* embryotoxicity tests.
- Whitlow, S., Bürgin, H. and Clemann, N. 2007. The embryonic stem cell test for the early selection of pharmaceutical compounds. *ALTEX - Alternatives to animal experimentation.* 24, 1 (Feb. 2007), 3-7.

6. EST-2: Individual effect curves and EC_{10} for all analogues

	Compound	structure	ID_{10} D3 µM	ID_{50} D3 µM	IC_{10} D3 µM	IC_{50} D3 µM	IC_{10} 3T3 µM	IC_{50} 3T3 µM	P.S	Class	Comments
	**Valproic acid**		275	378	415	1173	>30000	>30000	0,69	POS	strong induction seen with 3T3 cells
	**2-Ethylhexanoic acid**		547	1115	<312.5	4234*	9568	10084*	0,77	POS	*cytotox IC_{50} extrapolated
	**2-Methylhexanoic acid**		913	1166	>10000	>10000	6834	>10000	0,93	POS	Dose-dpt inhibition of differentiation but at concentrations higher than VPA
	**2-Methyl-pentanoic acid**		>30000	>30000	2713	>30000	1838	>30000	-	NEG	No effect up to 3mM
	**2-Propyl-heptanoic acid**		278	365	294	714	1084	1873	0,5	BL	Borderline positive result; apparent cpd loss >2mM; induction in 3T3
	**(+/-) 2-Propyl-4-pentenoic acid, 4ene VPA**		328	518	343	4385	354	>10000	1,11	POS	Some induction in 3T3 cells
	**4-Pentenoic acid**		72	86	<78	872	79	136*	0,6	POS	*Unusual plateau effect in 3T3 cells (confirmed in repeat expt); steep DRC in
	**2-Ethylbutyric acid**		>30000	>30000	2064	>30000	>30000	>30000	-	NEG	No effect up to 3mM
	**2,2-Dimethylvaleric acid**		>30000	>30000	>30000	>30000	>30000	>30000	-	NEG	No effect up to 3mM

	**Hexanoic acid (caproic acid)**		1651	2605	901	17110*	>10000	15318*	0,79	**(POS)**	*cytotox IC_{50} extrapolated; very high ID_{50}

7. UKN1-1: Method description UKN1 (DB-ALM)

Template for Data Content Last Update: September 2013 (v.9.4)

The present template is based on the Content Criteria for Protocols, which are designed for the provision of technical details that enable the documented alternative method to be transferred to other laboratories without the need of additional information.

The Content Criteria, on which the presented template is based, are generic and based on the analysis of common descriptors from hundreds of different non-animal experimental methods and techniques. However, not all parameters or sections indicated are applicable to all protocols. It is the responsibility of the author to provide content where relevant and as appropriate related to a precise protocol. The compilation is to be performed by completing the individual sections with the information related to your method. A review for consistency, completeness in relation to the technique described and compliance with the Content Criteria in place is always performed by the JRC staff. Furthermore, before any protocol is published via the DB-ALM, the final draft is reviewed and approved by designated contact person(s) (method's owner and/or experienced user).

A summary description of the main method features bringing it in a context regarding its intended purpose(s) and application(s), as well as the scientific rationale is always to be provided with each protocol. The content criteria for the method summary will be sent in due course.

The DB-ALM is operated by the European Reference Laboratory for Alternatives to Animal Testing of the Joint Research Centre.

Part A. Protocol Introduction

Protocol Name: UKN1 test system to test for disturbers of early neural development during differentiation from iPSCs to NEPs.

Abstract: This protocol provides information about the differentiation of human induced pluripotent stem cells to neural epithelial precursor cells (NEPs). The cells form neural rosettes on day 15 of differentiation that represents the *in vitro* counterpart of neural tube formation. The UKN1 test system uses these differentiating cells to identify disturbers of early neurodevelopment.

Résumé

The current protocol describes the differentiation of human induced pluripotent stem cells (hiPSCs) to human epithelial precursor cells (NEPs) adapted from the differentiation protocol that was published by Lorenz Studer (Chambers *et al.* 2009). The differentiation protocol was used to develop a test system that uses mainly gene expression as an endpoint (Balmer *et al.* 2012; Krug *et al.* 2013). Recently another morphological endpoint was developed to allow phenotypic anchoring. The ability to form neural rosettes is used as a readout for normal development. However under compound exposure (e.g. tool compounds

TSA or VPA) the cells do not form neural rosettes anymore. Such assay is performed on the top of viability assay, so that compounds that disturb development can be distinguished from general cytotoxic compounds.

Experimental Description

Biological Endpoint and Endpoint Measurement:

During early fetal development processes such as differentiation, proliferation, migration and many others take place and it is essential that these processes are tightly and exactly regulated. If something (e.g. toxicants) disturb the regulation diseases and malformations such as spina bifida, microcephaly and many others may occur.

The current protocol is used as an *in vitro* tool to detect compounds that disturb the early neural development by changing the differentiation direction.

The test captures endpoints like spina bifida or cleft palate also measured during developmental toxicity regulatory studies.

Endpoint Value:

Gene expression that can be anchored to a phenotype.

Experimental System:

The human iPS cells SBAD2 are differentiated to neuro epithelial precursor cells that form structures called neural rosettes. The differentiation itself is captured in the system.

Discussion

For the differentiation of neuro epithelial precursor cells in the UKN1 test system iPS cells are used, therefore no specific ethical approval is required. The stem cells as well as for the start of differentiation relies on feeder cells (MEFS) or on at least conditioned medium. Furthermore Matrigel is used and the medium has to be supplemented with serum replacement. The system showed for years that the differentiations work reliably and robustly and with little variance. However, the aim should be to have an animal-free, xeno-free, feeder-free cell culture to avoid any uncontrolled variance between lots. Of course also in our system a lot of effort is made to replace this material. The aim will be to use a chemically defined coating (e.g. Vitronectin, Laminin-521) instead of Matrigel and to use the E8/E6 medium system instead of feeder cells, conditioned medium and KSR supplemented with serum replacement. Experiments already showed promising results that could allow a replacement of the mentioned material. Since the mentioned media is very expensive and for this system a high amount is needed a lot of effort is made to produce the medium in house.

The test system is more complex than other tests and the operating person needs some training to produce reliable data (4 weeks). Especially the reseeding of cells on day 11 is a critical step that should be performed fast if many compounds are tested in the same experiment. However, it has been shown, that not only intra lab but also inter-lab transfer was recently initiated. Even with operators with poor experience in stem cell culture and differentiation.

qPCR analysis is a commonly used technique and can easily be done in other laboratories. For the imaging Cellomics automated microscope is used, however no special algorithm is

needed. Any other automated microscope may provide similar images. The data analysis is facilitated by the Konstanz information miner software protocol (KNIME). KNIME is an open source software and can be used by any other laboratory. However, a basic understanding of the software and the protocol is essential to also allow trouble shooting processes if needed.

The UKN1 model is no high throughput model since the differentiation has to be done in 6 well plates or (for viability pretests at least in 12 well plates). However, the results have a high value, since it does not only provide toxicity data for compounds. But, also provides a lot of data that allow a basic understanding of the developmental processes that are involved during early neuro development. Furthermore, the phenotypic anchoring leads to very reliable data that show only irreversible drug effects since the compound is washed out for a long time (d6-d15).

The prediction model is still under development since data of gene expression (Affymetrix gene chips) need to be correlated with rosette formation data. The aim is, to analyse which (gene) markers are the most important and predictive by using different kind of prediction models that are based on statistical methods as well as on biological background knowledge (rule based) and to constantly improve the model by simplification. However, a preliminary prediction model is provided.

Status

In Development:

The UKN1 test system was published until day 6 (Balmer *et al.* 2012; Krug *et al.* 2013). The new phenotypic endpoint (d15) was developed recently and will be published in 2018.

Known Laboratory Use:

University of Konstanz (used by different operators in this laboratory).

IfaDo Institut für Arbeitssicherheit Dortmund, Germany

Participation in Evaluation Study:

The test system was transferred recently to another laboratory. Intra-laboratory testing revealed a high robustness across different operators and assay runs.

Volatile compounds and substances that are not water soluble cannot be measured.

Participation in Validation Study:

No

Regulatory Accepted:

No

Proprietary and/or Confidentiality Issues

The distribution of the protocol or any protocol components is not limited.

Health and Safety Issues

General precautions:

No general precautions.

MSDS Information:

In addition to the safety measures regarding the compounds in use, there are no safety measures needed for the performance of this method.

Abbreviations and Definitions

UKN1: University of Konstanz 1

NEPs: Neuro epithelial precursor cells

iPSC: induced pluripotent stem cells

PBS: Phosphate-buffered saline

EGF: Epidermal growth factor

DMSO: Dimethyl sulfoxide

FBS: fetal bovine serum

KCM: conditioned medium

DMEM: Dulbecco's modified eagle medium

HESC: Human embryonic stem cells

RT: Room temperature

Last Update: 20/12/17

Part B. Technical Description

Procedure Details, Latest Version: 20/12/2017

Protocol Name: UKN1 test system to test for disturbers of early neural development in the differentiation from iPSCs to NEPs

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Materials and Preparations

CELL OR EXPERIMENTAL SYSTEM

The human iPSC line SBAD2 (Baud *et al.* 2017) is used to generate neural epithelial progenitor cells (NEPs). These NEPs are further cultivated, the cells' formation of rosettes is evaluated as a morphological endpoint.

EQUIPMENT

Fixed Equipment

- Biorad CFX96 Real Time PCR machine (C1000 Thermal Cycler)
- Cellomics Array Scan VTI HCS high content reader
- Centrifuge
- CFX96 Touch™ Real Time PCR Detection System
- Eppendorf Thermomixer compact
- Eppendorf Thermostat plus
- Freezer (-20°C and -80°C)
- Fridge (4°C)
- Filter bottles (0.22 µm)
- Humidified incubator (37°C, 5% CO_2 in air)
- Ice machine
- Infinite® M200 PRO Tecan
- Laminar flow hood for sterile atmosphere
- Light microscope
- Liquid nitrogen storage
- Micropipettes
- Multichannel pipettes
- Multichannel soaker
- Neubauer counting chamber
- PEQLAB Nanodrop 1000
- Tecan infinite M200 and "Tecan i-control 1.6" software
- Water bath

Consumables

- Freeze box, e.g. Nalgene™ Mr. Frosty™ (Thermo Fisher)
- Gloves

- Sterile 0.22 mm bottle-top vacuum filter system (Corning)
- Sterile 6 cm cell culture dishes (Sarstedt)
- Sterile 6-well plates (Falcon)
- Sterile 96-well plates (Falcon, flat bottom)
- Sterile Eppendorf tubes (1.5 ml)
- Sterile cryovials
- Hard-Shelll 96-Well PCR Plates (biorad)
- Safe seals
- Sterile plastic tubes (15 ml, 50 ml)
- Sterile single wrapped pipettes
- Sterile T25 cell culture flask (Corning)
- Sterile T75 cell culture flask (Corning)

MEDIA, REAGENTS, SERA, OTHERS

Material	Supplier	Catalogue Number
0.05% Trypsin-EDTA	Gibco	25300-062
2-mercaptoethanol	Gibco	31350
Accutase	PAA	L11-007
Apotransferin	Sigma	T-2036
Ascorbic Acid	Sigma	A4544-25G
Basic fibroblast growth factor (bFGF)	Invitrogen	13256029
Chloroform	Sigma	32211-1L
Dispase	Cell Systems GmbH	LS002104
DMEM / GlutaMax	Gibco	31966-047
DMEM/F12	Invitrogen	21331
Dimethylsulfoxid (DMSO)	Sigma	D2650
Dorsomorphin dihydrochloride	Tocris	3093
Epidermal growth factor (EGF)	R&D	236-EG
Ethanol	VWR Chemical	20821.296
Fetal bovine serum (FBS)	PAA	A15-101
broblast growth factor 2 (FGF2)	R&D	233-FB/CF
fibroblast growth factor 8b (FGF8b)	R&D	423-F8/CF
Formalin Solution 10% buffer	Sigma	HT5014-120ML
Gelatine	Sigma	G1890-100G
Glucose	Sigma	G7201
GlutaMax®	Invitrogen	35050-061
HEPES	Gibco	15630
Hoechst H-33342	Sigma	14533
Insulin	Sigma	I 9278
iScript	Bio-Rad	1708841
Isopropanol	Sigma	33539-1L-R
Knock out DMEM	Gibco	10829
Knockout serum replacement (KSR)	Gibco	10208
D-Mannitol	Riedel-de Haën	3446-100G
Matrigel	Corning	354234
Mouse embryonic fibroblasts (MEFs (CF6))	Amsbio	GSC-6005

Minimum essential medium non-essential amino acids (MEM NEAA)	nvitrogen	11140
Mitomycin C	Sigma	M0503
Noggin	R&D	719-NG
peqGOLD-TriFast	PEQLAB	30-2010
Phosphate buffered saline (PBS)	Invitrogen	14190
Poly-L-ornithine (PLO)	Sigma	P3655
Progesterone	Sigma	P-7556
Putrescine	Sigma	P-5780
Resazurin	Sigma	R7017-1G
ROCK inhibitor Y-27632	Tocris	1254
SB 4314542	Tocris	9A/199612
Selenium	Sigma	S-5261
Sonic hedgehog (Shh)	R&D	1845-SH-100/CF
Sodium acetate 3M	Thermo Scientific	R1181
Ssofast EvaGreen Supermix	Bio-Rad	172-5024
Tergazyme	Sigma	Z273287-1EA
Transforming growth factor beta (TGF-beta) inhibitor SB 43154	Tocris	1614
Trichostatin A (TSA)	Sigma	T1952-200UL
Triton X-100		
Trypan blue stain (0.4%)	Gibco	15250061
Trypsin	Gibco	25300-062
UltraPure distilled H2O	Invitrogen	10977-35
Valproic acid (VPA)	Sigma	P4543-10G
Zonula occludens protein 1 (ZO1) monoclonal antibody (IgG1, mouse)	Invitrogen	339100
Golgi matrix protein 130 (GM130) monoclonal antibody (rabbit)	Cell signalling	12480S
Alexa fluor 488-mouse IgG1 secondary antibody (goat)	Invitrogen	A21121
Alexa fluor 555-rabbit secondary antibody (donkey)	Life technology	A31572

Gene	Forward primer	Reversed primer
Pax6	CCGCCTATGCCCAGCTTCAC	AAGTGGTGCCCGAGGTGCCC
Otx2	GTTCAGAGTCCTTGGTGGGT	CCCTCACTCGCCACATCTAC
Nanog	GGTGAAGACCTGGTTCCAGAAC	CATCCCTGGTGGTAGGAAGAGTAAAG
Oct4	GCAAAGCAGAAACCCTCGTGC	ACACTCGGACCACATCCTTCTCG
Emx2	CCAAGGGAACGACACTAGCC	CCATACTTTTACCTGAGTTTCCGTG
Msx1	GGATCAGACTTCGGAGAGTGAACT	GCCTTCCCTTTAACCCTCACA
Cdh2	GGCGTCTGTAGAGGCTTCTG	CACGGCATACACCATGCCATC
FoxG1	AGAAGAACGGCAAGTACGAGA	TGTTGAGGGACAGATTGTGGC
Tfap2b	GGCCTCGGCCATCCCGGAATGG	CGGAGCAAAACACCTCGCCGGT
Sox1	CCGGGATAAGGGCCTCCCCA	ACACAGGGGAGCACAGGGGC
Rpl13a	CCTGGAGGAGAAGAGGAAAGAGA	TTGAGGACCTCTGTGTATTTGTCAA
TBP	GGGCACCACTCCACTGTATC	GCAGCAAACCGCTTGGGATTATATTCG

PREPARATIONS

Media and Endpoint Assay Solutions

- FGF2 (fibroblast growth factor 2) Aliquots

  Add 45.59 ml of 0.1% BSA/PBS to 1 mg of human recombinant FGF2 to a final concentration of 20 µg/ml. Aliquot FGF2 solution in 0.5 ml aliquots and store at 80°C.

- FGF8 (fibroblast growth factor 8) Aliquots

  Dissolve FGF8 in 0.1% BSA/PBS to a final concentration of 100 µg/ml. Store at -80°C.

- bFGF (basic fibroblast growth factor) Aliquots

  Dissolve 10 µg in 100 µl HESC medium (without bFGF) and store at -80°C or at 4°C for a maximum of 10 days.

- SB 43154 Aliquots

  Add 2404 µl ethanol (100%) to 10 mg of SB 43154 to a final concentration of 10 mM. Aliquot SB 43154 solution in 200 µl aliquots and store at -80°C.

- Noggin Aliquots

  Add 2 ml of 0.1% BSA/PBS to 500 µg of noggin to a final concentration of 250 µg/ml. Aliquot noggin solution in 25 µl aliquots and store at -80°C.

- Rock inhibitor Y-27632 Aliquots

  Add 15.185 ml sterile water to 50 mg of Y-27632 dihydrochloride to a final concentration of 10 mM. Aliquot Y-27632 solution in 500 µl aliquots and store at -20°C.

- Shh (sonic hedgehog) Aliquots

  Add 5ml of 0.1% BSA (in PBS) to 100 µg to a final concentration of 20 µg/ml. Store Aliquots of 100 µl at -80°C.

- Resazurin Solution

  Dissolve 1mg/ml of the Resazurin powder in PBS. Sterilise by filtrating through 22 µm filter.

- Ascorbic acid Aliquots

  Add 10 ml of PBS to 35 mg ascorbic acid to a final concentration of 20 mM. The solution is sterile filtered and stored as 1 ml aliquots at -80°C.

- Dorsomorphin dihydrochloride Aliquots

  Add 103.9 ml of sterile water to 50 mg of Dorsomorphine dihydrochloride to a final concentration of 1 mM. The solution is sterile filtered (0.22 µm) and stored as 400 µl aliquots at -80°C.

- Hoechst-33342 Aliquots

  Add 5 ml sterile water to 5 mg of Hoechst-33342 to a final concentration of 1 mg/ml. Aliquot Hoechst-33342 solution in 500 µl aliquots and store at +4°C.

- Accutase Aliquots

  Thaw out commercial Accutase bottle, prepare 10 ml aliquots and store at -20°C.

- Trypsin Aliquots

  Thaw out 0.05% Trypsin-EDTA bottle, prepare 12 ml aliquots and store at -20°C.

- Matrigel Aliquots

  Thaw out Matrigel by placing Matrigel bottle on ice until Matrigel becomes liquid. Aliquot Matrigel in 330 µl and 1 ml aliquots and store at -20°C.

- KnockOut serum replacement Aliquots

  Thaw out KnockOut serum replacement bottle, prepare 50 ml aliquots and store at -20°C.

- 0.1% gelatine solution

  Dissolve 0.25 g gelatine in 250 ml MiliQ water and sterile filtrate.

- Dispase aliquots

  Dissolve the powder in HESC medium (without bFGF) to an end concentration of 6 U/ml then sterile filtrate and store in 10 ml aliquots at -20°C.

- Preparation of Human embryonic stem cell medium (HESC) for hESC / iPSC maintenance culture

  Mix all the components in the table below in a bottle and sterilise it by filtering through a 0.22 µm filter bottle. Avoid repeated warming up of the medium.

Components of medium	Volume required per 100 ml
DMEM/F12	80 ml
Knock out serum replacement	20 ml
Hepes	1.5 ml
GlutaMax®	1.0 ml
MEM NEAA	1.0 ml
2-mercaptoethanol	0.18 ml
bFGF (0.1 µg / µl)	10 µl

- Preparation of MEF medium

  Mix all the components in the table below in a bottle and sterilise it by filtering through a 0.22 µm filter bottle. Store at 4°C up to 4 weeks. Avoid repeated warming up of the medium.

Components of medium	Volume required per 100 ml
DMEM / GlutaMax	90 ml
FBS	10 ml

- Preparation of Human stem cell medium (SC)

  Mix all the components in the table below in a bottle and sterilise it by filtering through a 0.22 µm filter bottle. Wrap in aluminum foil and store at + 4°Cup to one week maximum. Avoid repeated warming up of the medium.

Components of medium	Volume required per 100 ml
DMEM/F12	78.5 ml
Knock out serum replacement	20 ml
GlutaMax®	0.5 ml
MEM NEAA	1.0 ml
2-mercaptoethanol	0.1 ml

- Preparation of conditioned medium (KCM)

  Remove DMEM medium from mouse embryonic fibroblast (MEF) culture. Put HESC medium containing FGF2 (4 ng/ml) on MEFs for 24h, remove and filter through a 0.22 µm filter bottle, wrap bottle with aluminum foil and label with KCM and date. Medium can be used for up to 1 month when stored at 4°C or up to 6 month when stored at -20°C.

- Preparation of N2-S medium (N2-S)

  The stock solutions of the supplements putrescine (1 M), selenium (500 µM) and progesterone (100 µM) are prepared and aliquoted upon first use and stored at -80°C. Thawed aliquots can be stored at 4°C for up to two weeks. Weigh apotransferin and glucose into a plastic bottle and dissolve in DMEM/F12 medium. Mix all medium components from the table below and sterilise it by filtering through a 0.22 µm filter bottle. Wrap the bottle in aluminum foil, label with content and date. Keep it at 4°C for maximum 2 weeks. Medium should not be rewarmed, it is imperative to only warm up the medium amount to be used immediately.

Components of medium	Volume required per 100 ml
DMEM/F12	98.6 ml
Apotransferrin	10 mg
Glucose	155 mg
Insulin	400 µl
Putrescine	10 µl
Selenium	6 µl
Progesterone	20 µl
GlutaMax®	1.0 ml

- Preparation of Knockout serum replacement medium (KSR)

  Mix all the components in the table below in a bottle and sterilise it by filtering through a 0.22 µm filter bottle. Wrap the bottle in aluminum foil, label with content and date. Keep it at 4°C for maximum of 2 weeks. Medium should not be rewarmed, it is imperative to only warm up the medium amount to be used immediately.

Components of medium	Volume required per 100 ml
Knock out DMEM	83 ml
Knock out serum replacement	15 ml
GlutaMax®	1 ml
MEM NEAA	1 ml
2-mercaptoethanol	100

- Staining solutions
  - Permeabilisation: 0.3% Triton in PBS
  - Washing solution: PBS
  - Blocking solution: 5% bovine serum albumin, 0.1% Triton in PBS
  - Staining solution: Blocking solution + 1:400 primary antibodies (anti ZO1, IgG1 mouse; anti GM130, rabbit)

- Staining solution 2: Blocking solution+ 1:1000 secondary antibodies (488-mouse IgG1; 555-rabbit) +1:1000 Hoechst H-33342

- qPCR Master mix

  Prepare master mix on ice and keep light protected. Prepare duplicates for each sample.

Components	Volume required per sample
SSO Evagreen Supermix	5 µl
Water	3.6 µl
Forward primer	0.2 µl
Reversed primer	0.2 µl
cDNA	1 µl

Test Compounds

- Compounds are stored according to the manufacturer's instructions.
- Stock solutions should be dissolved in sterile water or DMSO, if possible 1000x more concentrated than the working solution. The used DMSO is stored in a lightproof, air-tight bottle at room temperature.
- The stock solutions are aliquoted into volumes sufficient for one experiment and discarded after first thawing. This avoids repeated freezing and thawing and therefore to damage the compounds stability and efficiency.
- Working solutions (to add to the cells) of toxicants are prepared fresh by dilution in KSR/N2-S medium and the final DMSO concentration in the wells is always 0 or 0.1% DMSO.

Positive Control(s)

The positive controls are compounds that are known to induce developmental neurotoxic effects *in vivo* and should induce changes in gene expression and rosettes formation in the present UKN1 test method without being cytotoxic.

