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OECD GUIDELINE FOR TESTING OF CHEMICALS

Determination of in vitro intrinsic clearance using cryopreserved rainbow trout hepatocytes (RT-HEP)

INTRODUCTION

1. In order to improve *in silico* predictions of chemical bioaccumulation in fish, methods are needed to estimate hepatic biotransformation and incorporate this information into established computational models. One promising approach involves the measurement of intrinsic clearance using *in vitro* metabolising systems derived from liver tissue (Nichols, Schultz and Fitzsimmons, 2006).

2. This Test Guideline (TG) describes the use of cryopreserved rainbow trout (*Oncorhynchus mykiss*) hepatocytes (RT-HEP) to determine the $CL_{\text{IN VITRO, INT}}$ of a test chemical using a substrate depletion approach.

3. The OECD Guidance Document RT-HEP and RT-S9 (OECD, 2018a) accompanying this Test Guideline and Test Guideline RT-S9 (OECD, 2018b) describes how to best perform these methods and how $CL_{\text{IN VITRO, INT}}$ can be used to inform *in silico* prediction models of bioaccumulation in fish.

4. In brief, liver intrinsic clearance is estimated from the determined $CL_{\text{IN VITRO, INT}}$ and may be used directly as an input to physiologically based toxicokinetic (PBTK) models for fish bioaccumulation assessment (Stadnicka-Michalak et al., 2014; Brinkmann et al., 2016). Alternatively, this value may be extrapolated to a whole-body (*in vivo*) biotransformation rate constant using an appropriate *in vitro* to *in vivo* extrapolation (IVIVE) model. An IVIVE model applicable to rainbow trout was recently described by Nichols et al. (Nichols et al., 2013) and is included as one example in OECD Guidance Document RT-HEP and RT-S9 (OECD, 2018a).

5. The *in vivo* biotransformation rate can be included into *in silico* models for prediction of bioconcentration factors (BCF). Several research groups have shown that incorporating biotransformation data derived with *in vitro* methods into one-compartment models for fish substantially improves model performance; i.e. predicted levels of accumulation are much closer to measured values than predictions obtained assuming no

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In accordance with the Decision of the Council on a Delegation of Authority to amend Annex I of the Decision of the Council on the Mutual Acceptance of Data in the Assessment of Chemicals [C(2018)49], this Guideline was amended by the OECD's Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology by written procedure on 25 June 2018.

metabolism (Cowan-Ellsberry et al., 2008; Dyer et al., 2008, 2009; Han et al., 2007; Laue et al., 2014; Mingoia et al., 2010; Fay et al., 2014a, 2014b). Additional discussion related to BCF prediction is included in OECD Guidance Document RT-HEP and RT-S9 (OECD, 2018a).

6. This guideline is based on the method used in a recent ring trial demonstrating its reproducibility by testing five chemicals and one reference chemical in six laboratories. Details on the test chemicals and the associated inter- and intra-laboratory variability are provided in the ring trial report (OECD, 2018c).

7. It is recognised that for the development of this TG a limited number of chemicals has been tested. However, $CL_{\text{IN VITRO, INT}}$ have been derived with comparable incubations protocols using fish hepatocytes, liver S9 sub-cellular fraction or microsomes, for a range of chemicals and many have been published in peer-reviewed literature (see Table R.7.10-6 of Appendix R.7.10-2 in the REACH guidance for a recent list; (ECHA, 2017)). Moreover, a fish *in vitro* biotransformation database has been developed covering intrinsic clearance data determined with *in vitro* methods (hepatocytes, S9, microsomes). The database is publicly available from the European Commission Joint Research Centre (Halder, Lostia and Kienzler, 2018) such that users can determine if their test chemical(s) fall within the applicability domain of the method.

8. Definitions of terms used in this document are provided in Annex 1.

INITIAL CONSIDERATIONS AND LIMITATIONS

9. A validated analytical method is mandatory to quantify the test chemical (see §22) (OECD, 2014).

10. In case, the $CL_{\text{IN VITRO, INT}}$ derived is used to inform *in silico* bioaccumulation models on biotransformation, the test chemical should be within the applicability domain of the corresponding model.

11. The total incubation time should not exceed 4 h due to loss of viability of the RT-HEP. This limits the use of the test for chemicals metabolised at very low rates. The lowest rate of *in vitro* activity which can be reliably quantified is a first-order elimination rate constant (k_e) of approximately 0.05 h^{-1} to 0.14 h^{-1} (Nichols et al., 2013; Chen et al., 2016). More details are provided in the OECD Guidance Document RT-HEP and RT-S9 (OECD, 2018a).

12. The incubation temperature should be at 11±1°C and since biotransformation rates are temperature sensitive, the test temperature should be strictly controlled at the acclimation temperature using a water bath, incubator, or thermomixer.

13. For volatile or otherwise difficult test chemicals, several alternative approaches are suggested in the OECD Guidance Document RT-HEP and RT-S9 (OECD, 2018a) such as use of tightly closed incubation vials (e.g. GC-vials with septa) for volatile chemicals as well as glass insert test tubes or passive dosing for chemicals with very low solubility. In addition, an alternative test set-up is provided in Annex 7, Test Set-Up 2.

14. For chemicals that are ionisable, the relevant dissociation constants (pK_a values) should be known prior to testing, since small changes in pH can alter the balance between the dissociated and non-dissociated forms of some chemicals. A discussion of recent studies and some considerations are included in the OECD Guidance Document RT-HEP and RT-S9 (OECD, 2018a).

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15. When considering testing of mixtures, difficult-to-test chemicals (e.g. unstable), or test chemicals not clearly within the applicability domain described in this Guideline, upfront consideration should be given to whether the results of such testing will yield results that are meaningful scientifically. The OECD Guidance Document RT-HEP and RT-S9 discusses the use of the method for testing mixtures (OECD, 2018a).

16. The methodology as described here only measures depletion of a parent chemical. The depletion approach could also be used to identify metabolites - if required by specific regulatory frameworks - as described in OECD Guidance Document RT-HEP and RT-S9 (OECD, 2018a).

17. Hepatocytes from fish species other than rainbow trout could be used, provided that primary hepatocytes can be successfully isolated and that protocols are adapted to species-specific considerations (OECD, 2018a).

SCIENTIFIC BASIS OF THE METHOD

18. Rainbow trout provide a relatively easy source of hepatocytes, and the resulting RT-HEP have been shown to cryopreserve well, with minimal loss of xenobiotic metabolising capability (Mingoia et al., 2010; Fay et al., 2014a). This feature makes it possible to freeze RT-HEP in one location and distribute them to other laboratories for later use, and to use one lot for several tests separated in time.

19. Fresh primary cultured hepatocytes obtained from rainbow trout largely maintain their epithelial phenotype, including functional glucose and lipid metabolism (Segner et al., 1994; Polakof et al., 2011), and the activities of Phase I (e.g. cytochrome P450 (CYP)) and Phase II (e.g. sulfotransferases (SULT), uridine 5'diphospho-glucuronosyltransferases (UGT), glutathione transferases (GST)) biotransformation enzymes (Segner and Cravedi, 2001). They possess functional membrane transporters, and have been studied using well-known transporter substrates and inhibitors (Bains and Kennedy, 2005; Hildebrand et al., 2009; Sturm et al., 2001; Žaja et al., 2008).

PRINCIPLE OF THE TEST

20. The $CL_{\text{IN VITRO, INT}}$ of a test chemical is determined by using a substrate depletion approach. The incubation system consists of RT-HEP (see Annex 2) suspended in Leibovitz L-15 medium (L-15) at a cell density of 1-2 $\times 10^6$ cells/mL. Introduction of the test chemical to the RT-HEP suspension initiates the reaction. In order to collect samples at various time points, the reaction is terminated by transferring an aliquot of the suspension to a stopping solution. The decrease of the test chemical concentration from the incubation vial is measured with a validated analytical method and used to determine the $CL_{\text{IN VITRO, INT}}$. Incubations using enzymatically inactive RT-HEP are carried out as negative control to distinguish between enzymatic biotransformation and abiotic decrease.

INFORMATION ON THE TEST CHEMICAL

21. Before carrying out this test, the following information about the test chemical should be known:

- Solubility in water (TG 105; [OECD, 1995a]);
- Solubility in cell culture medium;

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- Solubility in organic solvents (if needed for preparation of the test chemical) (OECD, 2018a: Section 3.2);
- n-Octanol-water partition coefficient (log K_{ow}) or other suitable information on partitioning behaviour (TGs 107, 117, 123; [OECD, 1995b, 2004a, 2006a]);
- Test chemical stability in water (TG 111; [OECD, 2004b]) and/or test medium;
- Vapour pressure (TG 104; [OECD, 2006b]);
- Information on biotic or abiotic degradation, such as ready biodegradability (TGs 301, 310; [OECD, 1992, 2006c]);
- Acid dissociation constant (pKa) for test chemicals that may ionise.

22. A validated analytical method, of known accuracy, precision, and sensitivity, for the quantification of the test chemical in the incubation mixture should be available, together with details of sample preparation and storage. The analytical limit of quantification (LOQ) of the test chemical in the incubation mixture should be known.

REFERENCE CHEMICAL AND PROFICIENCY TESTING

23. It is recommended to use an appropriate reference chemical as a positive control to check the performance of the test system. Points to be considered in choosing an appropriate reference chemical are addressed in the OECD Guidance Document RT-HEP and RT-S9 - Section 4.2 (OECD, 2018a).

24. Reference chemicals can also be used to establish the test system in a laboratory. In the ring trial, pyrene was used as reference chemical (OECD, 2018c).

VALIDITY OF THE TEST

25. For a test to be valid, the following criteria should be met:

- RT-HEP lots should be evaluated for the ability to catalyse Phase I and II metabolic enzymatic reactions as described in Annex 3.
- % yield of RT-HEP from cryopreservation (number of viable RT-HEP obtained after thawing compared to the number of RT-HEP cryopreserved initially) should be ≥ 25%.
- Cell viability in the RT-HEP suspension after thawing (Annex 5) should be ≥ 80%.
- The cell density of the final RT-HEP suspension used for the incubation should be measured and be within the nominal density ($1.0 \pm 0.25 \times 10^6$ cells / mL and $2.0 \pm 0.5 \times 10^6$ cells / mL, respectively).
- Negative (enzymatically inactive RT-HEP) controls should demonstrate no significant loss of parent chemical over the incubation time (i.e., <20% of loss determined in active RT-HEP incubations). Furthermore, negative controls should demonstrate no apparent increase (i.e., >20%) of the parent chemical over the incubation time.
- A minimum of six time points should be used to determine $CL_{\text{IN VITRO, INT}}$; i.e., to calculate the regression and derive the slope, with an R^2 value ≥0.85. In the case of chemicals that are more slowly metabolised (e.g. a very shallow slope), the R^2 may be <0.85. In this instance, careful consideration should be given to whether the slope is significantly different than zero before including or excluding the run.
- A minimum of two independent runs must be performed (see §35). If the calculated regression from the two runs with active RT-HEP are significantly

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different (e.g. t-test of the slopes with $p<0.05$), then a third run should be performed to obtain two confirmatory runs.

DESCRIPTION OF THE METHOD

Apparatus

26. The following equipment is required for RT-HEP incubation:

- 4°C refrigerator.
- -20°C freezer.
- Liquid nitrogen storage tank or ultralow temperature freezer (-150°C) for storage of cryopreserved RT-HEP.
- Analytical balance to measure reagents and test chemicals.
- pH meter.
- Vortex mixer.
- Refrigerated centrifuge for 50 mL tubes.
- 50 mL conical centrifuge tubes.
- Sample incubation equipment, e.g. shaking water bath with chiller, shaking incubator with heating and cooling functions, or thermomixer block with shaking capabilities.
- Glass ware for preparing solutions, reagents, etc.
- Glass vials for incubation test (e.g. 7 mL scintillation test tubes).
- 1.5 mL micro-centrifuge tubes.
- Small benchtop refrigerated centrifuge for micro-centrifuge tubes.
- Sample glass vials for HPLC/GC or other analytical instruments.
- Pipettes and tips.
- Appropriate equipment and reagents for the counting of RT-HEP.

Chemicals for analytical measurements

27. The following chemicals are required:

- Solvent to dissolve test chemical, analytical grade or equivalent (e.g. methanol, acetonitrile, acetone); the solvent must be miscible with the aqueous media used in the RT-HEP suspension.
- Stopping and extraction solvents, analytical grade or equivalent (e.g. methanol, acetonitrile, methylene chloride, methyl tert-butyl ether).

