Section 2
Effects on Biotic Systems

Test Guideline No. 249

Fish Cell Line Acute Toxicity: The RTgill-W1 cell line assay

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OECD Guidelines for the Testing of Chemicals

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OECD GUIDELINE FOR TESTING OF CHEMICALS

Fish Cell Line Acute Toxicity: The RTgill-W1 cell line assay

INTRODUCTION

1. This Test Guideline (TG) describes a 24-well plate format fish cell line acute toxicity test using the permanent cell line from rainbow trout (*Oncorhynchus mykiss*) gill, RTgill-W1. After 24 h of exposure to the respective test chemical, cell viability is assessed based on three fluorescent cell viability indicator dyes, measured on the same set of cells. One plate is required per one test chemical and one biological replicate. The test is designed to (i) predict fish acute toxicity in product testing; (ii) range-finding and pre-screening before conducting a full fish acute or other fish-based toxicity test; (iii) generation of toxicity information to be used for hazard assessment in combination with other lines of evidences (e.g., Quantitative Structure Activity Relationships (QSAR), weight of evidence (WoE)) within Integrated Testing Strategy (ITS)/Integrated Approach to Testing and Assessment (IATA).

2. The cell line and the assay are robust and transferable to laboratories without RTgill-W1 cell line and assay specific expertise [1, 2]. The reliability and reproducibility of the method has been demonstrated in several studies and its predictivity for acute fish toxicity shown by testing organic chemicals with a wide range of physico-chemical properties, modes of action and toxicity [3, 4]. Further details of the assay are provided in the associated validation report [1]. Furthermore, a comparable assay is available as ISO standard 21115 for water quality testing [5].

3. The RTgill-W1 cell line was purposefully selected for this assay [1 – 3.2.]. This cell line was initiated from gill filaments of a healthy rainbow trout [6], one of the standard test species listed in OECD TG 203 [7]. Importantly, this cell line sustains exposure over several days in a defined, protein- and, in fact, animal-component-free buffer, i.e. the exposure medium L-15/ex.

4. Definitions used in this TG are given in ANNEX 1

PRINCIPLE OF THE TEST

5. RTgill-W1 cells in confluent monolayers in 24-well cell culture plates are exposed to the test chemical for 24 h in the dark in a specifically designed, protein-free exposure medium (L-15/ex) and, thereafter, cell viability is assessed with a set of fluorescent cell viability indicator dyes (see paragraph 6 and 7). The assay is laid out such that exposure medium samples can be taken from wells of the culture plates at the onset (c_{0h}) and at the end (c_{24h}) of the exposure to determine the geometric mean of the measured test chemical concentrations [1 –3.3.5.]. The data are expressed as the percent cell viability of unexposed control values versus the test chemical concentration. The resulting concentration-response curves serve to determine the effective concentrations causing 50% loss in cell viability, i.e. the EC50 value. However, other effect parameters may be determined, such as non-toxic

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concentrations, derived by non-linear regression [8], or the lowest-observed or no-observed effect concentrations (LOEC/NOEC), derived by hypothesis testing (ANOVA).

6. The assay allows the detection of three cell viability measures on the same set of cells, indicating cellular (i.e. cyto-)toxicity. It uses a combination of the following fluorescent indicator dyes: a Resazurin-based dye for measuring cell metabolic activity; 5-carboxyfluorescein diacetate acetoxy methyl ester (CFDA-AM) for assessing the integrity of the cell membrane; and Neutral Red, which evaluates the integrity of the lysosomal membrane, (see Figure 1; [9]; [1 – 3.3.7.]). The assay is evaluated by measuring the fluorescence of these dyes in the medium and the results are expressed as percent cell viability in comparison to an untreated control group.

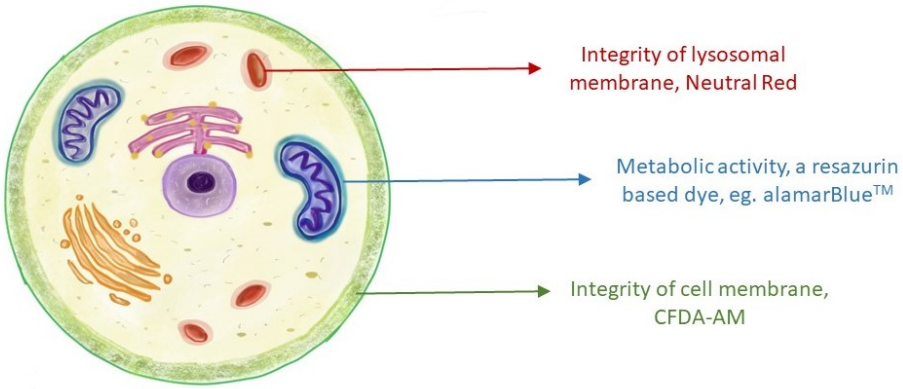

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Figure 1: Three fluorescent indicator dyes serve to determine cell viability based on different targets.

7. Resazurin enters the cells in its non-fluorescent form and is converted to the fluorescent product, resorufin, by mitochondrial, microsomal or cytoplasmic oxidoreductases. A reduction in the fluorescence of resorufin indicates a decline in cellular metabolic activity, including disruption of mitochondrial membranes [10]. CFDA-AM is an esterase substrate that is converted to 5-carboxyfluorescein in intact cells where it is retained [11]. A decline in CFDA-AM fluorescence therefore indicates disturbance of plasma membrane integrity. Neutral Red diffuses into the cells and accumulates in lysosomes [12]. Disruption of lysosomes therefore results in a decrease in Neutral Red fluorescence.

INITIAL CONSIDERATIONS

8. Applicability: While RTgill-W1 cell line assay-derived EC50 values have been demonstrated overall to be in excellent agreement with lethal concentrations (LC50 values) determined in fish acute toxicity tests for a wide range of chemicals [2-4] [1 – 3.5.], the following exceptions have been described [3] [1 – 1.2. and 7.]. Neurotoxic chemicals acting through specific ion channels or receptors typical of brain tissue appear less sensitive in the RTgill-W1 cell line assay compared to fish. As well, in one instance, allyl alcohol, and despite a proven ability of the cell line to biotransform chemicals, a notable underestimation of toxicity compared to fish was thought to be due to a lack of the cell line to convert allyl alcohol to the much more toxic biotransformation product, acrolein. However, *in vivo* toxicity of acrolein was very well represented by the RTgill-W1 cell line assay *in vitro*. Therefore, if indications are available that transformation products might be of relevance to toxicity, it is recommended to perform the test as well with that transformation product, in accordance with the Guidance Document 23 on aqueous-phase aquatic toxicity testing of difficult test chemicals [13].

9. Information on the test chemical: Available substance-specific properties, where relevant at temperatures as close as possible to the cell culture temperature of ~19°C, should be considered prior to chemical testing. These include the structural formula, molecular weight, purity, water solubility, vapour pressure, the n-octanol water partition coefficient (K_{OW}) (corrected for pH where indicated, see pKa), the Henry's law constant (HLC; note: HLC can be calculated from water solubility and vapour pressure), the acid dissociation constant (pKa – used to correct K_{OW} for chemicals that are pH-dependent at the L-15/ex pH [7-7.5, see Annex 5]), and stability in water and light. The assay procedure has been proven to work for chemicals with a wide range of physico-chemical properties, such as water solubility as surrogate for achievable aqueous concentrations (10^{-3} to 10^{6} mg/L), logK_{OW} as surrogate for hydrophobicity (-4 to 7) and logHenry's law constant as surrogate for volatility (0 to -13) [1 – 4. and 7.]. Conduct of this test guideline without the information listed above should be carefully considered as the study design will be dependent on the physico-chemical properties of the test chemical and could lead to meaningless or difficult to interpret results. For poorly water-soluble, or other difficult to test chemicals, the Guidance Document 23 on aqueous-phase aquatic toxicity testing of difficult test chemicals [13] should be consulted.

10. Quantification of exposure concentrations: Inasmuch as effective concentrations are derived based on measured test chemical concentrations, an analytical method with validated accuracy, limit of detection and quantification, and working range needs to be available. (Note: Nominal concentrations may be used in well justified cases. Moreover, if the chemical concentrations are being measured and verified to be within 20% of the nominal concentration at the end of the exposure, nominal concentrations can again be used for effect estimation, in line with other OECD test guidelines; see 1 – 3.3.5.).

11. Plate format and exposure time: While the assay has been developed and validated using the 24-well plate format in order to balance throughput and sufficient medium volume for chemical analysis without elaborate sample preparation, it can be carried out as well in other well-plate formats with proportional scaling of volumes and starting cell numbers [1 – 3.3.3.]. Likewise, while the assay has been optimized and validated with regard to predicting acute fish toxicity for 24 h exposures, longer exposure times, i.e. up to 72 h, are possible as this is the period for which the cells stay viable in the L-15/ex exposure medium [1 – 3.3.6.].

12. Chemical delivery: Since the assay is based on exposure of cells in aqueous solution (specifically the exposure medium L-15/ex), chemical delivery can be achieved by directly dissolving the test chemical in this medium. The Guidance Document 23 on aqueous-phase aquatic toxicity testing of difficult test chemicals [13] should be consulted for preparing difficult-to-test substances in this way. For routine use, however, in order to benefit from the low volume/test substance requirement of the assay, to reduce losses due to elaborate handling and the risk of microbial contamination (e.g. by prolonged stirring), an organic solvent (such as dimethyl sulfoxide (DMSO), methanol or ethanol) can be used for delivery. These organic solvents are known to be non-acutely cytotoxic to at least 1% v/v; nevertheless, ≤ 0.5% (v/v) are recommended (for further details see 1 – 3.3.2. and 3.3.8.2.).

13. Interferences of test chemicals with fluorescent dye assays: Certain chemicals (such as polycyclic aromatic hydrocarbons or textile dyes) may have an intrinsic background fluorescence (autofluorescence) that may interfere with the fluorescence of the indicator dye(s). Two cell-free control wells per 24-well test plate are dedicated to detect and quantify such interferences. If an interference is detected, a cell-free control reference plate is required in an additional test with this particular test chemical including all dilutions (cell-free plate treated in the same way as the exposure plate). Such interference appears rare [1 – 3.3.8.1.]. Available absorbance and emission spectra can be consulted to indicate if interference might have to be expected.

14. Replication: In this test, each test chemical concentration and solvent control are tested in three independent wells, containing a cell population derived from the same batch of cells (cell passage).

These independent wells are regarded as technical replicates. For biological replication, the test can be repeated independently with cells of a different passage number. A single testing run is usually sufficient for a test chemical given that the effective concentration is appropriately covered with the chosen concentration range (determined through a range-finder test) and variability among the technical replicates is admissible (i.e., the standard deviation between the three technical replicates does not exceed 20% for two or more consecutive concentrations) [1 – 3.3.9.].

15. Testing of mixtures and UVCB: The method described in this TG can be applied to aqueous mixtures of known or unknown composition. Indeed, the current TG has been validated earlier and proved to be applicable to water samples (i.e. surface waters or different kind of effluents) by applying certain modifications to sample preparation [5]. These include a strategy to identify potential matrix effects by testing the response of a spiked positive control and by examining whether precipitates have formed. In case of testing substances of Unknown or Variable composition, Complex reaction products or Biological materials (UVCB) or multi-constituent substances, its composition should, as far as possible, be characterised, e.g. by the chemical identification of its constituents, their quantitative occurrence and their chemical-specific properties (paragraph 9). Recommendations about the testing of difficult test chemicals, like mixtures, UVCB or multi-constituent substances are given in Guidance Document 23 on aqueous-phase aquatic toxicity testing of difficult test chemicals [13]. When considering testing of mixtures, difficult-to-test chemicals (e.g. unstable), or test chemicals not clearly within the applicability domain described in this TG, upfront consideration should be given to whether the results of such testing will yield outcomes that are scientifically meaningful.

DEMONSTRATION OF PROFICIENCY (POSITIVE CONTROL)

16. Prior to routine use of the validated test method, laboratories should test the positive control substance, here: 3,4-Dichloroaniline (3,4-DCA), repeatedly (i.e. at least in three independent tests) in a full concentration-response range to i) demonstrate their technical competence in performing the assay and ii) check the sensitivity / responsiveness of the RTgill-W1 cell line. The acceptable range of 3,4-DCA EC50 values for each of the fluorescent indicator dyes is given in paragraph 17.

VALIDITY CRITERIA OF THE TEST

17. For the test to be valid, the following criteria apply [1 – 3.3.8.]:

a) Excluding autofluorescence: cell-free control wells

The variation of raw fluorescence data of the two cell-free control wells (one carrying L-15/ex and the other carrying L-15/ex with the highest test chemical concentration) should not exceed 20%.

b) Excluding contamination of the organic solvent control

If an organic solvent, such as DMSO (see paragraph 12), is used to deliver the test chemical, the solvent control should not be more than 10% lower than the negative control in terms of cell viability, which is within the range of natural background variability.

c) Assay performance: quality control plate with the positive control 3,4-DCA

The mean EC50 values for the 3,4-DCA positive control are based on nominal concentrations (without analytical determination of the exposure concentration) and should lie between two-and-a-half standard deviations (SD) from the EC50 values as stated below.

- $EC50_{3,4\text{-}DCA}$ Resazurin: 43.6 mg/L ± 6.1 mg/L (2½ SD range: 28.4 mg/L to 58.9 mg/L)

- $EC50_{3,4\text{-}DCA}$ CFDA-AM: 62.5 mg/L ± 18.9 mg/L (2½ SD range: 15.3 mg/L to 109.8 mg/L)

- $EC50_{3,4\text{-}DCA}$ Neutral Red: 58.6 mg/L ± 18.6 mg/L (2½ SD range: 12.1 mg/L to 105.0 mg/L)

DESCRIPTION OF THE METHOD

18. An overview of required apparatus and materials is given in Annex 2. Required reagents and recipes for media and solutions can be found in Annex 3. An exemplary SOP for the test performance is presented in Annex 4. Test documentation templates are given in Annex 5. A complete overview of the day-by-day procedural steps of the method is presented in Figure 2.