Trichostatin A (20 nM), Valproic Acid (600 µM) are the positive controls in this test method. Both of them are reported to induce neural tube closure defects such as exencephaly (more prominent in animals) and spina bifida (more relevant to human). Both compounds are inhibitors of histone deacetylases (HDACi) and change gene expression patterns via epigenetic mechanisms.

Stock solutions are dissolved in sterile water for VPA (600 mM) and TSA in DSMO (5 mM). Working solutions (to add to the cells) of VPA is prepared freshly from the stock solutions by dilution in the medium of the corresponding day. The TSA stock solution is pre-diluted 1:1000 in medium of the corresponding day and then further diluted 1:250 in medium of the corresponding day of differentiation to a final concentration of 20 nM added to the cells.

Negative Control(s)

0.1% DMSO

Method

EXPERIMENTAL SYSTEM PROCUREMENT

The iPSC line SBAD2 are derived from fibroblasts (Lonza) and were provided by the laboratory of Paul Jennings (Baud *et al.* 2017).

The mouse embryonic fibroblasts CF6 are purchased from Amsbio Germany.

ROUTINE PROCEDURES

Preparation of MEF (mouse embryonic fibroblasts)

(1) Thawing of MEFs

- Prewarm 50 ml MEF medium at 37°C
- Coat 3 x T75 cell culture flasks with 10 ml 0.1% gelatin for 30 min. at 37°C in the incubator
- Add 10 ml warm MEF medium in a 15 ml Falcon tube
- Thaw 1 vial of purchased MEFs quickly in a 37°C water bath and pipet in the Falcon tube
- Centrifuge at 500g, 3 min
- Discard supernatant and dissolve the cell pellet in 3 ml MEF medium

(2) Seeding of MEFs

- Add 10 ml MEF medium in each T75 flask
- Add 1 ml of dissolved cell pellet in each T75 flask
- Culture the cells for 7 days in the cell culture incubator at 37°C / 5% CO2

(3) Splitting of MEFs

- Prewarm 200 ml MEF medium at 37°C
- Coat 15x T75 flask with 0.1% gelatin solution and incubate at 37°C for 30 min
- Aspirate medium from the day 7 MEFs from step (2) and wash once with 10 ml PBS
- Add 2 ml 0.05% trypsin and incubate for 2 min at 37°C
- Add 2 ml MEF medium to dissolve the MEFs and transfer the cells in a 15 ml Falcon tube
- Centrifuge at 500g for 3 min
- Discard the supernatant and dissolve the pellet in 15 ml MEFs medium
- Centrifuge at 500g for 3 min
- Discard the supernatant and dissolve the pellet in 15 ml MEFs medium
- Aspirate gelatin from the coated flask and add 10 ml MEF medium
- Add 1 ml of dissolved cells into each T75 flask

(4) Expansion of MEFs

- MEFs are cultivated for 5 – 7 days until 80 – 90% confluence
- Prewarm 450 ml MEFs medium at 37°C
- Coat 20x T175 flasks with 0.1% gelatin for 30 min at 37°C
- Trypsinise, wash and centrifuge the MEFs as described in step (3)
- Dissolve the pellet after the centrifugation step in 20 ml MEF medium
- Aspirate gelatin and add 30 ml MEF medium
- Add 1 ml of dissolved cell pellet into each T175 flask and mix
- Maintain the cells for 7 – 9 days in the cell culture incubator at 37°C / 5% CO_2 with no further actions.

(5) Preparation of MEFs

- Remove 18 ml medium from each flask and collect the medium in a sterile cell culture bottle (= 360 ml). Put the cells back into the incubator
- Dissolve 4 mg Mitomycin-C in 4 ml PBS, sterile filtrate it and add 3.6 ml of this solution into the 360 ml removed media
- Aspirate the remaining 12 ml medium from 6 T175 flasks cells and add 18 ml Mitomycin-C containing medium, note the exact time on the flask and put them back into the incubator. Then take the next 6 T175 flasks until all 20 flasks are treated. Incubate each flask batch for exactly 3 hours in the cell culture incubator
- Prewarm 2x 500 ml MEF medium at 37°C
- After exactly 3 hours aspirate the Mitomycin-C containing medium from the cells, wash twice with 20 ml PBS, add 10 ml MEF medium and put them back into the incubator until all flasks are washed
- Aspirate medium, wash once with 20 ml PBS and add 4 ml 0.05% trypsin for 2 min at 37°C
- Add 4 ml MEF medium and collect the cells in a 500 ml bottle on ice, wash the flasks again with 5 ml MEF medium and collect in the same 500 ml bottle
- Aliquot the cell suspension in 50 ml Falcon tubes and centrifuge at 500g for 3 min
- Dissolve all pellets in 20 ml MEF medium and count the cells in a Neubauer cell counting chamber and determine the total amount of cells
- Centrifuge at 500g for 3 min
- Adjust cell concentration to 1x 10^6 cells / ml in freezing medium (90% FBS / 10% DMSO) and add 1 ml of cell suspension to each cryo vial.
- Put the tubes in a Mr. Frosty freezing container and incubate at -80°C overnight
- Next day store the tubes in -80°C.

Maintenance of SBAD2

Day -1: Seeding of MEFs for one T25 flask or one 6 cm dish

(6) Prewarm 15 ml MEF medium at 37°C

(7) Add 4 ml 0.1% gelatin to T25 flask and incubate 30 min at 37°C in the incubator

(8) Thawing of MEFs

- Thaw 1 vial of frozen MEFs ($1*10^6$ cells / vial) in a 37°C water bath
- Add the thawn cells into 9 ml pre-warmed MEF medium
- centrifuge at 500g for 3 min
- dissolve the cell pellet in 1 ml of MEF medium

(9) Seeding of MEFs

- Aspirate gelatin from the T25 flask
- Add 4 ml of pre-warmed MEF medium into the flask
- Add dissolved cell pellet from step (8) (1 ml) into the flask
- Incubate at 37°C / 5% CO_2 in a cell culture incubator

Day 0: Splitting of SBAD2 (T25 flask)

(1) Pre-warm 1 ml dispase, 25 ml HESC medium (without bFGF) and 5 ml HESC medium at 37°C

(2) Aspirate medium from the cells

(3) Add 1 ml dispase and incubate for 6 min in the incubator (37°C / 5% CO_2)

- Meanwhile aspirate medium from the MEFs flask prepared on day -1 and add 4 ml PBS

(4) Add 2 ml HESC medium (without bFGF) to the dispase-treated cells from step (3) and pipet 4 -5 x up and down with a sterile 2 ml plastic pipette. The cells should stay in clumps.

(5) Transfer the cells into a 15 ml Falcon tube.

(6) Rinse the T25 flask with 9 ml HESC medium (without bFGF)

(7) Centrifuge at 500g for 3 min.

(8) Discard the supernatant and dissolve the pellet in 10 ml HESC medium (without bFGF).

(9) Centrifuge at 500g for 3 min.

- Meanwhile aspirate PBS from the MEFs from step (3) and add 3.5 ml HESC medium

(10) Discard the supernatant and dissolve the pellet in 4 ml HESC medium

(11) Add 0.5 ml dissolved pellet / T25 Flask from step (3)

Differentiation to neuro epithelial precursor cells (d6) and differentiation to rosette forming stage (d15)

Day -3:

(12) Prepare SC medium and KCM medium and prewarm them at 37°C

(13) Prewarm trypsin and PBS at RT

(14) Prepare Matrigel coated plate(s)

- Matrigel has to cover the plate bottom (therefore 1 ml suspension for one well of a 6-well plate is required)
- Add cold DMEM/F12 to frozen Matrigel pellet and dissolve it so that it is diluted 1:20
- Add 1ml of the Matrigel solution to each well and incubate for 1.5 h at RT or 30 min at 37°C
- After incubation time, remove Matrigel solution (you can add DMEM/F12 on the plate(s) as long as you need to prepare your cells)

(15) The SBAD2 cells are cultured on MEF cells until they are used for differentiation.

(16) Wash cells gently with 3 ml PBS at once

(17) Discard the PBS and add 1 ml of Trypsin and incubate 2 min at 37°C. Make sure that trypsin is covering all the cells area

(18) Wash feeder cells (MEFs) away by adding gently 3 ml SC medium

(19) Discard the supernatant which contains MEFs

(20) Add 3 ml of SC medium and detach SBAD2 cells from plate by gently pipetting up-anddown with a P1000 (make sure to have single cell suspension)

(21) Filter the cell suspension with a 40 µm cell strainer into a 50 ml tube

(22) Spin 3 min with 500 x g

(23) Aspirate supernatant

(24) Resuspend the pellet in 10 ml SC medium

(25) Spin again 3 min with 500 x g

(26) Aspirate supernatant

(27) Resuspend cells in 1 ml KCM containing 10 µM ROCK inhibitor and FGF2 (10 ng/ml)

(28) count cells in a Neubauer chamber using Trypan blue

(29) resuspend the cells in KCM containing 10 µM ROCK inhibitor and FGF2 (10 ng/ml) in order to have a seeded culture of 12 000 cells/cm^2 (for 6 well plate use 1.5 ml medium per well)

(30) Distribute the cells over the Matrigel coated wells

Day -2:

(31) Aspirate medium and gently add fresh KCM containing 10 µM ROCK inhibitor and FGF2 (10 ng/ml)

Day -1:

(32) Change medium to fresh KCM **without** ROCK inhibitor but with FGF2 (10 ng/ml)

Day 0:

(33) Cells should have **70-80**% confluence today. Discard plate if cells are too dense. Keep cells one day longer in KCM **without** ROCK inhibitor but with FGF2 (10 ng/ml) if cells are less dense.

Day 0-10:

(34) For day 0-10 change medium according to the table below:

Day	Medium		Supplements		
	KSR	N2-S	Noggin (35 ng/ml)	SB431542 (10 µM)	Dorsomorphin (0.6µM)
0	x		x	x	x
1	x		x	x	x
2	x		x	x	x
4	x (75%)	x (25%)	x	x	x
6	x (50%)	x (50%)	x	x	x
8	x (25%)	x (75%)	x	x	x
10		x	x	x	x

Day 11: (Reseeding of cells)

(1) Prepare Matrigel coated 96 well plate(s)

- Matrigel has to cover the plate bottom (therefore 50 µl suspension for one well of a 96-well plate is required)
- Add cold DMEM/F12 to frozen Matrigel pellet and dissolve it so that it is diluted 1:20
- Add 1ml of the Matrigel solution to each well and incubate for 1.5 h at RT or 30 min at 37°C

(2) Prewarm N2-S medium, PBS and Accutase at 37°C

(3) Discard medium and add 500 µl Accutase per each 6 well and incubate 20 min at 37°C

(4) Add 1 ml DMEM/F12 wash medium to each 6 well and detach cells from plate by pipetting with a P1000

(5) Transfer cells into 15 ml Falcon tube containing 10 ml wash DMEM/F12 medium

(6) Spin 3 min with 500 x g

(7) Remove supernatant

(8) Resuspend cells in 1 ml N2-S medium supplemented with ROCK inhibitor (10 µM), FGF2 (20 ng/ml), FGF8 (100 ng/ml), Ascorbic acid (20 µM) , and Sonic hedgehog (10ng/ml), count cells in a Neubauer chamber using Trypan blue

(9) Resuspend cells in N2S containing ROCK inhibitor (10 µM), FGF2 (20 ng/ml), FGF8 (100 ng/ml), Ascorbic acid (20 µM) and Sonic hedgehog (10ng/ml) in order to have a seeded culture of 150 000 cells/cm^2 (for 96 well plate use 100µl medium per well)

(10) Seed 6 replicates for test samples and at least 12 replicates for control samples

Day13:

(11) Change medium to N2S containing FGF2 (FGF2 (20 ng/ml), FGF8 (100 ng/ml), Ascorbic acid (20 µM) and Sonic hedgehog (10ng/ml); **without** ROCK inhibitor.

Day15 (Fixation of cells)

(1) Aspirate medium gently and add 50 µl of 4% Paraformaldehyde per well

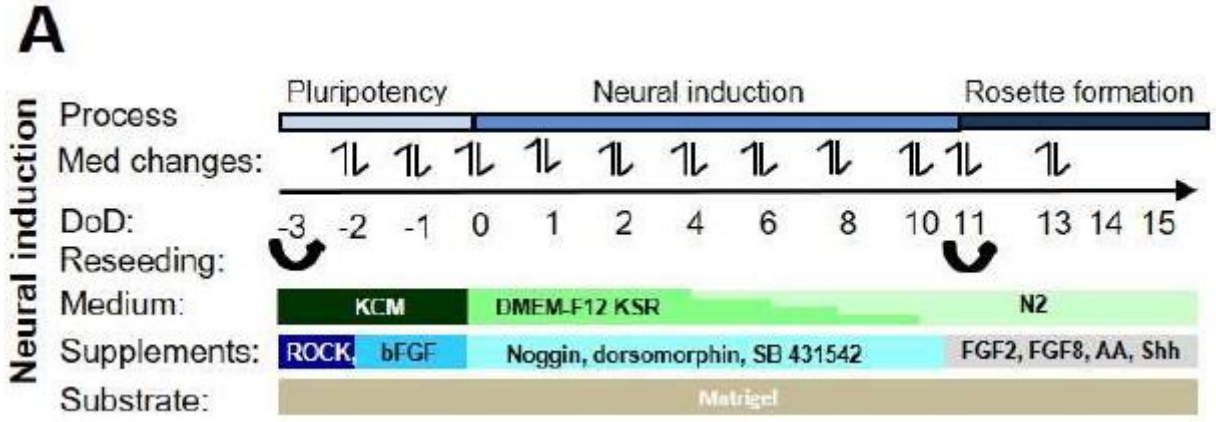
(2) Incubate for 10 minutes

(3) Remove Paraformaldehyde and add 100 µl PBS/well.

(4) Store at 4°C until use for staining endpoint.

Figure 1. Differentiation protocol from stem cell (d-3) to neuro epithelial precursor cells.

On d-3 stem cells are detached from their feeder cells, singelised and seeded in a low density. The cells are cultured under stem cell conditions until day0. At this time the confluence should be around 80%. At d0 differentiation is initiated by changing the medium from conditioned medium to KSR medium supplemented with Noggin, dorso and SB431542. The medium KSR is gradually replaced by N2S medium until d10. On day 11 of differentiation the cells are detached, singalised and seeded into 96well plates. The cells are then cultured in N2S supplemented with Fgf2, Fgf8, Ascorbic acid, Sonic hedgehog until they form neural rosettes on day 15.

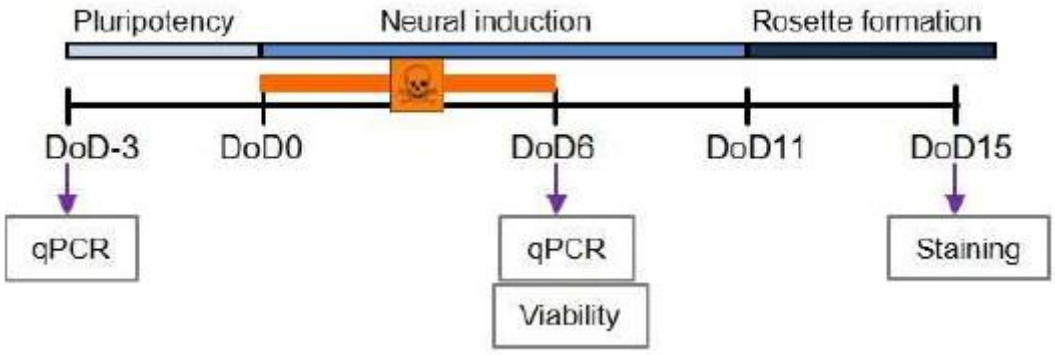


EXPOSURE PROCEDURE

Cells are exposed to test compound from d0-d6 during differentiation. Compound is added freshly with every medium change on d0, d1, d2, and d4. For negative control normal medium changes are performed without compound. As a positive control Trichostatin A (20 nM) or Valproic acid (600 µM) is used. Endpoints are measured either at day 6 (viability and gene expression) or at day 15 (morphology of rosettes formation). The exposure time (do-d6) does not change also if rosette formation on d15 is assessed. The compound is then washed out after day 6 and only irreversible changes in differentiation, that lead to an altered phenotype are assessed.

Figure 2. Exposure schema with the endpoints that are measured in the system

Differentiation of stem cells to endothelial precursor cells takes 6 days. During this period cells are treated with the respective test compounds. After 6 days viability as well as gene expression changes are assessed. For another morphological endpoint cells are further differentiated until they form neural rosettes on day 15. The rosette formation (number of rosettes/nuclei area) can be detected. However also in this case the treatment stops on day 6 and the compound is washed out. The gene expression can be anchored to the phenotype and the irreversible compound effects can be assessed.



ENDPOINT MEASUREMENT

There are three general endpoints measured in the UKN1 test method. Viability is assessed by resazurin assay on d6 (endpoint 1), differentiation is assessed by gene expression analysis of 10 genes (endpoint 2-Pax6, 3-Otx2, 4-Nanog, 5-Oct4, 6-Emx2, 7-Msx1, 8-Cdh2, 9-FoxG1, 10-Tfap2b, 11-Sox1) on d6 and cell morphology is assessed by measurement of the formation of neural rosettes on d15 using Cellomics automated microscope and KNIME software (endpoint 12).

Resazurin assay (d6):

Resazurin assay is based on the reduction of the non-fluorescent blue dye resazurin to the red-fluorescent resarufin. Resazurin is non-toxic and is able to cross the cell membrane. Inside the cells it is then reduced by cellular oxido-reductases. Fluorescence intensity is proportional to cell number and metabolic activity of the cells

(1) Differentiate cells until d6

(2) Dilute resazurin stock solution (1mg/ml) 1:10 with PBS. Add 200 µl of the resazurin solution to the cells to a final concentration of 10 µg/ml (without aspirating the old medium).

(3) Incubate the plate for 20 minutes at 37°C, under humidified conditions, 5% CO2.

(4) Measure Fluorescence at an excitation wavelength of 530 nm and an emission wavelength of 590 nm by Tecan infinite M200 and “Tecan i-control 1.6” software.

Use cell material further for mRNA isolation.

Gene expression analysis (d6):

Use at least the following samples: d-3 stem cells (for differentiation control), d6 untreated control, d6 test compound treated cells.

mRNA isolation

mRNA is isolated as described in the Trizol protocol (Invitrogen):

(1) Add 500µl/well Trizol to the cells and incubate for a few minutes. Transfer the samples to a 1.5 ml Eppendorf tube and store at -20°C.

(2) After thawing: Add 200 µl chloroform to the Trizol samples and mix roughly.

(3) Incubate 3 minutes at room temperature

(4) To separate the phases spin for 15 minutes at 20 000g at 4°C

(5) Transfer the upper aqueous phase to a fresh tube

(6) Add 500 µl isopropanol, mix roughly

(7) Incubate for 10 minutes at room temperature

(8) Spin for 10 minutes at 20 000g at 4°C

(9) Remove supernatant

(10) For washing: Add 1 ml of ice cold 75% EtOH to RNA pellet

(11) Spin for 5 minutes at 10 000g at 4°C. Remove supernatant.

(12) Repeat washing step (10 and 11)

(13) Remove ethanol completely, air-dry pellet for 25 minutes

(14) Dissolve in 40 µl RNAse free H_2O

(15) Measure RNA concentration by Nanodrop

(16) Store samples at -80°C.

Transcription to cDNA

mRNA is transcribed into cDNA as described in manufacturers protocol of iScriptTM Reverse Transcription Supermix for RT-qPCR (biorad)

(1) Use 1 µg of mRNA and adjust to 16 µl with RNAse free water

(2) Add 4 µl of iScript supermix

(3) Incubate 5 minutes at 25°C, for priming

(4) Incubate 20 minutes at 46°C

(5) Incubate 5 minutes at 95°C

(6) Add 80 µl of water

(7) Store samples at -20°C

qPCR

(1) Prepare qPCR mastermix

(2) Use duplicates for each sample

(3) Use primers for the test genes (Pax6, Otx2, Nanog, Oct4, Emx2, Msx1, FoxG1, Tfap2b, Sox1) and primers for the reference genes (TBP, Rpl13a)

(4) The sample are measured in CFX96 Real Time PCR machine with the following protocol:

98°C for two minutes to activate the Taq polymerase

40 amplification cycles:

- 2 sec at 98°C denaturising
- 5 sec at 60°C annealing and polymerisation
- plate readout

After the amplification cycles the temperature is increased in 0.5°C steps up to 95°C to provide a melting curve to determine the reaction specificity.

Figure 3. Typical plate layout for qPCR analysis.

It is important to measure the controls on the same plate as the testcompounds. Each sample is measured in duplicates.

		d-3	nc	Test1	Test2	Test3	Test4	Test5	Test6	Test7	Test8	Test9	Test10
		1	2	3	4	5	6	7	8	9	10	11	12
Pax6	A												
Pax6	B												
Otx2	C												
Otx2	D												
Nanog	E												
Nanog	F												
Rpl13a	G												
Rpl13a	H												

Rosette formation protocol (d15)

(1) Differentiate cells until d15, then fix cells as described in differentiation protocol.

(2) For measurement of rosette formation stain three replicates of the test wells and 6 replicates of the control wells.

(3) Add 50µl of 0.3% Triton (in PBS) to each well of the 96 well plate and incubate for 7 minutes at RT

(4) Remove Triton solution, wash once with 100 µl PBS

(5) Add 50 µl of Blocking Buffer (5% BSA, 0.1% Triton in PBS), incubate for 45 minutes at RT

(6) Wash once with PBS

(7) Add 50 µl of the Staining solution (1:400 GM130 antibody, 1:400 ZO1 antibody in blocking buffer), incubate for 1h at RT

(8) Wash once with PBS

(9) Add 50 µl of the antibody solution containing the secondary antibody (1:1000 alexa fluor-488 anti-mouse IgG1, 1:1000 alexa fluor-555 anti-rabbit, 1:1000 Hoechst in blocking buffer), incubate for 45 minutes

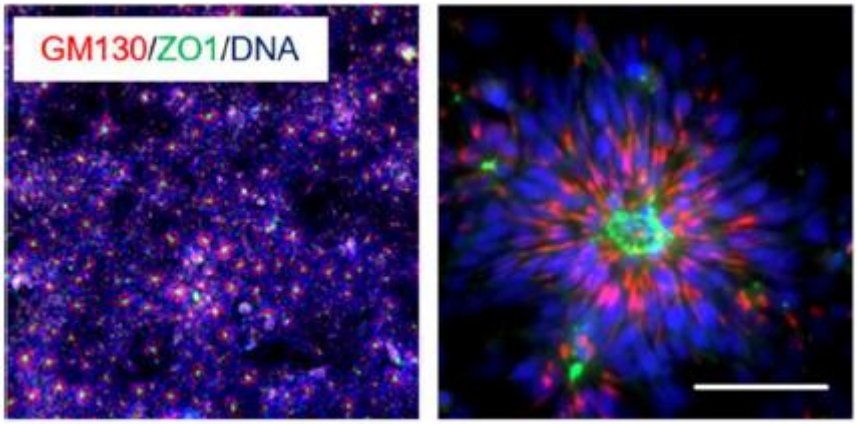
(10) Remove Staining solution, add 100 µl PBS and store at 4°C

(11) Take Fluorescence images of the whole well (channels 488, 555, Hoechst) with Cellomics automated microscope

(12) Analyse images with Konstanz information miner software workflow (described in detail in chapter data analysis)

Figure 4. Typical Rosette staining on d15: Left image is an overview image (10x magnification). In a normal differentiation rosettes form all over the well, equally distributed.

