DRAFT TEST GUIDELINE

DETERMINATION OF CYTOCHROME P450 (CYP) ENZYME ACTIVITY INDUCTION USING DIFFERENTIATED HUMAN HEPATIC CELLS

INTRODUCTION

1. The proposed Test Guideline (TG) describes the use of human derived metabolic competent hepatic test systems (e.g. cryopreserved differentiated HepaRG™ cells) to assess the potential of test chemicals to induce (i.e. increase the synthesis and activity) three Phase I biotransformation enzymes: the cytochrome P450 (CYP)1A2, CYP2B6 and CYP3A subfamily which are susceptible to induction and are highly expressed in human liver.
2. These CYP enzyme isoforms respond to activation of specific nuclear receptors/transcription factors[1] (pregnane X receptor (PXR), constitutive androstane receptor (CAR) and aryl hydrocarbon receptor (AhR)) associated with downstream signal transduction pathways. The proposed TG evaluates the potential of a test chemical for PXR, CAR and/or AhR activation, resulting in induction and increase of basal enzyme activity of the three CYP enzyme isoforms.
3. A chemical may have inherent toxicity or become toxic due to its metabolites produced by biotransformation enzymes (e.g CYPs) or its ability to induce biotransformation enzymes (e.g. CYPs) that affect its rate of metabolism (Tsaioun et al., 2016).
4. Besides detoxifying chemicals or increasing their toxicity due to formation of toxic metabolites, CYP enzymes play a key role in the biosynthesis of endogenous substrates (e.g. steroid hormones, prostaglandins and bile acids) (Hakkola et al., 2018). Chemical CYP enzyme activity induction may therefore cause dysregulation of normal metabolism and homeostasis, with potential toxicological effects (Staudinger et al., 2013; Amacher, 2010). For example, induction of CYP enzymes involving PXR, CAR and/or AhR activation has been demonstrated to be indirectly related to increased clearance of thyroid hormones (metabolised by other biotransformation enzymes), perturbing thyroid functions (OECD, 2014; ECHA and EFSA, 2018).
5. CYP enzyme activity induction alone does not provide information on the full spectrum of the metabolic processes but it may support integrated approaches to testing and assessment (IATA) where standardised data from various sources are used and interpreted in a structured way to better predict the toxicokinetic and toxicodynamic profile of chemicals (OECD, 2016).
6. The proposed TG is based on a validation study coordinated by the European Commission Joint Research Centre's European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM). The validation study demonstrated the reliability and relevance of

[1] pregnane X receptor (PXR), constitutive androstane receptor (CAR) and aryl hydrocarbon receptor (AhR) are often called 'nuclear' receptors, although AhR belongs actually to the family of basic-helix/loop/helix (bHLH)-receptors (bHLHe76) and only PXR and CAR belong to the family of nuclear receptors (NR1I2 for PXR and NR1I3 for CAR)

the CYP enzyme activity induction method using differentiated human hepatic cells (e.g. cryopreserved differentiated HepaRG™ cells) by testing ten chemicals and three reference chemicals (one for each CYP isoform) in three laboratories. To avoid the need for species extrapolation when evaluating the obtained *in vitro* CYP enzyme activity induction validation study data (EURL ECVAM, 2014, Bernasconi et al., 2019) only chemicals with high quality *in vivo* human CYP enzyme activity induction data (available from clinical studies) were selected.

7. Details of test chemicals and the evaluation of between cell batch reproducibility (BBR) and between laboratory reproducibility (BLR) are provided in the validation report (EURL ECVAM, 2014). The validation study was peer-reviewed by the ECVAM Science Advisory Committee (ESAC). The validation report, the peer-review report and the ESAC opinion are now available via the EURL ECVAM Tracking System for Alternative methods towards Regulatory acceptance (TSAR) (Ref https://tsar.jrc.ec.europa.eu/test-method/tm2009-14, *in vitro* method No. 194).
8. This was the first formally validated *in vitro* method (Bernasconi et al., 2019) providing both toxicokinetic and toxicodynamic information using human metabolic-competent hepatic cells (Hakkola *et al*. 2018), addressing the priority need for availability of *in vitro* metabolism methods (Coecke et al., 2006; OECD, 2008) and stimulating pursuit of additional *in vitro* methods for other critical metabolic processes (e.g. metabolite identification, induction and inhibition by other biotransformation enzymes, human hepatic metabolic clearance) (Coecke et al., 2013; Bessems et al., 2014, Gouliarmou et al., 2018; ECHA and EFSA, 2018).
9. Terminology and definitions used in the proposed TG are compliant with the Good *In Vitro* Method Practices (GIVIMP) OECD guidance document for the development and implementation of *in vitro* methods for regulatory use in human safety assessment, a joint activity between the OECD Working Group on Good Laboratory Practice (WG GLP) and the Working Group of the National Coordinators of the Test Guidelines Programme (WNT) (OECD, 2018). Abbreviations are provided in **Appendix 1**.

INITIAL CONSIDERATIONS AND LIMITATIONS

10. It is recognised that for the development of this proposed TG a limited number of chemicals has been tested in the validation study. A selection criterion for eligibility of a chemical was the availability of *in vivo* human data in order to determine the predictive capacity and evaluate the relevance of the test method. This limited the validation set of chemicals to 13 documented pharmaceuticals where high quality *in vivo* human clinical data were available. However, a chemical space analysis of the validation set of chemicals and the reference chemicals (with rifampicin used both as a test chemical and as a reference chemical) shows a high coverage of the chemical space formed by REACH registered substances, Drugbank approved drugs and some Tox21 chemicals (Bernasconi et al., 2019). This suggests that the *in vitro* method may be applicable to a structurally diverse range of chemicals.
11. In preliminary experiments, of the 13 chemicals initially selected as the validation set, two chemicals were excluded for cytotoxicity issues and one due to insolubility in assay medium (EURL ECVAM, 2014). Therefore 10 proficiency chemicals were selected for the proposed TG (**Appendix 2 – Table 2**).
12. The CYP2C9 enzyme isoform, regulated by CAR and PXR (Chen and Goldstein, 2009), is not part of the proposed TG. The FDA and EMA guidelines (FDA, 2017 and EMA, 2012) and

the Pharmaceuticals and Medical Devices Agency of Japan (PMDA, 2019[2]) do not include the CYP2C subfamily enzymes in the initial *in vitro* test. If a compound induces CYP2B6 or CYP3A subfamily, i.e. acting mainly via CAR or PXR, CYP2C9 could be tested for induction in subsequent in-depth investigations.

13. Other nuclear receptor have been reported to CYP 3A4 but are not part of the proposed TG (Hiebl et al., 2018).
14. When testing in submerged cultures, it should be determined (e.g. by visual inspection) that the test chemical is dissolved in the solvent (e.g. dimethyl sulfoxide (DMSO)) and soluble and stable in the exposure medium under experimental conditions (e.g. 37°±0.25 C with an atmosphere of 5±1% CO_2, 95±5% relative humidity, 24±0.3 h).
15. Only soluble and non-cytotoxic test chemical concentrations are compatible with the CYP enzyme activity induction method. If a test chemical is insoluble at an upper concentration (e.g. 1 mM), lower concentrations may be applicable.
16. DMSO is a generally applicable solvent for stock solutions, becoming diluted to 0.1% in assay medium to avoid interference with pregnane X receptor activity. Other solvents may also be compatible, assuming no impact on CYP enzyme activity induction and no cytotoxicity on the test system. Chemicals that are water soluble can be used as such.
17. The method is not intended for metabolite(s) identification and not for the determination of intrinsic human hepatic metabolic clearance.
18. CYP enzyme inhibition is not covered here as it involves other assays with their own test systems, standard operating procedures (SOPs) and evaluation criteria (Fowler et al., 2008).
19. Considering that only mono-constituent substances were used during the validation, the applicability to test mixtures has not been addressed. The test method is nevertheless theoretically applicable to the testing of multi-constituent substances and mixtures. When considering testing of mixtures, difficult-to-test chemicals (e.g. unstable), or test chemicals not clearly within the applicability domain described in the proposed TG, upfront consideration should be given to whether the results of such testing will yield results that are scientifically meaningful.

SCIENTIFIC BASIS OF THE METHOD

20. The three CYP enzyme isoforms (CYP1A2, CYP2B6, and CYP3A subfamily) included in this TG are recommended by the European Medicines Agency (EMA) and the United States (US) Food and Drug Administration (FDA) Guidelines for drug-drug interactions studies (FDA, 2017 and EMA, 2012) and are involved in the biotransformation of a wide variety of endogenous and exogenous substances in humans (and other animal species).
21. Chemical induction of CYP1A2, CYP2B6, and CYP3A subfamily enzymes may cause dysregulation of normal metabolism and homeostasis, with potential toxicological effects (Staudinger et al., 2013; Amacher, 2010).
22. The molecular initiating event of CYP1A2, CYP2B6, and CYP3A subfamily enzyme activity induction is the binding of a substance (endogenous or exogenous) to a specific receptor/transcription factor (CYP1A (AhR), CYP2B (mostly CAR), and CYP3A family (preferentially PXR and CAR)). CAR can be activated indirectly and cross-talk between the pathways is possible. Chemical activation of AhR/PXR/CAR following induction of the associated CYP isoform may impact various endogenous cell-related processes, such as cell differentiation and development, immune response, carcinogenesis (Hakkola et al., 2018) and

[2]https://www.pmda.go.jp/files/000228122.pdf

affect other kinetically relevant processes such as Phase II metabolism and transmembrane transport. These receptor/transcription factors regulate the expression of UDP-glucuronosyl transferases (UGTs) and glutathione-S-transferases (GSTs) and the transporters P-glycoprotein 1 (multidrug resistance protein 1-MDR1) and MRP2 (Hewitt et al., 2007). As such, CYP enzyme activity induction is regarded as the interface between toxicokinetics and toxicodynamics and serves as a biomarker of nuclear receptor activation.