Cell line

19. The RTgill-W1 cell line is commercially available at ATCC® (with the trademark of the product: CRL-2523™). After reaching 150 passages in the handling laboratory, the cells should be discarded and new cells of a lower passage number thawed. Detailed description of routine, sterile cell culture and freezing/thawing, along with lists of apparatus and materials, reagents, media and solutions and documentation forms are presented in Annex 6. The Guidance Document on Good In Vitro Method Practices (GIVIMP) is referred to for general cell culture consideration [14] [1 – 3.7.2.].

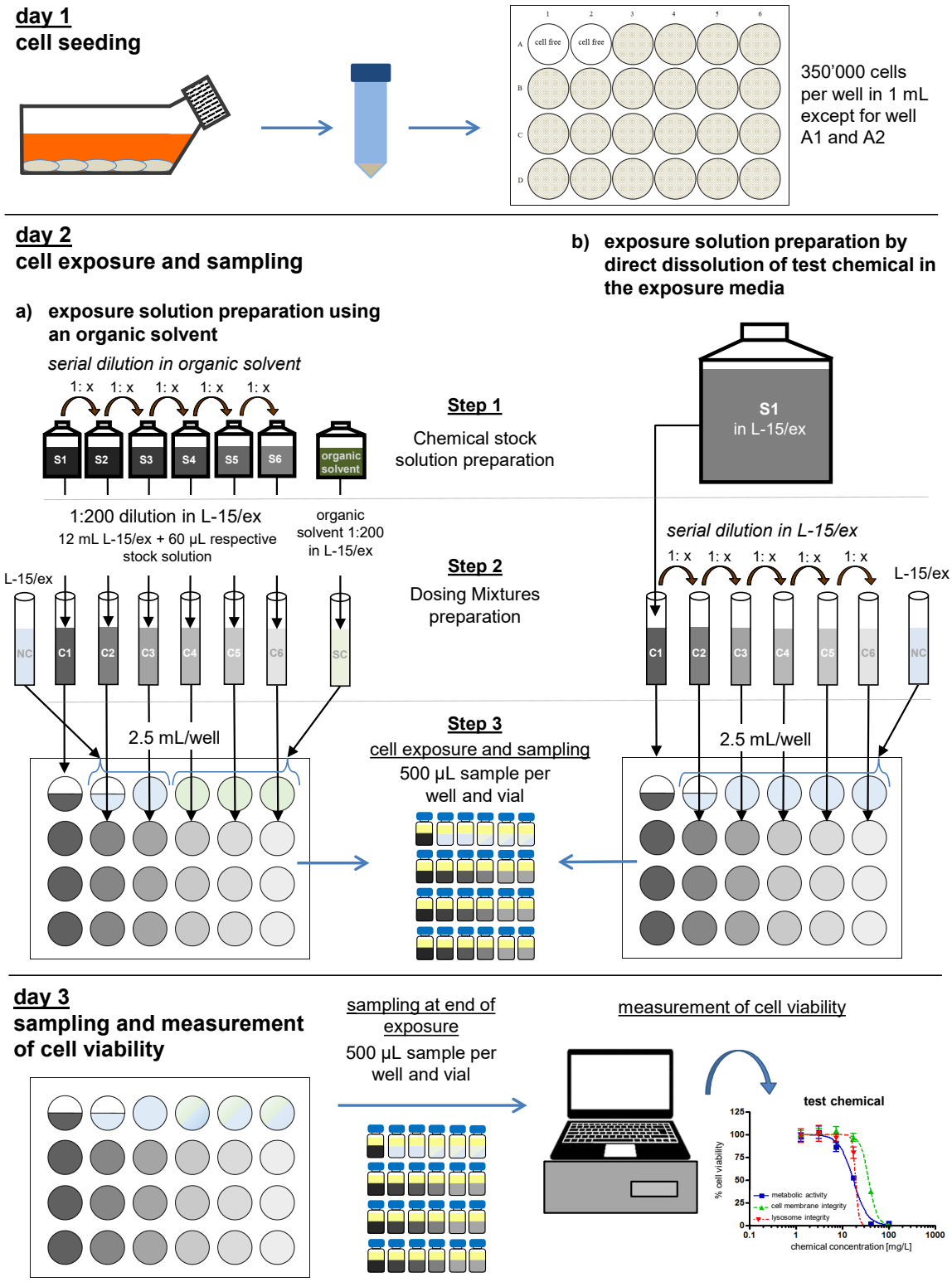


Figure 2: General overview of the test procedure. S1 to S6 refer to the test chemical stock solutions that are used for the preparation of dosing mixtures in the final test concentrations C1 to C6, with S1 and C1 being the highest concentration, respectively. For detailed information on the pipetting scheme of a test chemical, see Figure 5.

Seeding cells into 24-well plates (Figure 2, day 1)

20. For testing one chemical in one biological replicate, one well plate is needed. For each day of testing, an additional separate test plate needs to be prepared for the positive control (3,4-DCA). After harvesting the RTgill-W1 cells from the culture flask, which should yield about 10 million cells (for details see Annex 4), 350 000 cells in 1 mL L-15 complete culture medium per well are seeded into a 24-well plate, except for the "cell-free control" wells (A1 and A2, see pipetting scheme in Figure 3). During the seeding procedure, care should be taken to keep the cell suspension homogenous and avoid settling of cells. Twenty-four hours after plating, cells should have formed a confluent monolayer within each well (Figure 4), which is observed by microscopy. If confluence is not yet reached after 24 h of incubation, the plate can be incubated another 24 - 48 h before test chemical exposure.

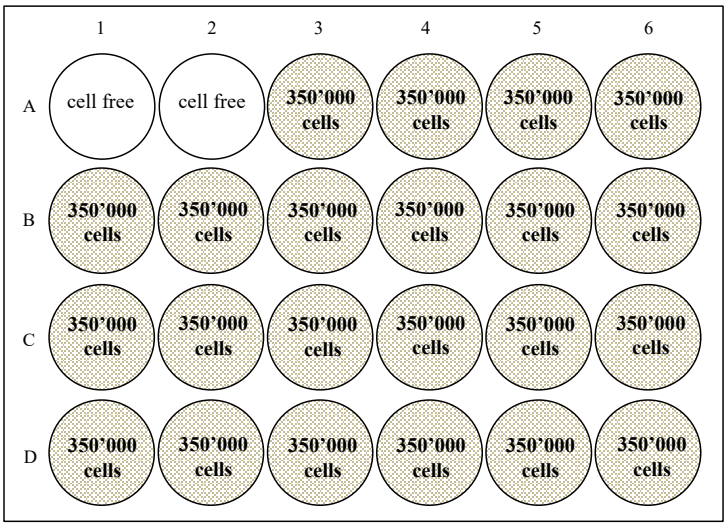


Figure 3: Pipetting scheme for cell seeding into 24-well plates (1 mL seeding volume per well).

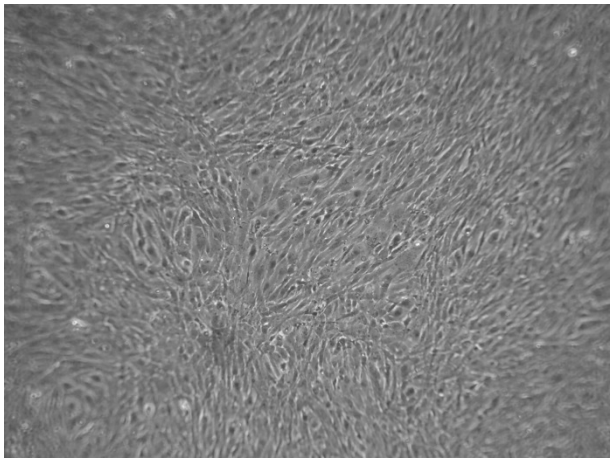


Figure 4: Confluent monolayer of RTgill-W1 cells.

Exposure of cells to a test chemical (Figure 2, day 2)

Selection of the six test concentrations

21. The highest concentration tested (wells B1, C1, D1, Figure 5) should preferably result in 0% cell viability compared to the negative or solvent control (wells A3-A6, Figure 5). The lowest test chemical concentration (wells B6, C6, D6, Figure 5) should preferably give no effect, and therefore result in 100% cell viability compared to the negative or solvent control, respectively. A range-finding test before the definitive test allows the selection of the appropriate concentration range. The range finding test is performed similar to the definitive test (see below), but with concentrations starting at water solubility with subsequent 10-fold dilutions. In addition to water solubility as criterion, the following Quantitative Structure-Activity Relationship (QSAR) [3] can be consulted to predict an EC50 to guide testing range selection around the lowest expected (baseline) toxicity:

$$\log EC50\ (mM) = -0.96\ (\pm 0.09)\ \log K_{OW} + 1.57\ (\pm 0.28) \qquad (1)$$

Preparation of stock solutions of the positive control and the test chemical (Figure 2, Step 1)

22. As positive control, *3,4-Dichloroaniline (3,4-DCA)*, dissolved in dimethyl sulfoxide (DMSO) as organic solvent is suggested. For use with DMSO, the test chemical stock solution needs to be 200 times more concentrated than the final exposure concentrations of the test chemical to gain a final (non-acutely cytotoxic) DMSO concentration in the exposure medium of 0.5% volume fraction. The highest exposure concentration of 3,4-DCA in the wells is 100 mg/L. Therefore, prepare a stock of 20 g/L in DMSO. This stock solution is now the starting point for a serial dilution in DMSO as depicted in Figure 2, Step 1. 3,4-DCA should be tested in six concentrations spaced by a constant factor of two, resulting in the exposure concentrations of 100, 50, 25, 12.5, 6.25 and 3.13 mg/L in the well. These concentrations were established to obtain a full concentration-response curve in the exposure medium (L-15/ex), leading to a minimal (0%) and maximal (100%) cell viability compared to the solvent control (Figure 5A) for the highest and lowest concentration, respectively. The 3,4-DCA dilution series shall be prepared freshly for each experimental day.

23. To prepare the *test chemical* stock solutions in an organic solvent (Figure 2, Step 1), the same procedure should be followed as for the preparation of the positive control, yielding six test chemical concentrations, with a constant dilution factor not exceeding 2.5. To prepare a *test chemical* stock solution without using an organic solvent, the test chemical is dissolved directly in L-15/ex, under sterile conditions, either directly producing the highest test concentration or a higher concentration.

24. Along with the stock solutions, material for the *negative* (cells exposed to L-15/ex only) and the *solvent-control* (cells exposed to solvent, i.e. either L-15/ex or organic solvent only) need to be prepared (Figure 2, Step 2).

Preparation of dosing mixtures in the exposure medium (L-15/ex) (Figure 2, Step 2)

25. Dosing mixtures are prepared by diluting the test chemical stock solutions (prepared in L-15/ex or an organic solvent, see paragraph 23) in L-15/ex exposure medium under sterile conditions (Figure 2, Step 2). In the case of use of an organic solvent, the already serially diluted stock solutions are individually diluted 1:200. In the case of L-15/ex as solvent, the stock solution is serially diluted in L-15/ex with a constant dilution factor not exceeding 2.5. The dosing mixtures should be prepared freshly immediately before dosing the cells.

Preparation of vials for exposure medium sampling for chemical quantification

26. For quantifying exposure concentrations, the cell exposure medium is sampled at the onset (c_{0h}) and the end (c_{24h}) of the exposure period and test chemical concentrations quantified to determine the geometric mean of the measured exposure concentrations (see paragraph 36). In exceptional cases, i.e. when there are strong indications that the test chemical is instable and disappears within a matter of four hours or less, the exposure duration might be lowered to 4 h, in which case exposure medium would be sampled at that time (c_{4h}) and cell viability be assessed immediately thereafter.

27. For exposure medium sampling at the start (c_{0h}) or upon termination (c_{24h}) of exposure, prepare the required number of sampling vials to sample all wells. The sampling vials might be pre-loaded with solvent according to the analytical protocol. All of the triplicate wells per concentration should be sampled. Two samples can initially be analysed while the third one serves as back-up and is used only in case of unexpected circumstances (e.g., failure of analytical instrument or largely differing values between the two replicates). Alternatively, all three samples can be analysed at once.

Test chemical exposure and sampling for chemical analyses at start of exposure (c_{0h}) (Figure 2, Step 3)

28. Exposure of cells to the test chemical, and sampling of the exposure medium containing the test chemical for analyses (i.e. to quantify test concentrations), are performed under sterile conditions.

29. Twenty-four hours after plating, cells should have formed a confluent monolayer within each well; only monolayers should be used for the assay (see paragraph 20, Figure 4) [1 – 3.3.1.]. This is observed by microscopy.

30. Remove the L-15 complete culture medium and wash all wells carefully with 1 mL exposure medium (L-15/ex), paying attention to not disturb the cell layer. Remove the L-15/ex and apply 2.5 mL of the respective medium (Figure 5): To well A1, carefully add the dosing mixture containing the highest test chemical concentration; to wells A2 and A3, add L-15/ex alone; to wells A4 through to D6, add the respective dosing mixture (paragraph 25) into each of the triplicate wells per concentration, paying attention to not disturb the cell layer. Immediately after dosing the plate, transfer a 500 µL sample from each of the three wells per test concentration and the controls into the respective, pre-prepared sampling vials (paragraph 27). Cover the plate with adhesive foil or other cover, see 1 – 3.3.4.), close the lid and incubate the plate for 24 h at 19 ± 1 °C in the dark in normal atmosphere.