Each ZO1 dot, surrounded by a halo of GM130 represents one rosette. The right image shows a rosette in high magnification (63x). In the centre of the rosette a detailed network of tight junctions (ZO1) is visible. The cells polarise around the rosette centre and also the golgi apparatus (stained by GM130) orients to the middle part.



ASSAY

ACCEPTANCE CRITERIA

The acceptance criteria for the cellular system are defined as follows:

- The cell density at d0 (start of differentiation and start of treatment) has to be between 70-80%. If cells are too dense, the test plates are not used. If the cells show a too low density, they are allowed to proliferate for another day (change medium to fresh KCM+10 ng/ml FGF2
- At d6 of differentiation (gene expression endpoint): expression of marker genes have to be checked whether differentiation was successful in the untreated control relative to stem cells at d-3. A successful differentiation is at least defined by the following rules:

  Pax6 is up regulated at least 30 fold

  Otx2 is up regulated at least 10 fold

  Nanog is at least down regulated to 0.01

  Oct 4 is at least down regulated to 0.1

  TFAP2B is not more than 50 fold up regulated

- At d6 the positive controls (20 nM TSA or 600 µM VPA) have to lead to an down regulation of Pax6 and Otx2, while Tfap2b is up regulated; relative to the untreated control
- At d6 test compounds that lead to decreased viability (less than 80% of control) have to be excluded from gene expression analysis. Since effects of general cytotoxicity should be avoided while the specific effects should occur in a semitoxic concentration range.
- At d15 the rosette formation has to take place in the untreated control while 10 nM TSA/ 600 µM VPA lead to a complete inhibition of rosette formation
- The nuclei are on d15 should not be influenced by a compound treatment since an inhibition of rosette formation due to lower amount of cells should be avoided.

Data Analysis

Viability (Resazurin assay)

Fluorescence is measured at an excitation wavelength of 530 nm and an emission wavelength of 590 nm by Tecan infinite M200 and "Tecan i-control 1.6" software (gain: optimal).

The value of a dead control (value at which cells are all dead) is subtracted from the measured values for normalisation. The Values are then expressed as % of control:

Viability(test)/viability(compound)*100

Gene expression (qPCR)

The results are analysed using the ΔΔCt method. Calculate the ΔCt by normalising the Ct values of the genes of interest (GOI) to the Ct value of the reference genes (TBP, Rpl13a).

ΔCt(GOI)=Ct(GOI)-Ct(biomean of reference gene)

Normalise the ΔCt values for the cells treated with test compounds to the ΔCt of the untreated control.

ΔΔCt= ΔCt(treatment) -ΔCt(control)

The relative gene expression levels are expressed as fold change relative to control

2^(-ΔΔCt).

Rosette formation assay

Images from Cellomics automated microscope are analysed by a workflow of Konstanz information miner software (KNIME).

The analysis of rosette images is divided into four parts.

- The total area of cells is determined which is used for normalisation
- The location of rosettes is detected and images which do not contain rosettes are filtered
- potential rosettes candidates are found and segmented

- it is determined for each segment if the segment is an actual rosette or not and the number of rosettes is counted.

Images are divided into three channels: DAPI, ZO1 (488), GM130 (555). Some images show uneven background illumination, therefore all images are pre-processed by calculating the background and subtraction from each image.

For calculation of the relative cell/nuclei area. Dapi channel images are segmented using the Otsu Global Threshold Algorithm. In order to calculate the area of cells the number of segmented pixels is calculated. Rosettes are located by small bright ZO1 spots as they are located in the centre of rosettes. These bright spots are found by applying Otsu thresholding and segmentation of the thresholded image.

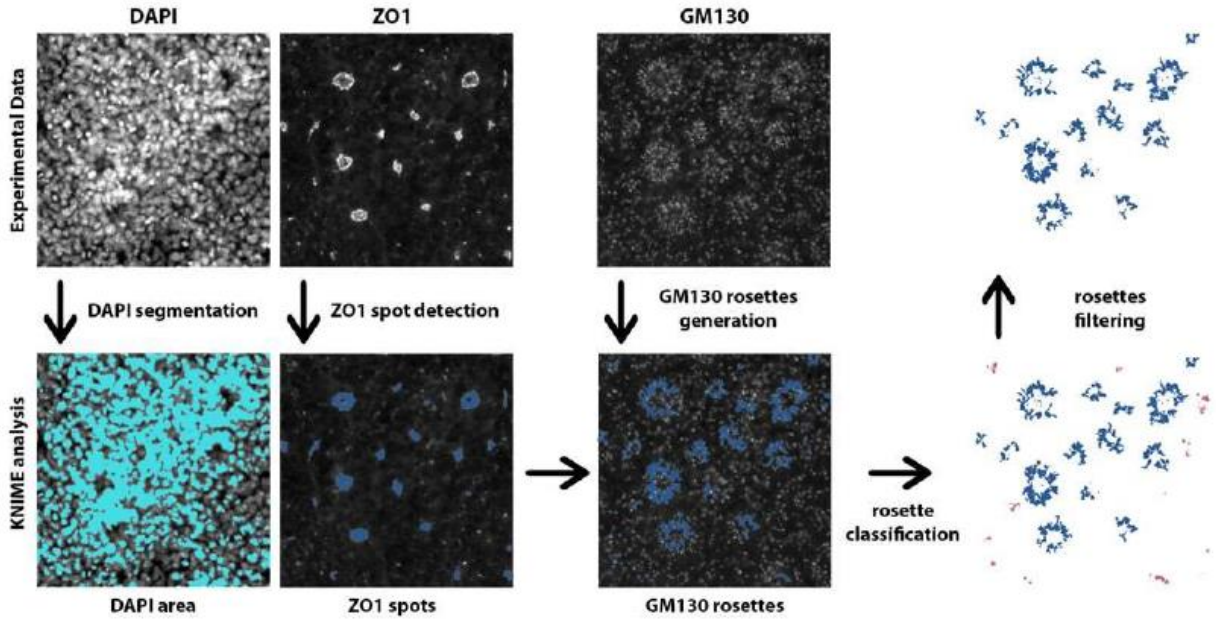
Test compounds may lead to formation of other cellular structure than neural rosettes such as epithelial and neuronal structures that are positive for ZO1. However, the software discriminates between images containing rosettes and images containing other structures. It is assumed that only rosettes display small bright ZO1 centres whereas other structures show bigger bright areas. These structures are not covered by the segmentation and can be filtered using the geometry of detected segments in ZO1. The information of possible rosette is used to generate a segmentation of the rosettes itself in the GM130 (555) channel. In order to do this Voronoi Segmentation is applied to the GM130 channel using the previously discovered locations as seed points. This leads to a segmentation of possible rosettes.

The results are improved by applying a machine learning algorithm which classifies the potential rosettes into positive and negative examples. That allows to filter out any wrong segments resulting from noise or other artefacts. The classification is done using a Random Forest which was learned on a manually defined training set. The features of the training set comprises of measures of segment geometry.

The number of rosettes per image is counted and summed up to the number/well. This is normalised to the nuclei area and the results are expressed relative to the untreated control.

Figure 5. KNIME analysis software.

The images that were measured by Cellomics automated microscope are analysed by KINIME workflow. For calculation of the cell number, the nuclei are in each well,(number of pixels of the Hoechst staining) is counted. In a next step the Zo1 spots are detected. For the classification of a ZO1 spot as a rosette, the Gm130 staining is detected and analysed. The Zo1 spots have to be surrounded by a halo of GM130. Furthermore the roundness and the size of the structures are evaluated and then the rosettes can be classified. Rosettes are counted and normalised to the nuclei area.



Prediction Model

UKN1 models the process of neural induction and patterning. The cells on DoD6 correspond to neuro ectodermal progenitors as present during neural plate and neural tube formation. Therefore disturbances of differentiation and patterning can be modelled by the test method. Compounds that induce neural tube defects should be detected. This includes e.g. spina bifida, anencephaly or encephalocele. The test captures endpoints also measured during developmental toxicity regulation studies like spina bifida.

The prediction model is still under development. Many compounds have been measured not only by qPCR analysis of single genes but also in whole gene chips (Affymetrix). Furthermore, rosette formation of this compounds has been analysed. Data of gene expression (Affymetrix gene chips) are still correlated with rosette formation data. The aim is, chose (gene) markers that are the most important and predictive ones.

This is facilitated by applying different kind of prediction models that are based on statistical methods (eg. Rempel *et al.* 2015) as well as on biological background knowledge (rule based) and to constantly improve the model by simplification of it.

A prediction model that is able to distinguish between relevant and irrelevant as well as reversible and irreversible gene expression changes will be built.

Annexes

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Summary of the development/validation status of the test method used in the project:

This material is to be used to document the status of test systems and test methods; moreover, it is intended to provide guidance for considerations around the use of test systems (or data therefrom) beyond the developer lab; the material will constitute a form of extended SOP, containing also elements important for test method transfer within the project, or within the commercialisation taskforce. Information is structured to provide an overview to interested stakeholders or to prepare for test (pre-)validation). This document is structured in three parts: A (identification); B (main part); C (background information).

***Important note**: Look at part C, before filling B. A test consists of four elements. If one is changed (e.g. the endpoint, or the timing of the test), it is another test. Thus, if you use cells in different 'ways', each way is another test!*

For the description here, decide on one standard protocol/application, and refer all information to this setup. Variations can be indicated in chapter 4.7. If they are used within the project, they will be deposited as new test.

Part A (general info)

Name of test method (original/published name, tradename).

Assigned name for EU-ToxRisk data base (following these rules):

UKN1_DART_iPSC_Diff_6D

Name (and acronym) of the project partner home organisation

University of Konstanz (UKN)

Name and email of contact person:

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Marion Kapitza: marion.kapitza@uni-konstanz.de

Name of further persons involved (e.g. PI of the lab; the person who does the experiments; etc.)

Prof. Dr. Marcel Leist: marcel.leist@uni-konstanz.de

Dr. Tanja Waldmann: tanja.waldmann@uni-konstanz.de

Number of additional files to be upload

General SOP (including cell culture maintenance)

Part B: Specific evaluation part

1. Description of general features of the test system (cells/tissue)

1.1 Cellular system component(s)

Give a brief overview of your biological system: e.g. the cells that you use; which cell type(s); monoculture/co-culture, differentiation state; 2D/3D; etc.

mc-iPSC

- mc-iPSC are induced pluripotent stem cells from human adipose stem cells (hASC). Pluripotency was induced using the minicircle technology (Jia *et al.*, 2010, Nature Methods Mar;7(3):197-199) and pluripotency is certified by gene and protein expression of pluripotency markers.
- In the presence of three SMAD inhibitors, noggin, dorsomorphine and SB431542 mc-iPSC cells can be differentiated into neuroectodermal progenitor cell (NEP) in 6 days.
- The maintenance is usually cultured in colonies on MEF feeder cells.
- The differentiation is usually cultured in a feeder-free **2D monolayer**.

1.2 Definition of cells used

Quantitative and semi-quantitative features that define your biological system:

STR signature (where available); Karyotype information, or e.g. gender (where available and relevant); ATCC number, passage number, source (supplier), subline (where relevant); source of primary material; purity of the cells; proliferation rate (doubling time), ...

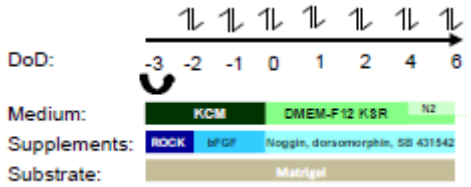
Describe defining biological features you have measured or that are FIRMLY established (use simple listing; limit to max. 0.5 pages): e.g. the cells express specific marker genes; have specific surface antigens; definitely do not have certain markers; have or have not a relevant metabolic or transporting capacity;

(Note: each method can use only one (transgenic) cell. Another cell is another method.)

- **Supplier:** System Biosciences Cat. # SC301A-1
- **Gender:** female
- **Morphology:** mc-iPSC are small and round cells that grow in colonies. Differentiated cells do not significantly change their morphology compared to iPSC.
- **Doubling time:** approx. 14 -20 h (depending on passage)
- **Phenotype:** mc-iPSC express all expected pluripotency markers, such as OCT4, NANOG, SSEA4 and SOX1. After 6 days of differentiation pluripotency markers are downregulated and the cells express markers of

neuroectoderm that suggest an anterior fate. These include, PAX6, OTX2, EMX2 and MSX1. Whole transcriptome analysis (Affymetrix gene chips) revealed a clear neural phenotype.

Figure 1. Schematic display of the differentiation protocol.



1.3 Give a reference/link to maintenance culture protocol (upload field)

How are the cells maintained outside the experiment (basic cell propagation). For primary cells, how are they obtained in general and what are they characterised for (and what are inclusion and exclusion criteria). Refer to a defined updated protocol. Upload file here.

External document is available

Maintenance Principle

mc-iPSC are grown in colonies on mouse embryonic fibroblasts (MEF) as feeder cells. The growth factor bFGF is added to the medium of proliferating cultures. Cells are passaged every 7 days. For splitting MEFs are removed by trypsin digest and the colonies are washed of the plates mechanically. Thereby the colonies get smaller and they are seeded in a 1:15 to 1:20 dilution in new flasks coated with fresh MEFs one day before splitting. The maintenance cultures are done in either T25 flasks or 6 cm plates.

2. Definition of the test system

2.1 Principles of the culture/differentiation protocol

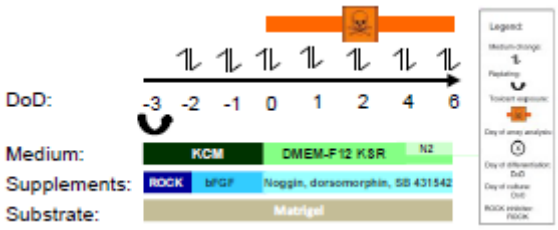
Describe here the particular process of generating the test system (i.e. producing the cells in the desired state, not maintenance culture as in chapter 1).

E.g. detail the general features/principles of the culture protocol (collagen imbedding, 3D structuring, addition of mitotic inhibitors, and addition of particular hormones/growth factors…)

E.g. give the principles of the selected differentiation protocol (provide scheme, indicating all phases, media, substrates, manipulation steps (medium change/re-plating, etc),)

Note: the exact experimental procedure is covered by a separate question.

UKN 1:



Coating of flasks and plates

Principle:

Plates and flasks are coated with matrigel for 30 minutes in the cell culture incubator. The coated plastic can be stored at 4°C up to 2 weeks.

Differentiation

Principle:

For differentiation, a quick trypsin digest removes MEFs and the colonies are manually dissociated into single cells. Then the cells are plated on matrigel coated plates in the presence of conditioned medium (KCM) supplemented with bFGF and ROCK inhibitor. Differentiation is started on DoD0 by adding the following factors: Noggin, dorsomorphine and SB431542 in DMEMF12-KSR medium.

2.2 Endpoints

Describe the endpoint(s) that you use to control your culture protocol (gene expression, neurite mass, P450 activity, ...)

Note: check part C for definition of 'endpoint'

Endpoints:

correct differentiation can be seen by changes of mRNA levels (in fully differentiated cells at DoD6) of the following markers:

- Upregulation of PAX6
- Upregulation of OTX2
- Upregulation of EMX2
- Downregulation of OCT4
- Downregulation of NANOG

2.3 Analytical endpoints

Describe the analytical/methods that you use to evaluate your culture endpoints (PCR, ATP measurement)

Actually used method:

- qPCR for differentiation (PAX6, OTX2, EMX2) and pluripotency (OCT4, NANOG) markers at day 6 of differentiation is routinely performed.

2.4 Acceptance criteria for test system (N.B. not for toxicity test method!)

Describe the acceptance criteria for your test system (this means the quality criteria for your cells/tissues/larvae..: which endpoints do you consider to describe the cells, which parameters are important?

Which values (e.g. degree of differentiation) need to be reached / should not be reached.

How does your test system perform with regard to these criteria? (when differentiation is performed 10 times, how do the values for the acceptance criteria parameters look like: average, variation)

Examples: neurite length is 50 ± 15 µm; experiments with average neurite length below 25 µm are discarded; Nestin induction is 200 ± 40 fold; experiments with inductions below 80-fold are discarded;….

Quantitative criteria have been defined using 17 different differentiations performed by three different experimenter as follows:

The lowest value for the upregulated genes PAX6, OTX2 and EMX2 and the highest value for downregulated genes OCT4 and NANOG were determined, 10% of these values were subtracted or added and rounded down or up, respectively. This revealed the following acceptance values:

- PAX6 > 40 fold upregulated compared to DoD-3
- OTX2 > 6 fold upregulated compared to DoD-3
- EMX2 > 40 fold upregulated compared to DoD-3
- OCT4 < 0,04 fold downregulated compared to DoD-3
- Nanog < 0,03 fold downregulated compared to DoD-3

2.5 Variability and troubleshooting

Known causes of variability (e.g. plate format and supplier);

Indicate critical steps;

Give recommendations to increase/ensure reproducibility and performance.

- **Causes of variability:**
  - Maintenance: size of cell clumps during splitting, density of colonies
  - Differentiation: seeding density on DoD-3
- General points for both:
  - lots of different plates/flasks: plastic might be different, if the manufacturer delivers from a different/new lot

- different lots of medium and supplements, especially matrigel and serum replacement lots.
- Medium change should be done always at the same time of the day +/- 2 hours.

2.6 Features relevant for cytotoxicity testing

Particular apoptosis sensitivity or resistance?

Cytotoxicity hard to capture for minor cellular subpopulations?

Issues with distinguishing slowed proliferation from cell death?

Etc.

Note: this is meant for a brief overview of main features, or for information from own experience that differs from the general literature.

As cells highly proliferate during differentiation, measurement of resazurin reduction cannot distinguish between cell death and reduced proliferation.

2.7 Metabolic capacity of the test system

What is known about endogenous metabolic capacity (CYP system)?

What is known about other systems relevant to xenobiotic metabolism?

What specific information is there on drug transport?

Note: this is meant for a brief overview of main features, or for information from own experience that differs from the general literature.

no information available

2.8 Omics characterisation of the test system (upload field)

Are there transcriptomics data or other omics data available that describe the basic state of the test system (characterisation of cells without compounds). Briefly list and describe such data.

Indicate, whether you want to upload data at a later time point.

Microarray analysis (Affymetrix gene chip) has been extensively used to characterise the differentiation procedure (Balmer, 2012; Balmer 2014).

Microarray analysis (Affymetrix gene chip) has been extensively used to characterise the effects of VPA and TSA (Balmer 2012; Krug 2013; Balmer 2014; Waldmann 2014; Rempel 2015; Shinde 2016; Waldmann 2016)

3. Test method description

3.1 Test documentation in published literature (web links)

Refer to published literature on the test AND indicate exactly deviations from published descriptions (e.g. plastic plate supplier, cell number, endpoint measurement, timing, ...)

Note: links can be added (e.g. to medline)

Note: protocol upload in point 4.6

Balmer, N. V., S. Klima, E. Rempel, V. N. Ivanova, R. Kolde, M. K. Weng, K. Meganathan, M. Henry, A. Sachinidis, M. R. Berthold, J. G. Hengstler, J. Rahnenfuhrer, T. Waldmann and M. Leist (**2014**). "From transient transcriptome responses to disturbed neurodevelopment: role of histone acetylation and methylation as epigenetic switch between reversible and irreversible drug effects." Arch Toxicol **88**(7): 1451-1468.

Balmer, N. V., M. K. Weng, B. Zimmer, V. N. Ivanova, S. M. Chambers, E. Nikolaeva, S. Jagtap, A. Sachinidis, J. Hescheler, T. Waldmann and M. Leist (**2012**). "Epigenetic changes and disturbed neural development in a human embryonic stem cell-based model relating to the fetal valproate syndrome." Hum Mol Genet **21**(18): 4104-4114.

Krug, A. K., R. Kolde, J. A. Gaspar, E. Rempel, N. V. Balmer, K. Meganathan, K. Vojnits, M. Baquie, T. Waldmann, R. Ensenat-Waser, S. Jagtap, R. M. Evans, S. Julien, H. Peterson, D. Zagoura, S. Kadereit, D. Gerhard, I. Sotiriadou, M. Heke, K. Natarajan, M. Henry, J. Winkler, R. Marchan, L. Stoppini, S. Bosgra, J. Westerhout, M. Verwei, J. Vilo, A. Kortenkamp, J. Hescheler, L. Hothorn, S. Bremer, C. van Thriel, K. H. Krause, J. G. Hengstler, J. Rahnenfuhrer, M. Leist and A. Sachinidis (**2013**). "Human embryonic stem cell-derived test systems for developmental neurotoxicity: a transcriptomics approach." Arch Toxicol **87**(1): 123-143.

Rempel, E., L. Hoelting, T. Waldmann, N. V. Balmer, S. Schildknecht, M. Grinberg, J. A. Das Gaspar, V. Shinde, R. Stober, R. Marchan, C. van Thriel, J. Liebing, J. Meisig, N. Bluthgen, A. Sachinidis, J. Rahnenfuhrer, J. G. Hengstler and M. Leist (**2015**). "A transcriptome-based classifier to identify developmental toxicants by stem cell testing: design, validation and optimization for histone deacetylase inhibitors." Arch Toxicol **89**(9): 1599-1618.

Shinde, V., L. Hoelting, S. P. Srinivasan, J. Meisig, K. Meganathan, S. Jagtap, M. Grinberg, J. Liebing, N. Bluethgen, J. Rahnenfuhrer, E. Rempel, R. Stoeber, S. Schildknecht, S. Forster, P. Godoy, C. van Thriel, J. A. Gaspar, J. Hescheler, T. Waldmann, J. G. Hengstler, M. Leist and A. Sachinidis (**2016**). "Definition of transcriptome-based indices for quantitative characterization of chemically disturbed stem cell development: introduction of the STOP-Tox and STOP-Tox tests." Arch Toxicol.

Waldmann, T., E. Rempel, N. V. Balmer, A. Konig, R. Kolde, J. A. Gaspar, M. Henry, J. Hescheler, A. Sachinidis, J. Rahnenfuhrer, J. G. Hengstler and M. Leist (**2014**). "Design principles of concentration-dependent transcriptome deviations in drug-exposed differentiating stem cells." Chem Res Toxicol **27**(3): 408-420.

Waldmann, T. and Grinberg, M. *et al.* (**2016**). "Stem cell transcriptome responses and corresponding biomarkers that indicate the transition from adaptive responses to cytotoxicity." Chem Res Toxicol doi: 10.1021/acs.chemrestox.6b00259

Deviations from published literature:

day -3: Feeder cells are removed by trypsin as follows: The cells are washed once with PBS, then trypsin (0.05%) is given for 2 min. MEFs are carefully removed by gently pipetting up and down. After removal of feeders, the stem cell colonies are removed from the flask mechanistically.

