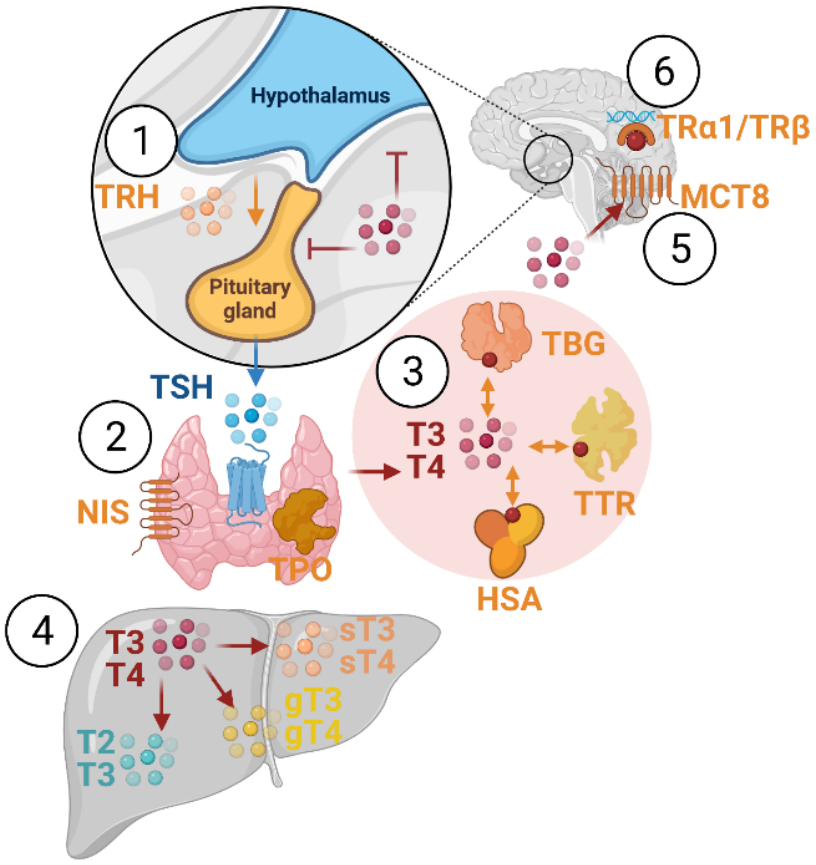
STANDARD OPERATING PROCEDURE

for determination of the potential inhibition of thyroid hormones glucuronidation in human liver microsomes, version 1.0

EURL ECVAM validation study of a battery of mechanistic methods relevant for the detection of chemicals that can disrupt the thyroid hormone system



2021

This Standard Operating Procedure (SOP) has been prepared within the context of a collaboration agreement signed in 2019 with the Joint Research Centre (JRC) Directorate for Health, Consumers and Reference Materials (Chemicals Safety and Alternative Methods Unit F3 / EURL ECVAM), for the validation of mechanistic methods to identify potential modulators of thyroid hormone signalling.

The SOP provided was used during Part 1 of the validation study. Later versions exist.

The method was developed and experimentally assessed by EU-NETVAL laboratory Accelera, Italy.

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PURPOSE

This SOP describes the method for the determination of test items' potential inhibitory capacity towards thyroid hormones glucuronidation in human liver microsomes.

1.1. Area of Application

The use of human liver microsome test systems modelling thyroid hormones (THs) metabolism can provide insight whether chemicals may affect uridine 5'-diphospho-glucuronosyltransferases (UGTs) activity, which may lead to disturbance of thyroid hormones glucuronidation.

The method described herein is applicable for the determination of inhibition of UGT enzymes in human liver microsomes upon exposure to test items.

The analysis is performed by LC-MS/MS to determine the concentrations of specific TH metabolites formed by UGT enzymes after incubation of T3 and/or T4 as substrates together with the test item.

1.2. Terms and Abbreviations

ACN	Acetonitrile
BG	Background
CAS	Chemical Abstracts Service
CoA	Certificate of Analysis
DMSO	Dimethyl sulfoxide
e.g.	For example
GLP	Good Laboratory Practice
h	Hour (s)
H_2O	Ultrapure or deionised water
HLM	Human Liver Microsomes
HPLC	High-performance liquid chromatography
HPT	Hypothalamus–pituitary–thyroid
ISTD	Internal Standard
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography coupled to tandem mass spectrometry

LLOQ	Lower limit of quantitation
MeOH	Methanol
MRM	Multiple Reaction Monitoring
m/z	Mass-to-charge ratio
min	Minute(s)
MU	4-Methylumbelliferone
MW	Molecular weight
NA	Not applicable
NC	Negative Control
PC	Positive Control
OECD	Economic Co-operation and Development
QC	Quality control
RT	Room temperature
sec	Second(s)
SS	Stock solution
SST	System suitability test
T3	L-Thyroxine
T4	3,3',5-Triiodo-L- thyronine
TH	Thyroid hormones
TSH	Thyroid stimulating hormone
UGT	UDP-glucuronosyltransferases
UDPGA	Uridine 5′-diphospho-glucuronic acid
ULOQ	Upper limit of quantitation
v/v	Volume per volume
WS	Working solution

1.3. Principles and Scientific Background

Thyroid hormones (THs) are important for many physiological processes including embryonic development, cellular differentiation, metabolism and regulation of cell proliferation.

