OECD GUIDELINE FOR THE TESTING OF CHEMICALS

DRAFT PROPOSAL FOR A NEW PERFORMANCE BASED TEST GUIDELINE

Human cytochrome P450 (CYP) n-fold induction *in vitro* test method

GENERAL INTRODUCTION

Performance-Based Test Guideline

1. This PBTG describes the methodology to assess the potential of test chemicals to induce three cytochrome P450 (CYPs) isoforms CYP1A2, CYP2B6, and CYP3A4 in two human-derived metabolically competent hepatic in vitro test systems:

(1) human primary hepatocytes (3 donors reflecting donor variability);
(2) HepaRG cell line (3 batches derived from one donor).

CYP induction, following multi-challenge exposure of the test systems to the test chemical (i.e. the medium with the test chemical is renewed every 24 hours over the duration of the *in vitro* test method), is assessed by analytical (LC/MS) measurement of the concentration of specific biotransformation products. A cocktail of the three specific CYP substrates is added and samples are then analyzed for the specific biotransformation products.

2. As risk assessment is moving towards greater use of quantitative kinetic data, the test methods in this PBTG will be of increasing value. The information/data produced with this PBTG will help to gain more insight into toxicological Mode of Action. The test method is biologically relevant for the induction of enzymatic activity for CYPs that may be associated with receptor-activated pathways. This information can be integrated into a test strategy that could help to assign a test chemical to a particular Adverse Outcome Pathway, where CYP induction is identified as a key event.

3. The fully validated reference *in vitro* test methods (Annex 2 and Annex 3) that provide the basis for this PBTG are:

- The cryopreserved human HepaRG® cells CYP n-fold induction *in vitro* test method (CryoHepaRG®)
- The cryopreserved human primary hepatocytes CYP n-fold induction *in vitro* test method (Cryoheps)

4. Performance standards (PS) are available to facilitate the development and validation of similar test methods (referred as “me-too” test methods) for the same endpoint and allow for timely amendment of this PBTG so that new similar test methods can be added to an updated PBTG. However, similar test methods will only be added after review and agreement that performance standards are met. The test methods included in this Test Guideline can be used indiscriminately to address countries’ requirements for test results on CYP induction while benefiting from the Mutual Acceptance of Data.

DEFINITIONS

5. Definitions are provided in Annex 1.

Background and principles of the tests method included in this PBTG

6. One of the most important functions of hepatocytes is the biotransformation of endogenous and exogenous (xenobiotics) compounds. These liver parenchymal cells are very rich in smooth endoplasmic reticulum which incorporates a large amount of biotransformation enzymes. The characteristically polarised structure of hepatocytes allows the excretion of the biotransformation products into the bile or the blood (1).

7. Biotransformation in the liver is accomplished by two classes of enzymes: phase I and phase II biotransformation enzymes (2). Cytochrome P450s are pivotal phase I mono-oxygenase enzymes involved in the synthesis and degradation of endogenous steroid hormones, vitamins and fatty acid derivates, but also in the transformation of xenobiotics such as drugs and environmental chemicals into more hydrophilic molecules, facilitating their excretion.

8. Exposure to xenobiotics can lead to the induction of biotransformation enzymes including CYPs. CYP induction is defined as an increase in the amount and activity of a biotransformation enzyme due to *de novo* CYP protein synthesis or stabilisation of CYP enzymes. CYP induction may lead to a significant variation in the concentration of the xenobiotic and its metabolites at the target site enhance clearance or toxic accumulation of the parent compound (or its metabolites) or produce toxic metabolites. Due to the relatively broad substrate specificity of CYPs, many metabolic routes of elimination can be induced by concomitant xenobiotic administration/exposure (i.e. mixtures, chemical-chemical, drug-drug).

9. The two human *in vitro* CYP induction test methods will contribute to knowledge gathering on:

a) Identification of compounds that have CYP induction as a key event in a toxicity pathway
b) Identification of compounds that lead to CYP induction and affect biotransformation of endogenous (=non-xenobiotics) substances and thus disturbing normal intermediary metabolism and physiological homeostasis.
c) Identification of compounds in mixtures that induce CYP

10. At the molecular level, CYP induction involves several cellular processes (3; 4; 5):

- The chemical binds to a specific receptor,
- The activated receptor forms heterodimer with factors, such as Ahrnt (Ahr nuclear translocator) and retinoid X receptor (RXR for both PXR and CAR) and migrates into the nucleus,
- The heterodimer binds to the target xenobiotic response elements (XRE) located in both the proximal and distal P450 gene promoters,
- The transcription of the respective CYP gene is enhanced, which is followed by the de novo protein synthesis and post-translational modification to a functional CYP enzyme.

11. Among the receptors, the Constitutive Androstane Receptor (CAR), the Pregnane X Receptor (PXR) and the Aryl Hydrocarbon Receptor (AhR) are involved in CYP-mediated metabolism. These receptors control the expression of CYP1A (AhR), CYP2, and CYP3A (PXR and CAR) families (6; 7; 8; 9; 10; 11; 12; 13), as well as UGTs and glutathione-S-transferases and the transporters MDR1 and MRP2 (14).

12. Use of a human relevant system is important because the AhR, PXR and CAR found in toxicological animal models, such as mouse and rat, exhibit significant differences in specificity (15). Differences of induction among species are explained by discrepancies in the ligand-binding domain of the receptors implying that their ligand specificities may differ dramatically between species. Therefore, extrapolation of animal data with respect to the inducibility of CYP enzymes in humans is not relevant.

13. The CYP n-fold induction *in vitro* test method can be used to assess inducing capacity of chemicals irrespective of their use class, i.e. including pharmaceutical ingredients, pesticides, cosmetic ingredients, ingredients of household products etc. Since the CYP induction method is based on xenobiotic-receptor binding, any class of compounds that can interact with such receptors is predicted to be qualified to be used in *in vitro* CYP induction test method. Test chemicals other than pharmaceuticals have been shown to induce CYPs (16; 17).

14. The validation study on which this PBTG is based was coordinated by EURL ECVAM and performed in accordance with the OECD Guidance Document No. 34 on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment (18). The validation study demonstrated the relevance and reliability of the human CYP n-fold induction *in vitro* test method for the intended purpose of assessing the induction of specific CYP isoforms by measuring enzymatic activity (19).

15. The validation study required reliable human clinical data on the induction of the four CYPs for a proper evaluation of the predictive capacity of the *in vitro* results. As human data of sufficient quality for the three CYPs are only available for pharmaceuticals, all the substances used in the validation study were pharmaceuticals. The test substances were tested at a wide range of concentrations in order to cover human clinically relevant concentrations of CYP inducers for comparison with the available human reference data.

16. In the validation study, CYP n-fold induction was assessed at the enzyme (CYP1A2, CYP2B6, and CYP3A4) activity level. Levels of mRNA were not measured since it is well documented that there is a discrepancy between mRNA induction and enzyme activity and thus a lack of positive correlation between CYP enzyme activity and the specific CYP mRNA level (20; 21; 22; 23). Abass (17) observed that in HepaRG® cells, phenobarbital induced the CYP activity in a dose dependent manner, in contrast with mRNA. As CYP induction is therefore not necessarily reflected by changes in mRNA levels, it was measured on the basis of the enzymatic activity for the isoforms after exposure of the cells to the test substance.

Scope and limitations of the human-derived test system based CYP induction in vitro method

17. While since 1997, the European Medicines Agency (EMA) and Food and Drug Administration (FDA) Guidelines (24; 25) require CYP induction assessment for new pharmaceuticals, human CYP induction for safety assessment of a broad spectrum of test chemicals (e.g. chemicals, cosmetics, food additives, pesticides, mixtures) is currently not systematically addressed by OECD Guidelines.

18. The human CYP induction *in vitro* test methods of this PBTG allow the identification of test chemicals that induce CYP1A2, CYP3A4 and CYP2B6, involving several cellular processes such as xenobiotic-receptor binding (CAR, PXR and AhR), *de novo* protein synthesis or protein stabilization.