23. Biotransformation enzyme (e.g. CYP) activity induction occurs when endogenous compounds or exogenous chemical compounds (xenobiotics) cause an increase in synthesis and activity of enzymes, thereby increasing the metabolism of parental compounds that are catalysed by those enzymes. Enzyme activity induction is as such a process in which a substance induces (i.e. enhances) the expression of an enzyme (at phenotypic level) by *de novo* protein synthesis (Coecke *et al.*, 1999). Hence, enzyme induction increases the specific activity of the enzyme (V_{max}) without changing its substrate specificity (K_m).
24. The measurement of functional CYP enzyme activity induction (i.e. catalytic activity) is considered more informative and relevant for chemical risk assessment than measurement of mRNA, since correlations between the CYP-selective activity and the specific CYP mRNA level are frequently poor or lacking (Abass et al., 2012; Choi et al., 2013; Mwinyi et al., 2011; Nakajima and Yokoi, 2011; Surapureddi et al., 2011). However, the parallel measurement of mRNA might be warranted in some specific cases, for example when the chemical is both a CYP inhibitor and inducer (Einolf et al., 2014).
25. A test chemical with ≥2-fold CYP enzyme activity induction with respect to negative control is classified as an *in vitro* positive inducer (Kanebratt and Andersson, 2008) for the specific CYP isoform investigated.
26. A 2-fold CYP enzyme activity induction is not based on a statistical treatment of data but on the relevance of the *in vitro* data for an induction response *in vivo*. The use of the concentration of a test chemical resulting in 2-fold CYP enzyme activity induction (called the F2 value) in the cell system has been described in detail by Kanebratt & Andersson (2008). By relating the F2 value to exposure, the relevance of the induction response can be evaluated.
27. Human derived metabolically competent test systems, such as cryopreserved differentiated human HepaRG™ cells, are of particular relevance for human safety assessment, since there are well described species differences in Phase I enzyme activity induction and metabolism (Martignoni et al., 2006, Pelkonen et al., 2009) and in CAR, PXR and AhR activation (Kretschmer and Baldwin, 2005; Kiyosawa et al., 2008; Köhle and Bock, 2009; Abass et al., 2012 and Fujiwara et al., 2012).
28. The proposed TG is based on data generated with cryopreserved differentiated human HepaRG™ cells, which maintain metabolic capacity qualitatively and quantitatively comparable to human hepatocytes, including expression of liver metabolising enzymes, nuclear receptors, and hepatic xenobiotic transporters (Aninat et al., 2006; Le Vee et al., 2006; Turpeinen et al., 2009; Andersson et al., 2012). Recent developments in cell cryopreservation and optimisation of seeding conditions have facilitated continuity of cryopreserved differentiated human HepaRG™ cells commercial supply.
29. HepaRG™ cells were first described in 2002 by Gripon (Gripon et al, 2002). HepaRG™ is a patented cell line (PCT/FR02/02391 of July 8.2002). Cryopreserved differentiated human HepaRG™ cells are now available from different suppliers worldwide.
30. Availability of a chemically-defined culture medium allows the test system to be maintained in the absence of foetal calf serum thereby avoiding undefined media components and increasing the reproducibility of culture conditions (OECD, 2018).

PRINCPLE OF THE TEST

31. The test system is seeded and cultured under optimised conditions and plate format (e.g. cryopreserved differentiated human HepaRG™ cells are seeded in a type I collagen coated (Davison-Kolter et al., 2019) in a 96-well plate).
32. In one plate cells are exposed to (I) test chemical(s) at least at six concentrations (II) reference chemicals at set concentrations (providing experimental positive controls; see chapter REFERENCE CHEMICALS AND PROFICIENCY TESTING**)** and (III) solvent-containing medium (e.g. 0.1 % DMSO) serving as the negative control (i.e. solvent control). If a test chemical is dissolved directly in medium, exposure to medium is considered as negative control. After the required incubation period, CYP enzyme activity is determined by applying fresh medium containing a combination ("cocktail") of the CYP-selective probe substrates (n-in-one approach or cassette dosing): phenacetin (CYP1A2), bupropion (CYP2B6) and midazolam (CYP3A subfamily). CYP1A2, CYP2B6 and CYP3A subfamily enzymes present in the test system will metabolise these probe substrates into the known metabolites acetaminophen, hydroxy bupropion and 1-hydroxy midazolam, which can be quantified with an appropriate analytical technique, such as liquid chromatography coupled to mass spectrometry (LC/MS).
33. LC/MS is a popular technique for the quantitation of xenobiotics because it provides excellent sensitivity and selectivity, with typically relatively quick short analysis times.
34. For each well of the plate, the supernatant is used for specific CYP probe substrate metabolites' quantitation (e.g. with LC/MS) while the cells are lysed for protein determination. **The amount of metabolites formed per unit of time and normalised to the protein content is a direct measurement of CYP enzyme activity (expressed for instance in pmol x min-1 x mg-1)**. Normalising the activity per protein content is considered more relevant as the number of cells seeded may not be the actual number of cells in the induction experiment. Moreover, in case of cryopreserved differentiated human HepaRG™ cells, hepatocyte-like colonies surrounded by clear epithelial cells corresponding to primitive biliary cells, form a confluent monolayer, thus the cell monolayers consist of two sub-populations of cells.

INFORMATION ON THE TEST CHEMICAL

35. Before carrying out the CYP enzyme activity induction method, the following information about the test chemical should be known:
    - Solubility in DMSO, other solvents, water or medium;
    - Solubility and stability in the induction serum free chemically-defined medium **(e.g. Appendix 3)** and in the induction experimental conditions;
    - Stability of the test chemical stock solution;
    - Cytotoxic effects on the test system.
36. A validated analytical method (e.g. LC/MS) with known accuracy, precision, and sensitivity, for the quantification of the metabolites acetaminophen, hydroxy bupropion and 1-hydroxy midazolam should be available, together with details of sample preparation and storage. The lower and upper limits of quantitation (LLOQ and ULOQ) of the metabolites have to be determined (OECD, 2018).

REFERENCE CHEMICALS AND PROFICIENCY TESTING

37. The purpose of the reference chemical(s) is to grade the response of the test system to the test chemical, while the purpose of the control item(s) is to check the proper performance of the test system. The reference chemical(s) is used to provide a basis for comparison with the test chemical or to validate the response of the test system to the test chemical i.e., provide a known measurable or observable response. Since the purpose of controls may be considered analogous to the purpose of a reference chemical, the definition of reference chemical may be regarded as covering the term 'positive control' (OECD, 2004, 2018). Reference chemicals at set concentrations should always be included (minimum in triplicates i.e. three wells) in each induction experiment.
38. For this method, the reference chemicals (**Appendix 2 - Table 1**) also act as positive controls. It is important that positive controls, as well as negative control, are run concurrently with the test chemicals each time the *in vitro* method is performed to check the variability between runs.
39. Prior to routine use this proposed TG, laboratories should demonstrate technical proficiency by correctly obtaining the expected *in vitro* results for the 10 proficiency chemicals listed in **Appendix 2 - Table 2**.

VALIDITY OF THE TEST

40. For an experimental run to be valid the following criteria should be met:

- The final solvent concentration during the CYP enzyme activity induction method should not affect cell viability and the induction of the specific isoform investigated (e.g. DMSO should not exceed 0.1 % v/v as DMSO induces CYP3A4)
- The top concentration (e.g. 1 mM) should subsequently be soluble upon further dilutions in induction medium and should be not cytotoxic
- Negative controls should demonstrate basal CYP enzyme activities in line with the data given on the test system' Certificate of Analysis (CoA)
- Solvent controls (if applicable) should demonstrate that the final concentration required has no effect on cell viability (must be within 15% of the untreated controls).
- Exposure to reference chemicals should lead to a ≥2-fold increase of CYP enzyme activity compared to negative control
- At least six test chemical concentrations have to be tested
- At least three cells batches have to be used in independent runs.

DESCRIPTION OF THE METHOD

41. Below general guidelines and key requirements are listed to perform the CYP enzyme activity induction *in vitro* method using cryopreserved differentiated human HepaRG™ cells or equivalent test systems after successful proficiency chemicals set testing. In the Appendixes more detailed procedures are provided as examples. They are based on the SOP(s) followed in the validation ring trial. The spreadsheets, as used in the validation ring trial, to facilitate data recording and results calculations are referenced in **Appendix 4** and available in TSAR (https://tsar.jrc.ec.europa.eu/test-method/tm2009-14)

Reagents and Media

42. For the analytical metabolite quantitation reagents are recommended to be of analytical grade.
43. Media and media supplements are available at different suppliers (**Appendix 3** provides, as examples, media and media preparation procedures followed in the validation ring trial). Follow supplier's instruction.
44. For protein determination any suitable method to detect low amounts (e.g. 0.5µg/mL (2 µg/mL in microplate format)) of protein can be used (e.g. micro-bicinchoninic acid assay).
45. For cytotoxicity determination suitable method to detect cell viability can be used (e.g. cell titer blue, GIVIMP (OECD, 2018)).

Test system

46. The proposed TG is based on the use of a human derived metabolic competent test system (e.g. cryopreserved differentiated HepaRG™ cells). Other test systems having the same functionalities or other formulations of HepaRG™ cells may be used provided they generate correct classification of the 10 proficiency chemicals.
47. The test system should be supplied with the CoA informing on cell yield per vial, post thaw viability, basal metabolic activities and cell quality control (see **Appendix 5**).
48. Whenever this information is not available on the CoA, the analysis has to be performed.

Test set up

49. Before planning the CYP enzyme activity induction method, preliminary experiments that include (I) solubility of test chemical in solvent, (II) solubility and stability of test chemical in the serum-free chemically defined induction medium/0.1% v/v DMSO and in the induction experimental conditions, (III) stability of test chemical stock solutions and (IV) cytotoxicity of test chemical towards the test system have to be conducted.
50. A sufficient number of concentration points (e.g. six), starting from an upper soluble and non-cytotoxic concentration (e.g. 1 mM), should be tested to produce a sigmoid CYP enzyme activity induction dose-response curve.
51. Each test chemical concentration, reference chemical and negative control have to be tested at least in triplicate (i.e. 3 individual wells).
52. Reference chemicals at set concentrations and negative controls should always be included in the plate.
53. Each test consists of three independent runs. 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) different cell batches independently thawed are used.
54. In the following paragraphs, the procedures for one run are detailed.

Cells thawing and seeding

55. Cryovials containing the test system should be shipped on dry ice (less than a total of 10 days in this storage condition).
56. Upon receipt, the cryovials should be immediately stored in liquid nitrogen. Supplier instructions should be followed.
57. Recommended cryopreserved differentiated human HepaRG™ cells concentration for a 96 well plate is 0.72 x 10^6 viable cells/ml. For other plate formats and/or test systems an adequate amount of cell seeding density should be determined.

Test chemical solubility and stability assessment

58. Solubility can be assessed by visual inspection or by other methods (e.g. nephelometry, OECD, 2018).
59. Test chemical's stock solutions may only be used if the test chemical is dissolved completely.

