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OECD GUIDELINE FOR TESTING OF CHEMICALS

Determination of in vitro intrinsic clearance using rainbow trout liver S9 sub-cellular fraction (RT-S9)

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* metabolizing systems derived from liver tissue (Nichols, Schultz and Fitzsimmons, 2006).

2. This Test Guideline (TG) describes the use of liver S9 sub-cellular fraction (RT-S9) of rainbow trout (*Oncorhynchus mykiss*) to determine the *in vitro* intrinsic clearance ($CL_{\text{IN VITRO, INT}}$) 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-HEP (OECD, 2018b) describes how to best perform these methods and how the $CL_{\text{IN VITRO, INT}}$ 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 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.

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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.

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 recognized 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 (ECHA, 2017: see Table R.7.10—6 of Appendix R.7.10-2 in the REACH guidance for a recent list). 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 2 h due to progressive loss of enzyme activity of the RT-S9. However, longer incubation times (up to max. 4 h) may be considered for very slowly biotransformed chemicals. This limits the use of the test for chemicals metabolized 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 11±1°C, and since biotransformation rates are temperature sensitive, the test temperature should be strictly controlled 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 6, Test Set-Up 2.

14. For chemicals that are ionizable, 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).

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).

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16. The methodology as described here only measures depletion of the 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. Liver S9 sub-cellular fractions from fish species other than rainbow trout could be used, provided that they can be successfully prepared and that protocols are adapted to species-specific considerations (OECD, 2018a).

SCIENTIFIC BASIS OF THE METHOD

18. Rainbow trout liver S9 sub-cellular fraction is relatively easy to prepare and to use (Johanning et al., 2012; ANNEX 2). In a frozen state (-80°C), samples can be shipped to different locations and stored for at least up to two years. Furthermore, it is possible to use the same lot of RT-S9 for several tests separated in time.

19. The RT-S9 contains enzymes responsible for both Phase I (e.g., cytochrome P450 (CYP)) and Phase II biotransformation (e.g., sulfotransferases (SULT), uridine 5'-diphospho-glucuronosyltransferases (UGT), glutathione transferases (GST)) (Ekins et al., 1999).

PRINCIPLE OF THE TEST

20. The $CL_{,\ \text{IN VITRO, INT}}$ of the test chemical is determined by using a substrate depletion approach. The incubation system consists of RT-S9 and potassium phosphate buffer supplemented with enzymatic cofactors and alamethicin to support both Phase I and II biotransformations. The reaction is initiated by the addition of the test chemical. 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 over time is measured with a validated analytical method (see §22) and used to determine the $CL_{,\ \text{IN VITRO, INT}}$. Incubations using enzymatically inactive RT-S9 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 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 behavior (TGs 107, 117, 123; [OECD, 1995b, 2004a, 2006a);
- Test chemical stability in water (TG 111; [OECD, 2004b]) and/or test medium;
- Vapor 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 ionize.

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,

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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 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:

- The RT-S9 lots should be evaluated for the ability to catalyze Phase I and II metabolic enzymatic reactions as described in ANNEX 3;
- The protein concentration of the RT-S9 lots should be determined (see ANNEX 2). The substrate (test chemical) and protein concentrations should be determined in preliminary experiments achieving first-order kinetics as described in ANNEX 5. The protein concentration in the final incubation mixture may range from 0.25 - 2 mg/mL, with 1 mg/mL most commonly used;
- Negative (enzymatically inactive RT-S9) controls should demonstrate no significant loss of parent chemical over the incubation time (i.e., <20% of loss determined in enzymatically active RT-S9 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 the $CL_{\text{IN VITRO, INT}}$; 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 metabolized (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-S9 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.

DESCRIPTION OF THE METHOD

Apparatus

26. The following equipment is required:

- 4°C refrigerator;
- -20°C freezer;
- -80°C freezer;
- Analytical balance to weigh out reagents, cofactors and test chemicals;
- pH meter;
- Vortex mixer;

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- Refrigerated centrifuge for micro-centrifuge tubes or alternative test tubes used in the incubation and/or stopping reaction step;
- 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;
- Sample glass vials for HPLC/GC or other analytical instruments;
- Pipettes and tips.

Cofactors, chemicals to prepare reagents

27. The following are required (analytical grade or equivalent):

- Nicotinamide adenine dinucleotide 2'-phosphate, tetrasodium salt (NADPH)
- Uridine 5'-diphosphoglucuronic acid, trisodium salt (UDPGA)
- L-Glutathione reduced (GSH)
- Adenosine 3'-phosphate 5'-phosphosulfate lithium salt hydrate (PAPS)
- Alamethicin from *Trichoderma viride*
- Potassium phosphate dibasic (K_2HPO_4)
- Potassium phosphate monobasic (KH_2PO_4)

Chemicals for analytical measurements

28. 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 potassium phosphate reaction mixture.
- Stopping and extraction solvents, analytical grade or equivalent (e.g., methanol, acetonitrile, methylene chloride, methyl-tert-butyl ether).

RT-S9

29. RT-S9 can be obtained from commercial sources, if available, or prepared following the example protocol in ANNEX 2.

30. The protein concentration of each RT-S9 lot should be determined (see ANNEX 2) and each RT-S9 lot should be evaluated for its ability to catalyze Phase I and II biotransformation reactions. Standardized assays to determine Phase I and Phase II enzyme activity are briefly described in ANNEX 3. These characterization assays or known reference chemicals should be used to test a new lot of RT-S9 at the beginning of the test or before the lot is used for the first time. They should also be used occasionally to monitor possible activity losses during storage.

31. The inclusion of enzymatically inactive (e.g., heat-inactivated) RT-S9 is mandatory. Incubations using enzymatically inactive RT-S9 serve as a negative control to distinguish between enzymatic biotransformation and abiotic decrease by adsorption to the incubation vial, volatilization, 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.

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Test set-up

32. Preliminary experiments that include range finding conditions (e.g., substrate concentration, protein concentration and incubation time) should be conducted to establish incubation conditions needed to reliably measure intrinsic *in vitro* hepatic clearance of the test chemical. ANNEX 5 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 6. This test set-up 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) at one test concentration. It generally produces the least variable results and is simplest to perform. For volatile or very hydrophobic test chemicals, the multiple vial approach is recommended (ANNEX 6, 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-S9 is used; however, RT-S9 may come from the same lot. If the calculated regression from the two runs with active RT-S9 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-S9 and enzymatically inactive RT-S9 is spiked with the test chemical, and one vial for active RT-S9 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. For incubations with enzymatically inactive RT-S9, sampling time points may be reduced if warranted based on preliminary experiments (ANNEX 5).

37. In the following paragraphs, the single vial approach is described whereas the multiple vial approach is detailed in ANNEX 6.