19. The purpose of the human CYP induction *in vitro* test method of this PBTG is to evaluate the potential of a test chemical to induce CYP mediated via PXR/CAR (CYP3A4, CYP2B6) and the Ah-receptor (CYP1A2). The selection of these three CYP isoforms, which are recommended by the EMA and FDA Guidelines, is based on the fact that in humans they are involved in the biotransformation of a wide variety of endogenous and exogenous chemicals and that they are target CYPs for classical model inducers: CYP1A2 for dioxins and PAHs, CYP2B6 for phenobarbital and CYP3A4 for rifampicin.

20. Data generated during the validation study showed that the test method performed well in terms of within laboratory reproducibility (WLR) and between laboratory reproducibility (BLR) and they can serve as references in developing similar methods (26; 27).

21. The human CYP induction test method has been validated using test substances that are well-known PXR/CAR or Ah-receptor ligands, and for which human clinical data are available. However, the CYP n-fold induction test method can be used to assess the CYP induction capacity of any test chemical, (e.g. pharmaceutical ingredients, pesticides, cosmetic ingredients, ingredients of household products etc.) able to bind to one of the above mentioned receptors.

22. Cultured human primary hepatocytes (fresh or cryopreserved) are the most accepted (industry, academia) *in vitro* test system for assessing the potential for xenobiotics to induce human CYP isoforms and are still the gold standard for FDA Guidelines on drug-drug interaction studies (28; 24). One important advantage of using human hepatocytes is that one can get information on CYP induction in different donors. In the validation study on which this PBTG is based, information was generated for human CYP induction using three different donors, according to EMA and FDA guidelines.

23. Many researchers use pools of hepatocytes for other applications such as human hepatic metabolic stability/clearance. The use of separate hepatocyte donors or pools depends on the purpose of the study. Individual donors or pools could be used for the study of a phenomenon of human CYP induction, but the use of individual preparations enriches the information by providing at least some idea about intraspecies variability.

24. Primary human hepatocytes derived from 3 separate donors were considered in the past as a kind of gold standard for CYP induction studies. However, known donor variability and their limited availability stimulated a search for alternative test systems. HepaRG® cells represent a promising alternative to primary human hepatocytes as they combine long-term stability of chemical-metabolising

enzymes and transporters with the correct plasma membrane polarization. HepaRG is a cell line derived from one donor allowing the generation of comparable historical data that is considered by current industrial end-users to function like an adult liver cell, with unique characteristics such as the whole Hepatitis C cycle and ADME gene expression.

25. The human CYP n-fold induction *in vitro* method does not provide information on the number or nature of possible (reactive/non-reactive) metabolites or about human hepatic clearance/stability of the chemicals tested. To address this aspect, *in vitro* hepatic metabolic clearance/stability, metabolite identification and reactivity assessments would be needed. However, the two *in vitro* test systems used in the human *in vitro* CYP n-fold induction method might be employed, using a suitably modified protocol, for estimating an *in vitro* intrinsic human hepatic metabolic clearance, which is then used for the extrapolation of *in vivo* human hepatic metabolic clearance.

26. Before use of the test guideline on a mixture for generating data for an intended regulatory purpose, it should be considered whether, and if so why, it may provide adequate results for that purpose. Such considerations are not needed, when there is a regulatory requirement for testing of the mixture.

CYP N-FOLD INDUCTION TEST METHOD COMPONENT

Methodological standards

27. Standard methodology used to provide specific essential information for, and evidence of good characterisation of the test systems for the basal and induced enzymatic activities for the three CYPs.

- Exposure to a prototypical inducers β-naphthoflavone (BNF; 25 µM), phenobarbital (PB; 500 µM), and rifampicin (RIF; 10 µM) must lead to a ≥ 2-fold increase of enzymatic activity at the defined fixed concentrations.
- Reference substances should be classified as inducers or non-inducers according to Table 2 Decision classification of proficiency substances.

28. Such standards will guide the end-users of the OECD performance-based test guideline ultimately to obtain the information necessary for harmonised reporting formats for *in vitro* methods.

Essential test method component

29. The PBTG applies to methods using metabolically competent human-derived *in vitro* test systems. They should express the nuclear receptors PXR, CAR and AhR[1] as well as transporters and the three CYP isoforms, CYP1A2, CYP2B6, and CYP3A4, should be functionally active and inducible. Other receptors, transporters and Phase I and II enzymes may be expressed. These are essential test method components.

Control substances

[1] Strictly speaking, AhR does not belong to nuclear receptor family, but because of its role in mediating induction of some xenobiotic-metabolizing CYP and other enzymes, it is grouped here with PXR and CAR.

30. The basis for the proposed reference substances and controls should be described. Concurrent controls (negative, solvent, and prototypical inducers), as appropriate, serve as an indication that the test method is operative under the test conditions and provide a basis for experiment-to-experiment comparisons; they are usually part of the acceptance criteria for a given experiment (18).

Standard Quality Procedures

31. Standard quality control procedures should be performed to ensure that the CYPs investigated are inducible in the test system used. Table 1 shows the prototypical inducers to be included in every study. Exposure to a prototypical inducer must lead to a ≥ 2-fold increase of enzymatic activity at the defined fixed concentrations.

Table 1: Prototypical inducers

Isoenzyme	CYP1A2	CYP2B6	CYP3A4
Inducer	β-Naphthoflavone (BNF) [25 µM]	Phenobarbital (PB) [500 µM]	Rifampicin (RIF) [10 µM]

Cells should be evaluated with regard to contamination of mycoplasma and other contaminants (e.g. fungi, yeast). Established acceptance criteria for morphology must be met.

Proficiency substances

32. Prior to using this PBTG for testing unknown chemicals, each laboratory should demonstrate proficiency in using the test method by first testing reference test substances. This proficiency testing will also confirm the responsiveness of the test system. The list of proficiency substances is a subset of the substances used in the CYP n-fold induction Validation Study. They represent different MoA covering the three main initiating molecular events (binding to the PXR, CAR or AhR receptors). These substances, all pharmaceuticals, are commercially available.

Table 2: Decision classification of proficiency substances

Proficiency substance	Test concentration range [µM]	LOEL [µM][a]			Decision classification		
		CYP1A2	CYP2B6	CYP3A4	CYP1A2	CYP2B6	CYP3A4
Omeprazole	0.48-115.8	12.9	38.6	38.6	N-fold induction	N-fold induction	N-fold induction
Carbamazepine	0.70-169.3	54.6	2.09	6.27	N-fold induction	N-fold induction	N-fold induction
Phenytoin	0.45-145.8	16.2	0.60	1.80	N-fold	N-fold	N-fold

					induction	induction	induction
Rifampicin	0.20-48.6	0.2	0.2	0.2	N-fold induction	N-fold induction	N-fold induction
Sulfinpyrazone	0.41-98.9	3.66	3.66	1.22	N-fold induction	N-fold induction	N-fold induction
Bosentan	0.07-70.2	0.29	0.29	0.07	N-fold induction	N-fold induction	N-fold induction
Metoprolol	0.62-149.6	NA	NA	NA	No n-fold induction	No n-fold induction	No N-fold induction
Sotatol	0.53-129.5	NA	NA	NA	Non-fold induction	No n-fold induction	No n-fold induction
Penicillin G	0.46-112.2	NA	NA	NA	No n-fold induction	No n-fold induction	No n-fold induction

[a]Lowest F2 value from either CryoHepaRG or Cryoheps. NA = applicable because the CYP isoform was not induced.

33. Reference compounds, concurrent controls (negative, solvent, and prototypical inducers) and unknown compounds should be tested in triplicate wells.

34. Testing of these reference substances should be replicated once. If the acceptance criteria are met, no repetition is required. Proficiency is demonstrated by correct classification (inducer/non inducer) of each proficiency reference item. Proficiency testing should be repeated by each technician when learning the test method.