Cryopreserved RT-HEP

28. Cryopreserved RT-HEP can be obtained from commercial sources, if available, or prepared following the example protocol in Annex 2.

29. Each RT-HEP lot should be evaluated for its ability to catalyse Phase I and II biotransformation reactions. Standardised assays to determine Phase I and Phase II enzyme activity are briefly described in Annex 3. These characterisation assays or known reference chemicals should be used to test a new lot of RT-HEP at the beginning of the study or before the lot is used for the first time. They should also be used occasionally to monitor possible activity losses during storage.

30. The inclusion of enzymatically inactive RT-HEP is mandatory. Incubations using inactive RT-HEP serve as a negative control to distinguish between enzymatic

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biotransformation and other potential loss processes, such as adsorption to the incubation vial, volatilisation, and abiotic degradation (further details are addressed in OECD Guidance Document RT-HEP and RT-S9; [OECD, 2018a]). A protocol for enzyme inactivation by heating is provided in Annex 4. Other methods to inactivate the RT-HEP can be used, but were not explored in the ring trial (OECD, 2018c).

Cell culture medium and reagents

31. Cell culture medium and reagents needed for thawing and incubation of RT-HEP are included in Annex 5.

Test set-up

32. Preliminary experiments that include range finding conditions (e.g. test chemical concentration, RT-HEP cell density and incubation time) should be conducted to establish incubation conditions needed to reliably measure intrinsic *in vitro* hepatic clearance of the test chemical. Annex 6 details how conditions that result in first-order depletion kinetics can be determined.

33. A sufficient number of sampling time points should be obtained to develop a high-quality regression of log-transformed chemical concentration data. At least six time points should be used to generate this regression.

34. An example test set-up using a single vial approach with seven time points is shown in Figure 1 of Annex 7. This test set-up is recommended to test chemicals that are not difficult-to-test (e.g. non-volatile, do not bind to vessel walls, and distribute rapidly through the incubation system) at one test concentration. It generally produces the least variable results and is simplest to perform. For volatile test chemicals or very hydrophobic, the multiple vial approach is recommended (Annex 7; Figure 2).

35. Each test consists of at least two independent runs to determine the $CL_{\text{IN VITRO, INT}}$. Each independent run is performed on a different day or on the same day provided that for each run: a) independent fresh stock solutions and working solutions of the test chemical are prepared and b) independently thawed RT-HEP are used; however, RT-HEP may come from the same lot. If the calculated regression from the two runs with active RT-HEP are significantly different (e.g. t-test of the slopes with $p<0.05$), then a third run should be performed to obtain two confirmatory runs.

36. For each run, one vial each for active RT-HEP and enzymatically inactive RT-HEP is spiked with the test chemical, and one vial for active RT-HEPs is spiked with a reference chemical. Samples are collected at each time point (e.g. 2, 10, 20, 30, 60, 90, 120 min). In some cases, additional vials (e.g. duplicates for each vial) may be required to ensure accuracy of the analytical method for the given test chemical.

37. In the following paragraphs, the single vial approach is described whereas the multiple vial approach is detailed in Annex 7.

Preparation of test chemical, media and stopping solutions

38. Stock solution(s) of the test chemical should be prepared in reaction buffer (L-15 medium) or an adequate solvent that is previously tested. Typical solvents include acetone, acetonitrile, and methanol. The stability of the test chemical in the stock solution should be evaluated in advance of the test if stock solutions are not prepared daily (see OECD Guidance Document RT-HEP and RT-S9 - Section 3.2, [OECD, 2018a]).

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39. On the day of the test, the desired spiking concentration of the test chemical is prepared by diluting the stock solution with reaction buffer (L-15 medium) or an organic solvent based on the results of the preliminary experiments (see §32; Annex 6; [OECD, 2018a]). If an organic solvent is used, the total amount in the incubation RT-HEP suspension should be kept as low as possible and not exceed 1% to avoid inhibition of enzyme activity. In general, the test chemical concentration should be approximately 10-fold higher than the LOQ of the analytical method as long as it results in first-order depletion kinetics as determined in the preliminary experiments (see §32; Annex 6).

40. A stopping solution (e.g. methanol, acetonitrile, methylene chloride, methyl tert-butyl ether) is prepared which may include an internal standard. For most tests, 1.5 mL micro-centrifuge tubes may be filled with the stopping solution in advance (e.g. 100 µL sample terminated in 400 µL stopping solution) and stored on ice. For volatile solvents (e.g. solvents that vaporise at room temperature, such as methylene chloride, methyl tert-butyl ether), the tubes should remain capped and kept cool, or the solvents should be added directly prior to collection of the time point. For solvents which interact with plastic, glass tubes should be used to stop the reactions (see also OECD Guidance Document RT-HEP and RT-S9 - Section 3.3; [OECD, 2018a]).

Preparation of RT-HEP suspension

41. The volume of RT-HEP suspension (active or enzymatically inactive) required for each incubation vial depends on the number of desired time points as well as the volume to be removed for each time point. To ensure good mixing at the final time point, the volume of the RT-HEP incubation remaining should be still large enough to cover the bottom of the vial.

42. For the single vial approach (Figure 1, Annex 7), two vials with active and one vial with enzymatically inactive RT-HEP suspensions are used per run. In preparing the two suspensions, an additional 25% is recommended to provide a modest excess of biological material, taking into account the number of time points in the test set-up. For example, if 1.0 mL per vial will be used in the test, a total of about 2.5 mL (2.0 mL + 25%) should be prepared for the active RT-HEP suspension and 1.25 mL (1.0 + 25%) for the enzymatically inactive RT-HEP suspension.

43. Incubations are conducted using 1-2 x 10^6 viable hepatocytes/mL. RT-HEP counting should be performed using a basic methodology as described in Fay et al. (2015). After thawing (Annex 5), the number of viable cells is determined and adjusted to the desired cell density in L-15 medium, pH 7.8. To obtain highly accurate estimates of the RT-HEP cell density, additional cell counts may be performed on the RT-HEP suspension once it has been diluted to its incubation concentration. Three additional cell counts (post-dilution) are recommended (using separate dilutions with trypan blue) on the incubation suspension. This second set of counts may be performed during or after the incubations as time allows. If the three cell counts vary by more than 20% CV, an additional cell count may be needed to obtain an accurate estimation of the active RT-HEP cell density.

44. The desired volume (e.g. 1 mL for seven time points) of active and enzymatically inactive RT-HEP suspension is added to the respective incubation vials. After loosely capping the vials, they are pre-incubated at the test temperature (11±1°C) for 10 min with gentle shaking. RT-HEP should not be shaken vigorously or vortexed.

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Incubation with test chemical and stopping of reaction

45. Test chemical (usually 5µL; however, this depends on the concentration of the spiking solution) is directly added into the suspension of each incubation vial (usually 1 mL) to initiate the reaction. The vials are swirled to distribute the chemical and loosely capped. Throughout the incubation, RT-HEP should be gently shaken as above (§44) at the test temperature (11±1°C).

46. For sampling at a specified time point, the incubation vial is removed from the water bath or incubator, gently swirled or shaken to form a homogenous suspension, and an aliquot (e.g. 100 µL) is removed with a pipette and directly dispensed into the corresponding 1.5 mL micro-centrifuge tube containing ice-cold stopping solution kept on ice (see §40). To ensure quantitative transfer of the sample, pipetting up and down in the solvent three times is recommended.

47. The micro-centrifuge tubes are kept on ice until samples from all time points have been collected. It may be useful to refrigerate samples overnight to facilitate complete protein precipitation prior to centrifugation if a water miscible solvent is used as stopping solution. If volatile solvents like methylene chloride, and methyl tert-butyl ether are used, the samples must be extracted if possible directly after stopping the reaction. Preliminary experiments should be performed to confirm complete precipitation of proteins upon termination of the reaction (Annex 6).

48. After the sampling is completed or for volatile solvents at each sampling point, micro-centrifuge tubes are vortexed (e.g. for 3 min at 1 500 to 2 000 rpm) and centrifuged (e.g. 15 min at 20 000 × g and 4°C). Some test chemicals may require overnight refrigeration to ensure maximal extraction with the solvent. Additional considerations are provided in OECD Guidance Document RT-HEP and RT-S9 – Section 3.3 (OECD, 2018a). The supernatant is transferred to analytical HPLC/GC sample vials and stored at -20±1°C until analysis.

ANALYTICAL MEASUREMENTS

49. The concentration of the test chemical is determined in the samples using a validated analytical method. More details are provided in OECD Guidance Document RT-HEP and RT-S9 – Section 3.1 (OECD, 2018a).

50. Since the whole procedure is governed essentially by the accuracy, precision and sensitivity of the analytical method used for the test chemical, the accuracy, precision and reproducibility, as well as recovery (80-120%) of the test chemical from the incubation mixture should be verified experimentally.

DETERMINATION OF IN VITRO INTRINSIC CLEARANCE

51. The $\log_{10}$-transformed substrate concentrations are plotted against time and should demonstrate a log-linear decline (R^2 value ≥0.85).

52. If a visual inspection of the regression shows obvious outliers, a statistically valid outlier test may be applied to remove spurious data points (e.g. as described in [OECD, 2006d]) as well as documented justification for their omission. In some cases, non-linear behaviour may be observed at the beginning or end of a test, which could be due to problems with dissolution of the test chemical or loss / inhibition of enzyme activity.

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However, the depletion rate should be determined from the linear portion of the curve, with a minimum of six data points.

53. If there is an abiotic loss of test chemical from enzymatically inactive RT-HEP which cannot be avoided by optimisation of assay conditions (i.e. abiotic decrease >20%), the rate of this loss process may be subtracted from the measured rate of depletion in active samples to obtain a corrected *in vitro* intrinsic clearance (OECD Guidance Document RT-HEP and RT-S9 §52; [OECD, 2018a]). In this case, however, it must be verified that the abiotic loss process follows first-order kinetics.

54. A first-order elimination rate constant, k_e (h^{-1}), is determined as -2.3 × slope of the log-linear decline.

55. k_e is divided by the measured viable (e.g. as determined by trypan blue exclusion) RT-HEP concentration to obtain the $CL_{\text{IN VITRO, INT}}$ (mL/h/10^6 cells) rate. If RT-HEP were counted pre- and post-dilution of RT-HEP suspension, the post-dilution counts should be used.

TEST REPORT

56. The test report should include the following:

Test chemical

- Mono-constituent substance:
  - physical appearance, water solubility, and additional relevant physicochemical properties; chemical identification, such as IUPAC or CAS name, CAS number, eSMILES or InChI code, structural formula, purity, chemical identity of impurities as appropriate and practically feasible, etc.
- Multi-constituent substance, unknown or variable composition, complex reaction products or of biological materials (UVCBs) and mixtures:
  - characterised as far as possible by chemical identity (see above), quantitative occurrence and relevant physicochemical properties of the constituents
- Analytical method for quantification of the test chemical.

RT-HEP

- If purchased:
  - commercial source
  - rainbow trout supplier
  - rainbow trout strain
  - acclimation temperature
  - fish weight
  - liver weight
  - gonadosomatic index (for determination of sexual maturity)
- If prepared in-house, see Table 3: Reporting Template in Annex 2.
- Characterisation (see Annex 3).
- % yield after thawing.
- Viability of RT-HEP in suspension.

Test conditions

- Concentration of test chemical and reference chemical.

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- Method of preparation of stock solution(s) of test chemical and reference chemical (name and concentration of solvent, if applicable).
- Cell culture medium characteristics (temperature, pH).
- Incubation temperature.
- RT-HEP cell density in suspension for incubations (nominal and measured).
- Test set-up (single vial or multiple vial approach).
- Number of replicates (if more than one is used per run).
- Number of independent runs.
- Time points.
- Description of preliminary experiments.

Analytical method

- Complete description of all test chemical analysis procedures employed including limits of detection and quantification, variability and recovery efficiency, matrix used for standard preparations, internal standard, etc.
- Statistical method.
- Description and statistical method used for exclusion of time points and/or runs.

