DB-ALM Protocol n° 194 : Cytochrome P450 (CYP) induction *in vitro* test method using cryopreserved human HepaRG™ cell line

Biotransformation, Hepatotoxicity / Metabolism-mediated Toxicity

This method assesses the potential of test items to induce the activity of three cytochrome (CYP) enzymes (CYP1A2, CYP2B6 and CYP3A) in cryopreserved human HepaRG™ cell line (cryoHepaRG).

Résumé

The human CYP induction *in vitro* method assess the potential of a test item to induce the activity of cytochrome (CYP) enzymes (CYP1A2, CYP2B6, and CYP3A4) in two human *in vitro* metabolic competent test systems: the cryopreserved primary human hepatocytes (PHH) and the cryopreserved human HepaRG™ cell line.
The selected CYP enzymes which are expressed in the liver and are inducible by reference items (Lehmann et al., 1998; Gibson et al., 2002; Chen et al., 2004; Wang et al., 2004; Sueyoshi et al., 1999; Goodwin et al., 2003) are recommended for drug-drug interaction studies by the regulatory agencies (US FDA, 2017; EMA, 2012).

CYP induction has been selected as biological endpoint to validate the cryopreserved primary human hepatocytes and the cryopreserved human HepaRG™ cell line as reliable hepatic metabolic competent test systems, as it is a slow process controlled by a set of nuclear receptors followed by downstream signal transduction pathways (EURL ECVAM, 2014). To measure CYP induction indeed the whole molecular machinery from receptors and transporters expression to transcription, translation and expression of CYP enzyme should be present and functional in the test system.

At molecular level, CYP induction is initiated by the binding of endogenous or exogenous ligand to the nuclear receptors/transcription factors aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR) and pregnane X receptor (PXR) . PXR primarily induces the transcription of CYP3A family, CAR of CYP2B family and AhR of CYP1A. Traditionally CAR, PXR and AhR are thought to be specialised for the detoxification processes, but it has become increasingly apparent that they have much broader functions in various physiological processes. Consequently, their functions have to be assessed not merely in the context of xenobiotic metabolism and toxicokinetics but also in the context of possible disruption of physiological functions (Kretschmer et al, 2005; Sueyoshi et al, 2014; Wang and Tompkins, 2008) leading to adverse effects (e.g. inflammation, cholestasis, steatosis, hepatotoxicity and carcinogenesis and thyroid disruption) (Rubin et al., 2015; Christmas P, 2015; Woolbright and Jaeschke, 2015; Hakkola,et al. 2018; Gómez-Lechón, et al. 2009; De Mattia et al, 2016; Fucic et al, 2017; Pondugula et al, 2016).

Where rodents are used, species differences in liver metabolism present a challenge when extrapolating to humans (Kiyosawa et al., 2008; Tsaioun et al., 2016). Differences in the receptors' ligand-binding domain imply that their ligand specificities may differ between species. The potency of compounds to activate receptors can also vary between species (Martignoni et al, 2006; Kedderis and Lipscomb, 2001). The two validated methods are based on human-derived test systems and therefore are of relevance for evaluating potential toxicity for humans.

In DB-ALM collection both CYP induction in vitro test methods are available:

• the current protocol, **DB-ALM No.194**, describes the method for the determination of test items potential for cytochrome P450 enzyme induction in cryopreserved HepaRG™ cells as monolayers in 96-well plates.

• **DB-ALM protocol No.193** describes the method for the determination of test items potential for cytochrome P450 enzyme induction in cryopreserved human hepatocytes that are provided as cryovials to be thawed and seeded as monolayers in 96- or 48-well plates.

Experimental Description

Endpoint and Endpoint Measurement:

Enzyme activity: Cytochrome P450 (CYP) CYP1A2, CYP2B6 and CYP3A4 induction.
CYP induction is defined as *de novo* synthesis of CYP enzyme (protein) as a result of increased transcription of the respective gene following an appropriate stimulus (binding of endogenous or exogenous compound to CAR, PXR, or AhR receptors). Measuring mRNA does not necessarily determine whether induction results in actual elevated CYP activity.

The use of human hepatic cell systems modelling 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 potency to induce CYP1A2, CYP2B6 and CYP3A4. The method described herein is applicable for the determination of induction of cytochrome P450 enzymes in cryopreserved HepaRG™ monolayers after exposure to test items. The analysis is performed by LC-MS measurement of the concentrations of specific products formed by P450 enzymes after cocktail incubation with specific substrates of the respective P450 enzymes.

Following exposure of the cells to the test item, CYP induction is measured by applying a cocktail of 3 specific CYP substrates (i.e. phenacetin, bupropion and midazolam) at the same time (n-in one), followed by the measurement of the 3 specific metabolites (see **Table 1**) by the analytical liquid chromatography/mass spectrometry (LC-MS) technique.

Table 1. CYP isoforms investigated, reference inducers, substrates and metabolites.

CYP	Reference inducer	Substrate	Metabolite
1A2	β-Naphthoflavone (BNF) [25 µM]	Phenacetin	Acetaminophen
2B6	Phenobarbital (PB) [500 µM]	Bupropion	Hydroxybupropion
3A4	Rifampicin (RIF) [10 µM]	Midazolam	1-OH-midazolam

Endpoint Value:

n-fold induction: results are expressed as n-fold induction which is calculated by normalising the enzymatic activity in presence of the test item to the basal enzymatic activity (without test items). Results are expressed as n-fold induction. If a chemical causes two-fold induction for at least two consecutive concentrations, it is classified as positive.

Experimental System(s):

Cryopreserved human HepaRG™ Cells (CryoHepaRG™ human hepatic cell line).

Status

Participation in Validation Studies:

The human Cytochrome P450 (CYP) induction *in vitro* test method using HepaRG™ cells has undergone an EURL ECVAM-coordinated validation study (EURL ECVAM, 2014) which was followed by independent peer review by the EURL ECVAM Scientific Advisory Committee (ESAC). More details about the status of this test method and supporting documents are available on EURL ECVAM **Tracking on Alternative Methods towards Regulatory acceptance (TSAR)**.

Proprietary and/or Confidentiality Issues

HepaRG™ is a patented cell line (PCT/FR02/02391 of July 8, 2002) licensed to the company BIOPREDIC INTERNATIONAL. In the validation study (EURL ECVAM, 2014), cells have been provided from the Biopredic International (Rennes, France) as cryopreserved, differentiated cells. Other cells providers are currently available.

Health and Safety Issues

General Precautions

General safety instructions should be followed at all times. Appropriate protective safety equipment should be worn. Unknown and coded test items should be considered as potential toxic and must be handled with maximum care.

MSDS Information

MSDS should be consulted.

Abbreviations and Definitions

OH-diclofenac	4‘-hydroxydiclofenac
OH-midazolam	1‘-hydroxymidazolam
ACN	acetonitrile
ADD	additive (medium supplement)
AHR	aryl hydrocarbon receptor
BNF	beta-naphthoflavone
CAS	Chemical Abstracts Service
CAR	Constitutive Androstane Receptor
CYP	cytochrome P450 enzyme
DMSO	dimethylsulfoxide
EtOH	ethanol
H2O	deionised water (e.g. MilliQ water)
h	hour (s)
iPrOH	isopropanol
ISTD	internal standards
LC-MS	liquid chromatography-mass spectrometry
LOQ	limit of quantitation
LOD	limit of detection
LLOQ	lower limit of quantitation
MeOH	methanol
m/v	weight per volume
MW	molecular weight
min	minute(s)
NaOH	sodium hydroxide
OD	optical density
PB	phenobarbital
PXR	Pregnane X Receptor
QC	quality control
q.s.	quantum satis
RIF	rifampicin
RFU	relative fluorescence units
RT	room temperature
sec	second(s)
ULOQ	upper limit of quantification
v/v	volume per volume

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PROCEDURE DETAILS, 01 June 2012

Cytochrome P450 (CYP) induction *in vitro* test method using cryopreserved human HepaRG™ cell line
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FORMS used for calculations are made available from the **Downloads** section of this protocol.

Hepatotoxicity / Metabolism-mediated Toxicity (1 Results)

1. **Cytochrome P450 (CYP) induction *in vitro* test method using cryopreserved human HepaRG™ cell line**
Protocol no. 194
This method assesses the potential of test items to induce the activity of three cytochrome (CYP) enzyme (CYP1A2, CYP2B6 and CYP3A) in cryopreserved human HepaRG™ cell line (cryoHepaRG).
Further Applications: Biotransformation
Contact Person: Camilla Bernasconi, Sandra Coecke
Status: Scientifically validated
Documents: see on TSAR (EURL ECVAM Tracking System for Alternative methods towards Regulatory acceptance)

Downloads (12)

FORM-01 (Solubility)
Template for documenting the solubility of test items.