Preparation of test chemical, buffers, cofactors and stopping solution

38. Stock solution(s) of the test chemical should be prepared in potassium phosphate buffer (ANNEX 7) or in 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 freshly (OECD, 2018a: Section 3.2).

39. On the day of the test, the desired spiking concentration of the test chemical is prepared by diluting the stock solution with potassium phosphate buffer (ANNEX 7) or an organic solvent based on the results of the preliminary experiments (see §25; ANNEX 5; OECD, 2018a). If an organic solvent is used, the total amount in the incubation mixture should be kept as low as possible and should not exceed 1% to avoid inhibition of enzyme activity. It has to be kept in mind that the alamethicin solution contains a significant proportion of organic solvent which contributes to the final solvent concentration (i.e., 0.25%). In general, the test concentration should be approximately 10-

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fold higher than the LOQ of the analytical method as long as it results in first order kinetics as determined in the preliminary experiment (§25; ANNEX 5).

40. ANNEX 7 describes the preparation of the buffers, cofactors and alamethicin solutions needed. Stock solutions can be prepared in advance, e.g., one day before the test, whereas spiking solutions should be prepared on the day of the test.

41. 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 vaporize 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 (OECD, 2018a: Section 3.3).

Preparation of RT-S9 and incubation mixtures

42. A sufficient volume of active RT-S9 and enzymatically inactive RT-S9 are thawed and diluted with potassium phosphate buffer (see ANNEX 7) to a 10x higher protein concentration (e.g., 10 mg/mL) compared to the protein concentration in the final incubation mixture (e.g., 1 mg/mL). ANNEX 7 includes an example calculation. The active RT-S9 should be thawed in an ice-water bath. An additional volume of e.g., 25-30% is recommended to provide a modest excess of biological material for both active and enzymatically inactive RT-S9. For example, if a total of 300 µL diluted RT-S9 is needed for each set of active and inactive RT-S9 respectively, then prepare about a total of 400 µL.

43. For the single vial approach as shown in Figure 1 of ANNEX 6, two vials with active and one vial with enzymatically inactive RT-S9 are used, taking into account the number of time points in the test set-up. *Note:* Excess active RT-S9 should not be re-frozen for later use in depletion tests but may be used for as the preparation of enzymatically inactive RT-S9. Excess inactive RT-S9 may be re-frozen for later use.

44. Incubation vials are prepared by adding 400 µL of potassium phosphate buffer pH 7.8 (see ANNEX 7) to each of the three vials. The diluted active or enzymatically inactive RT-S9 (100 µL each) is added to the corresponding incubation vials. Then, 100 µL of the alamethicin working solution (250 µg/mL) is added to each of the incubation vials and the vials are pre-incubated on ice for 15 min.

45. A master mix of cofactors (NADPH, UDPGA, GSH, and PAPS) is prepared as described in ANNEX 7. The master mix is mixed well and stored on ice for the least amount of time before addition to the RT-S9 mixture. PAPS degrades rapidly and therefore should be prepared during the pre-incubation with alamethicin and added immediately prior to §46.

46. 400 µL of the cofactors master mix is added to each of the pre-incubated vials on ice. Each vial is gently swirled until thoroughly mixed. Refer to Figure 1 in ANNEX 7 for details of the contents in the incubation mixture.

47. The incubation vials of active and enzymatically inactive RT-S9 are placed into a shaking water bath or incubator and pre-incubated at 11± ℃ for 10 minutes with gentle shaking.

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Incubation with test chemical and stopping of reaction

48. Test chemical (usually 5 µL; however, this depends on the concentration of the spiking solution) is directly added into the incubation mixture of each vial (usually 1 mL) to initiate the reaction. The vials are swirled to distribute the chemical and loosely capped.

49. For sampling at a specified time point, the incubation vial is removed from the water bath or incubator, gently swirled or shaken, 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 §41). To ensure quantitative transfer of the sample, pipetting up and down in the solvent three times is recommended.

50. 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 have to 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.

51. After the sampling is completed or for volatile solvents at each sampling point, micro-centrifuge tubes are vortexed (e.g., for 3 min at 1500 to 2000 rpm) and centrifuged (e.g., 5 min at 20,000 × g and 4°C). If recovery of the test chemical in the preliminary experiment is poor, longer vortexing time (e.g., 10 min) may be needed. 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

52. 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).

53. 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 of the chemical analysis, as well as recovery (80-120%) of the test chemical from the incubation mixture should be verified experimentally.

DETERMINATION OF IN VITRO INTRINSIC CLEARANCE

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

55. 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 behavior 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.

56. If there is an abiotic loss of test chemical from enzymatically inactive RT-S9 which cannot be avoided by optimization 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, 2018a). In this case, however, it must be verified that the abiotic loss process follows first-order kinetics.

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

58. k_e is divided by the measured protein concentration to obtain the $CL_{\text{IN VITRO, INT}}$ (mL/h/mg protein).

TEST REPORT

59. 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:
  - characterized 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-S9

- 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 Reporting Template in ANNEX 2
- Characterization (see ANNEX 3)

Test conditions

- Concentration of test chemical and reference chemical
- Method of preparation of stock solution(s) of test chemical and reference chemical (name and concentration of solvent, if applicable)
- Preparation and composition of reagents
- Preparation and composition of incubation mixture

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- Incubation temperature
- Amount of protein in the incubation mixture
- Test set-up: method of incubation (single vial or multi 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 experiments performed
- Data from individual vials, time points for each independent run (e.g.; test chemical, reference chemical, active and enzymatically inactive RT-S9)
- If measured, appearance of formed metabolites (including as optional reporting: identification of metabolites and metabolic pathways)
- Calculated $CL_{\text{IN VITRO, INT}}$ from independent incubations with active and enzymatically inactive RT-S9 (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 $CL_{\text{IN VITRO, INT}}$ 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.

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ANNEX 1

Abbreviations & Definitions

BCF	Bioconcentration factor (L/kg)
$CL_{\text{IN VITRO, INT}}$	*in vitro* intrinsic clearance (mL/h/10^6 cells or mL/h/mg protein)
CYP	Cytochrome P450
DTT	DL-Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EROD	Ethoxyresorufin-O-deethylase
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
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HPLC	High Performance Liquid Chromatography
IVIVE model	*In vitro* to *in vivo* extrapolation model
k_e	Elimination rate constant (h^{-1})
K_{ow}	n-Octanol-water partition coefficient
K_M	Michaelis-Menten constant
LOQ	Limit of quantification
MS-222	Tricaine methanesulfonate
NADPH	Nicotinamide adenine dinucleotide 2'-phosphate
PAPS	Adenosine 3'-phosphate 5'-phosphosulfate
pKa	Acid dissociation constant

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rpm	Revolutions per minute
RT-S9	Rainbow trout liver S9 subcellular fraction
SULT	Sulfotransferase
S9 sub-cellular fraction	Supernatant fraction obtained from an organ (usually liver) homogenate by centrifuging at 9000 × g for 20 minutes in a suitable medium; this fraction contains cytosol and microsomes; note S9 from fish is centrifuged at 13 000 × g
TG	Test Guideline
UDPGA	Uridine 5'-diphosphoglucuronic acid
UGT	Uridine 5'-diphospho-glucuronosyltransferase
V_{max}	Maximum enzymatic rate at saturating test chemical concentration

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ANNEX 2

Example Protocol for Preparation of Rainbow Trout Liver S9 Sub-Celullar Fraction (RT-S9)

NOTE: RT-S9 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 (Johanning et al., 2012) and was used in the ring trial (*OECD, 2018*).*

Fish

1. RT-S9 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., 2012, 2010; Fay et al., 2014). RT-S9 can therefore be collected without regard to gender.