Preparation of test chemicals, reference chemicals and controls for the CYP enzyme activity induction assessment

60. Stock solution(s) of the test chemical(s) and reference chemicals are prepared in solvent and further diluted in the culture induction medium to achieve a final solvent concentration of 0.1% (v/v) (e.g. **Appendix 4 -** FORM-03: pages 1 and 2).
61. Test chemical stock solution is aliquoted and stored at -20°C (≤ 1 month, if not indicated otherwise, e.g. by the supplier) [3]. Information about the stability in stock solution is part of the pre-requisite information.
62. Working solutions have to be prepared freshly every day.
63. The upper test chemical starting concentration (e.g. 1 mM) is determined based on its solubility at a non-cytotoxic concentration (it might happen that the presence of cells helps solubilisation).
64. Reference chemical working solutions are prepared freshly every day. Initial weight and preparation of stock and working solutions of reference chemicals are documented (e.g. **Appendix 4 -** FORM-03: pages 1 and 2).

Test chemical cytotoxicity assessment

65. Cytotoxicity of test chemical(s) towards the test system is determined before starting the induction experiments.
66. During the validation trial on which the proposed TG is based, cell titer blue method was used. Other cell viability method can be used provided same results are obtained (OECD, 2018).
67. Potential cytotoxicity of test chemical for the test system has to be determined starting from the upper soluble concentration and the cytotoxicity incubation time should reflect conditions used for the CYP enzyme activity induction method.
68. A proper cytotoxicity positive control should always be included (e.g. 8 µM Doxorubicin, CAS: 25316-40-9, MW 579.98 g/mol, serves as positive control reducing cryopreserved differentiated HepaRG™ cells' viability of 30-70 % compared to negative control).
69. The highest test chemical concentration with a FS≥90% is eligible as starting concentration for the induction method.

Test chemical CYP enzyme activity induction assessment

70. CYP enzyme induction experiments are performed in a 96-well format (see **Appendix 6** for plate layout example). Other plate formats may be applied after successful proficiency chemicals set testing.
71. Each plate should include: test chemical(s) at least six different concentrations (n=3); solvent-treated control corresponding to the solvent of the test chemical (each n=3); reference

[3] Aliquots of the stock solution to be used on each incubation day have to be stored under suitable conditions in order to avoid chemical instabilities due to multiple freeze-thaw cycles.

chemicals at set concentration (n=3 per reference chemical); solvent-treated control corresponding to reference chemicals (n=3); solvent-free-control (n=3).

72. The upper test chemical concentration (e.g. 1 mM) is based on solubility and cytotoxicity results. Suggested test chemical dilution factors are 1:1.5; 1:2 or 1:2.5.
73. Briefly the main steps of the CYP enzyme activity induction method (more detailed procedure is provided in **Appendix 10)**.
74. Induction is initiated by the addition of the induction solution (t = 0 h). The induction solution is replaced at time point t = 24 ± 0.3 h by freshly prepared induction solutions (in order to partially cover loss of test chemical due to metabolism during the induction period) and incubated for additional 24 ± 0.3 h. Thus the cells are exposed to the inducer for 48 ± 0.6 h in total.
75. After 48 ± 0.6 h cells induction solution is replaced by the cocktail of 3 CYP probe substrates (phenacetin 26 μM, bupropion 100 μM and midazolam 3 μM) and after one hour incubation at 37°C samples are analysed. For each well, the supernatant is used for quantification of specific CYP probe metabolites with an appropriate analytical technique (e.g. LC/MS) and the cells are lysed for protein determination (e.g. the bicinchoninic acid method). The amount of metabolites formed per unit of time and normalised to the protein content is a direct measurement of CYP enzyme activity (expressed for instance in **pmol x min-1 x mg-1**).
76. Suitable acceptance criteria should be defined to demonstrate that the analytical assay fit for purpose. The reader can refer to the following publications for guidance: EMA, 2011, FDA, 2018, OECD, 2018.

CALCULATION OF *IN VITRO* CYP ENZYME ACTIVITY INDUCTION POTENTIAL

77. For each CYP isoform, the potential enzyme activity induction is calculated for each concentration of the test chemical and for the reference chemicals and expressed as n-fold CYP isoform enzyme activity (expressed as **pmol CYP specific isoform metabolite x min-1 x mg protein-1**) relative to the negative control enzyme activity (expressed as **pmol CYP specific isoform metabolite x min-1 x mg protein-1**) averaged over the three replicates:

$$\text{n} - \text{fold CYP enzyme activity induction} = \frac{\text{test chemical (or reference chemical) CYP enzyme activity}}{\text{negative control CYP enzyme activity}}$$

78. To classify a test chemical as an *in vitro* CYP enzyme activity inducer the following criteria should be met:
    - Minimum one out of three cell batches should generate an *in vitro* induction result for at least one of the 3 CYP isoforms.
    - A least two consecutive concentrations in the dose-response curve generating ≥2-fold induction response should be observed to reduce the risk of false positive.

TEST REPORT

79. The test report should include the following:

Test chemical

Mono-constituent substance: physical appearance, water solubility, and additional relevant physicochemical properties; chemical identification (i.e. IUPAC or CAS name, CAS number), 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.

Test system

- Supplier, quality control and characterisation (see **Appendix 5**)
- Cell passage number, if applicable
- Method for cell culturing

Test conditions

- Concentrations of test chemical(s) and reference chemicals
- Solvent used and volume used (v/v). If solvent other than DMSO is used provide information on its potential effects on cells and CYP induction should be known and reported
- Method of preparation of stock solution(s) of test chemical and reference chemicals (name and concentration of solvent, if applicable)
- Method used for cytotoxicity assessment
- Cell culture medium characteristics and additional media components
- Cell recovery after thawing and cell density (pictures of the cells are recommended)
- Incubation conditions (e.g. temperature, humidity, $\%CO_2$)
- Test set-up
- Number of replicates (if more than one is used per run)
- Number of independent runs
- Description of preliminary experiments

Analytical method

- Complete description of the appropriate analytical technique and of all test chemical analysis procedures employed including limits of detection and quantification, variability and recovery efficiency, internal standard, etc.
- Statistical method. The analysis of the data shall include detection of possible outliers and if detected, appropriate statistical methods shall be used by biostatistician.

Results

- Results from any preliminary experiment performed
- Results on solubility of the test chemical in solvent or medium
- Results on stability of the test chemical, reference chemicals and probe substrates stock solutions
- Results on solubility and stability of the test chemical in induction medium and induction experimental conditions
- Results on cytotoxicity assessment through cryopreserved differentiated human HepaRG™ cells

- Data from individual wells, time points for each independent run (e.g. test chemical, reference chemical)
- Data from individual wells on protein content
- Calculated n-fold CYP enzyme activity induction results for each well
- Statistical analyses, if any, together with a measure of error (e.g. SD, %CV or 95% confidence interval) and a description of how these values were obtained
- Average and standard deviation values from independent, not significantly different, runs, as well as results from t-tests to compare average n-fold induction 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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APPENDIX 1- ABBREVIATIONS

ACN: acetonitrile
ADD: additive (medium supplement)
ADME: absorption, distribution, metabolism, and excretion.
AhR: aryl hydrocarbon receptor
BBR: between batches reproducibility.
BLR: between laboratory reproducibility.
BNF: beta-naphthoflavone
CAS: chemical abstracts service
CAR: constitutive androstane receptor
CYP: cytochrome P450 enzyme
DMSO: dimethylsulfoxide
EMA: European Medicines Agency
FDA: United States (US) Food and Drug Administration
H_2O: deionised water (e.g. MilliQ water)
h: hour (s)
IATA: integrated approach to testing and assessment
K_m: concentration of substrate which permits the enzyme to achieve half V_{max}
LC-MS: liquid chromatography-mass spectrometry
LOQ: limit of quantitation
LLOQ: lower limit of quantitation
MeOH: methanol
MIE: molecular initiating event
m/v: weight per volume
MW: molecular weight
min: minute(s)
NaOH: sodium hydroxide
OD: optical density
OH-midazolam: 1‘-hydroxymidazolam
PB: phenobarbital
PBPK: physiologically-based pharmacokinetics.
PBS: phosphate buffered saline.
PXR: pregnane X receptor
QC: quality control
RIF: rifampicin
RFU: relative fluorescence units
s: second(s)
TSAR: EURL ECVAM Tracking System for Alternative methods towards Regulatory acceptance.
ULOQ: upper limit of quantification
v/v: volume per volume
V_{max}: the rate of reaction when the enzyme is saturated with substrate is the maximum rate of reaction

APPENDIX 2 –TABLES

Table 1. Reference chemicals. Reference chemicals and their concentration tested in the CYP enzyme activity induction *in vitro* method during the validation ring trial (EURL ECVAM, 2014). Enzymatic probe substrates, applied as a cocktail, and the respective metabolites measured by LC/MS are reported.

CYP	Reference chemical	Enzymatic probe substrate	Concentration in the cocktail (μM)	Metabolite measured
1A2	β-naphthoflavone (BNF)[4] 25 μM	phenacetin	26	acetaminophen
2B6	Phenobarbital (PB) 500 μM	bupropion	100	OH-bupropion
3A subfamily	Rifampicin (RIF) 10 μM	midazolam	3	1-OH-midazolam

Table 2. Proficiency chemicals. Proficiency chemicals, proposed starting concentrations and expected *in vitro* results based on the validation ring trial. CAS: CAS number, MW: molecular weight. Y: yes (or inducer); N: no (non-inducer). * Excluded from proficiency testing based on the validation ring trial results (EURL ECVAM, 2014).

#	Chemical Name	CAS#	MW	Proposed starting concentration (mg/ml)	CYP1A2	CYP2B6	CYP3A subfamily
1	Omeprazole	73590-58-6	345.4	40	Y	N*	N
2	Carbamazepine	298-46-4	236.3	40	Y	Y	Y
3	Phenytoin sodium	630-93-3	274.3	30[5]	Y	Y	Y
4	Penicillin G sodium	69-57-8	356.4	40	N	N	N
5	Sulfinpyrazone	57-96-5	404.5	40	Y	Y	Y
6	Bosentan hydrate	157212-55-0	569.6	40	Y	Y	Y
7	Artemisinin	63968-64-9	282.3	40	N	Y	N
8	Rifampicin	13292-46-1	822.9	40	Y	Y	Y
9	Metoprolol	51384-51-1	267.4	40	N	N	N
10	Sotalol hydrochloride	959-24-0	308.8	40	N	N	N

Table 3: Example of standards for protein determination according to the Micro-BCA Protein Assay used in the validation ring trial (EURL ECVAM, 2014).

Standard name	Final BSA concentration [mg/ml]	0.05 M NaOH [µl]	BSA solution to use	Volume [µl]
Blank	0 (blank standard)	500	-	0
S1	0.2000	900	2 mg/ml stock	100
S2	0.0400	800	S1	200

[4] β-naphthoflavone (BNF) 25 μM is used as reference chemical for CYP1A2 since at the beginning of the validation study (2008), FDA supported BNF.

[5] The solvent to be used is a 1:1 blend DMSO:water.