FORM-02 (Cytotoxicity)
Templates for plate layout design and calculation of results of cytotoxicity assay.

FORM-03 (Weighing compounds and solutions-Induction)
Templates for reporting the weight of compounds and the preparation of stock and working solutions for induction assay.

FORM-04 (Weighing compounds and solutions-Cytotoxicity)
Templates for reporting the weight of compounds and the preparation of stock and working solutions for cytotoxicity assay.

FORM-05 (Thawing and seeding)
Template for documenting the procedure of thawing and seeding cells.

FORM-06 (P450 assay procedure)
Template for reporting the CYP P450 induction assay procedure.

FORM-07 (LC-MS analysis)
Template for the preparation of reagents for liquid chromatography-mass spectrometry (LC-MS) analysis.

FORM-08 (Calculation of results)
Template for calculating results of CYP induction in vitro test method.

FORM-09 (Flowchart-Cytotoxicity)
Flowchart outlining the steps of cytotoxicty assay.

FORM-10 (Flowchart-Induction)
Flowchart outlining the steps of cytotoxicty assay

FORM-11 (HepaRG photos)
Example of photos to evaluate HepaRG™ cells morphology.

FORM-12 (Outlier test)
Template for calculating outliers.

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Materials and Preparations

The materials and equipment described herein were applied when this protocol was prepared. Alternative materials have to be tested for appropriateness.

Cell or Test System

The cryopreserved HepaRG™ cells used in the validation study (EURL ECVAM, 2014) were provided by Biopredic International, Saint-Grégoire, France. HepaRG cells are human hepatic progenitor cells able to give rise to adult fully differentiated hepatocytes in appropriate culture conditions, and are derived from a human hepatoma.

HepaRG displays hepatocyte-like functions and functionally expresses drug detoxifying enzymes at relatively high levels compared to cell lines like HepG2 (Kanebratt, 2008), drug transporter proteins and nuclear receptors.

When passaged at low density, HepaRG cells recover characteristic features of progenitor cells able to differentiate in both hepatocytes and biliary epithelial cells and able to form a coculture system, resulting in long-term maintenance of liver-specific functions at high levels (Aninat et al., 2006, LeVee et al., 2006). A few days after thawing and culture of HepaRG, the cells form a coculture of hepatocytes and of biliary-like epithelial cells.

HepaRG cells were first described in 2002 by Gripon (Gripon et al, 2002). Since 2007, Biopredic granted a worldwide exclusive license. Nowadays the cryopreserved HepaRG cells are available from different suppliers in Europe, USA, Japan and Brazil.

Equipment

Fixed Equipment

- 8-channel pipette
- Cell counting chamber and coverslips (Neubauer or equivalent)
- Centrifuges (Hettich, Universal 320R; Eppendorf, 5417C, or equivalent)
- Cell culture incubator with a 5±1% CO2 atmosphere and 95±5 % relative humidity (Binder, or equivalent)
- Flanging pliers (Roth, or equivalent)
- Fluorometer (Victor Wallac3, Perkin Elmer, or equivalent)
- Laminar flow workbench
- LC-MS system. Each laboratory may use an LC-MS system of its choice for the analysis of the probes as long as it meets performance criteria.
- Liquid nitrogen refrigerators
- Microscope (Motic, AE20, or equivalent)
- Pipettes 2-20 µl, 20-200 µl, 100-1000 µl (e.g. Eppendorf, or equivalent)
- Pipet-aid (e.g. Pipetboy Integra, or equivalent)
- m) Repeater pipette (e.g. Eppendorf Multistepper or equivalent)
- Ultrasonic bath
- UV/VIS spectroscope: Spectramax Plus384 (Molecular Devices, or equivalent); data handling with the standard software SoftmaxPro 3.1.2 or equivalent.
- Vortex-Mixer (Scientific Industries Vortex Genie 2, or equivalent)
- Water bath (PD Industriegesellschaft bmH, or equivalent)

Consumables

- 0.45 µm sterile filter
- 1.5 ml, 15 ml und 50 ml centrifugation tubes, conically shaped, polypropylene, sterile
- 50 ml polystyrene reservoir
- 92 mm Ø petri dish, sterile
- 96-well plates with lid, uncoated, polypropylene
- 96-well plates for analysis, uncoated, polypropylene
- 96-well plates coated with collagen I, qualified for seeding and culture of cryopreserved HepaRG™ (e.g. Biopredic International, PLA136 were used in the validation study; ThermoFisher Scientific, A1142803)
- Suitable cover foil for 96-well plates for analysis (e.g. AxyMat™ sealing mat for 96-well plate with round wells, Axygen via VWR, 736-0340) or equivalent
- Suitable cover foil for 96-well plates for storage at -20°C (organic-solvent resistant, e.g. Costar 6570 Thermowell sealing tape) or equivalent
- LC-MS sampler vials, brown glass (alternative to 96-well-plates for analysis) or equivalent
- LC-MS sampler vial inserts (white glass) and caps - crimp cap or equivalent - (alternative to 96-well-plates for analysis)
- Sterile serological pipettes individually wrapped, polystyrene
- Sterile tips

Media, Reagents, Sera, others

- Acetonitrile (HPLC gradient grade)
- DMSO (analytical grade)
- Ethanol (analytical grade)
- Formic acid (purum ≥ 98%)
- Distilled H2O (e.g. NANOpure DIamond Life Science Water purification system or MilliQ water)
- Isopropanol (analytical grade)
- Methanol (HPLC gradient grade)

- 1 M NaOH (sodium hydroxide standard solution,1.0 N in H2O)
- Dulbecco's phosphate buffered saline w/o Ca, Mg (e.g. Sigma, D5652 or PAN, Po4-36500)
- Commercial Kits:
  - Protein determination Kit: Pierce™Micro-BCA™ Protein Assay Kit (Thermo Scientific, 23235) or other suitable system to detect low amounts of protein
  - Cytotoxicity determination kit: Cell Titer Blue® (Promega, G8081) or other suitable method to detect viability as end-point

Preparations

Media and Endpoint Assay Solutions

Media

Note. *Media as well as media supplements are available at different suppliers. Media and media supplements are prepared and stored according to the respective manufacturer's instructions.*

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 (Invitrogen, 12551032 or Lonza BE12-761F) and and stable glutamine (Ultraglutamine 200 mM from Lonza, BE17-605E/U1 or GlutaMax™ 200 mM, nvitrogen 35050061). For instance, 1 ml 200 mM glutamine were added to 99 ml WME.
- Basal Medium can be stored at 4°C for 4 weeks.
- Alternatively, the following product can be applied: William's E Medium with GlutaMax™ (Invitrogen, 32551020).

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

It consists of Basal medium + additive (ADD)

- HepaRG™ Thaw, Seed and General Purpose Supplement (ThermoFisher Scientific, 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 (ThermoFisher scientific, HPRG640) 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 P450 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) (Invitrogen, 25030-024) to 500 ml Williams E without phenol red (Invitrogen, A12176-01).
- Supplement with 12.5 ml 1 M HEPES solution (Invitrogen, 15630-056).

Solutions

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

Reference induction solutions

The stability of the stock solution has to be demonstrated over the given time period (lead laboratory internal validation).

a) Beta-naphthoflavone (CAS 6051-87-2, MW 272.3 g/mol, Sigma-Aldrich N3633)
A 25 mM stock solution in DMSO is prepared, aliquoted and stored at -20°C (FORM-03) for up to 21 days.

b) Phenobarbital sodium salt (CAS 57-30-7, MW 254.2 g/mol, Sigma-Aldrich, P5178)
A 500 mM stock solution in DMSO is freshly prepared every day (FORM-03).

c) Rifampicin (CAS 13292-46-1, MW 822.94 g/mol, Sigma R3501)
A 10 mM stock solution in DMSO is prepared, aliquoted and stored at -20°C (FORM-03) for up to 21 days.

Cytochrome P450 substrates

The stock solutions are stored at -20°C and can be used for 1 month.

a) Phenacetin (CAS: 62-44-2, MW 179.22 g/mol, Sigma-Aldrich, 77440)
A 10 mM stock solution is prepared in MeOH (FORM-03).

b) Bupropion HCl (CAS: 31677-93-7, MW 276.20 g/mol, Sigma-Aldrich, B102)
A 10 mM stock solution is prepared in MeOH (FORM-03)

c) Midazolam HCl (CAS: 59467-96-8, MW 362.2 g/mol, Sigma-Aldrich, UC429)
A 10 mM stock solution is prepared in MeOH (FORM-03).