2. If fish are obtained from a supplier, they should be acclimatized 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 10-15°C. 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 2).

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 2). 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. It is strongly recommended to use fresh liver tissue for S9 preparation. Previous work has shown that freezing and thawing fish liver tissue reduces the activities of CYP enzymes (Förlin and Andersson 1985; Lindström-Seppä and Hänninen, 1988).

7. Generally, pooling RT-S9 from several fish (three to six) is recommended. This approach will diminish the influence of a single fish, and better represent a population.

8. After humanely killing of the fish (see §19), the hepatic portal vein is cannulated, and the liver is perfused with a Ca^{2+}/Mg^{2+}-free balanced salt buffer (clearing buffer); see Table 1) to clear the liver of blood.

9. The liver is excised from the fish, homogenized in homogenization buffer (Table 1) and centrifuged at 13,000 - 15,000 x g for 20 min at 4°C.

10. The resulting supernatant from the centrifugation is aspirated and aliquoted out in micro-centrifuge tubes or cryovials and stored at -80 ±1°C.

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Apparatus and Material

11. Apparatus

- Vessels to expose fish to anesthetic
- Digital balance (1 g – 2000 g), weigh boats
- Forceps, large and small sharp surgical scissors, one-edge razor blades
- Refrigerated centrifuge (e.g., for 50 mL tubes)
- Conical centrifuge tubes, e.g., 50 mL
- 23-G × ¾ safety winged infusion set (butterfly catheter)
- 30 mL disposable plastic syringes
- Peristaltic pump
- Pump tubing
- 6 cm glass petri dishes, pre-chilled
- Glass beakers
- 30 mL Wheaton Potter-Elvehjem mortar with Teflon pestle
- Multi-speed bench-top drill press
- Pipets
- Pipet tips
- Pipet aid for serological pipets
- Serological pipets
- 1.8 mL working volume cryogenic storage tubes
- Micro-centrifuge tubes

12. Chemicals and reagents

- Tricaine Methanesulfonate (MS-222)
- Sodium bicarbonate ($NaHCO_3$)
- Hanks' Balanced Salt Solution (HBSS) without Ca^{2+} and Mg^{2+}
- 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES)
- Tris[hydroxymethyl]aminomethanehydrochloride) (Tris-Cl), 1 M; pH 7.8
- Potassium chloride (KCl), 1 M
- Ethylenediaminetetraacetic acid disodium salt (EDTA-Na_2; 0.5 M)
- DL-Dithiothreitol (DTT), 100 mM)
- Protease inhibitor cocktail (optional)
- Sucrose
- Sodium hydroxide (NaOH), 1 M
- Potassium hydroxide (KOH), 1 M
- Commercial protein assay to determine RT-S9 protein content.

Preparation of Reagents and Solutions

13. The *tricaine methanesulfonate* (MS-222; 150 mg/L) should be prepared with water from the same source used to maintain the fish prepared; e.g., 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 3 times that of the MS-222.

14. Two buffers (clearing buffer and homogenization buffer) are prepared as listed in Table 1. The amounts provided are sufficient to perfuse approximately 15 fish.

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Table 1. Reagents and concentrations for clearing and homogenization buffers

	Reagent	Per 1000 mL preparation	Concentration
Clearing buffer pH 7.8; store at 4°C	1 × HBSS (without Ca^{2+}/Mg^{2+} salts)	985.4 mL	
	0.5 M EDTA	4.6 mL	2.3 mM
	1 M HEPES	10 mL	10 mM
Homogenization buffer* pH 7.8; store at 4°C	1 M Tris-HCl pH= 7.8	800 mL	50 mM
	1 M KCl	150 mL	150 mM
	0.5 M EDTA	4 mL	2 mM
	100 mM DTT	10 mL	1 mM
	sucrose	85.55 g	250 mM

* Protease inhibitors may be added to the homogenization buffer

15. Clearing buffer is prepared as follows: Hanks Balanced Salt Solution (HBSS containing 4.2 mM $NaHCO_3$ (0.35 g/L); without Ca^{+2} and Mg^{+2}) is supplemented with EDTA (target concentration 2.3 mM) and HEPES (target concentration 10 mM). For example: to 985.4 mL of HBSS (containing $NaHCO_3$) 4.6 ml of 0.5 M EDTA and 10 ml of 1 M HEPES are added. The pH is adjusted to 7.8 using 1 M NaOH and the clearing buffer is stored at 4°C. Buffers are prepared fresh monthly and discarded if visibly contaminated (i.e., floating particles and cloudy).

16. Homogenization buffer (e.g., 1 L) is prepared by combining first 50 ml of 1 M Tris-HCl and 950 ml of ultrapure water. In a 1 L volumetric flask 800 ml of 50 mM Tris-HCl is combined with 150 ml 1 M KCl, 4 ml 0.5 M EDTA, 10 ml 100 mM DTT, and 85.55 g sucrose. The pH is adjusted to 7.8 with 1 M KOH, then brought up to the final volume with 50 mM Tris-HCL. The homogenization buffer is stored at 4°C. Buffers are prepared fresh monthly and discarded if visibly contaminated (i.e., floating particles and cloudy). If used, protease inhibitors should be added as a last step.

Detailed Description

Preparation of fish and surgery

17. Fish should fast 24 h prior to sacrifice.

18. Because RT-S9 proteins degrade rapidly at room temperature, every effort should be made to carry out these procedures using ice-cold buffers, instruments, and glassware.

19. Using a net, fish are captured and transferred to a tank or bucket containing 8 L of anesthetic solution (MS-222) prepared earlier. The fish should be immersed in the MS-222 solution for at least 1 min. The fish is properly anesthetized 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). Subsequently, the fish should be humanely killed with a sharp blow to the head.

20. The weight of the fish is recorded.

21. The fish is placed with the ventral surface facing up. As illustrated in Figure 1, 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.