Results

- Results from any preliminary experiment performed.
- Data from individual vials, time points for each independent run (e.g. test chemical, reference chemical, active and enzymatically inactive RT-HEP).
- If measured, appearance of formed metabolites (including as optional reporting: identification of metabolites and metabolic pathways).
- Calculated CL, IN VITRO, INT from independent incubations with active and enzymatically inactive RT-HEP (test and reference chemical, as appropriate).
- Average and standard deviation values from independent, not significantly different, runs, as well as results from t-tests to compare average CL, IN VITRO, INT from the runs.
- Any excluded time points or runs.
- Anything unusual about the test, any deviation from the test guideline and any other relevant information.

References

Bains, O.S. and C.J. Kennedy (2005), Alterations in respiration rate of isolated rainbow trout hepatocytes exposed to the P-glycoprotein substrate rhodamine 123, *Toxicology* 214:87-98.

Brinkmann, M. et al. (2016), Cross-species extrapolation of uptake and disposition of neutral organic chemicals in fish using a multispecies physiologically-based toxicokinetic model framework, *Environmental Science & Technology* 50: 1914-23.

Chen, Y. et al. (2016), Which molecular features affect the intrinsic hepatic clearance rate of ionizable organic chemicals in fish?, *Environmental Science & Technology* 50: 12722-12731.

Cowan-Ellsberry, C.E et al. (2008), Approach for extrapolating in vitro metabolism data to refine bioconcentration factor estimates, *Chemosphere* 70:1804-1817.

Dyer et al. (2008), In vitro biotransformation of surfactants in fish. Part I: Linear alkylbenzene sulfonate (C12-LAS) and alcohol ethoxylate (C13EO8), *Chemosphere* 72:850-862.

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Dyer et al. (2009), In vitro biotransformation of surfactants in fish. Part II: Alcohol ethoxylate (C13EO8) and alcohol ethoxylate sulfate (C14EO2S) to estimate bioconcentration potential, *Chemosphere* 76:989-998.

ECHA (2017), *Guidance on Information Requirements and Chemical Safety Assessment*, Chapter R.7c: Endpoint specific guidance, Version 3.0.

Fay, K.A. et al. (2014a), Optimizing the use of rainbow trout hepatocytes for bioaccumulation assessments with fish, *Xenobiotica* 44:345-351.

Fay, K.A. et al. (2014b), Intra- and interlaboratory reliability of a cryopreserved trout hepatocyte assay for the prediction of chemical bioaccumulation potential, *Environmental Science & Technology* 48:8170-8178.

Fay, K.A. et al. (2015), Determination of metabolic stability using cryopreserved hepatocytes from rainbow trout (Oncorhynchus mykiss), *Current Protocols in Toxicology* 65:4.42.1-29.

Halder, M., A. Lostia and A. Kienzler (2018), *EURL ECVAM Fish In Vitro Intrinsic Clearance Database*, European Commission, Joint Research Centre (JRC) [Dataset] PID: http://data.europa.eu/89h/jrc-eurl-ecvam-fish-in-vitro-intr-clear-db.

Han, X. et al. (2007), Determination of xenobiotic intrinsic clearance in freshly isolated hepatocytes from rainbow trout (Oncorhynchus mykiss) and rat and its application in bioaccumulation assessment, *Environmental Science & Technology* 41:3269-3276.

Hildebrand, J. et al. (2009), Functional and energetic characterization of P-gp-mediated doxorubicin transport in rainbow trout (Oncorhynchus mykiss) hepatocytes, Comparative Biochemistry and Physiology Part C: *Toxicology and Pharmacology* 149:65-72.

Laue, H. et al. (2014), Predicting the bioconcentration of fragrance ingredients by rainbow trout using measured rates of in vitro intrinsic clearance, *Environmental Science & Technology* 48:9486-9495.

Mingoia, R.T. et al. (2010), cryopreserved hepatocytes from rainbow trout (Oncorhynchus mykiss): A validation study to support their application in bioaccumulation assessment, *Environmental Science & Technology* 44:3052-3058.

Nichols, J.W. et al. (2013), Towards improved models for predicting bioconcentration of well-metabolized compounds by rainbow trout using measured rates of in vitro intrinsic clearance, *Environmental Toxicology and Chemistry* 32: 1611-1622.

Nichols, J.W., I.R. Schultz and P.N. Fitzsimmons (2006), In vitro-in vivo extrapolation of quantitative hepatic biotransformation data for fish: I. A review of methods, and strategies for incorporating intrinsic clearance estimates into chemical kinetic models, *Aquatic Toxicology* 78:74-90.

OECD (2018a), Guidance Document on the Determination of in vitro intrinsic clearance using cryopreserved hepatocytes (RT-HEP) or liver S9 sub-cellular fractions (RT-S9) from rainbow trout and extrapolation to in vivo intrinsic clearance, *Series on Testing and Assessment* No. 280, OECD Publishing, Paris.

OECD (2018b), Test Guideline No.319B: Determination of in vitro intrinsic clearance using rainbow trout liver S9 sub-cellular fraction (RT-S9), *OECD Guidelines for the Testing of Chemicals*, Section 3, OECD Publishing, Paris.

OECD (2018c), Multi-laboratory ring trial to support development of OECD test guidelines on determination of in vitro intrinsic clearance using cryopreserved rainbow trout hepatocytes and liver S9 sub-cellular fractions, *Series on Testing and Assessment* No. 281, OECD Publishing, Paris.

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OECD (2014), Guidance Document for Single Laboratory Validation of Quantitative Analytical Methods – Guidance used in Support of Pre-and-Post-Registration Data Requirements for Plant Protection and Biocidal Products, *Series on Testing and Assessment* No. 204, OECD Publishing, Paris, http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV/JM/MONO(2014)20&doclanguage=en.

OECD (2006a), Test No. 123: Partition Coefficient (1-Octanol/Water): Slow-Stirring Method, OECD *Guidelines for the Testing of Chemicals*, Section 1, OECD Publishing, http://dx.doi.org/10.1787/9789264015845-en.

OECD (2006b), Test No. 104: Vapour Pressure, *OECD Guidelines for the Testing of Chemicals*, Section 1, OECD Publishing, http://dx.doi.org/10.1787/9789264069565-en.

OECD (2006c), Test No. 310: Ready Biodegradability - CO2 in sealed vessels (Headspace Test), *OECD Guidelines for the Testing of Chemicals*, Section 3, OECD Publishing, http://dx.doi.org/10.1787/9789264016316-en.

OECD (2006d), Current approaches in the statistical analysis of ecotoxicity data: A guidance to application, *OECD Series on Testing and Assessment* No. 54, OECD Publishing, Paris, http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?doclanguage=en&cote=env/jm/mono%282006%2918.

OECD (2004a), Test No. 117: Partition Coefficient (n-octanol/water), HPLC Method, *OECD Guidelines for the Testing of Chemicals*, Section 1, OECD Publishing, http://dx.doi.org/10.1787/9789264069824-en.

OECD (2004b), Test No. 111: Hydrolysis as a Function of pH, *OECD Guidelines for the Testing of Chemicals*, Section 1, OECD Publishing, http://dx.doi.org/10.1787/9789264069701-en.

OECD (1995a), Test No. 105: Water Solubility, *OECD Guidelines for the Testing of Chemicals*, Section 1, OECD Publishing, http://dx.doi.org/10.1787/9789264069589-en.

OECD (1995b), Test No. 107: Partition Coefficient (n-octanol/water): Shake Flask Method, *OECD Guidelines for the Testing of Chemicals*, Section 1, OECD Publishing, http://dx.doi.org/10.1787/9789264069626-en.

OECD (1992), Test No. 301: Ready Biodegradability, *OECD Guidelines for the Testing of Chemicals*, Section 3, OECD Publishing, http://dx.doi.org/10.1787/9789264070349-en.

Polakof, S. et al. (2011), The metabolic consequences of hepatic AMP-kinase phosphorylation in rainbow trout, *PLoS one* 6:e20228.

Segner, H. and J.P. Cravedi (2001), Metabolic activity in primary cultures of fish hepatocytes, *Alternatives to Laboratory Animals* 29:251-257.

Segner, H. et al. (1994), Cultured trout liver cells: utilization of substrates and response to hormones, in *Vitro Cellular and Developmental Biology* 30:306-311.

Stadnicka-Michalak, J. et al. (2014), Measured and modeled toxicokinetics in cultured fish cells and application to in vitro-in vivo toxicity extrapolation, *PLoS One*. 9, e92303.

Sturm, A. et al. (2001), Expression and functional activity of P-glycoprotein in cultured hepatocytes from Oncorhynchus mykiss, *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* 281:R1119-R1126.

Žaja, R. et al. (2008), Cloning and molecular characterization of apical efflux transporters (ABCB1, ABCB11 and ABCC2) in rainbow trout (Oncorhynchus mykiss) hepatocytes, *Aquatic Toxicology* 90:322-332.

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ANNEX 1

Abbreviations & definitions

BCF	Bioconcentration factor (L/kg)
BSA	Bovine serum albumin
$CL_{\text{IN VITRO, INT}}$	*in vitro* intrinsic clearance (mL/h/10^6 cells or mL/h/mg protein)
CV	Coefficient of variation
CYP	Cytochrome P450
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco's Modified Eagle Medium
EDTA	Ethylenediaminetetraacetic acid
EROD	Ethoxyresorufin-O-deethylase
FBS	Fetal bovine serum
First-order depletion kinetics	A chemical reaction in which the rate of decrease in the number of molecules of a substrate is proportional to the concentration of substrate molecules remaining
GC	Gas Chromatography
GSH	L-Glutathione
GSI	Gonadosomatic index
GST	Glutathione transferase
HBSS	Hanks' Balanced Salt Solution
HPLC	High Performance Liquid Chromatography/
IVIVE model	*In vitro* to *in vivo* extrapolation model
k_e	Elimination rate constant (h^{-1})
K_M	Michaelis-Menten constant
K_{ow}	n-Octanol-water partition coefficient
L-15	Leibovitz-15
LOQ	Limit of quantification
MS-222	Tricaine methanesulfonate
pKa	Acid dissociation constant
rpm	Revolutions per minute
RT-HEP	cryopreserved rainbow trout hepatocytes
SULT	Sulfotransferase
TG	Test Guideline
UGT	Uridine 5'-diphospho-glucuronosyltransferase
V_{max}	Maximum enzymatic rate at saturating test chemical concentration

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ANNEX 2

Example Protocol for Isolation and Cryopreservation of Rainbow Trout Hepatocytes (RT-HEP)

*NOTE: Cryopreserved RT-HEP can be obtained from commercial sources, if available, or prepared following the **example** protocol provided in this Annex. The protocol is adapted from a published protocol (Fay et al., 2015) and was used in the ring trial (see OECD ring trial report; [OECD, 2018]).*

Fish

1. RT-HEP should be obtained from sexually immature rainbow trout since previous work has shown that sexually immature rainbow trout (*Oncorynchus mykiss*) do not differ with respect to their metabolic capabilities in relation to their gender (Johanning et al., 2010, 2012; Fay et al., 2014). RT-HEP can therefore be collected without regard to gender.

2. If fish are obtained from a supplier, they should be acclimatised in the laboratory for at least 2 weeks prior to use. Fish should not receive treatment for disease in the two-week acclimation period and any disease treatment by the supplier should be completely avoided if possible. Fish with clinical signs of disease should not be used.

3. Rainbow trout are typically raised at 10-15ºC. The temperature of the holding tank in the laboratory should be similar and maintained at ± 2°C. Holding density of fish should be low enough to ensure optimal growth and welfare.

4. Measure and record water chemistry characteristics at periodic intervals, including: pH, total alkalinity (as mg/L $CaCO_3$), dissolved oxygen (mg/L, converted to percent saturation), and total ammonia (mg/L) (Table 3).

5. Record fish maintenance details as well, including: photoperiod, feeding regime, feed type, water temperature, holding density (kg fish/liter tank volume), and number of fish/tank (Table 3). This specific information should be reported to allow for isolation-specific parameters to be used in subsequent applications, such as BCF prediction models.

Procedure Summary

6. The procedure for isolating hepatocytes from rainbow trout largely follows techniques used to obtain hepatocytes from mammals (Fay et al., 2015, 2014; Mommsen et al., 1994; Mudra and Parkinson, 2001; Seglen, 1976; Segner, 1998).