Cytochrome P450 products

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

a) Acetaminophen (CAS: 103-90-2, MW 151.16 g/mol, Sigma-Aldrich, A5000)
A 10 mM stock solution is prepared in ACN (FORM-07). 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, TRC, H830675)
A 10 mM stock solution is prepared in MeOH (FORM-07). 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, (Sigma, UC430)
A 0.5 mM stock solution is prepared in MeOH (FORM-07). 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.

Stop solution / Internal standard (ISTD)

An ISTD suitable for acetonitrile precipitation, griseofulvin (1 µM stop solution final concentration),is applied. 10 mM stock solutions in ACN are prepared.

Griseofulvin (CAS: 126-07-8, MW 352.8 g/mol, Sigma, G4753):

- A 10 mM stock solution is prepared in ACN and stored at -20°C. This stock solution can be used for 6 months (FORM-07).
- 10 µl griseofulvin stock solution is diluted with 990 µl ACN to give a 100 µM working solution (V1).
- 100 µl working solution (V1) is diluted with 9900 ml ACN to give the stop solution, 1 µM griseofulvin (FORM-05). The stop solution can be used for 4 weeks.

Note. As an *alternative suitable internal standard (e.g. 5,5-Diethyl-1,3-diphenyl-2-iminobarbituric acid (DDIBA); stop solution with 1µM finaconcentration) or the corresponding stable labelled CYP products can be used. The applicability has to be ensured, e.g. based on qualification of validation of the applied LC-MS method.*

Trypan blue reagent (0.5%) for cell counting

a) 0.9% NaCl solution (CAS 7647-14-5, MW 58.44 g/mol)
A 0.9% (m/v) solution is prepared in H_2O (FORM-05).The solution can be stored for 12 months at room temperature.

b) Trypan blue (CAS 72-57-1, MW 960.81 g/mol, Sigma, T6146)
A 0.5% (m/v) solution is prepared in NaCl solution 0.9 % (FORM-05) and filtered through a 0.45 µm sterile filter. The solution can be stored for 6 months at room temperature.

Positive Control(s)

For cytotoxicity

Doxorubicin (CAS: 25316-40-9, MW 579.98 g/mol, Sigma, D1515) is used as positive control for cytotoxicity assay. A 8 mM stock solution is prepared in DMSO (FORM-04) and can stored at -20°C for 4 weeks. The stock solution can be subjected to one freeze-thaw cycle only; therefore it is recommended to aliquote it.

Negative Control(s)

Solvent treated controls and solvent-free controls

Method

Differentiated cryopreserved HepaRG™ are thawed and cultured before exposure to inducers. Known chemical inducers (i.e. β-naphthoflavone, phenobarbital, rifampicin), called reference items, are included in every experiment. The cells are exposed to the reference items at a defined concentration for 48 hours in parallel to the exposure of the test items. Exposure to reference items has to lead to a > 2-fold increase of enzymatic activity at a concentration of < 500 µM in order to allow a classification of the test item (Draft Guidance for Industry, US FDA, 2017).

The intended concentration range of the test items depends on their solubility and toxicity. Before planning the induction assay, solubility and toxicity towards cryopreserved HepaRG™ cells have to be determined in separate experiments (see **Determination of solubility of test items**, page 11 and **Determination of cytotoxicity of test items**, page 13).
The final solvent concentration during the induction period should not exceed 0.1% v/v DMSO. The test item concentrations of unknown compounds will have to be specified depending on their solubility in HepaRG™ serum-free induction medium/0.1% v/v DMSO and on their cytotoxic potential. The highest concentration will be selected based on the results of the previously performed assays, and must not decrease the cellular viability < 90% within 48 ± 0.3 h of incubation.

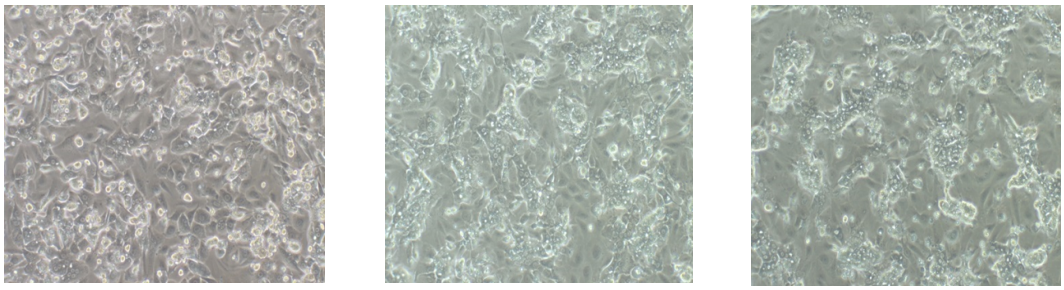
The main steps to perform the induction ***in vitro* test method using cryopreserved HepaRG™ cell line** are summarised here below. A detailed description of the experimental procedure is available in the following sections of this protocol.

- **Determination of solubility of test items** (page 11)
- **Determination of cytotoxicity of test items** (page 13)
- **Determination of induction potential of test items** (page 20)

Test System Procurement

The cryopreserved HepaRG ™ human hepatic cell line was supplied by Biopredic International (Biopredic International, Saint-Grégoire, France). Nowadays the cryopreserved HepaRG™ cells are available from different suppliers in Europe, USA, Japan and Brazil (e.g. HepaRG™ Cells, Cryopreserved, ThermoFisher scientific, HPRGC10). In the validation study, the cryovials provided by Biopredic International containing cryopreserved differentiated HepaRG ™ cells (≥ 8 x 10^6 viable cells per vial) were shipped on dry ice.
Immediately upon on receipt, the cryovials were placed into the vapour phase of liquid nitrogen for storage using a suitable liquid nitrogen refrigerator.
After thawing, the cell morphology has to meet the following **acceptance criteria** (FORM-05, FORM-11 and **Figure 1**):

- 6 h after thawing, hepatocyte-like cells are attached and appear in small, differentiated colonies, individualized (**Figure 1A**).
- After 3-4 days of culture, a restructuration of cell monolayer can be observed with hepatocyte-like cells' organisation in clusters (**Figure 1B**).



A **B** **C**

Figure 1. Cell morphology of HepaRG6h after thawing (A), before induction (B) and at the end of the induction period (C), respectively (untreated control wells).

Determination of solubility of test items

The test item has to be dissolved in a suitable solvent at a suitable concentration. The intended concentration for CYP induction depends on the solubility (and cytotoxicity) of the test item. Since the final solvent concentration during the induction period (exposure of cells to the test item for 48 ± 0.3 h) should be ≤ 0.1% v/v DMSO, the stock solution of the test item in DMSO has to be at least 1000-fold, e.g. for an inducer to be tested at a starting concentration of 40 µg/ml, the concentration of the stock solution in pure DMSO has to be 40 mg/ml. The procedure for solubility testing is documented in FORM-01.

Preparation of test item stock solutions (FORM-01)

By default, test items are dissolved in pure DMSO. In order to increase the compounds solubility, the resulting stock solutions can be heated gently to 37°C in a water bath. Sonication of the tightly closed vial in an ultrasonic bath can be used to accelerate the compounds solubility. The stock solutions may only be used if the test item is dissolved completely.

1. Weigh about 20 mg of test item into a screw cap glass vial. Add DMSO (or alternative solvent) according to the following equation to prepare a starting concentration of 40 mg/ml:

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

2. Vortex-mix or shake for 1 min and visually inspect the solubilisation of the compound.
3. In case of any undissolved particles, repeat steps 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 can not be applied.
8. Add additional DMSO (or alternative solvent) to obtain a twofold lower strength (20 mg/ml) and repeat steps 1-6.
9. In case of any undissolved particles, the intended concentration can not be applied.
10. Add additional DMSO to obtain a twofold lower strength (10 mg/ml) and repeat steps 1-6.
11. In case of undissolved particles, the intended concentration can not be applied. Add additional DMSO (or alternative solvent) to obtain a twofold lower strength (5 mg/ml) and repeat steps 1-6.
12. In case of any undissolved particles, the intended concentration can not be applied.
13. Add additional DMSO to obtain a twofold lower strength (5 mg/ml) and repeat steps 1-6.
14. In case of any undissolved particles, the intended concentration can not be applied using DMSO as solvent.
15. Repeat steps 1-14 using HepaRG Serum-free Induction Medium as a solvent but reduce starting concentration to 5 mg/ml.
16. In case of any undissolved particles, the test item cannot be applied for induction as such.

Dilution and stability of test items in incubation medium

A pre-test is performed to determine whether the test item remains in solution in the media used for induction and cytotoxicity assays by diluting the test item stock solution in HepaRGSerum-free Induction Medium (= cytotoxicity incubation medium) in a 1:1000 ratio for DMSO as solvent.

In case of using the medium as solvent (absence of organic solvents), a concentration of at least 40 µg/ml should be used. Record all data in FORM-01.