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22. By folding back and cut away the resulting flap, the body cavity is exposed and the liver should be dark red (Figure 2). 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.

23. It is optional to loosely loop silk suture material (4/0) under the hepatic portal vein before cannulating it (Figure 2) using a safety winged infusion needle set, 23-G × 3/4-in., attached to a 30 mL syringe filled with ice-cold clearing buffer (see Table 1). The 4/0 silk suture is tightly drawn around the needle and tied off to prevent leakage of the clearing buffer from the insertion site. The hepatic vein(s) leading from the liver to the heart is severed to permit drainage of the tissue.

Liver perfusion

24. The liver is perfused (about 10 to 15 mL/min) with 20 to 30 mL of ice-cold clearing buffer until the tissue is pale in color (i.e., blood is removed; Figure 3). The liver is gently massaged while perfusing to aid flow of the blood, especially areas where the blood is concentrated. Removal of blood is required to ensure that RT-S9 is free of blood-borne metabolizing enzymes (e.g., plasma proteases).

25. After removal of the blood, the liver is excised and placed on an ice-cold petri dish, taking care not to cut open the gall bladder (a thin-walled sac, usually containing dark green or brown bile). The gall bladder is carefully removed by using scissors to sever the connective tissue that attaches it to the liver. The liver is rinsed with 5 to 10 mL of ice-cold clearing buffer, kept on hand in a 30 mL syringe for this purpose. *Note:* It is important that gall bladder bile does not contaminate the liver RT-S9 since this will denature and inactivate metabolic enzymes. This rinse step is employed as a precaution to deal with possible contact of the liver with small amounts of bile. Livers that come in contact with large amounts of bile should be rejected from further processing if possible.

26. Before weighing the livers to the nearest 0.01 g, excess fluid is removed from the liver with a paper towel. The liver weight is recorded (Table 2). The liver is placed in a 250 mL beaker containing 150 mL ice-cold homogenization buffer kept on ice until all the fish for that specific batch are sampled and livers collected.

27. 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 (Table 2). 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). Detailed descriptions of gonadal development in trout may be found in (Billard and Escaffre, 1975; Gomez et al., 1999; Le Gac et al., 2001).

28. The desired number of fish is sampled as described in §§17-27. It is important to proceed quickly. The total length of time from netting a fish to placing a liver in the homogenization buffer should be <15 min.

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RT-S9 processing and preparation

29. After sampling and weighing the livers, these are treated as follows. The livers are cut into small (<0.5 cm^2) pieces with a pair of scissors and/or one edge razor blades.

30. The minced tissue in homogenization buffer is transferred to a Potter-Elvehjem mortar (pre-chilled and kept on ice) and the beaker rinsed with an additional volume (equivalent to the liver weight) of homogenization buffer. This volume along with any remaining tissue is transferred to the mortar. The tissue is homogenized using a Teflon pestle (normal clearance 0.1 to 0.15 mm) attached to a bench-top drill press set at low speed (e.g., 500 rpm). This should be done by forcing the pestle slowly (10 to 15 sec per stroke) to the bottom of the mortar and should be repeated four to five times. Additional homogenization is not required and may cause protein denaturation.

31. The liver homogenate is poured into a 50 mL round-bottom centrifuge tube (pre-chilled and kept on ice). All livers are processed as described in §§ 24-28. Assuming the fish size range (400 to 600 g) and sample number (four to five) recommended here, the expected volume of raw liver homogenate is approximately 50 to 70 mL.

32. The liver homogenates are centrifuged for 20 min at 13,000 × g, 4°C. The tubes are gently removed from the centrifuge. Depending on the lipid content of livers being sampled, a layer of yellow lipid may form on the surface of the supernatant. This layer is aspirated with a Pasteur pipet and discarded. The remaining supernatant (i.e., the pooled RT-S9) is decanted or aspirated with a serological pipet into a chilled 150 mL beaker taking care not to transfer any material from the pellet. The pellet at the bottom of the centrifuge tube should be relatively firm and brown in color. A lighter-colored layer may form on the surface of the pellet.

33. The pooled RT-S9 is mixed using a glass rod or equivalent, then aliquots (e.g., 0.5 mL) of the solution are transferred to pre-labeled 1.8 mL cryogenic storage tubes or micro-centrifuge tubes and immediately snap frozen in liquid nitrogen (or equivalent). *Note:* It is extremely important to keep RT-S9 on ice at all times. Four to five fish of the recommended size will yield 35 to 45 mL of RT-S9. Assuming that this is divided into 0.5 mL aliquots for storage, it will be necessary to pre-label 70 to 90 cryogenic storage tubes. Labeled tubes should be chilled before samples are added and stored not more than 24 h in a −20°C freezer until just prior to use.

34. The RT-S9 containing storage tubes are collected and transferred to liquid nitrogen or a -80 ±1°C freezer for storage (for at least 2 years).

35. The total volume of RT-S9 obtained from a sampling effort is recorded. This value is used to calculate the concentration of RT-S9 protein per gram of liver tissue.

Determination of RT-S9 protein content

36. Three RT-S9 samples are thawed in an ice-water bath (e.g., micro-centrifuge tube holders floating in a beaker containing ice and water) and placed on ice once the ice crystals are dissolved. The protein content is determined in a well-mixed homogenous RT-S9 sample using standard assay (e.g., [Bradford, 1976; Lowry et al., 1951]) or a commercially available protein assay kit following the manufacturer's instructions (e.g., 96-well flat-bottom plates and a microplate reader, bioanalyzer, cuvettes and spectrophotometer or other instrumentation and methodologies that allow accurate protein concentration determination). The dilution of RT-S9 used for the protein concentration determination should be such that the value falls in the linear section of the standard

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curve to avoid under or overestimations of protein content. The protein content of the given RT-S9 lot is the average of replicate sample vials.

Reporting Template

Table 2. Record of fish information, maintenance conditions, and individual fish observations.