7. The fish is anesthetised (but alive) during the isolation procedure to take advantage of the dilated hepatic vascular system, which allows for increased perfusion efficiency. The hepatic portal vein is cannulated, and the liver is perfused with a Ca^{2+}/Mg^{2+}-free balanced salt buffer (Buffer I; see Table 1) to clear the liver of blood and loosen desmosomes. The liver is then perfused by a balanced salt solution (with Ca^{2+} and Mg^{2+}) containing the enzyme collagenase (Buffer II; see Table 1). After digestion, the collagenase reaction is terminated by perfusion with cell medium containing bovine serum albumin (Buffer III; see Table 1), and the hepatocytes are mechanically separated from the liver capsule.

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8. The primary hepatocytes are washed and purified using a density gradient. All buffers are adjusted to the pH of the fish's blood and chilled to its acclimation temperature. For rainbow trout, this is typically pH 7.8 at 11±1°C.

9. Generally, pooling hepatocytes from several fish (three to six) is recommended. This approach will diminish the influence of a single fish, and better represent a population. Elapsed time from isolation to use, or cryopreservation, should be minimised. Quickly isolating hepatocytes from several fish may be accomplished by operating several isolation stations simultaneously, or by splitting the perfusate line to accommodate more than one fish. This latter scenario is described below.

Apparatus and Material

10. Apparatus

- Vessels to expose fish to anaesthetic.
- Digital balance (1 g – 2000 g), weigh boats.
- Refrigerated centrifuge (e.g. for 50 mL tubes).
- Conical centrifuge tubes, e.g. 50 mL.
- Forceps, large and small sharp surgical scissors.
- Mesh (e.g. 100 µm).
- Tall glass beaker, 150 mL.
- 21G × ¾ safety winged infusion set (butterfly catheter).
- Micro-bulldog clamps (optional; Harvard Apparatus, NP 52-3258).
- Perfusion apparatus (Figure 3) including:
  - recirculating water bath capable of chilling water to 11±1°C
  - peristaltic pump
  - pump tubing
  - water-jacketed glass coil condenser (45 mm × 260 mm or similar)
  - water-jacketed glass bubble trap, with stopcock
  - surgical platform with catch basin for blood and perfusate (optional; a tray lined with paper towelling is also sufficient).
- Serological pipets and pipetman.
- Buckets with ice.
- Cryogenic container for cell storage.
- Liquid nitrogen.
- Equipment for counting cells.

11. Buffers, cell culture media, chemicals

- Tricaine Methanesulfonate (MS-222).
- Sodium bicarbonate (NaHCO3).
- Ethanol, 70% (v/v).
- Percoll.
- Hydrochloric acid (HCl), 1 N.
- Leibovitz L-15 medium (L-15).
- Hanks' Balanced Salt Solution (HBSS).
- Ethylenediaminetetraacetic acid disodium salt (EDTA).
- Bovine Serum Albumin (BSA).
- Fetal Bovine Serum (FBS).
- Dimethyl sulfoxide (DMSO).

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- Dulbecco's Modified Eagle Medium (DMEM).
- Dulbecco's Phosphate Buffered Saline (DPBS).
- Collagenase (type IV).

Preparation of Reagents and Solutions

12. The *tricaine methanesulfonate* (MS-222; 150 mg/L) should be prepared with water from the same source used to maintain the fish prepared. For example, for 8 L, 1.2 g MS-222 is added to the water and mixed until dissolved. A predetermined amount of $NaHCO_3$ is used to maintain the source water pH. If the water is low-alkalinity, the required mass of $NaHCO_3$ is approximately three times that of the MS-222.

13. The three *perfusion buffers* are prepared as listed in Table 1. The amounts provided are sufficient to perfuse three to four fish.

Table 1. Perfusion buffers I, II, and III

	Reagent	Per 600 mL preparation	Concentration
Buffer I	1 × HBSS (without Ca^{2+}/Mg^{2+} salts)	600 mL	
pH 7.8	EDTA	510 mg	2.3 mM
	$NaHCO_3$	212 mg	4.2 mM
Buffer II	1 × HBSS (with Ca^{2+}/Mg^{2+} salts)	600 mL	
pH 7.8	Collagenase, type IV	150 mg**	0.25 mg/mL**
	$NaHCO_3$	212 mg	4.2 mM
Buffer III	DMEM	600 mL	
pH 7.8	BSA	6.0 g	1% (w/v)

Note: ** Buffer II: Collagenase activity varies from lot to lot, and is not a pure preparation of enzyme, but contains other proteases, polysaccharidases, and lipases. It may be necessary to adjust the amount used depending on how well the liver digests.

14. The *90% Percoll solution* for cell purification should be prepared using a bio-hood and sterile technique. The temperature of the Percoll should match the test conditions (the temperature at which the fish are acclimated). 90 mL of chilled Percoll is added to a graduated cylinder and the volume adjusted up to 100 mL with DPBS 10 × solution. After mixing well, the solution is adjusted to pH 7.8 by slowly adding 1 N HCl. If the pH drops below 7.8, NaOH should not be added since it forms a precipitate and turns the solution cloudy. Instead, additional Percoll/DPBS should be added to increase the pH. The solution can be stored at 2-8°C for up to 14 d.

15. The *cryopreservation medium* may be prepared the day before use, but the pH should only be adjusted 1-2 h prior to hepatocyte isolation. The ingredients are provided in Table 2. The pH may need to be adjusted to fully dissolve the albumin. Allow the solution to sit overnight at 1-10°C to reduce foam. On the day of use, adjust the buffer pH to 7.8 at 11±1°C and sterile filter through a 0.2 µm polyethersulfone filter.

16. Table 2 further indicates how cryopreservation medium with 12% and 16% DMSO, respectively, are prepared.

Table 2. Cryopreservation medium recipes

	Reagent	Per 200 mL preparation	Concentration
Cryopreservation buffer	DMEM	160 mL	

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pH 7.8 at 11±1°C	FBS	40 mL	20% (v/v)
	BSA	0.5 g	0.25% (w/v)
Cryopreservation medium with 12% DMSO	Combine 1.8 mL of DMSO for every 13.2 mL cryopreservation buffer		
Cryopreservation medium with 16% DMSO	Combine 7.2 mL of DMSO for every 37.8 mL cryopreservation buffer		

Detailed Description

Preparation of apparatus and buffers on the day of RT-HEP isolation and cryopreservation

Apparatus

17. A recommended set up for the perfusion apparatus is shown in Figure 1. Alternate set-ups may also be used (e.g. peristaltic pump). The perfusate flow is set to approximately 10 mL/min. Note that the flow rate may need adjustment for fish of different sizes (e.g. 5 mL/min for 100 g fish).

18. If perfusing two fish with one apparatus, the line exiting the bubble trap may be split. A clamp or valve should be used to control the perfusate flow through the second line while starting perfusion on the first fish. In this case, the pump rate needed for 10 mL/min flow through in the first line when the second is clamped should be determined and the necessary increase in pump rate required to maintain the flow rate when both perfusion lines are open.

19. The temperature of perfusate exiting the cannula should be approximately 11±1°C (or the temperature at which the fish was acclimated) and the temperature setting of the chiller and circulator should be adjusted accordingly.

20. Prior to the start of the liver perfusion (see §33), the tubing and bubble trap of the perfusion apparatus is flushed with 70% ethanol for approximately 10 min, followed by 10 min flushing with distilled water, and finally with perfusion buffer I for 3 min.

21. To flush the bubble trap, open the top and front valves to empty. With the front valve closed, fill the bubble trap with fluid until it spills out the top valve. Next, discharge the majority of the fluid by releasing the front valve. Flush in this manner several times.

22. For filtering the hepatocytes suspension, a piece of nylon mesh (e.g. 100 µm) is secured over the rim 125 mL tall glass beaker using a rubber band. The beaker should be placed on ice. The mesh should not be tight across the top of the beaker, but depressed in the center to filter the RT-HEP suspension. Note that poor quality nylon may not be sufficient for use in filtering hepatocytes. Stitching with ragged edges may damage hepatocytes.

23. All surgical supplies should be set out, including: forceps, large and small scissors, weigh boat, butterfly catheter set, and micro-bulldog clamp or sutures.

Perfusion buffers and cryopreservation media

24. Within 2 h prior to use, the pH of pre-prepared perfusion buffers should be adjusted to the target pH at the acclimation temperature of the fish (e.g. pH 7.8 at

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11±1°C), if necessary. Perfusion buffers I and II, especially, will decrease in pH if prepared too far in advance due to the dissolution of CO_2 and formation of carbonic acid.

Preparation of fish and surgery

25. Fish should fast 24 h prior to isolation of hepatocytes.

26. Using a net, fish are captured and transferred to a tank or bucket containing 8 L of anaesthetic solution (MS-222) prepared earlier. The MS-222 solution can be used to anesthetise several fish without loss of anaesthetic efficacy, but this number may depend on the size of the fish.

27. The fish should be immersed in the MS-222 solution for at least 1 min. The fish is properly anesthetised when opercular movement has ceased, there is a total loss of equilibrium and muscle tone, and no response to stimuli (a firm squeeze at the base of the tail may be used to determine response to stimuli). After finalisation of the liver perfusion (§§ 34-36), the fish should be humanely killed with a sharp blow on the head.

28. The weight of anesthetised fish is recorded (see Table 3: Reporting Template).

29. The fish is placed on the surgical platform with the ventral surface facing up. As illustrated in Figure 2, the following incisions are recommended: a) midline incision from the vent to the isthmus, taking care not to cut too deeply into the body cavity; followed by b) a lateral incision at the caudal end of the midline incision extending about half way up to the dorsal surface and c) a similar lateral incision just caudal to the operculum.

30. By folding back and cutting away the flap of tissue resulting from the incisions described above, the body cavity is exposed and the liver should be dark red and the heart should still be beating (Figure 3). The ventral branch of the hepatic portal vein (running from the intestine to the liver hilus) should be located and carefully cleared from any obscuring connective tissue.

31. The perfusate pump set to 10 mL/min is turned on. If perfusing 2 fish with one apparatus, the second perfusion line should be clamped initially. Both fish should be prepared to the point where they are ready for liver perfusion: anesthetised, weighed, body cavity exposed, and hepatic portal veins located.

32. With perfusion buffer I flowing, a 21-G butterfly catheter is carefully inserted into the portal vein in the direction of the liver, and secure in place with a micro-bulldog clamp (Figure 4). Different gauge catheters may be preferred depending upon the size of the fish. If the micro-bulldog clamp is not available, sutures or pressure applied by fingers may be substituted, as described in (Johanning et al., 2012).

33. The blood vessels leading from the anterior aspect of the liver to the heart are severed, or, alternatively, the heart may be severed or removed completely to allow efflux of perfusate (Figure 4). If perfusing two fish from one apparatus, the second perfusion line should be unclamped just prior to cannulating the portal vein of the second fish, and the pump rate increased to provide the target perfusion flow rate (e.g. 10 mL/min) in both perfusion lines. The liver of the first fish is perfused with perfusion buffer I while the second fish is prepared (approximately 1 min).

Liver Perfusion

34. Liver perfusion is started with perfusion buffer I for 8-12 min. Blanching of the liver should be evident within the first minute of perfusion (Figure 4). It is followed by

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perfusion buffer II for 12-15 min until the liver visibly softens. After approximately 5 min of perfusion with perfusion buffer II, the liver may periodically be prodded gently with blunt forceps to test for softening. Generally, perfusion with perfusion buffer II beyond 15 min will result in over-digestion and is not recommended.

35. Once the liver has softened sufficiently, the collagenase digestion is terminated by perfusing for 3 min with perfusion buffer III.

36. Switching perfusates may be accomplished by quickly transferring the draw tubing to the reservoir containing the next buffer, or by using a split line with a valve on the draw tubing to switch between buffers. The inclusion of a bubble trap in the perfusion apparatus will prevent any air introduced to the perfusion line during the buffer transition from reaching the liver.

RT-HEP isolation

37. The flow of perfusion buffer III is stopped and the catheter removed. Using small, sharp scissors, the liver is excised along with the intact gall bladder. The gall bladder is carefully cut away the without rupturing and the liver transferred to a weigh boat containing ~30 mL of ice-cold perfusion buffer III (Figure 5). If the gall bladder ruptures during this process, the liver should be rinsed with perfusion buffer III to remove any bile prior to its transfer to the weigh boat containing perfusion buffer III.

38. Using sharp forceps or the ends of small, sharp scissors, the Glisson's capsule is torn open and the liver gently shaken in perfusion buffer III to release the hepatocytes (Figure 6). The liver may be gently raked with forceps or scissors to facilitate recovery of hepatocytes. The scraping and gentle shaking of the liver capsule may take several minutes to collect a sufficient number of hepatocytes.

