DB-ALM Protocol n° 193 : Cytochrome P450 (CYP) induction *in vitro* test method using cryopreserved primary human hepatocytes

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 primary human hepatocytes (PHH).

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.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.
- **DB-ALM protocol 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.

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.
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	ß-Naphthoflavone (BNF) [25µM]	Phenacetin	Acetaminophen
2B6	Phenobarbital (PB) [500µM]	Bupropion	Hydroxybupropion
3A4	Rifampicin (RIF) [10µM]	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):

HEPATOCYTES (human): primary human hepatocyte cultures

Status

Participation in Validation Studies:

The Cytochrome (CYP) induction *in vitro* test method 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)**.

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 as appropriate.

Abbreviations and Definitions

ACN acetonitrile

AHR aryl hydrocarbon receptor

BNF β-naphtoflavone

BG background

CAS Chemical Abstracts Service

CAR constitutive androstane receptor

CPZ chlorpromazine

CYP cytochrome P450 enzyme

DPBS Dulbecco’s phosphate buffered saline

DMEM Dulbecco's modified eagle medium

DMSO dimethylsulfoxide

FCS fetal calf serum

HBSS Hank's balanced salt solution

HMM hepatocytes maintenance medium

H2O deionised water

h hour (s)

ITS insulin transferrin selenium mix

LC-MS liquid chromatography- mass spectrometry

LOQ limit of quantitation

LOD limit of detection

LLOQ lower limit of quantitation

MeOH methanol

MDZ midazolam

MW molecular weight

min minute(s)

OD optical density

OME omeprazole

PB phenobarbital

PXR Pregnane X receptor

QC quality control

RFU relative fluorescence units

RIF rifampicin

RT room temperature

sec second(s)

ULOQ upper limit of quantitation

Last update: 3 September 2018

PROCEDURE DETAILS, 5 November 2012

Cytochrome P450 (CYP) induction *in vitro* test method using cryopreserved primary human hepatocytes
DB-ALM Protocol n° 193

The experimental procedures described in the sections of this protocol are summarised in **Figure 1**.

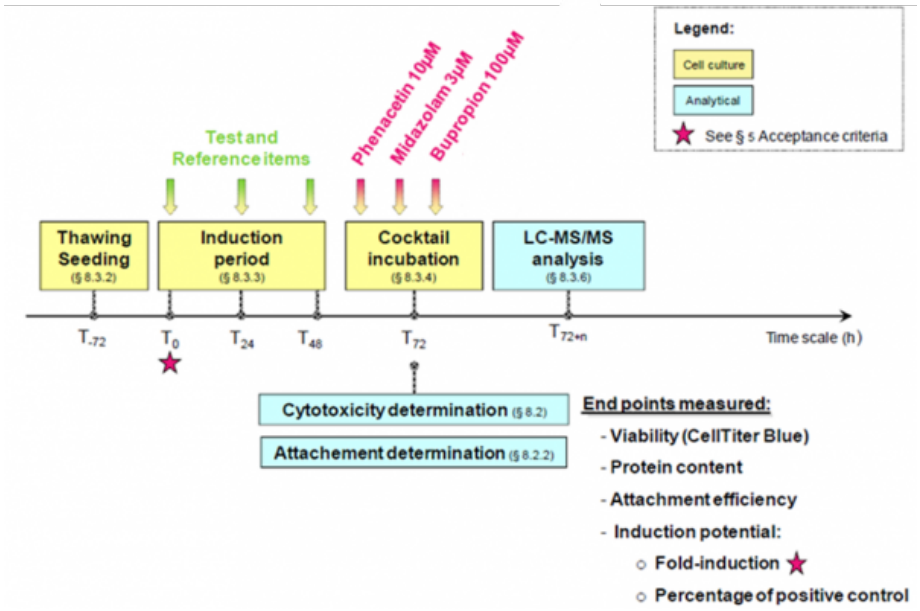


Figure 1. Experimental design of the CYP induction in vitro test method using PHH.

Acceptance criteria to be met are

Acceptance criteria with regards to the cells:

- After thawing, cell viability will need to be in the range +/- 10% of that given by KaLy-Cell, and attachment rate, measured by morphological observation of the cell monolayer, needs to be in the range +/- 10% of that given by KaLy-Cell.
- 70% confluent hepatocyte monolayer minimum after the 24h attachment period (morphological observations, see **Figure 3,** page 13).
- Less than 50% protein lost at the end of the 72-h induction period (T_{72} versus T_0).
- Known chemical inducers (e.g. β-naphtoflavone, phenobarbital, and rifampicin) are included in every study. The cells are exposed to the reference items at a defined concentration for 72 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 (of statistical significance) at the defined fixed concentrations (US FDA, 2017).

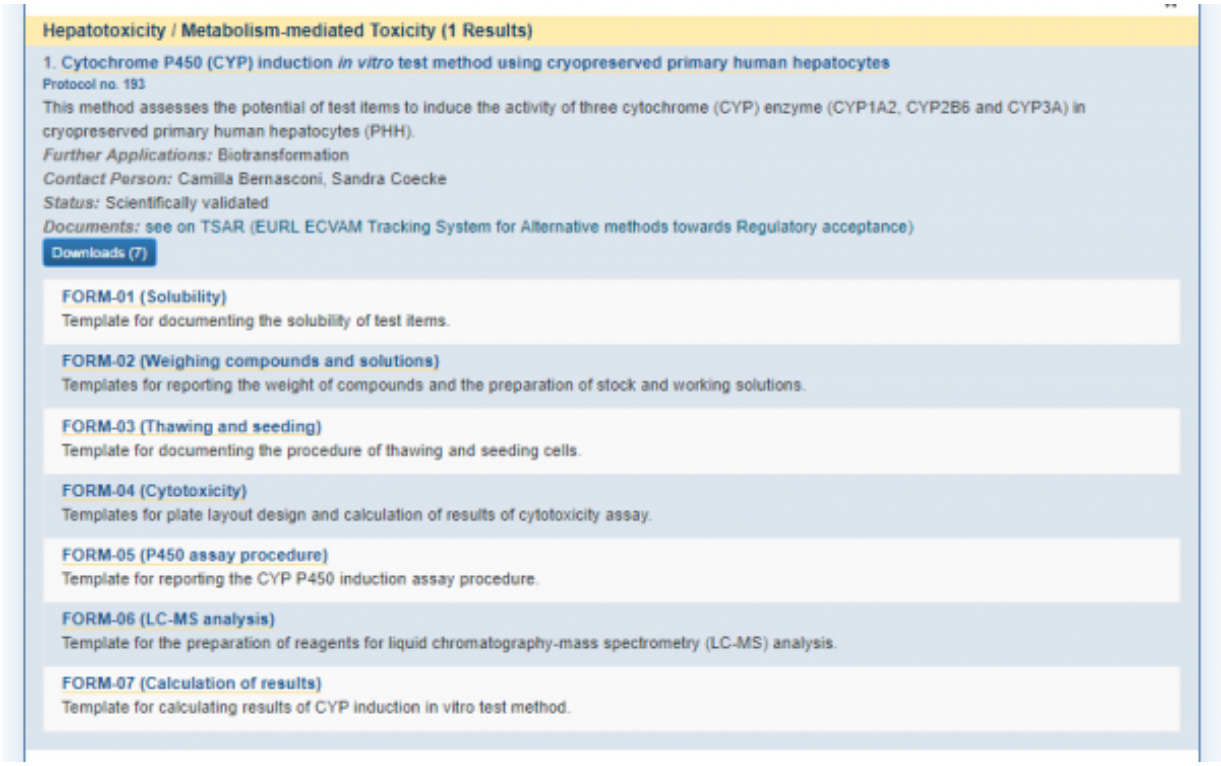
Acceptance criteria for selection of appropriate test concentrations:

- 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 80% after 72 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 at 6 levels.