1. Add 10 µl test item stock solution to 9990 µl HepaRGSerum-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 HepaRGSerum-free Induction Medium w/o test item 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.

Determination of cytotoxicity of test items

Cytotoxicity of unknown test items towards HepaRG 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).
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 assay is performed according to the recommendations given by the manufacturer with modifications described in this protocol.
The solvent concentration must not exceed 0.1% v/v DMSO. According to the induction assay procedure, the cells are exposed to the test item for 48 ± 0.3 h. After 24 ± 0.3 h of exposure, the test item solution is renewed as the cytotoxicity assay should mimic the induction assay. The time schedule for cytotoxicity testing in HepaRG is shown in **Table 2**.

Table 2. Exemplary time schedule for cytotoxicity in HepaRG cells.

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

Cell culture (FORM-02, FORM-04, FROM-05)

Thawing and seeding

HepaRG^{TM} cells are delivered as differentiated cryopreserved cells on dry ice. Upon delivery, the cryovials are transferred to a liquid nitrogen refrigerator for storage until thawing. For each experiment, one vial, containing > 8 x 10^6 viable cells, is thawed and seeded into the inner wells of one collagen I coated 96-well plate.

The following ***acceptance criteria*** have to be fulfilled ***after thawing and seeding*** :

- minimum cell viability : 80 % after thawing
- minimum recovery per vial : 4.5 x 10^6 cells/vial

If more than one cryovial is used for the experiments, the following steps are performed individually for each vial.

1. The HepaRG Thaw, Seed and General Purpose Medium is prepared according to ***Preparations-Media-b,*** page 7 and pre-warmed using a 37°C water bath.
2. A sterile 50 ml polystyrene tube containing 9 ml of pre-warmed HepaRG Thaw, Seed and General Purpose Medium per cryovial (containing 1 ml cell suspension) is prepared.
3. An absorbent paper is prepared with 70% ethanol or isopropanol.
4. The cryovial is removed from the liquid nitrogen.
5. Under the laminar flow hood, the cap of the vial is briefly twisted a quarter turn to release the internal pressure and closed again. However, the vial is not opened completely.
6. 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. Furthermore, small ice crystals should remain when the vial is removed from the water bath.

7. The outside of the cryovial is disinfected with the isopropanol or ethanol containing absorbent paper and placed under the laminar flow hood.
8. The HepaRG cell suspension (1 ml) is poured out into the tube containing pre-warmed (37°C) HepaRGThaw, Seed and General Purpose Medium for a 1/10 dilution. This process has to be performed under aseptic conditions.
9. 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.
10. The HepaRG cell suspension is centrifuged for 2 min at 350-360 x g at room temperature.
11. The supernatant is aspirated and the cell pellet is resuspended in 5 ml HepaRGThaw, Seed and General Purpose Medium (pre-warmed to 37°C).
    First, the cell pellet is loosened by rotating the vial before adding 5 ml medium.
    The pellet is carefully resuspended using a 5 ml serological pipette.
    Remaining aggregates are loosened by gentle up- and down pipetting with a 1000 µl pipette.
12. Trypan Blue (prepared as described in ***Preparation-Solutions,*** page 8) is used to count the cells: 25 µl of the cell solution is mixed with 75 µl Trypan Blue solution and gently homogenized. An aliquot is introduced into a Neubauer counting chamber covered with a cover slip. (A Neubauer chamber consists of 9 large squares. As the area of each of these large squares is 1 mm² and the height of each square is 0.1 mm, the resulting volume is 0.1 µl. A scheme is shown in **Figure 2**).

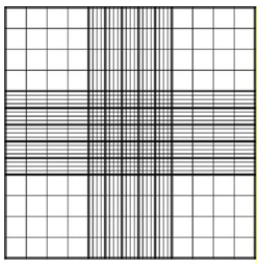


Figure 2. Neubauer counting chamber (scheme).

13. Cell observation and counting is performed under the microscope.Cells in 4 (upper right, upper left, lower right, lower left) of the large squares are counted. 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 FORM-05.
    Cell viability [% viability] and concentration [viable cells/ml] are calculated:

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

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

14. For one 96-well plate, 8 ml of a cell suspension containing 0.72×10^{6} viable cells/ml has to be prepared.
    The HepaRG cell suspension is diluted using HepaRG Thaw, Seed and General Purpose Medium to 0.72×10^{6} viable cells/ml using the following formula:

$$\text{Volume CryoHepaRG cell suspension [ml]} = \frac{0.72 * 10^{6}\ [\text{viable cells/ml}] \times 8\ \text{ml}}{\text{cell concentration [viable cells/ml]}}$$

15. The diluted cell suspension is transferred into a sterile 92 Ø mm Petri dish and gently agitated.
16. 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 (e.g. Biopredic International, qualified for seeding and culturing of HepaRG). The Petri dish is gently agitated in-between the pipetting steps.
    The outer wells of the 96-well plates are not seeded with cells in order to avoid any problems due to evaporation of the medium. The following wells contain HepaRGcells: B2-B11, C2 C11, D2-D11, E2-E11, F2-F11, G2-G11.
17. The outer wells are filled with Dulbecco's PBS subsequently to cell seeding.
18. 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°C with an atmosphere of 5±1% CO_2/95±5% relative humidity.
19. 6 h after plating, cells are observed for their morphology under a phase-contrast microscope. Photographs are taken, if possible.
20. 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.
21. Plated HepaRGcells are incubated in HepaRG Thaw, Seed and General Purpose Medium for at least 70 h (6h + further 64 h) before the cytotoxicity or induction assay starts.
22. 70 h after plating, the cells have to be visually inspected and have to meet the **acceptance criteria** given in **Test System Procurement** (page 10) and FORM-11. Wells in which the acceptance criteria are not met or the integrity of the monolayers is not given have to be excluded for experiments.

Preparation of test items

Test items are dissolved as described in ***Determination of solubility of test items*** (page 11). Compounds should be prepared as stock solutions, ideally at 40 mg/ml (if applicable), aliquoted and stored at -20°C (≤ 1 month, if not indicated otherwise, e.g. by the supplier).

Note. *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.*

Working solutions have to be prepared freshly every day. The content of organic solvents should be kept as low as possible. The concentration of organic solvents must not 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.

Plate layout

Two test items can be tested on a 96-well plate. Each test item is analysed at eight concentrations in triplicates. For each test item a corresponding negative control (containing medium w/solvent, if solvent is used for dissolving of the test item) is included (n=3). Doxorubicin (8 µM, n=6) serves as positive control and has to lead to 30-70% decrease in viability.

Additionally, not only the background fluorescence (n=8 per test item) of the reagent is measured, but also the fluorescence of the test item in medium at each tested concentration.

Thus, on every HepaRG plate, the following parameters are tested, as shown in **Figure 3**:

- CellTiter-Blue ® reagent background fluorescence without cells (A1-H1 and A12-H12) in HepaRG Serum-Free Induction Medium (absence of any solvent or test item dilution), which is transferred from the dilution plate
- Fluorescence of test item 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 untreated/solvent treated controls (B7-G7)
- Fluorescence of 8 test item dilutions (B2-G6 and B9-G11, i.e. B2-D6 and B9-D11 and E2-G6 and E9-G11 for the two test items).

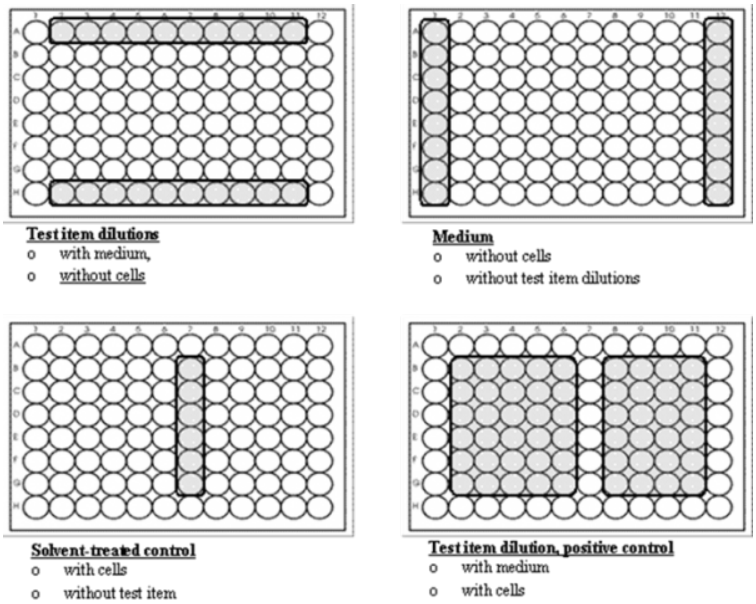


Figure 3. Layout of 96 -well plate for cytotoxicity testing of two test items (“HepaRG plate”).