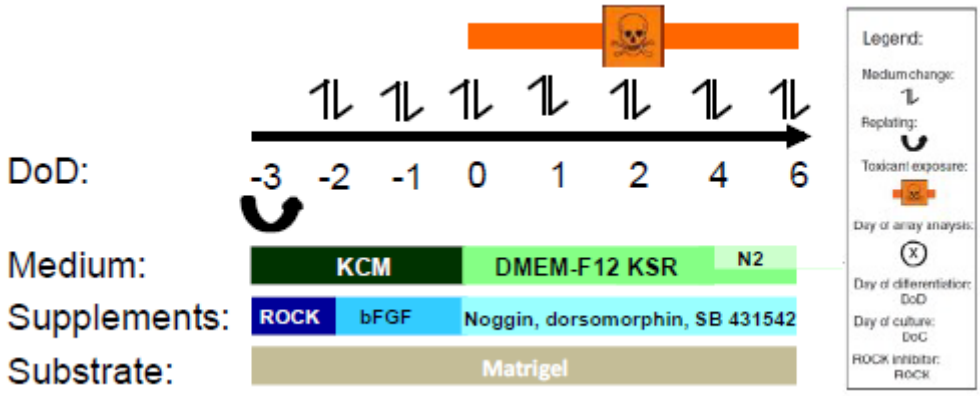
3.2 Exposure scheme for toxicity testing

Define exposure scheme (graphically), within the context of the overall cell culture scheme (e.g. freshly re-plated cells or confluent cells at start, certain coatings…);

Include medium changes and how/whether compounds are re-added in cases of medium change.

Note: there can only be one exposure scheme, no options! Check part C for definitions. See for point 4.7 for indicating variations.

UKN 1:



day -3: MEFs from maintenance culture are removed, iPSC are seeded in single cells on matrigel coated plates in conditioned medium (KCM) supplemented with bFGF and ROCK inibitor.

day -2: Medium is changed to KCM supplemented with bFGF without ROCK inhibitor

day -1: Medium is changed to KCM supplemented with bFGF without ROCK inhibitor

day 0: Start of differentiation and toxicant treatment: Medium is changed to DMEM-F12 KSR supplemented with noggin, dorsomorphine, SB431542 and toxicant.

day 1: Medium is changed to DMEM-F12 KSR supplemented with noggin, dorsomorphine, SB431542 and toxicant for sufficient nutrient supply.

day 2: Medium is changed to DMEM-F12 KSR supplemented with noggin, dorsomorphine, SB431542 and toxicant for sufficient nutrient supply.

day 4: Medium is changed to 75% DMEM-F12 KSR / 25% N2 supplemented with noggin, dorsomorphine, SB431542 and toxicant for sufficient nutrient supply

day 6: Measurements of cytotoxicity by resazurin reduction, isolation of RNA samples.

3.3 Test method endpoint

Define the specific endpoint(s) of the test system that you use for toxicity testing, and potential reference endpoints (e.g. cytotoxicity).

Note: check part C for definition of endpoint!

Test endpoints: 1) cytotoxicity

2) differentiation capacity

3.4 Analytical endpoint(s) overview

Define the analytical endpoints used on samples from the test system. Give here a general overview of principles and key steps.

For imaging endpoints: explain in general how quantification algorithm or how semi-quantitative estimates are obtained.

- **Resazurin reduction assay (Cytotoxicity)**

  Resazurin (7-hydroxy-3H-phenoxazin-3-one-10-oxide) reduction assay, also called Alamar blue assay is a commonly used assay to measure cell viability via measurement of fluorescence intensity. It is based on the reduction of the non-fluorescent blue dye resazurin to the redfluorescent resarufin. Resazurin is non-toxic and is able to cross the cell membrane. Inside the cells it is then reduced by cellular oxido-reductases. Fluorescence intensity is proportional to cell number and metabolic activity of the cells Resazurin is added to the cells to a final concentration of 10 µg/ml and incubated at 37°C for 30 minutes under humidified conditions, 5% CO_2. Fluorescence measurement was performed at an excitation wavelength of 530 nm and an emission wavelength of 590 nm by Tecan infinite M200 and "Tecan i-control 1.6" software.

- **Gene expression analysis (differentiation capacity)**

  - **by RT-qPCR**

  On DoD6 medium is removed and the cells are dissolved in 500 µl TRIzol® / 6 well and whole RNA is purified according to the manufactures protocol. After resolving the RNA in water, a quality control is performed by measuring OD 230 nm, 260 nm and 280 nm. RNA preparation is accepted if 280 / 230 and 280 / 260 ratio are between 1.8 and 2.2.

  Next RNA is translated into cDNA using Biorad IScript reverse transcription Kit.

  qPCR is performed using primers for specific neural and pluripotency markers OCT4, NANOG, PAX6, OTX2 and EMX2

  - **by Affymetrix gene chip**

  RNA is purified as described above using the TRIzol reagent. Then the samples are shipped to the laboratory of Prof. Agapios Sacchinides and further processed for hypbridisation.

3.5 Analytical endpoint(s) technical details

Upload a protocol that also informs on machine settings, analytical standards, data processing and normalisation procedures. For imaging endpoints: give detailed algorithm.

Cytotoxicity by resazurin reduction

Resazurin (7-hydroxy-3H-phenoxazin-3-one-10-oxide) reduction assay, also called Alamar blue assay is a commonly used assay to measure cell viability via measurement of fluorescence intensity. It is based on the reduction of the non-fluorescent blue dye resazurin to the red-fluorescent resarufin (O'Brien, Wilson *et al.* 2000). Resazurin is non-toxic and is able to cross the cell membrane. Inside the cells it is then reduced by cellular oxido-reductases (Gonzalez and Tarloff 2001). Fluorescence intensity is proportional to cell number and metabolic activity of the cells (Gonzalez and Tarloff 2001; Zhang, Du *et al.* 2004).

Resazurin is added to the cells to a final concentration of 10 µg/ml and incubated at 37°C for 30 minutes under humidified conditions, 5% CO_2. Fluorescence measurement is performed at an excitation wavelength of 530 nm and an emission wavelength of 590 nm by Tecan infinite M200 and "Tecan i-control 1.6" software. Values are calculated relative to untreated control.

RNA isolation, transcription to cDNA and qPCR

The mRNA is purified according to the Trizol protocol (Invitrogen). 0.5 ml/well Trizol was added to the cells and incubated for a few minutes. The samples are transferred to an Eppendorf tube and can be stored at -20°C.

After thawing 100 µl chloroform is added to the samples and mixed roughly followed by 3 minutes incubation at room temperature. The liquid phases are separated by spinning for 15 minutes at 20 000g at 4°C. The upper aqueous phase is transferred to a fresh tube. 200 µl isopropanol is added to the phase and mixed roughly. After an incubation for 10 minutes at room temperature the samples are centrifuged for 10 minutes at 20 000g at 4°C. The supernatant is removed and the RNA pellet is washed twice with 0.5 ml ice cold 75% ethanol. The pellet is air-dried for 25 minutes and dissolved in RNAse free H_2O. The RNA concentration is measured by Nanodrop and the samples are stored at -80°C.

The transcription of mRNA into cDNA is facilitated using iScriptTM Reverse Transcription Supermix for RT-qPCR (biorad). For one reaction 1 µg of mRNA is used, the volume is adjusted to 16 µl with RNAse free water and then 4 µl of 5 fold iScript supermix is added. Priming is performed 5 minutes at 25°C, the reverse transcription 30 minutes at 42°C and the inactivation 5 minutes at 85°C. cDNA is stored at -20°C.

For qPCR the Sso FastTM Fast EVAGreen Supermix is used which contains EVAGreen dye as a fluorescent dye (Mao, Leung *et al.* 2007).

The cDNA samples are diluted 1:5. For each reaction to 1 µl of cDNA template 0.2 µl of each primer (10µM; forward and reverse), 5 µl Sso FastTM Fast EVAGreen Supermix and 3.6 µl H_2O is added. The qPCR is performed in CFX96 Real-time PCR detection System with C1000 Thermal Cycler (Bio-Rad) using the following conditions:

The samples are heated to 98°C for two minutes to activate the Taq polymerase which is followed by 40 amplification cycles of 2 sec at 98°C denaturising, 5 sec at 60°C annealing

and polymerisation steps and after each cycle a plate readout is performed. After the amplification cycles, the temperature is increased in 0.5°C steps up to 95°C to provide a melting curveof the qPCR product to determine the reaction specificity.

The results are analysed using the ΔΔCt method. First the ΔCt is calculated by normalising the Ct values of the genes of interest (GOI) to the (geometric mean of the) Ct value of the reference gene(s) which are here in this case RPL13a and TBP.

ΔCt(GOI)=Ct(GOI)-Ct(reference gene)

The ΔCt values for the cells treated with the test compounds are further normalised to the ΔCt of the untreated control

ΔΔCt= ΔCt(treatment) -ΔCt(control)

The relative gene expression levels are expressed logarithmically as 2-ΔΔCt since the amount of DNA should double with every PCR cycle under perfect conditions.

Gene expression by Affymetrix gene chip

The RNA was quantified using a NanoDrop N-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA), and the integrity of RNA was confirmed with a standard sense automated gel electrophoresis system (Experion, Bio-Rad, Hercules, CA, USA). The samples were used for transcrip- tional profiling when the RNA quality indicator (RQI) number was [8. First-strand cDNA was synthesised from 100 ng total RNA using an oligo-dT primer with an attached T7 promoter sequence, followed by the complementary second strand. The double-stranded cDNA molecule was used for *in vitro* tran- scription (IVT, standard Affymetrix procedure) using Gene- chip 30 IVT Express Kit. During synthesis of the aRNA (amplified RNA, also commonly referred to as cRNA), a biotinylated nucleotide analogue was incorporated, which serves as a label for the message. After amplification, aRNA was purified with magnetic beads and 15 lg of aRNA was fragmented with fragmentation buffer as per the manufacturer's instructions. Then, 12.5 lg fragmented aRNA was hybridised with Affymetrix Human Genome U133 plus 2.0 arrays as per the manufacturer's instructions. The chips were placed in a GeneChip Hybridization Oven-645 for 16 h at 60 rpm and 45 °C. For staining and washing, Affymetrix HWS kits were used on a Genechip Fluidics Station-450. For scan- ning, the Affymetrix Gene-Chip Scanner-3000-7G was used, and the image and quality control assessments were performed with Affymetrix GCOS software. All reagents and instruments were acquired from Affymetrix (Affymetrix, Santa Clara, CA, USA).

3.6 Endpoint-specific controls

Do you use endpoint-specific controls that show biologically plausible changes of the endpoint; List such controls (up to 10), indicate why you consider them as endpoint-specific controls, and how data on such controls look like?

Example 1: U0126 (ERK signalling pathway inhibitor). Neurite outgrowth in the CNS is controlled by ERK, inhibitors should therefor block this endpoint: U0126 blocks neurite growth at concentrations that block ERK activation

Example 2: Cytochalasin D (actin depolymeriser). Cell movement requires actin reorganisation. Disturbance of actin structure should attenuate cell migration. Cytochalasin D inhibits cell migration at non-cytotoxic concentrations.

Example 3: BMP4 (endogenous protein, ligand of BMP receptor). Cell differentiation towards neuroectoderm is disturbed by BMP-SMAD signalling. Therefore the test is based on SMAD inhibition by noggin (a protein scavenging/neutralising BMP4). Addition of additional BMP4 should outcompete noggin and lead to SMAD signalling, therefore preventing neuronal differentiation. BMP4 prevents the normal differentiation, this test is based on.

- **Positive control for differentiation disturbances:**
  - 600 µM valproic acid, a known DNT compound.
  - 2 µM CHIR99021, a GSK3 inhibitor, wnt pathway is activated and therefore the anterior patterning of the cells is disturbed.
  - 2 µM Bio, a GSK3 inhibitor, wnt pathway is activated and therefore the anterior patterning of the cells is disturbed.

3.7 Negative, positive controls

What do you use as negative, positive controls? How do data on such controls typically look like (signal and its uncertainty).

Are the positive controls of toxicological relevance. Do they have in vivo anchoring

What is the rationale for the concentration setting of negative controls.

- Negative control:
  - solvent (0.1% DMSO final concentration)
- Positive control:
  - 600 µM valproic acid (VPA), a known DNT compound *in vivo* induces congenital malformations of the neural tube (e.g. spina bifida). *In vitro* it induces massive changes in gene expression patterns that indicate disturbances of the proper differentiation track.
  - 10 nM trichostatin A (TSA), a known DNT compound and also known to induce changes in gene expression patterns.

3.8 Acceptance criteria

Acceptance criteria for test runs: when is a test discarded?

Which rule do you apply to test whether a test run is within the normal performance frame; How do you document this decision?

- No formal acceptance criteria have been defined.

3.9 Test parameters

Indicate here basic performance features or possibly preliminary estimates (label as such): Baseline variation (noise) within assays /between assays.

Signal/noise ratio (signal = standard positive control); (z-factor determined?)

Detection limit (required change of endpoint to become measurable);

Interpretation of changes to both sides of controls (up/down)?

Inter-operator variation.

Historical controls over longer time period?

Note: assay parameters can only apply to one assay version. A standard version must be defined and referred to in all answers

UKN2 Antwort --- ist denke ich nicht in dem Sinne übertragbar auf UKN1, wie könnten wir das ausrechnen?

1. The variation of controls between the plates of one experiment is ≤ 5% and the variation between different experiments is ≤ 20%.

   The variation between technical replicates of viability is about 5% and the variation of migration is about 15%.

4. Migration can be accelerated (e.g. by FCS) or decreased (Fig.2).

5. After two weeks the inter-operator variations can be reduced to a moderate level.

3.10 Throughput estimate

Real data points per month *(not per weak/per quarter…; count three working weeks per month). Each different concentration is a data point, but necessary controls that are required for calibration and for acceptability criteria are NOT counted as data points.*

All technical replicates of one condition are counted as one single data point.

Indicate possibility/extent of repeated measures (over time) from same dish.

Give rationale/explanation of your estimate.

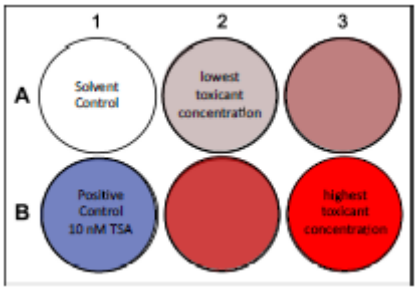
Data point = one biological replicate (→ usually 1 technical replicates); each concentration/condition of a compound counts as data point

UKN 1:

360 data points per month

- 6 plates per week can be measured.
- 1 plate has 6 data points → 36 data points per week
- 3 weeks per month → 108 data points per month

Typical plate layout:



Typical week schedule for three biological replicates:

	Replicate	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
week 1	Diff_#1	DoD-3 Seeding on matrigel	DoD-2 Medium change	DoD-1 Medium change	DoD0 Medium change	DoD1 Medium change	DoD2 Medium change	DoD3 -------

		Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
week 2	Diff_#1	DoD4 Medium change	DoD5 -------	DoD6 Resazurin assay Cells in Trizol	RNA prepartion cDNA synthesis	qPCR data analysis		
	Diff_#2	DoD-3 Seeding on matrigel	DoD-2 Medium change	DoD-1 Medium change	DoD0 Medium change	DoD1 Medium change	DoD2 Medium change	DoD3 -------

		Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
week 3	Diff_#2	DoD4 Medium change	DoD5 -------	DoD6 Resazurin assay Cells in Trizol	RNA prepartion cDNA synthesis	qPCR data analysis		
	Diff_#3	DoD-3 Seeding on matrigel	DoD-2 Medium change	DoD-1 Medium change	DoD0 Medium change	DoD1 Medium change	DoD2 Medium change	DoD3 -------

		Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Week 4	Diff_#3	DoD4 Medium change	DoD5 -------	DoD6 Resazurin assay Cells in Trizol	RNA prepartion cDNA synthesis	qPCR data analysis		

3.11 Concentration settings

How is the concentration range of test compounds usually defined (e.g. only single concentrations; always 10 x dilutions, or variable dilution factors. Is there a rule for defining starting dilutions.

- The first step is always a concentration response study using cytotoxicity as an endpoint.
- Then the EC_{10} is determined as the concentration to be used for gene expression analysis.

3.12 Special instrumentation

Does the method require specialised instrumentation, not found in standard labs.

Is there a need for custom-made instrumentation or material?

Is there a need for equipment not commercially available (anymore)?

- The method requires a standard qPCR machine present in most laboratories.
- For whole transcriptomics analysis a special equipment from Affymetrix platform is needed:
  - Gene Chip hybridization oven-645
  - Genechip Fluidics Station-450
  - Affymetrix Genechip Scanner-3000-7G

4. Handling details of the test method

4.1 Preparation / addition of test compounds

How are stocks prepared (fold concentration, verifiction, storage?)

How are dilutions prepared?

How does the final addition to test systems take place?

Give an overview on range of volumes, particular labware/instruments for dispensing, temperature/lighting considerations, particular media/buffers for dilution, decison rules for the solvent; tests of solubility.

Details of addition of test compounds to test systems, (give details: e.g. in which compartment of compartmentalised cultures, in which volume, before after or during medium change, ...

Note: Strike a reasonable balance between information overview (focus here) and technical detail (to be detailed in an attached protocol file)

- Compounds are stored according to the manufacturer's instructions (e.g. 4°C, room temperature, -20°C).
- Preferable solvent is DMSO. The used DMSO is stored in a lightproof, air-tight bottle at room temperature.
- Final DMSO concentration on the cells is 0.1%
- After dissolving the compounds which are delivered in a solid/powder form, all compound solutions are aliquoted into volumes sufficient for one experiment (i.e. one biological replicate). In this way repeated freezing and thawing and therefore damaging the compound's stability and efficiency can be avoided.
- on DoD0 medium is prepared according to the SOP and supplemented with the EC_{90} concentration of the compound.

4.2 Day-to-day documentation of test execution

Note: lab book entries are metadata that need to be linkable to test results on demand!

How are actual day-to-day procedures documented (type of 'lab book' organisation)

Define lab-specific procedures used for each practical experiment to calculate test compound concentrations (and to document this)

How are plate maps defined and reported

- Plate maps are defined prior to the experiment and documented in the lab book and files (Excel files) are stored on the work group server.
- Concentrations and compound dilutions are calculated prior to the experiment.
- Experimental procedures are noted manually in a paper lab book.

4.3 Practical phase of test compound exposure

How is the time plan of pipetting established, followed, documented;

How is adherence to plate maps during pipetting documented.

What are the routine procedures to document intermediate steps with potential errors, mistakes and uncertainties.

How are errors documented (e.g. pipetting twice in one well). How are the plate wells used sequentially – following which pattern, ….

- The exact time points of start of differentiation, start of treatment and harvesting RNA samples are documented in the lab book.
- Pipetting errors are marked directly on the plate maps and are documented in the lab book.
- The paper lab book is taken to cell culture rooms and errors are documented in there right away.

4.4 Outliers

How are outliers defined and handled?

How are outliers documented

General frequency of outliers.

Note: if only processed data are transferred (initial project phase), outlier information gets lost

- Mathematical procedures to define outliers have not been defined. Data points that 'look' very far off are discarded. Biological outliers do practically not exist, most far data points are the result of technical problems (RNA preparation not pure or failed, contaminated primers)

- All raw data (incl. outliers) are stored.
- No information about the frequency of outliers available.

4.5 Uncertainties, problems

Types of compounds that give trouble;

Experimental variables that are hard to control;

Critical steps of assay performance

Robustness issues: known variations of test performance due to operator training, season, use of certain consumable or unknown causes, ...

Known pitfalls (or potential operator mistakes);

- Compound solubility in stock and during dilution is too low (stock solved in 100% DMSO, final concentration of the solvent on the cells is 0.1% DMSO)
- Operators have to be well trained in cell culture handling as on DoD-3 the survival of cells is essential for the density on DoD0, which in turn is essential for robust differentiation.
- Plastic quality can be problem if plastic lots even from the same supplier change, cells detach during differentiation.
- Operators can get trained within 2-4 month. Cell seeding and medium change should be performed as fast as possible to keep cells as short as possible at room temperature. The more practice an operator has, the faster the critical steps can be performed.

4.6 Detailed protocol (upload field)

Reference to additional file (containing info covered in chapters 3+4), but containing all details and explanations. Upload!

Has the SOP been deposited in an accessible data base? Has the SOP been reviewed externally – how?

Note: updated protocols / protocol version can be uploaded later.

- SOP is available and will be uploaded

4.7 Possible variations

Describe variations, modifications and extensions of the test method documented in the current protocol:

a) other endpoints,

b) other analytical methods for same endpoint,

c) other exposure scheme, e.g. repeated exposure, ...

d) experimental variation (e.g. use of a specific medium, presence of an inhibitor or substrate that affects test outcome, ...)

Note: This should not be everything that COULD be done, but only points that have been done successfully, or that have high likelihood to be done in a defined project context and after some appropriate evaluation.

- **Additional endpoints:**
  - analysis of differentiation markers by immunostaining
  - functional endpoint of rosette formation see UKN1b
- **Exposure timing**

4.8 Cross-reference to related test methods (upload field)

Indicate the names of related tests and short description.

Note: updated protocols / protocol versions can be uploaded later.

Of particular importance: test method may have been used for high throughput transcriptomics or deep sequencing as alternative endpoint. Such reference would need to be added here.

- The rosette formation assay (UKN1b) is based on UKN1 and represents a phenotypic anchoring of gene expression changes on DoD6.

5. Prediction model/Translation

5.1 Rationale of your test method (= scientific purpose)

Which process(es) or toxicological events are modelled/reflected by your test method?

To which human adverse outcome(s) is your test method related or could be related?

Does the test method capture an endpoint of current regulatory studies?

Note: not more than 200 words.

- UKN1 models the process of neural induction and patterning. The cells on DoD6 correspond to neuroectodermal progenitors as present during neural plate and neural tube formation. Therefore disturbances of differentiation and patterning can be modelled by the test method.
- Compounds that induce neural tube defects should be detected. This includes e.g. spina bifida, anencephaly or encephalocele.
- The test captures endpoints also measured during developmental toxicity regulation studies like spina bifida.

5.2 Prediction model

Provide your prediction model: when do you consider the result as toxic or not toxic.

What is a 'hit' if the test is used in screening mode (if different from above)?

- The prediction model development is ongoing.

5.3 Prediction model setup

How was the prediction model set up (which training chemicals)?

Has the prediction model been tested (testing set of chemicals; list or give n, if n > 50)?

Is the process documented (Publication)

What sensitivity and specificity was obtained for testing set of chemicals)

- The prediction model development is ongoing.

5.4 IVIVE (In vitro – in vivo extrapolation)

Describe parameters important for determination of free compound concentrations in the medium (lipid content, protein content);

Has the test been used earlier for IVIVE?

Are there special considerations relevant for IVIVE (e.g. potential for compound accumulation due to frequent medium changes and compound re-addition, pgp expression, metabolic capacity..)

- Estimated lipid content and albumin concentration in *in vitro* test media and human plasma:

Medium	Lipid content (mg/l)	Albumin concentration (µM)
UKN1	92	184.7
Human plasma	6000	600

- The test has been used for IVIVE (see Krug *et al.*, 2013; Arch. Toxicol. 87:123-143)
- No special considerations known.

5.5 Applicability domain

Which compounds is the test likely to pick up correctly, where is it likely to fail?

Are there areas (according to industry sector, compound chemistry, phys-chem properties) that need to be excluded from testing, or that are particularly suitable?

- HDAC inhibitors and WNT activators have been detected.

- The exact applicability domain is not yet clear.
- Volatile compounds and substances that are not water-soluble cannot be measured.

5.6 Incorporation in test battery

Indicate potential strengths and weaknesses of the system in a test battery.

Compare performance to similar tests.

Which gaps in a known battery is your test filling.

Preferential use in first tier or later tiers, requiring complementary assays or being stand-alone.

- UKN1 is a low throughput assay
- UKN1 can detect pathways that are involved in compound-specific toxicity
- The assay has been used as part of the ESNATS screen battery
- The negative hits are little defined and require other hits in a developmental toxicity test battery

6. Data management

6.1 Data format (multiple upload field)

What is the data format? A: raw data: give general explanation. You may upload an example now or later. B: processed data at a level suitable for general display and comparison of conditions and across experiments and methods: focus on this level for now and give example.