Acceptance criteria for sequence analysis:

- 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).
- At least 50% of a level of QC (QC1, QC2 and QC3) must be accepted within a sample list.
- All blocks of QC must have at least 1 QC accepted.

The followings **FORMS** are used during the different phases of this protocol. They are made available for download from the **Downloads** section of this protocol.



Hepatotoxicity / Metabolism-mediated Toxicity (1 Results)

1. Cytochrome P450 (CYP) induction *in vitro* test method using cryopreserved primary human hepatocytes

Protocol no. 193

This method assesses the potential of test items to induce the activity of three cytochrome (CYP) enzyme (CYP1A2, CYP2B6 and CYP3A) in cryopreserved primary human hepatocytes (PHH).

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 (7)

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

FORM-02 (Weighing compounds and solutions)
Templates for reporting the weight of compounds and the preparation of stock and working solutions.

FORM-03 (Thawing and seeding)
Template for documenting the procedure of thawing and seeding cells.

FORM-04 (Cytotoxicity)
Templates for plate layout design and calculation of results of cytotoxicity assay.

FORM-05 (P450 assay procedure)
Template for reporting the CYP P450 induction assay procedure.

FORM-06 (LC-MS analysis)
Template for the preparation of reagents for liquid chromatography-mass spectrometry (LC-MS) analysis.

FORM-07 (Calculation of results)
Template for calculating results of CYP induction in vitro test method.

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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

PHH are internationally commercially available. The PHH used in the validation study (EURL ECVAM, 2014) were provided by the lead laboratory KaLy-Cell (Plobsheim, France) and grown according to the supplier's instruction.

Equipment

Fixed Equipment

- 8-channel pipette
- Balance
- Camera
- Cell culture incubator
- Centrifuges
- Chemicals cabinet
- Class II safety cabinet
- Counting chamber
- 20°C Freezer
- 80°C Freezer
- Fluorimeter
- Inverted research microscope (Nikon Diaplot THD, or equivalent)
- 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.
- Multiplate Reader 5 BioTek Synergy HT°, or equivalent
- Multiscan Ex (ThermoScientific, or equivalent)
- Pump
- Pipettes P10, P50, P200, P1000
- +4°C Refrigerator
- Refrigerated Centrifuge
- Pipetboy
- Vortex
- Water Bath

Consumables

- 0.2 µm sterile filters
- 1.5 ml, 15 ml and 50 ml centrifugation tubes
- LC-MS sampler vials with inserts and sampler caps
- LC-MS 96-well plates with lid (Nunc 249944 or equivalent)
- Stepper tips
- Sterile serological pipettes
- Cover foil for 96-well plates
- Uncoated 96- and 48-well polystyrene tissue culture plates with lid (e.g. Dutscher catalog numbers 064040 and 055621)

Media, Reagents, Sera, others

- 5,5-diethyl-1,3-diphenyl-2-iminobarbituric acid as Internal standard (e.g. Sigma S518891)
- Acetonitrile (HPLC gradient grade)
- BSA 1mg/mL, Protein standard (e.g. Sigma P0914-10AMP)
- Dexamethasone water-soluble (e.g. Sigma D2915)
- DMSO (analytical grade)
- Dulbecco's phosphate buffered saline w/o Ca, Mg (e.g. Lonza 17513)
- Formic acid (purum ≥ 98%)
- FCS (e.g. InVitrogen 16000-044, or equivalent)
- Gentamycin (e.g. 50mg/mL, InVitrogen 15750)
- Glacial Acetic Acid (e.g. 99-100%, Sigma 338826)
- H2O (e.g. MilliQ water)
- Insulin (e.g. 4mg/mL, InVitrogen 12585)
- Insulin-Transferrin-Selenium (ITS -G) (100X) (e.g. InVitrogen 41400-045)
- Geltrex or Matrigel (InVitrogen 12760 or Corning 356231 respectively)
- Methanol (HPLC gradient grade)
- NaOH (Sigma S8045)
- Percoll (e.g. Sigma P-1644-1L, pH 8.5-9.5 (25°C), density 1.125 - 1.135 g/ml at 25°C)
- PBS 10X with calcium and magnesium (e.g. InVitrogen 14080-048)
- Rat tail collagen (e.g. BD 354236)
- Trypan blue (e.g. Sigma T8154) used to 1:4 dilution in DPBS.
- Commercial Kits:
  - Protein determination: Pierce® BCA™ Protein Assay Kit (Sigma #BCA1) or equivalent
  - Cytotoxicity determination:Cell Titer® Blue (Promega, G8081) or equivalent

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) Heat Inactivation of FCS

- After thawing at RT, place the FCS bottle in a water-bath maintained at 56°C for 30 min.
- Divide into 10 aliquots of 50 mL.
- Freeze aliquots at -20°C and store for up to the shelf life stated by the supplier.

b) Thawing medium/washing medium

DMEM (Invitrogen 21063). The medium is supplemented with heat inactivated FCS (10% v/v final), 1 µM dexamethasone, 4 µg/mL insulin, and gentamycin 50 µg/mL.
Store at +4°C for no more than 1 month.

c) Seeding medium

DMEM (Invitrogen 21063). The medium is supplemented with heat inactivated FCS (5% v/v final), 1 µM dexamethasone, 4 µg/mL insulin, and gentamycin 50 µg/mL.
Store at +4°C for no more than 1 month.

d) KaLy-Cell's additive

1h before renewing medium, place the additive aliquots on ice to thaw gently (30 min to an hour). As soon as the additive is thawed, add it into warm seeding medium (23.3 µLof Geltrex or 25 µL of Matrigel/mL of seeding medium).

e) Induction medium

HMM (Lonza CC-3197), DMSO and serum free. Add to the medium ITS (1% v/v final), dexamethasone 1 mM (100 nM final) and gentamycin 50 mg/mL (50 µg/mL final). The medium has to be prepared fresh each day.

f) Incubation medium (for P450 activity determination)

HBSS + Ca^{2+} (Lonza BE10-527F). The medium does not contain DMSO or serum.

Solutions

Reference induction solutions (FORM-02)

All reference inducers stock solution can be prepared once and kept at +4°C during the 3 days of induction. The working solution are prepared freshly every day.

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.*

a) β-naphthoflavone (CAS 6051-87-2, MW 272.3 g/mol)
A 25 mM stock solution is prepared in DMSO.

b) Phenobarbital sodium salt (CAS 57-30-7,MW 254.2 g/mol)
A 500 mM stock solution is prepared in DMSO.

c) Rifampicin (CAS 13292-46-1, MW 822.94 g/mol)
A 10 mM stock solution is prepared in DMSO.

Cytochrome P450 substrates (FORM-02)
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)
A 10 mM stock solution is prepared in MeOH.

b) Bupropion HCl (CAS: 31677-93-7, MW 276.20 g/mol)
A 100 mM stock solution is prepared in MeOH.

c) Midazolam HCl (CAS: 59467-96-8, MW 362.2 g/mol)
A 3 mM stock solution is prepared in MeOH.