S3	0.0200	500	S2	500
S4	0.0100	500	S3	500
S5	0.0050	500	S4	500
S6	0.0025	500	S5	500
S7	0.0010	600	S6	400
S8	0.0005	500	S7	500

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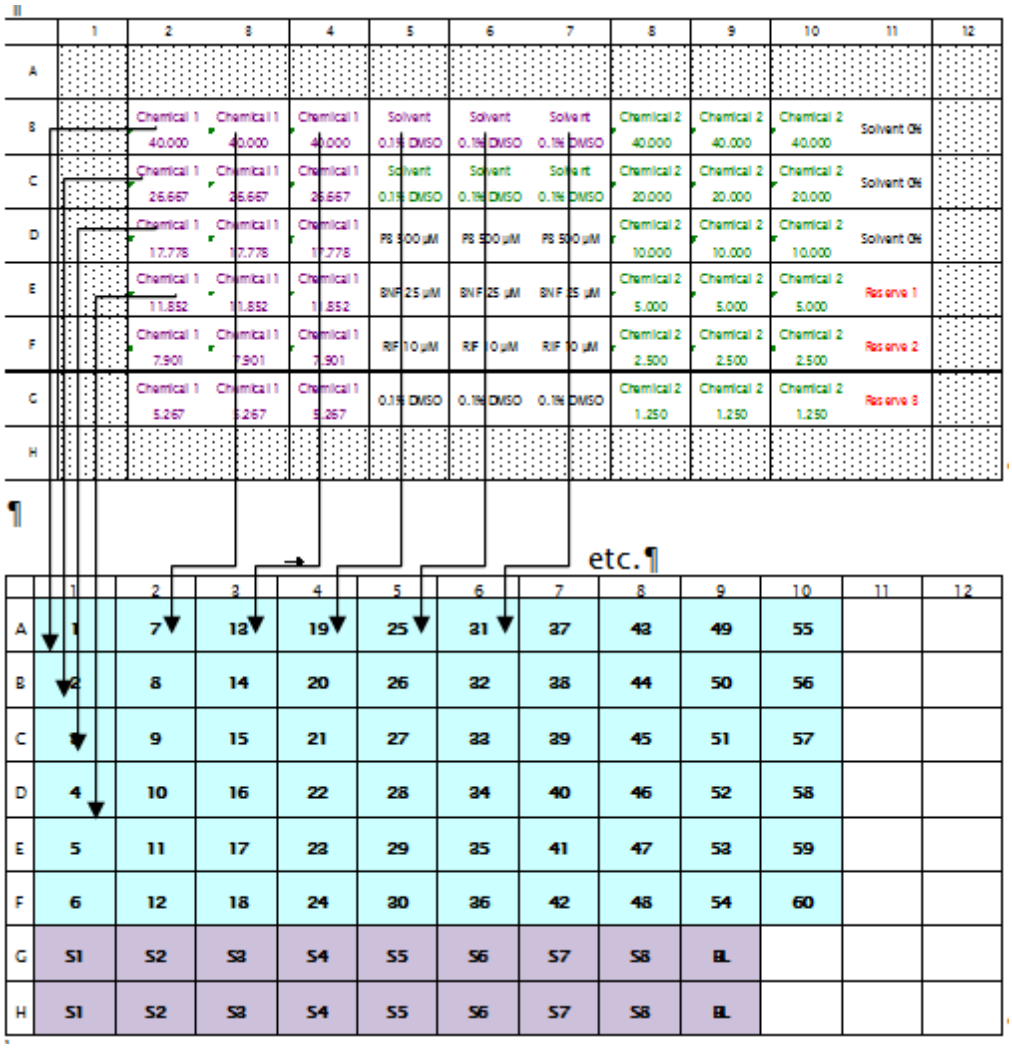
605 **Table 4**: Example of Micro-BCA transfer and pipetting scheme.



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APPENDIX 3 – **MEDIA, ENDPOINT ASSAY SOLUTIONS AND CONTROLS**

Media

Media and media supplements are prepared and stored according to the respective manufacturer's instructions. Here, as examples, the media preparation procedures followed in the validation ring trial

a) HepaRG™ Basal Medium

Basal medium is used both for Thaw, Seed and General Purpose Medium (see b) and for Serum-Free Induction medium (see c).

Basal Medium consists of William's E Medium and and stable glutamine. For instance, 1 ml 200 mM glutamine are added to 99 ml William's E Medium.

Basal Medium can be stored at 4°C for 4 weeks. Alternatively, the following product can be applied: William's E Medium with GlutaMax™.

b) HepaRG™ Thaw, Seed and General Purpose Medium.

It consists of Basal medium + additive (ADD)

HepaRG™ Thaw, Seed and General Purpose Supplement (e.g. HPRG670) is thawed by placing the vial into a 37°C water bath. Thaw, Seed and General purpose Medium is reconstituted by addition of one vial of the supplement (12.5 ml. *Note*: volume may vary depending on supplier) to 100 ml Basal Medium.

Reconstituted HepaRG™ Thaw, Seed and General Purpose Medium can be stored at 4°C for 4 weeks.

c) HepaRG™ Serum-Free Induction medium

It consists of Basal medium + ADD

HepaRG™ Induction Supplement (e.g. HPRG650) is thawed by placing the vial into a 37°C water bath. HepaRG™ Serum-Free Induction Medium is reconstituted by addition of one vial of the supplement (0.6 ml) to 100 ml Basal Medium.

Reconstituted HepaRG™ Serum-Free Induction medium can be stored at 4°C for 4 weeks.

d) Incubation medium (for CYP enzyme activity determination)

It consists of Williams E without phenol red supplemented with 25 mM HEPES pH 7.4 and 2 mM Lglutamine prior to use.

Add 5 ml L-Glutamine 200 mM supplement (100x) to 500 ml Williams E without phenol red. Supplement with 12.5 ml 1 M HEPES solution.

Solutions

Note. *An equivalent product from other suppliers can be used, if the foreseen product can not be purchased. In this case make sure that the selected product has the same CAS number as the suggested.*

Note. *Phenobarbital and midazolam: an equivalent product from other (local) suppliers can be used, if due to federal regulations with respect to handling of controlled drugs, the foreseen product can*

not be purchased. In this case the study director has to make sure that the selected product has got the same CAS number as the suggested product.

a) Reference chemicals' solutions (Appendix 4 – FORM 03)

The stability of the stock solution has to be demonstrated over the given time period.

a) Beta-naphthoflavone (CAS 6051-87-2, MW 272.3 g/mol). A 25 mM stock solution in DMSO is prepared, aliquoted and stored at -20°C for up to 21 days.

b) Phenobarbital (CAS 50-06-6, MW 232.23 g/mol). A 500 mM stock solution in DMSO is freshly prepared every day.

c) Rifampicin (CAS 13292-46-1, MW 822.94 g/mol). A 10 mM stock solution in DMSO is prepared, aliquoted and stored at -20°C for up to 21 days.

b) Cytochrome P450 (CYP) probe substrates (Appendix 4 – FORM 03)

The stock solutions are stored at -20°C and can be used for 1 month. The stability of the stock solution has to be demonstrated over the given time period.

Phenacetin (CAS: 62-44-2, MW 179.22 g/mol). A 10 mM stock solution is prepared in MeOH.

Bupropion (CAS: 34911-55-2, MW 239.74 g/mol). A 10 mM stock solution is prepared in MeOH.

Midazolam (CAS: 59467-70-8, MW 325.77 g/mol). A 10 mM stock solution is prepared in MeOH

c) Cytochrome P450 (CYP) metabolites (Appendix 4 – FORM 07)

The stock solutions can be stored at -20°C and be used for 21 days, unless otherwise stated.

a) Acetaminophen (CAS: 103-90-2, MW 151.16 g/mol). A 10 mM stock solution is prepared in ACN Alternatively, a suitable amount is weighed into a 10 ml volumetric flask and the solvent is added. In this case, the resulting concentration has to be changed in FORM-07.

b) Hydroxybupropion (CAS: 92264-81-8, MW 255.74 g/mol). A 10 mM stock solution is prepared in MeOH. Alternatively, a suitable amount is weighed into a 10 ml volumetric flask and the solvent is added. In this case, the resulting concentration has to be changed in FORM-07.

c) 1'-Hydroxymidazolam (CAS: 59468-90-5, MW 341.77 g/mol). A 0.5 mM stock solution is prepared in MeOH. Alternatively, a suitable amount is weighed into a 10 ml volumetric flask and the solvent is added. In this case, the resulting concentration has to be changed in FORM-07.

APPENDIX 4 – **FORMS**

Examples of the spreadsheets for data recording and calculation of results used in the validation ring trial are provided.

Active spreadsheets can be downloaded at Ref https://tsar.jrc.ec.europa.eu/test-method/tm2009-14, *in vitro* method No. 194.

FORM-01_R01 (Solubility).xls
- Solubility assay

FORM-02_R01 (Cytotoxicity).xls
- Plate layout (page 1/3)
- Calculation of results test chemical 1 (page 2/3)
- Calculation of results test chemical 2 (page 3/3)

FORM-03_R01 (Weighing of compounds and preparation of solutions for induction).xls
- Induction solutions positive control (page 1/3)
- Induction solutions test chemical (page 2/3)
- P450 substrates (page 3/3)

FORM-04_R00 (Weighing of compounds and preparation of solutions for cytotoxicity).xls
- Cytotoxicity test chemical and positive control (page 1/1)

FORM-05_R01 (Cryopreserved differentiated human HepaRG™ cells culture).xls
- Cryopreserved differentiated human HepaRG™ cells culture (page 1-3/3)

FORM-06_R01 (CYP induction assay procedure.xls)
- Assay set up (page 1/4)
- Micro BCA protein determination (pages 2-3/4)
- Remarks (page 4/4)

FORM-07_R01 (Preparation of CYP metabolites for LC/MS analysis).xls
- Stock solutions (page 1/3)
- ISTD (page 2/3)
- Workup calibration standards and QCs (page 3/3)

FORM-08_R01 (Calculation of results for induction).xls
- Protein determination (page 1/7)
- CYP1A2 test chemical 1 (page 2/7)
- CYP2B6 test chemical 1 (page 3/7)
- CYP3A4 test chemical 1 (page 4/7)
- CYP1A2 test chemical 2 (page 5/7)
- CYP2B6 test chemical 2 (page 6/7)
- CYP3A4 test chemical 2 (page 7/7)

FORM-09_R01 (Flowchart_Cytotoxicity).pdf (pages 1-2)

FORM-10_R01 (Flowchart_Induction).pdf (pages 1-4)

APPENDIX 5 – TEST SYSTEM CHARACTERISATION AND QUALITY CONTROL

As an example, recommended minimal required information on test systems (e.g. cryopreserved differentiated human HepaRG™ cells) are:

- Test system characterisation, e.g. species, origin (GIVIMP, OECD 2018)
- Safety data: absence of hepatitis B, hepatitis C and HIV1 viruses (recommended PCR analysis on the cell suspension)
- Biosafety level: e.g. during the validation ring trial, the manufacturer guaranteed the supplied cryopreserved differentiated human HepaRG™ cells as level 2, as recommended in the CDC-NIH Manual, 2009
- Passage number (as an example, the HepaRG™ passage number is a record of number of times the cell culture has been subcultured, e.g. harvested and reseeded into a daughter cell culture supports; the manufacturer recommended passage number 16 for cryopreserved differentiated human HepaRG™ cell)
- Number of cells per vial
- Post thaw viability[6]: (e.g. assessed by trypan blue exclusion test) ≥90%
- Cell density[7] (microscopic observation 6 h after thawing and seeding cells): ≥80%
- Cell morphology (microscopic observation 4 days after thawing and seeding cells): 50 % of typical hepatocyte-like cells are organised in well delineated clusters with bright canaliculi-like structures

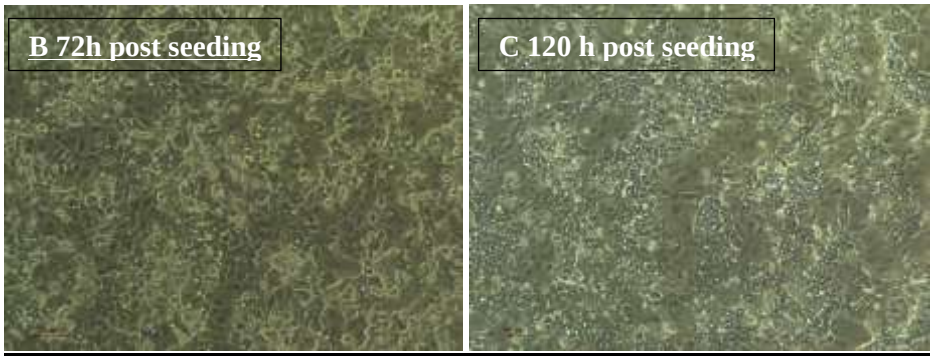
  A Six hours after plating, cells attach and spread to form a monolayer

  B After 72-96 hours in culture, restructuring of the cell monolayer to a hepatocyte-like cell cluster organization can be observed

  C 120 hours after plating, hepatocyte - like cells are organised in well - delineated trabeculae with many bright canaliculi - like structures

[6] After thawing, differentiated human HepaRG™ cells viability is determined by Trypan blue (0.05% in PBS) exclusion.

[7] Differentiated human HepaRG™ are seeded on collagen-coated multi-well culture plates in the HepaRG Thawing/Plating/General Purpose medium using ADD, according to manufacturer's description and use guide for thawing, culture and use of cryopreserved differentiated HepaRG.



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739 • Contamination screening e.g. mycoplasma, viruses and microorganisms (GIVIMP, OECD
740 2018)
741 • Basal activity of CYP1A2, CYP2B6 and CYP3A
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APPENDIX 6 – PLATE LAYOUT FOR CYP ENZYME ACTIVITY *IN VITRO* METHOD

As an example, the 96 well plate format layout is provided. This is the plate layout used in the validation ring trial (EURL ECVAM, 2014).

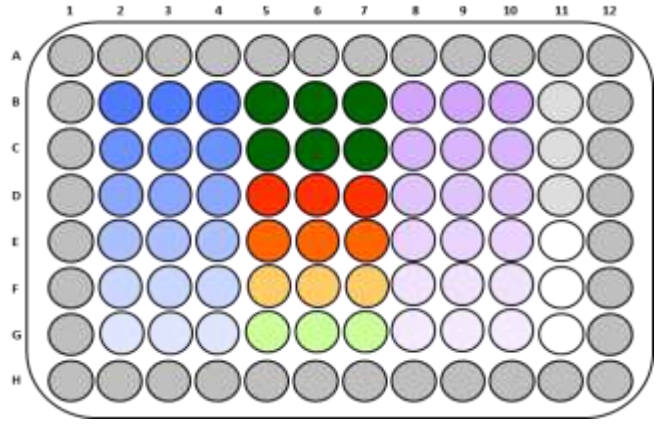
The outer wells of the 96-well plates are not seeded with cells in order to avoid any edge effect resulting from evaporation of the medium.

The outer wells are filled with phosphate buffered saline (PBS) subsequently to cell seeding.

The following wells contain cryopreserved differentiated human HepaRG™ cells: B2-B11, C2-C11, D2-D11, E2-E11, F2-F11, G2-G11.

- The outer wells A1 to A12, B1 and B12, C1 and C12, D1 and D12, E1 and E12, F1 and F12, G1 and G12, H1 to H12 (dark grey) do not contain cells and are not used for testing. They are filled with PBS subsequently to cell seeding.
- Wells E11-F11-G11 (white) are not foreseen for testing in the experimental design; they can be used as reserve wells if cells in one of the wells foreseen for induction does not pass the cell quality control due to inhomogeneity or disintegration of the monolayer.
- Wells B2-G4 (blue): test chemical A (tested at six concentrations).
- Wells B8-G10 (purple): test chemical B (tested at six concentrations)..
- Wells B5-B7 (green): solvent control test chemical A.
- Wells C5-C7 (green): solvent control test chemical B.
- Wells D5-D7 (red): reference chemical (positive control CYP2B6): phenobarbital 500 µM.
- Wells E5-E7 (orange): reference chemical (positive control CYP1A2): β-naphthoflavone 25 µM.
- Wells F5-F7 (yellow): reference chemical (positive control CYP3A subfamily): rifampicin 10 µM.
- Wells G5-G7: (pale green) solvent control reference items.
- Wells B11-D11: (pale grey) solvent free control.

Below a schematic example of the induction plate layout. See text for explanation and colour shading key.



APPENDIX 7 – CELL THAWING AND SEEDING AND QUALITY CONTROL OF THE CELLS

An example protocol for thawing and seeding cryopreserved differentiated human HepaRG™ cells is provided. The described procedure was followed in the validation ring trial (EURL ECVAM, 2014) using cryopreserved differentiated human HepaRG™ cell.

This protocol is used for thawing and seeding cells both for the cytotoxicity assay and for the CYP enzyme induction assay.

If more than one cryopreserved differentiated human HepaRG™ cell cryovial is used for the experiment, the following steps are performed individually for each vial.

1. The HepaRG™ Thaw, Seed and General Purpose Medium is pre-warmed using a 37°C water bath.
2. Prepare a 50 ml polystyrene tube with 9 ml of pre-warmed HepaRG™ Thaw, Seed and General Purpose Medium is prepared.
3. The cryovial is removed from the liquid nitrogen and under the laminar flow hood, the cap of the vial is briefly twisted a quarter turn to release the internal pressure and closed again.
4. The vial is quickly transferred to the 37°C water bath. While holding the tip of the cryovial, the vial is gently agitated for 1 to 2 minutes. It is of highest importance that the vial is not submerged completely to avoid water penetration into the cap. Small ice crystals should remain when the vial is removed from the water bath.
5. The outside of the cryovial is wiped with the isopropanol or ethanol.
6. The cryopreserved differentiated human HepaRG™ cell suspension (e.g. 1 ml) is transferred into the 50 ml polystyrene tube containing pre-warmed (37°C) HepaRG™ Thaw, Seed and General Purpose Medium for a 1/10 dilution.
7. To recover all cells, 1 ml HepaRG™ Thaw, Seed and General Purpose Medium (pre-warmed to 37°C) is used to rinse out the cryovial once. The resulting suspension is returned to the 50 ml tube.
8. The cryopreserved differentiated human HepaRG™ cells suspension is centrifuged for 2 min at 350-360 x g at room temperature.
9. The supernatant is aspirated and the cell pellet is resuspended in 5 ml HepaRG™ Thaw, Seed and General Purpose Medium (pre-warmed to 37°C).
10. First, the cell pellet is loosened by rotating the vial before adding 5 ml medium and then is carefully resuspended using a 5 ml serological pipette.
11. Remaining aggregates are loosened by gentle up- and down pipetting.
12. To count cells, 25 µl of the cell solution is mixed with 75 µl Trypan Blue solution and gently homogenised and an aliquot is introduced into a Neubauer counting chamber.
13. Cells in 4 (upper right, upper left, lower right, lower left) of the large squares are counted under microscope. Living cells exclude the dye while dead cells take it up and appear blue. The living and dead cells are counted in each of the selected 4 large squares and recorded in (e.g. **Appendix 4** - FORM-05).
14. Cell viability [% viability] and concentration [viable cells/ml] are calculated:

$$viability\ [\%] = \frac{\sum viable\ cells \text{ in } 4\ squares}{\sum dead\ +\ viable\ cells\ in\ 4\ square} * 100$$

$$cell\ concentration\ [\ viable\ cells/ml\ ]\ = \sum viable\ cells \text{ in } 4\ squares \text{ x } 10$$

15. For one 96-well plate, 8 ml of a cell suspension containing 0.72 x 10^6 viable cells/ml has to be prepared.
16. The cryopreserved differentiated human HepaRG™ cell suspension is diluted using HepaRG™ Thaw, Seed and General Purpose Medium to 0.72 x 10^6 viable cells/ml using the following formula:

$$Volume\ HepaRG™\ cell\ suspension\ [ml] = \frac{0.72\text{x}106\ [\text{viable cells/ml}]\ \text{x}\ 8\ \text{ml}}{\text{cell concentration [viable cells/ml] square}}$$

17. The diluted cell suspension is transferred into a sterile 92 Ø mm Petri dish or in a pipette try and gently agitated.
18. Using a 8-channel pipette (6 channels equipped with pipette tips only), 100 µl of this cell solution is transferred to the inner wells of a collagen-I coated 96-well plate. The Petri dish or pipette try is gently agitated in-between the pipetting steps. The outer wells of the 96-well plates are not seeded. The following wells contain cryopreserved differentiated human HepaRG™: B2-B11, C2 C11, D2-D11, E2-E11, F2-F11, G2-G11. The outer wells are filled with PBS subsequently to cell seeding.
19. The plate is carefully moved in order to evenly distribute the cells across the surface of the wells and placed in a humidified incubator maintained at 37°±0.25 Cwith an atmosphere of 5±1% CO_2 /95±5% relative humidity.
20. Six h after plating, observe cell morphology under a phase-contrast microscope. When possible, take photographs for laboratory record.
21. HepaRG™ Thaw, Seed and General Purpose Medium in the cell-containing wells is aspirated and replaced by 100 µl fresh HepaRG™ Thaw, Seed and General Purpose Medium per well.
22. Plated cryopreserved differentiated human HepaRG™ cells are incubated in HepaRG™ Thaw, Seed and General Purpose Medium for at least 70 h (6h + further 64 h) before starting the cytotoxicity or induction method.
23. 70 h after plating, visually inspect the cells: restructuring of the cell monolayer to a hepatocyte-like cell cluster organization should be observed (**Appendix 5**). Wells in which the acceptance criteria are not met or the integrity of the monolayers is not given have to be excluded for experiments.
24. The seeded cryopreserved differentiated human HepaRG™ cells have to meet the following quality control parameters:
    - cell viability after thawing: ≥80 %
    - cell recovery per vial: ≥ 90%
    - Six hours after thawing, hepatocyte-like cells should be attached and appear in small, individual differentiated colonies (**Appendix 5 - Figure A**)
    - After 72±0.9 h of culture, a restructuration of about 80% confluent cryopreserved differentiated human HepaRG™ monolayer has to be observed with hepatocyte-like cells' organisation in clusters (**Appendix 5 - Figure B**).