Figure 4 shows a plate layout for cytotoxicity analysis of two test items. The starting concentration depends on the solubility of the test item in presence of 0.1% v/v organic solvent. For these test items, the highest applicable test concentration is assumed to be 40 µg/ml in presence of 0.1% v/v DMSO.

	1	2	3	4	5	6	7	8	9	10	11	12
A		chemical 1 40.000	chemical 1 13.333	chemical 1 4.444	chemical 1 1.481	chemical 1 0.494	Neg. Control 0.1%	Doxorubicin 8 µM	chemical 1 0.1646	chemical 1 0.0549	chemical 1 0.0183	
B		chemical 1 40.000	chemical 1 13.333	chemical 1 4.444	chemical 1 1.481	chemical 1 0.494	Neg. Control 0.1%	Doxorubicin 8 µM	chemical 1 0.1646	chemical 1 0.0549	chemical 1 0.0183	
C		chemical 1 40.000	chemical 1 13.333	chemical 1 4.444	chemical 1 1.481	chemical 1 0.494	Neg. Control 0.1%	Doxorubicin 8 µM	chemical 1 0.1646	chemical 1 0.0549	chemical 1 0.0183	
D		chemical 1 40.000	chemical 1 13.333	chemical 1 4.444	chemical 1 1.481	chemical 1 0.494	Neg. Control 0.1%	Doxorubicin 8 µM	chemical 1 0.1646	chemical 1 0.0549	chemical 1 0.0183	
E		chemical 40.000	chemical 13.333	chemical 4.444	chemical 1.481	chemical 0.494	Neg. Control 0.1%	Doxorubicin 8 µM	chemical 0.1646	chemical 0.0549	chemical 0.0183	
F		chemical 40.000	chemical 13.333	chemical 4.444	chemical 1.481	chemical 0.494	Neg. Control 0.1%	Doxorubicin 8 µM	chemical 0.1646	chemical 0.0549	chemical 0.0183	
G		chemical 40.000	chemical 13.333	chemical 4.444	chemical 1.481	chemical 0.494	Neg. Control 0.1%	Doxorubicin 8 µM	chemical 0.1646	chemical 0.0549	chemical 0.0183	
H		chemical 40.000	chemical 13.333	chemical 4.444	chemical 1.481	chemical 0.494	Neg. Control 0.1%	Doxorubicin 8 µM	chemical 0.1646	chemical 0.0549	chemical 0.0183	

Figure 4. General layout of 96-well plate for cytotoxicity testing of two .test items (concentrations are expressed as µg/ml) (FORM-02).

CellTiter-Blue ® reagent (or other system to detect viability)

The CellTiter-Blue® reagent is stored frozen at -20°C and protected from light.
For use, the reagent has to be thawed and brought to room temperature. The reagent should be protected from direct light. For frequent use, the product may be stored tightly capped at 4°C or at ambient temperature (22-25°C) for 6-8 weeks. The product is stable for at least 10 freeze-thaw cycles.

Preparation of test item dilutions ("dilution plate")

The dilutions of the test items 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 (**Figure 5**) is carefully transferred to the plate containing the HepaRG monolayers using an eight-channel pipette.

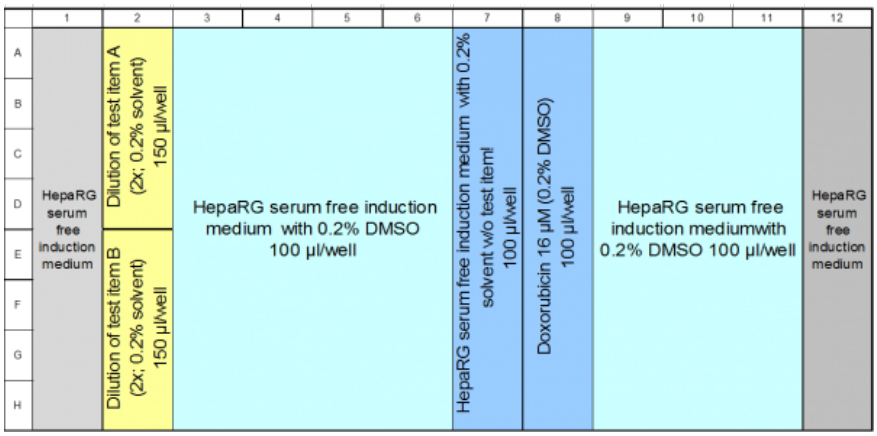


Figure 5. General layout of 96-well plate for test item dilutions ("Dilution plate").

1. Wells A1-H1 and A12-H12 of a new, uncoated, sterile 96-well plate are filled with Serum-free induction medium without solvent using an eight-channel pipette.
2. Dispense 100 µl HepaRG Serum-Free Induction Medium containing solvent to all other wells except wells A2-H2 (highest test item 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 level. (*Example*: If the intended final concentration is 0.1% v/v, the medium has to contain 0.2 % v/v solvent).
3. 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 as described in FORM-04.
4. Doxorubicin, serving as positive control for cytotoxicity, is prepared as follows:
   a 16 µM solution is prepared freshly from an 8 mM stock solution which is initially prepared in DMSO according to FORM-04.
   100 µl of this solution is transferred to wells A8-H8.
5. To wells A2-H2 add 150 µl HepaRG Serum-free induction medium containing the test item at a twofold higher concentration than the intended final concentration.
   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.
6. Transfer 50 µl from wells A2-H2 are transferred to wells *A3-H3* through A6-H6.
   Mix by pipetting 4 times in each well.
7. 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.
8. The test item dilution plate is warmed at 37°C in a cell culture incubator for 10 min.

Cytotoxicity assay

1. HepaRG Thaw, Seed and General Purpose Medium is removed from all the wells.
2. All wells of the HepaRG plate are filled with 50 µl HepaRG Serum-Free Induction Medium (the non-seeded, outer wells as well).
3. The cytotoxicity assay is initiated by the transfer of 50 µl of the test item dilutions from the separate 96-well plate (see ***Preparation of test item dilutions*** ("dilution plate"), page 17) to the HepaRG plate containing 50 µl medium using an 8-channel pipette (fresh tips for each concentration).
4. The HepaRG plate is then placed in a cell culture incubator (5±1% CO2 / 95±5% relative humidity) at 37°C for 24 ± 0.3 h.
5. After 24 ± 0.3 h of incubation, the incubates in the wells are changed:

   a) The test item dilutions and positive control are freshly prepared as described in ***Preparation of test item dilutions*** ("dilution plate") page 16.

   b) The incubates are removed after 24 ± 0.3 h and replaced by 50 µl of fresh, prewarmed HepaRG Serum-Free Induction Medium 650.

   c) Transfer of 50 µl of the freshly prepared test item dilutions and positive control using fresh tips for each concentration and continue incubation at 37°C for additional 24 ± 0.3 h.

6. The viability measurement is started 45 ± 0.3 h after initiation of the cytotoxicity assay.

Viability measurement

1. CellTiter-Blue ® reagent is thawed as described in chapter ***CellTiter-Blue ® reagent*** (page 17) and adapted to room temperature for 10 min.
2. Dispense 20 µl (= 20% of incubation volume) to each well after 45 ± 0.3 h of incubation.
3. Incubate for additional 3 ± 0.3 h.
4. 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.
5. 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),

Calculation of results

Results are expressed as fractional survival (% FS) and are calculated using the relative fluorescent units (RFU). FORM-2 can be applied.

All wells containing test item as well as control wells are corrected by the mean background fluorescence (rows A1-H1 and A12-H12). Fractional survival is calculated according to the following formula:

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

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

Acceptance criteria for cytotoxicity assays

1. For the negative control, RFU (relative fluorescence units) > 100,000 have to be detected after 3 h of reagent incubation (specification for Perkin Elmer Wallac Victor multiwell-plate fluorimeter).
   If the optical density of the negative control wells is found < 100,000 RFU, the metabolic activity of the cell batch cannot be guaranteed and the assay needs to be repeated using a new cell batch.
2. 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.
3. The positive control doxorubicin at 8 µM has to induce at least 30-70% of cell viability reduction (arithmetic mean) compared to the negative control.
4. If the background fluorescence of the test item 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 item 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 item 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.

Determination of induction potential of test items

Test item

Test items are usually tested at six concentrations for their CYP induction potential. The highest test concentration should be in the range of Cmax in vivo. The following test concentrations represent generic concentration sets:

40 µg/ml – 26.7 µg/ml – 17.8 µg/ml – 11.9 µg/ml – 7.9 µg/ml – 5.3 µg/ml (1:1.5 dilution)

40 µg/ml – 20 µg/ml – 10 µg/ml – 5 µg/ml – 2.5 µg/ml – 1.25 µg/ml (1:2 dilution)

40 µg/ml – 13.3 µg/ml – 4.44 µg/ml – 1.48 µg/ml – 0.49 µg/ml – 0.19µg/ml (1:3 dilution)

Ideally, a starting concentration of 40 µg/ml should be applied, but depending on the study requirements and the solubility of the test items, the test concentrations may vary.