Test run acceptability criteria

35. Acceptance or rejection of a test run is based on the evaluation of the n-fold induction results obtained for the prototypical inducers, the reference substances and the controls. The 2-fold induction value for the reference substances should meet the acceptability criteria and all positive/negative controls should be correctly classified in each accepted run.

Analysis of data

36. The n-fold induction potential of a test chemical is calculated as *n-fold induction of solvent-treated control* (0.1% DMSO), i.e. calculated by normalizing the enzymatic activity in the presence of the test chemical to the enzymatic activity in the absence of the test chemical,

n-fold induction = activity of treated cells/activity of control cells,

A compound is classified an inducer in the *in vitro* system when ≥2-fold increase in enzyme activity of probe substrate at inducer concentrations ≤ 500 μM is observed.

37. The CYP enzyme potency of a test compound in the *in vitro* model needs to be related to *in vivo* exposure to predict the propensity of the compound to be an inducer *in vivo*. For drug compounds the blood plasma concentrations are often known and can thus be used to evaluate the relevance of an n-fold induction response in the *in vitro* system. A simple correlation analysis using Cmax *in vivo* and the concentration in the cell system that results in a 2-fold (F2) induction can be applied. A ratio of Cmax/F2 above 2 indicates an inducer *in vivo*. By applying this model the Sensitivity and the Specificity of the *in vitro* models can be analyzed (see Proficiency standards).

38. Standard deviation (SD) or coefficient of variation (CV) for the means of the reference substances curve fitting parameters from multiple experiments may be used as a measure of within-laboratory reproducibility.

The concentrations tested should remain within the solubility range of the test chemical and not demonstrate cytotoxicity.

The defined data interpretation procedure for each test method should be used for classifying a compound as inducer or non-inducer.

Meeting the acceptability criteria with the reference/proficiency substances indicates that the assay system is properly operating but it does not ensure that the test method will produce accurate data. Replicating the results of the first test is the best indication that accurate data have been produced.

General data interpretation

39. N-fold induction response is not an all-or-none response but a graded response that is both time- and inducer-dependent and results from a number of steps; consequently the criteria for defining inducer status consist of both objective statistically definable measures (extent of n-fold induction, statistically significant increase) and observations (shape of the concentration – response curve, possible outliers, distribution of statistically significant increases along concentration points, fit to Hill curve). Theoretically, n-fold induction response should preferably be characterized by quantitative measures as EC50, F2, Emax, Tmax, and perhaps a threshold concentration such as BMDL10. These quantitative coefficients could be determined for any response, including receptor binding, mRNA, enzyme protein, activity, or more distal biomarker. It is noteworthy that repeated experiments and/or extended range of concentrations for the test chemicals considered may provide information to determine the amplitude of induction eventually observed.

40. There is currently no universally agreed method for interpreting the CYP n-fold induction data. The 2-fold induction cutoff for these test methods has been a general consensus among the scientific community based on past experience. The 2-fold induction response is a useful point for calculations of statistical significance and is applicable in assessing, interpreting and extrapolating the response, but it is possible to select other thresholds. It is also important to observe the shape of the dose response curve. It is suggested that at least 2 of the 6 concentrations tested should be above the background response to ensure relevant data.

Test report

41. The test report should include the following information:

Test method:

− Test method used;

Test chemicals:

− Mono-constituent substance:

physical appearance, water solubility, and additional relevant physicochemical properties;

chemical identification, such as IUPAC or CAS name, CAS number, SMILES or InChI code, structural formula, purity, chemical identity of impurities as appropriate and practically feasible, etc. (including the organic carbon content, if appropriate).

− Multi-constituent substance, UVBCs and mixtures:

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

− storage conditions and stability of the test chemical;

– concentration tested

Solvent/Vehicle:

− solvent used and ratio of its constituents, if applicable;

− chemical identification and CAS number;

− physical appearance and purity and physicochemical properties in case other solvent/vehicle than that mentioned in the Test Guideline is used and to the extent available;

- storage conditions and stability of the test chemical

− justification for choice of solvent/vehicle;

− solubility and stability of the test chemical in solvent/vehicle, if known;

Cells:

− type and source of cells:

– Phase I and II enzymes expression; inducibility of CYPs (provided on the CoA for each cell batch)

− passage number of cells at thawing;

− methods for maintenance of cell cultures;

Test conditions:

− solubility limitations;

- cytotoxicity limitations;

− description of the methods applied for assessing viability;

− composition of media;

− concentrations of test chemical;

− volume of vehicle and test chemical added;

− incubation temperature and humidity;

− duration of treatment;

− cell density and morphology at the start of, during and at the end of treatment;

− positive and negative reference substances;

− criteria for considering tests as positive, negative or equivocal;

LC/MS-MS:

− Type of LC/MS instrument. Parameter relevant to the analysis. LC/MS operation and processing methods.

Acceptability check:

− n-fold inductions for each assay plate and whether they meet the minimum required by the test method based on historical controls;
− *Results*:
− raw and normalised data;
− the n-fold induction level;
− cytotoxicity data;
− concentration-response relationship, where possible;
− statistical analyses, if any, together with a measure of error and confidence (e.g. SEM, SD, CV or 95% CI) and a description of how these values were obtained;
Discussion of the results
Conclusion

ANNEX 1

Definitions and Abbreviations

Acceptability criteria: Minimum standards for the performance of experimental controls and reference standards. All acceptability criteria should be met for an experiment to be considered valid.

Accuracy (concordance): The closeness of agreement between test method results and an accepted reference values. It is a measure of test method performance and one aspect of relevance. The term is often used interchangeably with "concordance" to mean the proportion of correct outcomes of a test method.

AOP (Adverse Outcome Pathway): An AOP is a sequence of events from the exposure of an individual or population to a chemical substance through a final adverse (toxic) effect at the individual level (for human health) or population level (for ecotoxicological endpoints).

Cell morphology: The shape and appearance of cells grown in a monolayer in a single well of a tissue culture plate. Cells that are dying often exhibit abnormal cell morphology.

Cytotoxicity: Harmful effects to cell structure or function that can ultimately cause cell death and can be reflected by a reduction in the number of cells present in the well at the end of the exposure period or a reduction of the capacity for a measure of cellular function when compared to the concurrent vehicle control.

CV: Coefficient of variation

DMSO: Dimethyl sulfoxide

EC50: The half maximal effective concentration of a test chemical.

ICCVAM: The Interagency Coordinating Committee on the Validation of Alternative Methods.

Between-laboratory reproducibility: A measure of the extent to which different qualified laboratories, using the same protocol and testing the same substances, can produce qualitatively and quantitatively similar results. Between-laboratory reproducibility is determined during the validation processes, and indicates the extent to which a test method can be successfully transferred between laboratories.

"Me-too test": A colloquial expression for a test method that is structurally and functionally similar to a validated and accepted reference test method. Interchangeably used with similar test method.

PBTG: Performance-Based Test Guideline

Performance standards: Standards, based on a validated test method, that provide a basis for evaluating the comparability of a proposed test method that is mechanistically and functionally similar. Included are (1) essential test method components; (2) a minimum list of reference substances selected from among the chemicals used to demonstrate the acceptable performance of the validated test method; and (3) the comparable levels of accuracy and reliability, based on what was obtained for the validated test method,

that the proposed test method should demonstrate when evaluated using the minimum list of reference substances.

Proficiency substances: A subset of the reference substances included in the Performance Standards that can be used by laboratories to demonstrate technical competence with a standardized test method. Selection criteria for these substances typically include that they represent the range of responses, are commercially available, and have high quality reference data available.

Proficiency: The demonstrated ability to properly conduct a test method prior to testing unknown substances.

Reference standard: a reference substance used to demonstrate the adequacy of a test method.

Reference test methods: The test methods upon which this PBTG is based.