39. The crude hepatocyte suspension is filtered through the nylon mesh and hepatocytes are collected in the beaker (Figure 7) prepared as indicated in §22. The remaining liver connective tissue may be gently pushed against the mesh to increase hepatocyte recovery; however, excessive handling will produce poorer quality hepatocytes (e.g. caused by formation of blebs).

40. The mesh should be primed with a small amount of perfusion buffer III prior to pouring the hepatocytes to minimise initial sheer stress. Alternatively, nylon mesh tube inserts may be purchased for use with 50 mL conical tubes. These tube inserts should also be primed with a small amount of perfusion buffer III.

41. Prior to transfer of the filtered hepatocytes to 50 mL centrifuge tubes, the beaker is gently swirled to distribute the hepatocytes evenly. The crude hepatocyte suspension is centrifuged for 3 min at 50 × g, 4°C.

42. The gonads (ovaries or testes) are removed in their entirety and weigh to the nearest 0.01 g. The gonadosomatic index (GSI) of the donor animal is determined by calculating the gonad weight divided by the whole animal weight (GSI = (100 x the gonad mass) /whole animal mass). Both the gonad weight and GSI are recorded (see Table 3: Reporting Template). The gonads (testes or ovaries) appear as two strands of tissue that run along the length of the peritoneal cavity on the ventral side of the kidney. Sexual maturity in trout may be determined by the measured GSI. Generally, males with a GSI < 0.05 and females with a GSI < 0.5 may be considered sexually immature. Alternatively, sexual maturity may be determined using histology (Blazer, 2002).

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Detailed descriptions of gonadal development in trout may be found in (Billard and Escaffre, 1975; Gomez et al., 1999; Le Gac et al., 2001).

43. The supernatant obtained in §41 is aspirated to the point where the centrifuge tube begins to taper (~4 mL mark) without disturbing the hepatocyte pellet. The supernatant may be aspirated either manually by using a serological pipette, or by using a vacuum pump, but should not be poured.

44. Next, 5 mL of perfusion buffer III are added and the hepatocytes are suspended by holding the centrifuge tube at approximately a 60º angle, and gently tapping the bottom of the centrifuge tube on the back of the opposite hand. After visually inspecting the tube for complete hepatocyte suspension (no visible clumps), the final volume is brought up to 32 mL with perfusion buffer III.

45. From each 50 mL centrifuge tube, 16 mL of hepatocyte suspension is transferred to a new 50 mL centrifuge tube (so that all tubes contain 16 mL of hepatocyte suspension). To each tube, 14 mL of 90% Percoll solution (4°C or ice) are added and mixed well by gentle inversion.

46. The mixture is centrifuged for 10 min at 96 × g, 4°C. The supernatant is immediately removed by aspirating to just above the pellet, and the hepatocytes are suspended in approximately 20 mL of L-15 medium (pH 7.8 at 11±1°C). Two tubes of hepatocytes are combined into 1.

47. After centrifugation of the suspension for 3 min at 50 × g, 4°C to sediment hepatocytes, and re-suspension of the hepatocyte pellet in 20 mL of L-15, two tubes may be combined together into one. Depending upon the number of tubes required for the amount of hepatocytes isolated. This step may be repeated.

48. Then the hepatocyte pellet is suspended in 20–40 mL of L-15 depending on the expected hepatocyte concentration. All suspensions should be combined into one tube at this point.

49. The hepatocyte yield and viability is determined by using a hemocytometer with e.g. 0.04% trypan blue solution. Hepatocyte counts and viability should be recorded (see Table 3: Reporting Template).

50. The total yield from the isolation procedure is calculated as viable hepatocyte concentration (hepatocytes/mL) × suspension volume (prior to cell counting; mL) = total yield of hepatocytes. The total number of hepatocytes available for cryopreservation is similarly calculated as viable hepatocyte concentration (hepatocytes/mL) × suspension volume (post-cell counting; mL).

RT-HEP cryopreservation

51. During the entire procedure, the primary hepatocytes and cryopreservation media should be kept on ice unless specifically stated otherwise.

52. The pH of all cryopreservation media should be adjusted at the fish maintenance temperature (e.g. 11±1°C) within 2 h prior to use and maintain on ice or in a 4°C refrigerator.

53. The procedure described in the following is designed for 50 cryogenic vials containing 1.5 mL of 10×10^6 hepatocytes/mL each (15×10^6 hepatocytes per cryogenic vial). Fifty vials will be required for 750×10^6 hepatocytes, but the number of vials may

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be scaled up or down depending upon the number of hepatocytes available for cryopreservation.

54. Determine the suspension hepatocyte concentration and calculate the volume required for 375×10^6 hepatocytes. Hepatocytes should be concentrated such that the required volume is less than 50 mL (minimum hepatocyte concentration of 7.5×10^6 hepatocytes/mL).

$$375 \times 10^6 \text{ hepatocytes / suspension concentration (hepatocytes/mL)}$$

$$= \text{volume required (mL)}$$

55. Transfer 375×10^6 hepatocytes into two clean 50 mL centrifuge tubes, and bring each tube to a final volume of 50 mL with cryopreservation buffer (see Table 2).

56. Centrifuge hepatocytes for 5 min at 50 × g, 4°C to sediment hepatocytes. For each tube, aspirate the supernatant to just above pellet, and then add cryopreservation medium up to 12.5 mL. Assuming no loss of hepatocytes in this step, the density will be 30×10^6 hepatocytes/mL.

57. Suspend hepatocytes by gentle inversion and tapping as described above. Slowly add 6.25 mL of cryopreservation medium containing 12% DMSO while gently swirling hepatocytes.

58. Maintain the hepatocytes on ice for 5 min, then slowly add 18.75 mL of cryopreservation buffer containing 16% DMSO while gently swirling hepatocytes. The final volume is 37.5 mL at 10×10^6 hepatocytes/mL.

59. Maintain the hepatocytes on ice for 5 min, then suspend by gentle inversion. Transfer 1.5 mL aliquots of the bulk hepatocyte suspension to the 1.8 mL cryogenic vials. To ensure proper hepatocyte density, gently mix or swirl the hepatocytes in the bulk suspension between each transfer.

60. Cryopreserve hepatocytes by placing the vials into the vapour phase of liquid nitrogen.

61. Store vials in the vapour phase of liquid nitrogen.

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Reporting Template

Table 3. Reporting Template

Species (e.g. *O. mykiss*): _____

Strain (if applicable) _____

Fish Source (e.g. hatchery name): _____

Photoperiod regime (e.g. natural photoperiod): _____

Water Temperature (°C): _____

Water source (e.g. well): _____

Water Flow rate (L/min): _____

pH: _____

Dissolved Oxygen (mg/L): _____

Fish holding density (kg/liter tank volume: _____ or

Number fish/tank: _____

Feeding Regime (e.g., % body weight): _____

Feed Type (e.g.. Nelson's Silver Cup trout feed): _____

Fish No.	Fish Weight (g)	Sex (Female or Male) (if possible)	Liver Weight(g)	Gonad Weight (g)	GSI (Gonad weight/fish weight
1					
2					
3					
4					
5 and so on					

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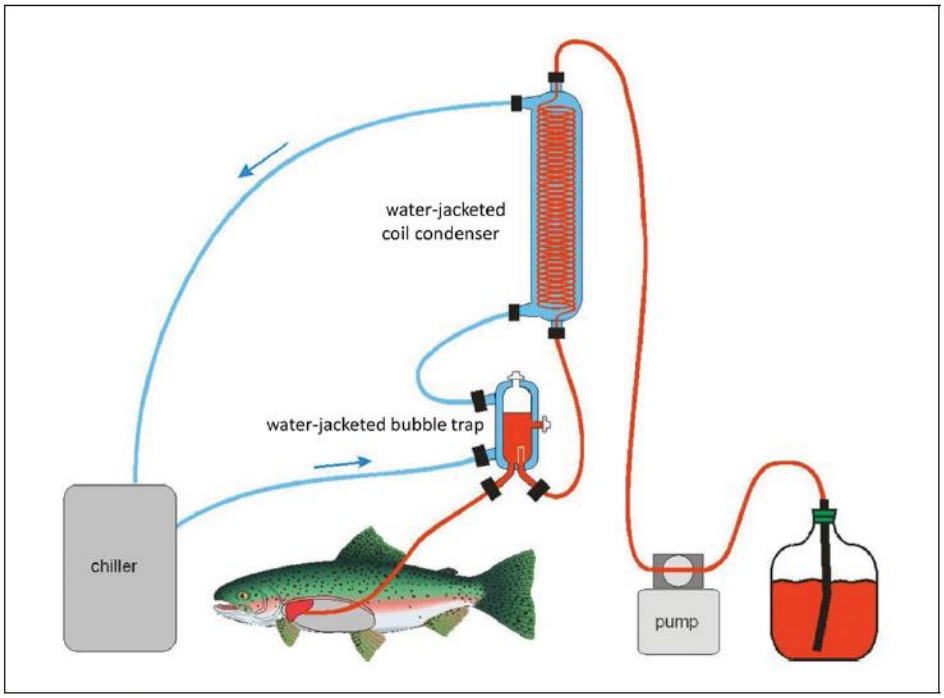
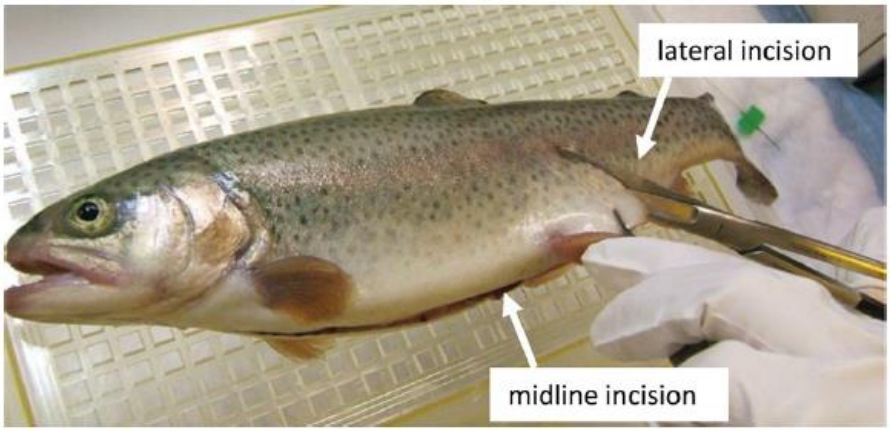
Figures

Figure 1. Set-up for perfusion of a fish liver to obtain primary hepatocytes



Note: Perfusate is pumped first through a water-jacketed coil condenser followed by a water-jacketed bubble trap before perfusing the liver. Water is cooled by a chiller so that the perfusate exiting the bubble trap is maintained at the temperature in which the fish was acclimated. The perfusion line exiting the bubble trap may be split to perfuse two fish, simultaneously.
Source: Fay et al. (2015).

Figure 2. Photograph showing a midline incision from the vent to the isthmus, and a lateral incision extending dorsally from the vent



Source: Fay et al. (2015).

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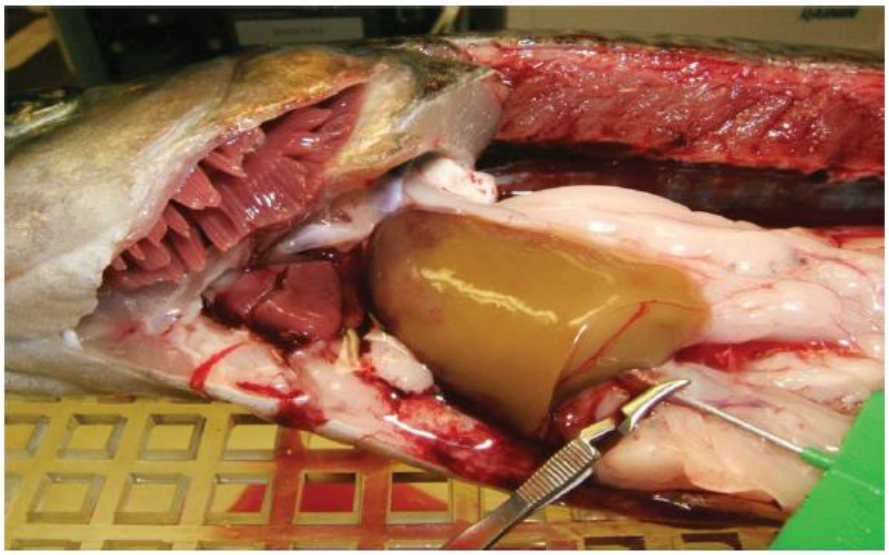
Figure 3. Photograph showing the fish's liver, exposed by cutting away the body wall

Note: The arrow indicates a ventral branch of the hepatic portal vein.
Source: Fay et al. (2015).