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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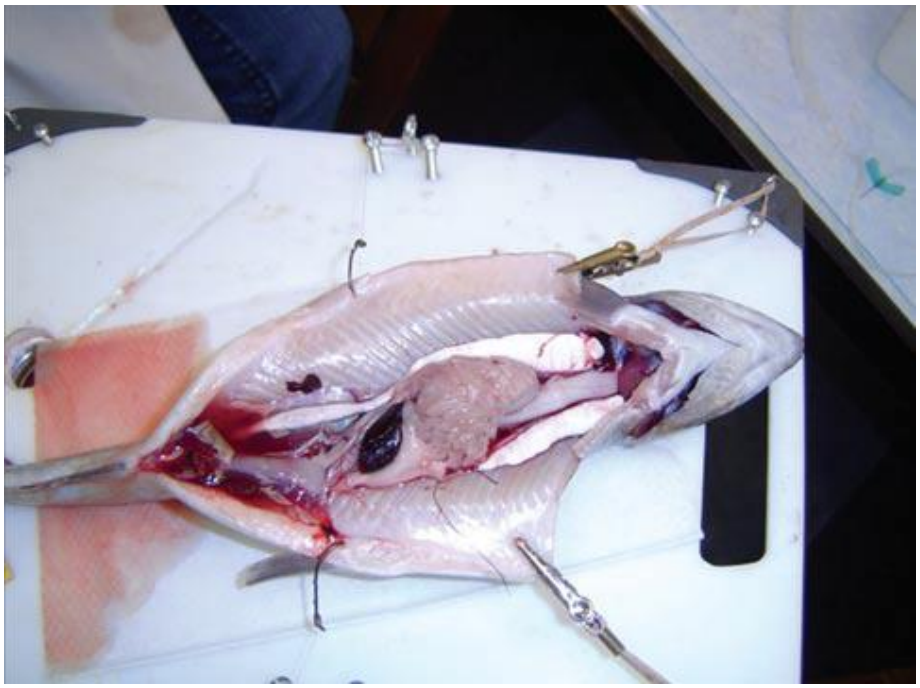
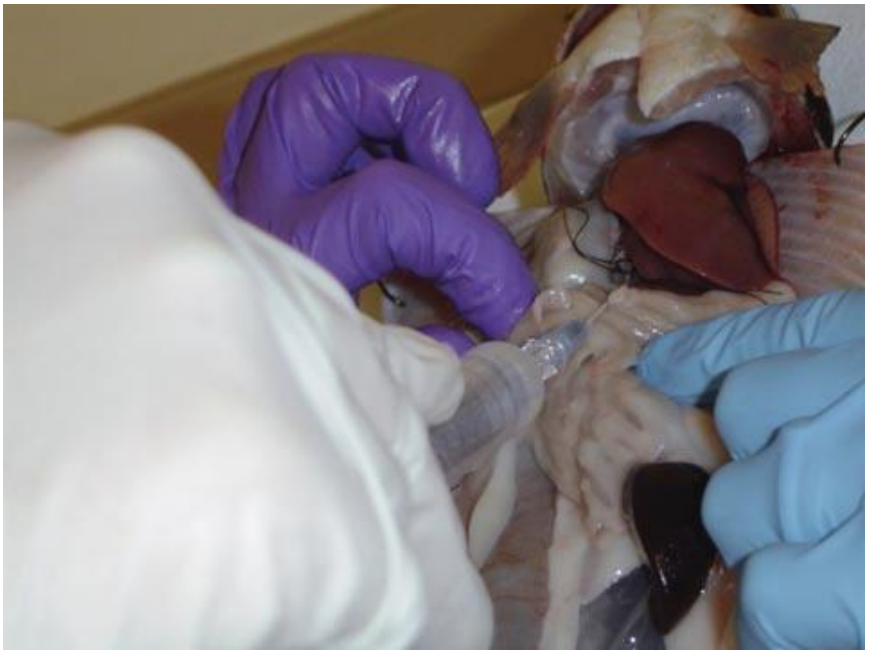
Figures

Figure 1. Incisions required exposing the internal organs of the fish after it has been euthanized.



Note: The fish in this picture is sexually mature and is shown for illustrative purposes only.
Source: Johanning et al. (2012).

Figure.1. Cannulation of the hepatic portal vein to clear the liver of blood.



Source: Johanning et al. (2012).

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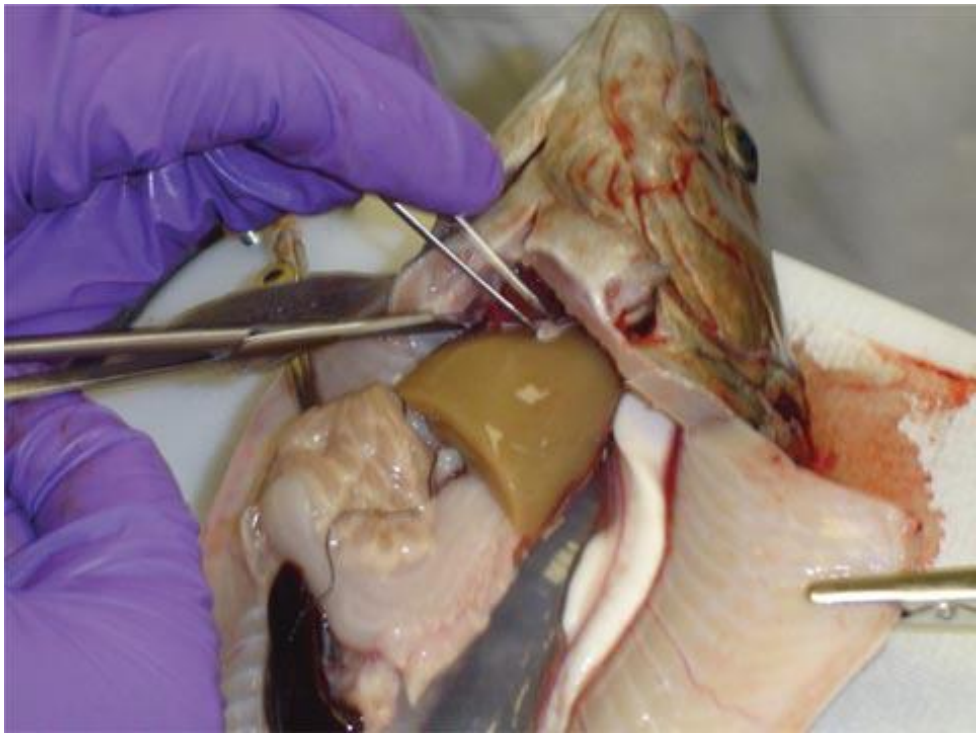
Figure 3. Blanched appearance of a liver that has been successfully cleared of blood.



Note: The fish in this picture is sexually mature and is shown for illustrative purposes only. Sexually immature animals should be used to obtain RT-S9.
Source: Johanning et al. (2012).

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ANNEX 3

Characterization of Rainbow Trout Liver S9 Sub-Cellular Fraction (RT-S9)

1. Each isolated RT-S9 lot should be evaluated for the ability to catalyze Phase I and II biotransformation reactions. These characterization assays may be performed on freshly prepared or thawed RT-S9 fractions.

2. Suggested standardized assays for measuring Phase I and Phase II biotransformation activity are listed in Table 1 and 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.

3. Activity of RT-S9 may also be evaluated using well-characterized, known test chemicals.

4. Generally, the RT-S9 is diluted to the necessary protein concentration specified by the particular assay. The results are then normalized to protein content, determined as described in ANNEX 2.

5. 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 standardized 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) utilized should be considered.

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Table 1. List of commonly-used enzyme activity assays, substrates, and references that can be used to characterize activity of RT-S9.