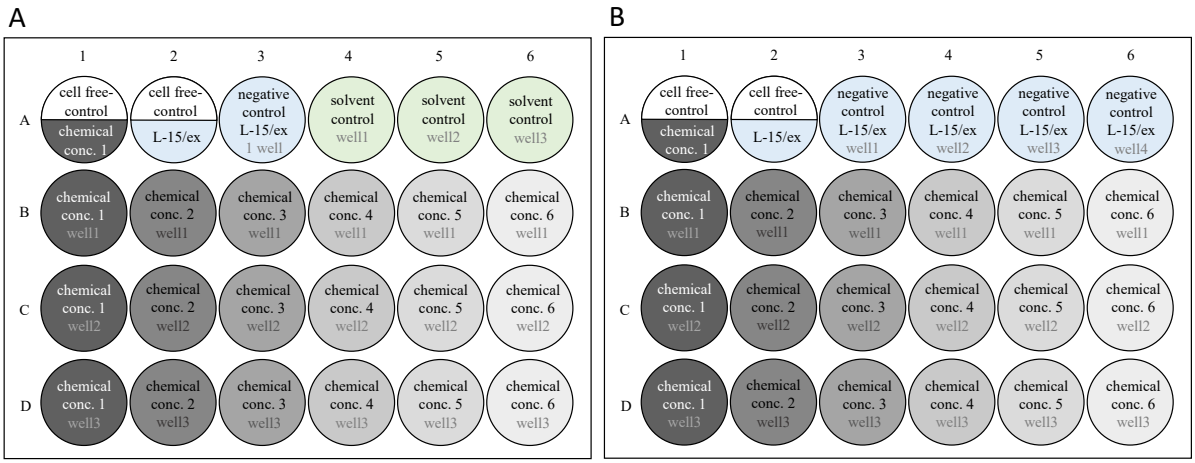


Figure 5: Pipetting scheme for a test chemical (one test chemical, six test concentrations and three technical replicates per concentration with "conc. 1" signifying the highest test concentration). Panel A represents a pipetting scheme for a test chemical where an organic solvent is used for exposure solution

preparation. Panel B represents a pipetting scheme for a test chemical where exposure solution is prepared in exposure medium L-15/ex without an organic solvent.

Determination of cell viability

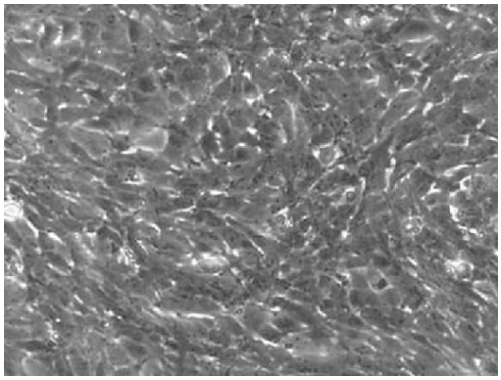
Preparation of working materials and solutions

31. Prepare the Resazurin/CFDA-AM and the Neutral Red working solutions (see Annex 3 to 5).

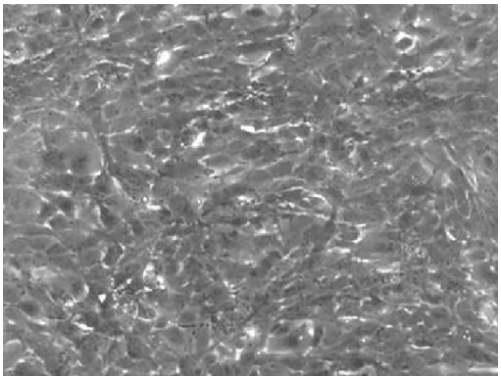
32. Prepare sampling vials for chemical analysis (c_{24h}) according to paragraph 27.

Visual control of cell damage

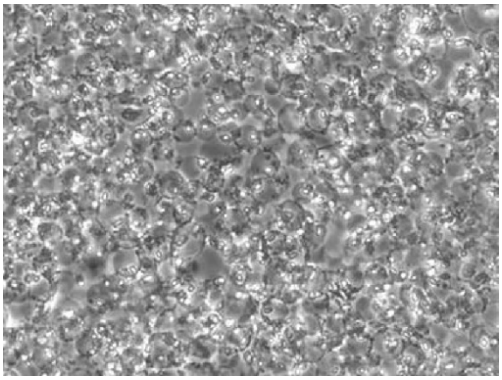
33. The cells in the culture plate should be visually observed, paying particular attention to the degree of damage as a first control of the test performance. It is recommended to perform the visual observation when the adhesive foil and test chemical are already removed from the plate and 1 mL of phosphate buffered saline (PBS) per well is added to the plate (see paragraph 34). This allows for better observation of cells. In Figure 6, cells are shown with a different grade of damage after exposure to the positive control 3,4-DCA in the exposure medium L-15/ex. These images serve as a guide to ensure optimal test performance.



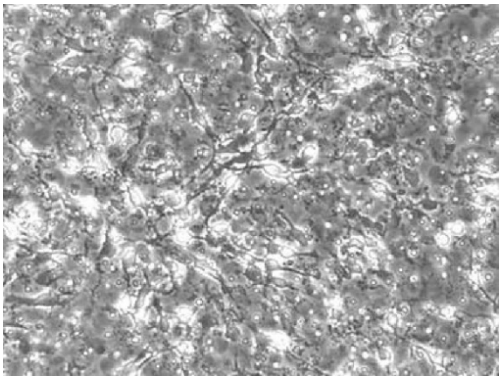
a) negative control (100% metabolic activity)



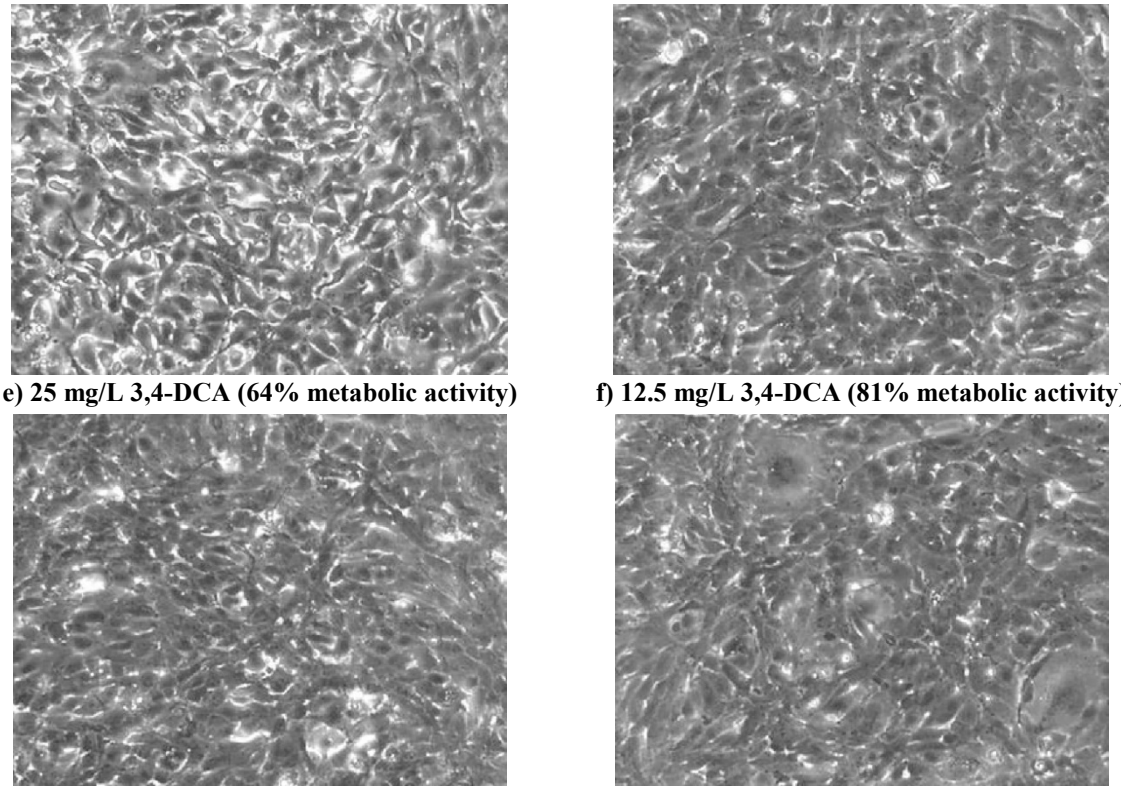
b) solvent control (100% metabolic activity)



c) 100 mg/L 3,4-DCA (2% metabolic activity)



d) 50 mg/L 3,4-DCA (12% metabolic activity)



e) 25 mg/L 3,4-DCA (64% metabolic activity) **f) 12.5 mg/L 3,4-DCA (81% metabolic activity)**

g) 6.25 mg/L 3,4-DCA (91% metabolic activity) **h) 3.13 mg/L 3,4-DCA (96% metabolic activity)**

NOTE Percentages refer to the percentage of metabolic activity compared to the control as measured by the alamarBlue™ ready-to-use resazurin dye preparation.

Figure 6: Images of RTgill-W1 cells with different grades of damage after 24 h of exposure to the positive control 3,4-Dichloroaniline.

Measuring cell viability and sampling for chemical analyses at the end of exposure (c_{24h}), 24 h after initiating the exposure period (Figure 2, day 3)

34. In a first step, transfer 500 µL of the exposure medium from each well into the respective sampling vials for chemical analysis (paragraph 26, 27). The remaining exposure solution is discarded from the plate and each well carefully washed with 1 mL PBS, paying attention to not disturb the cell layer. Replace the washing solution with 400 µL per well of the Resazurin/CFDA-AM working solution. After 30 min of incubation at 19 ± 1 °C in the dark, fluorescence of the Resazurin-based dye (exc: 530 nm, em: 595 nm) and thereafter the fluorescence of CFDA-AM (exc: 493 nm, em: 541 nm) are measured as raw fluorescent units with the fluorescent plate reader. The Resazurin/CFDA AM working solution is discarded and 400 µL per well of Neutral Red working solution added. After 60 min of incubation at 19 ± 1 °C in the dark, Neutral Red working solution is discarded and each well washed with 400 µL fixative. Fixative is removed and 400 µL extraction solution added per well. The plate is incubated for 10 min while gently shaking on a plate shaker at room temperature and thereafter, the fluorescence of Neutral Red (exc: 530 nm, em: 645 nm) measured as raw fluorescent units with the fluorescent plate reader. Further details can be found in Annex 3 to 5.

DATA AND REPORTING

Data treatment

35. The raw fluorescence data (arbitrary units) gained is used to calculate the toxicity as a percentage of cell viability compared to the respective control (see below). In case that the test chemical was dissolved in an organic solvent (such as DMSO), the organic solvent control is used as reference. In case the test chemical was dissolved in L-15/ex without an organic solvent, the negative (i.e. exposure medium) control is used as a reference (see Figure 5).

a) Subtract the corresponding average "cell-free control" fluorescence units from the fluorescence units of each well of the corresponding test plate (see paragraphs 13 and 17 a).

b) Calculate for each replicate well of each chemical test concentration the percentage of cell viability with regard to the respective control. Therefore, the average of the fluorescence units of the control is set to 100% (indicating that 100% of the cells are viable) and corresponding cell viability calculated as shown by formula (2):

$$\%\ cell\ viability = \frac{fluo.units\ chemical\ x\ 100\%}{fluo.units\ respective\ control} \qquad (2)$$

c) Calculate the average and standard deviation of the %cell viability per chemical test concentration and use the percentage of cell viability for concentration-response curve analysis.

Analysis of data / Evaluation of test results

36. The average of the measured test chemical concentrations (see paragraph 26) is used as the common exposure concentration for all three replicate wells per test chemical concentration. Information from well A1 (Figure 5) can be used as indication for abiotic (cell-free) chemical loss, if desired. For the cell containing wells, in the event that a test chemical is undergoing so much loss as to no longer be detectable at the end of the exposure period, c_{24h} should be expressed as ½ of the limit of quantification, in line with the Guidance document 23 on aqueous-phase aquatic toxicity testing of difficult test chemicals [13]. Use the geometric mean based measured concentrations for the x-axis. Thus, for each test concentration, calculate the geometric mean of the concentrations measured at the start (c_{0h}) and the end (c_{24h}) of the test. Plot the percentage of cell viability values on the y-axis.

37. EC50 values are calculated based on non-linear regression sigmoidal concentration−response curve fitting with constrains set for Bottom (0.0) and Top (100.0) using appropriate statistical methods [15, 16].

38. Other effect parameters may be determined from the concentration-response curve, such as the non-toxic concentrations [8], or the lowest-observed or no-observed effect concentrations (LOEC/NOEC) [1 – 3.4.].

39. If the aim is to predict, from the cell viability data, the lethal concentration of acute chemical exposure to fish, first, all EC50 values should be reported and of these, the lowest is used as most conservative estimate [1 – 3.3.7.]. This EC50 value is then taken as the predicted 96 h LC50 value in fish [1 – 3.5.].

Test report

40. The test report should include the following information. An example test report template is presented in Annex 7.

Test chemical

- Mono-constituent substance
  - Chemical identification, such as IUPAC or CAS name(s), CAS number(s), SMILES or InChI code, structural formula, molecular weight and/or other identifiers, like batch/ lot number and expiry date;
  - Physical appearance, water solubility, solubility in the organic solvent if used for test performance, molecular weight and additional relevant physicochemical properties, such as K_{OW} and Henry's law constant, to the extent available;
  - Statement on (in)solubility or stable dispersion in exposure media;
  - Purity, chemical identity of impurities as appropriate and practically feasible;
  - Treatment prior to testing, if applicable (e.g. warming, grinding);
  - Concentration(s) tested;
  - Storage conditions and stability to the extent available.
- Multi-constituent substance, UVCB and mixture:
  - Characterisation as far as possible by e.g. chemical identity (see above), purity, quantitative occurrence and relevant physicochemical properties (see above) of the constituents, to the extent available;
  - Molecular weight or apparent molecular weight in case of mixtures/polymers of known compositions or other information relevant for the conduct of the study;
  - Statement on (in)solubility or stable dispersion in exposure media;
  - Treatment prior to testing, if applicable (e.g. warming, grinding);
  - Concentration(s) tested;
  - Storage conditions and stability to the extent available.