Figure 4. Photograph showing a cannulated liver



Note: The liver will blanch immediately upon insertion of the catheter and perfusion with buffer. Sever the vessels to the heart or the chambers of the heart to allow for perfusate efflux.
Source: Fay et al. (2015).

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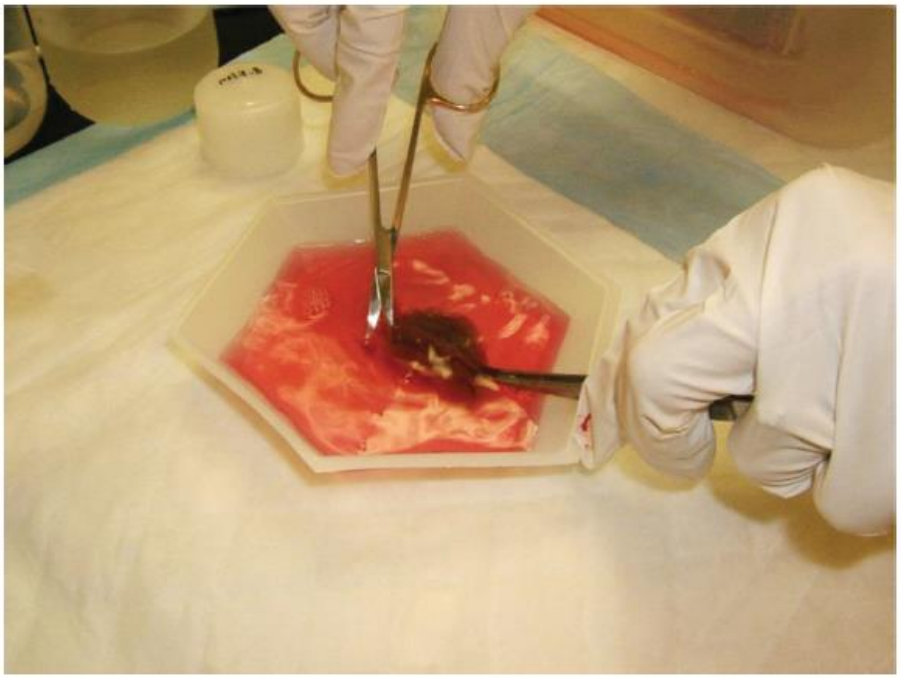
Figure 5. Photograph showing removal of the liver

Source: Fay et al. (2015).

Figure 6. Photograph showing the mechanical collection of hepatocytes



Source: Fay et al. (2015).

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Figure 7. Photograph showing the crude hepatocyte suspension after filtration through a 100µm nylon mesh

Source: Fay et al. (2015).

References

Billard, R. and A. Escaffre (1975), Identification of spermatogenesis stages in the rainbow trout based on gonad morphology and spermiation (in French), *Bulletin français de la pêche et de la pisciculture* 256:111-118.

Blazer, V. (2002), Histopathological assessment of gonadal tissue in wild fishes, *Fish Physiology and Biochemistry* 26:85-1201.

Fay, K.A. et al. (2014), Optimizing the use of rainbow trout hepatocytes for bioaccumulation assessments with fish, *Xenobiotica* 44:345-351.

Fay, K.A. et al. (2015), Determination of metabolic stability using cryopreserved hepatocytes from rainbow trout (Oncorhynchus mykiss), *Current Protocols in Toxicology* 65:4.42.1-29.

Gomez, J.M. et al. (1999), Growth hormone receptors in ovary and liver during gametogenesis in female rainbow trout (Oncorhynchus mykiss), *Journal of Reproduction and Fertility* 115:275-285.

Johanning, K. et al. (2010), In vitro rainbow trout (Oncorhynchus mykiss) liver S9 metabolism assay: optimizing the assay conditions to determine chemical bioaccumulation potential, *SETAC Europe 20th Annual Meeting*, Seville, Spain.

Johanning, K. et al. (2012), Assessment of metabolic stability using the rainbow trout (Oncorhynchus mykiss) liver S9 fraction. *Current Protocols in Toxicology* 53:14.10.1-28.

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Le Gac, F. et al. (2001), In vivo and in vitro effects of prochloraz and nonylphenol ethoxylates on trout spermatogenesis, *Aquatic Toxicology* 53:187-200.

Mommsen, T. et al. (1994), Hepatocytes: isolation, maintenance and utilization, in *Biochemistry and molecular biology of fishes*, Vol. 3, Elsevier, Amsterdam, pp. 355-372.

Mudra, D. R. and A. Parkinson (2001), Preparation of hepatocytes, *Current Protocols in Toxicology* 8:14.2.1-13.

OECD (2018), Multi-laboratory ring trial to support development of OECD test guidelines on determination of in vitro intrinsic clearance using cryopreserved rainbow trout hepatocytes and liver S9 sub-cellular fractions, *Series on Testing and Assessment* No. 281, OECD Publishing, Paris.

Seglen, P. (1976), Preparation of isolated rat liver cells, *Methods in Cell Biology* 13:29-83.

Segner, H. (1998), Isolation and primary culture of teleost hepatocytes, *Comparative Biochemistry and Physiology Part A*: 120:71-81.

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ANNEX 3

Characterisation of RT-HEP

1. Freshly isolated RT-HEP should be characterised for % viability, as determined by e.g. trypan blue exclusion (see Annex 2). The % yield (=% viable hepatocytes obtained post-thaw compared to the number of viable cryopreserved RT_HEP, initially) and % viability of RT-HEP thawed after cryopreservation should be determined (see Annex 5).

2. Each hepatocyte lot should be evaluated for the ability to catalyse Phase I and II biotransformation reactions. Assuming the RT-HEP retain their activity upon cryopreservation, ideally these characterisation assays should be performed on both freshly isolated and thawed RT-HEP.

3. Suggested standardised assays for measuring Phase I and Phase II activity are listed in Table 1 and are briefly described in (Johanning et al., 2012). Table 1 provides an overview of the methods most commonly used, the substrates, and key references. Results from these assays should be included in the test report.

4. Activity of biological material may also be evaluated using well-characterised, known test chemicals.

5. When applied to RT-HEP, these assays are generally performed on the lysate obtained from a sonicated suspension of hepatocytes. Generally, RT-HEP are diluted to a concentration of $2x10^6$ hepatocytes/mL and briefly sonicated using a hand-held ultrasonic cell disruptor (Fay et al., 2014), although necessary hepatocyte concentration may change depending on the assay specifications. The results are then normalised to protein content, determined using a standard assay (e.g. [Bradford, 1976; Lowry et al., 1951]) or a commercially available protein assay kit following the manufacturer's instructions.

6. If the likely pathway for biotransformation of a particular test chemical is known, it may be advisable to evaluate this pathway in advance, assuming that a standardised assay for measuring this activity is available. Assays that evaluate endpoints (e.g. enzyme activity at 30 min) or kinetic activity determination can be used. If comparing results with other laboratories, the exact conditions (e.g. substrate concentration, protein concentration, incubation time(s), endpoint or time points) utilised should be considered.

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Table 3. List of commonly-used enzyme activity assays, substrates, and references that can be used to characterise RT-HEP activity, usually performed with cell lysates.

	Assay / Activity	Enzyme	Reaction type	Substrate	Reference(s)
Phase I	Ethoxycoumarin O-deethylation (ECOD)	CYP1A	O-deethylation	7-Ethoxycoumarin	Edwards et al., 1984; Cravedi, Perdu-Durand and Paris, (1998); Leguen et al., 2000
	7-ethoxyresorufin O-dealkylation (EROD)	CYP1A	O-dealkylation	7-Ethoxyresorufin	Nabb et al., 2006
	7-methoxyresorufin O-dealkylation (MROD)	CYP1A	O-dealkylation	7-Methoxyresorufin	Nabb et al., 2006
	7-pentoxyresorufin O-dealkylation (PROD)	CYP2B	O-dealkylation	7-Pentoxyresorufin	Nabb et al., 2006
	Testosterone 6˙ hydroxylation	CYP3A	Aromatic ring hydroxylation	Testosterone	Oesch et al., 1992
	Chlorzoxazone 6-hydroxylation	CYP2E1	Aromatic ring hydroxylation	Chlorzoxazone	Peter et al., 1990
	Lauric acid 11-hydroxylation	CYP2K1	Long-chain aliphatic hydroxylation	Lauric acid	Nabb et al., 2006
	p-nitrophenyl acetate hydrolysis	Carboxyl-esterase	Hydrolysis	p-nitrophenyl acetate	Wheelock et al., 2005
	CDNB-glutathione conjugation	GST	Glutathione conjugation	1-chloro-2, 4-dinitrobenzene	Habig, Pabst and Jakoby, 1974
Phase II	p-Nitrophenol glucuronidation	UGT	Glucuronidation	p-Nitrophenol	Castren and Oikari, 1983; Ladd, Fitzsimmons and Nichols, 2016

References

Bradford, M. M. (1976), A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Analytical Biochemistry* 72:248–254.

Castren, M. and A. Oikari (1983), Optimal assay conditions for liver UDP-glucuronosyltransferase from the rainbow trout, Salmo gairdneri, *Comparative Biochemistry and Physiology Part* C 76:365–369.

Cravedi, J.P., E. Perdu-Durand and A. Paris (1998), Cytochrome P450-dependent metabolic pathways and glucuronidation in trout liver slices, *Comparative Biochemistry and Physiology Part C* 121:267-275.

Edwards, A.M. et al. (1984), 7-ethoxycoumarine deethylase activity as a convenient measure of liver drug metabolizing enzymes: *Regulation in cultured hepatocytes, Biochemical Pharmacology* 33:1537–1546.

Fay, K.A. et al. (2014), Intra- and interlaboratory reliability of a cryopreserved trout hepatocyte assay for the prediction of chemical bioaccumulation potential, *Environmental Science & Technology* 48:8170-8178.

Habig, W.H., M.J. Pabst and W.B. Jakoby (1974), Glutathione S-transferases. The first enzymatic step in mercapturic acid formation, *Journal of Biological Chemistry* 249:7130–7139.

Johanning, K. et al. (2012). Assessment of metabolic stability using the rainbow trout (Oncorhynchus mykiss) liver S9 fraction. *Current Protocols in Toxicology* 53:14.10.1-28.

© OECD 2018

Ladd, M.A., P.N. Fitzsimmons and J.W. Nichols (2016), Optimization of a UDP-glucuronosyltransferase assay for trout liver S9 fractions: activity enhancement by alamethicin, a pore-forming peptide, *Xenobiotica* 46:1066-1075.

Leguen, I. et al. (2000), Xenobiotic and steroid biotransformation activities in rainbow trout gill epithelial cells in culture, *Aquatic Toxicology* 48:165–176.

Lowry, O. H. et al. (1951), Protein measurement with the Folin phenol reagent, *Journal of Biological Chemistry*. 193:265–75.

Nabb, D.L. et al. (2006), Comparison of basal level metabolic enzyme activities of freshly isolated hepatocytes from rainbow trout (Oncorhynchus mykiss) and rat, *Aquatic Toxicology* 80:52–59.

Oesch, F. et al. (1992), Improved sample preparation for the testosterone hydroxylation assay using disposable extraction columns, *Journal of Chromatography* 582:232–235.

Peter, R. et al. (1990), Hydroxylation of chlorzoxazone as a specific probe for human liver cytochrome P-450IIE1, *Chemical Research in Toxicology* 3:566–573.

Wheelock, C. E. et al. (2005), Individual variability in esterase activity and CYP1A levels in Chinook salmon (Oncorhynchus tshawytscha) exposed to esfenvalerate and chlorpyrifos, *Aquatic Toxicology* 74: 172-192.

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ANNEX 4

Heat-Inactivation of RT-HEP

1. It is suggested that laboratories consider preparing a large volume of enzymatically inactive RT-HEP in advance, and freeze them as aliquots (e.g. 5 mL).