	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-pentoxyresorufinO-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
Phase II	CDNB-glutathione conjugation	GST	Glutathione conjugation	1-chloro-2,4-dinitrobenzene	Habig, Pabst and Jakoby, 1974
	p-Nitrophenol glucuronidation	UGT	Glucuronidation	p-Nitrophenol	Castren and Oikari, 1983; Ladd, Fitzsimmons, and Nichols, 2016

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ANNEX 4

Preparation of Enzymatically Inactive Rainbow Trout Liver S9 Sub-Cellular Fraction (RT-S9)

1. It is suggested that laboratories consider preparing a large volume of enzymatically inactive RT-S9 in advance, and freeze them as aliquots (e.g., 0.5 mL).

2. RT-S9 is enzymatically inactivated to provide negative control material for the substrate depletion test. Inactivation is routinely achieved by heating at 100°C (in boiling water) in a capped vial. The resulting enzymatically inactive material may be processed using a hand-held homogenizer to produce a sample that can be easily pipetted if there is excessive agglutination in the material (Johanning et al., 2012).

3. If there is a substantial decrease (>20%) of the test chemical in the enzymatically inactive RT-S9 control, an alternative negative control may be used (see OECD Guidance Document RT-HEP and RT-S9, Annex 4 [OECD, 2018]).

4. Equipment:

- Hot plate
- Beaker with water (water bath)
- Vessel for boiling the RT-S9 within the water bath with a cap
- hand-held tissue homogenizer (e.g., 15 mL) (Optional)
- 1.5 mL micro-centrifuge tubes

5. Material:

- RT-S9 with known protein content

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

7. A beaker of water is heated on a hotplate and the water brought to boiling. A capped vessel with the RT-S9 suspension is placed into the boiling water bath, and the suspension brought to a slow boil for 15 min.

8. After the RT-S9 has cooled down, this is transferred to a graduated cylinder and the volume adjusted by adding 100 mM potassium phosphate buffer to maintain the desired concentration. OPTIONAL: If excess agglutination is observed, transfer to a handheld homogenizer. Homogenize the sample by hand until the solution is homogeneous (Johanning et al., 2012).

9. The RT-S9 should be diluted with the 100 mM potassium phosphate buffer to the desired concentration (e.g., 10 mg/mL) before use in a test.

10. Aliquots (e.g., 0.5 mL) of the enzymatically inactive RT-S9 are transferred into 1.5 mL micro-centrifuge tubes and stored at -20 ±1°C until used.

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References

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 5

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 RT-S9 that has been characterized 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% to 90% depletion of the test chemical over the course of the test. Variables that can be tested to achieve this goal include: protein concentration, starting test chemical concentration, and total incubation time (Johanning et al., 2012).

4. In addition to these range finding conditions, 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 (refer to ANNEX 4 and [OECD, 2018]).

5. It is generally recommended that substrate depletion experiments, including preliminary experiments may be conducted at protein concentrations of 0.25-2 mg/mL, with 1 mg/mL most commonly used.

6. 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 (OECD, 2018).

7. 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).

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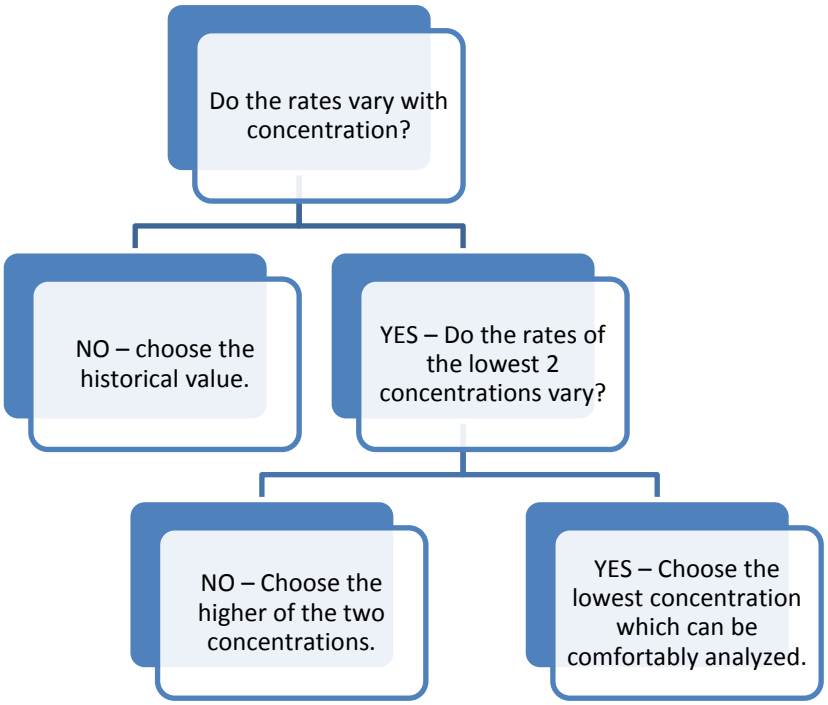
8. A decision tree may be employed to make the final decisions regarding the starting incubation concentration for each test chemical.



9. Preliminary experiments are generally conducted with a limited number of time points (e.g. 6, 30 and 60 min). The initial test chemical concentration resulting in the most rapid depletion rate is usually preferred for the definitive test. If several test chemical concentrations generate similar depletion rates, the higher concentration is preferred as it minimizes detection limit challenges.

10. Depending on the need, the sampling scheme may span <10 min up to usually up to 2 h, for very slowly biotransformed test chemicals up to 4 h may be possible incorporating the recommended six or more individual sampling time points.

11. First-order depletion rate constants derived from these tests cannot be expected to vary in direct proportion to the protein concentration or test chemical concentration. Increasing the protein concentration above 2 mg/mL is not recommended to avoid saturation of enzymes.

12. 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 et al., 2012).

13. 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 accumulation of metabolites inhibits enzymatic activity at later time points, cofactor limitation or enzyme saturation. Reduction of both the starting chemical concentration

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and protein concentration may be attempted in an effort to eliminate this problem (Johanning et al., 2012).