Controls

- Cell-free control
  - Difference in fluorescence of the cell-free control wells A1 and A2 for each cell viability indicator dye (see paragraph 13 and 17 a).
- Solvent/negative control
  - Chemical identification, such as IUPAC or CAS name(s), CAS number(s), and/or other identifiers;
  - Supplier, order number, Lot number;
  - Purity, chemical identity of impurities as appropriate and practically feasible, etc;
  - Physical appearance, molecular weight, and additional relevant physicochemical properties to the extent available;
  - Storage conditions and stability to the extent available;
  - Justification for choice of organic solvent, final solvent concentration in the exposure medium and percent difference of the solvent compared to the negative control (see paragraph 12 and 17 b).
- Positive control, e.g. 3,4-DCA
  - Supplier, order number, Lot number;
  - Purity, chemical identity of impurities as appropriate and practically feasible;
  - Concentration(s) tested;

- Resulting EC50 values for each cell viability indicator dye (see paragraph 17 c).

Test method conditions

- Name and address of test facility and study director;
- Cell line source (e.g. the facility from which they were obtained);
- Passage number and level of confluence of cells used for testing;
- Cell counting method used for seeding prior to testing and measures taken to ensure homogeneous cell number distribution (see paragraph 20);
- Fluorescent plate reader used (e.g. model), including instrument settings;
- Method of preparation of stock solutions and dosing mixtures;
- Analytical method used for quantification of exposure concentrations with accuracy, limit of detection and quantification and working range;
- Justification in case nominal concentrations are used for EC50 calculation;
- The procedure used to demonstrate proficiency of the laboratory in performing the test method (e.g. by testing the positive control 3,4-Dichloroaniline, see paragraph 16) or to demonstrate reproducible performance of the test method over time.

Test procedure

- Test chemical concentrations, application procedure and exposure time used (if different than the one recommended);
- Description of any modifications of the test procedure (see below).

Results

- Tabulation of measured concentrations at start and end of exposure and resulting geometric mean (see paragraph 36);
- Tabulation of the average and standard deviation of % cell viability values per test concentration (see paragraph 35);
- Description of EC50 evaluation (e.g. program and method used, see paragraph 37);
- Tabulation of EC50 values along with 95% confidence intervals obtained for the test chemical and for the positive control;
- A graph depicting concentration-response curves for the cell viability indicator dyes;
- Description of any other relevant observations, if applicable.

Discussion of the results

- Discussion and interpretation of the results obtained.
- Any deviations from the guideline should be reported. Consequences of these deviations should be considered in relation to the reliability of the test data and these considerations should be included in the report.

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ANNEX 1 - Definitions

alamarBlue™: an example of a ready-to-use resazurin-based dye preparation for assessment of cell metabolic activity (CAS Number Resazurin 550-82-3).

ATCC: American Type Culture Collection.

c_{0h}: analytically determined exposure concentration at the start of the exposure.

c_{24h}: analytically determined exposure concentration at the end of the exposure.

Cell culture freezing solution: Leibovitz L-15 media supplemented with 10% FBS and 10% DMSO used for freezing of cell cultures (see Annex 6).

Cell-free control: are two wells per test plate without cells to determine background fluorescence; one receiving L-15/ex and the other receiving L-15/ex with the highest concentration of the test chemical (see paragraph 13 and 17).

Cell viability assessment: quantifying the % of cells that are in a healthy state, i.e. are metabolically active, with intact cell membranes and organelles. Reduced viability (or cytotoxicity) is caused by harmful effects of a test chemical on cell structure and function, evaluated by comparison to the concurrent negative or solvent control.

CFDA-AM (CAS Number 124412-00-6): 5-carboxyfluorescein diacetate acetoxy methyl ester, indicator dye for assessment of cell membrane integrity.

3,4-DCA (CAS Number 95-76-1): 3,4-Dichloroaniline is used as positive control (see paragraph 17 c) to check the assay performance and for demonstration of proficiency (see paragraph 16).

DMSO (CAS Number 67-68-5): dimethyl sulfoxide

ECx (Effective concentration): is the concentration of the test chemical that causes an x percentage change in cell viability compared to the negative or solvent control during a specified time interval. EC50 is for example the concentration at which the cell viability is 50% compared to the (solvent) control (see paragraphs 5 and 37).

FBS: Fetal bovine serum

HLC: Henry's law constant, a physico-chemical property of a chemical describing its tendency to partitioning between the aqueous and the gas phase.

IATA: Integrated Approach to Testing and Assessment

ISO: International Standardization Organization

ITS: Integrated Testing Strategy

K_{OW}: a physico-chemical property of a chemical describing its octanol/water partition coefficient.

L-15 complete culture medium: Leibovitz L-15 medium supplemented with 5% FBS and optional antibiotics, such as 0.5% Gentamicin (see Annex 3 and 6).

L-15/ex: is a protein free medium, containing the same amounts of salts, galactose and pyruvate as Leibovitz L-15 medium, used for exposure ("/ex") of RTgill-W1 cells to a test chemical. L-15/ex is prepared according to Annex 3 to 5.

LC50 (Lethal concentration 50): test chemical concentration which kills 50% of test population.

LOEC: Lowest-Observed Effect Concentration (hypothesis-derived concentration through ANOVA or non-parametric counterpart)

mos: months

Negative control: refers to the exposure medium L-15/ex without test chemical and organic solvent.

Neutral Red (CAS Number 553-24-2): indicator dye for assessment of lysosomal membrane integrity.

NOEC: No-Observed Effect Concentration (hypothesis-derived concentration through ANOVA or non-parametric counterpart)

PBS: Phosphate buffered saline

pKa: a physico-chemical property of a chemical describing its acid dissociation constant.

Positive control: refers to any well-characterized reference substance that, when evaluated by a specific test method, demonstrates the suitability of the test system to yield a reproducible, appropriate response. The protocols presented in this document were validated using 3,4-Dichloroaniline (3,4-DCA) as a positive control because it is easy to handle, and is used as positive control in ISO 15088 and OECD 236. Other substances can be suitable as positive control as long as they yield a reproducible, appropriate response in the test system (see 1 – 3.3.8.3.).

PrestoBlue®: an example of a ready-to-use resazurin-based indicator dye preparation for assessment of cell metabolic activity (CAS Number resazurin 550-82-3).

QSAR: Quantitative Structure-Activity Relationship

RTgill-W1: a permanent cell line from rainbow trout (*Oncorhynchus mykiss*) gill (see ref. [6])

SD: Standard Deviation

Solvent control: refers here to the exposure medium (L-15/ex) plus the respective concentration of the organic solvent used in the test, (e.g. dimethyl sulfoxide (DMSO)). Solvent control only applies in case an organic solvent, such as DMSO, is used to dissolve the test chemical.

SOP: Standard Operating Procedure

TG: Test Guideline

UVCB: substances of Unknown or Variable composition, Complex reaction products or Biological materials.

VR: Validation Report

WoE: weight of evidence

ANNEX 2 - Apparatus and materials for cell viability assessment

General equipment		
Material		**Example Supplier**
1	Biosafety cabinet	HeraSafe; Kendro
2	Incubator	BK-700, (3 – 40 °C); Heraeus Instruments
3	Microscope	Axiovert 40C; ZEISS
4	Vacuum pump	Mini-Vac eco; Axonlab
5	Pasteur pipettes (sterile)	230 mm; VWR
6	Glass pipettes (sterile)	5 mL, 10 mL, 20 mL; VWR
7	Pipetting help	Pipetus-akku; Hirschmann Laborgeräte
8	Pipette box to sterilize pipettes	612-2089; VWR
9	Pipette tips (sterile)	10 µL, 100 µL, 1 mL, 2 mL, 5 mL, 10 mL; Socorex
10	Pipettes	10 µL, 100 µL, 1 mL, 2 mL, 5 mL, 10 mL; Socorex
11	Multi-channel pipette	50 - 1200 µL; Research pro, Eppendorf
12	Pipette tips for multi-channel pipette	50 - 1000 µL; Eppendorf (Germany); 613-3505 (e.g. through VWR)
13	Reagent reservoirs (autoclavable or sterile)	613-2671; VWR
14	Vortex	Vortex Genius 3; IKA

Cell seeding and plate dosing		
Material		**Example Supplier**
15	Cell culture flasks	75 cm^2 with vent screw cap; 90075; TPP
16	Centrifuge	Heraeus Labofuge 400R (0 – 40 °C; 17 -3900 g); Thermo Science
17	Plastic centrifuge tubes (15 mL, sterile)	I91015; TPP
18	Plastic centrifuge tubes (50 mL, sterile)	91050; TPP
19	Cell counting chamber	Neubauer chamber (improved); VWR
or	Cell counter	Casy1, Schärfe System (e.g. through Roche) CasyTon, 10 L, 05651808001; Roche Casy cups; 2 x 900 cups, 05651794001; Roche

20	Multi-well plates (24well)	662160; Greiner-Bio-one. Use sterile plates, with hydrophilic surface (TC surface treatment) for improved cell adhesion, high clarity and low autofluorescence.

Preparation/dosing of stock solutions		
Material		**Example Supplier**
21	Fume hood	
22	Scale	AE50; Mettler Toledo
23	4 mL amber glass vials	27116-U; Supelco; Sigma-Aldrich
24	Osmometer	Vapro 5600; Wescor
25	pH-Meter	605 pH-Meter with electrode 6.0220.100; Metrohm
26	Screw caps for 4 mL amber glass vials, aluminium lined	27142; Supelco; Sigma-Aldrich
27	20 mL amber glass vials (sterilized)	EPA20-B 20ml EPA Screw Vial (amber) with 24-400 Screw Neck, 57 x 27.5mm, pk.100; BGB
28	Screw caps for 20 mL amber glass vials (sterilized)	SV-EPA PP Screw Cap with Hole (white) for 24-400 Screw Neck, Septa Silicone/PTFE, pk.100; BGB. Avoid caps where septa are attached with glue
29	Vial shaker (orbital shaker)	IKA KS125; IKA
30	Duran glass bottles	100 mL, 250 mL, 500 mL, 1 L; e.g. Schott
31	Plastic pipette	50 mL
32	Measuring cylinder (sterilized)	500 mL
33	Adhesive foil	Polyester film with acrylic adhesive, nunc, 732-2610 (e.g. through VWR)

Sampling		
Material		**Example Supplier**
34	Sample vials	1.5 mL short thread vial, 32 x 11.6 mm, clear glass, 1st hydrol. Class, wide opening, label + filling lines; 548-0029; VWR
35	Vial caps with fixed septa	9mm UltraBond Combination Seal: PP Short Thread Cap, black, centre hole; Silicone white/PTFE red, 45° shore A, 1.3mm; 09 04 1220; La-Pha-Pack or 548-0371; VWR. Vials with fixed septa are especially good for transportation/shipping of samples. Otherwise, caps with non-fixed septa might be used as well.
36	Cyclohexane	puriss. p.a., ACS reagent, ≥99.5% (GC); 33117; Sigma-Aldrich
37	Microtube storage boxes	HEATHS120035; VWR

Detection of reduced cell viability		
Material		**Example Supplier**
38	Fluorescent plate reader	Infinite M200; Tecan
39	Plastic centrifuge tubes (50 mL)	91050; TPP
40	Plate shaker	TIMIX; Johannes Otto GmbH
41	Aluminium foil	Alternatively, a box might be used to protect test plates from light during dye incubation.
42	Kitchen paper	

ANNEX 3 - Reagents, media and solutions for cell viability assessment

As far as available, use only cell culture tested grade chemicals.

Ready-for-use purchased reagents:

	Example Supplier	Storage
Bovine serum (FBS)	fetal bovine serum CVFSVF00-01 (Eurobio) **Note:** do not heat inactivate the FBS [Nims R.W. and Harbell J.W. (2017). In Vitro Cell.Dev.Biol.—Animal., 53:682–690.]	thaw before use and prepare 25 mL aliquots in adequate sterile tubes **store at -20 °C**
Gentamicin	10 mg/mL e.g. LONZ02-012E (Lonza) or BCHRA2712 (Biochrom)	**store according to manufacturer's instructions**
Trypsin	0.25% in PBS w/o Ca^{2+}, Mg^{2+}, L11-002 (PAA)	thaw before use and prepare 10 mL aliquots in adequate sterile tubes **store at -20 °C**
Versene (0.2 g/L EDTA in PBS)	1:5000, 15040 (Gibco)	**store at 4 °C**
Leibovitz L-15 media	with Glutamine and without Phenolred 21083 (Gibco)	**store at 4 °C**
37% (w/v) Formaldehyde	F1635-500ML; Sigma-Aldrich	**store at room temperature**
Acetic acid	*BioUltra*, ≥99.5% (GC/T), 45726-1L-F; Sigma-Aldrich	**store at room temperature**
Ready-to-use Resazurin dye preparation	alamarBlue™ solution - DAL1100; Invitrogen	**store at 4 °C**
CFDA-AM	C1345; Invitrogen	**store at -20 °C**
Neutral Red solution	N2889; Sigma-Aldrich	**store at 4 °C**
10 x Dulbeccos PBS	with Ca^{2+} and Mg^{2+}; 14080-089, Invitrogen	**store at room temperature**
Dimethyl sulfoxide (DMSO)	≥99.9%, for molecular biology D8418-250ML; Sigma-Aldrich	**store at room temperature**
Ethanol (absolute)	pro analysis, 1.00983.1000; MERCK	**store at room temperature**
Calcium Chloride ($CaCl_2$)	≥96%, anhydrous, cell culture tested C5670-100G; Sigma-Aldrich	**store at room temperature**
Sodium Chloride (NaCl)	≥99%, cell culture tested S5886-1KG; Sigma-Aldrich	**store at room temperature**