Cytochrome P450 products (FORM-06).
The stock solutions are stored at -20°C and can be used for one month, unless otherwise stated.

a) Acetaminophen (CAS: 103-90-2, MW 151.16 g/mol)
A 10 mM stock solution is freshly prepared in H_2O.

b) Hydroxybupropion (CAS: 92264-81-8, MW 255.74 g/mol)
A 10 mM stock solution is prepared in MeOH.

c) 1'-Hydroxymidazolam (CAS: 59468-90-5, MW 341.77 g/mol)
A 1 mM stock solution is prepared in MeOH.

Internal standard (ISTD) (FORM 06)

5,5-diethyl-1,3-diphenyl-2-iminobarbituric acid (Sigma, S518891; MW 335.409 g/mol)
A stock solution in ACN is prepared, aliquoted (79.5 µL per aliquots) and stored at -20°C for 6 months.

Positive Control(s)

For cytotoxicity: Chlorpromazine hydrochloride (CAS number 69-09-0, MW 355.33 g/mol). A 25 mM stock solution is prepared in DMSO (see FORM-02) and stored at -20°C for no longer than one month.

Negative Control(s)

Solvent-treated controls, i.e. samples without test item (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 were used for calculation of induction potential of test and reference items (n-fold induction). Additionally, solvent-free negative controls were run in parallel to observe possible effects of organic solvent on the cells.

Method

The intended concentration range of the test items depends on their solubility and toxicity. Before planning the induction assay, solubility and cytotoxicity towards PHH have to be determined in separate experiments. The final solvent concentration during the induction period should not exceed 0.1 % (v/v) DMSO.
The test item concentrations will have to be specified depending on its solubility in induction medium/0.1% (v/v) DMSO and on its cytotoxic potential. The highest concentration will be selected based on the results of the previously performed assays, and must not decrease the cellular viability < 80% within 72 h of incubation.

The main steps to perform the **CYP induction in vitro test method using cryopreserved primary human hepatocytes** are summarised here below. A detailed description of the experimental procedure is available in the following sections:

- **Determination of solubility of test items** (page 11)
- **Determination of cytotoxicity of test items** (page 12)
- **Determination of induction potential of test items** (page 18)

Test System Procurement

Technical specifications provided by the supplier for the storage of cryopreserved primary human hepatocytes should be followed.

In this protocol we refer to the specifications provided by KaLy-Cell, the supplier of PHH in the validation study (EURL ECVAM, 2014). KaLy-Cell PHH are stored in cryovials at -150°C. For use in other laboratories, cells are shipped by overnight transportation in a liquid nitrogen container (dry shipper). After arrival, cells should be kept at -150°C or at least in liquid Nitrogen until thawing.

Determination of solubility of test items

The test item has to be dissolved in a suitable solvent at a suitable concentration. Since the final solvent concentration during the induction period (exposure of cells to the test item for 72 h) should be 0.1% v/v DMSO, the stock solution of the test item in DMSO has to be at least 1000-fold strength 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
By default, test items are dissolved in pure DMSO or in a suitable solvent. 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 dissolution.The stock solutions may only be used if the test item is dissolved completely.

1. Weigh in ~20 mg test item into a screw cap brown glass vial and add DMSO so that the starting concentration should be 40 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 vortex-mixing.
4. Visually inspect the solubilisation of the compound. In case of any undissolved particle, place vial into an ultrasonic bath and sonicate for 2 min.
5. Visually inspect the solubilisation of the compound. In case of any undissolved particles, vortex-mix for 10 sec and sonicate for 5 min.
6. Visually inspect the solubilisation of the compound. In case of any undissolved particles, place the vial into a 37°C water bath for 10 min.
7. Visually inspect the solubilisation of the compound.
8. In case of any undissolved particles, the intended concentration cannot be applied.
9. Add additional DMSO (or alternative solvent) to obtain a solution of twofold lower strength.
10. (20 mg/mL) and repeat steps 2-7.
11. In case of any undissolved particles, the intended concentration cannot be applied.
12. Add sufficient additional solvent to obtain a solution of twofold lower strength (10 mg/mL) and repeat steps 2-7.
13. In case of undissolved particles, the intended concentration cannot be applied.
14. Add sufficient additional solvent to obtain a solution of twofold lower strength (5 mg/mL) and repeat steps 2-7.
15. In case of any undissolved particles, the intended concentration cannot be applied.
16. Add sufficient additional solvent to obtain a solution of twofold lower strength (2.5 mg/mL) and repeat steps 2-7.
17. In case of any undissolved particles, the intended concentration cannot be applied. Repeat steps 1-14 also using DMSO as solvent but reduce starting concentration to 1 mg/mL.
18. In case of any undissolved particles, the test item cannot be applied for induction.

Dilution and stability of test items in induction medium
A pre-test is performed by diluting the test item stock solution in induction medium in a 1:1000 ratio for DMSO. Any observations are reco rded in FORM-01.

1. Add 10 µl test item stock solution in DMSO to 9990 µl induction medium, (i.e. 1:1000 ratio in DMSO for test item dilution).
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. Tubes are incubated at 37°C for 24 h.
5. At the end of the incubation, the reaction tubes are centrifuged (16 100g, 5 min, RT).
6. The tubes are visually inspected for compound precipitation.
7. In case of compound precipitation, steps 1-6 have to be repeated using stock solutions of two-fold lower strength.

Determination of cytotoxicity of test items

Cytotoxicity of test items towards PHH is determined previously 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 fluorimeter. Since cytotoxicity is not significantly dependent on cell culture format, in order to save cells, the assay is performed in 96-well plates according to the recommendations given by the manufacturer with modifications described in this SOP. 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 72 h. After 24 h of exposure, the test item solution is renewed.

Reference inducers
Reference inducers are chemically defined compounds with a known induction potential.
To ensure that the concentration of the reference inducers used during the induction assay will not be cytotoxic, reference inducers are tested (in triplicate) in parallel with test items, using the same batch of cryopreserved human hepatocytes, and cultured under identical conditions.
Concentration chosen for reference inducers must not decrease cellular viability under 80% after 72h of incubation.
Reference inducers are displayed in **Table 2**, but alternative reference items can be applied, depending on the requirements of the test plan.

Table 2. Reference inducers.

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

Plate layout
Cytotoxicity experiments are performed in a 96-well plate format which allows testing two test items per plate. Each test item is analysed at seven concentrations in triplicates.
For each test item 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 item) of the reagent is measured, but also the fluorescence of the test item in medium at each tested concentration.