Acceptance criteria for selection of appropriate test concentrations are:

- Test item has to be dissolved at all concentrations chosen for induction in induction medium (see ***Determination of solubility of test items,*** page 11).
- The highest concentration chosen for induction must not decrease cellular viability below 90% after 48 ± 0.3 hours of incubation (see ***Determination of cytotoxicity of test items,*** page 12).
- 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.

Acceptance criterion before starting of the study is:

- About 80% confluent HepaRG monolayer after the 72 h attachment period (morphological observation, see **Figure 1**).

Reference items

Reference items (or reference inducers) are chemically defined compounds with a known induction potential. For each study, a reference item is tested in parallel for induction of each tested P450 isoform in triplicate on each HepaRG plate, cultured under identical conditions.

β-Naphthoflavone, phenobarbital and rifampicin are included in every study. The cells are exposed to the reference items at a defined concentration for 48 hours in parallel to the exposure of the test items. Exposure to reference items has to lead to a > 2-fold increase of enzymatic activity at the defined fixed concentrations (25 µM, 500 µM and 10 µM, respectively). Reference inducers are displayed in **Table 3**.

Table 3. Reference inducers.

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

Plate layout

Induction experiments are performed in a 96-well format. An example for an assay set-up is given in **Figure 6**. It is recommended to perform the experiments at least in duplicate.
The following treatment groups are included on each plate:

- test item(s) at six different concentrations (n=3)
- solvent-treated control corresponding to the solvent of the test item (each n=3)
- reference items at one specific concentration (n=3 per reference item)
- solvent-treated control corresponding to reference items (n=3) solvent-free-control (n=3)(FORM-06)

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 with HepaRG Thaw, Seed and General Purpose Medium subsequently to cell seeding.

	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												

Figure 6. Example set-up for induction testing (2 test items, full dose-response range)

The following wells contain HepaRG : **B2-B11, C2-C11, D2-D11, E2-E11, F2-F11, G2-G11** . Determination of functional CYP enzyme activity is performed in a cocktail (n-in-one) approach. Wells **E11-G11** are not foreseen for testing in the experimental design, as shown in **Figure 6,** 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.

Time schedule

An exemplary time schedule for induction in HepaRG cells is given in **Table 4**. In general, 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 items 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 ± 0.3 h, the probe substrate reaction is carried out by addition of the probe substrate cocktail. The time schedule in **Table 4** demonstrates the induction assays performed with one 96-well-plate from one batch (one dispatch).

Table 4. Exemplary time schedule for P450 induction in HepaRG cells (example for 1 assay to be performed with one batch).

Day	***Action***
Fri (day 1)	*Morning:* Thawing and seeding of HepaRG in HepaRG Thaw, Seed and General Purpose Medium *Late afternoon (6 h 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 item (t=0 h)
Tue (day 5)	Medium exchange: HepaRG Serum-Free Induction Medium + test item (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

Cell culture

Thawing and counting of HepaRG cells

HepaRG cells are delivered by Biopredict International as differentiated cryopreserved cells on dry ice. Upon delivery, the cryovials are transferred to a liquid nitrogen refrigerator for storage until thawing. For each experiment, one vial, containing > 8 x 10^6 viable cells, is thawed and seeded into the inner wells of one collagen I coated 96-well plate.

The following **acceptance criteria** have to be fulfilled after thawing and seeding:

- minimum cell viability: 80 % after thawing
- minimum recovery per vial: 4.5 x 106 cells/vial

If more than one cryovial is used for the experiments, the following steps are performed individually for each vial.

1. The HepaRG Thaw, Seed and General Purpose Medium is prepared according to ***Preparation-Media -b,*** page 7 and pre-warmed using a 37°C water bath.
2. A sterile 50 ml polystyrene tube containing 9 ml of pre-warmed HepaRG Thaw, Seed and General Purpose Medium per cryovial (containing 1 ml cell suspension) is prepared.
3. An absorbent paper is prepared with 70% ethanol or isopropanol.
4. The cryovial is removed from the liquid nitrogen.
5. Under the laminar flow hood, the cap of the vial is briefly twisted a quarter turn to release the internal pressure and closed again. However, the vial is not opened completely.
6. 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. Furthermore, small ice crystals should remain when the vial is removed from the water bath.
7. The outside of the cryovial is disinfected with the isopropanol or ethanol containing absorbent paper and placed under the laminar flow hood.
8. The HepaRG cell suspension (1 ml) is poured out into the tube containing pre-warmed (37°C) HepaRG Thaw, Seed and General Purpose Medium for a 1/10 dilution. This process has to be performed under aseptic conditions!
9. 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.
10. The HepaRG cell suspension is centrifuged for 2 min at 350-360 x g at room temperature.
11. 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). First, the cell pellet is loosened by rotating the vial before adding 5 ml medium. The pellet is carefully resuspended using a 5 ml serological pipette. Remaining aggregates are loosened by gentle up- and down pipetting with a 1000 µl pipette.
12. Trypan Blue (prepared as described in ***Preparation-Solutions,*** page 8) is used to count the cells: 25 µl of the cell solution is mixed with 75 µl Trypan Blue solution and gently homogenized. An aliquot is introduced into a Neubauer counting chamber covered with a cover slip. (A Neubauer chamber consists of 9 large squares. As the area of each of these large squares is 1 mm² and the height of each square is 0.1 mm, the resulting volume is 0.1 µl).
13. Cell observation and counting is performed under the microscope. Cells in 4 of the large squares are counted. 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 FORM-05. Cell viability [% viability] and concentration [viable cells/ml] are calculated:

$$\text{viability}[\%] = \frac{\Sigma \text{ viable cells in 4 squares}}{\Sigma \text{ dead + viable cells in 4 squares}} \times 100$$

$$\text{Cell concentration [viable cells /ml]} = \Sigma \text{viable cells in 4 squares} \times 10^4$$

14. For one 96-well plate, 8 ml of a cell suspension containing 0.72×10^6 viable cells/ml has to be prepared: The HepaRG cell suspension is diluted using HepaRGThaw, Seed and General Purpose Medium 670 to 0.72×10^6 viable cells/ml using the following formula:

$$\text{Volume HepaRG cell suspenion [ml]} = \frac{0.72 \times 10^6 \text{ [viable cells /ml]} \times 8\text{ml}}{\text{cell concentration [viable cells /ml]}}$$

15. The diluted cell suspension is transferred into a sterile 92 Ø mm Petri dish and gently agitated.

16. 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 (Biopredic International, qualified for seeding and culturing of HepaRG). The Petri dish is gently agitated in-between the pipetting steps. The outer wells of the 96-well plates are not seeded with cells in order to avoid any problems due to evaporation of the medium. The following wells contain HepaRG: **B2-B11, C2-C11, D2-D11, E2-E11, F2-F11, G2-G11** .

1. The outer wells are filled with Dulbecco's PBS subsequently to cell seeding.
2. 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°C with a atmosphere of 5±1% CO2/95±5% relative humidity.
3. 6 h after plating, cells are observed for their morphology under a phase-contrast microscope. Photographs are taken, if possible.
4. 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.
5. Plated HepaRG are incubated in HepaRG Thaw, Seed and General Purpose Medium for at least 70 ± 1 h (6h + further 64 h) before the cytotoxicity assay starts.
6. 70 h after plating, the cells have to be visually inspected and have to meet the **acceptance criteria** given in **Test System Procurement** (page 10) and in FORM-11. Wells in which the acceptance criteria are not met or the integrity of the monolayers is not given, have to be excluded for experiments.

Induction

Preparation of solutions for induction

Test items

Each test item is dissolved in an appropriate solvent. Working solutions are prepared in HepaRG Serum-Free Induction Medium, to reach the concentrations for induction.
The solvent concentration should be kept as low as possible during the induction period (DMSO ≤ 0.1% v/v), since the solvent itself has induction potential (e.g. DMSO induces CYP3A4).

Compounds should be prepared as stock solutions, ideally at 40 mg/ml (if applicable), aliquoted and stored at -20°C (≤1 month, if not indicated otherwise, e.g. by the supplier). Working solutions have to be prepared freshly every day.

Initial weight and preparation of stock and working solutions must be recorded in FORM-03.

Note. *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.*

Reference items

Reference items used are beta-naphthoflavone (BNF, CYP1A2), rifampicin (RIF, CYP3A4) and phenobarbital (PB, CYP2B6).
The working solutions of PB, BNF and RIF are prepared freshly every day. Initial weight and preparation of stock and working solutions is documented in FORM-03 pages 1 and 2.