Potassium Chloride (KCl)	≥99%, cell culture tested P5405-250G; Sigma-Aldrich	**store at room temperature**
Magnesium Sulfate ($MgSO_4$)	≥98%, cell culture tested M2643-500G; Sigma-Aldrich	**store at room temperature**
Magnesium Chloride ($MgCl_2$)	≥97%, cell culture tested M4880-100G; Sigma-Aldrich	**store at room temperature**
Galactose	≥96%, anhydrous, cell culture tested G5388-100G; Sigma-Aldrich	**store at room temperature**
Sodium pyruvate	≥99%, cell culture tested P5280-100G; Sigma-Aldrich	**store at 4 °C**
Sodium Phosphate Dibasic (Na_2HPO_4)	≥99%, cell culture tested S5136-100G; Sigma-Aldrich	**store at room temperature**
Potassium Phosphate Monobasic (KH_2PO_4)	≥99%, cell culture tested P5655-100G; Sigma-Aldrich	**store at room temperature**
Deionized water	resistivity: ≤ 18.2 MΩ*cm	
3,4-Dichloroaniline	35827, PESTANAL®, analytical standard; Sigma-Aldrich	**store at room temperature**

Freshly prepared solutions:

L-15 complete culture medium	add to 500 mL L-15: 25 mL FBS 2.5 mL gentamicin (**Note:** experienced labs may work without addition of antibiotics)	**store at 4 °C** (durability 3 mos)

Salt Solution A (10x concentrated for L-15/ex)	80 g NaCl 4.0 g KCl 0.98 g $MgSO_4$ 0.94 g $MgCl_2$ fill up with deionized water to 600 mL	autoclave **store at room temperature** (durability 6 mos)
Salt Solution B (10x concentrated for L-15/ex)	1.4 g $CaCl_2$ fill up with deionized water to 100 mL	autoclave **store at room temperature** (durability 6 mos)
Salt Solution C (10x concentrated for L-15/ex)	1.9 g Na_2HPO_4 0.6 g KH_2PO_4 fill up with deionized water to 300 mL	autoclave **store at room temperature** (durability 6 mos)

Galactose solution (10x concentrated for L-15/ex)	9.0 g galactose fill up with deionized water to 100 mL	filter-sterilize (0.2 µm) dispense in 12 mL aliquots **store at -20 °C** (durability 6 mos)
Sodium pyruvate solution (10x concentrated for L-15/ex)	5.5 g sodium pyruvate fill up with deionized water to 100 mL	filter-sterilize (0.2 µm) dispense in 12 mL aliquots **store at -20 °C** (durability 6 mos)

L-15/ex (prepare aseptically)	60 mL Salt solution A 10 mL Salt solution B 30 mL Salt solution C 10 mL galactose solution 10 mL sodium pyruvate solution fill up with sterilized deionized water to 1000 mL	**store at room temperature** (durability 3 mos)

CFDA-AM 4 mM stock solution	5 mg CFDA-AM 2.32 mL DMSO	prepare 200 µL aliquots **store at -20 °C**

PBS	900 mL deionized water 100 mL 10x Dulbeccos PBS	stirr and adjust the pH to 7.1 (with 10 N NaOH) **store at room temperature**

Resazurin and CFDA-AM working solution [e.g., 5% (v/v) alamarBlue™ and 4 µM CFDA-AM in PBS]	11.4 mL PBS 600 µL alamarBlue™ 12 µL CFDA-AM stock solution	prepare freshly for testing of cell viability

Neutral Red working solution	11.82 mL PBS 180 µL Neutral Red solution	prepare freshly for testing of cell viability
Fixative	5 g $CaCl_2$ 6.75mL 37% (w/v) Formaldehyde fill up with deionized water to 1000 mL	**store at room temperature** (durability 6 mos)
Extraction Solution	500 mL ethanol (abs.) 10 mL acetic acid (100%) fill up with deionized water to 1000 mL	**store at room temperature** (durability 6 mos)

ANNEX 4 – Exemplary SOP for the test performance using DMSO as organic solvent for test chemical delivery

A4.1. Recurrent quality assurance measures

The rainbow trout gill cell line, RTgill-W1, used for this assay, is cultivated according to TG Annex 6 and should be free of microbial or chemical contamination. The different steps of test performance are to be recorded in the lab book with the help of the annexed templates (see TG Annex 5).

A4.2. Basics of the cytotoxicity assay

A4.2.1. Test controls

cell-free control: wells without cells to determine background fluorescence (see plate set up Figure Annex 4-5)

Inasmuch as the three cell viability indicators applied rely on fluorescent measurements, one needs to make sure that there is no interference from the test chemical due to autofluorescence. Thus, in order to detect potential autofluorescence, two cell-free wells are used, thereby one well receives medium only and the second receives medium containing the highest test chemical concentration in the respective organic solvent (i.e. to detect the maximum autofluorescence that can theoretically be observed). A threshold of > 20% fluorescence values compared to the test chemical-free control was arbitrarily set to signal potential interference by autofluorescence. By the nature of this quality criterion, it should consistently stand out in each biological replicate per dye. If autofluorescence is consistently indicated in this way, a separate cell-free control plate should be prepared with the full test chemical concentration range to obtain concentration-specific background values. If no interference is detected, both cell-free control wells are treated as cell-free control values. (see paragraph A4.8.1.)

negative control: exposure medium (L-15/ex) added to one cell-containing well

solvent control: exposure medium (L-15/ex) + the respective concentration of the used organic solvent (e.g., dimethyl sulfoxide (DMSO)) added to cell-containing wells

The solvent control serves to detect potential chemical cross-contamination of the organic solvent from procedural problems leading to a reduction in cell viability. Troubleshooting (i.e., identification of the source of cross-contamination) shall be done if the solvent control yields fluorescence values that are > 10% lower than the negative control. (see paragraph A4.8.2)

positive control: 3,4-Dichloroaniline (3,4-DCA) is used to check assay performance *(see paragraph A4.8.3)*

A4.2.2. Chemical Analytics

Quantification of test chemical exposure concentrations serves to allow the expression of test results based on actual exposure concentrations. Therefore, at the start of cell exposure (0 h) and the end (24 h), i.e. immediately before initiating the cell viability assays, samples for chemical analyses are taken out of the exposure wells. The total handling time for sampling should be as short as possible to avoid test chemical evaporation from the wells.

A4.3. Apparatus and materials

For apparatus and materials see TG Annex 2.

A4.4. Reagents, media and solutions

For reagents, media and solutions see TG Annex 3.

A4.5. Test performance

A general overview about the entire test procedure is given in TG Figure 2.

A4.5.1. Seeding cells into 24-well plates

Per biological replicate, one 24-well plate of cells is seeded. One confluent 75 cm^2 flask of RTgill-W1 contains around 10 million cells. Thus, one plate can be generated out of one confluent flask of RTgill-W1.

A4.5.1.1. Preparation of working materials and solutions for cell seeding

Thaw the trypsin. Adjust trypsin, Versene and the L-15 complete culture medium to 19 ± 1 °C in the incubator for at least one hour before performing the cell seeding. Turn the biosafety cabinet on, wipe the cabinet with disinfectant according to the sterility procedures in your facility and let the air flow for approximately 10 -15 min. Clean the pipette boxes (Pasteur and glass), trypsin, Versene, L-15 complete culture medium bottles and the cell culture flasks according to the sterile procedures in your facility and put them under the biosafety cabinet.

Depending on the cell counting method you use, prepare a cell counting chamber for manual cell counting or prepare the electric cell counter for automatic counting. In this SOP examples for the *Neubauer chamber* and the *Casy1 cell counter* are given.

A4.5.1.2. Seeding cells: step-by-step performance

Description for seeding of one plate (cells taken from one confluent cell culture flask):

a) check cells in the cell culture flask under the microscope
b) if confluency is reached (see TG Figure 4), prepare cells for seeding as follows:
c) draw up the medium using sterile Pasteur pipette and vacuum pump
d) gently add 1 mL Versene (**note**: do not pipette Versene directly onto cells, detachment of cells may occur)
e) wash cells by gentle agitation of the cell culture flask
f) draw off the Versene and repeat washing step (d, e and f)
g) after drawing off Versene for the second time, add 0.7 mL trypsin

h) cell detachment occurs within 0.5 to 5 min and can be promoted by gently tapping and agitating the cell culture flask (check detachment visually, don't let the trypsin incubate longer than 5 min)

i) add 3-5 mL L-15 complete culture medium to stop the action of trypsin

j) suspend cell clumps and remove remaining attached cells by gently pipetting the solution several times up and down and also over the surface of the cells (strictly avoid the formation of foam – shear forces can disrupt the cells)

k) transfer the cell solution into a plastic tube (15 mL centrifuge tube)

l) centrifuge for 3 min with 875 g at 18 – 21 °C

m) draw off the supernatant as low as possible without removing the cell pellet

n) dissolve the cell pellet in 5 mL L-15 complete culture medium by gently pipetting the solution several (3 to 5) times up and down or against the tube wall for better cell suspension without foam formation

o) determine the cell number of the cell suspension in two independent runs (repeat resuspension step n before collection of the second sample) with the counting method of choice:
   - Neubauer chamber: add 10 µL of the cell suspension and count the cells of at least two quadrates (marked in grey, Figure Annex 4-1)
   - Casy1 cell counter: add 10 µL of the cell suspension to 10 mL CasyTon and count the cells ranging between 10 – 20 µm in size according to the manufactures instructions

Example Neubauer chamber:

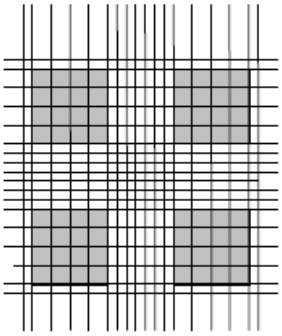


Figure Annex 4-1: Simplified picture of the counting areas of a Neubauer chamber. Areas for counting are marked grey. The number of cells in each grey quadrate is noted.

evaluate the number of cells per 1 mL:

<u>example</u>:

- counted number of cells in two quadrates: 198 and 192 ⇨ average: 195

- 100 counted cells correspond to 1 000 000 cells/ mL

- $\frac{195 \cdot 1000000}{100} = 195 \cdot 10^4 \, cells / mL$

Cell counter example for Casy1:

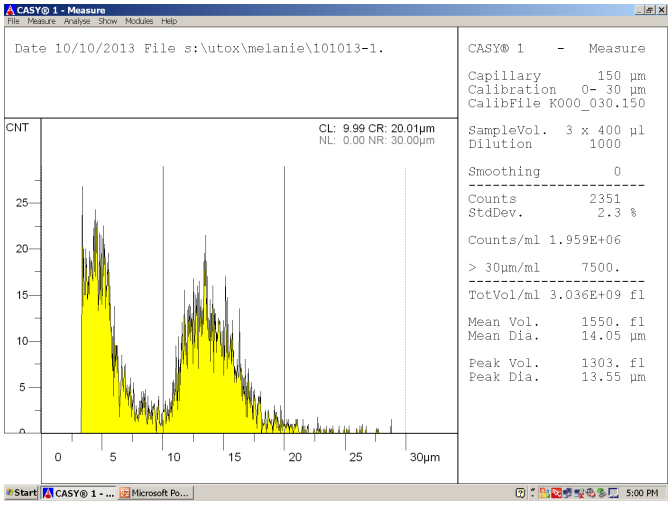


Figure Annex 4-2: Distribution of a 10 µL cell suspension in 10 mL CasyTon. RTgill-W1 cells range in size from 10 to 20 µm.

example:

In a range of 10 to 20 µm 1.959 * 10^6 cells per mL were counted.

a) if the difference between the two independent measurements of the cell number differ by more than 10%, take another count and use the average of the two closest values

b) calculate the volume of cell suspension prepared under n) needed to prepare 25 mL of a seeding solution with a cell density of 350 000 cells/mL for seeding into one 24-well plate:

example:

In one well (of a 24-well plate) 350 000 cells should be seeded (1 mL seeding volume).

$$\frac{cellNo_{needed} \times volume_{needed}}{cellNo_{counted}} = required \cdot Volume \cdot for \cdot seeding \cdot solution$$

$$\Rightarrow \frac{350000 cells \times 25 mL}{1.959 \cdot 10^6 / mL} = \frac{35 \cdot 10^4 / mL \times 25 mL}{195.9 \cdot 10^4 / mL} = 4.47 mL$$

a) transfer the respective volume of cell suspension into a new 50 mL plastic tube and fill up with L-15 complete culture medium to 25 mL (seeding solution). Mix by gently pipetting the solution up and down at least 3-4 times

b) transfer 1 mL of the seeding solution into each well of a 24-well plate (individually or by using a multi-channel pipette), except for the “cell-free control” wells (A1, A2, see pipetting scheme in Figure Annex 4-3). Take care to gently sway the cells in the seeding solution throughout the pipetting procedure to avoid settling of cells. Close the well plate with the lid.

c) incubate the plate at 19 ± 1 °C for 24 h

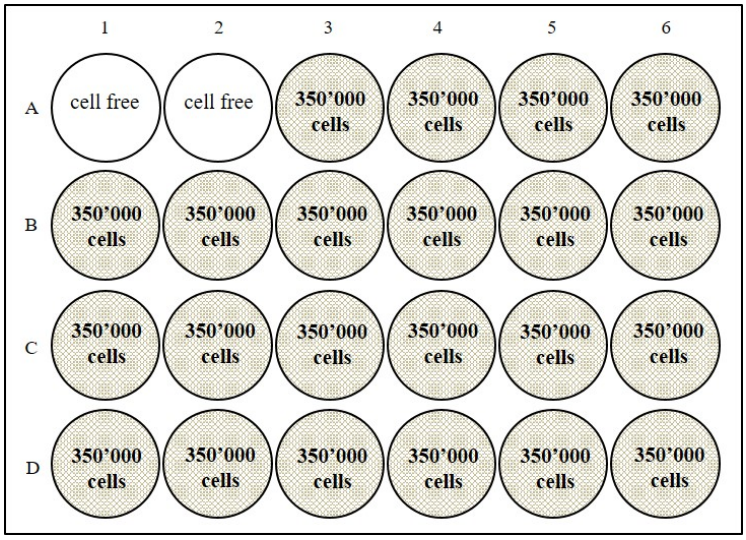


Figure Annex 4-3: Pipetting scheme for cell seeding into 24-well plates (1 mL seeding volume).