2. Active RT-HEP unused at the end of a test may become material for subsequent heat-inactivation.

3. Equipment:

- Hot plate.
- Beaker with water (water bath).
- Vessel for boiling the cells within the water bath.
- Graduated cylinder.

4. The RT-HEP suspension should be diluted in L-15 medium to the desired cell density for test use (e.g. 2×10^6 hepatocytes/mL). When using cryopreserved RT-HEP, an abbreviated thawing protocol may be sufficient.

5. The volume of the suspension should be recorded and the suspension transferred to a heat-safe vessel (preferably glass).

6. A beaker of water is heated on a hotplate and the water brought to boiling. The vessel with the RT-HEP suspension is placed into the boiling water bath, and the suspension brought to a slow boil for 15 min.

7. After the suspension has cooled, the suspension is transferred to a graduated cylinder and the volume adjusted by adding L-15 medium or water to maintain the desired hepatocyte density.

8. The enzymatically inactive RT-HEP suspension is stored at -20±1 or -80±1°C.

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ANNEX 5

Thawing of Cryopreserved RT-HEP

1. Cryopreserved RT-HEP should be thawed following the instructions of the supplier.

2. If the RT-HEP were isolated and cryopreserved according to the protocol given in Annex 2, the procedure described here should be followed to thaw the RT-HEP. High viability (>80%) is usually obtained after thawing of the cryopreserved RT-HEP, whereas the yield (% recovery; i.e., number of viable RT-HEP obtained after thawing compared to the number of viable RT-HEP cryopreserved initially) is usually 25-45% (Fay et al., 2014a, 2014b; Markell et al., 2014; Mingoia et al., 2010).

Apparatus and Material

3. The following apparatus and material is needed:

- Refrigerated centrifuge (e.g. for 50 mL tubes).
- Conical centrifuge tubes, e.g. 50 mL.
- Water bath (room temperature).
- 1000 µL pipette with tips.
- Serological pipets (2.5 - 25 mL) and pipet-aid.
- Cryogenic vials containing 1.5 mL of cryopreserved RT-HEP at 10×10^6 cells/mL.
- 1.5 mL micro-centrifuge tubes and rack.
- Sterile vacuum filters (0.2 µm polyethersulfone [PES] membrane).
- RT-HEP recovery medium (see Table 1).
- Dulbecco's Modified Eagle Medium (DMEM), low glucose with phenol red
- Fetal Bovine Serum (FBS), non heat-inactivated.
- Bovine Serum Albumin (BSA).
- Leibovitz L-15 medium (L-15) with glutamine, without phenol red.
- Trypan blue, 0.4%.
- 1 N NaOH, and HCL (for pH adjustment).

Preparation of media

Determination of the total number of RT-HEP and volume of media needed

4. Assuming a 25-45% yield (§2), 2 vials containing 1.5 mL of 10×10^6 cells/mL suspension each can be expected to provide ~ $7.5 – 13.5 \times 10^6$ viable RT-HEP.

5. The total number of cryogenic vials needed should be determined to provide enough RT-HEP taking into account that the substrate depletion method described in this Test Guideline is conducted at $1\text{-}2\times10^6$ cells/mL.

6. It is recommended that the user thaw 2-3 vials together in one 50 mL tube with recovery medium for superior yield. Thawing one vial alone may result in <25% yield from cryopreservation.

7. The volume of recovery medium should be determined assuming that 44-45 mL of recovery medium are needed for one tube (see below). The total volume of L-15 medium can be determined calculating a volume of ~100 mL per tube.

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Recovery medium preparation (prior the test day)

8. The recovery medium should be prepared prior to the test day using the ingredients provided in Table 1. The medium is sterile filtered and may need to be pH adjusted. Allow the solution to sit overnight at 1-10°C to reduce foam.

Table 4. Recovery medium

Reagent	Per 100 mL preparation	Final concentration
DMEM	90 mL+	
FBS	10 mL	10% (v/v)
BSA	0.25 g	0.25% (w/v)

Media preparation (test day)

9. On the day of the test, the pH of the recovery medium should be re-adjusted to 7.8 ±0.1 at 11±1°C.

10. 42 mL of recovery medium should be added into each 50 mL tube. An additional tube with recovery medium for cryovial washes (see below) should be prepared. The recovery medium is kept at room temperature.

11. The pH of ca. 100-150 mL L-15 medium (incubation medium) is adjusted to pH 7.8 at 11±1°C using 1 N HCl or NaOH. L-15 is kept on ice.

Thawing procedure

12. The cryogenic vials are removed from the liquid nitrogen vapour and immediately placed in a room temperature water bath, holding them by their caps above the water level so that the frozen suspensions are below the water line. The vials are gently moved side to side until contents freely move and a small ice crystal remains. Typically, the thawing process takes about 2 min 15 sec. If the location of the cryopreserved RT-HEP and the laboratory used for thawing are different, it is recommended to transport the vials on dry ice.

13. The content of 2-3 vials is poured into one 50 mL centrifuge tube containing 42 mL of recovery medium (room temperature).

14. Emptied cryovials are rinsed with recovery medium to suspend any remaining RT-HEP: 1 mL of recovery medium from the extra tube is added to the empty cryovials, the cryovials are then capped and inverted once to mix. The contents from the rinse are added to the 50 mL tube.

15. The centrifuge tubes are capped and gently inverted, followed by centrifugation for 5 min at 50 × g, 4°C.

16. The supernatant should be aspirated to the point where the centrifuge tube begins to taper (around 4 mL), being careful not to disturb the cell pellet. Aspirating close to the pellet may decrease yield. To obtain consistent results, the supernatant should be aspirated to the conical portion of the tube for all wash steps. The supernatant can be aspirated manually by using a pipette, or by using a vacuum pump. It should not be discarded by pouring.

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17. 5 mL of L-15 medium (pH 7.8 ±0.1, 4°C or ice cold) is added to the centrifuge tube and the cell pellet suspended. This should be done gently by tapping the side of the centrifuge tube against the back of the opposite hand. Suspensions from 2 tubes are combined into 1, if applicable, and all tubes are brought to a final volume of 45 mL with the L-15. The tubes are capped and inverted once and centrifuged at 50 × g for 5 min at 4°C.

18. Steps described in 12 & 13 are repeated if more vials are thawed and used. Again, RT-HEP are combined into 1 centrifuge tube, if applicable, and add L-15 to a final volume of 45 mL.

19. The tube is capped and inverted once and centrifuged at 50 × g for 3 min at 4°C.

20. The supernatant is aspirated to just below (~2 mm) the conical portion of the centrifuge tube, and approximately 0.75 mL of L-15 is added per cryogenic vial thawed. The RT-HEP pellet is suspended.

21. The volume of suspension is measured by using a serological pipet of appropriate size.

22. The viable and dead RT-HEP are counted using a hemocytometer and e.g. 0.04% trypan blue and dilute to the desired cell density) as described in (Fay et al., 2015). Alternative methods to count cells may be used (e.g. cell counters).

23. The yield (% of RT-HEP recovered from cryopreservation) is calculated based on the volume of the cell suspension (see §21above) and cell count results.

Number of viable RT-HEP after thawing	=	Average counted viable RT-HEP density (see §22)	x	Suspension volume prior to RT-HEP counting

Yield (% RT-HEP recovery)	=	100 x (Number of viable RT-HEP after thawing / Number of viable RT-HEP initially cryopreserved)

24. The RT-HEP suspension is diluted to the desired final cell density by adding an appropriate volume of L-15 medium.

25. The RT-HEP suspension should be kept on ice until use.

References

Fay, K.A. et al. (2014a), Optimizing the use of rainbow trout hepatocytes for bioaccumulation assessments with fish, *Xenobiotica* 44:345-351.

Fay, K.A. et al. (2014b), Intra- and interlaboratory reliability of a cryopreserved trout hepatocyte assay for the prediction of chemical bioaccumulation potential, *Environmental Science & Technology* 48:8170-8178.

Fay, K.A. et al. (2015), Determination of metabolic stability using cryopreserved hepatocytes from rainbow trout (Oncorhynchus mykiss), *Current Protocols in Toxicology* 65:4.42.1-29.

Markell, L.K. et al. (2014), Endocrine disruption screening by protein and gene expression of vitellogenin in freshly isolated and cryopreserved rainbow trout hepatocytes, *Chemical Research in Toxicology* 27:1450-1457.

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Mingoia, R.T. et al. (2010), Cryopreserved hepatocytes from rainbow trout (Oncorhynchus mykiss): A validation study to support their application in bioaccumulation assessment, *Environmental Science & Technology* 44:3052-3058.

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ANNEX 6

Preliminary Experiments to Establish Reaction Conditions

1. The primary goal of conducting preliminary experiments is to determine reaction conditions that result in first-order depletion kinetics. These experiments are used to establish a sampling schedule that captures the depletion of the test chemical (as significantly different from the negative controls) while preserving the ability to quantify the test chemical concentration in the system at final points. Preliminary experiments are performed with biological material (i.e. cryopreserved RT-HEP) that has been characterised for Phase I and II metabolic enzyme activity (refer to Annex 3).

2. An appropriate analytical method, of known accuracy, precision, and sensitivity, for the quantification of the test chemical in the test medium should be available, together with details of sample preparation and storage. The analytical limit of quantification (LOQ) of the test chemical in the test medium should be known.

3. To obtain a chemical depletion rate for use in the *in vitro* to *in vivo* extrapolation model, it is generally desirable to achieve 20-90% depletion of the test chemical over the course of a test. Variables that can be tested to achieve this goal include: the cell density of RT-HEP, starting test chemical concentration, and total incubation time (Fay et al., 2015).

4. In addition to these preliminary experiments, other considerations for achieving an accurate substrate depletion measurement include: sensitivity of the analytical method and the need for an internal standard, solvent selections to dissolve the test chemical, introduction into the system and reaction termination, and the use of positive (reference chemical) and negative controls (see Annex 4 and [OECD, 2018]).

5. It is generally recommended that substrate depletion experiments, including preliminary experiments should be conducted at densities of 1-2.0 x 10^6 hepatocytes/mL.

6. The test chemical concentration should not demonstrate cytotoxicity. Therefore, viability of RT-HEP exposed to the test concentration should be evaluated, e.g. by using trypan blue exclusion. RT-HEP viability should be ≥ 80%.

7. The starting test chemical concentration is determined by the need to achieve first-order kinetics as well as the sensitivity of the analytical method, keeping in mind the possible need to measure concentrations substantially lower than starting values (i.e., at later time points). The sensitivity of the analytical method should be able to accurately measure all time points or 10% of the initial test chemical concentration. Theory dictates that the likelihood of first-order kinetics increases as the starting concentration is decreased below the Michaelis-Menten constant, $K_{M.}$ The K_M is the substrate concentration at which the reaction rate is ½ V_{max} (maximum rate achieved by the system at substrate maximum saturation concentration). Realistically, it is not always possible to achieve these concentrations due to detection limitations of the analytical method for the test chemical. Practical experience suggests that a starting concentration in the very low micromolar/high nanomolar range (e.g. ≤ 1.0 µM) often yields satisfactory results, although users should try to perform depletion experiments at the lowest reasonable test chemical concentration. Further discussion of chemical concentrations is included in the OECD Guidance Document RT-HEP and RT-S9 - Section 3.4 (OECD, 2018).

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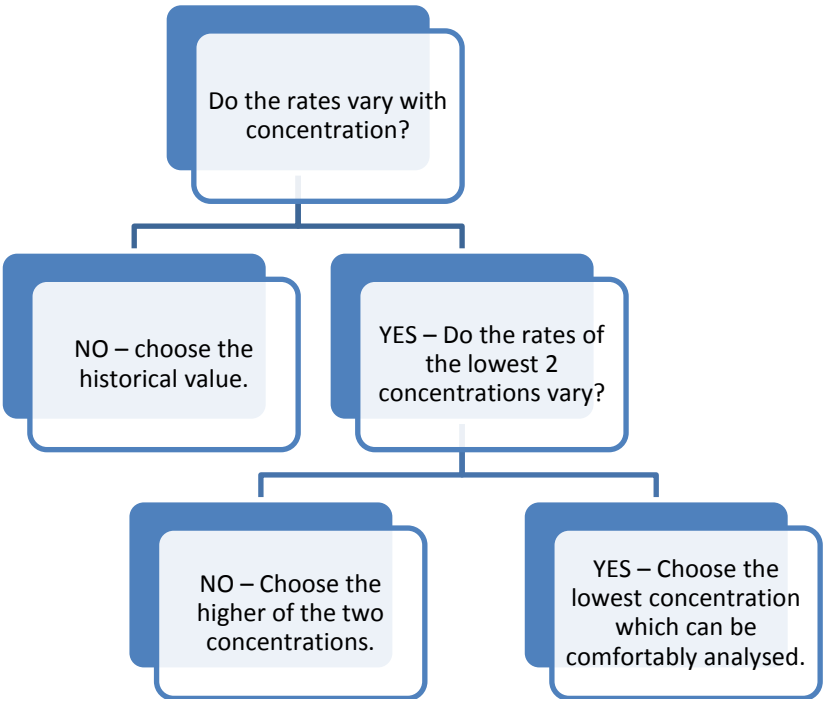
8. In order to choose appropriate starting test chemical concentration, three concentrations may be evaluated: a) 1.0 µM or other concentration based on previous information, if available; b) lowest concentration-quantifiable assuming 50% depletion, and c) a concentration in between a) and b).