On every plate, the following parameters are tested, as shown in **Figure 2**:

a) Fluorescence of test item dilutions in medium (without cells) (A2-A8 and H2-H8)

b) CellTiter-Blue® reagent background fluorescence (A1-H1 and A12-H12)

c) Fluorescence of 7 test item dilutions (B2-G8, i.e. B2-D8 and E2-G8 for the two test items). Fluorescence of the CYP induction reference items (i.e. B10-D10 for β-naphtoflavone at 25 µM, E10-G10 for phenobarbital at 500 µM, and B11-D11 for rifampicin at 10 µM)

e) Fluorescence of the positive control (25 µM chlorpromazine, E11-G11)

d) Fluorescence of the negative control (solvent treated controls, B9-G9)

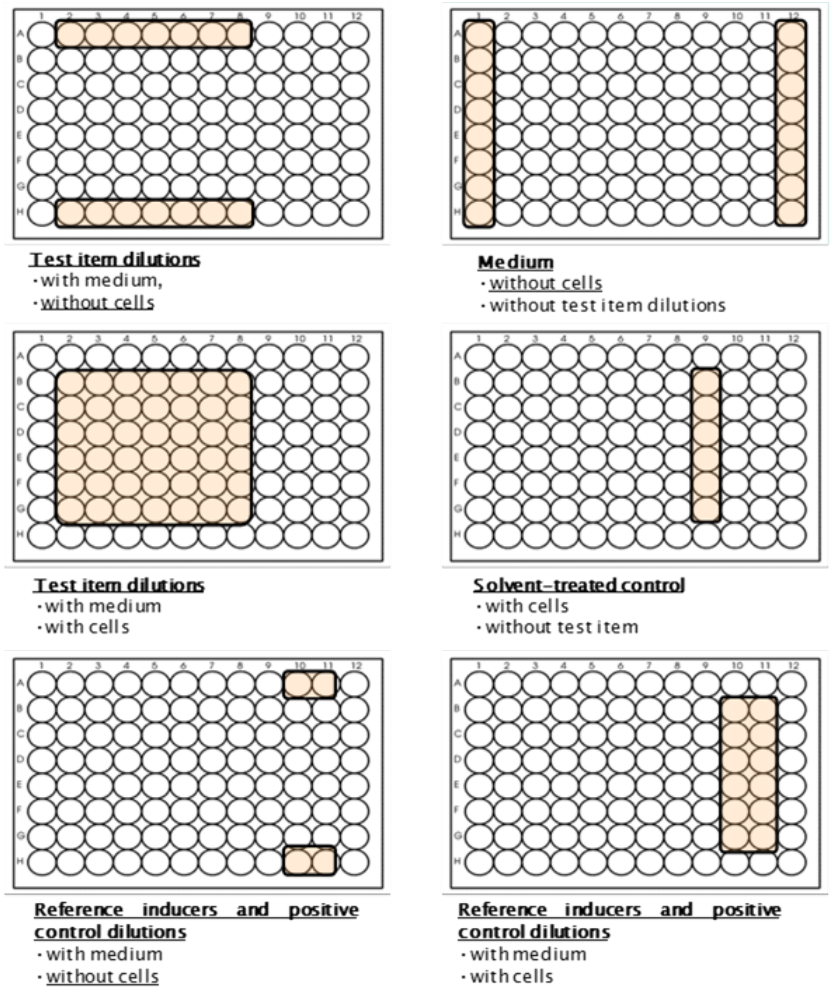


Figure 2. General layout of a 96-well plate for cytotoxicity testing.

Figure 3 shows an example plate layout for cytotoxicity analysis of two test items. The starting concentration is depending on the solubility of the test item in presence of 0.1% organic solvent.

	1	2	3	4	5	6	7	8	9	10	11	12
A	induction medium	test item A 0.625	test item A 1.25	test item A 2.5	test item A 5	test item A 10	test item A 20	test item A 40	DMSO 0.1%	β-Naphto-flavone 25 µM	Rifampicin 10 µM	induction medium
B	induction medium	test item A 0.625	test item A 1.25	test item A 2.5	test item A 5	test item A 10	test item A 20	test item A 40	DMSO 0.1%	β-Naphto-flavone 25 µM	Rifampicin 10 µM	induction medium
C	induction medium	test item A 0.625	test item A 1.25	test item A 2.5	test item A 5	test item A 10	test item A 20	test item A 40	DMSO 0.1%	β-Naphto-flavone 25 µM	Rifampicin 10 µM	induction medium
D	induction medium	test item A 0.625	test item A 1.25	test item A 2.5	test item A 5	test item A 10	test item A 20	test item A 40	DMSO 0.1%	β-Naphto-flavone 25 µM	Rifampicin 10 µM	induction medium
E	induction medium	test item B 0.625	test item B 1.25	test item B 2.5	test item B 5	test item B 10	test item B 20	test item B 40	DMSO 0.1%	Phenobarbital 500 µM	Chlorpromazine 25 µM	induction medium
F	induction medium	test item B 0.625	test item B 1.25	test item B 2.5	test item B 5	test item B 10	test item B 20	test item B 40	DMSO 0.1%	Phenobarbital 500 µM	Chlorpromazine 25 µM	induction medium
G	induction medium	test item B 0.625	test item B 1.25	test item B 2.5	test item B 5	test item B 10	test item B 20	test item B 40	DMSO 0.1%	Phenobarbital 500 µM	Chlorpromazine 25 µM	induction medium
H	induction medium	test item B 0.625	test item B 1.25	test item B 2.5	test item B 5	test item B 10	test item B 20	test item B 40	DMSO 0.1%	Phenobarbital 500 µM	Chlorpromazine 25 µM	induction medium

Figure 3 . Layout of a 96-well plate for cytotoxicity testing of two test items grey wells do not contain cells).

Time schedule

An exemplary time schedule for Cytotoxicity assay in PHH is given in **Table 3**. After delivery, cells are kept at -150°C or at least in liquid nitrogen until thawing for the assay. The time schedule demonstrates two independent assays performed with plates from one batch (one dispatch).

Note. *It is possible to test up to 10 compounds a day, performing 5 plates. Procedure in each cell plate should be started 10 minutes after the previous one.*

Table 3*. Exemplary time schedule cytotoxicity in PHH (example for 2 assays to be performed with the same batch).*

Month :	Action	
	Plate 1	Plate 2
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 item (t=0 h ± 0.5h)	Medium exchange: induction medium + test item (t=0 h± 0.5h)
Week 2 Tue	Medium exchange: induction medium + additive + test item (t=24 h± 0.5h)	Medium exchange: induction medium + additive + test item (t=24 h± 0.5h)
Week 2 Wed	Medium exchange: induction medium + test item (t=48 h± 0.5h)	Medium exchange: induction medium + test item (t=48 h± 0.5h)
Week 2 Thu	t=72 h± 0.5h enzyme cytotoxicity assay	t=72 h± 0.5h enzyme cytotoxicity assay

Cell culture

PHH are delivered in cryovials and have to be thawed and seeded in in-house-collagen-coated 96-well plates. 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 PHH:

B2-B11, C2-C11, D2-D11, E2-E11, F2-F11, G2-G11

The procedure to coat plates, thaw and seed cells, is documented using FORM-03.

Coating of 96-well plates

1. Add 10 mL glacial acetic acid solution to 490 mL deionized water and filter through a 0.2 µm filter. This provides a 0.02 N stock coating solution that can be kept at room temperature for one month.
2. Prepare a 50 µg/mL working solution of rat tail collagen type I by diluting the commercial rat tail collagen type 1 stock solution in 0.02 N glacial acetic acid. This solution should be prepared freshly.
3. Under laminar flow, add 100 µL of the collagen working solution to each of a 96-well plate:
4. Keep the plate for minimum 1 h at RT. Remove the collagen solution and wash the wells twice with DPBS solution (100 µL/well).
5. Plates may be used immediately or dried and stored at +2/8°C for up to one week under sterile conditions until the thawing procedure.