- beta-naphthoflavone (MW 272.3 g/mol, induction concentration 25 µM, final solvent concentration 0.1% v/v DMSO, 25 mM stock solution).
- rifampicin (MW 822.94 g/mol, induction concentration 10 µM, final solvent concentration 0.1% v/v DMSO, 10 mM stock solution).
- phenobarbital (MW 254.2 g/mol, induction concentration 500 µM, final solvent concentration 0.1% v/v DMSO, stock solution 500 mM).

Addition of induction solution

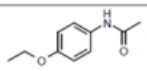
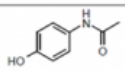
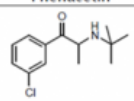
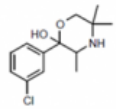
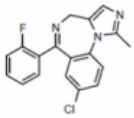
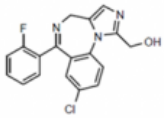
The induction solutions are prepared freshly every day (stock and working solutions of PB, working solutions of BNF and RIF).
The **induction** is initiated by the addition of the induction solution (100 µl/well), which corresponds to time point **t = 0 h.**
The induction solution is replaced at time point **t = 24 ± 0.3 h** by **freshly prepared induction solutions** in order to maintain constant inducer concentrations during the induction period.
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** .

Time points of start of incubation and medium exchange are documented in FORM-06.

Functional enzyme activity assay

The functional activity is analysed after 48 ± 0.3 h exposure of the cells to the inducers. P450 isoenzyme activities are tested using Incubation medium. The specific enzyme reactions are summarized in Table 4. A cocktail of three P450 substrates is added to each well and incubated for 60 ± 3 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 shown in **Table 5** by means of LC-MS.

Table 5. Specific P450 reactions.

Isoenzyme	Probe Substrate	Product	Final test concentration [µM]	Incubation time [min]
CYP1A2	 Phenacetin	 Acetaminophen	26	60 ± 3
CYP2B6	 Bupropion	 Hydroxybupropion	100	
CYP3A4	 Midazolam	 1-Hydroxymidazolam	3	

Preparation of substrate solutions

The stock solutions of the P450 substrates are prepared as described in **Preparations -Solutions** (page 8). The data are recorded in FORM-03. All substrates are dissolved in MeOH at a concentration of 10 mM and further diluted in MeOH to obtain working solutions of 4-fold higher strength than the intended final substrate concentrations in experimental incubations (Phenacetin 26 µM, Midazolam 3 µM, Bupropion 100 µM), as shown in Table 5). Thus the working solutions have the following concentrations: Phenacetin 104 µM, Midazolam 12 µM, Bupropion 400 µM.

Activity assay (FORM-03, FORM-05, FORM-06, FORM-07)

1. Prepare the substrate cocktail by dilution of stock solutions with MeOH to obtain working solutions of 4-fold higher strength than the intended final test concentrations (FORM-03).
2. Mix 1.5 ml of 4-fold concentrated working solutions of each 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.
3. Pre-warm incubation medium, prepared as described in chapter ***Preparations-Media-d*** (page 7) in a water bath to 37°C (20 ml per plate).
4. The dried residue of the substrate cocktail in the centrifugation tube is reconstituted in 6 ml Incubation medium.
5. Pre-warm the substrate cocktail (**Table 5**) in a water bath to 37°C.
6. Prepare the stop solution (page 8) and place it on ice (FORM-07).
7. 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 100 µl pre-warmed incubation medium. An 8-channel pipette is used for the washing step.
8. The washing step is repeated once.
9. 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 in FORM-06. Perform this step for all rows in timed intervals (e.g. start every row after 20 or 30 sec).
10. Carefully move the plate in order to equally distribute the substrate cocktail in the wells. Transfer the plate into the cell culture incubator.
11. Incubate for 60 ± 3 min in total. (Incubation time starts with the addition of the cocktail to the first well).
12. Prepare start solution samples: 40 µl substrate solution (n = 2) are added to 1.5 ml reaction tubes containing an equal volume acetonitrile/ISTD (page 8). 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. **Note:** Aliquots of the starting solution are checked for unspecific formation of the products from the probe substrates.
13. Shortly before the end of the incubation period, add 40 µl stop solution to a separate 96-well-plate (= “stop solution plate”).
14. At the end of the incubation time, the medium (40 µl) is removed from the wells in the same timed intervals (see 9) and transferred to the stop solution plate, correspondingly labelled.

15. Transfer the start solution samples (see 11) to 2 empty wells of the stop plate, too.
16. 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 H2O (final acetonitrile content: 15% v/v). An 8-channel pipette can be applied. This plate is subjected to LC-MS analysis (see 17).
    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.

    Note *.In case of analysis of the backup samples, the plate will be removed from storage at - 20°C, thawed to room temperature, gently mixed, and diluted with 70 l H2O (final acetonitrile content: 15% v/v). If the remaining volume of the backup sample is lower, the volume of H2O has to be adapted accordingly.*

17. The other plate is covered with a suitable cover mat for LC-MS and subjected to LC-MS analysis. If the samples can not be analysed directly, the plate is sealed with a suitable foil and stored at -20°C until analysis as well. Due to potential instabilities of the formed probe product 4-OH-diclofenac at -20°C, the samples have to be analysed within 2 days). 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.
18. 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).
19. The residual Incubation medium (~ 10 µl) is aspirated off the cell plate. Subsequently, cells in all wells are lysed by the addition of 50 µl 1 M 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).
20. Aliquots of the lysates are removed from the plate, diluted 1:20 (i.e. 10 µl lysate with 190 µl H2O) and stored at -20°C until analysis for protein content. Prior to the performance of the protein determination assay, the execution of one freeze-thawcycle 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.

Endpoint Measurement

At the end of the incubation with substrate cocktail solution **protein determination** and **LC-MS analysis** of the induction assay samples are performed as follows.

Protein determination

The protein determination is performed according to the manual of the Pierce ® Micro-BCA™ Protein Assay Kit (Thermo Scientific #23235) with minor modifications

1. Prepare diluted Albumin (BSA) standards: For preparation of standard solution S1 the Albumin Standard stock (BSA) ampule [2 mg/ml] supplied with the kit is diluted in 0.05 M NaOH **(Table 6).** The standard solutions S2-S7 are prepared by serial dilution. ***Note.*** 0.05 M NaOH is prepared by mixing 0.5 ml NaOH 1M and 9.5 ml H_2O.
2. 150 µl sample (single determinations, diluted cell lysates, see ***Activity assay*, step 19**) 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 **Figure 7**.

Table 6. Standards for protein determination.

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
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

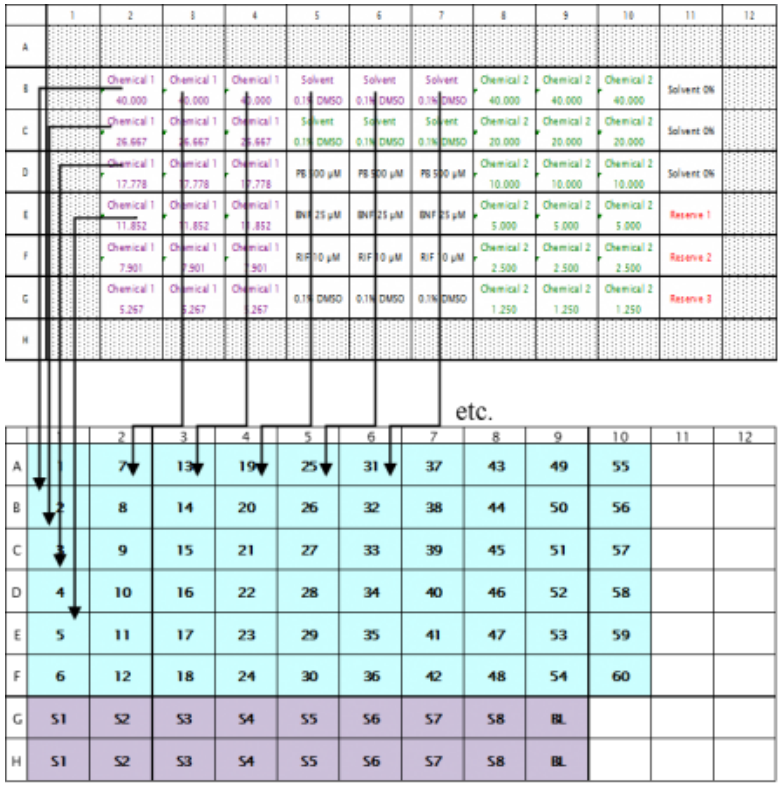


Figure 7. Micro-BCA transfer and pipetting scheme

3. Prepare Pierce® Working Reagent 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).
   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.
4. 150 µl Pierce 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.
5. Cover the plate and incubate at 37°C for 2h.
6. Cool the plate to room temperature.
7. Read the plate at OD562 nm within 10 min.
8. Calculation of results: the absorbance of the blank standard is subtracted from the absorbance of all other individual standard and unknown sample replicates.
9. A standard curve is prepared by plotting the average blank-corrected absorbance for each standard vs. its concentration in mg/ml. Unknowns are extrapolated from the standard curve using linear regression (see FORM-08).