Relevance: Description of relationship of the test to the effect of interest and whether it is meaningful and useful for a particular purpose. It is the extent to which the test correctly measures or predicts the biological effect of interest. Relevance incorporates consideration of the accuracy (concordance) of a test method.

Reliability: Measure of the extent that a test method can be performed reproducibly within and between laboratories over time, when performed using the same protocol. It is assessed by calculating intra- and inter-laboratory reproducibility.

SD: Standard deviation.

Sensitivity: The proportion of all positive/active substances that are correctly classified by the test. It is a measure of accuracy for a test method that produces categorical results, and is an important consideration in assessing the relevance of a test method.

Specificity: The proportion of all negative/inactive substances that are correctly classified by the test. It is a measure of accuracy for a test method that produces categorical results, and is an important consideration in assessing the relevance of a test method.

Substance: Used in the context of the UN GHS as chemical elements and their compounds in the natural state or obtained by any production process, including any additive necessary to preserve the stability of the product and any impurities deriving from the process used, but excluding any solvent which may be separated without affecting the stability of the substance or changing its composition.

Validated test method: A test method for which validation studies have been completed to determine the relevance (including accuracy) and reliability for a specific purpose. It is important to note that a validated test method may not have sufficient performance in terms of accuracy and reliability to be found acceptable for the proposed purpose.

Validation: The process by which the reliability and relevance of a particular approach, method, process or assessment is established for a defined purpose.

421 **VC (Vehicle control)**: The solvent that is used to dissolve test chemicals and control substances is tested
422 solely as vehicle without dissolved chemical/substance.

423 **Within-laboratory reproducibility**: A determination of the extent that qualified people within the same
424 laboratory can successfully replicate results using a specific protocol at different times.

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ANNEX 2

Cytochrome P450 n-fold induction in CryoHepaRG® cells (N-in-one incubation on 96-well plate)

INITIAL CONSIDERATIONS AND LIMITATIONS (see also GENERAL INTRODUCTION, page 1)

1. Cytochrome P450 n-fold induction in CryoHepaRG® cells (n-in-one incubations on 96-well plates) is an *in vitro* test method which assesses the potency of chemicals to induce Cytochrome P450 (CYP) isoforms CYP1A2, CYP2B6, and CYP3A4 in the human cryopreserved HepaRG® cell line.

2. The test method does not provide information on the number or nature of possible (reactive/non-reactive) metabolites nor about human hepatic clearance/stability of the chemicals investigated.

3. The "GENERAL INTRODUCTION" and the "CYP N-FOLD INDUCTION TEST METHOD COMPONENTS" should be read before using this test method for regulatory purpose

PRINCIPLE OF THE TEST METHOD (see also GENERAL INTRODUCTION, page 1)

Procedure: summary

4. Before performing the n-fold induction experiment, the test chemical is assessed for its solubility in DMSO (solvent) and in the induction medium under experimental conditions (37°C, 24 h). The highest soluble concentration is used as a starting concentration for cytotoxicity assessment on cryopreserved HepaRG® cells. The highest non-cytotoxic concentration is then used as the starting concentration in n-fold induction experiments.

5. The *in vitro* CYP n-fold induction method is performed with one 96-well-plate (CryoHepaRG®) for one cell batch. Cells are exposed to the test chemicals and prototypical inducers in serum-free induction medium which is renewed after 24 ± 0.3 h (multi - challenge exposure). After a total induction time of 48 ± 0.3 h (CryoHepaRG®), the probe substrate reaction is carried out. A cocktail of three P450 substrates is added to each well and incubated for 60 ± 3 min (CryoHepaRG®), at 37 ± 1°C. The reaction is then quenched by the addition of acetonitrile supplemented with internal standards for bioanalysis and the samples are analysed for the specific reaction products by means of LC/MS-MS.

Test System: CryoHepaRG®6. CryoHepaRG®cells originate from a hepatocarcinoma of a human female patient. They maintain an efficient proliferation differentiation interplay accompanied by morphological changes leading to hepatocyte-like cells and a stable expression of P450 enzymes, phase II enzymes, transporters, and nuclear transcription factors for up to 6 weeks in cultures. They respond to prototypical inducers of CYP1A1, CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2E1 and CYP3A4 (29; 30; 31; 32; 33). Due to these features, the CryoHepaRG® cell line is considered a valuable human-relevant *in vitro* model for investigating P450 induction properties of drug compounds.

Data analysis

7. The endpoint value is n-fold induction defined as

$$\text{n-fold induction} = \frac{\text{activity of treated cells}}{\text{activity of solvent-treated control (0.1\% DMSO) cells}}$$

8. Exposure to prototypical inducers must lead to a ≥ 2-fold increase of enzymatic activity (of statistical significance) at a concentration of ≤ 500 µM to classify the test chemical as an inducer (35).

9. A test chemical is considered an inducer if a ≥2-fold increase of enzymatic activity (of statistical significance) is measured. Because a ≥2-fold increase is just one point of information, it is also important to observe the shape of the dose response curve. It is suggested that at least 2 of the 6 concentrations tested should be above the background response to ensure relevant data.

PROCEDURE

Cell line

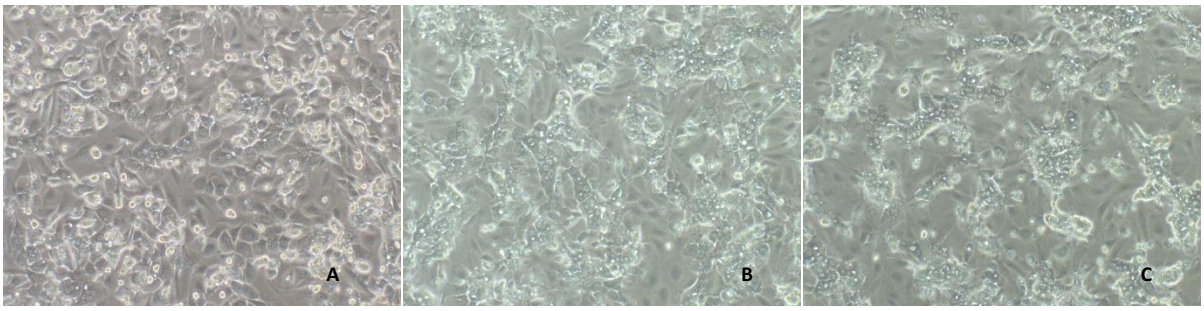
10. CryoHepaRG® cells were first described in 2002 by Gripon (30). For commercial purposes, cells and all media are available from Life Technologies or Millipore.

Cell plating and culture

11. CryoHepaRG® cells are delivered as differentiated cryopreserved cells in vials on dry ice. For n-fold induction assay, they are thawed according to the manufacturer's instructions, counted (Trypan Blue dye exclusion method) and seeded into the inner wells of one collagen I coated 96-well plate at a density of 7.2 x 10^4 cells per well in 100 µl CryoHepaRG® Thaw, Seed and General Purpose Medium. The outer wells are filled with Dulbecco's PBS. Seeded cells are maintained in a CO_2 incubator (5% CO_2) at 37°C and the following acceptance criteria should be met: (I) minimum cell viability: 80 % after thawing; (II) minimum recovery per vial: 4.5 x 10^6 cells/vial.

6 h after plating, cell morphology is checked and medium is renewed. 70 h after plating, cells are visually inspected and have to meet the morphological acceptance criteria (Figure 1). A few days after thawing and culture of CryoHepaRG®, the cells form a coculture of hepatocytes and of biliary-like epithelial cells.

Figure 1: Cell morphology of CryoHepaRG® 6h after thawing (A), before induction (B) and at the end of the induction period (C), respectively (untreated control wells).



-Approximately 80% confluent CryoHepaRG® monolayer after the 72 h attachment period (morphological observation).