If the file format is not proprietary or binary, please upload a template. This will help other users to provide their data in a similar way to the general data infrastructure. Additionally, we will use this as starting point for the development of a standard file format.

Example for a data format suitable for many cell assays (need not be followed, but recommended):

column 1: 'line number' (identifier of condition within one experiment)

column 2: date

column 3: experiment number or identifier for reference to partner lab book

column 4: compound

column 5: concentration (in: -log (M))

column 6: which control does this value refer to (indicate 'line number'); type 'nr', if not relevant; important for PCR, microarray or for evaluation within one plate!

column 7: assay name

column 8: number of technical replicates = a

column 9: number of endpoints = b

column 10 til 10+a: data for endpoint 1 (all technical replicates)

column 11+a til 11+2a: data for endpoint 2

....

column 10+ba+1 til 10+b+1+ba: data for endpoint b

Files available for upload.

6.2 Internal data handling pipeline

How are raw data processed to obtain the above data level in your lab

Cytotoxicity by resazurin reduction:

- Resazurin reduction is measured as fluorescence signal by the Tecan reader
- Calculations (normalisation to untreated control) are done in Excel as % of control values.
- Graphical representation of concentration response curves are done in GraphPad Prism

Gene expression by qPCR:

- The qPCR machine gives the Ct values as an Excel file
- Calculations (normalisation to reference genes and untreated control) are done using Excel
- Graphical representation and statistics are done using GraphPad Prism

Gene expression by Affymetrix Gene Chips:

- Affymetrix Gene-Chip Scanner-3000-7G measures the fluorescence intensity of hybridised probes and generates the CEL files. CEL files are used for further statistical analysis using R.
- For background correction, log2 transformation, quantile normalisation and linear model fit the Robust Multi-array Average (RMA) algorithm is used.
- For differential gene expression and p-value the R package limma is used

6.3 Standard data handling pipeline

How are the above data normally handled to obtain overall test result (e.g. concentration response fitting by xx method; determination of EC_{50} by yy method,use of EC_{50} as final data)?

For determination of the EC_{10} value of cytotoxicity the following steps are done in GraphPad Prism:

- Logarithmic transformation of the x values (concentrations)
- The conc./response curve is done by a sigmoidal dose response (variable slope) fit.
- The table of x/y coordinates is created with 550 curve points and the corresponding x value (= concentration) of the 90% of control value is read off the table.

For determination of the fold change value of gene expression (by RT-qPCR) the delta-delta Ct (ddCt) method is used and the following calculations are done in Excel:

- Calculation of the geometric mean of two reference genes (TBP, RPL13)
- Subtraction of the Ct value of the gene of interest from this GEO Mean = delta Ct value (dCt)
- Subtraction of dCt treated from dCt control = ddCt
- A doubling of DNA is assumed that in each PCR cycle and therefore the deltadelta Ct value is taken 2^ddCt

The calculations from Affymetrix gene chips is done using the following R-packages:

- For background correction, log2 transformation, quantile normalisation and linear model fit the Robust Multi-array Average (RMA) algorithm is used.
- For differential gene expression and p-value the R package limma is used

6.4 Internal data storage

How are data stored?

What backup procedures are used (how frequently)?

How are data versions identified?

Data are exported and stored on group servers. The servers are back-upped once per week.

Approaches for data versions has not been identified.

6.5 Metadata

Note: meta data examples are: laser power, microscope objective, binning of camera, slit/filter of optical units; temperature cycle of PCR; all data that refer to instrument settings during data recording; suppliers of chemicals; software versions for data processing; etc.

How are metadata documented and stored (lab book, excel files, left in machine, etc;

What metadata are stored/should be stored

Metadata of qPCR machine settings as well as the TECAN reader settings are strored within the raw data file.

Please upload a template of the metadata file (if available). Please also consider in this template to include fields, which are variable, cannot be completely specify in the protocol and could be changed without changing the read out (e.g. suppliers of chemicals, see also question 4.7).

Information will be added.

6.6 Metadata files for transfer

Do instruments (e.g. automated microscope) automatically generate files, that could be transferred. How are such files linked to experimental data (e.g. ID, date, etc.)

Information will be added.

7. Test method transferability

7.1 Operator training

What experience is required? How are new operators trained in your laboratory?

How long training/experience is required for smooth assay performance

- Basic cell culture experience is essential.
- Operators can get trained for the assay within 2 – 3 month. Cell seeding and medium change should be performed as fast as possible to keep cells as short as possible at room temperature. The more practice an operator has, the faster the critical steps can be performed.
- Apart from the test system itself (the differentiation to NEPs) the operator need to be able to maintain iPCS cells which can be learned also in 2 – 3 months.

7.2 Transfer

Has the test system (cells) been transferred to other labs?

Has the test method been used by various operators (over a large time period)?

Has the test method been transferred to other labs?

Procedures and performance (experience) of the transfer?

The test method has been used by various operators in the same lab. A transfer is planned in 2017 to IfaDo.

8. Safety, ethics and specific requirements

8.1 Specific hazards; issues of waste disposal

Are there special legal requirements to run the test in your lab; are special hazards associated to the test that may affect operators, bystanders, others (e.g. through waste)

No specific requirements.

8.2 Material safety data sheet (MSDS)

Are the MSDS available for all hazardous reagents used in the test method?

Are the MSDS stored for all hazardous test compounds?

Describe where and how are the MSDSs stored internally. How is safe handling ensured.

MSDS are available in the university DaMaRIS database.

8.3 Specific facilities/licenses

Are there special permits (e.g. genetic work, stem cells, radioactivity,…).

Are special facilities required?

Is special ethical approval necessary (indicate approval document).

- Work requires S1 cell culture laboratories (genetically modified cells).
- No specific facilities are required.
- for the iPSC-derived NEPs no specific ethical approval is required.

9. Publication/validation

9.1 Availability of key publications (web links)

If possible please provide here max. 3 most relevant publications that describe / give a comprehensive overview of (a) your test system and / or (b) your test method. Describe what aspects are covered therein.

Balmer, N. V., M. K. Weng, B. Zimmer, V. N. Ivanova, S. M. Chambers, E. Nikolaeva, S. Jagtap, A. Sachinidis, J. Hescheler, T. Waldmann and M. Leist (**2012**). "Epigenetic changes and disturbed neural development in a human embryonic stem cell-based model relating to the fetal valproate syndrome." Hum Mol Genet **21**(18): 4104-4114.

→ *first description of the biological system, positive, negative and endpoint-specific controls*

Krug, A. K., R. Kolde, J. A. Gaspar, E. Rempel, N. V. Balmer, K. Meganathan, K. Vojnits, M. Baquie, T. Waldmann, R. Ensenat-Waser, S. Jagtap, R. M. Evans, S. Julien, H. Peterson, D. Zagoura, S. Kadereit, D. Gerhard, I. Sotiriadou, M. Heke, K. Natarajan, M. Henry, J. Winkler, R. Marchan, L. Stoppini, S. Bosgra, J. Westerhout, M. Verwei, J. Vilo, A. Kortenkamp, J. Hescheler, L. Hothorn, S. Bremer, C. van Thriel, K. H. Krause, J. G. Hengstler, J. Rahnenfuhrer, M. Leist and A. Sachinidis (**2013**). "Human embryonic stem cell-derived test systems for developmental neurotoxicity: a transcriptomics approach." Arch Toxicol **87**(1): 123-143.

→ *comparisons of different test methods from the ESNATS consortium, broad charaterisation of transcriptomics data between test methods and different compounds based on gene expression data*

Waldmann, T., E. Rempel, N. V. Balmer, A. Konig, R. Kolde, J. A. Gaspar, M. Henry, J. Hescheler, A. Sachinidis, J. Rahnenfuhrer, J. G. Hengstler and M. Leist (**2014**). "Design principles of concentration-dependent transcriptome deviations in drug-exposed differentiating stem cells." Chem Res Toxicol **27**(3): 408-420.

→ *Concentration response characterisation of transcriptomics data*

9.2 Full coverage of the literature (update later)

Optional: Name a range of (or all) publications that refer to your test method.

Give short comments on which type of information can be obtained from these publications (e.g. containing test chemical lists; containing more positive/negative controls; containing validation against other tests, containing incorporation in test battery, demonstrating use by other lab, ...)

Balmer, N. V., S. Klima, E. Rempel, V. N. Ivanova, R. Kolde, M. K. Weng, K. Meganathan, M. Henry, A. Sachinidis, M. R. Berthold, J. G. Hengstler, J. Rahnenfuhrer, T. Waldmann and M. Leist (**2014**). "From transient transcriptome responses to disturbed neurodevelopment: role of histone acetylation and methylation as epigenetic switch between reversible and irreversible drug effects." Arch Toxicol **88**(7): 1451-1468.

→ *time point and exposure duration dependency of transcriptomics data*

Balmer, N. V., M. K. Weng, B. Zimmer, V. N. Ivanova, S. M. Chambers, E. Nikolaeva, S. Jagtap, A. Sachinidis, J. Hescheler, T. Waldmann and M. Leist (**2012**). "Epigenetic changes and disturbed neural development in a human embryonic stem cell-based model relating to the fetal valproate syndrome." Hum Mol Genet **21**(18): 4104-4114.

→ *first description of the biological system, positive, negative and endpoint-specific controls*

Krug, A. K., R. Kolde, J. A. Gaspar, E. Rempel, N. V. Balmer, K. Meganathan, K. Vojnits, M. Baquie, T. Waldmann, R. Ensenat-Waser, S. Jagtap, R. M. Evans, S. Julien, H. Peterson, D. Zagoura, S. Kadereit, D. Gerhard, I. Sotiriadou, M. Heke, K. Natarajan, M. Henry, J. Winkler, R. Marchan, L. Stoppini, S. Bosgra, J. Westerhout, M. Verwei, J. Vilo, A. Kortenkamp, J. Hescheler, L. Hothorn, S. Bremer, C. van Thriel, K. H. Krause, J. G. Hengstler, J. Rahnenfuhrer, M. Leist and A. Sachinidis (**2013**). "Human embryonic stem cell-derived test systems for developmental neurotoxicity: a transcriptomics approach." Arch Toxicol **87**(1): 123-143.

→ comparisons of different test methods from the ESNATS consortium, broad charaterisation of transcriptomics data between test methods and different compounds based on gene expression data

Rempel, E., L. Hoelting, T. Waldmann, N. V. Balmer, S. Schildknecht, M. Grinberg, J. A. Das Gaspar, V. Shinde, R. Stober, R. Marchan, C. van Thriel, J. Liebing, J. Meisig, N. Bluthgen, A. Sachinidis, J. Rahnenfuhrer, J. G. Hengstler and M. Leist (**2015**). "A transcriptome-based classifier to identify developmental toxicants by stem cell testing: design, validation and optimization for histone deacetylase inhibitors." Arch Toxicol **89**(9): 1599-1618.

→ Development of an algorithm that may identify HDAC inhibitors, providing biomarkers for HDAC inhibitors

Shinde, V., L. Hoelting, S. P. Srinivasan, J. Meisig, K. Meganathan, S. Jagtap, M. Grinberg, J. Liebing, N. Bluethgen, J. Rahnenfuhrer, E. Rempel, R. Stoeber, S. Schildknecht, S. Forster, P. Godoy, C. van Thriel, J. A. Gaspar, J. Hescheler, T. Waldmann, J. G. Hengstler, M. Leist and A. Sachinidis (**2016**). "Definition of transcriptome-based indices for quantitative characterization of chemically disturbed stem cell development: introduction of the STOP-Tox and STOP-Tox tests." Arch Toxicol.

→ Development of different Toxicity indices to quantify transcriptomics data

Waldmann, T., E. Rempel, N. V. Balmer, A. Konig, R. Kolde, J. A. Gaspar, M. Henry, J. Hescheler, A. Sachinidis, J. Rahnenfuhrer, J. G. Hengstler and M. Leist (**2014**). "Design principles of concentration-dependent transcriptome deviations in drug-exposed differentiating stem cells." Chem Res Toxicol **27**(3): 408-420.

→ Concentration response characterisation of transcriptomics data; treatment with VPA in the standard UKN1 SOP (DoD0 – DoD6)

Waldmann, T. and Grinberg, M. *et al.* (**2016**). "Stem cell transcriptome responses and corresponding biomarkers that indicate the transition from adaptive responses to cytotoxicity." Chem Res Toxicol **submitted**.

→ Concentration response characterisation of transcriptomics data; treatment with VPA and MeHg with different exposure scheme DoD4 – DoD6.

9.3 (Potential) linkage to AOPs

Indicate whether the test method has been linked to an AOP (or several AOPs) and in which form (e.g. test of KE activation) and where (e.g. in AOPwiki).

- Test is not linked to AOP but may deliver information on KE such as gene expression changes

9.4 Steps towards mechanistic validation

Has it been explored in how far the system reflects human biology, signalling, tissue organisation relevant to the form of toxicity to be explored. (E.g. nigrostriatal neurons should contain dopamine; liver tests relevant to cholestasis may need to contain bile canalicular structures, ...

Are there interventions (knock-out, knockdown, chemical inhibitors, specific pathway triggering that support the use of the test for certain toxicological questions, and that corroborate expectations to the test system.

Is there a form of mechanistic validation?

Can the test method cover an AOP MIE/KE?

(Examples: 1.. If a test measures neurite growth, then biological signals known to control neurite growth and growth cone collapse should be present in the system and their modulation should affect the test endpoint. 2. If a test measures the DNA damage response, then DNA damage sensors should be expressed and functioning, and knockdown of DNA damage sensors should affect the test endpoint; …

- GSK3 inhibitors BIO and CHIR were used to investigate WNT signalling. A clear disturbance of anterior patterning was observed.
- A formal mechanistic validation has not been performed.

9.5 Has the test undergone pre-validation, validation or a related evaluation procedure?

Indicate e.g. ring trials, full (pre)validations. Give an overview of compounds or libraries that have been tested.

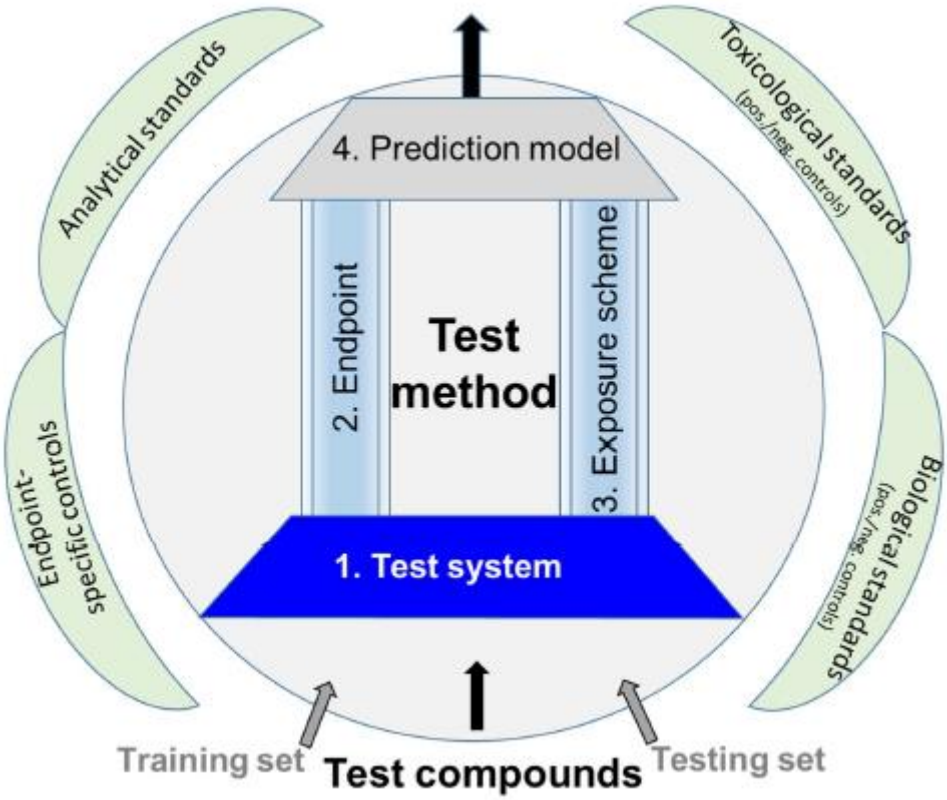
No ring trials with the test system have been done.

9.6 Linkage to (e.g. OECD) guidelines

Indicate whether the test method is linked to an OECD test guideline (how, and which) or other regulatory guidance (e.g. EMA).

Test is not linked to regulatory guidelines.

Part C: Background/glossary



Test (or test method): This term is used in many disciplines, and it is little defined in colloquial language. In toxicology, it is the term used to describe a procedure based on a test system, used to obtain information on the potentially hazardous effects of a substance. A toxicological test method consists of four major components (i.e. 1. test system, 2. endpoint, 3. exposure scheme, 4. Prediction model), and it produces a test result (information regarding the ability of a substance or agent to produce a specified biological/toxicological effect under specified conditions). The term is used interchangeably with "test" and "assay" in the literature. A test method can have several analytical endpoints.

Test system / biological system: This term is often confused with 'test method', but it has a different definition. A test system is a cellular (or biochemical) system used in a study (e.g. "proliferating neural stem cells", or "neuronally-differentiating PC-12 cells", or "organotypic hippocampal slices"). The term is often used interchangeably with "*in vitro* system". The term test system is equivalent to "biological model" as far as test setup is concerned. From this follows that the test system is only one component of a test or 'test method'. Good performance of a test system does not imply good functioning of a test method. Acceptability criteria for test systems (e.g. at least 75% of the differentiated cells staining positive for nestin under control conditions) are different from acceptability criteria for the test method using the test system (e.g. inhibition of differentiation by a specified positive control by at least 35%, and alteration of normal differentiation by a defined negative control by less than 10%).

Endpoint / Test endpoint: The term endpoint has two implications and it is essential to understand the differences. Within the context of a toxicological test, the endpoint is the biological or chemical process, response, or effect assessed in a test system by a specific analytical method/assay. For instance, "cell viability" or "cell proliferation" or "electrical network activity" are endpoints. Each endpoint may be assessed by different 'analytical methods'. For instance, 'viability' may be assessed by LDH-release, resazurin reduction, cell counting or measurement of ATP. "Differentiation" may be measured by PCR quantification of a differentiation marker or by morphometry (e.g. dendritic tree arborisations or synaptic spine density).

Analytical endpoint: An endpoint of a test system (e.g. proliferation, or differentiation, or viability) may be quantified by different analytical methods (measurement endpoints). It is important to distinguish such analytical endpoints (referring to the analytical methods used) from (test system) endpoints that refer to the biological concept evaluated. The test endpoint and analytical endpoints require independent optimisation, characterisation and use of control compounds.

Exposure scheme: A drug may be added to a test system continuously, or for certain time periods, in a certain solvent, with or without medium change, at a specified temperature, etc. All this information is contained in the exposure scheme. As each of the other three elements of a test, an exposure scheme needs to be optimised independently. For instance, with all other test parameters fixed, the test outcome can dramatically change with the time period of exposure. Depending on the point-of-view, the analytical endpoint may be regarded as part of the exposure scheme. Optimisation of the exposure scheme may require switching analytical endpoints, even if the same test endpoint is evaluated.

Prediction model: The prediction model (PM) is a formula or algorithm (e.g., formula, rule or set of rules) used to convert the results generated by a test method into a prediction of the (toxic) effect of interest. Also referred to as decision criteria. A prediction model contains four elements: (1) a definition of the specific purpose(s) for which the test method is to be used; (2) specifications of all possible results that may be obtained, (3) an algorithm that converts each study result into a prediction of the (toxic) effect of interest, and (4) specifications as to the accuracy of the prediction model (e.g., sensitivity, specificity, and false positive and false negative rates). The PM is often neglected in test setup. In its narrow sense, it defines the procedure how data are being processed, and how technical data (instrument readings) are translated into toxicological information. For instance: if calcium oscillations are measured, the PM determines what type of change is considered as relevant to toxicity.

Another important example is a change of gene expression, measured by PCR or a transcriptomics approach. A heatmap of gene expression is a technical set of data, but not toxicological information. A PM transforms this into a test statement of compound hazard. A first consideration about PM is whether there is a binary outcome (toxic – non toxic) or are there more than 2 classes (mild, moderate, severe irritants, and how are the boundaries defined). For instance, many *in vitro* tests give information if a compound is hazardous or non-hazardous, but not on the strength of effect or the potency of a chemical.

Another important issue is: if there are two or more assay endpoints (e.g. viability and neurite growth), how are they combined to a final toxicity statement? During test optimisation and validation, the prediction model needs scrutiny and the questions asked are as follows: Is there a threshold (different from the statistical threshold) for when an effect can be considered biologically relevant? How is the outcome interpreted when more than one endpoint is measured (e.g. general cytotoxicity and functional impairment or

effects on two different cell types)? Is an increase compared to normal good, when a decrease is bad? How should data be interpreted when a compound alters the baseline values for the endpoint (e.g. coloured compound in spectrophotometric assays)? What is the correct reference value, if the test system changes over time? The PM defines these decision points and then translates the test result into a prediction, e.g. converting the luminometer reading of an ATP assay into a toxicological statement (prediction) whether the compound is cytotoxic (at a given concentration). In practical terms, a test is set up to be predictive for unknown compounds (test compounds), but to achieve this goal, the different elements of the test usually require optimisation and fine tuning. This is performed by anchoring the test or its elements to a frame of known information, i.e. defined controls and standards as outlined below:

Analytical standards: each analytical method requires calibration by the use of standards (positive and negative controls). This can include physicochemical approaches (e.g. to make sure that the balances and the spectrophotometer are working), or scaling approaches (e.g. to obtain absolute values in microscopic morphometric measurements or counts). On the next level, the analytical endpoint needs to be calibrated in the context of the test system. For instance, if LDH-release is used as a measure of viability, then it needs to be evaluated, how much LDH is released under conditions of all cells dying (e.g. detergent lysis; not necessarily = 100%), and the overall assay needs to be normalised to such values. An important example is viability measurement by resazurin or tetrazolium dye reduction. This works only after normalisation for cells that are 100% dead or alive, as the instrument readings as such have no dimension.

Endpoint-Specific Controls: Chemicals known to reliably and consistently alter the endpoint of a test system at a mechanistic level. These are also referred to as 'endpoint-selective controls' or 'mechanistic tool compounds'. This would be the first set of compounds, used during test system setup to obtain information on the biological/toxicological behaviour of the test system and its dynamic range. Such control compounds can be used to define acceptance criteria.

Positive/negative control (PC/NC) or 'toxicological standards': A NC for a 'test method' is a compound or condition that should not trigger a response, i.e. it should not change the endpoint from baseline. A PC is a compound or condition that triggers a response, i.e. a change of the endpoint from baseline in the right direction and to a certain specified extent. The performance of PC and NC can be used to define 'acceptance criteria' of a test.

Acceptance criteria: Criteria defined before performing an assay to determine whether it is "valid", i.e. whether the data can be used. Typical issues of acceptance criteria comprise: 'has the actual run or plate of the test method functioned (e.g. are the endpoint values for PC and NC in the right range)', 'is the test method performing within the desired range of variability (e.g. are the standard deviations of PC and NC in the right range)'.