APPENDIX 8: ASSESSMENT OF SOLUBILITY OF TEST CHEMICAL IN SOLVENT AND IN THE INDUCTION MEDIUM (e.g. APPENDIX 4 - FORM-01)

DMSO is a generally applicable solvent. Solubility assessment is performed by visual inspection for precipitation. In case of apparent insolubility in DMSO or precipitation in medium, dissolution is attempted by incremental two-fold dilution (e.g. 0.5 mM, 0.25 mM). The absence of precipitation in the medium is checked pre- and post-incubation (24 hours) by centrifugation of the sample and observation of any precipitation (pellet residue).

In order to increase compound solubility, stock solutions can be heated gently to 37°C or sonicated.

Examples of the protocols used in the validation ring trial (EURL ECVAM, 2014) to assess the solubility of test chemical in DMSO and in the induction medium are provided.

Assessmemt of solubility of test chemical in solvent

1. Weigh test chemical into a screw cap glass vial. Add DMSO according to the following equation to prepare the starting concentration (e.g. 1 mM):

$$\text{Volume solvent [µl]} = \frac{\text{initial weight[mg]} * 1000}{\text{desired concentration [mM]}}$$

2. Vortex-mix or shake for 1 min and visually inspect the solubilisation of the compound.
3. In case of any undissolved particles, repeat step 2. Visually inspect the solubilisation of the compound.
4. In case of undissolved particles place the tightly closed vial into an ultrasonic bath and apply ultrasonic for 2 min.. Visually inspect the solubilisation of the compound.
5. In case of undissolved particles vortex-mix for 10 sec and apply ultrasonic for 5 min. Visually inspect the solubilisation of the compound.
6. In case of undissolved particles, place the vial into a 37°C water bath for 10 min. Visually inspect the solubilisation of the compound.
7. In case of any undissolved particles, the intended concentration cannot be applied.
8. Add additional DMSO to obtain a twofold lower strength concentration and repeat steps 1-6.
9. In case of any undissolved particles, the intended concentration cannot be applied.
10. Add additional DMSO to obtain a twofold lower strength concentration and repeat steps 1-6.
11. In case of undissolved particles, the intended concentration cannot be applied using DMSO as solvent.

Assessment of solubility and stability of test chemicals in the induction medium

A pre-test is performed to determine whether the test chemical remains in solution in the media used for induction method by diluting the test chemical stock solution in HepaRG™ Serum-free Induction Medium in a 1:1000 ratio for DMSO as solvent.

1. Add 10 µl test chemical stock solution to 9990 µl HepaRG™ Serum-free Induction Medium (i.e. 1:1000 ratio in case of DMSO)
2. The resulting incubation solution (highest concentration of intended testing range) is visually inspected for compound precipitation.
3. The solution is transferred to 1.5 ml reaction tubes (500 µl, n=3).
4. One additional reaction tube is prepared using HepaRG™ Serum-free Induction Medium w/o test chemical for comparison.
5. Tubes are incubated at 37°C for 24 ± 0.3 h.

6. At the end of the incubation, the reaction tubes are centrifuged (4,400 – 4,700g, 10 min, RT).
7. The tubes are visually inspected for compound precipitation.
8. In case of compound precipitation, steps 1-7 have to be repeated using stock solutions of two-fold lower strength.

APPENDIX 9: CYTOTOXICITY ASSESSMENT OF TEST CHEMICAL(s)

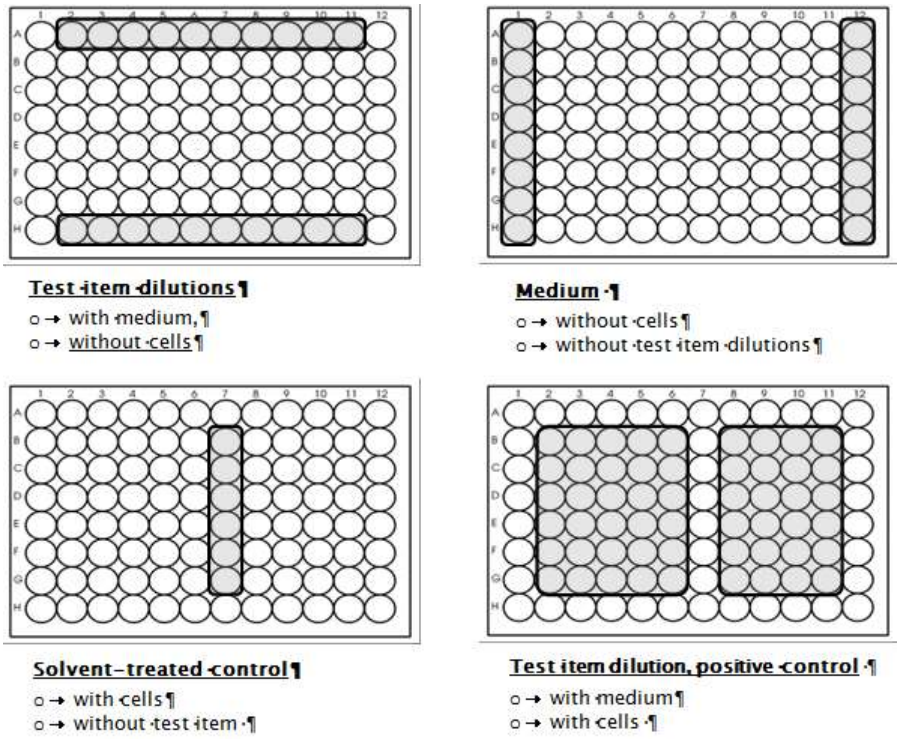
1. The described assay (i.e. CellTiter-Blue), used in the validation ring trial (EURL ECVAM, 2014), is based on the ability of living cells to convert a redox dye (resazurin) into a fluorescent end product (resorufin). Non-viable cells rapidly lose metabolic capacity and thus will not generate a fluorescent signal. The advantage of this assay is that it can be efficiently performed in multi-well plates and that it is not invasive and highly sensitive. Some test chemicals might interfere with the assay and interference testing is recommended (OECD, 2018).
2. Only those concentrations of a test chemical reducing cell viability of maximum 10 % are eligible for induction. Potential cytotoxicity of test chemical for cryopreserved differentiated human HepaRG™ cells has to be determined starting from the highest soluble concentration, followed, for example, by 1:3 dilution.

The example of the time-scheduled followed in the validation ring trial (EURL ECVAM, 2014) is reported:

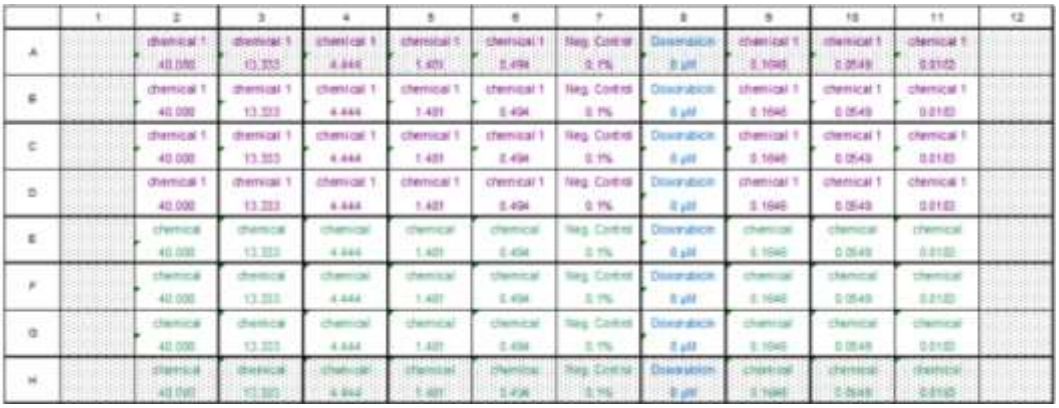
Day	Action
Fri (day 1)	Morning: Thawing and seeding of cryopreserved differentiated human HepaRG™ cells in HepaRG™ Thaw, Seed and General Purpose Medium Late afternoon (6 h ± 5 min after plating): Renewing of HepaRG™ Thaw, Seed and General Purpose Medium
Sat (day 2)	
Sun (day 3)	
Mon (day 4)	Medium exchange: HepaRG™ Serum-Free Induction Medium + test chemical (t=0 h)
Tue (day 5)	Medium exchange: HepaRG™ Serum-Free Induction Medium + test chemical (t=24±0.3 h)
Wed (day 6)	t=45±0.5 h addition of Cell Titer Blue reagent t=48±0.6 h cytotoxicity assay

3. Working solutions of test chemical(s) have to be prepared freshly every day. The content of organic solvents should be kept as low as possible. The concentration of DMSO has not to exceed 0.1% v/v DMSO in order to reduce unspecific effects on cellular growth and viability. The corresponding controls contain the same amount of organic solvent for normalization of potential unspecific effects.
4. In the proposed protocol, two test chemicals can be tested on a 96-well plate. Each test chemical is analysed at eight concentrations in triplicates (i.e. three different wells). For each test chemical a corresponding negative control (containing medium w/solvent, if solvent is used for dissolving of the test chemical) is included (n=3).
5. Doxorubicin (8 µM, n=6) serves as positive control.
6. The background fluorescence (n=8 per test chemical) of the reagent is measured and the fluorescence of the test chemical in medium at each tested concentration.
7. On a 96 well plate, the following parameters are tested:

- Cell Titer-Blue reagent background fluorescence without cells (A1-H1 and A12-H12) in HepaRG™ Serum-Free Induction Medium (absence of any solvent or test chemical dilution),
- Fluorescence of test chemical dilutions in HepaRG™ Serum-Free Induction Medium (without cells) (A2-A11 and H2-H11)
- Fluorescence of the positive control (8 µM Doxorubicin, B8-G8)
- Fluorescence of solvent treated controls (B7-G7)
- Fluorescence of 8 dilutions of test chemical(s) (B2-G6 and B9-G11; for examples in case of two test chemicals B2-D6 and B9-D11 for test chemical A and E2-G6 and E9-G11 for test chemical B).