TEST CHEMICALS, PROTOTYPICAL INDUCERS AND CONTROLS

Vehicle

12. Dimethyl sulfoxide (DMSO), or an appropriate solvent should be used for positive and negative controls and for the test chemicals. The final DMSO (or other solvent) concentration should not exceed 0.1 % (v/v) since the solvent itself may have induction potential (e.g., DMSO induces CYP3A4). For any other vehicle, it should be demonstrated that the maximum concentration used is not cytotoxic and does not interfere with assay performance in terms of induction or inhibition of functional CYP activities.

Preparation of reference test substances

13. Reference test substances should be dissolved in DMSO or other suitable solvent and serially diluted with the same solvent in order to prepare solutions for dilution with CryoHepaRG® Serum-Free induction Medium. In order to cover a full-dose response range, the highest concentration is serially diluted by a factor of 1:1.5, 1:2, 1:2.5 or 1:3 at 6 levels.

Solubility: considerations for range findings

14. Acceptance criteria for selection of appropriate test concentrations:

- Test chemical must be dissolved at all concentrations chosen for induction in induction medium.
- The highest concentration chosen for induction must not decrease cellular viability below 90% after 48 hrs.

Working solutions must be prepared freshly every day. Preliminary tests should be carried out to assess the appropriate concentration range of test chemical to be tested and to ascertain whether the test chemical may have any solubility and/or cytotoxicity problems. Test chemicals are tested up to a concentration in the range of C_{max} in vivo, if data are available. Unless prior information such as information on solubility limits or cytotoxicity provides a basis for selecting test concentrations, it is recommended to use 1 mM as maximum concentration. Vortex-mix, ultrasound, and warming at 37°C might be used to facilitate the solubility. In case of un-dissolved particles, add additional solvent to a test chemical to obtain a dissolved concentration.

Prototypical inducers

15. β-naphthoflavone (25 µM), phenobarbital (500 µM), and rifampicin (10 µM) are chemically defined compounds with a known n-fold induction potential. For each study, these reference substances are tested in parallel for n-fold induction of each tested P450 isoform in triplicate on each CryoHepaRG® plate, cultured under identical conditions.

Exposure to reference substances must lead to a $\geq$ 2-fold increase of enzymatic activity at the defined fixed concentrations.

Negative Control(s)

16. Solvent-treated controls, i.e., samples without test chemical (or control inducers) but organic solvent (e.g. 0.1% v/v DMSO) are included to determine the generation of specific products without inducing effects. Basal values of the respective negative controls are used for calculation of the n-fold induction potential of test and reference substances (n-fold induction). Additionally, solvent-free negative controls are run in parallel to observe possible effects of organic solvent on the cells.

Cytotoxicity and assay plate layout

17. Cytotoxicity of unknown test chemicals toward CryoHepaRG® cells is determined before starting the induction experiments. The assay is based on the ability of living cells to convert a redox dye (resazurin) into a fluorescent end product (resorufin). Nonviable cells rapidly lose metabolic capacity and do not generate a fluorescent signal. The homogeneous assay procedure involves adding the single reagent (e.g., Cell Titer Blue reagent, Promega) directly to cells. The assay is performed according to the recommendations given by the manufacturer. The solvent concentration must not exceed 0.1% v/v DMSO. As the cytotoxicity assay should mimic the n-fold induction assay, the cells are exposed to the test chemcial for 48 h with the test chemical solution renewed after 24 h.

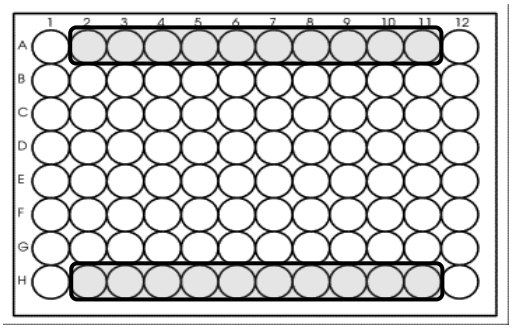
Table 3: Exemplary time schedule for cytotoxicity in CryoHepaRG® cells

Day	Action
Fri (day 1)	Morning: Thawing and seeding of CryoHepaRG® in CryoHepaRG® Thaw, Seed and General Purpose Medium Late afternoon (6 h after plating): Renewing of CryoHepaRG® Thaw, Seed and General Purpose Medium
Sat (day 2)	
Sun (day 3)	
Mon (day 4)	Medium exchange: CryoHepaRG® Serum-Free induction Medium + test chemical (t=0 h)
Tue (day 5)	Medium exchange: CryoHepaRG® Serum-Free induction Medium + test chemical (t=24 h)
Wed (day 6)	t=45 h addition of Cell Titer Blue reagent (Promega) t=48 h cytotoxicity assay

General layout of 96-well plate for cytotoxicity testing

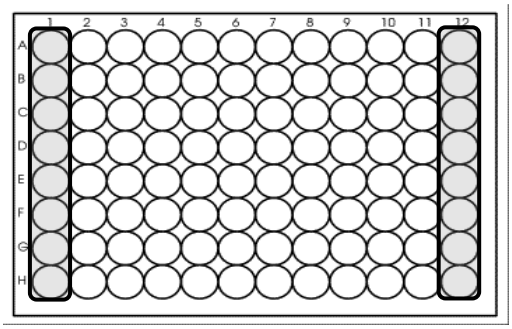
18. 96-well plate: two test chemicals can be tested at eight concentrations in triplicates. The negative control for each test chemical (medium w/solvent) and the positive control (8 µM doxorubicin) are included.

Figure 2: Plate layout for cytotoxicity assay



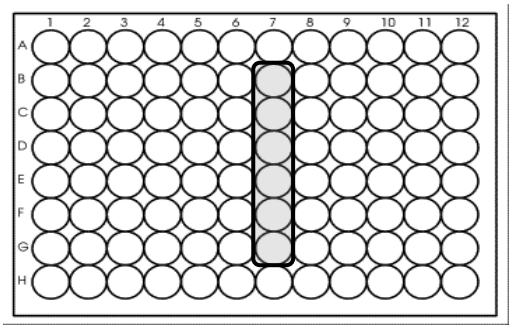
Test chemical dilutions

- with medium,
- without cells



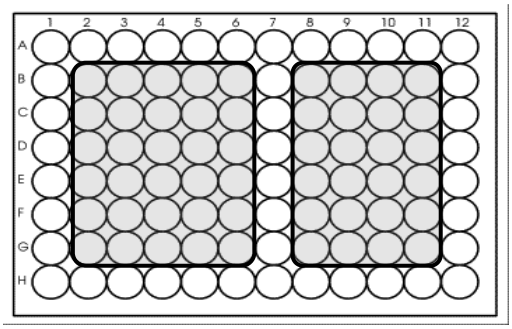
Medium

- without cells
- without test chemical dilutions



Negative control (solvent)

- with cells
- without test chemical



Test chemical dilution, positive control (B8-G8)

- with medium

546

Cytotoxicity assay

19. 50 µl of the test chemical dilutions are transferred to the CryoHepaRG® plate containing 50 µl medium. Freshly prepared test chemical dilutions are renewed after 24h. The CellTiter-Blue® method is used according manufacturer's instruction. By dispensing 20 µl (= 20% of incubation volume) to each well after 45 hours (±0.3h) of incubation and incubating at 37°C for additional 3 hours (±0.3h).

Calculation of results

20. Results are expressed as fractional survival (% FS) and are calculated using the relative fluorescent units.

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

In case of a fluorescence impact of the test chemical (wells A2-A11 and H2-H8), the Cell Titer Blue assay cannot be applied. 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.
The negative control acceptance criterion should be established based on the analysis of historical data set for the equipment used.
8 µM doxorubicin must induce at least 30-70% of cell viability reduction (arithmetic mean) compared to the negative control.

N-fold induction assay

21. N-fold induction experiments are performed in a 96-well plate format. It is recommended to perform each experiment at least twice (once on each of two days).
The following treatment groups are included on each plate: Test chemical(s) at six different concentrations (n=3); solvent-treated control corresponding to the solvent of the test chemical (each n=3); prototypical inducers at one specific concentration (n=3 per compound); solvent-treated control corresponding to prototypical inducers (n=3) solvent-free-control (n=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, are not used for testing and are filled with CryoHepaRG® Thaw, Seed and General Purpose Medium.