A4.5.1.3. Preparation of sampling for chemical analytics

Before stock solutions and dosing mixtures are set-up, prepare the sampling vials to take samples for chemical analyses. It is crucial to label the sample vials in a readable and waterproof manner. Therefore, water- and lightproof marker or resistant adhesive labels, which can be imprinted with the computer using a laser printer, are to be used. However, it should be noted that an adhesive label should not cover the entire vial as it is important to be able to see inside the vial.

A4.5.2. Preparation of test chemical stock solutions in DMSO:

Using DMSO as an organic solvent, the test chemical stock solutions need to be 200-times higher concentrated than the final exposure concentrations of the test chemical to gain a final DMSO concentration in the exposure media of 0.5% (v/v), i.e. a clearly non-cytotoxic concentration. After weighing the appropriate amount of test chemical in a 4 mL amber glass vial and addition of the corresponding DMSO volume, the first stock solution should be extensively mixed by using a shaker for at least 15 min. This stock solution is now the starting point for a serial dilution in DMSO, with a constant dilution factor not exceeding 2.5 (see TG Figure 2 day 2). The test chemical dilution series should be prepared freshly for each experimental day. Special attention should be drawn to keep the DMSO free of contaminations. It is recommended to use a new bottle and prepare smaller aliquots, which are then used to prepare the test chemical dilution series. Doing so avoids frequent re-use of the original DMSO bottle and thereby decreases the risk of DMSO contamination.

Test chemicals that are in solid form at room temperature can simply be weighed in a 4 mL amber glass vial.

For test chemicals that are liquid at room temperature, take a defined volume of the test chemical, transfer it by pipette to the 4 mL amber glass vial and weigh it. For calculation of the corresponding amount of DMSO to add, subtract the volume of pipetted chemical.

<u>example:</u> - 80 µL chemical weigh 103.6 mg

- To obtain a nominal concentration of 40 g/L, 2.51 mL of DMSO need

to be added (103.6 mg : 40 mg/mL = 2.59 mL DMSO – 0.08 mL

Volume of the chemical)

A4.5.3. Preparation of dosing mixtures in L-15/ex

Steps A4.5.3. and A4.5.4. are performed under sterile conditions. Thus, turn the biosafety cabinet on and let the air flow for approximately 10 -15 min. Clean the cabinet and the material needed for preparation of dosing mixtures and exposure according to the sterile procedures of your facility and put them under the biosafety cabinet.

Dosing mixtures are prepared by diluting the stock solutions in L-15/ex exposure medium under sterile conditions and the use of sterile 20 mL amber glass vials. The dosing mixtures should be prepared freshly immediately before dosing onto the cells.

For the preparation of the test chemical dosing mixtures from the DMSO stock solutions, place seven sterilized 20 mL glass vials under the biosafety cabinet. Add to each glass vial 12 mL L-15/ex and 60 µL of the respective test chemical-DMSO stock solution or DMSO alone (TG Figure 2 day 2). Shake the dosing mixture vigorously for 10 min using an orbital shaker.

A4.5.4. Test chemical exposure and sampling for chemical analyses at start of exposure (c0h): step-by-step performance

After 24 h incubation, cells should have formed a confluent layer within each well (Figure Annex 4-4). This is observed by microscopy. If confluence is not yet reached after 24 h of incubation, the plate can be incubated for another 24 - 48 h before test chemical exposure.

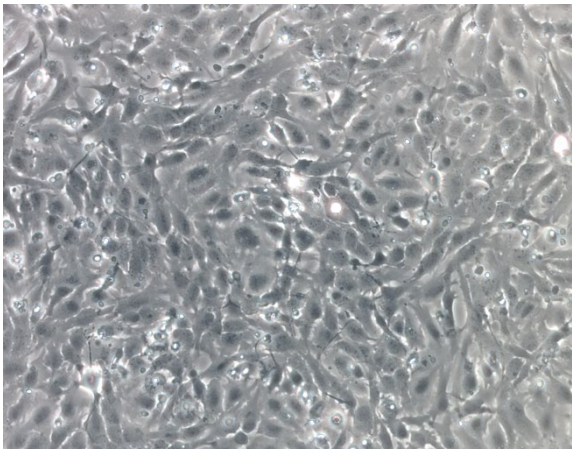


Figure Annex 4-4 — Images of RTgill-W1 cells recorded 24 h after seeding (seeding density: 350 000 cells per mL). Even distribution of the cells across the whole well can be observed.

If required by the analytical method of choice, preload the 1.5 mL sampling vials with solvent and cap the vials such that they can be easily opened to add the medium samples. It is recommended to arrange the sampling vials in the rack according to the plate set-up to avoid confusion.

a) Draw of the L-15 complete culture medium from each well. Tilt the plate to allow for easier removal and take care not to touch the cells with the Pasteur pipette. Alternatively, the plate can be emptied by pouring the content into a collection tray and dabbing the plate dry onto kitchen paper. Immediately proceed to the next step.

b) Gently add 1 mL L-15/ex to each well using the multi-channel pipette (**note**: add L-15/ex along the wall of the wells and do not pipette directly onto the cells, otherwise cells will detach from the bottom) and wash wells by manual, gentle swaying of the plate.

c) Draw of L-15/ex or pour the plate and dab dry onto kitchen paper.

d) Add 2.5 mL of the respective dosing mixture into each well except for the negative control with L-15/ex and the cell-free control wells where 2 mL of medium are added (see Figure Annex 4-5 and TG Figure 2 day 2; **note**: add exposure medium along the wall of the wells and do not pipette directly onto the cells, otherwise cells will detach from the bottom). During dosing procedure, put the lid on the plate and open only the wells to pipette in, to protect the cell layers in not yet dosed wells from drying out.

e) *Immediately* remove a 500 µL sample from each of the three wells per concentration and solvent control and transfer it into the respective sampling vial, preloaded or not, with a solvent according to the analytical protocol. Immediately close the vial tightly.

f) Cover the plate with adhesive foil.

g) Incubate the plate for 24 h at 19 ± 1 °C in the dark.

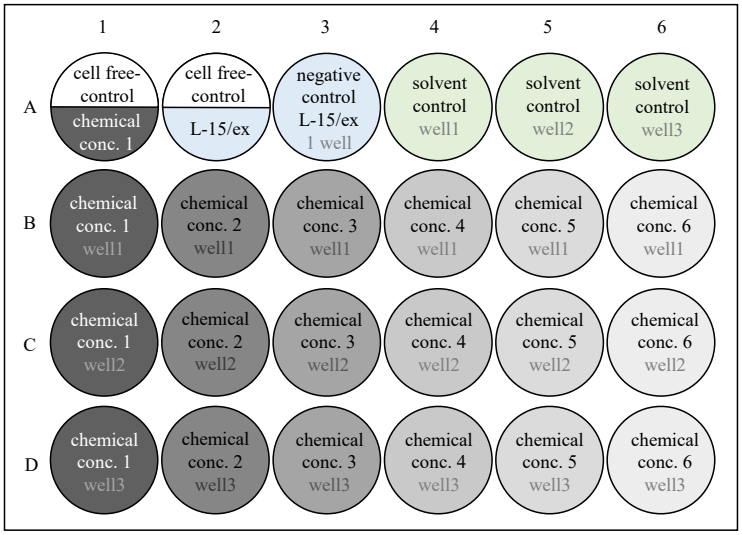


Figure Annex 4-5: Pipetting scheme for a test chemical (one chemical, 6 test concentrations and 3 replicates per plate)

"cell-free control" wells:

These cell-free wells are treated in the same way as the wells containing cells. To one of the two cell-free control wells, 2 mL of L-15/ex are added. This well is needed to account for the background fluorescence of the dyes. The second cell-free control is used for detecting possible interferences between the test chemical and fluorescent dyes. To this well, 2 mL of the dosing mixture containing the highest test chemical concentration is added. If an interference is detected (e.g. a higher/lower fluorescence by > 20% as compared to the other cell-free control well), a cell-free control reference plate is required for the next tests with this respective test chemical (cell-free plate treated in the same way as the exposure plate). If no interference is detected, both cell-free control wells are treated as cell-free control values.

A4.5.5. Determination of cytotoxicity

A4.5.5.1. Preparation of working materials and solutions for cytotoxicity measurement

The dye Resazurin is available in ready-to-use preparations (O'Brien et al., 2000); all procedures in this document are based on one of them: alamarBlue™. Other preparations, such as PrestoBlue®, can be used interchangeably without any adaptations.

Prepare the alamarBlue™ (= example of ready-to-use resazurin dye)/CFDA-AM and the Neutral Red working solutions (see TG Annex 3). For measuring several plates at the same time, prepare respective multiple volumes of the dye working solutions.

A4.5.5.2. Visual control of cell damage

The cells in the culture plate should be visually observed, paying particular attention to the degree of damage as a first control of the test performance. It is recommended to perform the visual observation at step d) of the procedure for cytotoxicity measurement (see paragraph A4.5.5.3), i.e., when the adhesive foil and test chemical is already removed from the plate. This allows for better observation result.

In TG Figure 6, cells are shown with a different grade of damage after exposure to the positive control, 3,4-DCA. These pictures should serve as a guide to ensure optimal test performance.

A4.5.5.3. Measuring cytotoxicity and sampling for chemical analyses at end of exposure (c_{24h}): step-by-step performance

If required by the analytical method of choice, preload the 1.5 mL sampling vials with solvent and cap the vials such that they can be easily opened to add the medium samples. It is recommended to arrange the sampling vials in the rack according to the plate set-up (Figure Annex 4-5) to avoid confusion.

a) Remove the adhesive foil from the test plate and take 500 µL of the exposure medium from each well and transfer it to the respective sampling vial for chemical analysis for the 24 h exposure time point. Immediately close the vial tightly.
b) Discard the remaining exposure solution. Tilt the plate to allow for easier removal and take care not to touch the cells with the Pasteur pipette. Alternatively, the plate can be emptied by pouring the content into a collection tray and dabbing the plate dry onto kitchen paper. Immediately proceed to the next step.
c) Wash each well with 1000 µL PBS (see TG Annex 3; **note**: add PBS along the wall of the wells and do not pipette directly onto the cells, otherwise cells will detach from the bottom).
d) Place the plate lid and observe the cells visually (see paragraph A4.5.5.2.).
e) Discard the washing solution.
f) Add to each well 400 µL of the alamarBlue™/CFDA-AM working solution (**note**: add dye working solution along the wall of the wells and do not pipette directly onto the cells, otherwise cells will detach from the bottom).
g) Cover the plate with the plate lid and incubate at 19 ± 1 °C for 30 min in the dark (e.g. wrap the plates in aluminum foil).
h) Measure first the fluorescence of alamarBlue™ (for wavelengths see below) and after that the fluorescence of CFDA-AM (for wavelengths see below) with the fluorescent plate reader.
i) Discard the alamarBlue™, CFDA-AM working solution.

j) Add to each well 400 µL Neutral Red working solution (**note**: add dye working solution along the wall of the wells and do not pipette directly onto the cells, otherwise cells will detach from the bottom).

k) Cover the plate with the plate lid and incubate at 19 ± 1 °C for 60 min in the dark (e.g. wrap the plates in aluminum foil).

l) Discard the Neutral Red working solution.

m) Wash each well carefully with 400 µL fixative (see TG Annex 3) and discard the fixative (**note**: add fixative solution along the wall of the wells and do not pipette directly onto the cells, otherwise cells will detach from the bottom).

n) Add to each well 400 µL extraction solution (see TG Annex 3), place the plate lid and incubate for 10 min at room temperature in the dark (e.g. wrap the plates in aluminium foil) and gently shake on a plate shaker.

o) Measure the fluorescence of Neutral Red (for wavelengths see below) with the fluorescent plate reader.

Parameters for the fluorescence measurement:

Endpoint measurement will be performed. It is assumed that the formed fluorescent dyes are dissolved and evenly distributed in the media.

alamarBlue™:	exc: 530 nm	em: 595 nm
CFDA-AM:	exc: 493 nm	em: 541 nm
Neutral Red:	exc: 530 nm	em: 645 nm

A4.6. Waste disposal

Dispose the exposure solutions, remaining dye working solutions/fixative/extraction solution, well plates and remaining stock solutions and dosing mixtures according to your facility standards.

A4.7. Calculation of results

The raw fluorescence data gained is used to calculate the toxicity as a percentage of cell viability compared to the solvent control.

a) Subtract the corresponding average “cell-free control” fluorescence units from the fluorescence units of each well of the corresponding test plate.

b) Calculate for each replicate well of each chemical test concentration the percentage of cell viability with regard to the respective control.

c) Therefore, the average of the fluorescence units of the control is set to 100% (indicating that 100% of the cells are viable) and corresponding cell viability is calculated.

$$\%\ cell\ viability = \frac{fluo.units\ chemical * 100\%}{fluo.units\ solvent\ control}$$

d) Calculate the average and standard deviation of the %cell viability per chemical test concentration and use this for concentration response curve analysis.

e) Use the geometric mean based measured concentrations of each test concentration at the start (c_{0h}) and the end (c_{24h}) of the test for the x-axis. Plot the percentage of cell viability values on the y-axis. EC50 values are calculated based on non-linear regression sigmoidal concentration−response curve fitting with constrains set for Bottom (0.0) and Top (100.0) (e.g. using a four parameter logistic equation with variable slope or the profile likelihood method according to Raue et al. (2009)).

A4.8. Validity criteria of the test

A4.8.1. Excluding autofluorescence

The variation of fluorescence between the cell-free control wells should not exceed 20%. If an interference is detected (e.g. a fluorescence higher/lower than 20% as compared to the other cell-free control well, see paragraph A4.2.1. and A4.5.4), a cell-free control reference plate is required in an additional test (cell free plate treated in the same way as the exposure plate) for concentration-dependent fluorescence background subtraction of the dyes.