References

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 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 6

Test Set-Ups

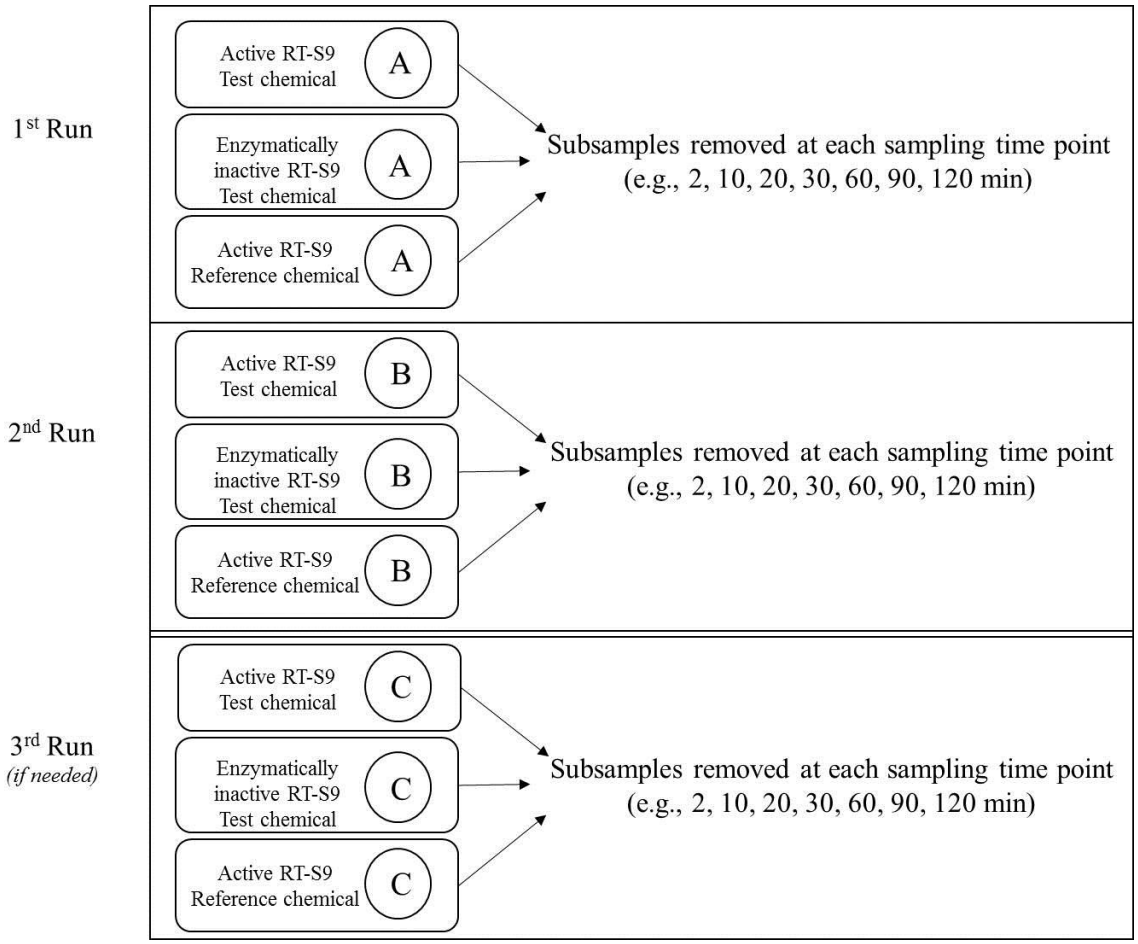
Test Set-up 1: Single vial approach (Figure 1)

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-S9 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 1. Test Set-up 1: Independent runs using the single vial approach



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

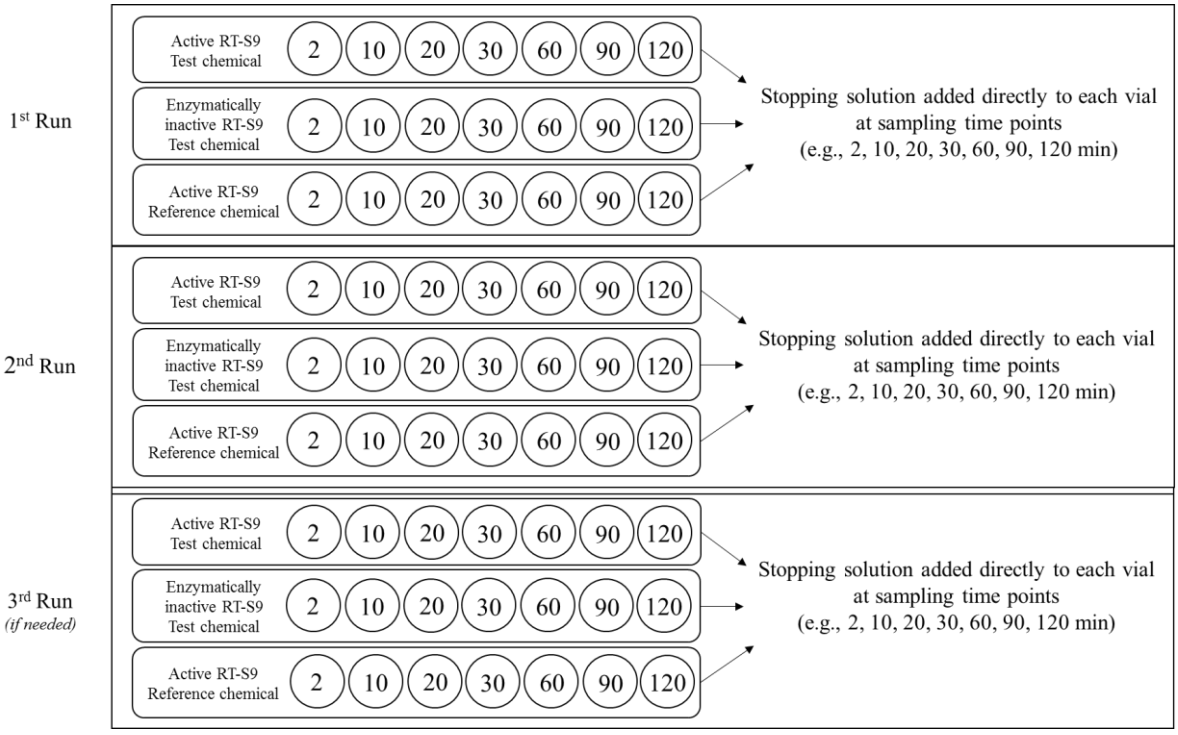
4. This set-up involves incubations in individual vials and is recommended for volatile or very hydrophobic test chemicals.

5. Incubations with volatile test substances can be performed by using closed GC vials containing e.g., 200 µL of RT-S9 incubation mixtures 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-S9 is used. For each run, the pre-determined number of vials is prepared for active RT-S9 (e.g., total of 14, 7 for test chemical and 7 for the reference chemical) and for enzymatically inactive RT-S9 (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 (Johanning et al., 2012).