Table 4: Exemplary time schedule for n-fold induction in CryoHepaRG® cells (1 assay to be performed with one batch)

Day	Action
Fri (day 1)	Morning: Thawing and seeding of CryoHepaRG® in CryoHepaRG® Thaw, Seed and General Purpose Medium 6 h after plating: renewing of CryoHepaRG® Thaw, Seed and General Purpose Medium
Sat (day 2)	
Sun (day 3)	
Mon (day 4)	Medium exchange: CryoHepaRG® Serum-Free induction Medium + test chemical (t=0 h)
Tue (day 5)	Medium exchange: CryoHepaRG® Serum-Free induction Medium + test chemical (t=24 h)
Wed (day 6)	t=48 h: end of induction addition of probe substrate cocktail in incubation medium (1 h incubation) cell lysis, BCA assay

Figure 3: Example set-up for n-fold induction testing (2 test chemicals, full dose-response range)

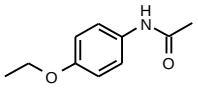
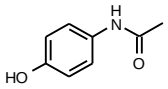
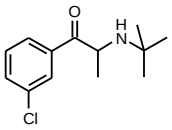
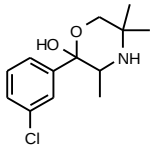
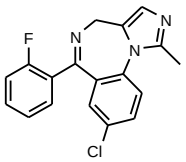
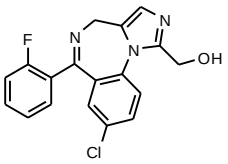
	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		chemical 1	chemical 1	chemical 1	Solvent / chemical1	Solvent / chemical1	Solvent / chemical1	chemical 2	chemical 2	chemical 2	Solvent 0%	
C		chemical 1	chemical 1	chemical 1	Solvent / chemical2	Solvent / chemical2	Solvent / chemical2	chemical 2	chemical 2	chemical 2	Solvent 0%	
D		chemical 1	chemical 1	chemical 1	PB 500 µM	PB 500 µM	PB 500 µM	chemical 2	chemical 2	chemical 2	Solvent 0%	
E		chemical 1	chemical 1	chemical 1	BNF 25 µM	BNF 25 µM	BNF 25 µM	chemical 2	chemical 2	chemical 2		
F		chemical 1	chemical 1	chemical 1	RIF 10 µM	RIF 10 µM	RIF 10 µM	chemical 2	chemical 2	chemical 2		
G		chemical 1	chemical 1	chemical 1	DMSO 0.1%	DMSO 0.1%	DMSO 0.1%	chemical 2	chemical 2	chemical 2		
H												

ENDPOINT MEASUREMENT

22. The CYPs functional activity is analysed after 48 h exposure of the cells to the inducers in incubation medium. The specific enzyme reactions are summarized in the table below. A cocktail of three P450 substrates is added to each well and incubated for 60 min at 37 ± 1°C. At the end of the incubation time, the reaction is quenched by the addition of stop solution (ACN + ISTD) and the samples are analysed for the specific products by means of LC/MS.

Table 5: CYP Probe Substrates and Products

Isoenzyme	Probe Substrate	Product	Final test concentration [µM]	Incubation time [min]

CYP1A2	 Phenacetin	 Acetaminophen	26	60
CYP2B6	 Bupropion	 Hydroxybupropion	100	
CYP3A4	 Midazolam	 1-Hydroxymidazolam	3	

587

588 Protein determination is performed for each well e.g. according to the manual of the PierceMicro-BCA™
589 Protein Assay Kit (Thermo Scientific #23235) using BSA calibration standards in NaOH.

590 Each laboratory may use an LC/MS system of its choice for the analysis of the probes as long as it meets
591 performance criteria, including the limit of quantification (2.30 nM for acetaminophen, 1.15 nM for
592 hydroxybupropion and 1.15 nM for 1-hydroxymidazolam).

593 **Calculation of results**

594 23. The absorbance of the blank standard is subtracted from the absorbance of all other individual
595 standard and unknown sample replicates.

596 A standard curve is prepared by plotting the average blank-corrected absorbance for each standard vs. its
597 concentration in mg/ml. Unknowns are extrapolated from the standard curve using linear regression.

598 $$\text{protein}[\text{mg/ml}] = \frac{\text{absorbance}_{\text{sample}} - \text{axis intercept}_{\text{standard curve}}}{\text{slope}_{\text{standard curve}}}$$

599 Results [mg/ml] are corrected for the dilution factor.

600 The n-fold induction potential of a test chemical is calculated by normalising the enzymatic activity in
601 presence of the test chemical to the enzymatic activity in absence of the test chemicals. Results are
602 expressed as n-fold induction.

603 $$\text{n-fold induction} = \frac{\text{P450 activity}_{\text{induced well}}}{\text{mean P450 activity}_{\text{untreated control wells}}}$$

604 The standard curve should have a correlation coefficient (r^2) equal or greater than 0.9.

ANNEX 3

Cytochrome P450 n-fold induction in human cryopreserved hepatocytes (n-in-one incubations on 48-well plates)

INITIAL CONSIDERATIONS AND LIMITATIONS (see also GENERAL INTRODUCTION, page 1)

1. The method described herein is applicable for the determination of n-fold induction of cytochrome P450 enzymes in Cryoheps monolayers after exposure to test chemicals. The analysis is performed by LC-MS/MS measurement of the concentrations of specific products formed by P450 enzymes after cocktail incubation with specific substrates of the respective P450 enzymes. The use of human hepatic cell systems modeling xenobiotic biotransformation, in addition to the use of a user-friendly substrate cocktail, will contribute to start building an in vitro platform for assessing metabolism and toxicity including assessment of compounds potency to induce CYP1A2, CYP2B6, and CYP3A4.

2. The test method does not provide information on the number or nature of possible (reactive/non-reactive) metabolites nor about human hepatic clearance/stability of the chemicals investigated.

3. The "GENERAL INTRODUCTION" and the "CYP N-FOLD INDUCTION TEST METHOD COMPONENTS" should be read before using this test method for regulatory purpose.

PRINCIPLE OF THE TEST METHOD (see also GENERAL INTRODUCTION, page 1)

Procedure: summary

4. The intended concentration range of the test chemicals depends on their solubility and toxicity. Before planning the n-fold induction assay, solubility and toxicity towards Cryoheps have to be determined in separate experiments. The final solvent concentration during the induction period should not exceed 0.1 % (v/v) DMSO. The highest test concentration for the n-fold induction assay will based on the results of solubility and cytotoxicity tests, and must not decrease the cellular viability < 80% within 72 h of incubation.

5. The functional activity is analyzed after 72 h (± 0.5 h) exposure of the cells to the inducer. P450 iso-enzyme activities are tested in Incubation medium. A cocktail of three P450 substrates is added to each well and incubated for 30 min. At the end of the incubation time, the reaction is quenched by transfer of each cell supernatant to an Eppendorf tube. The samples are analyzed for the specific products by means of LC/MS, or stored at -20°C until analysis.

Test System: Cryoheps

6. The cryopreserved human hepatocytes functionally express drug detoxifying enzymes, drug transporter proteins and nuclear receptors at a level equivalent to freshly isolated human hepatocytes. Human hepatocytes are the gold standard for the in vitro evaluation of CYP n-fold induction and prediction of risk for drug-drug interactions in humans. Cryoheps have been shown to retain CYP expression, activity and response to inducers of their fresh counterparts (34, 35).