Note that acceptance criteria can (and should) also be defined for an 'analytical endpoint' or for a 'test system'. For instance, for a test system, the acceptance criteria may say that it is only valid if at least 400 cells were in the region of interest, or if at least 80% neurons were present in mixed cultures, or if the average neurite length was at least 4 cell diameters. Such test system acceptance criteria are not at all related to those used for the test method. In this context, it is important to rationalise that endpoints that are meaningful for the description of the biological system/test system may not be useful for the test method and vice versa. For instance, a person's body weight can be measured well on scales (to give a good readout on general growth characteristics of a person = biological system), but this endpoint will hardly respond to acute poisoning of the person. Instead, blood pressure or

vomiting activity may be good measures of human poisoning (toxicological test), but they in turn give little information on the growth activity over time. In a neurotoxicity test for network activity, the extent of synaptic staining may be a good acceptability criterion for the test system, but it will not react to a glutamate receptor agonist; on the other hand, electrical activity pattern will be a very sensitive measure for glutamate receptor-affecting toxicants, but the synapse number will not change (upon acute exposure).

Once the first three elements of the test system have been established, optimised and assembled to a test, the prediction model can be established to complete the test system setup. One standard procedure is to use a training set of chemicals. These would be known positive and negative controls, and run them through the test. Based on the test data, a prediction model would be established that suits best the known information about which of the compounds should test positive or negative. In a second round of testing, a test set of compounds would be used (i.e. a new set of positive and negative controls). The data of these substances would be run through the prediction model to determine accuracy, specificity and sensitivity of the test system. Possibly, further adaptations would then follow.

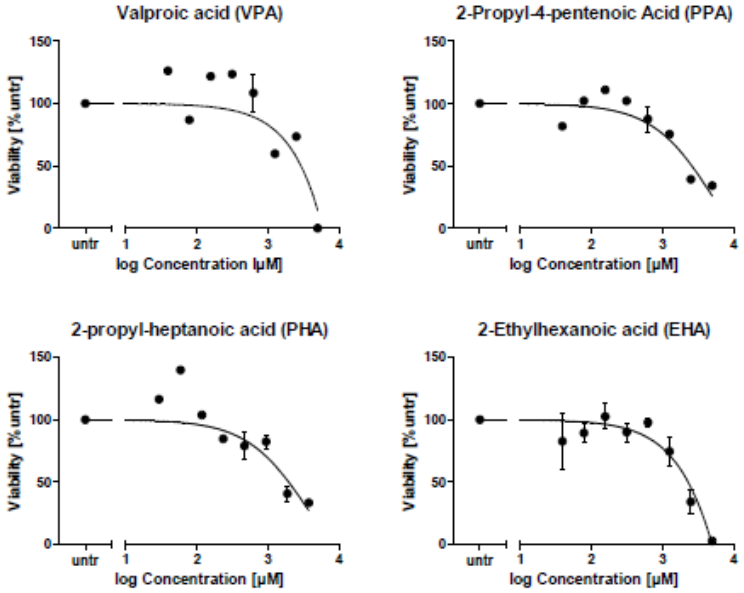
Training Set Chemicals: This set should include chemicals known (preferably from *in vitro* systems) to reliably elicit a response, or no response, with respect to the endpoint of interest. The goal of using this set is proof-of-concept that the test method can rapidly and efficiently screen moderate numbers of chemicals with reasonable predictivity. A training set of chemicals can be used to optimise an assay (test method), to set acceptability criteria, and to build a prediction model.

Testing Set Chemicals: This set would be used to validate and possibly improve the prediction model. The goal of using 'testing set chemicals' is also to demonstrate the ability to test larger numbers of chemicals.

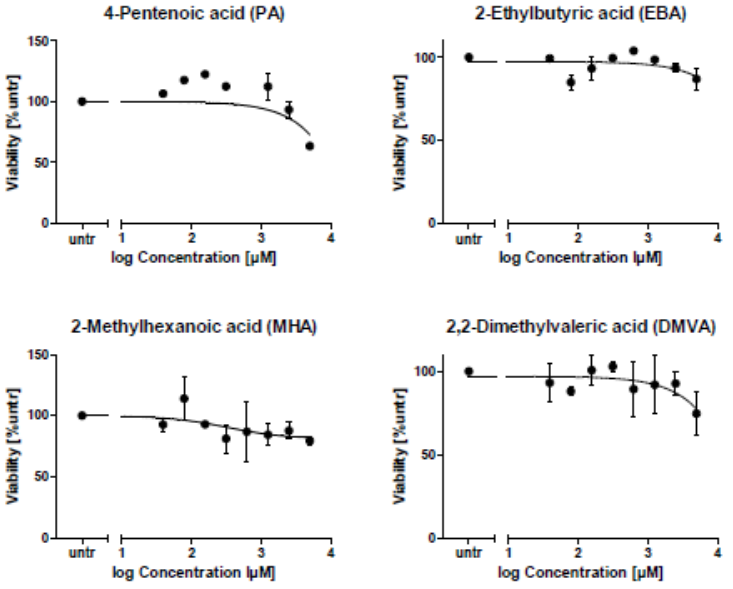
8. UKN1-1: Individual effect curves and EC_{10} for all analogues

Concentration response curves for endpoint 1 (viability)

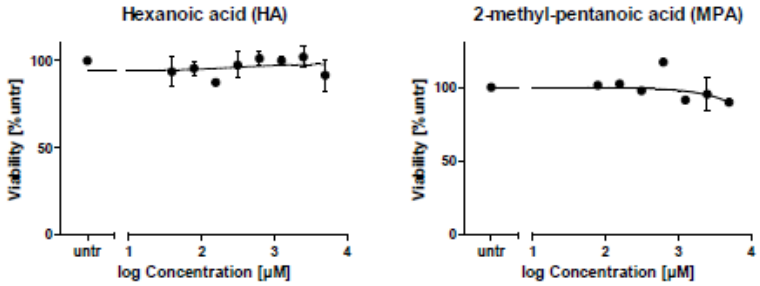
in vivo positive



in vivo negative

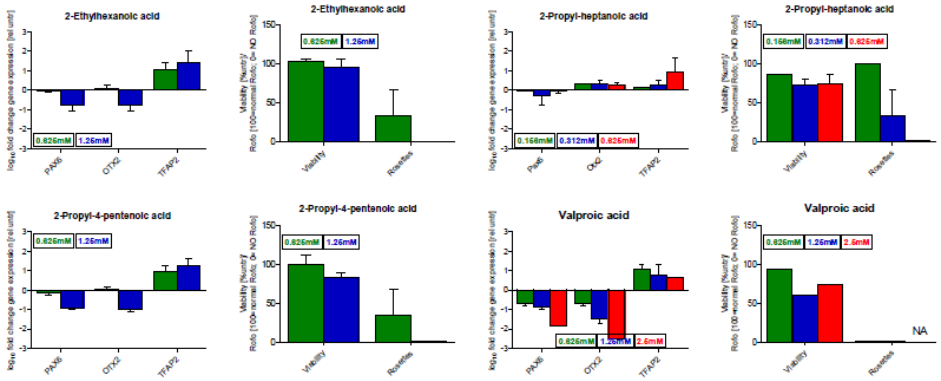


in vivo unknown

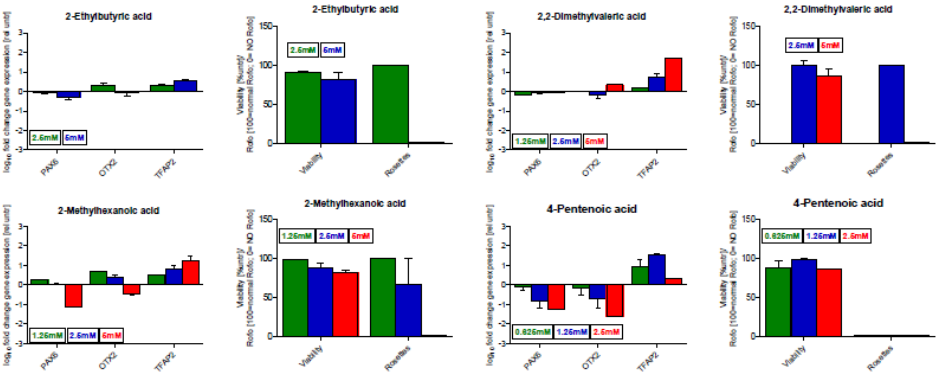


Endpoints 2 and 3: Gene expression and rosettes formation

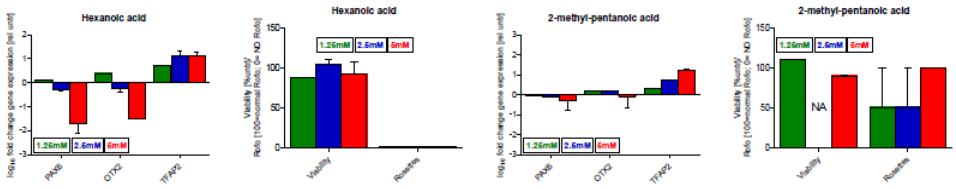
in vivo positive



in vivo negative



in vivo unknown



9. HDAC-1: Method description HDAC test

- UNK_DART_HDACi –

General information

Name of test method (original/published name, tradename).

No information available

Assigned name for EU-ToxRisk database:

UNK_DART_HDACi

Variant of/ parent test method

/

Organisation

University of Konstanz (UKN)

Contact person for the test method

Jaffar Kisitu (jaffar.kisitu@uni-konstanz.de)

Person involved with the test system

Marcel Leist, PI (marcel.leist@uni-konstanz.de)

Tanja Waldmann, Experiments (tanja.waldmann@uni-konstanz.de)

Specific evaluation part

This protocol uses cell lysates from the following test systems:

- UKN1a_DART_IPC_Diff_6D
- mESC…….(test method description not available)

1. Description of general features of the test system (cells/tissue)

1.1 Cellular system component(s)

- See **DART test methods**

1.2 Definition of cells used

- See **DART test methods**

1.3 Give a reference/link to maintenance culture protocol (upload field)

- See **DART test methods**

2. Definition of the test system

2.1 Principles of the culture/differentiation protocol

See **DART test methods**

Extracting HDAC enzymes

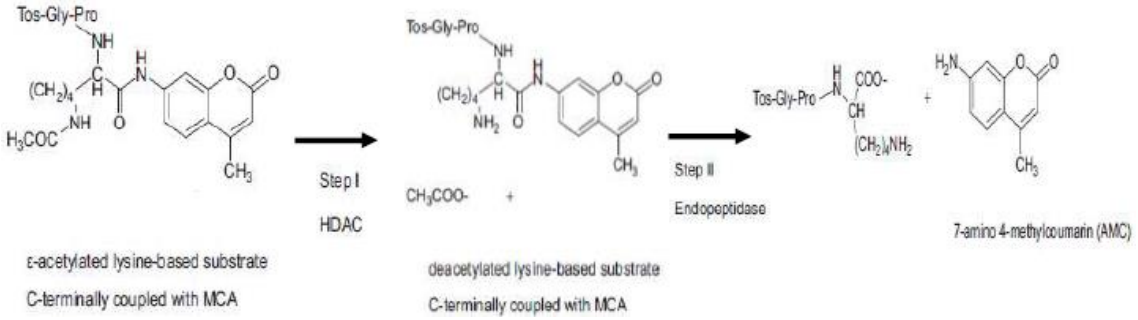
Lyse ~2.5 million cells in 500 µl lysis buffer (20 mM Hepes, pH 7; 150 mM NaCl, 0.5% NP40) supplemented with complete (protease inhibitor). Centrifuge at 10,000g for 10 minutes and collect the supernatant containing the required histone deacetylase (HDAC) proteins.

2.2 Endpoints

Potent inhibition of histone deacetylase enzymes in the cell lysate is measured by the amount of released fluorogenic subgroup 7-amino-4-methylcoumarin (AMC)

2.3 Analytical endpoints

Used method: Deacetylation of the short peptide is measured indirectly in a two-stage reaction resulting into the release of a fluorogenic subgroup 7-amino-4-methylcoumarin (AMC)



2.4 Acceptance criteria for test system

In vitro data using whole embryo cultures shows that valproic acid analogues inducing developmental effects are active at concentrations ≤ 2mM. Therefore, active analogues in this HDAC assay would have an EC_{50} inhibitory value below or equal to this concentration. Additionally, for every assay run, there should be almost ~95% inhibition by the positive control, TSA (1µM).

2.5 Variability and troubleshooting

Causes of variability

- Differences in the number of cells from which the HDAC protein is extracted
- The plates used for the assay have different settings for the z- position and optimal gain in the fluorescence reader: these readout parameters must always be optimised for a specific plate.

General point

- Always use the same concentration of positive control for baseline correction

2.6 Features relevant for cytotoxicity testing

/Not applicable

2.7 Metabolic capacity of the test system

/Not applicable

2.8 Omics characterisation of the test system

/Not applicable

3. Test method description

3.1 Test documentation in published literature (web links)

Wegener D, Hildmann C, Riester D, Schwienhorst A. Improved fluorogenic histone deacetylase assay for high-throughput-screening applications. Anal Biochem. 2003;321(2):202–8.

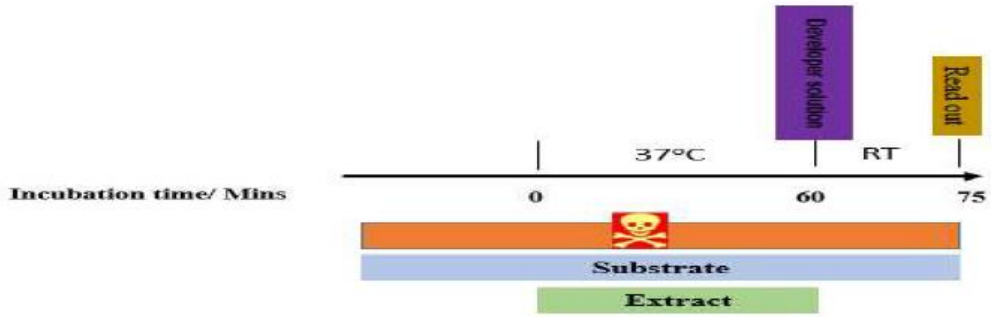
Heltweg B, Trapp J, Jung M. *In vitro* assays for the determination of histone deacetylase activity. Methods. 2005;36(4):332–7.

Krasteva S, Heiss E, Krenn L. Optimization and application of a fluorimetric assay for the identification of histone deacetylase inhibitors from plant origin. Pharm Biol. 2011;49(6):658–68.

Deviation

Differences may arise from the buffer used for the developer solution; the activity of the lysine endopeptidase and the composition of the developer solution. In this case, the developer solution is composed of 1µM Trichostatin A (TSA) and the endopeptidase in a 2 mmol/L Tris-HCl buffer, pH 8.0. The other differences are in the activities of the activities of the HDAC and endopeptidase enzymes, which ultimately result in different incubation times for the two-stage reactions. Such differences in enzyme activity may also arise from repeated freeze thaw cycles.

3.2 Exposure scheme for inhibitory testing



- Fluorescence assay performed in a black 96 well plate
- At 0 minutes: addition of toxicant/ inhibitor and enzyme substrate
- 0 minute: addition of extract

- 60 minutes: stoppage of reaction with 1µM TSA and addition of endopeptidase
- 75 minutes: read-out: excitation at 370 nm and fluorescence at 460 nm

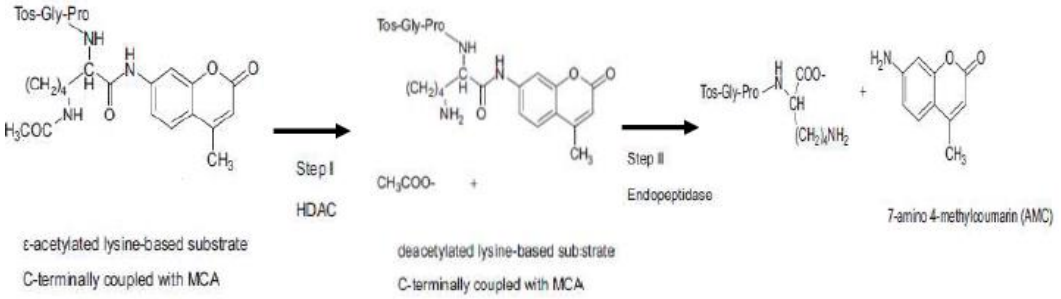
3.3 Test method endpoint

Endpoint

HDAC inhibition

3.4 Analytical endpoint(s) overview

The HDAC Inhibition assay is based on the release of a fluorogenic sub-group AMC from a short deacetylated peptide. Once the primary substrate (BOC-(Ac)Lys-AMC) is deacetylated by the HDAC enzymes, it is only after then that it becomes a substrate for an endopeptidase enzyme (step 2) that is contained in the developer solution. The endopeptidase has specificity for hydrolysing bonds at the lysine carboxyl terminal. This reaction ultimately results in the release of the fluorescent subgroup 7-amino-4-methylcoumarin (AMC) which has an absorption range of light between 340-370nm and an emission range between 440 - 465nm. The amount of emitted light by the fluorescent substrate AMC is a function of the extent of the deacetylation reaction. HDAC inhibitors will block the rate-limiting step 1 of this cascade and ultimately result in less fluorescence readout. HDAC inhibition is then quantified as the amount of fluorogenic substance released in the presence of inhibitor relative to a negative control well, in which no inhibitor is added. Background correction is done by subtracting a blank reading (substrate + assay buffer) and baseline correction is by subtracting the reading from positive control wells, to which 1µM TSA is added as inhibitor. TSA is a known potent HDAC inhibitor, which at a 1µM concentration reduces the activity by more than 95%.



3.5 Analytical endpoint(s) technical details

Endpoint overview and exposure scheme

3.6 Endpoint-specific controls

Positive control: TSA is a known potent HDAC inhibitor that inhibits both class I and class II HDAC enzymes within a nano-molar concentration range.

3.7 Negative, positive controls

Negative control: for each experiment run, at least two blank wells are included on the plate to correct for background noise from the assay reagents.

Positive control: 1µM TSA. In this assay, separate wells are prepared for this compound. At this concentration, no residual enzyme activity is expected.

3.8 Acceptance criteria

Minimal blank readings and at least more than 95% inhibition in the positive control wells.

3.9 Test parameters

To be confirmed in each assay run:

- Within-run variation of the control well is less than 5%
- Between-run variations of all assay readings are less than 15%
- Baseline correction with a positive control well. Inhibition in this well should be ≥ 95% of enzyme control well without inhibitor.

3.10 Throughput estimate

- One technical replicate also counted as a biological replicate: on a 96 well plate, at least seven (7) compounds can be tested each with at least six (6) concentration levels. In one week, at least ten compounds can be tested at six concentration levels in triplicate.
- Only a single measure can be obtained from the same plate.

Rational: Assay preparation from test compound and solution preparation can take about 1.5 - 3 hours.

3.11 Concentration settings

The concentration range of the test compounds is obtained through a concentration range finding. This is done for each compound in a range between 0 to 10mM. However, in most cases, the test concentration range will lie within the range in which *in vitro* effects for a compound class were reported in literature or a laboratory assay.

3.12 Special instrumentation

The method requires a Tecan infinite M200 PRO fluorescence reader and pH meter for buffer preparation, all tools expected in most standard laboratories.

4. Handling details of the test method

4.1 Preparation/addition of test compounds

	Negative control well	Test well	Positive control well
Assay buffer (ml)	40	-	-
Substrate (ml)	10	10	10
Inhibitor (ml)	-	10	-
TSA (ml)	-	-	10
Lysate (ml)	30	30	30
Developer solution (ml)	50	50	50

- All compounds are stored according to the manufactures recommendations i.e. room temperature, 4°C and -20°C. Solid compounds are dissolved in DMSO.
- They are stored as pure compounds in volumes sufficient for a single experimental run to avoid repeated freeze-thaw effects that would ultimately affect the compound's stability.

- For the experiments, the substrate, test compounds and controls are diluted in assay buffer (25 mM Tris-HCl pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$) to the appropriate concentrations.
- For each concentration, 10µL of test compound and 10µL of substrate are added to each test well. To the blank well, 40µL of assay buffer and 10µL of substrate are added while the positive control well is treated as the test compound well with the final concentration of TSA in the assay well being 1µM.
- In the experimental setup, the lysate is the last added reagent and timing starts immediately.
- The developer solution which is an endopeptidase solution containing 1µM TSA is prepared in due course of the incubation phase. To stop the deacetylation reaction, 50µL of developer solution is added to each reaction well.

4.2 Day-to-day documentation of test execution

- Plate maps are defined to each experiment in a lab book or files stored on a group server
- Test concentrations are calculated prior to each experiment
- Any planned variations in the experimental protocol are manually recorded in a lab book.

4.3 Practical phase of test compound exposure

- The times for initiation of the assay reaction is documented in a lab book
- Any errors during the experiments are mapped directly on to the plate for follow up and also recorded in the lab book for consideration during data analysis.

4.4 Outliers

- All raw data including outliers is stored
- Based on the expected reproducibility of the assay, data points in a replicate analysis that are > 3SD from the mean are considered outliers. Otherwise, some outliers are outrightly obvious.

4.5 Uncertainties, problems

- Some compounds are volatile and so can be lost during storage, which affects the working concentrations
- The test is easy to run and operators are trained within a few months. The most challenging part is to do with cell culture.
- The critical steps in the experiment are the addition of the reaction initiator (lysate containing HDAC enzyme) and developer solution (containing reaction stop reagents) as there are slight variations in time. The more practice the operator has, the better they can perform the most critical steps

4.6 Detailed protocol (upload field)

SOP available – to be uploaded

4.7 Possible variations

- Variation in the timings for the two incubation steps
- Composition of buffers: substances with similar absorption-emission characteristics as the fluorogenic moiety AMC may interfere with the final readout

4.8 Cross-reference to related test methods (upload field)

Not applicable

5. Prediction model/Translation

5.1 Rationale of your test method (= scientific purpose)

- There is a high weight of evidence that HDAC inhibition is the molecular initiating event in the development of neural tube defects such as spina bifida and anencephaly
- Compounds which exhibit significant HDAC inhibition are likely inducers of such developmental defects

5.2 Prediction model

- Depending on the compound class, *in vitro* studies have shown different compound to induce developmental effects within a specific concentration range. A compound that displays significant HDAC inhibition within the respective concentration range is deemed as toxic.
- However, efforts tailored for a better prediction model are ongoing

5.3 Prediction model setup

- Specifically tailored efforts for developing a prediction model are ongoing

5.4 IVIVE (In vitro – in vivo extrapolation)

HDAC inhibition has been used to predict *in vivo* behaviour of certain compounds (see Eikel *et al.*, 2005; Chem Res Toxicol. 19(2):272–8)

5.5 Applicability domain

The current assay is set up for detecting inhibitors of the zinc-dependent HDAC enzymes. It can, however, be extended to other HDAC groups after necessary factors have been added.

5.6 Incorporation in test battery

- This method is a high throughput assay

- It can be used along with other test methods involving embryonic stem cells from zebrafish and mouse embryonic stem cells or other stem cells from other organisms used in DART case studies.
- The assay needs special tuning to detect inhibitors for the NAD-dependent HDAC enzymes
- For some compounds, the acceptance criteria is much dependent on the results from other *in vitro* assays

6. Data management

6.1 Data format (multiple upload field)

Raw data

/

Processed data

/

6.2 Internal data handling pipeline

- The extent of deacetylation is measured indirectly as the amount of AMC released which is measured as a fluorescence signal by the Tecan reader
- The following initial data processing is done in excel
- Subtraction of average blank signal from all assay well signals (background correction)
- Subtraction of TSA positive control signal from the average signal of enzyme wells without toxicant (baseline correction)
- The extent of inhibition (normalisation to enzyme wells without toxicant) calculated as a percentage value
- Graphical representation of concentration-response curves using r software

6.3 Standard data handling pipeline

For determination of either EC_{10}/EC_{50}: curve fitting is done using a four parameter log-logistic regression in using the *drm* package. The points of departure are the recalled from the optimal fitting curve data using an appropriate function code.