Thawing of cells

1. Prepare isotonic 90% Percoll (50 mL 10X PBS in 450 mL of Percoll) and mix thoroughly. This stock solution can be stored up to 8 weeks at +4°C.
2. For up to two PHH vials to thaw, mix 16 mL 90% Percoll with 25 mL thawing medium in a 50mL tube. Warm the Percoll/thawing medium at 37°C.
3. Carefully remove the vial from the storage container and transfer to the water-bath as quickly as possible. If the vial is stored in liquid nitrogen phase, remove the cap and poor off any liquid nitrogen. Close the cap firmly before placing the vial into the water bath.
   If the storage room and the lab are distant, it is important to keep frozen cells in refrigerated condition (preferably a small container with dry ice or at least liquid nitrogen) until placed into the water bath at 37°C.

4. Immediately immerse the vial(s) into the water-bath. Shake gently until the ice crystals are completely melted.
5. Transfer the vial(s) and the thawing medium tube(s) to the laminar flow. Pour the content of the vial(s) into the thawing medium tube(s). To wash out all the cells from the cryovial(s), gently poor a small volume of the pre-warmed thawing medium into it and then back into the tube(s). Complete up to 50 mL with thawing medium.
6. Close the lid and invert the tube slowly approximately 10 times to resuspend the cells.
7. Centrifuge at 170g for 20 min at RT.
8. At RT, pour off the supernatant and loosen the cell pellet before adding 50 mL/pellet warm washing medium.
9. Centrifuge the cell suspension at 100g for 5 min at RT.
10. At RT, poor off the supernatant and loosen the cell pellet before adding 2-3mL/pellet warm seeding medium.
11. Determine the total cell amount and the number of viable cells using Trypan Blue exclusion assay:

    - dilute commercial trypan blue to a 1:4 ratio in DPBS,
    - mix 20 µL of cells suspension to 20µL of the diluted trypan blue,
    - transfer a small volume of this solution into a counting chamber
    - evaluate the amount of cell by counting how many cells (both alive and dead) are present in the counting chamber using an inverted research microscope.

Seeding of cells

1. A 70-90% confluent monolayer is formed when cells from a given batch (from KaLy-Cell) are diluted in seeding medium according to KaLy-Cell's recommendations (cell density) for the specific batch.
2. The outer wells are filled with 100 µL Dulbecco's PBS in order to avoid evaporation.
3. Cells will be distributed into in-house-collagen I-coated 96-well plates in a final volume of 100 µl/well. The cell density will be that recommended by the supplier (e.g. 0.7 million cells/ml for Kaly-Cell supplier).
4. Gently rotate the plate in a figure of 8 in order to evenly distribute the cells across the surface of the wells.
5. Place for minimum 4h in a humidified incubator maintained at 37°C and under atmosphere of 5% CO2/95% air.
6. At least 4h after seeding, seeding medium is renewed in all wells and replaced by fresh seeding medium supplemented with KaLy-Cell's additive (see ***Preparation of media, point c***, page 8). 100 µL of this medium is distributed to each well.
7. Place all over the week-end in a humidified incubator maintained at 37°C and under atmosphere of 5% CO2/95% air.

Determination of cell attachment

After 72h of attachment, cell morphology is checked microscopically. Confluence should be minimum 70% (**Figure 4**).
If the integrity of the cells is not given, cells have to be discarded.If the monolayers are in good condition, cells are washed twice with warm DPBS (100 µL/well). Cytotoxicity testing is initiated by replacing DPBS with induction medium containing the test item, positive control or the corresponding solvents, respectively, as detailed under the paragraph ***Plate layout*** (see page 12) and **Figure 2.**

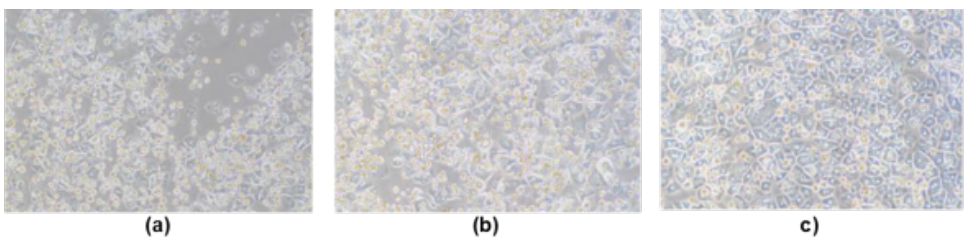


Figure 4. Examples of the morphology at 50% (a), 70% (b) and 90% (c) confluency of PHH seeded in 96-well plate for cytotoxicity assays.

Cytotoxicity assay

Preparation of solutions

Test items are dissolved as described in **Determination of solubility of test items,** page 11. Compounds should be prepared as stock solutions > 4 mg/mL and stored at -20°C. Organic solvents concentration has to be kept as low as possible. The concentration of organic solvents should not exceed 0.1% 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.

The dilutions of the test items and the control media (containing solvent at the appropriate concentration) are prepared in sterile microtubes by serial dilution. The content of the microtubes is carefully transferred to the plate containing the human cryopreserved hepatocytes monolayers using a pipette.

1. Prepare the 1000x test items stock solutions in DMSO (unless otherwise stated; in this case, a description of the whole preparation should be available).
2. For each test item align 7 sterile microtubes as described in FORM-08.
3. Transfer 40 µL of the test item stock solution in the first tube. In the 6 following tubes, add 20 µL of DMSO, and transfer 20 µL from the first tube to the second one, and so on through the last one (serial dilution 1/2). Mix by pipetting more or less 4 times in each tube.
4. Align 7 new sterile tubes per test item in front of the 7 tubes with test items DMSO diluted solutions. Add 999 µL of induction medium to each microtube and transfer 1 µL from the DMSO diluted solutions to the tubes in front, containing the induction medium. The content of solvent corresponds to the intended final concentration, *e.g*. a final solvent content of 0.1% (v/v).

Negative control, positive control and reference inducers (quantity for one plate)

- For **negative control**, prepare a sterile tube containing 999 µL of induction medium, and add 1 µL DMSO (or appropriate solvent) *e.g.* final solvent content of 0.1% (v/v) (volume for one plate to treat).
- For analysis of the toxicity potential of the **reference inducers** (BNF, RIF and PB), 1mL of solutions are prepared according to FORM-08 (Flowchart Cytotoxicity-Preparation of solutions) at the intended final test concentration and solvent concentration into sterile microtubes.
- Chlorpromazine, **positive control for cytotoxicity** is prepared as follows:
  a 25 µM solution is prepared freshly by diluting 1 µL of a 25 mM stock solution which is initially prepared in DMSO into 999 µL of induction medium.

  Warm these test item dilution microtubes at 37°C in a cell culture incubator for 10 min (±5min).

Addition of solutions

1. Seeding medium supplemented with KaLy-Cell's additive is removed from all wells after 72 h of attachment.
2. Wash cells twice with warm DPBS (100 µL/well) and take a picture from one well/plate at this stage to have a record of cells morphology, if the microscope is equipped with a camera
3. The incubation phase is initiated by taking off the DPBS from all wells and transferring 100 µl of the test item dilutions from the microtubes (see ***Preparation of solutions,*** page 8) to the hepatocytes plate, from the lowest concentration to the highest (allows to keep the same tip).
4. The hepatocytes plate is then placed in a cell culture incubator at 37°C and the 24 hours incubation time can be started.
5. After 24 ±0.5 h the incubation medium in the wells is changed. The test item dilutions are freshly prepared as described in chapter *Preparation of test item dilutions* , except that incubation medium **is supplemented with KaLy-Cell's additive** (see ***Preparation of media, point e,*** page 8) prior to test item dilution.
6. The incubation medium is removed and replaced by transfer of 100 µl of the freshly prepared test item dilutions from the lowest concentration to the highest (allows to keep the same tip). Continue incubation at 37°C for additional 24 h.
7. After 48 h of incubation (±0.5h), the incubation medium in the wells is renewed with freshly prepared test item dilutions exactly as described in ***Determination of solubility of test items***, page 11**.** Continue incubation at 37°C for additional 21 h.