642
643 **Data analysis**

644 7. The n-fold induction potential of a test chemical is calculated by normalizing the enzymatic
645 activity in presence of the test chemical to the enzymatic activity in absence of the test chemical. Results
646 are expressed as n-fold induction.

$$n-\text{fold induction} = \frac{\text{P450 activity}_{\text{induced well}}}{\text{mean P450 activity}_{\text{control wells}}}$$

647

648 Exposure to prototypical inducers must lead to a ≥ 2-fold increase of enzymatic activity (of statistical
649 significance) at a concentration of ≤ 500 µM to classify the test chemical as an inducer (31; 32).

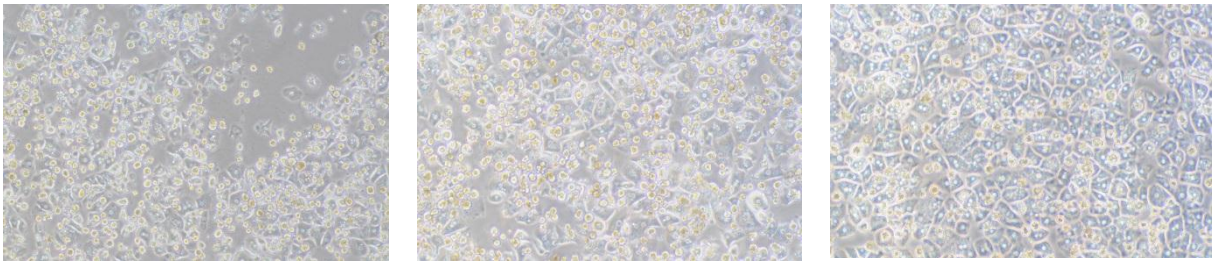
650 8. A test chemical is considered an inducer if a ≥2-fold increase of enzymatic activity (of statistical
651 significance) is measured. As a ≥2-fold increase is just point information, also it is important to observe
652 the shape of a dose response n-fold induction curve and it is suggested that at least 2 out of the 6
653 concentrations should be above the background to ensure relevant data.

654 **PROCEDURE**

655
656 **Primary cells**
657 9. Human Hepatocytes are isolated, frozen and provided by KaLy-Cell (20 rue du Général Leclerc ,
658 67115 Plobsheim, France).

659 **Cell plating and culture**

660 10. Cryoheps are delivered in cryovials, and have to be thawed and seeded in in-house-collagen-
661 coated 96-well plates. The procedures to coat plates thaw and seed cells are according manufacturer's
662 instructions. After 72h of attachment, the morphology of the cells is checked microscopically. Confluence
663 should be minimum 80%. If the integrity of the cells is not given, the cells have to be discarded. If the
664 monolayers are in good condition, cells are washed twice with warm DPBS (100 µL/well). Then
665 cytotoxicity testing is initiated by replacing DPBS by induction medium containing the test chemical,
666 positive control or the corresponding solvents, respectively.



(a) **(b)** **c)**

Figure 4: example of picture taken to check morphological observations of Cryoheps seeded in 96-well plate for cytotoxicity assays, respectively at 50% (a), 70% (b) and 90% (c) confluency.

TEST CHEMICALS, PROTOTYPICAL INDUCERS AND CONTROLS

Vehicle

11. Dimethyl sulfoxide (DMSO), or an appropriate solvent should be used for positive and negative controls and for the test chemicals. The final DMSO (or other solvent) concentration should not exceed 0.1 % (v/v) since the solvent itself may have induction potential (e.g., DMSO induces CYP3A4). For any vehicle, it should be demonstrated that the maximum concentration used is not cytotoxic and does not interfere with assay performance.

Preparation of reference test substances

12. Reference test substances are chemically defined compounds with a known induction potential. To ensure that the concentration of the reference inducers used during the n-fold induction assay will not be cytotoxic, reference inducers are tested (in triplicate) in parallel with test chemicals, using the same batch of cryopreserved human hepatocytes, and cultured under identical conditions. Concentration chosen for reference inducers must not decrease cellular viability to <80% after 72h of incubation.

Solubility: considerations for range findings

Acceptance criteria for selection of appropriate test concentrations:

- Test chemical has to be dissolved at all concentrations chosen for induction in induction medium
- The highest concentration chosen for n-fold induction must not decrease cellular viability below 80% after 72 hours of incubation.
- In order to cover a full-dose response range, the highest concentration is serially diluted at 6 levels.

13. Working solutions must be prepared freshly every day. Preliminary tests should be carried out to assess the appropriate concentration range of test chemical to be tested and to ascertain whether the test chemical may have any solubility and/or cytotoxicity problems. Test chemicals are tested up to a concentration in the range of Cmax in vivo, if data are available. Unless prior information such as information on solubility limits or cytotoxicity provides a basis for selecting test concentrations, it is recommended to use 1 mM as maximum concentration. Vortex-mix, ultrasounds and warming at 37°C might be used to facilitate the solubility. In case of un-dissolved particles, add additional solvent up to a test chemical to obtain a dissolved concentration.

Prototypical inducers

14. Prototypical inducers (e.g. β-naphthoflavone, phenobarbital, and rifampicin) are included in every study. The cells are exposed to the reference substances at a defined concentration for 72 hours in parallel to the exposure of the test chemicals. Exposure to reference substances has to lead to a > 2-fold increase of enzymatic activity (of statistical significance) at the defined concentrations.

Negative Control(s)

15. Solvent-treated controls, i.e., samples without test chemical (or control inducers) but organic solvent (e.g. 0.1% v/v DMSO) are included to determine the generation of specific products without inducing effects. Basal values of the respective negative controls are used for calculation of the n-fold induction potential of test and reference substances (n-fold induction).

Cytotoxicity and assay plate layout

16. Cytotoxicity of unknown test chemicals towards Cryoheps is determined prior to the induction experiments. The assay 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 do not generate a fluorescent signal. The homogeneous assay procedure involves adding the single reagent directly to cells. After an incubation step, data are recorded using a plate-reading fluorometer. The solvent concentration must not exceed 0.1% (v/v) DMSO. According to the n-fold induction assay procedure, the cells are exposed to the test chemical for 72 h. After 24 h of exposure, the test chemical solution is renewed.

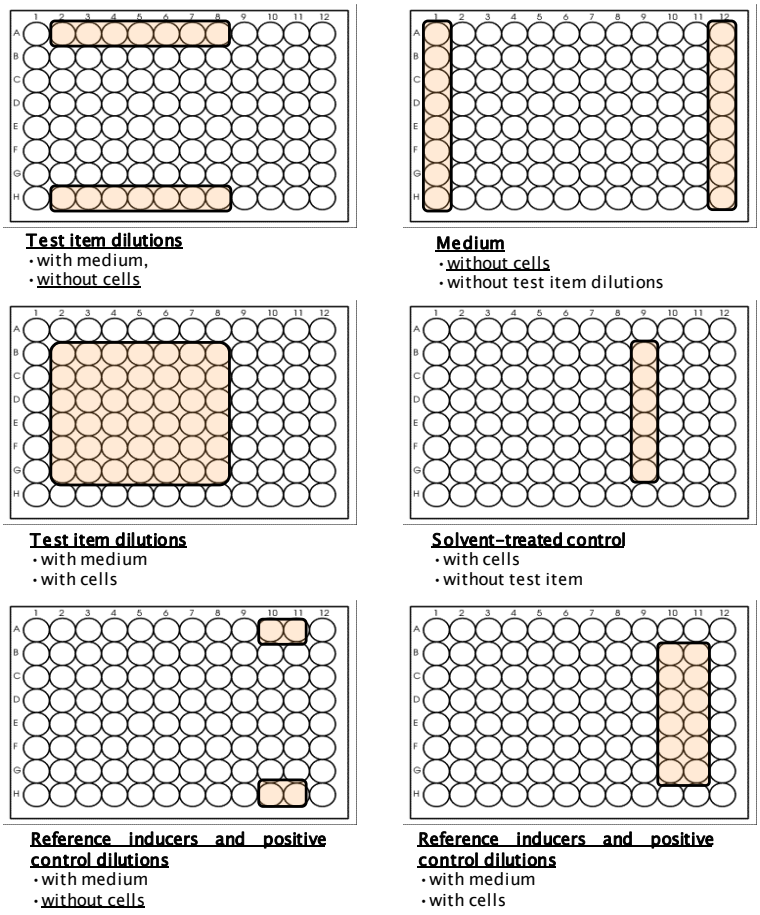
Table 6: Exemplary time schedule for cytotoxicity in Cryoheps