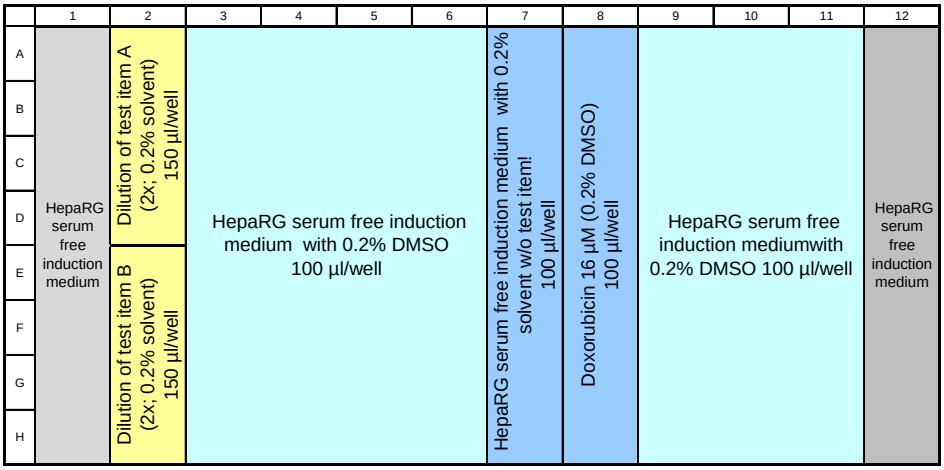


8. An example of plate layout for the cytotoxicity assay of two test chemicals (test chemical A = purple, test chemical B= green) is provided below. The starting concentration of test chemical depends on the solubility of the test chemical in presence of 0.1% v/v organic solvent. (e.g. **Appendix 4** - FORM-02). In this example, which reflect what was done during the validation ring trial (EURL ECVAM, 2014), concentrations are expressed in µg/ml. For these test chemicals, the highest applicable test concentration is assumed to be 40 µg/ml in presence of 0.1% v/v DMSO.



Test chemical(s) dilution plate layout

9. The dilutions of the test chemicals and the control media (containing solvent at the appropriate concentration) are prepared in a separate, sterile 96-well plate by serial dilution (all steps performed under the laminar flow hood). The content of the separate plate is carefully transferred to the plate containing the cryopreserved differentiated human HepaRG™ cell monolayers using an eight-channel pipette. The following section describes the 96 well dilution plate layout and procedure followed during the validation ring trial (EURL ECVAM, 2014).



10. Wells **A1-H1** and **A12-H12** of sterile 96-well plate are filled with Serum-free induction medium without solvent using an eight-channel pipette.
11. Dispense 100 µl HepaRG™ Serum-Free Induction Medium containing solvent to all other wells except wells **A2-H2** (highest test chemical concentration) and wells **A7-H7** (solvent control) and **A8-H8** (positive control). The medium has to contain the corresponding solvent in a concentration 2-fold higher than the intended final concentration. This procedure ensures that the solvent content in all wells is at the same concentration. (Example: If the intended final concentration is 0.1% v/v, the medium has to contain 0.2 % v/v solvent.)
12. To wells **A7-H7**, add 100 µl HepaRG™ Serum-Free Induction Medium supplemented with solvent at a concentration two-fold higher than the intended final test concentration. (Example: If the intended final concentration is 0.1% v/v, the medium in wells A7-H7 has to contain 0.2% v/v solvent.) The solution is prepared (e.g. **Appendix 4** - FORM-04).
13. For doxorubicin, a 16 µM solution is prepared freshly from an 8 mM stock solution which is

initially prepared in DMSO (e.g. **Appendix 4** - FORM-04). 100 µl of this solution is transferred to wells **A8-H8.**

14. To wells **A2-H2**, add 150 µl HepaRG™ Serum-free induction medium containing the test chemical at a twofold higher concentration than the intended final concentration is added. The content of solvent corresponds to the twofold of the intended final concentration, e.g. 0.2% v/v for a final solvent content of 0.1% v/v:
15. Transfer 50 µl from wells **A2-H2** to wells **A3-H3** through **A6-H6**. Mix by pipetting 4 times in each well.
16. Transfer 50 µl from wells **A6-H6** to wells **A9-H9** through **A11-H11** using fresh tips for each concentration. Mix by pipetting 4 times in each well. Wells **A7-H7** and **A8-H8** have to be skipped, since A7-H7 serve as solvent control and already contain 100 µl medium + 0.2% v/v solvent and A8-H8 contains 100 µl medium + 16 µM doxorubicin + 0.2% v/v solvent
17. Warm this test chemical dilution plate at 37°±0.25 C in a cell culture incubator for 10 min.

Cytoyoxicity assay

18. To perform the cytotoxicity assay, 72±0.9 hours after seeding of cryopreserved differentiated human HepaRG™ cells the plate is removed from incubator and cells are exposed to test chemical(s) as follows:
19. HepaRG™ Thaw, Seed and General Purpose Medium is removed from all the wells.
20. All wells are filled with 50 µl HepaRG™ Serum-Free Induction Medium (the non-seeded, outer wells as well).
21. The cytotoxicity assay is initiated by the transfer of-50 µl of the test chemical dilutions from the separate 96-well dilution plate ~~(~~described above~~)~~ are transferred to this plate using an 8-channel pipette (fresh tips for each concentration).
22. The plate is then placed in a cell culture incubator (5±1% CO_2 / 95±5% relative humidity) at 37°±0.25 C for 24 ± 0.3 h.
23. After 24 ± 0.3 h of incubation, the incubates in the wells are changed:
24. Remove the incubates after 24 ± 0.3 h and replaced by 50 µl of fresh, pre-warmed HepaRG™ Serum-Free Induction Medium.
25. Transfer of 50 µl of the freshly prepared test chemical dilutions and positive control using clean tips for each concentration and continue incubation at 37°±0.25 C for additional 24 ± 0.3 h. The viability measurement is started 45 ± 0.5 h after initiation of the cytotoxicity assay.

Viability measurement

26. The CellTiter-Blue reagent is stored frozen at -20°C and protected from light. For use, the reagent is thawed and brought to room temperature (10 min). The reagent should be protected from direct light.
27. Dispense 20 µl (= 20% of incubation volume) to each well after 45 ± 0.3 h of incubation.
28. Incubate for additional 3 ± 0.3 h.
29. At the end of the incubation time, remove the plate from the incubator and gently shake it in order to distribute the fluorescent dye equally.
30. Read the plate in a multiwell fluorometer at e.g. 544 nm excitation/590 nm emission (Options for fluorescence filter sets include 530-570 nm for excitation and 580-620 nm for fluorescence emission.)

Cytotoxicity results calculation

31. Results are expressed as fractional survival (% FS) with respect to untreated controls and are calculated based on the measured relative fluorescent units (RFU) corrected by the background signal *(rows A1-H1 and A12-H12)* (e.g. **Appendix 4** - FORM-2).

$$\%FS = \frac{RFU_{treated\ cells} - \text{mean } RFU_{background}}{\text{mean } RFU_{untreated cells} - \text{mean } RFU_{background}} \times 100$$

32. Mean % FS values of the individual test chemical concentrations are plotted against the corresponding concentrations. In case of a fluorescence impact of the test chemical (wells A2-A11 and H2-H8), the Cell Titer Blue assay can not be applied for cytotoxicity assessment. Such an impact is given if the auto-fluorescence of the test chemical is depending on its concentration and is > 1.5 higher at the highest concentration than at the lowest test chemical concentration.

Acceptance criteria for cytotoxicity assays

33. In the negative controls, the resulting RFU has to demonstrate the metabolic activity of the cells in the experiment. The negative control acceptance criterion should be established based on the analysis of historical data set for the equipment used. The study director is responsible to assure applicability of the established criterion.
34. The positive control, doxorubicin (8 µM), has to reduce cell viability of 30-70% ~~of reduction~~ (arithmetic mean) compared to the negative control.
35. If the background fluorescence of the test chemical interferes with the fluorescence measurement of the assay, the CellTiter Blue viability assay cannot be applied. Interference is produced, if the fluorescence of the test chemical is found to be higher than the RFU values of the negative control and if concentration dependence of the fluorescence is given, i.e. the fluorescence (RFU) of the test chemical increases with increasing concentrations. If the fluorescence (RFU) of the highest test concentration is >1.5 fold higher than the fluorescence (RFU) of the lowest test concentration, the CellTiter Blue viability assay cannot be applied.

APPENDIX 10: CYP INDUCTION ASSESSMENT OF TEST CHEMICAL(s)

1. An example of the time-scheduled followed in the validation ring trial (EURL ECVAM, 2014) is reported:

Day	Action
Fri (day 1)	Morning: Thawing and seeding of cryopreserved differentiated human HepaRG™ cells in HepaRG™ Thaw, Seed and General Purpose Medium Late afternoon (6 h ± 5 min after plating): Renewing of HepaRG™ Thaw, Seed and General Purpose Medium
Sat (day 2)	
Sun (day 3)	
Mon (day 4)	Medium exchange: HepaRG™ Serum-Free Induction Medium + test chemical (t=0 h)
Tue (day 5)	Medium exchange: HepaRG™ Serum-Free Induction Medium + test chemical (t=24 ±0.3h)
Wed (day 6)	t=48 h: end of induction addition of probe substrates cocktail in incubation medium (1 h incubation) LS/MS analysis of the CYP metabolites and in parallel cell lysis for protein content determination (e.g. BCA assay)