8. Just before initiating the viability measurement, if the microscope is equipped with a camera, it is recommended to take a picture from one well/condition at this stage to have a record of the aspect of the cells.
9. The viability measurement is started 69 hours after initiation of the incubation phase.

Cytotoxicity measurement

The CellTiter-Blue ® method is used according manufacturer's instruction. 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.

CellTiter-Blue ® reagent is thawed as described above and adapted to room temperature for 10 min.

1. After 69 hours (±1h) of incubation dispense 20 µl (= 20% of incubation volume) to each well.and incubated at 37°C for additional 3 hours (±1h).
2. 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.
3. Read the plate in a multiwell fluorimeter at e.g. 530 nm excitation/590 nm emission (according to KaLy-Cell's Biotek Synergy HT fluorimeter).

Calculation of results

Results are expressed as fractional survival (% FS) and are calculated using the relative fluorescent units (RFU) as measured by the fluorometer. FORM-04 is used for calculations.

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:

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

Mean % FS values of the individual test item concentrations are plotted against the corresponding concentrations.

Acceptance criteria

The assays will only be accepted, if the following acceptance criteria are met:

1. The positive control 25 µM chlorpromazine has to produce equal to or less than 70% fractional survival (FS) of the cells (calculated based on an arithmetic mean of replicates).
2. At least, two non toxic concentrations should be found or the cytotoxic assay should be repeated with lower test item concentrations.
3. Negative control and reference inducers should be ≥80%FS.
4. For the negative control, RFU > 100 have to be detected after 3 h of reagent incubation (specification for KaLy-Cell Multiplate Reader BioTek Synergy HT fluorimeter). If the optical density of the negative control wells is found < 100, the metabolic activity of the cell batch cannot be guaranteed and the assay needs to be repeated using a new cell batch.

Determination of induction potential of test items

Test item
Test items are usually tested at six concentrations for their CYP induction potential. The following test concentrations represent generic concentration sets:

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 – 16 µg/ml – 6.4 µg/ml – 2.56 µg/ml – 1.02 µg/ml – 0.41 µg/ml	(1:2.5 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.

Reference inducers
For each study, a reference inducer is tested (in triplicate) in parallel for induction of each tested P450 isoform using the same batch of cryopreserved human hepatocytes, cultured under identical conditions. The increase of the functional activity of each particular P450 isoenzyme after incubation with the reference inducer has to be significant in order to allow a classification of the test item's potential for induction. Reference inducers are displayed in **Table 2** (see page 12) but alternative reference items can be applied, depending on the requirements of the test plan.

Plate layout
Induction experiments are performed in a 48-well format. An example for an assay set-up is given in **Figure 5.** It is recommended to perform the experiments at least in duplicate .

The following treatment groups are included on each plate (FORM-04):

- **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 compound).

Determination of functional CYP enzyme activity is performed in a cocktail (n-in-one) approach.

	1	2	3	4	5	6	7	8
A	Omeprazole 0.512	Omeprazole 0.512	Omeprazole 0.512	DMSO 0.1%	Phenobarbital 500 µM	Troglitazone 0.09375	Troglitazone 0.09375	Troglitazone 0.09375
B	Omeprazole 1.28	Omeprazole 1.28	Omeprazole 1.28	DMSO 0.1%	Phenobarbital 500 µM	Troglitazone 0.1875	Troglitazone 0.1875	Troglitazone 0.1875
C	Omeprazole 3.2	Omeprazole 3.2	Omeprazole 3.2	DMSO 0.1%	Phenobarbital 500 µM	Troglitazone 0.375	Troglitazone 0.375	Troglitazone 0.375
D	Omeprazole 8	Omeprazole 8	Omeprazole 8	β-Naphto-flavone 25 µM	Rifampicin 10 µm	Troglitazone 0.75	Troglitazone 0.75	Troglitazone 0.75
E	Omeprazole 20	Omeprazole 20	Omeprazole 20	β-Naphto-flavone 25 µM	Rifampicin 10 µm	Troglitazone 1.5	Troglitazone 1.5	Troglitazone 1.5
F	Omeprazole 50	Omeprazole 50	Omeprazole 50	β-Naphto-flavone 25 µM	Rifampicin 10 µm	Troglitazone 3	Troglitazone 3	Troglitazone 3

Figure 5. Example set-up for induction testing (2 test items, full dose-response range).

Time schedule
An exemplary time schedule for induction in PHH is given in **Table 4**. After delivery, cells are kept at -150°C or at least in liquid Nitrogen until thawing for the assay. The time schedule in **Table 4** demonstrates three independent induction assays performed with plates from one batch (one dispatch).

Note. *It is possible to test up to 10 compounds a day, performing 5 plates. Procedure in each cell plate should be started 10 minutes after the previous one.*

Table 4. Exemplary time schedule for P450 induction in PHH (example for 3 assays to be performed in parallel).

Month :	**Action**		
	Plate 1	**Plate 2**	**Plate 3**
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 item **(t=0 h± 0.5h)**	*Medium exchange:* induction medium + test item **(t=0 h± 0.5h)**	*Medium exchange:* induction medium + test item **(t=0 h± 0.5h)**
Week 2 Tue	*Medium exchange*: induction medium + additive + test item **(t=24 h± 0.5h)**	*Medium exchange:* induction medium + additive + test item **(t=24 h± 0.5h)**	*Medium exchange:* induction medium + additive + test item **(t=24 h± 0.5h)**
Week 2 Wed	*Medium exchange* : induction medium + test item **(t=48 h± 0.25h)**	*Medium exchange:* induction medium + test item **(t=48 h± 0.5h)**	*Medium exchange:* induction medium + test item **(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	-	-	-

Cell culture

PHH are delivered in cryovials and have to be thawed and seeded in in-house-collagen-coated 48-well plates.

Coating of 48-well plates

1. Add 10 mL glacial acetic acid solution to 490 mL deionized water and filter through a 0.2 µm filter**.** This provides a 0.02 N stock coating solution that can be kept at room temperature for one month.
2. Prepare a 50 µg/mL working solution of rat tail collagen type I by diluting the commercial rat tail collagen type 1 stock solution in 0.02 N glacial acetic acid. This solution should be prepared freshly.
3. Under laminar flow, add 250 µL of the collagen working solution to each of a 48-well plate.
4. Keep the plate for minimum 1 h at RT. Remove the collagen solution and wash the wells twice with DPBS solution (250 µL/well).
5. Plates may be used immediately or dried and stored at +2/8°C for up to one week under sterile conditions until the thawing procedure.