Day:	**Action**
Week 1 Thu	96-well plate coating
Week 1 Fri	Morning: Thawing and seeding of cells Late afternoon (≥4h after seeding): medium renewing (seeding medium+additive)
Week 1 Sat	
Week 1 Sun	
Week 2 Mon	Medium exchange: induction medium + test chemical (t=0 h ± 0.5h)
Week 2 Tue	Medium exchange: induction medium + additive + test chemical (t=24 h± 0.5h)
Week 2 Wed	Medium exchange: induction medium + test chemical (t=48 h± 0.5h)
Week 2 Thu	t=72 h± 0.5h enzyme cytotoxicity assay

General layout of 96-well plate for cytotoxicity assays

17. For each test chemical a corresponding negative control (containing medium with 0.1% DMSO) is included (n=3). Chlorpromazine (25 µM, n=3) serves as positive control and has to produce equal to or less than 70% fractional survival (FS) of the cells (calculated based on an arithmetic mean of replicates. Additionally, not only the background fluorescence (n=8 /test chemical) of the reagent is measured, but also the fluorescence of the test chemical in medium at each tested concentration.

Figure 5: Plate layout for cytotoxicity assay



725

726

727 **Cytotoxicity assay**

728 18. The CellTiter-Blue® method is used according manufacturer's instruction. By dispensing 20 µl
729 (= 20% of incubation volume) to each well after 69 hours (±1h) of incubation and incubating at 37°C for
730 additional 3 hours (±1h).

731 **Calculation of results**

732 19. Results are expressed as fractional survival (% FS) and are calculated using the relative
733 fluorescent units (RFU) as measured by the fluorometer.

734 $$\%FS = \frac{RFU_{treated\ cells} - mean\ RFU_{background}}{mean\ RFU_{solvent\ treatedcells} - mean\ RFU_{background}} x100$$

735 **Acceptance criteria**

736 20. The assays will only be accepted, if the following criteria are met:

737 – The positive control chlorpromazine at 25 µM has to produce equal to or less than 70% fractional
738 survival (FS) of the cells (calculated based on an arithmetic mean of replicates).
739 – At least, two non toxic concentrations should be found or the cytotoxic assay should be repeated with
740 lower test chemical concentrations.
741 – Negative control and reference inducers should be ≥80% FS.

742

743 **N-fold induction assay**

744 21. N-fold induction experiments are performed in a 48-well format. It is recommended to perform
745 the experiments for each treatment group at least in duplicates. The following treatment groups are
746 included on each plate: Test chemical(s) at six different concentrations (n=3); reference substances at one
747 specific concentration (n=3); medium control (0.1% (v/v) solvent) (n=3).

748 **Table 7: Exemplary time schedule for P450 n-fold induction in Cryoheps**

Day:	**Action**		
Week 1 Thu	48-well plates coating		
Week 1 Fri	Morning: Thawing and seeding of cells Late afternoon (≥4h after seeding): medium renewing (seeding medium+additive)		
Week 1 Sat	-		
Week 1 Sun	-		
Week 2 Mon	Medium exchange: induction medium + test chemical (t=0 h± 0.5h)	Medium exchange: induction medium + test chemical (t=0 h± 0.5h)	Medium exchange: induction medium + test chemical (t=0 h± 0.5h)
Week 2 Tue	Medium exchange: induction medium + additive + test chemical (t=24 h± 0.5h)	Medium exchange: induction medium + additive + test chemical (t=24 h± 0.5h)	Medium exchange: induction medium + additive + test chemical (t=24 h± 0.5h)
Week 2 Wed	Medium exchange: induction medium + test chemical (t=48 h± 0.25h)	Medium exchange: induction medium + test chemical (t=48 h± 0.5h)	Medium exchange: induction medium + test chemical (t=48 h± 0.5h)
Week 2 Thu	t=72 h ± 0.5h enzyme activity assay	t=72 h ± 0.5h enzyme activity assay	t=72 h ± 0.5h enzyme activity assay
Week 2 Fri	-	-	-

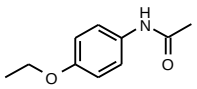
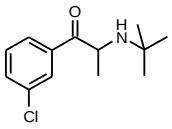
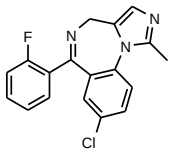
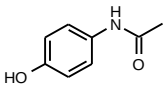
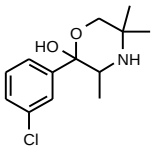
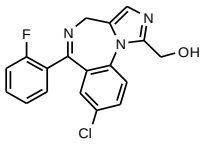
749 **Figure 6: Example set-up for n-fold induction testing (2 test chemicals), full dose-response range)**

	1	2	3	4	5	6	7	8
	chemical 1	chemical 1	chemical 1	Solvent/ chemical 1 and 2	PB 500 µM	chemical 2	chemical 2	chemical 2
	chemical 1	chemical 1	chemical 1	Solvent/ chemical 1 and 2	PB 500 µM	chemical 2	chemical 2	chemical 2
	chemical 1	chemical 1	chemical 1	Solvent/ chemical 1 and 2	PB 500 µM	chemical 2	chemical 2	chemical 2
	chemical 1	chemical 1	chemical 1	BNF 25 µM	RIF 10 µM	chemical 2	chemical 2	chemical 2
	chemical 1	chemical 1	chemical 1	BNF 25 µM	RIF 10 µM	chemical 2	chemical 2	chemical 2
	chemical 1	chemical 1	chemical 1	BNF 25 µM	RIF 10 µM	chemical 2	chemical 2	chemical 2

Endpoint measurement

22. The CYPs functional activity is analyzed after 72 h (± 0.5 h) exposure of the cells to the inducer in Incubation medium. The specific enzyme reactions are summarized in the table below. A cocktail of three P450 substrates is added to each well and incubated for 30 min. At the end of the incubation time, the reaction is quenched by stop solution (ACN + ISTD). The samples are analyzed for the specific products shown in table below by means of LC/MS, or stored at -20°C until analysis.

Table 8: **Specific P450 reactions**

Isoenzyme	CYP1A2	CYP2B6	CYP3A4
Substrate	 Phenacetin	 Bupropion	 Midazolam
Product	 Acetaminophen	 Hydroxybupropion	 1-Hydroxymidazolam
Concentration of substrate [µM]	10	100	3
Incubation time [min]	30	30	30

760 23. Protein determination is performed for each well e.g. according to the manual of the PierceMicro-
761 BCA™ Protein Assay Kit using BSA calibration standards in NaOH.

762 24. Each laboratory may use an LC/MS system of its choice for the analysis of the probes as long as
763 it meets performance criteria, including the limit of quantification (2.30 nM for acetaminophen, 1.15 nM
764 for hydroxybupropion and 1.15 nM for 1-hydroxymidazolam).

765 **Calculation of results**

766 25. A standard curve is prepared by plotting the average absorbance for each standard vs. its
767 concentration (in mg/ml). Unknowns are extrapolated from the standard curve using linear regression.

768 $$protein[mg/ml] = \frac{absorbance_{sample} - axis\ intercept_{standard\ curve}}{slope_{standard\ curve}}$$

769 The standard curve should have a correlation coefficient (R^2) equal or greater than 0.95

770 The n-fold induction potential of a test chemical is calculated by normalizing the enzymatic activity in
771 presence of the test chemical to the enzymatic activity in absence of the test chemical. Results are
772 expressed as n-fold induction.

773 $$n-fold\ induction = \frac{P450\ activity_{induced\ well}}{mean\ P450\ activity_{control\ wells}}$$

774

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