6.4 Internal data storage

Data is exported and stored on a group server, which is backed up at least once a week.

6.5 Metadata

Metadata from the Tecan reader settings is stored within the raw data files.

6.6 Metadata file format (upload field)

/

6.7 Metadata files for transfer

/

7. Test method transferability

7.1 Operator training

- The operator should have some minimal experience in basic cell culture
- Training in cell culture on average would be between 1-4 months depending on the cell line
- The operator should have basics lab experience like solution/ buffer preparation

7.2 Transfer

The test method has been used for a short time now in the lab. However, transfer to another lab is easy

7.3 Safety, ethics and specific requirements

////////////

7.3 Specific hazards; issues of waste disposal

Not specific requirements

7.4 Material safety data sheet (MSDS)

MSDS are available in the university DaMaRIS database

7.5 Specific facilities/licenses

Cell culture laboratories: depends on the cell line to be lysed. But in any case, S1 will suffice. No specific facilities are required

8. Publication/validation

8.1 Availability of key publications (web links)

1. Krasteva S, Heiss E, Krenn L. Optimization and application of a fluorimetric assay for the identification of histone deacetylase inhibitors from plant origin. Pharm Biol. 2011;49(6):658–68.
2. Wegener D, Hildmann C, Riester D, Schwienhorst A. Improved fluorogenic histone deacetylase assay for high-throughput-screening applications. Anal Biochem. 2003;321(2):202–8.

Describes the assay principle and common reagents for the assay.

8.2 Full coverage of the literature (update later)

1. Krasteva S, Heiss E, Krenn L. Optimization and application of a fluorimetric assay for the identification of histone deacetylase inhibitors from plant origin. Pharm Biol. 2011;49(6):658–68.

2. Active Motif fluorescence HDAC Assay Kit. 2008;(56210).

8.3 (Potential) linkage to AOPs

Test method is not linked to any formal AOP but may deliver information on the proposed molecular initiating event of developmental and reproductive toxicity for neural tube defects.

8.4 Steps towards mechanistic validation

- A formal validation has not been performed
- The test method can cover the molecular initiating event in the Valproic acid-associated developmental and reproductive toxicity AOP.

8.5 Has the test undergone pre-validation, validation or a related evaluation procedure?

No trials have been done

8.6 Linkage to (e.g. OECD) guidelines

Test not linked to regulatory guidelines

10. CALUX-1: Methods description CALUX Reporters (Combined DB-ALM/OECD GD 211)

Combined DB-ALM/OECD GD 211 template for reporting individual (nonguideline) *in vitro* methods

Part A. Protocol introduction

Protocol name

Automated CALUX reporter gene assay procedure - Effects on Endocrine System (DB-ALM 197)

Abstract

This protocols describes a reporter gene assays for the detection of nuclear hormone receptor modulation, or the activation of cell signalling pathways, using high throughput screening.

Résumé

The current protocol describes the performance of BioDetection Systems Chemically Activated LUciferase eXpression (BDS CALUX) reporter gene assays in automated high throughput screening of pure compounds. It has been published before in the context of several screening studies (Piersma *et al.* 2013, van der Burg *et al.* 2013, van der Burg *et al.* 2015a, van der Burg *et al.* 2015b, van Vugt-Lussenburg *et al.* 2018).

Experimental description

Biological endpoint and endpoint measurement:

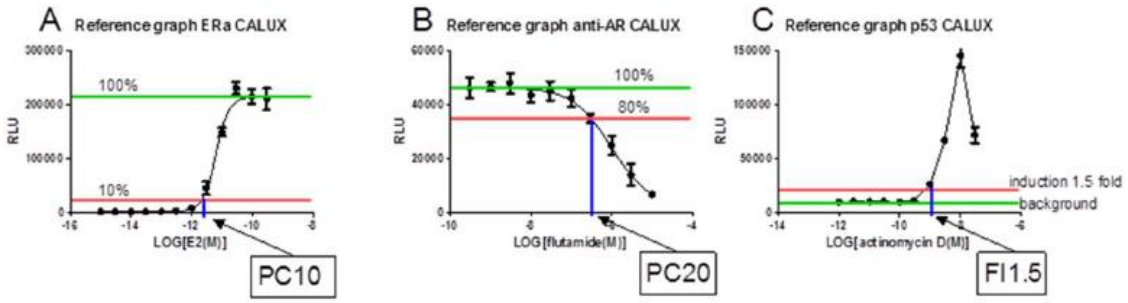
Receptor agonism, antagonism or cell signalling pathway activation (see the section Annexes, Table 1), assessed by luminescence.

Endpoint value:

Reported values are lowest effect concentrations (LEC) in Log(M). Depending on the assay, LECs are defined as:

- the concentration where the test compound causes an activation/agonist effect equal to 10% of the maximum effect elicited by the test's reference compound (PC_{10}, figure A);
- the concentration where the test compound causes an antagonist effect equal to 20% of the maximum effect elicited by the test's reference compound (PC_{20}, figure B);
- the concentration where the test compound elicits pathway activation 1.5-fold above background (FI 1.5, figure C)

Figure. definition of lowest effect concentrations in mechanistically different CALUX assays.



Experimental system:

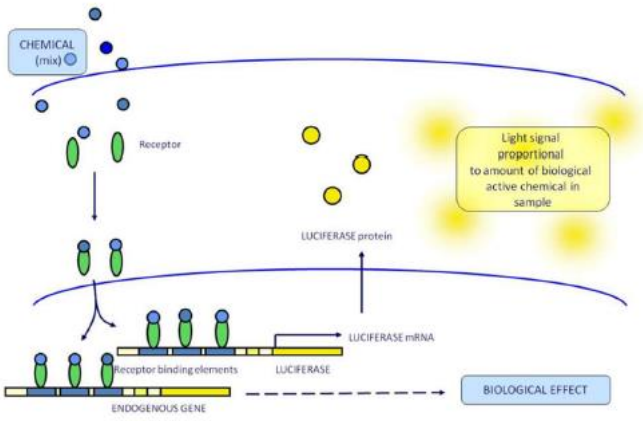
Assays are based on the human osteosarcoma cell line U2-OS (ATCC HTB-96), or the rat hepatoma H-4-II-E (ATCC CRL-1548) (AhR CALUX only).

From OECD GD 211

2.1 Purpose of the test method

2.2 Scientific principle of the method

The CALUX panel consist of 26 cell based reporter gene assays. Each cell line measures the activation or inhibition of one specific nuclear hormone receptor or cell signalling pathway (figure). The assay results can be used to identify compounds activating similar MIE's, and is therefore well suited for read across studies. Furthermore, the results provide clues for the MoA of the different compounds. Therefore, a direct link with an AOP (if available) can be made.



Cell line	Endpoint
ERa CALUX (ago/anta)	Estrogen receptor (ant)agonists
AR CALUX (ago/anta)	Androgen receptor (ant)agonists
PR CALUX (ago/anta)	Progesterone receptor (ant)agonists
GR CALUX (ago/anta)	Glucocorticoid receptor (ant)agonists
TRb CALUX (ago/anta)	Thyroid receptor (ant)agonists
RAR CALUX	Retinoic acid receptor agonists
LXR CALUX	Liver X receptor agonists
PXR CALUX	Pregnane X receptor agonists
PPARa CALUX	Peroxisome proliferator activated receptor agonists
PPARg2 CALUX	Peroxisome proliferator activated receptor agonists
PPARd CALUX	Peroxisome proliferator activated receptor agonists
AhR CALUX	Aryl Hydrocarbon receptor agonists
Hif1a CALUX	Chemical hypoxia response
TCF CALUX	wnt/TCF pathway activation
AP-1 CALUX	AP1 pathway activation / cell cycle control
ESRE CALUX	Endoplasmic reticulum stress
NFkB CALUX	Activation of NF-kB pathway (immune response)
Nrf2 CALUX	Oxidative stress
p21 CALUX	Transcription of p21 inhibitor of cell cycle progression
p53 CALUX	p53-dependent pathway activation / genotoxicity
Cytotox CALUX	Cytoxicity

2.4 Metabolic competence of the test system

Main metabolic genes (p450s, GSTs, UGTs, SULTs) have been assessed using both activity measurements and RT-qPCR analysis; no metabolic activity could be detected using either method (van Vugt-Lussenburg *et al.* 2018).

2.8 Known technical limitations and strengths

Strengths:

Since the U2-OS cells have very low expression levels of endogenous receptors, no crosstalk with other receptors can occur and therefore the assay is highly specific and responsive.

Also, U2-OS cells have no or little metabolic capacity, enabling the specific analysis of the test (parent) compound, rather than a mixture of parent compound plus metabolites. If information on bio(in)activation is required, a metabolic module can be added to the CALUX assay to study the activity of metabolites.

The CALUX panel consists of 26 assays, covering a broad range of nuclear receptors and cell signalling pathways, all in the same cellular background. This makes the results more easily comparable to each other than when using assays with several different cellular backgrounds.

Limitations:

Several sources of variation are known and are carefully controlled at BDS:

- Serum
- Plastics / labware
- Cell passage number

Compounds with high volatility or low water solubility are less suitable to test; it should be taken into account in those cases that the nominal (dosed) concentration is much higher than the actual bioavailable concentration. PBK-models can be used to identify compounds where bioavailability-issues may arise, and correction factors can be derived.

Compounds that aspecifically influence the expression, activity or stability of the luciferase reporter gene can result in false- positive or false-negative signals. Therefore, a cell line constitutively expressing luciferase (Cytotox CALUX) is used as a control for aspecific effects for all test compounds.

4.4 Scope and limitations of the assay, if known

See also section 2.8 above.

Scope of the assay panel:

It has been shown to be applicable for a wide range of applications including, but not limited to, chemicals, cosmetic ingredients, hormones and pharmaceuticals, pesticides, either alone or in complex (environmental) mixtures, like surface and waste water, body fluids, and tissue extracts.

Discussion

- The method described here is an adaptation of BDS CALUX reporter gene assays, some of them already formally validated. Although the validated assays are identical to the assays used in the current protocol, the experimental protocol used is slightly different, namely on the type of cell culture plates used (384-wells instead of 96-wells plates). Details like detection limits and

acceptable standard deviations may therefore differ, while the overall performance of the cell lines (robustness, stability, predictivity) remains the same.

- No ethical issues arise from the use of these immortalised cell lines.
- Equipment: absolute requirements are a laminar flow cabinet, a cell culture incubator and a luminometer.
- For the automated procedure, as used for the EU-ToxRisk case studies, additionally a cell washer-dispenser, a liquid handling machine and a stacker coupled to the luminometer are required.
- The cell lines are genetically modified organisms (GMOs). Therefore, a GMO permit is required (Dutch regulations: safety level Microbiologisch Laboratorium Klasse I (ML-I)).
- Training sessions consist of 1 or 2 weeks, depending on the prior experience of the trainee.
- The CALUX assays are performed over the duration of three days; day 1: seeding; day 2: exposure; day 3: harvesting and measurement.
- The CALUX assays are very sensitive; especially the nuclear receptor hormone assays and the aryl hydrocarbon receptor assay can suffer from background signals caused by e.g. plasticisers leaching from plastics, or hormones present in foetal calf serum. Therefore it is recommended to use only BDS-approved brands of plastic consumables, and to use only BDS-approved dextran coated charcoal (DCC)-stripped foetal calf serum.

From OECD GD 211

4.1 Robustness of the method

CALUX assays are licensed world-wide by BioDetection Systems BV, Amsterdam, The Netherlands. This company also provides world-wide training and support. The assays can be easily transferred to and performed at other labs all over the world; training generally takes 1-2 weeks. Examples for inter- and intralab validations can be found in the following references, where cv values on EC50/IC50's were 1-30% (intralab), and standard deviations on EC50/IC50's were 6-13% (interlab) (van der Burg *et al.* 2010b); cv values on EC50/IC50's 3-20% (intralab), and standard deviations on EC50/IC50's 18-26% (interlab) (van der Burg *et al.* 2010a).

4.2 Reference chemicals/chemical libraries, rationale for their selection and other available information

The CALUX panel described here consists of 26 different assays; specific information on reference compounds and performance per assay can be found at https://eutoxrisk.douglasconnect.com/test-methods. A table of all assays and their reference compound is also given in the section Annexes, Table 1. In general, the assays have been validated with relevant known positive and negative controls for the specific endpoint (nuclear receptor or cell signalling pathway) (Gijsbers *et al.* , Garrison *et al.* 1996, Sonneveld *et al.* 2005, Sonneveld *et al.* 2006, van der Burg *et al.* 2010b, van der Burg *et*

al. 2010a, Gijsbers *et al.* 2011, Sonneveld *et al.* 2011, Gijsbers *et al.* 2013, van der Linden *et al.* 2014, OECD 2016).

4.3 Performance measures/predictive capacity (if known)

Dose-response curves are analysed in Prism (four parameter sigmoidal, variable slope).

QA/QC criteria / typical performance:

Z-factor >0.6

Negative control within set limits (relative induction <10%)

Positive control: induction factor within set limits (differs per assay); EC50 value within set limits (differs per assay)

Standard deviation on triplicates within 15%

Status

In development:

All assays have passed their developmental phase and have already been used in several high throughput screening programs (van der Burg *et al.* 2010b, Sonneveld *et al.* 2011, Piersma *et al.* 2013, van der Burg *et al.* 2015b).

Known laboratory use:

CALUX assays are marketed by BDS. As such, many assays are in use in other laboratories (see the section Annexes, Table 1, column 'external use'). The method has been used in the EU-ToxRisk context.

Participation in evaluation study:

All assays have undergone internal validation with respect to robustness and stability, and predictiveness with respect to known positive controls.

Participation in validation study:

Various CALUX assays have undergone validation studies, and/or participated in interlaboratory ring studies (see the section Annexes, Table 1, column 'validation'). Currently, two assays are in OECD Test Guideline 455 (OECD 2016), four more assays are being evaluated according to OECD-guidelines, and one assay is ISO-certified. Although the validated assays are identical to the assays used in the current protocol, the experimental protocol used is slightly different: the current protocol describes automated 384-wells screening, while the validation was performed manually on 96-wells plates. Details like detection limits and acceptable standard deviations may therefore differ, while the overall performance of the cell lines (robustness, stability, predictivity) remains the same.

Regulatory accepted:

Currently, two assays are in OECD Test Guideline 455 (OECD 2016), four more assays are being evaluated according to OECD-guidelines, and one assay is ISO-certified.

Proprietary and/or confidentiality issues

The name CALUX is a registered trademark; CALUX assays are marketed by BioDetection Systems (BDS; http://www.bds.nl/).

Health and safety issues

General precautions:

Standard laboratory procedures apply. Always use gloves and labcoat; when working with dangerous or unknown compounds, always work in a fume hood. If the dilution/exposure work is performed by a liquid handling machine, the risk for the employee is greatly reduced.

MSDS information:

In addition to the safety measures regarding the compounds in use, there are no safety measures needed for the performance of this method.

Abbreviations and definitions

ATP: Adenosine triphosphate

AhR: Aryl hydrocarbon receptor

BDS: BioDetection Systems bv

CALUX®: Chemically Activated LUciferase eXpression

CDTA: Trans-1,2-diaminocyclohexane-N,N,N'N'-tetra acetic acid monohydrate

DCC: dextran coated charcoal

DCC-Stripped FCS: active charcoal-stripped FCS (a process to remove steroids from the serum)

DMEM/F12: Dulbecco's modified eagle medium supplemented with F12

DMSO: Dimethyl sulfoxide

DTT: Dithiothreitol

EDTA: Ethylenediaminetetraacetic acid

FCS: Fetal calf serum

GMOs: Genetically modified organisms

NADPH: b-Nicotinamide adenine dinucleotide phosphate tetrasodium salt

PBS: Phosphate buffered saline

PC: Positive Control

Trypsinate: Enzymatic treatment of cells with trypsin to remove the intercellular and surface attachment resulting in single rounded cells

Subculture: The transfer of a cell suspension into a new culture flask

Last update

15-January-2018

Part B. Technical description

Procedure details, latest version:

15-January-2018

Protocol name:

Automated CALUX reporter gene assay procedure

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Materials and preparations

Assays are based on the human osteosarcoma cell line U2-OS (ATCC HTB-96), or the rat hepatoma H-4-II-E (ATCC CRL-1548) (AhR CALUX only).

Each cell line is stably transfected with a reporter gene plasmid containing luciferase under control of the responsive elements of a specific receptor (e.g. oestrogen receptor) or cell signalling pathway (e.g. NFκB). Activation of the transfected pathway or receptor by a test chemical results in luciferase production, which can be measured as light production by adding the substrate luciferin. Since most nuclear hormone receptors are not endogenously expressed in U2-OS cells, these receptors have been co-transfected when applicable (see the section Annexes, Table 1).

Equipment

Fixed Equipment

- Cell culture incubator (37°C, 5% CO_2)
- Cell washer-dispenser (BioTek EL406)

- Centrifuge
- Freezers (-20 and -80 and liquid nitrogen)
- Inverted phase contrast Microscope
- Laminar flow cabinet
- Liquid handling machine (Hamilton STARlet)
- Luminometer with stacker

Consumables

Several CALUX assays are sensitive to background signals arising from contaminations present in, or leaching from, reagents and consumables. When a specific brand and order number is quoted below, the advised brand and type is preferred. In cases where the brand and order numbers are only indicative, it is preceded by 'e.g.'.

- 15 ml tubes (Greiner, 188271)
- 384 wells plates (Greiner, 781080)
- 50 ml tubes (Greiner, 210261)
- 96-deepwell plates (Greiner, 780261)
- Adhesive Plate Seal (e.g. Thermo Fisher, 11524794)
- BreathEasy plate seals (Molecular Devices, E1005)
- Brown autosampler Glass conical vials (VWR, BROW153810)
- Cell culture flasks (75 cm2, e.g. Greiner, 658 175)
- Cryovials (e.g. Greiner, 121261)
- Filter units pore size 0.2 micron (e.g. Whatman 10462200)
- Mr FrostyTM container (e.g. Thermo Fisher, 10110051)
- Pipetting tips 1000, 300 and 50 µl (Hamilton, 235904, 235902 and 235966)
- Sterile reservoirs (50ml) (e.g. VWR, 613-1184)
- Sterile serological pipettes 5ml, 10ml and 25ml (Greiner, 606180, 607180 and 760180)

Media, reagents, sera, others

- 1M HCl solution (pH meter) (e.g. Sigma, H1758)
- 1M NaOH solution (pH meter) (e.g. Sigma, S5881)
- Adenosine triphosphate (ATP) (e.g. Ducheva Biochemie BV, A 1335.0010)
- Alcohol 70% (e.g. BioSolve, 5210502)
- b-Nicotinamide adenine dinucleotide phosphate tetrasodium salt (NADPH) (e.g. Applichem, A1395,0500)
- Dithiothreitol (DTT) (e.g. Ducheva Biochemie BV, D1309-0025)

- D-Luciferine (e.g. Resem bv, D-luciferin 500mg)
- Dulbecco's modified eagle medium supplemented with F12 (DMEM/F12) with phenol red as pH-indicator (e.g. Thermo Fisher, 11514436)
- Dulbecco's modified eagle medium supplemented with F12 (DMEM/F12) without phenol red (e.g.Thermo Fisher, 11580546)
- Ethylenediaminetetraacetic acid (EDTA) (e.g. Acros, 147855000)
- Fetal calf serum (FCS) (South American origin, Thermo Fisher, 11573397)
- Glucose-6-phosphate (e.g. Biosynth, G-3340)
- Glucose-6-phosphate dehydrogenase type VII (e.g. Sigma, G7877)
- Glycerol (e.g. Baker, 7044)
- Magnesium carbonate hydroxide (e.g. VWR, AAA18070-0B)
- Magnesium sulphate(e.g. Sigma, M7506)
- $MgCl_2$ hexahydrate (e.g. Sigma, M2393)
- Non-essential amino acids (100x) (e.g. Thermo Fisher, 11140-035)
- Penicillin-streptomycin (e.g. Thermo Fisher Scientific, 15070-063; 5000 penicillin units per ml / 5000 µg streptomycin per ml)
- Phosphate buffered saline pH 7.2 (PBS, e.g. Thermo Fisher, 20012-019)
- Rat liver S9 (e.g. MolTox Trinova, 11-105.5)
- Trans-1,2-diaminocyclohexane-N,N,N'N'-tetra acetic acid monohydrate (CDTA) (e.g. Sigma, 32869)
- Tricine (e.g. Sigma, T0377)
- Tris (e.g. Sigma, T1378)
- Triton X-100 (e.g. Sigma, T8787)
- Trypsin (e.g. Thermo Fisher, 27250-042)

Preparations

Media and endpoint assay solutions

Cell Culture Medium (all solutions should be sterile and handled in a sterile environment):

Take a new bottle of DMEM/F12 with phenol red in the laminar flow cabinet.

Add a tube of FCS (41 ml) to the flask DMEM/F12 (7.5%).

Add 5 ml of non-essential amino acids (100x NEAA).

Add 1 ml of penicillin-streptomycin solution.

Store at 4 o C for maximum 2 months.

Assay Medium (all solutions should be sterile and handled in a sterile environment):

Open a 500 ml bottle of DMEM/F12 without phenol red in the laminar flow cabinet

Add 26.6 ml DCC-FCS (5% v/v).

Add 5 ml of non-essential amino acids (100x NEAA).

Add 1 ml of penicillin-streptomycin solution.

Store at 4 ºC for maximum 2 months.

Trypsin solution:

Trypsin solution should be diluted with PBS containing 0.2 g/L EDTA until the trypsin solution has a concentration of 0.05% trypsin (g/L). Filter-sterilise using a 0.2 micron pore size filter. Store the tubes of trypsin at -20 o C until use. After thawing, store at 4 o C for maximum 2 months.

Freezing medium (all solutions should be sterile and handled in a sterile environment):

Open a 500 ml bottle of DMEM/F12 with phenol red in the laminar flow cabinet.

Remove and discard 143ml of DMEM/F12 medium.

Add 5.5 ml of non-essential amino acids (MEM 100x).

Add 100 ml of FCS.

Add 37.5 ml of DMSO.

Mix gently and distribute as 40 ml aliquots in 50 ml Greiner tubes.

Label the plastic tubes containing the freezing medium (preparation date; expiring date (1 year)).

Store freezing medium at -20 °C.

Triton Lysis Buffer:

Dissolve Tris (25mM), DTT (2 mM) and CDTA (2 mM) in demineralised water.

Add 10% (v/v) glycerol and 1% (v/v) Triton® X-100.

Adjust the pH to 7.8 using 1 M HCl and/or 1 M NaOH solutions.

Transfer aliquots of approximately 40 ml into 50 ml tubes.

Store at -20°C for a maximum of 1 year or at 4°C for a maximum of 1 month.

BDS Illuminate Mix:

Dissolve in demineralised (demi) water: tricine (20 mM), magnesium carbonate hydroxide (1 mM), magnesium sulphate (2.7 mM), EDTA (0.1 mM), DTT (1.5 mM) and D-luciferine (539 µM).

After adding the D-Luciferine, the BDS illuminate-mix should be kept in the dark and further preparation may last no longer than 0.5h due to the instability of the compounds used.

Add ATP (5.49 mM).

Adjust the pH to 7.8 using 1 M HCl and/or 1 M NaOH solutions.

Divide the BDS illuminate mix into 100 ml portions in HDPE bottles.

Close and label the bottles.

Store at -20°C for a maximum of 3 months or at -80 °C for one year.