Thawing of cells

1. Prepare isotonic 90% Percoll (50 mL 10X PBS in 450 mL of Percoll) and mix thoroughly. This stock solution can be stored up to 8 weeks at +4°C .
2. For up to two PHH vials to thaw, mix 16 mL 90% Percoll with 25 mL thawing medium in a 50mL tube. Warm the Percoll/thawing medium at 37°C.
3. Carefully remove the vial from the storage container and transfer to the water-bath as quickly as possible. If the vial is stored in liquid nitrogen phase, remove the cap and poor off any liquid nitrogen. Close the cap firmly before placing the vial into the water bath.
   If the storage room and the lab are distant, it is important to keep frozen cells in refrigerated condition (preferably a small container with dry ice or at least liquid nitrogen) until placed into the water bath at 37°C.
4. Immediately immerse the vial(s) into the water-bath. Shake gently until the ice crystals are completely melted.
5. Transfer the vial(s) and the thawing medium tube(s) to the laminar flow. Pour the content of the vial(s) into the thawing medium tube(s). To wash out all the cells from the cryovial(s), gently poor a small volume of the pre-warmed thawing medium into it and then back into the tube(s). Complete up to 50 mL with thawing medium.
6. Close the lid and invert the tube slowly approximately 10 times to resuspend the cells.
7. Centrifuge at 170g for 20 min at RT.
8. At RT, pour off the supernatant and loosen the cell pellet before adding 50mL/pellet warm washing medium.
9. Centrifuge the cell suspension at 100g for 5 min at RT.
10. At RT, poor off the supernatant and loosen the cell pellet before adding 2-3mL/pellet warm seeding medium.
11. Determine the total cell amount and the number of viable cells using Trypan Blue exclusion assay*:*

    - dilute commercial trypan blue to a 1:4 ratio in DPBS,
    - mix 20µL of cells suspension to 20µL of the diluted trypan blue,
    - transfer a small volume of this solution into a counting chamber
    - evaluate the amount of cell by counting how many cells (both alive and dead) are present in the counting chamber using an inverted research microscope.

Seeding of cells

1. A 70-90% confluent monolayer is formed when cells from a given PHH batch (from KaLy-Cell) are diluted in seeding medium according to KaLy-Cell's recommendations (cell density) for the specific batch.
2. The outer wells are filled with 250 µL Dulbecco's PBS in order to avoid evaporation.
3. Cells will be distributed into in-house-collagen I-coated 48-well plates in a final volume of 250 µl/well. The cell density will be that recommended by the supplier (e.g. 0.8 million cells/ml for cells supplied by KaLy-Cell).
4. Gently rotate the plate in a figure of 8 in order to evenly distribute the cells across the surface of the wells.
5. Place for minimum 4h in a humidified incubator maintained at 37°C and under atmosphere of 5% CO2/95% air.
6. At least 4h after seeding, seeding medium is renewed in all wells and replaced by fresh seeding medium supplemented with KaLy-Cell's additive (*see* ***Preparation of media, point c,*** page 8). 250 µL of this medium is distributed to each well.
7. Place all over the week-end in a humidified incubator maintained at 37°C and under atmosphere of 5% CO_2/95% air.

Determination of cell attachment

After 72h of attachment, cell morphology is checked microscopically. Confluence should be minimum 70% (**Figure 4,** page 15). If the integrity of the cells is not given, cells have to be discarded. If the monolayers are in good condition, induction assay is initiated by replacement of seeding medium by induction medium containing the test item, positive control or the corresponding solvents.

Induction

Preparation of solutions

Each test item is dissolved in an appropriate solvent. Working solutions are prepared in induction medium (*see* ***Preparation of media, point e,*** page 8) 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 solvents themselves have induction potential (e.g. DMSO induces CYP3A4). Stock and working solutions of the test items have to be prepared freshly every day.
In the exceptional case of low amounts of compounds, the stock solution can be prepared once, but in this case three aliquots of the stock solution (one for each incubation day) have to be stored under suitable conditions in order to avoid chemical instabilities due to multiple freeze-thaw cycles. Initial weight and preparation of stock and working solutions have to be documented in FORM-02.

Reference items are β-naphtoflavone (BNF, CYP1A2), rifampicin (RIF, CYP3A4) and phenobarbital (CYP2B6). Their stock solutions are prepared according to ***Preparation of solutions*** *(*see page 8) and working solutions are prepared freshly every day. Initial weight and preparation of stock and working solutions is documented in FORM-03.

a) β-naphtoflavone (MW 272.3 g/mol, induction concentration 25 µM, final solvent concentration 0.1% (v/v) DMSO, 25 mM stock solution)

b) rifampicin (MW 822.94 g/mol, induction concentration 10 µM, final solvent concentration 0.1% (v/v) DMSO, 10 mM stock solution)

c) phenobarbital (MW 254.22 g/mol, induction concentration 500 µM, final solvent concentration 0.1% (v/v) DMSO, stock solution 500 mM)

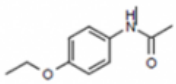
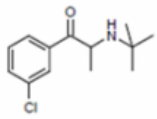
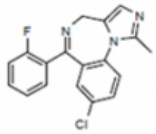
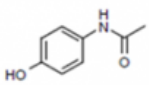
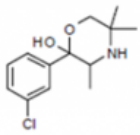
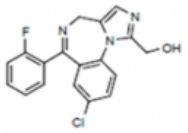
Addition of induction solution

Stock solutions are prepared according to point ***Preparation of media and solutions*** *(*see page 8-9).
The induction is initiated by the addition of the induction solution (250 µl/well), which corresponds to time point t = 0 h. In order to maintain inducer concentrations constant during the induction period, the induction solution is replaced at time point t = 24 h (±0.5h) by freshly prepared induction solutions, as described in Cytotoxicity/Preparation of solutions/Test items, except that incubation medium is **supplemented with KaLy-Cell's additive** (***Preparation of media, point e,*** page 8) prior to test item dilution.
The medium added at time point t = 24 h (±0.5h) is incubated for additional 24 h. At time point t = 48 h (±0.5h), the induction solution is again replaced (***Preparation of media, point e,*** page 8) and cells are again incubated for additional 24 h (±0.5h), thus the cells are exposed to the inducers for 72h (±0.5h) in total. Time points of start of incubation and medium exchange are documented in FORM-05.

Functional enzyme activity assay

The functional activity is analysed after 72 h (±0.5h) exposure of the cells to the inducer. P450 iso-enzyme activities are tested in Incubation medium. The specific enzyme reactions are summarized in **Table 5**. 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 analysed for the specific products shown in **Table 5** by means of LC-MS, or stored at -20°C until analysis.

Table 5. Specific CYP 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

Preparation of substrate solutions

The stock solutions of the P450 substrates are prepared as described in ***Preparation of solutions*** (see pages 8-9). The procedure is documented in FORM-02.
All substrates are dissolved in MeOH to obtain a working solution of 1000-fold higher strength than the intended final substrate concentrations in experimental incubations (Phenacetin 10 µM, Midazolam 3 µM, Bupropion 100 µM), as shown in **Table 5**. Thus the working solutions have the following concentrations: Phenacetin 10 mM, Midazolam 3 mM, Bupropion 100 mM.