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



References

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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ANNEX 7

Preparation of Buffers, Cofactor and Alamethicin Solutions, and Dilution of RT-S9 to Working Protein Concentration

1. Potassium phosphate mono and dibasic buffer stock solutions should be prepared as listed in Table 1.

Table 2. Potassium phosphate mono and dibasic buffer stock solutions

Reagent	mM	mg	Volume ultrapure water (mL)	Storage temperature & time
Dibasic (K_2HPO_4) potassium phosphate buffer	100	1.742	100	4°C; max 1 month
Monobasic (KH_2PO_4) potassium phosphate buffer	100	0.681	50	4°C; max 1 month

2. Potassium phosphate buffer (KPO_4 buffer; pH 7.8, 100 mM) should be prepared as described in Table 2.

Table 3. Preparation of potassium phosphate100 mM (KPO4 buffer) buffer, pH 7.8 at 11±1°C

Reagent	Concentration	Dibasic (K_2HPO_4) buffer (mL)	Monobasic (KH_2PO_4) buffer (mL)	Total volume (mL)	pH at 11°C	Storage temperature
Potassium phosphate buffer (KPO_4), pH 7.8 +/- 0.1	100 mM	88	12	100	7.8	4°C; max 1 month

3. Additional dibasic or monobasic potassium phosphate buffer can be added to adjust to pH 7.8 at 11± 1°C.

4. Preparation of alamethicin stock solution and working solution:

- Alamethicin stock solution (10 mg/mL) is prepared in methanol by adding 0.1 mL methanol per mg of alamethicin to the vial. The vial is carefully vortexed and the alamethicin stock solution stored as 25 µL aliquots in micro-centrifuge tubes (e.g., 1.5 mL Eppendorf) at -20 ±1°C until use.
- Alamethicin working solution (250 µg/mL) in KPO_4 buffer is prepared by diluting one 25 µL aliquot of 10 mg/mL stock solution with 975 µL KPO_4 buffer (Table 2)

5. Preparation of cofactors solutions: The three cofactors listed in Table 3 may be pre-weighed prior to the test and stored in vials (e.g., 1.5 mL Eppendorf) at -20 ±1°C. On the day of the test, 1 mL of ice-cold KPO_4 buffer is added to the respective vials, the solutions are vortexed until completely dissolved and then stored on ice until use.

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Table 4. Preparation of cofactors solution

Cofactors	mM	mg	mL of KPO_4 buffer (ice-cold)
β-nicotinamide adenine dinucleotide 2'-phosphate, tetrasodium salt (NADPH)	20	16.67	1
uridine 5' -diphosphoglucuronic acid, trisodium salt (UDPGA)	20	12.93	1
L-glutathione (GSH)	50	15.37	1

6. Since the purity of the fourth cofactor 3'-phosphoadenosine 5'-phosphosulfate (PAPS) will likely be <95%, adjustment for solution preparation is required. It is further recommended to prepare one concentrated solution (10 mM) prior to the test day and freezing it as 50 µL aliquots. On the day of the test, one aliquot may be thawed and diluted to the working concentration (1 mM).

- Calculate the amount of chemical required to prepare a 10 mM PAPS solution. An accurate adjustment for chemical purity would take into account the lot-specific information of the purity of the anhydrous acid, % water, % lithium and % solvent. However, for the purposes here, a simple adjustment for purity of anhydrous acid and water content will be sufficient.
  - Chemical mass × anhydrous acid content × (1 - water content)/ 507.26 g/mol = mol anhydrous acid
  - Volume of KPO_4 buffer (mL) to add = (mmol of anhydrous acid/10 mM) × 1000 mL/L
- Adjust a small volume (e.g., 25 mL) of ice-cold KPO_4 buffer to pH 8.0 by addition of dibasic potassium phosphate solution (see Table 1)
- Dissolve PAPS in the calculated volume of ice-cold pH 8.0 KPO_4 buffer (PAPS is most stable when frozen at pH 8).
- Aliquot 50 µL each into pre-chilled micro-centrifuge tubes and immediately freeze at -80 ±1°C.

7. A master mix of cofactors is prepared just prior to the pre-incubation step with each of the pre-weighed cofactor (NADPH, UDPGA, GSH) and PAPS.

8. Immediately before addition of the master mix, one aliquot of the 10 mM PAPS solution is thawed and diluted with 450 µL of KPO_4.

9. All the cofactors are combined in a master mix as follows:

- 500 µL of 20 mM NADPH
- 500 µL of 20 mM UDPGA
- 500 µL of 50 mM GSH
- 500 µL of 1 mM PAPS

The master mix should be vortexed prior to adding it to the reaction vials.

10. Dilution of active and enzymatically inactive RT-S9 to working protein concentration: the active and the enzymatically inactive RT-S9 are diluted with KPO_4

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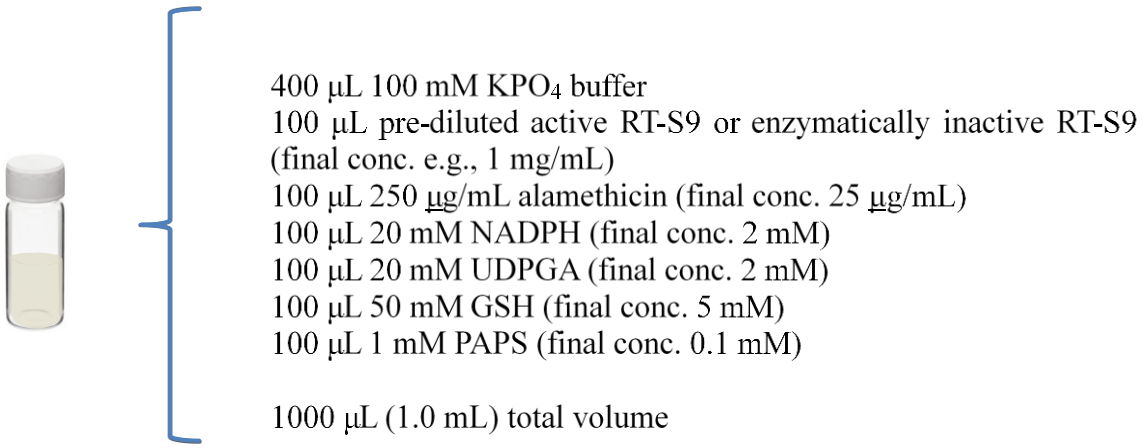
buffer to 10-fold concentrated working solutions so that the appropriate concentration of protein is delivered to the reaction system in 100 µL (active or enzymatically inactive RT-S9).

- The amount of KPO_4 buffer needed to achieve the appropriate RT-S9 working concentration (i.e., 10-fold) is determined.
- Example: concentration of active RT-S9 = 23.6 mg/mL (protein/mL); the target concentration for the given chemical is 1 mg/ml; the volume of the frozen RT-S9 tube is for example 150 µl
    - Total protein content: 23.6 mg/mL x 150 µL = 3540 µg protein
    - Total volume needed: 3540 µg protein/10 mg/mL = 354 µL
    - Buffer to add: 354 µL-150 µL = 204 µL KPO_4

Figure 3. Description of reaction mixture contents in a 7 mL scintillation vial prior to spiking with the test chemical.



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