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

Results [mg/ml] are corrected for the dilution f actor (FORM-08) .

LC-MS analysis of induction assay samples

Preparation of product standards

For quantitative analysis of the enzymatic activities of P450 enzyme, the formation of specific products by the respective isoenzyme is quantified by LC-MS measurement (see ***LC-MS method*** *below*).
Standard solutions containing defined concentrations in the range of the expected product concentrations are prepared in Incubation medium as described in FORM-07.
Make sure that for the preparation of the calibration standards and the assay samples, the identical stop solution is applied. The preparation of the standards is recorded using FORM-07.

1. Prepare stock solutions of P450 products according to ***Preparations -Solutions*** *(*page 8*)* (FORM-07 page 1).
2. Prepare predilutions of the 3 metabolites at 500 µM (predilution 1); 62.5 µM (predilution 2) from the stock solutions and at 7.8 µM (predilution 3) from predilution 1 according to FORM-07 page 1.
3. Mix 200 µl of each 500 µM metabolite solution (predilution 1) with 200 µl ACN, resulting in a total volume of 1000 µl = WS 1 (FORM-07 page 3).
4. Serially dilute into working solutions 2 and 3 by mixing with ACN (FORM-07 page 2).
5. Mix 200 µl of each 62.5 µM metabolite solution (predilution 2) with 200 µl ACN, resulting in a total volume of 1000 µl = WS 4 (FORM-07 page 3).
6. Serially dilute into working solutions 5 and 6 by mixing with ACN (FORM-07 page 3).
7. Mix 200 µl of each 7.8 µM metabolite solution (predilution 3) with 200 µl ACN, resulting in a total volume of 1000 µl = WS 7(FORM-07 page 3).
8. Serially dilute into working solutions 8 and 9 by mixing with ACN (FORM-07 page 3).
9. Prepare standard solutions by addition of 5 µl of WS 1-9 to 245 µl Incubation Medium (FORM-07 page 3).
10. Vortex-mix for 10 sec.
11. Add 250 µl Stop solution (ACN + ISTD) to each calibration standard solution.
12. Prepare ISTD sample by adding 250 µl Stop solution to 250 µl incubation medium
13. Prepare blank sample by adding 250 µl pure ACN to 250 µl incubation medium.
14. Vortex-mix all samples for 10 sec.
15. Centrifuge for 5 min at ~4800 x g at room temperature.
16. 60 µl of the particle-free supernatant is diluted with 140 µl H2O (final ACN content: 15% v/v) and transferred to analysis plate (96-well plate) or sampler vials.

General LC-MS method performance requirements

Each laboratory may use an LC-MS system of its choice for the analysis of the probes as long as it meets performance criteria, including the Limit of Quantitation (LOQ) as specified below.

Prior to initiation of experiments, the laboratory should demonstrate that the probe metabolites can be measured with sufficient accuracy and precision to meet the Quality Control (induction of prototypical inducers ≥ 2).

Performance criteria

The method developed and implemented for LC/MS quantification of the probe products must be validated for accuracy, precision, limit of detection, limit of quantitation and method linearity according to accepted methods such as described by e.g. European Medicines Agency (Guideline on bioanalytical method validation 2012) or Food and Drug Administration (Guidance for Industry: Bioanalytical Method Validation, 2018). At least, a "fit-for-purpose" validation has to be performed.

During method development, accuracy of the developed method is determined by analysis of controls (external or internal). Measured results are evaluated against expected results. Accuracy is acceptable if results obtained by the new method are within ± 25% of expected value.

- **Limit of detection (LOD)** is the lowest concentration of the analyte present in the sample matrix that is detected, although not necessarily quantitated, under the method acceptance criteria.

- **Limit of Quantitation (LOQ)** is the lowest concentration of the analyte present in the sample matrix that is detected under the method acceptance criteria at a concentration within + 20% of target concentration. LOQ of 2.30 nM for acetaminophen, 1.15 nM for hydroxybupropion and 1.15 nM for 1hydroxymidazolam is required.
- **Upper Limit of Quantitation (ULOQ)** is the highest concentration of the analyte present in the sample matrix that is detected under the method acceptance criteria at a concentration within + 20% of target concentration.

LC-MS system

An HPLC system consisting of a suitable pump and a 96-well-compatible auto-sampler is required. Mass spectrometry is performed on a qualified instrument. The use of high-resolution mass spectrometry is preferred but not a prerequisite in the analysis of the probe substrate cocktail. Tandem mass spectrometry can be used if the sensitivity of the method is sufficient. A suitable software for LC-MS data evaluation is required.

Analytes (i.e. probe products and internal standard) are separated on suitable HPLC column with a hydrophobic column material (e.g. PFP, RP18, etc.) using gradient elution. Chromatographic conditions have to be optimized in a way that adequate chromatographic retention is ensured.

Furthermore, adequate chromatographic separation has to be ensured such that the resolution of two quantifiable peaks is at least 2.0 for a robust assay to overcome the potential for peak area integration errors.

Ideally, chromatographic peaks should be symmetrical, but asymmetry of an analyte peak is acceptable provided that the degree of asymmetry observed in the incurred samples is reflected in the calibration and quality control (QC) samples.

The chromatographic response at the lower limit of quantitation (LLOQ) should be at least five times the response compared to the blank response which is often (incorrectly) interpreted to be a measure of the assay signal to noise (S/N).

Conditions of the mass spectrometer have to be established based on the respective instrument type and might require specific optimization in order to assess all three analytes in a cassette approach. Typically, full scan mass spectra are acquired in the positive mode using syringe pump infusion to identify the protonated quasimolecular ions $[M+H]^+$. Auto-tuning is carried out for maximising ion abundance followed by the identification of characteristic fragment ions. Ions with the highest S/N ratio are used to quantify the analyte in a suitable instrument mode (such as selective monitoring mode (SRM)) and as qualifier, respectively

Runs may be accepted if LLOQ or ULOQ standards fail as long as the QCs pass and are bracketed by standards. If the assay range is truncated by the removal of LLOQ and/or ULOQ standards, samples with reported concentrations below the lowest or above the highest acceptable standard should be flagged for repeat analysis. Extrapolation below the lowest or highest standards is not permitted.

Chromatographic failure of individual samples can be considered outliers and be flagged for repeat analysis. Chromatographic failure of QCs or standards is not an acceptable reason for excluding these samples from the calculation of general run acceptance criteria.

Acceptance Criteria

As recommended by the FDA (US FDA, 2018), the sequence analysis validation results from the acceptance of analysis, the calibration range, the sequence (with samples of quality control), and ultimately the result.

For chromatographic methods, each chromatogram must be checked to ensure:

1. the absence of any interference

2. the good recognition of peaks (e.g. retention time absolute and relative)

3. the quality of chromatographic conditions (resolution, asymmetry, etc..)

4. the proper integration of the peaks.

Samples from quality control allow accepting or rejecting the analysis sequence. If the value deviates by more than ± 15% of the nominal value, they are unacceptable. The sequence analysis is validated if:

1. No more than 33.3% (2 of 6, 3 of 9, 4 of 12) of QC should be excluded (for all the reasons as: loss of sample QC, poor injection, a value greater than ± 15 % of the nominal value).

2. At least 50% of a level of QC (QC1, QC2 and QC3) must be accepted.

3. All blocks of QC must have at least 1 QC accepted.

If these criteria are not met, the results of the series of samples are rejected and the analysis is to be rebuilt.

Data Analysis

The induction potential of a test item is calculated by normalising the enzymatic activity in presence of the test item to the enzymatic activity in absence of the test items. Results are expressed as n-fold induction.

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

Standard curve acceptance criteria: The standard curve should have a correlation coefficient (r^2) equal or greater than 0.9.

Outlier test

To check the calculated results for supposed outliers, FORM-12 (standardized test method for calculation of outliers) has to be used: Values are inserted in the “List of results” column. If a value is marked as an outlier according to the Grubb, the Dean-Dixon as well as the Nalimov tests, it has to be excluded from calculation (parameters: α = 1%, number of digits: 3). Excluded values were marked with an annotation.

Prediction Model

A test item is considered an inducer if a >2-fold increase of the baseline levels at two consecutive concentrations in vitro is observed.

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