Assay procedure (FORM-02, FORM-05)

1. Warm incubation medium in a water bath to 37°C.
2. Remove induction medium from the wells. Wash the cells twice with 250 µl warm DPBS. An 8-channel pipette or a stepper can be used for the washing step.
3. Prepare the substrate cocktail by mixing 1µL each 1000-fold concentrated stock solution per mL HBSS (0.3% (v/v) MeOH final concentration) in a Falcon tube (e.g. 5.5 mL of solution should be prepared per assay plate).
4. Warm the substrate cocktail (**Table 5**) in a water bath to 37°C.
5. Add 100 µl substrate cocktail to the wells using a pipette and document starting time in FORM-05 (start the incubation time with the addition in the first well).
6. Carefully move the plate in order to equally distribute the substrate cocktail. Transfer the plate into the cell culture incubator.
7. Incubate for 30 min.
8. At the end of the incubation time, the supernatant (100 µl) is removed from the wells and transferred to correspondingly labelled Eppendorf tubes filled with 100 µL of ice cold acetonitrile containing ISTD if required for the analysis (filling of Eppendorf tubes can be done during incubation time).
9. If the samples cannot be analysed directly, the Eppendorf tubes are stored at -20°C until analysis (stable for 9 months).
10. After supernatant has been transferred, cells in all wells are lysed by the addition of 200 µl 0.1 M NaOH (stable 6 months at RT) on the monolayers and mixed for 5min at RT.
    Then, scrape the entire bottom of each well with the tip of the pipette (use a new one for each well, see **Figure 6** *below*) so that all cells are lysed, and transfer the lysates in correspondingly labelled Eppendorf tubes.

    Store at -20°C until analysis for protein content. Prior to the performance of the **protein determination** assay, the execution of one freeze-thaw-cycle of the lysates is mandatory (freezing period not less than 1 hour to support the lysis process).

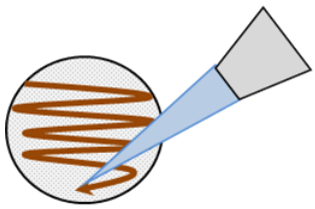


Figure 6. Lysis of the cells for protein determination.

Endpoint Measurement

At the end of the incubation with substrate coktail 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 Bicinchoninic Acid Kit for Protein Determina tion (Sigma #BCA1) with minor modifications (see steps 1-8) :

1. Preparation of diluted albumin (BSA) standards: the BSA stock ampoule [1 mg/ml] supplied with the kit is diluted in 0.1 M NaOH and the standard solutions are prepared by serial dilution as in **Table 6**. Alternatively, standards can be prepared from BSA fraction V (e.g Sigma A9647) . In this case, a 1 mg/ml stock solution is prepared in 0.1 M NaOH. All standards are stable at -20°C for approximately 6 months.

Table 6. Standards for protein determination.

Standard name	Final BSA concentration [mg/ml]	0.1 M NaOH [µl]	BSA solution to use	BSA Volume [µl]
Blank	0 (blank standard)	500	-	-
S1	0.1	450	1 mg/ml stock	50
S2	0.2	400	1 mg/ml stock	100
S3	0.3	350	1 mg/ml stock	150
S4	0.4	300	1 mg/ml stock	200
S5	0.5	250	1 mg/ml stock	250
S6	1	0	1 mg/ml stock	500

2. Transfer 20 µL of each sample (*see* ***Induction/Assay procedure/step 10,*** page 23) or sample standard, in duplicate in a 96-well plate.
3. Prepare Pierce ® Working Reagent by mixing (for a 96-well plate): 20 mL of BCA and 400 µL of copper sulfate 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. 200 µl Pierce Working Reagent is added per well, mix plate thoroughly on a shaker for 30 sec.
5. Cover the plate and incubate at 37°C for 30 min.
6. Cool the plate to room temperature.
7. Read the plate at OD 595nm within 10 min.

Calculation of results

A standard curve is prepared by plotting the average absorbance for each standard vs. its concentration (in mg/ml). Unknowns are extrapolated from the standard curve using linear regression (see FORM-07).

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

Standard curve acceptance criteria: The standard curve should have a correlation coefficient (r2) equal or greater than 0.95.

LC-MS analysis of induction assay samples

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.** Standard solutions containing defined concentrations in the range of the expected product concentrations are prepared in Incubation medium as described in FORM-06 and documented accordingly.

Preparation of stock solution

1. Prepare predilutions of the 3 metabolites to 0,5 (PD1); 0,0625 (PD2) and 0,0078 mM (PD3)
2. Mix 200 µL of each 0,5 mM metabolite solution with 400 µL ACN - total 1000 µL - WS A
3. Serial dilute into working solutions (B-C) by mixing with ACN
4. Mix 200 µL of each 0,0625 mM metabolite solution with 400 µL ACN - total 1000 µL - WS D
5. Serial dilute into working solutions (E-F) by mixing with ACN
6. Mix 200 µL of each 0,0078 mM metabolite solution with 400 µL ACN - total 1000 µL - WS G
7. Serial dilute into working solutions (H-I) by mixing with ACN
8. Prepare standard solutions by addition of 5 µL of WS (1-9) to 245 µL Incubation Medium
9. Vortex mix for 10 sec
10. Take 200 µL of each st. solution and add 200 µL Stop solution (1 µM ISTD in ACN)
11. Prepare ISTD sample by adding 200 µL Stop solution to 200 µL of Inc. Medium
12. Prepare blank sample by adding 200 µL ACN to 200 µL of Inc. Medium
13. Vortex-mix all for 10 sec
14. Centrifuge for 5 min at 16,100 g at room temperature
15. 30 µL of the particle-free supernatant is diluted with 70 µL H2O supplemented with a total content of 0.1% (v/v) formic acid (final ACN content: 15% v/v) and transferred to LC-MS vials
16. Quick vortex mix

Preparation of samples

1. If samples have been stocked at -20°C until analysis, let them thaw at room temperature for approximately half an hour (±15min).
2. Vortex, and centrifuge for 5 min at 16,100g at room temperature.
3. After centrifugation, 30 µl of the particle-free supernatant is transferred to LC-MS sample vials (200 µl insert) correspondingly labelled and diluted with 70 µl H2O+0.1% (v/v) formic acid (final acetonitrile content: 15% (v/v)). The vials are closed using screw caps (analytical 96-well plates with lid can be used if the autosampler allows it).
4. After LC-MS measurement the remaining quantities of the samples should be stored at -20°C for possible further analysis.

General LC-MS method performance requirements

Each laboratory may use an LC-MS system of its choice for the analysis of the CYP products as long as it meets performance criteria, including the Limit of Quantitation (LOQ) as specified below. (9.0 nM for acetaminophen, 1.8 nM for hydroxybupropion and 1.8 nM for 1-hydroxymidazolam).

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 (EMA, Guideline on bioanalytical method validation, 2012) or Food and Drug Administration (US FDA, 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 results are expressed as P450 activities in pmol/min/mg protein or pmol/min/1*10^6 cells, respectively (FORM-07).

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

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

If outliers are found within triplicates, an exclusion of a value might become necessary. In this case, the respective value has to be marked with an apostrophe (‘), e.g. *‘500* instead of *500* has to be typed in the respective cell in FORM-07. Hence, the value will no longer be recognized as a number and will be excluded from the subsequent calculations automatically.

Attention: *The determination of outliers cannot be performed in FORM-07; it has to be calculated separately!*

The percent response can be calculated as a percentage of the positive control (PC), according to FDA guidance for Industry (US FDA, 2017) as follows:

$$\text{Percentage of PC} = \frac{(\text{activity of treated cells} - \text{activity of control cells})}{(\text{activity of positive control cells} - \text{activity of control cells})} \times 100$$

Treated cells: cells treated with the test compound
Control: cells treated with 0.1 % (v/v) DMSO
Positive control cells: cells treated with the reference inducer

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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