A4.8.2. Excluding contamination of the organic solvent

To exclude contaminations of the used organic solvent, cell viability in the solvent-free negative vs. solvent containing control is checked on each test plate. The criteria is set to a no more than 10% reduction in raw fluorescent values between the negative and solvent control (see paragraph A4.2.1).

A4.8.3. Assay performance (positive control)

In each round of testing, a separate plate carrying a concentration range of the positive control, 3,4-Dichloroaniline, should be tested. The mean EC50 value for each dye based on nominal concentrations should lie between 2½ standard deviations (SD) from the EC50 determined during the testing of 3,4-DCA within the round robin study (n=27; Fischer et al., 2019).

alamarBlue™:	43.6 mg/L ± 6.1 mg/L (2½ SD range: 28.4 - 58.9 mg/L)
CFDA-AM:	62.5 mg/L ± 18.9 mg/L (2½ SD range: 15.2 - 109.8 mg/L)
Neutral Red:	58.6 mg/L ± 18.6 mg/L (2½ SD range: 12.1 - 105.0 mg/L)

A4.9. References to ANNEX 4

Fischer M, Belanger SE, Berckmans P, Bernhard MJ, Bláha L, Coman Schmid DE, Dyer SD, Haupt T, Hermens JLM, Hultman MT, Laue H, Lillicrap A, Mlnaříková M, Natsch A, Novák J, Sinnige TL, Tollefsen KE, von Niederhäusern V, Witters H, Županič A, Schirmer K. (2019). Repeatability and reproducibility of the RTgill-W1 cell line assay for predicting fish acute toxicity. Toxicological Sciences, 169(2): 353-364. doi: 10.1093/toxsci/kfz057

O'Brien J, Wilson I, Orton T, Pognan F. (2000). Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. European Journal of Biochemistry, 267(17): 5421–5426. doi: 10.1046/j.1432-1327.2000.01606.x

Raue A, Kreutz C, Maiwald T, Bachmann J, Schilling M, Klingmüller U, Timmer J. (2009). Structural and practical identifiability analysis of partially observed dynamical models by exploiting the profile likelihood. Bioinformatics, 25: 1923–1929. doi.org/10.1093/bioinformatics/btp358

ANNEX 5 - Test documentation templates

PREPARATION OF 10 x concentrated L-15/ex STOCK SOLUTIONS

Stock solution*	Substance	Supplier** Lot-No.	Final conc. [g/L]	Amount to add	Actual weight	Sterilized ?
Salt Solution A (600 mL)	NaCl		133.3	**80.0 g**		☐ autoclaved
	KCl		6.66	**4.0 g**		
	$MgSO_4$		1.63	**0.98 g**		
	$MgCl_2$		1.56	**0.94 g**		
	Deionized water		**Fill up to 600 mL**		☐	
Salt Solution B (100 mL)	$CaCl_2$		14.0	**1.4 g**		☐ autoclaved
	Deionized water		**Fill up to 100 mL**		☐	
Salt Solution C (300 mL)	Na_2HPO_4		6.33	**1.9 g**		☐ autoclaved
	KH_2PO_4		2.0	**0.6 g**		
	Deionized water		**Fill up to 300 mL**		☐	
Galactose Solution (100 mL)	Galactose solution		90.0	**9.0 g**		☐ sterile filtered (0.2 µm)
	Deionized water		**Fill up to 100 mL**		☐	
Pyruvate Solution (100 mL)	Sodium pyruvate		55.0	**5.5 g**		☐ sterile filtered (0.2 µm)
	Deionized water		**Fill up to 100 mL**		☐	

* Salt stock solutions should be kept at room temperature and Galactose and Pyruvate solutions in the freezer, max. for 6 mos.
**Indicate Supplier and Cat# if different from guidance document.

Date of preparation:	
Date of expiry:	
Prepared by:	

signature of operator:

The preparation of L-15/ex stock solutions should be mentioned in the lab book and recorded in an extra file folder. Salt stock solutions should be kept at room temperature and Galactose and Pyruvate solutions in the freezer, max. for 6 mos.

PREPARATION OF L-15/ex* from 10 x concentrated stock solutions

For a final Volume of 1000 mL in sterile container:

Substance	Date of preparation	Volume to add	Added?
Salt solution A		60 mL	☐
Salt solution B		10 mL	☐
Salt solution C		30 mL	☐
Galactose solution		10 mL	☐
Pyruvate solution		10 mL	☐
Sterilized water		880 mL	☐

* L-15/ex should be kept at 19 ± 1 °C, max. for 3 mos
* pH and osmolality of the L-15/ex should range between 7 – 7.5 and 290 – 320 mmol/kg, respectively

Date of preparation:	
Date of expiry:	
Prepared by:	

Signature of operator:

The preparation of L-15/ex should be mentioned in the lab book and recorded in an extra file folder. L-15/ex should be kept at 19 ± 1 °C, max. for 3 mos.

PREPARATION OF TEST CHEMICAL STOCK SOLUTION SERIES IN DMSO and PREPARATION OF DOSING MIXTURES IN L-15/ex

PREPARATION OF TEST CHEMICAL STOCK SOLUTION SERIES IN DMSO

STOCK SOLUTION

Substance	**Supplier Lot-No.**	**Nominal conc.**	**Initial weight**	**DMSO added? How much?**
				☐ mL

DILUTION SERIES in DMSO

Name	**Concentration [g/L]**	**Volume DMSO [mL]**	**Added vol. from solution [mL]**	**Dilution factor**
Stock 1 (S1)		= stock solution		
Stock 2 (S2)			S1:	
Stock 3 (S3)			S2:	
Stock 4 (S4)			S3:	
Stock 5 (S5)			S4:	
Stock 6 (S6)			S5:	
Stock 7 (S7)	0.0	= DMSO only		

PREPARATION OF DOSING MIXTURES IN L-15/ex

Name	**Volume L-15/ex [mL]**	**Volume of Stock added [µL]**		**Final conc. of Dosing Mixture [mg/L]**
Concentration 1 (C1)	12	S1	60	
Concentration 2 (C2)	12	S2	60	
Concentration 3 (C3)	12	S3	60	
Concentration 4 (C4)	12	S4	60	
Concentration 5 (C5)	12	S5	60	
Concentration 6 (C6)	12	S6	60	
Solvent Control (SC)	12	S7	60	0.0

Test identification code:	
Date of preparation:	

signature of operator:

The preparation of test chemical stock solutions should be mentioned in the lab book and recorded in an extra file folder.

PREPARATION OF TEST CHEMICAL STOCK SOLUTION DOSING MIXTURE DILUTION SERIES IN L-15/ex

PREPARATION OF TEST CHEMICAL STOCK SOLUTION IN L-15/ex

STOCK SOLUTION

Substance	**Supplier Lot-No.**	**Nominal conc.**	**Initial weight**	**L-15/ex added? How much?**
				☐ mL

DILUTION SERIES in L-15/ex

Name	**Concentration [mg/L]**	**Volume L-15/ex [mL]**	**Added vol. from solution [mL]**	**Dilution factor**
Concentration 1 (C1)		= stock solution		
Concentration 2 (C2)			C1:	
Concentration 3 (C3)			C2:	
Concentration 4 (C4)			C3:	
Concentration 5 (C5)			C4:	
Concentration 6 (C6)			C5:	
Negative Control (NC)	0.0	= L-15/ex only		

Test identification code:	
Date of preparation:	

signature of operator:

The preparation of test chemical stock solution and dilution series should be mentioned in the lab book and recorded in an extra file folder.

PREPARATION OF FIXATIVE AND EXTRACTION SOLUTION

Solution*	Chemical	Supplier** Lot-No.	Final conc.	Nominal value	Actual value
Fixative (1 L)	$CaCl_2$		5 g/L	5 g	
	37% (w/v) formaldehyde		0.25%	6.75 mL	
	Water			993 mL	
Extraction (1 L)	Acetic Acid		1.0%	10 mL	
	Ethanol		50%	500 mL	
	Water			490 mL	

* Solutions should be kept at room temperature, max. for 6 mos.
**Indicate Supplier and Cat# if different from guidance document.

Date of preparation:	
Prepared by:	

Signature of operator:

The preparation of Fixative and Extraction solution should be mentioned in the lab book and recorded in an extra file folder. Solutions should be kept at room temperature, max. for 6 mos.

SOLUTIONS USED FOR TEST

Test identification code:	

Preparation date of L-15/ex:	

PBS – Supplier* / Lot-No.	

Preparation of dye working solutions:**			
Working solution:	alamarBlue™ + CFDA-AM		Neutral Red
Supplier*: Lot-No.:			
Final concentration:	- 5% (v/v) alamarBlue™ - 4 µM CFDA-AM		- 1.5% (v/v)
For 12 mL dye solution:	- 600 µL alamarBlue™ - 12 µL 4 mM CFDA-AM in DMSO - 11.4 mL PBS		- 180 µL Neutral Red - 11.82 mL PBS
Required Volume for dye solution:	mL		mL
Required Vol. of the dye:	alamarBlue™: µL	CFDA-AM: µL	Neutral Red: µL
Required Volume of PBS:	mL		mL
Preparation date of dye working solutions:			

* Indicate Supplier and Cat# if different from guidance document.
** durable for one day, when stored in the dark at room temperature

Preparation date of Fixative solution:	

Preparation date of Extraction solution:	

Signature of operator:

Dye solutions should be freshly prepared each day. They are durable for one day, when stored in the dark at room temperature.

ANNEX 6 - Maintenance of the RTgill-W1 cells

This annex describes the routine cell culture procedures (sub-culturing, freezing and thawing of cells) for the RTgill-W1 cell line.

A6.1 Recurrent quality assurance measures

Reporting: Thawing of cells, propagation, freezing and the mycoplasma testing will be recorded in a lab book.

Mycoplasma Testing: Permanent cell lines are a common target of mycoplasma contamination. Mycoplasma interfere with the cell cycle and metabolism of the cells and subsequently may have an influence on the sensitivity of the cell viability test. Therefore, every culture must be checked regularly (every third month) for mycoplasma. In suspicious cases (e.g. unusual microscopic appearance or altered sensitivity of the test system) cell cultures should be tested immediately. Contaminated cultures should be discarded and replaced by a newly thawed batch of cells.

A6.2 Basics of RTgill-W1 cell culturing

RTgill-W1 cells grow adherent and are cultivated as monolayer cultures. The cell line is propagated in cell culture flasks at 19 ± 1 °C, without any additional CO_2 supply or steam saturation.

Cells are able to grow to a defined degree of confluence. Therefore, it is necessary to propagate the cells on a regularly base. After reaching 150 passages, the cells are discarded and new cells of a lower passage number are thawed.

Cells should be split once they have reached 90 - 100% confluency, i.e. when the cell culture surface is completely covered. They are routinely split 1:2 (generate 2 flasks out of 1). Under these conditions, cells can be passaged every 10 to 12 days.

The presence of bacteria, fungi and yeast is mostly indicated by an opaque and muddy culture media and dying/detaching cells. In that case all contaminated culture flasks and associated media and solutions should be eliminated immediately and the working place cleaned thoroughly with disinfectant according to your facility standards (e.g. 70% (v/v) ethanol or isopropyl alcohol).

A6.3 Morphology of RTgill-W1

At low density the cells have an irregular spindly shape, but with reaching confluence, the cells exhibit a polygonal shape and are tightly packed (Figure Annex 6-1; see also Bols et al., 1994).

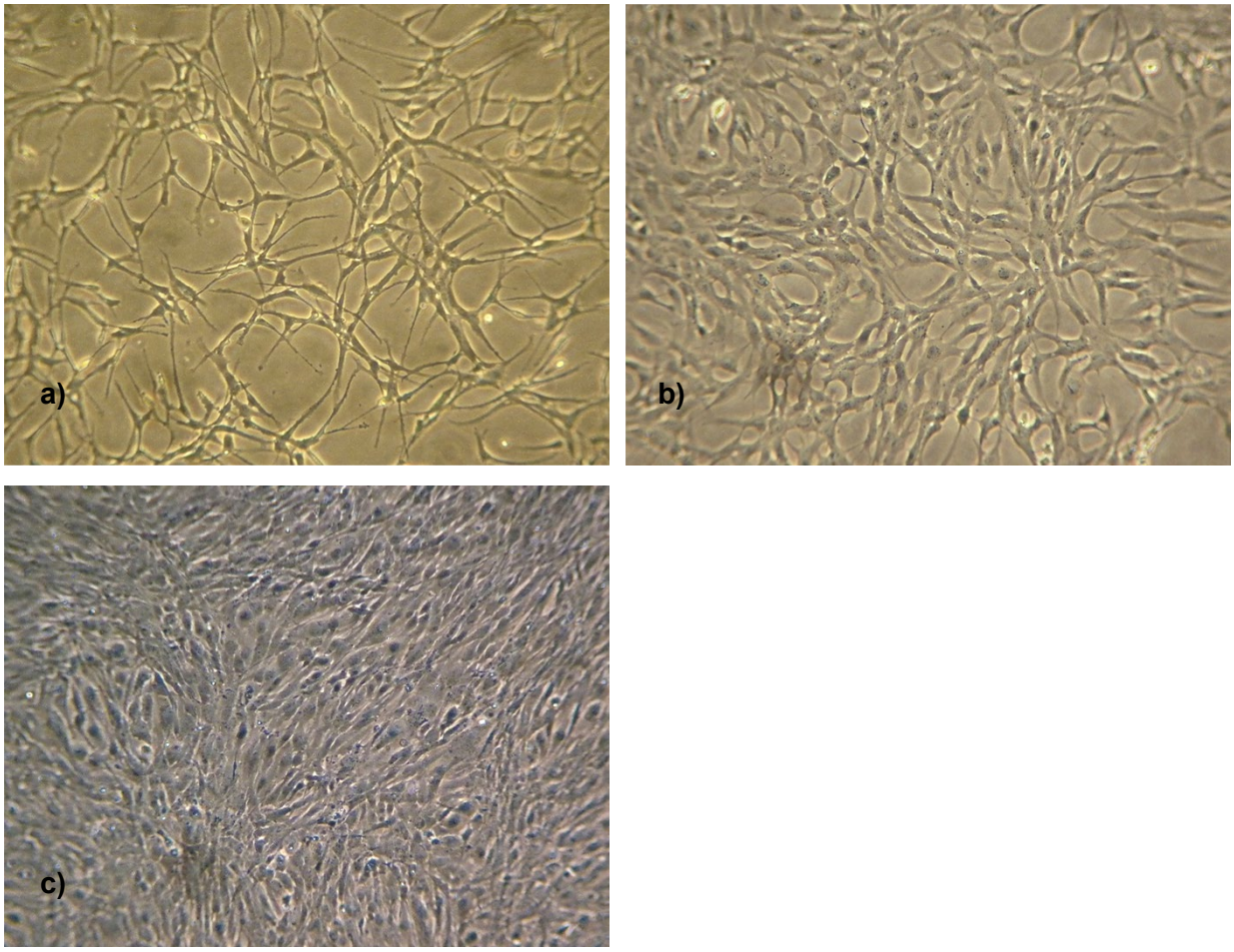


Figure Annex 6-1 — non-confluent (a, b) and confluent (c) monolayers of RTgill-W1 cells; a) one day after passaging, b) five days after passaging, c) 12 days after passaging

A6.4 Apparatus and materials for cell culturing

Note that the given providers are exemplarily. These apparatus and materials that are listed have been proven to be reliable. Deviations for the material are possible.