2. Briefly: cells are thawed on a Friday morning and allowed to attach for 6 hours. The HepaRG™ Thaw, Seed and General Purpose Medium is refreshed and the cells are allowed to recover for 65-72 hours. On Monday morning, the HepaRG™ Thaw, Seed and General Purpose Medium is replaced by the test chemical and reference compounds in HepaRG™ Serum-Free Induction Medium and the induction solutions are renewed after 24 ± 0.3 h. After a total induction time of 48 ± 0.6 h, the probe substrate reaction is carried out by addition of the probe substrate cocktail.
3. Wells A1 to A12, B1 and B12, C1 and C12, D1 and D12, E1 and E12, F1 and F12, G1 and G12, H1 to H12 do not contain cells and are not used for testing. They are filled HepaRG™ Thaw, Seed and General Purpose Medium subsequently to cell seeding. The following wells contain cryopreserved differentiated human HepaRG™: B2-B11, C2-C11, D2-D11, E2-E11, F2-F11, G2-G11. Wells E11-G11 are not foreseen for testing in the experimental design, as shown below in Figure, but they can be used as reserve wells, if one of the wells foreseen for induction must not be used due to inhomogeneity or disintegration of the monolayer.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		Chemical 1 40.000	Chemical 1 40.000	Chemical 1 40.000	Solvent 0.1% DMSO	Solvent 0.1% DMSO	Solvent 0.1% DMSO	Chemical 2 40.000	Chemical 2 40.000	Chemical 2 40.000	Solvent 0%	
C		Chemical 1 26.667	Chemical 1 26.667	Chemical 1 26.667	Solvent 0.1% DMSO	Solvent 0.1% DMSO	Solvent 0.1% DMSO	Chemical 2 20.000	Chemical 2 20.000	Chemical 2 20.000	Solvent 0%	
D		Chemical 1 17.778	Chemical 1 17.778	Chemical 1 17.778	PB 500 µM	PB 500 µM	PB 500 µM	Chemical 2 10.000	Chemical 2 10.000	Chemical 2 10.000	Solvent 0%	
E		Chemical 1 11.852	Chemical 1 11.852	Chemical 1 11.852	BNF 25 µM	BNF 25 µM	BNF 25 µM	Chemical 2 5.000	Chemical 2 5.000	Chemical 2 5.000	Reserve 1	
F		Chemical 1 7.901	Chemical 1 7.901	Chemical 1 7.901	RIF 10 µM	RIF 10 µM	RIF 10 µM	Chemical 2 2.500	Chemical 2 2.500	Chemical 2 2.500	Reserve 2	
G		Chemical 1 5.267	Chemical 1 5.267	Chemical 1 5.267	0.1% DMSO	0.1% DMSO	0.1% DMSO	Chemical 2 1.250	Chemical 2 1.250	Chemical 2 1.250	Reserve 3	
H												

4. Determination of functional CYP enzyme activity is performed in a cocktail (n-in-one) approach.
5. The induction is initiated by the addition of the induction solution (100 µl/well), which corresponds to time point t = 0 h.

6. The induction solution is replaced at time point t = 24 ± 0.3 h by freshly prepared induction solutions.
7. The medium added at time point t = 24 h is incubated for additional 24 ± 0.3 h, thus the cells are exposed to the inducer for 48 ± 0.3 h in total.
8. Time points of start of incubation and medium exchange are documented (e.g. **Appendix 4** - FORM-06).

Functional CYP enzyme activity assay

9. The functional activity is analysed after 48 ± 0.6 h exposure of the cells. CYP isoenzyme activities are tested using Incubation medium. A cocktail of 3 CYP probe substrates ((phenacetin 26 µM, bupropion 100 µM and midazolam 3 µM) is added to each well and incubated for 60 ± 3 min at 37° ± 1C. At the end of the incubation time, the reaction is quenched by the addition of stop solution (e.g. ACN with ISTD) and the samples are analysed for the specific metabolites (**Appendix 2 – Table 1**) with an appropriate analytical technique (e.g. LC/MS).
10. CYP probe substrates 10 mM stock solutions are prepared in MeOH (e.g. **Appendix 3)**. They are further diluted in MeOH to obtain working solutions of 4-fold higher strength than the intended final probe substrate concentrations (e.g. **Appendix 4** - FORM-03) in experimental incubations (phenacetin 26 µM, midazolam 3 µM, bupropion 100 µM), (**Appendix 2 – Table 3**). Thus the working solutions have the following concentrations: Phenacetin 104 µM, Midazolam 12 µM, Bupropion 400 µM.
11. Mix 1.5 ml of 4-fold concentrated working solutions of each probe substrate in a centrifugation tube and evaporate the solvent under a stream of nitrogen at room temperature. The centrifugation tube has to be wrapped with aluminum foil, since bupropion is light-sensitive.
12. Prewarm incubation medium, in a water bath to 37°C (20 ml per plate).
13. The dried residue of the substrate cocktail in the centrifugation tube is reconstituted in 6 ml Incubation medium.
14. Prewarm the probe substrate cocktail in a water bath to 37°C.
15. Prepare the stop solution and place it on ice (e.g. **Appendix 4** - FORM-07).
16. Remove Induction solutions from the wells of the cell plate (2-3 columns can be aspirated at once, a small volume should remain in the wells) and carefully wash all wells with 100µl prewarmed incubation medium. An 8-channel pipette is used for the washing step.
17. The washing step is repeated once.
18. Remove the washing solution of the second washing step from the first column and add 50 µl substrate cocktail to the respective wells using an 8-channel pipette (column by column) and document starting time (e.g. **Appendix 4** - FORM-06**)**. Perform this step for all rows in timed intervals (e.g. start every row after 20 or 30 sec).
19. Carefully move the plate in order to equally distribute the substrate cocktail in the wells. Transfer the plate into the cell culture incubator.
20. Incubate for 60 ± 3 min in total. (Incubation time starts with the addition of the cocktail to the first well.)
21. Prepare time point zero samples (or substrate control solutions): 40 µl substrate solution (n = 2) are added to 1.5 ml reaction tubes containing an equal volume acetonitrile/ISTD. The samples are vortexed for 10 seconds and stored at RT until the end of the incubation phase. 1 ml of the remaining substrate cocktail is immediately placed at -20°C to serve as a backup sample.

22. Shortly before the end of the incubation period, add 40 µl stop solution to a separate 96-well-plate (= "stop solution plate").
23. At the end of the incubation time, the medium (40 µl) is removed from the wells in the same timed intervals (see 8.) and transferred to the stop solution plate, correspondingly labelled.
24. Transfer the time point zero samples (or substrate control solutions) (see 11.) to 2 empty wells of the stop plate, too.
25. The content of the wells is thoroughly mixed using a multichannel pipette and the plate is subsequently centrifuged (10 min at ≥ 2,200 g, centrifuge equipped with a multi-well-plate rotor). 30 µl of the particle-free supernatant is transferred to a new 96-well plate (correspondingly labelled, the "LC/MS analysis plate") and diluted with 70 µl H_2O (final acetonitrile content: 15% v/v). An 8-channel pipette can be applied. This plate is subjected to LC/MS analysis (see 27), the analytical method applied during the validation ring trial. Other appropriate analytical technique might be used.
26. Another 30 µl of the acetonitrile precipitated samples is transferred to a new plate. This plate is covered with solvent-resistant aluminium foil and stored (undiluted) at -20°C as backup sample[8]
27. The other plate is covered with a suitable cover mat for LC/MS and subjected to LC/MS analysis. If the samples cannot be analysed directly, the plate is sealed with a suitable foil and stored at -20°C until analysis as well. After LC/MS measurement, the remaining quantities of the samples have to be stored at -20°C for possible further analysis until the study director decides to discard them. Frozen samples have to be thawed at room temperature and resuspended by up- and down pipetting before re-analysis.
28. Alternatively, the mixture is transferred to LC/MS sample vials (200 µl insert). The vials are closed using flanging pliers and subjected to LC/MS. (If the samples can not be analysed directly, the supernatants are transferred to 1.5 ml reaction tubes and stored at -20°C until analysis, see above.)
29. The residual Incubation medium (~ 10 µl) is aspirated off the cell plate. Cells in all wells are lysed by the addition of 50 µl 1M NaOH, incubated for 5 min and by mixing ten times with a multichannel pipette: half of the volume in the wells is pipetted up and down (i.e. 25-30 µl).
30. Aliquots of the lysates are removed from the plate, diluted 1:20 (i.e. 10 µl lysate with 190 µl H_2O) and stored at -20°C until analysis for protein content. Prior to the performance of the protein determination assay, the execution of one freeze-thaw-cycle of the lysates is mandatory (freezing period not less than 1 hour to support the lysis process). The cell plate containing the residual undiluted lysates is stored frozen at -20°C.

Protein content determination

31. For the protein determination we give, as an example, the procedure performed in the validation ring trial (i.e. Micro - Bicinchoninic Acid method, which was considered the most sensitive method for low amount of cells) (EURL ECVAM, 2014). Perform the assay according to the manufacturer's instructions.
32. Prepare diluted Albumin (BSA) standards: for preparation of standard solution S1 the Albumin Standard stock (BSA) ampule [2 mg/ml] is diluted in 0.05 M NaOH[9]. The standard solutions S2-S7 are prepared by serial dilution (**Appendix 2- Table 3**).

[8] In case of analysis of the backup samples, the plate can be removed from storage at -20°C, thawed to room temperature, gently mixed, and diluted with 70 µl H_2O (final acetonitrile content: 15% v/v). If the remaining volume of the backup sample is lower, the volume of H_2O has to be adapted accordingly.

[9] Prepared by mixing 0.5 ml NaOH 1M and 9.5 ml H_2O.

33. 150 µl sample (single determinations, diluted cell lysates, see step18) or sample standard (in duplicate) are pipetted from the cell plate into a clear-bottomed 96-well plate using an 8-channel pipette according to the scheme in **Appendix 2 Table 4**.
34. Working Reagent is prepared by mixing 25 parts of BCA Reagent A with 24 parts of BCA Reagent B and 1 part of BCA Reagent C (25:24:1, A:B:C) according to the manifacturer. When Reagent C is first added to Reagent A/B mixture, turbidity is observed that quickly disappears upon mixing to yield a clear, green Working Reagent. The Working Reagent is stable for one day when stored in a closed container at room temperature.
35. 150 µl Working Reagent is added per well, the plate is covered using an adhesive foil, and the plate is mixed thoroughly on a shaker for 30 sec.
36. Cover the plate and incubate at 37°C for 2h.
37. Cool the plate to room temperature.
38. Read the plate at OD_{562} nm within 10 min.

Protein content results calculation

39. The absorbance of the blank standard is subtracted from the absorbance of all other individual standard and unknown sample replicates
40. A standard curve is prepared by plotting the average blank-corrected absorbance for each standard vs. its concentration in mg/ml. Unknown samples are extrapolated from the standard curve using linear regression (e.g. **Appendix 4** - FORM-08).

$$protein\ \left[\frac{mg}{ml}\right] = \frac{absorbance_{\ sample} - axis\ intercept_{standard\ curve}}{slope_{standard\ curve}}$$

41. Results [mg/ml] are corrected for the dilution factor (**e.g. Appendix 4 -** FORM-08).
42. The results are expressed as CYP enzyme activities in **pmol x min-1 x mg protein-1 or pmol x min-1 x (1*10^6 cells)-1**, respectively (**e.g. Appendix 4** - FORM-08).

Standard curve acceptance criteria

43. The standard curve should have a coefficient of determination (r^2) equal or greater than 0.9.