9. A decision tree may be employed to make the final decisions regarding the starting incubation concentration for each test chemical.



10. Preliminary experiments are generally conducted with a limited number of time points (e.g. 0.1, 1 and 2 h). The initial test chemical concentration resulting in the most rapid depletion rate is usually preferred for the definitive study. If several test chemical concentrations generate similar depletion rates, the higher concentration is preferred for the definitive study as it minimises detection limit challenges.

11. Depending on the need, the sampling scheme may span <10 min up to 4 h incorporating the recommended six or more individual sampling time points.

12. First-order depletion rate constants derived from these tests cannot be expected to vary in direct proportion to the cell density or test chemical concentration. Very low rates of biotransformation can be addressed by increasing the incubation time up to 4 h. Increasing the RT-HEP density above 2 x 10^6 cells/mL to promote increased levels of activity is not recommended to avoid saturation of enzymes.

13. A departure from first-order kinetics can be expected if the starting chemical concentration saturates the activity of enzymes responsible for chemical clearance. For reaction pathways that exhibit classical Michaelis-Menten kinetics, this saturation will result in zero-order elimination. The appearance of zero-order kinetics suggests that the starting chemical concentration should be reduced (Johanning, 2012).

14. Alternatively, log-transformation of the data may yield a pattern suggesting bi-exponential kinetics with an initial “fast” depletion phase followed by a “slow” terminal depletion phase. This pattern can be caused by product inhibition, wherein the

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accumulation of metabolic products inhibits enzymatic activity at later time points, cofactor limitation or enzyme saturation. Reduction of both the starting chemical concentration and cell density may be attempted in an effort to eliminate this problem (Fay et al., 2015).

References

Fay, K.A. et al. (2015), Determination of metabolic stability using cryopreserved hepatocytes from rainbow trout (Oncorhynchus mykiss), *Current Protocols in Toxicology* 65:4.42.1-29.

Johanning, K. et al. (2012), Assessment of metabolic stability using the rainbow trout (Oncorhynchus mykiss) liver S9 fraction, *Current Protocols in Toxicology* 53:14.10.1-28.

OECD (2018), Guidance Document on the Determination of in vitro intrinsic clearance using cryopreserved hepatocytes (RT-HEP) or liver S9 sub-cellular fractions (RT-S9) from rainbow trout and extrapolation to in vivo intrinsic clearance, *Series on Testing and Assessment* No. 280, OECD Publishing, Paris.

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ANNEX 7

Test Set-Ups

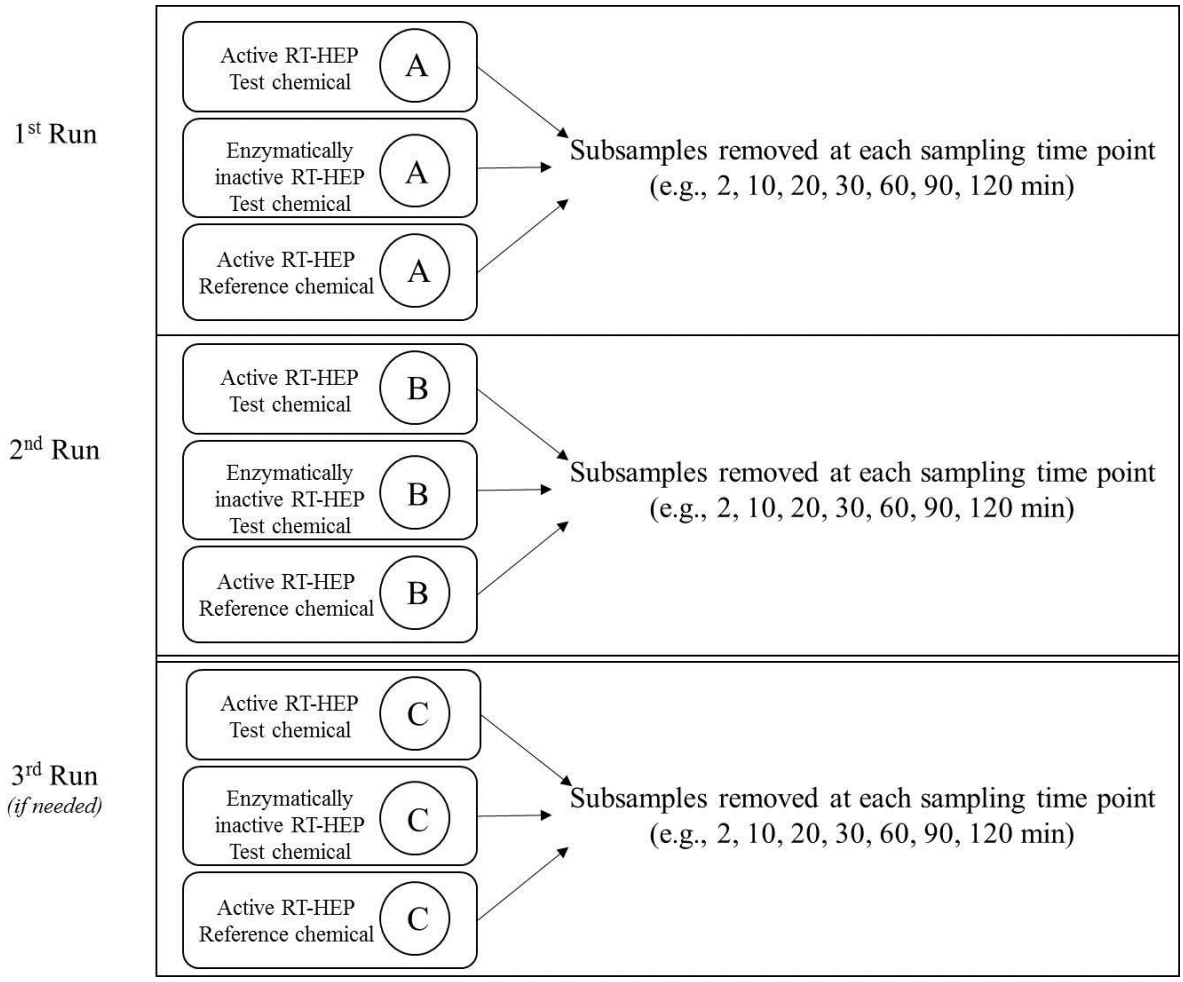
Test Set-up 1: Single vial approach (Figure 1)

1. The single vial approach is recommended to test chemicals that are not difficult-to-test (e.g. non-volatile, does not bind to vessel walls, and distributes rapidly through the incubation system). It generally produces the least variable results and is simplest to perform.

2. As described in §34ff of the main text, incubations are carried out in a single vial containing e.g. 1 mL of RT-HEP suspension. Samples (100 µL) are taken at the defined time points from this vial and are transferred into a micro-centrifuge tube containing stopping solution.

3. A minimum number of 6 time points is required to determine the $CL_{\text{IN VITRO, INT}}$; therefore, the test set-up should include ≥6 time points (e.g. 2, 10, 20, 30, 60, 90, 120 min).

Figure 8. Test Set-up 1: Independent runs using the single vial approach



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Test Set-up 2: Multiple vial approach (Figure 2)

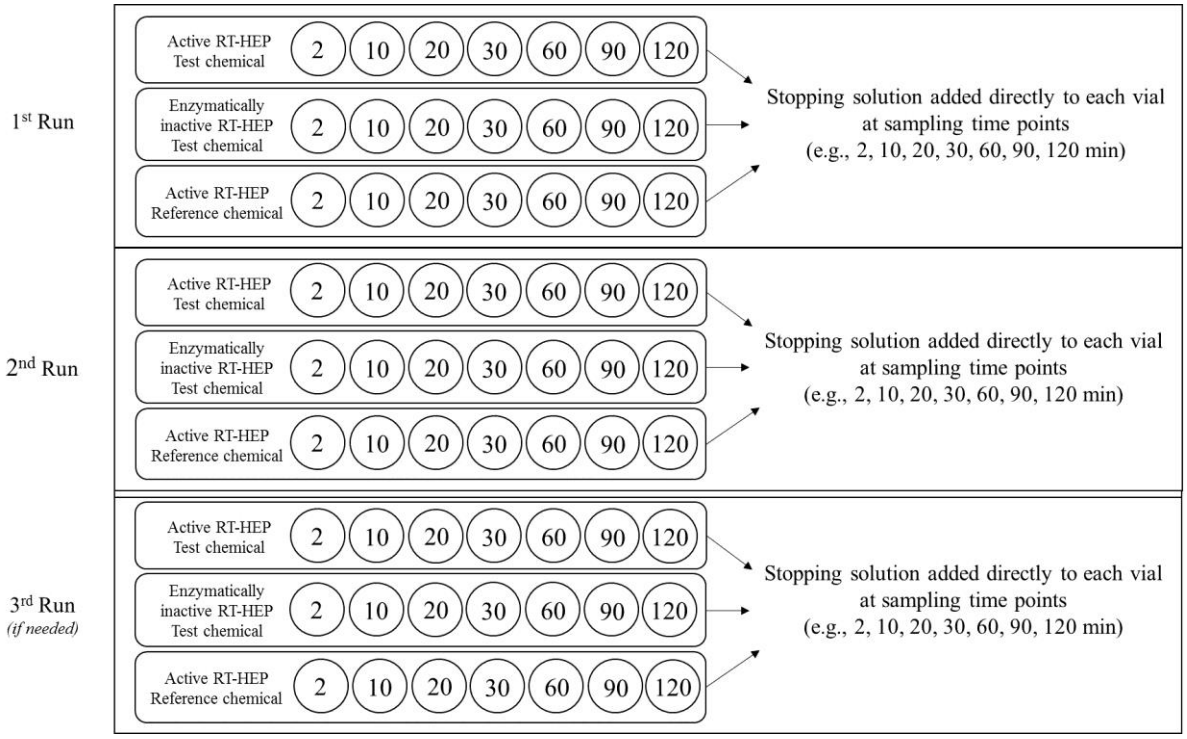
4. This set-up involves incubations in individual vials and is recommended for volatile or very hydrophobic test chemicals.

5. Incubations with volatile test substances can be performed by using closed GC vials containing e.g. 200 µL of RT-HEP suspension with a septum-lined cap after the pre-incubation period. A syringe may then be used to introduce both the test chemical and stopping solution. Alternatively, vials may be closed directly after adding the test chemical with a pipette and opened just prior to adding the stopping solution.

6. Hirschmann glass insert vials can be used for very hydrophobic chemicals.

7. As for the single-vial approach, each test consists of at least two independent runs to determine the $CL_{\text{IN VITRO, INT}}$. Each independent run is performed on a different day or on the same day, provided that for each run: a) independent fresh stock solutions and working solutions of the test chemical area prepared and; b) independently thawed RT-HEP are used. For each run, the pre-determined number of vials is prepared for active RT-HEP (e.g. total of 14, 7 for test chemical and 7 for the reference chemical) and for enzymatically inactive RT-HEP (e.g. 7). The vials are spiked with the test chemical and the reference chemical as shown in Figure 2. Stopping solution is added directly to each sample vial at the various time points (e.g. 2, 10, 20, 30, 60, 90, 120 min). Additional details are provided in (Fay et al., 2015).

Figure 9. Test Set-up 2: Independent runs using the multiple vial approach



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References

Fay, K.A. et al. (2015), Determination of metabolic stability using cryopreserved hepatocytes from rainbow trout (Oncorhynchus mykiss), *Current Protocols in Toxicology* 65:4.42.1-29.

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