	Apparatus and Material	Example Supplier
1	Bio-working bench	HeraSafe; Kendro
2	Incubator	BK-700, (3 – 40 °C); Heraeus Instruments
3	Cell culture flasks	75 cm^2 with vent screw cap, 90075; TPP. Do not use filter caps as difficulties were observed during culturing of RTgill-W1 cells with filter caps capped cell culture flasks.

4	Glass pipettes (sterile)	5 mL, 10 mL, 20 mL; VWR
5	Pasteur pipettes (sterile)	230 mm; VWR
6	Pipetting help	Pipetus-akku; Hirschmann Laborgeräte
7	Pipette box to sterilize pipettes	612-2089; VWR
8	Microscope	Axiovert 40C; ZEISS
9	Vacuum pump	Mini-Vac eco; Axonlab
10	Pipette tips (sterile)	10 µl ,1000 µl; Socorex
11	Pipettes	10 µl ,1000 µl; Socorex
12	Centrifuge	Heraeus Labofuge 400R (0 – 40 °C; 17 -3900 g); Thermo Science
13	Plastic centrifuge tubes (15 mL, sterile)	l91015; TPP
14	Plastic centrifuge tubes (50 mL, sterile)	91050; TPP
15	Cryo vials	1.2 mL, 479-0802; VWR

A6.5 Reagents, media and solutions for cell culturing

Note that the given providers are exemplarily. The solutions from the given suppliers have provided long-standing positive results but it is possible to use cell culture grade solutions from other providers as long as the final concentrations/compositions of the solutions used for sub-cultivation are similar to the given examples.

FBS from other providers need to be tested for compatibility. This can be done by culturing the cells for three passages in culture medium with this FBS and observing cell viability and growth by microscopy. If the FBS does not work, the cells grow more slowly or even start detaching. If a suitable FBS is found, several bottles of this respective batch should be ordered to ensure the use of the same FBS throughout the testing period.

A6.5.1 Ready-for-use purchased solutions

	Example Supplier	**Storage**
Bovine serum (FBS)	CVFSVF00-01 (Eurobio) **Note:** do not heat inactivate the FBS [2]	thaw before use and prepare 25 mL aliquots in adequate sterile tubes **store at -20 °C**
Gentamicin	10 mg/mL e.g. LONZ02-012E (Lonza) or BCHRA2712 (Biochrom)	**store according to manufacturer's instructions**
Trypsin	0.25% in PBS w/o Ca^{2+}, Mg^{2+}, L11-002 (PAA)	thaw before use and prepare 10 mL

		aliquots in adequate sterile tubes **store at -20 °C**
Versene (0.2 g/L EDTA in PBS)	1:5000, 15040 (Gibco)	**store at 4 °C**
Leibovitz L-15 medium	with Glutamine and without Phenolred 21083 (Gibco)	**store at 4 °C**
DMSO	Dimethyl sulfoxide, for molecular biology, suitable for plant cell culture, ≥99.9%, D8418; Sigma-Aldrich	**store at room temperature**

A6.5.2 Self-prepared solutions

L-15 complete culture medium: add to 500 mL L-15:

25 mL FBS

2.5 mL Gentamicin solution (**Note:** The use of antibiotics, such as 0.5% Gentamicin, is optional. Experienced labs may work without addition of antibiotics.)

store at 4 °C (durability 3 mos)

cell culture freezing solution: add to L-15 media (cold, 4 °C): *e.g. 10 mL*

10% (v/v) FBS + *1 mL*

10% (v/v) DMSO + *1 mL*

(adjust to 4 °C when freshly prepared)

A6.6 Preparation of working materials and solutions for sub-culturing

Thaw the trypsin. Adjust trypsin, Versene and the L-15 complete culture medium to 19 ± 1 °C in the incubator for at least one hour before performing the sub-culturing. Turn the working bench on, wipe the bench with disinfectant according to the sterility procedures in your facility and let the air flow for approximately 10-15 min. Clean the pipette boxes (Pasteur and glass), trypsin, Versene, L-15 complete culture medium bottles and the cell culture flasks according to the sterile procedures in your facility and put them on the bench.

A6.7 Step by step performance: subculturing

The sub-culturing is performed at room temperature and therefore should be done quickly to not expose the cells to high temperatures for too long. Do not subculture the cells at room temperatures ≥ 25 °C.

Description for one cell culture flask:

a) check cells in the cell culture flask under the microscope
b) if confluency is reached (see Figure Annex 6-1c) prepare them for splitting as follows:
c) draw off (aspirate) the media using sterile Pasteur pipette and vacuum source
d) gently add 1 mL Versene (**Note**: do not pipette Versene directly onto cells, detachment of cells may occur)
e) wash cells by gentle agitation of the cell culture flask
f) draw off the Versene and repeat washing step (d, e and f)
g) after drawing off Versene for the second time, add 0.7 mL trypsin (**Note** that the cell detachment after adding the trypsin occurs quite fast (usually within 1 minute)). Therefore, it is recommended to split not more than eight flasks at once to avoid long handling time during trypsination which can lead to cell digestion by the trypsin.
h) promote the cell detachment by gently tapping and agitating the cell culture flask (check detachment visually)
i) add 5 mL L-15 complete culture medium to stop the action of trypsin
j) dissolve cell clumps and remove remaining attached cells gently by pipetting the solution several times up and down and also over the surface of the cells (strictly avoid the formation of foam – shear forces can disrupt the cells)
k) transfer the cell solution into a plastic tube (15 or 50 mL centrifuge tube)
l) centrifuge for 3 min with ≤ 875 g at 18 - 21 °C
m) draw off the supernatant as much as possible without removing the cell pellet
n) dissolve the cell pellet in 10 mL L-15 complete culture medium by pipetting the solution several times up and down or against the tube wall (strictly avoid the formation of foam – shear forces can disrupt the cells)
o) subculture the cells in two cell culture flasks (cell culture flasks can be used for two passages of cells)

- add 5 mL of the cell solution to each cell culture flask
- add 5 mL complete culture medium to reach a final volume of 10 mL
- close the flasks and incubate the cells at 19 ± 1 °C in the dark.

Cells should be checked regularly (at least every three days) by microscopy for their morphology and for microbial contaminations. The minimum/maximum cultivation temperature should be controlled every working day and deviations beyond ± 1 °C should be documented. Cells should be sub-cultivated approximately every 10 - 12 days. An exchange of culture medium should be performed after 5-7 days.

The sub-culturing procedure is described for using one cell culture flask. If several flasks are sub-cultured at once (use only cells from the same passage), the amount of Versene and trypsin per flask remains the same. The volume of L-15 complete culture medium to stop the trypsination can be varied (e.g. pooling cells of 4 flasks into 15 mL medium) and the cell suspension of several flasks from the same passage can be collected in one tube for centrifugation. The volume of L-15 complete culture medium to re-suspend the cell pellet after centrifugation can also be varied in a suitable way as long as the cells are split 1:2 and the flask contains 10 mL of culture medium at the end of the procedure.

A6.8 Step by step performance: freezing and thawing of RTgill-W1 cultures

Freezing:

a) It is recommended to re-suspend the cell pellet in cold freezing medium and to freeze them quickly for higher success at thawing.
b) prepare cell culture freezing solution freshly (see paragraph A3.5.2 above) and place at 4 ± 1 °C or on ice until use
c) label cryo vials and place them into a pre-cooled rack or a rack that sits on ice
d) use flasks of confluent cells
e) handle cells like described in paragraph A3.7 step a – h above
f) after trypsination add 5 mL L-15 complete culture medium per flask and re-suspend cells by gently pipetting the solution several times up and down and also over the surface of the cells
g) centrifuge for 3 min with ≤ 875 g at 18 - 21 °C
h) draw off the supernatant
i) dissolve the pellet in 1 mL cell culture freezing solution per used flask of cells and aliquot into the pre-cooled cryo vials (1 mL per vial)
j) store at –80 °C for at least 24 h (max. 6 mos)
k) finally store in liquid nitrogen (liquid or vapor phase) ideally after a 24 h storage period at -80 °C

Thawing:

a) prepare L-15 complete culture medium and adjust it to 19 ± 1 °C
b) remove cryo vials with frozen cells from liquid nitrogen or -80 °C
c) thaw frozen cells rapidly in a mildly warm water bath (~20 °C)
d) transfer cells gently to a 15 mL centrifuge tube
e) add gently and drop by drop 5 mL L-15 complete culture medium under gentle agitation
f) transfer cell suspension in a cell culture flask (using a glass pipette) (one 75 cm^2 or 2x 25 cm^2 flasks) and add medium to reach final volume of 10 mL (75 cm^2) or 5 mL (25 cm^2) medium
g) incubate cells in the incubator at 19 ± 1 °C
h) change medium within 24 h

Documentation form:

PREPARATION OF L-15 COMPLETE CULTURE MEDIUM *

For a final volume of 527.5 mL:

Substance	**Supplier *** Lot-No.**	**Final conc.**	**Volume to add**	**Added?**
L-15 medium			500 mL	
FBS		4.7%	25 mL	☐
Gentamicin (10 mg/L) **		50 µg/L	2.5 mL	☐

* can be kept for 3 mos at 4 °C

** The use of antibiotics, such as 0.5% Gentamicin, is optional. Experienced labs may work without addition of antibiotics.

*** Indicate Supplier and Cat# if different from guidance document.

Date of preparation:	
Date of expiry:	
Prepared by:	

signature of operator:

A.6.9. References to ANNEX 6

Bols NC, Barlian A, Chirinotrejo M, Caldwell SJ, Goegan P, Lee LEJ. (1994). Development of a Cell Line from Primary Cultures of Rainbow-Trout, *Oncorhynchus mykiss* (Walbaum), Gills. Journal of Fish Disease, 17: 601-611.

Nims R.W. and Harbell J.W. (2017). Best practices for the use and evaluation of animal serum as a component of cell culture medium. In Vitro Cellular and Developmental Biology—Animal, 53: 682–690.

ANNEX 7 – Test report template RTgill-W1 cell line assay (example)

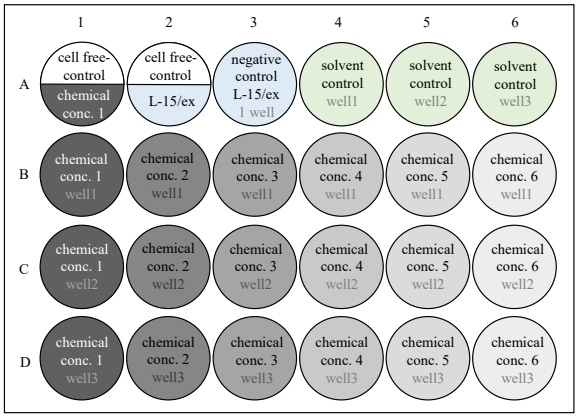
Test date:	
Identification code of the test:	

Test item information:

Tested chemical:	
Chemical supplier/ order No./ Lot-No.:	
Molecular weight:	
Purity:	
K_{OW}:	
Vapour pressure:	
pKa:	
Water solubility:	
HLC:	
Organic solvent used for stock preparation:	
Solvent supplier/ order No./ Lot-No./ purity:	
Test concentration range (in mg/L):	
Preparation date of dilution series:	
3,4-Dichloroaniline supplier/ order No./ Lot-No./ purity:	

Test information:

Used RTgill-W1 cells: - passage nr. - cell suspension (cells/mL) - calculation of required seeding solution (volume cell suspension/L-15 complete culture media)	 - - -
Samples for chemical analysis: - analytical method: - limit of quantification: - solvent pre-load required?: - samples taken at beginning of test (c_{0h}): - samples taken at end of test (c_{24h}):	
Plate dosed according to pipetting scheme (see below):	

Exposure temperature:	
Remarks:	
Chosen pipetting scheme (see Figure 5):	
Program and method used for EC50 value calculation:	
Concentration-response curves:	
EC50 values:	

	Resazurin	CFDA-AM	Neutral Red
Test item EC50 [mg/L]			
Test item 95%-CI [mg/L]			
3,4-DCA EC50 [mg/L]			
3,4-DCA 95%-CI [mg/L]			

		Resazurin-based dye	CFDA-AM	Neutral Red
Test valid according to criteria (tick box if yes):	fluorescence difference cell-free controls < 20% (see paragraph 17 a. of TG)	☐	☐	☐
	% cell viability solvent control not > 10% lower than negative control (see paragraph 17 b. of TG)	☐	☐	☐
	EC50 positive control within acceptance range (see paragraph 17 c. of TG)	☐	☐	☐
Date:	**Signature of operator:**			