Test compounds

1. Prepare a 0.1M stock of the test compound in DMSO (if not soluble: go down in 0.5 log unit increments until dissolved).
2. Divide into 100 µl aliquots and store at -20°C.
3. Use glass vials only (Brown autosampler vials)!
4. Before use: thaw one aliquot.
5. The liquid handling robot prepares a dilution series in a 96-deep-well plate; the following 14 concentrations (M) with 0.5Log increments are prepared: 1.0E-04; 3.0E-05; 1.0E-05; 3.0E-06; 1.0E-06; 3.0E-07; 1.0E-07; 3.0E-08; 1.0E-08; 3.0E-09; 1.0E-09; 3.0E-10; 1.0E-10; 0
6. The compound is exposed by the Hamilton STARlet; the deep-well plate containing the dilutions is discarded.
7. If the compound is tested again later (as a duplicate measurement, or on different assays), a fresh aliquot is used. EXCEPTION: if a compound is only very scarcely available, the deep-well plates with dilution series will be re-used, and stored at -20 ºC, covered with a plate lid.

Note. For volatile compounds, the 96w-dilution plates are covered with a non-breathable seal until exposure. The assay plates containing the cells exposed to volatile compounds are covered with a breathable seal immediately after exposure.

Note 2. Compounds insoluble in DMSO cannot be tested using the routine automated HTSmethod, and have to be tested separately. For compounds dissolved in water, PBS or medium, a method is available. Compounds in EtOH, MeOH or other solvents will have to be tested manually on 96-wells plates.

Note 3. No specific protocol for very viscous compounds is in place; they may have to be diluted until they can be pipetted by the robotic system. However, within the EU ToxRisk case studies, compounds with such high viscosity have not been encountered yet (CS1 to 5).

Positive Control(s)

For the positive controls (see the section Annexes, Table 1), aliquots of 1000x stock solutions in DMSO are frozen at -20 ºC. For each experiment, the relevant positive controls are thawed, and diluted by the liquid handling machine together with the test compounds (see the section Annexes, Table 1).

Negative Control(s)

DMSO is used as a negative control for all cell lines. A fresh aliquot of DMSO is used for each experiment.

Method

Experimental system procurement

Freezing of cells:

- Prepare 75 cm^2 culture flasks with cells (start 1.5 week in advance).
- Check whether the flasks are approximately 90% confluent before starting the freezing procedure.
- Trypsinise all cells according to the procedure described in the subculturing of cells section (below) including the removal of trypsin.
- Suspend the cells in 10ml growth medium per flask.
- Pool and count the cells from the different flasks. Calculate how many cryovials can be prepared at a concentration of 1.5 x 10^6 cells/vial.
- Divide the cells over 50 ml Greiner tubes and centrifuge (250 g, 5 minutes).

Note. After the centrifugation step, keep the vials and 50 ml Greiner tubes on ice at all times and work as quickly as possible to minimise the toxic effect of DMSO at room temperature!

- Discard medium and re-suspend the cells in freezing medium. The volume in ml of freezing medium to be used equals the number of vials that can be prepared. Each cryovial should contain 1 ml of cell suspension.
- Divide the re-suspended cells in freezing medium over the number of cryovials calculated (1 ml per cryovial) and close the cryovials.
- Put the cryovials in the Mr. Frosty freezing containers and place it at -80 °C (only overnight) to achieve acooling rate of 1 °C/minute.
- Transfer cryovials to liquid nitrogen for storage.

Thawing of cells:

- Heat water bath to 37°C.
- Take a flask of growth medium from the refrigerator and heat it in the water bath.
- Retrieve a cryovial of CALUX cells from the liquid nitrogen.

Note. Wear safety glasses and protective gloves.

- Thaw the cells quickly by gently moving the vial in a water bath of 37°C until the ice has almost melted.
- Clean the outside of the cryovial with 70% alcohol.
- Pipette 0.5 ml of growth medium from the culture flask into the cryovial using a sterile pipette.
- Transfer cells to a 15 ml sterile plastic tube.
- Add drop wise 10 ml of cold cell culture medium and mix gently (4°C).

- Spin down the cells in the centrifuge at approximately 250x g, 5 minutes.
- Remove the medium.
- Resuspend the pellet in 10 ml of culture medium (RT to 37°C), transfer to a culture flask (75 cm^2), and transfer to the CO_2-incubator. Indicate the type of cells, date of preparation, name and passage number.

Routine procedures

Subculturing of cells:

- Subculture preferably every Monday and Friday.
- Thaw a tube of trypsin.
- Take a flask growth medium from the refrigerator.
- After pre-warming the solutions in a 37 ºC water bath, place the trypsin tube and the growth medium and a PBS flask in the safety-cabinet and open them.
- Take a culture flask (75 cm^2) with cells to be subcultured from the CO_2 incubator.
- Transfer the growth medium from the culture flask into the waste bottle by sterile pipetting.
- Carefully pipette 5 ml of PBS into the culture flask by sterile pipetting. Place the tip of the pipette just below the neck of the culture flask. Ensure that the pipette-tip does not to touch the neck of the culture flask.
- Swirl the culture flask approximately 5 times.
- Transfer the PBS from the culture flask into the waste bottle using a sterile 10 ml pipette.
- Rinse again with 5 ml PBS.
- Pipette 2 ml of trypsin into the culture flask by sterile pipetting.
- Swirl the culture flask approximately 5 times.
- Transfer all the trypsin from the culture flask into the waste bottle by sterile pipetting.
- Gently slap the bottom of the culture flask against the palm of your hand after 5 minutes to see if the cells are detaching from the bottom of the culture flask.
- Pipette sterile 10 ml of growth medium supplemented with FCS in the culture flask using a new sterile pipette.
- Swirl the culture flask 5 times allowing the cells to go into suspension. Ensure the cells just below the neck of the flask are in suspension too.
- Re-suspend the cells by performing 10 careful up and down cycles in growth medium using 10 ml pipette. Ensure no flocks of cells are visible anymore.
- Transfer the proper amount of cell suspension into the new culture flask (75 cm^2).

- The amount of cells to be transferred depends on the growth-rate of the cells and the number of days until the day it is intended to subculture them. Generally, a ratio of 1:6 or 1:8 is appropriate.
- After transfer of cell suspension, fill up the new culture flasks to a final volume of 10 ml with growth medium.
- Label the culture flasks. Indicate the type of cells, date of subculturing, passage number, dilution factor and your name.
- Place the culture flask in the CO_2 incubator.

Test material exposure procedures

- Prepare a cell suspension of 1×10^5 cells/ml in white assay medium (see above), and seed white 384-wells plates with 30 µl cell suspension/well using an automatic multidispense multichannel pipette.
- 24 h after seeding, (or, in the case of assays BDS21a and BDS21b, 48h after seeding, see below), prepare exposure medium: prepare a dilution series in 0.5log unit increments of each test compound (in DMSO), in 96W-deepwell plates, and add 1 µl of each concentration to a 96-wells plate containing 500 µl assay medium (or, in the case of assays BDS21a and BDS21b, 10 µl of each concentration, see below).
- Of this exposure medium, add 30 µl, in triplicate, to the assay plates containing the CALUX cells (final DMSO-concentration: 0.1% (or 1% in the case of assays BDS21a and BDS21b, see below)).
- Additionally, prepare DMSO blanks and a full dose-response curve of the relevant reference compounds in similar fashion (for reference compounds and concentrations, see the section Annexes, Table 1).
- At BDS, the preparation of the compound dilution series as well as the exposure of the cells are performed on a Hamilton STARlet liquid handling robot coupled to a Cytomat incubator. This automated CALUX assays have been described in the publication (van der Burg *et al.* 2015a).

Note 1. In order to be able to detect receptor antagonism, six CALUX assays were also performed in antagonistic mode. The assay procedure was as described above, with the only exception that the cells were supplemented with an EC_{50} concentration of the reference agonist before exposure (for concentrations and compounds: see the section Annexes, Table 1, column 'EC_{50} agonist').

Note 2. The assays BDS21a and BDS21b have a slightly modified protocol to ensure good performance. They are seeded 48 h before exposure instead of 24 h; and they are exposed at 1% (DMSO v/v) instead of 0.1%.

Additionally, immediately after exposure 6 µl/well of a 10x concentrated S9 mix is added to BDS21b_Geno_RGA_p53S9_act_24h in order to allow metabolic (in)activation by hepatic enzymes. 10x S9 mix consisted of: 3 mg/ml PB-BNF induced Sprague-Dawley rat liver S9, 2 mM NADPH, 30 mM glucose-6-phosphate, 50 mM $MgCl_2$, and 3 units/ml glucose-6-phosphate dehydrogenase. After 3 h, the exposure medium is replaced by assay medium, and cells were allowed to recover for 21 h. After a total of 24 h, cells are harvested and analysed as described in the following paragraph.

Endpoint measurement

- After 24 h exposure, remove the exposure medium, e.g. using an EL406 cell washerdispenser (Biotek).
- Add 10 µl/well triton lysis buffer.
- Measure the luminescent signal in a luminometer (e.g. InfinitePro coupled to a Connect Stacker (both TECAN)), by injecting BDS Illuminate Mix and measuring light output.
- Machine settings:
  - Injection speed: 200 µl/second.
  - Injection volume: 35 µl/well.
  - Measurement time (luminescence): 1 sec/well.
  - Temperature settings: none (ambient).

Acceptance criteria

Acceptance criteria have been established for triplicate datapoints as well as for the entire 384 wells plates. First, determine for each triplicate on the plate whether it meets the acceptance criteria; then, determine for the entire plate if it meets the acceptance criteria.

- Discard triplicates with a standard deviation >15%
- Discard datapoints where the compound is cytotoxic (<80% cell viability as determined using a separate cytotoxicity assay)
- Using only datapoints that passed the two first criteria: negative control sample (DMSO) should be within set limits (relative induction <10%)
- Using only datapoints that passed the two first criteria: positive control sample (reference compound) maximum induction factor and EC_{50} value should be within set limits (relative induction >30%; EC_{50} +/- 0.5 Log units; for EC_{50} values, see the section Annexes, Table 1)

Perform two analyses as independent (biological) duplicates. If the results (PC_{10} or PC_{20} values) of the first and second experiment deviate for >0.5 log unit, perform a third replicate.

From OECD GD 211

2.7 Quality/acceptance criteria

- All data, both raw and calculated, is saved on our servers; data is backed-up daily. Raw data consists of text files (.txt or .asc) containing 384 numbers (24 columns x 16 rows), which are the Relative Light Units (RLUs) measured by the luminometer. Calculated data is reported in % activity compared to reference, and induction factor above blank, calculated by and stored in a custom database tool on the BDS servers. Calculated datafiles also contain information on test compound names, concentrations, and test compound stock solution numbers (internally assigned).
- The database tool performs baseline subtraction and recalculates test compound data into '% of reference compound' and 'fold induction above background'. EC_{10}, EC_{50}, PC_{10}, NOEL and LOEL and maximum induction (fold, %) are calculated and

summarised in a results-table. Quality control calculations are also performed (% SD on triplicates).

- Each plate contains a suitable reference compound (assay-specific, see the section Annexes, Table 1), and a negative control (DMSO); plates are rejected if the following criteria are not met: negative control within set limits (relative induction <10%); positive control: induction factor within set limits (differs per assay); EC_{50} value within set limits (differs per assay). Individual datapoints are discarded if standard deviation on triplicates >15%.

Data analysis

Data is uploaded into our custom designed database tool. This tool performs baseline subtraction and recalculates test compound data into '% of reference compound'. EC_{10}, EC_{50}, PC_{10}, NOEL and LOEL and maximum induction (fold, %) are calculated based on a sigmoidal fit (four parameters, variable slope) and summarised in a results-table.

Prediction model

The prediction model differs per assay, and can be found in detail on https://eu-toxrisk.douglasconnect.com/test-methods. In short, the CALUX assays measure transactivation of a specific receptor, or activation of a cell signalling pathway. Since the U2-OS cells have very low expression levels of endogenous receptors, no cross-talk with other receptors can occur and therefore the assay is highly specific and responsive. A compound is considered a 'hit' if it gives a response that equals 10% of that of the positive control reference compound, 20% of that of the positive control reference compound, or 1.5-fold induction above background (DMSO). See also the picture in the section 'Experimental description' and in the section Annexes, Table 1, column 'reported value'.

Annexes

Table 1. CALUX assay characteristics

Name	Endpoint	EC_{50} or max	[Stock] (M)	Reported value	Reference compound	EC_{50} agonist (M)	Validation	Ext. use
BDS1a_EDC_RGA_ERa_ago_24h	Estrogen receptor alpha agonism	6.0E-12	1.0E-06	PC10	estradiol		TG455 / van der Burg (2010b)	yes
BDS1b_EDC_RGA_ERa_anta_24h	Estrogen receptor alpha antagonism	2.4E-08	1.0E-03	PC20	Tamoxifen	Estradiol 6E-12	TG455 / van der Burg (2010b)	yes
BDS6a_EDC_RGA_ERb_ago_24h	Estrogen receptor beta agonism	1.6E-10	1.0E-05	PC10	estradiol		internal	yes
BDS6b_EDC_RGA_ERb_anta_24h	Estrogen receptor beta antagonism	5.0E-08	1.0E-03	PC20	tamoxifen	Estradiol 2E-10	internal	yes
BDS2a_EDC_RGA_AR_ago_24h	Androgen receptor agonism	4.5E-10	1.0E-05	PC10	dihydrotestosterone (DHT)		OECD in progress / van der Burg (2010a)	yes
BDS2b_EDC_RGA_AR_anta_24h	Androgen receptor antagonism	3.4E-07	1.0E-02	PC20	flutamide	DHT 7E-10	OECD in progress / van der Burg (2010a)	yes
BDS3a_EDC_RGA_PR_ago_24h	Progestin receptor agonism	3.2E-10	1.0E-05	PC10	Org2058		Sonneveld (2011)	yes
BDS3b_EDC_RGA_PR_anta_24h	Progestin receptor antagonism	9.0E-11	1.0E-05	PC20	Ru486	Org2058 3E-10	Sonneveld (2011)	yes
BDS4a_EDC_RGA_GR_ago_24h	Glucocorticoid receptor agonism	1.7E-09	1.0E-04	PC10	dexamethasone		internal	yes
BDS4b_EDC_RGA_GR_anta_24h	Glucocorticoid receptor antagonism	1.4E-09	1.0E-05	PC20	Ru486	Dexamethasone 2E-9	internal	yes
BDS5a_EDC_RGA_TRb_ago_24h	Thyroid receptor beta agonism	8.2E-10	1.0E-04	PC10	3,3',5-trioiodo-L-thyronine (T3)		OECD in progress	yes
BDS5b_EDC_RGA_TRb_anta_24h	Thyroid receptor beta antagonism	4.0E-07	1.0E-02	PC20	deoxynivalenol	T3 8E-10	OECD in progress	yes
BDS7a_repro_RGA_RAR_ago_24h	Retinoic acid receptor agonism	2.0E-07	1.0E-02	PC10	retinoic acid		internal	no
BDS8a_metab_RGA_LXR_ago_24h	Liver X receptor agonism	1.0E-07	1.0E-03	PC10	GW3965 hydrochloride		internal	no
BDS9a_xenob_RGA_PXR_ago_24h	Pregnane X receptor agonism	2.6E-07	1.0E-02	PC10	nicardipine		internal	no
BDS10a_metab_RGA_PPARa_ago_24h	PPAR alpha receptor agonism	2.3E-09	1.0E-04	PC10	GW7647		internal	yes
BDS11a_metab_RGA_PPARg_ago_24h	PPAR gamma receptor agonism	6.7E-08	1.0E-02	PC10	rosiglitazone		internal	yes
BDS12a_metab_RGA_PPARd_ago_24h	PPAR delta receptor agonism	1.0E-07	1.0E-02	PC10	L-165,041		internal	yes

BDS13a_xenob_RGA_AhR_ago_24h	Aryl hydrocarbon receptor agonism	1.0E-11	1.0E-06	PC10	2,3,7,8-Tetrachlorodibenzo-p-dioxin	ISO	yes
BDS14a_hypox_RGA_Hif1a_act_24h	Hif1a pathway activation	3.2E-04	5.7E-01	PC10	cobaltous(II)chloride	internal	no
BDS15a_Wnt_RGA_TCF_act_24h	Wnt pathway activation	3.2E-02	1.2E+01	Fold=1.5	lithium chloride	internal	no
BDS16a_regul_RGA_AP1_act_24h	Activator Protein 1 pathway activation	7.0E-10	1.0E-05	PC10	Phorbol 12-myristate 13-acetate	internal	no
BDS17a_stres_RGA_ESRE_act_24h	ER stress pathway activation	3.0E-08	1.0E-03	PC10	tunicamycin	internal	no
BDS18a_stres_RGA_NFkB_act_24h	NFkB pathway activation	3.0E-10	1.0E-05	PC10	Phorbol 12-myristate 13-acetate	internal	no
BDS19a_OxStr_RGA_Nrf2_act_24h	Nrf2 pathway activation	1.0E-05	1.0E-01	Fold=1.5	curcumine	van der Linden (2014)	no
BDS20a_Geno_RGA_p21_act_24h	p21 Pathway activation	1.0E-08	1.0E-04	PC10	actinomycin D	van der Linden (2014)	no
BDS21a_Geno_RGA_p53_act_24h	p53 pathway activation	1.0E-08	1.0E-04	Fold=1.5	actinomycin D	van der Linden (2014)	yes
BDS21b_Geno_RGA_p53S9_act_24h	p53 pathway activation (+S9)	3.2E-04	1.0E-01	Fold=1.5	cyclophosphamide	van der Linden (2014)	yes
BDS22a_Tox_RGA_cytotox_act_24h	Cytotoxicity	2.7E-07	1.0E-02	PC20	tributyltinacetate	van der Linden (2014)	no

Legend.
Name: name of the assay.
Endpoint: receptor or pathway addressed.
EC_{50} / max: concentration where half-maximal activation is achieved (for assays showing a full dose-response curve), or concentration where maximum activation is achieved (for assays not showing a full dose-response curve).
Stock (M): stock concentration of the reference compound.
Reported: the output parameter of the assay (PC_{10}, PC_{20} or FI).
Reference compound: name of the reference compound used for the assay.
EC_{50} agonist (M): for antagonist assays, an agonist is added to the assay in order to be able to detect repression of this agonist. The column shows the name and concentration of this agonist.
Validation: how is the assay validated (+ reference)?
External (Ext.) use: is the assay used by third parties in other labs yes/no?.

Downloads

Section linked to the protocol and made available through DB-ALM independently of the file containing Part A and Part B. Serves to accommodate supporting content that cannot be adequately reproduced in a PDF version of the protocol. May include:

- templates for data storage and analysis used in the laboratories,
- data samples,
- scripts or source code used for analysis,
- tabulated lists of reference substances and/or results,
- high quality graphs and figures,
- other supplementary documents.

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11. CALUX-2: Individual effect curves and EC_{10} for all analogues

Results CALUX for VPA analogues / CS2 (EU-ToxRisk)

Chemical ID		Source 1	Source 2	Source 3	Target	Source 4	Source 5	Source 6	Outlier 1	Outlier 2	Outlier 3
CAS		88-09-5	142-62-1	97-61-0	4536-23-6	149-57-5	99-66-1	31080-39-4	1185-39-3	1575-72-0	591-80-0
Name		EBA	HA	MPA	MHA	EHA	VPA	PHA	DMPA	4-ene-VPA	PA
Supporting data related to the target endpoint(s)											
In vivo	Toxicogenomics										
	…										
In vitro	CALUX reporter gene assays	LEC in Log (M), CALUX (DBALM 197)	LEC in Log (M), CALUX (DBALM 197)	LEC in Log (M), CALUX (DBALM 197)	LEC in Log (M), CALUX (DBALM 197)	LEC in Log (M), CALUX (DBALM 197)	LEC in Log (M), CALUX (DBALM 197)	LEC in Log (M), CALUX (DBALM 197)	LEC in Log (M), CALUX (DBALM 197)	LEC in Log (M), CALUX (DBALM 197)	LEC in Log (M), CALUX (DBALM 197)
	Cytotox CALUX	>-3	>-3	>-3	>-3	>-3	>-3	>-3	>-3	>-3	>-3
	ERa CALUX	>-3	>-3	>-3	>-3	>-3	>-3	>-3	>-3	>-3	>-3
	Anti-ERa CALUX	>-3	>-3	>-3	>-3	>-3	>-3	>-3	>-3	>-3	>-3
	AR CALUX	>-3	>-3	>-3	>-3	>-3	>-3	>-3	>-3	>-3	>-3
	Anti-AR CALUX	>-3	>-3	>-3	>-3	-3.4	>-3	-3.5	-3.5	>-3	>-3
	PR CALUX	>-3	>-3	>-3	>-3	>-3	>-3	>-3	>-3	>-3	>-3
	Anti-PR CALUX	>-3	-3.0	>-3	>-3	-3.0	-3.1	-3.6	>-3	>-3	>-3
	GR CALUX	>-3	>-3	>-3	>-3	>-3	>-3	>-3	>-3	>-3	>-3
	Anti-GR CALUX	>-3	>-3	>-3	>-3	>-3	>-3	>-3	>-3	>-3	>-3
	TRb CALUX	>-3	>-3	>-3	>-3	>-3	-3.0	-3.5	>-3	-3.0	-3.4
	Anti-TRb CALUX	>-3	>-3	>-3	>-3	>-3	>-3	>-3	>-3	>-3	>-3
	RAR CALUX	>-3	>-3	>-3	>-3	>-3	>-3	>-3	>-3	>-3	>-3
	LXR CALUX	>-3	>-3	>-3	>-3	>-3	>-3	>-3	>-3	>-3	>-3
	PXR CALUX	>-3	-3.2	>-3	>-3	-3.6	-4.1	-4.3	>-3	-3.5	-3.5
	PPARa CALUX	-3.5	-3.5	-3.5	-4.5	-4.0	-4.0	-4.5	-3.7	-4.1	-3.5
	PPARd CALUX	>-3	>-3	>-3	>-3	>-3	>-3	-3.0	>-3	>-3	>-3
	PPARg CALUX	>-3	>-3	>-3	>-3	>-3	>-3	-3.9	>-3	>-3	>-3

	AhR CALUX	>-3	>-3	>-3	>-3	>-3	>-3	-3.0	>-3	>-3	>-3
	Hif1a CALUX	>-3	>-3	>-3	>-3	>-3	>-3	>-3	>-3	>-3	>-3
	TCF CALUX	>-3	>-3	>-3	>-3	-3.5	-4.0	-4.3	>-3	-3.5	-3.5
	AP1 CALUX	>-3	>-3	>-3	>-3	>-3	>-3	-4.0	>-3	>-3	>-3
	ESRE CALUX	>-3	>-3	>-3	>-3	-3.0	-4.0	-4.0	>-3	-3.0	-3.5
	NFkB CALUX	>-3	>-3	>-3	>-3	>-3	>-3	>-3	>-3	>-3	>-3
	Nrf2 CALUX	>-3	-3.0	>-3	>-3	-3.0	>-3	-4.0	>-3	>-3	-3.2
	p21 CALUX	>-3	>-3	>-3	>-3	-3.0	-3.2	-3.7	>-3	-3.1	>-3
	p53 GENTOX CALUX	>-3	-3.0	>-3	>-3	-3.1	-3.6	-4.0	>-3	-3.3	-3.5
	p53 S9 GENTOX CALUX	>-3	>-3	>-3	>-3	>-3	>-3	>-3	>-3	>-3	>-3
	Defind approach of IATA										
	…										