TH production and secretion by the thyroid gland is regulated by the hypothalamus–pituitary–thyroid (HPT) axis. As a matter of fact, thyroid stimulating hormone (TSH) acts at the level of the pituitary gland where it controls the release in circulation of THs. The predominant TH in circulation in the euthyroid situation is the prohormone thyroxine (T4), which is the precursor of the biological active form triiodothyronine (T3) (Yamanaka et al. 2007).

Deiodination, glucuronidation and sulfation of thyroid hormones are the main pathways in the metabolism of T3 and T4. Figure 1 shows the metabolism pathways of thyroxine in humans.

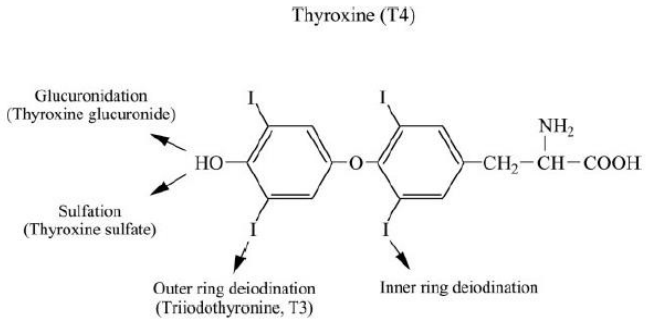


Figure 1 - Major metabolic pathways of thyroxine in human

The enzymes involved in glucuronidation are called UDP-glucuronosyltransferases (UGTs) (Murk et al. 2013).

It is important to highlight that there is a large inter-species variability in both expression and function of UGT enzymes; for this reason, the validated method is based on human-derived hepatic test systems (mixed sex pooled human liver microsomes) and therefore it is of relevance for the human situation.

The modulation of the two hormones has been identified as one of the priority indicators of thyroid system toxicity (OECD, 2017). As a consequence, glucuronidation inhibition has been selected as the biological endpoint to assess the capability of test items to interfere with the intra-cellular metabolism and excretion of THs.

Inhibition of TH glucuronidation is a proposed mechanism of interference for a number of xenobiotics and it may result in impairment of TH homeostasis.

The potential inhibition of the compounds towards glucuronidation of T3 and T4 will be determined by measuring and comparing the TH glucuronide conjugates formation (see Table 1) after incubation with and without the test item by the analytical liquid chromatography - mass spectrometry (LC-MS) technique.

1.4. Responsibilities

All study personnel are responsible for recording raw data promptly and accurately and in compliance with the Principles of GLP and/or OECD GIVIMP (OECD, 2018), and are responsible for the quality of the data.

1.5. Health Safety and Environment

Study personnel should exercise health precautions to minimize risk and to ensure the integrity handling related to this assay. They should communicate to the appropriate people any relevant known health or medical condition in order that they can be excluded from operations that may affect the performance of this method.

2. EXPERIMENTAL METHOD

2.1. General requirements

Commercially available cryopreserved human liver microsomes are used to evaluate the UGT activity in presence of cofactor UDPGA. The UGT activity of new batches of human liver microsomes has to be determined by quantification of methylumbelliferone in the "MU fluorescence assay".

In the inhibition assay test items are incubated at six different concentrations with a known UGT inhibitor (Mefenamic Acid) and a no inhibitor (Fluconazole) at one concentration together with the corresponding solvent controls.

Substrates T3 and T4 are incubated in separated plates with the test system in the presence and in the absence of the known inhibitor and of the test items.

The glucuronide conjugates are quantified in the presence and in the absence of the inhibitor to assess its inhibition potential.

The inhibition activities of test items are evaluated in a "range finding assay" using a wide range of concentrations. Once the six concentrations of the test items are chosen, the "main inhibition assay". Final data must be performed in three valid runs.

In all inhibition assays the UGTs activity is evaluated in parallel by "MU fluorescence assay". This fluorescence assay is performed also to characterize a new batch of microsomes.

The overall experimental design is summarized in *Figure 2.*

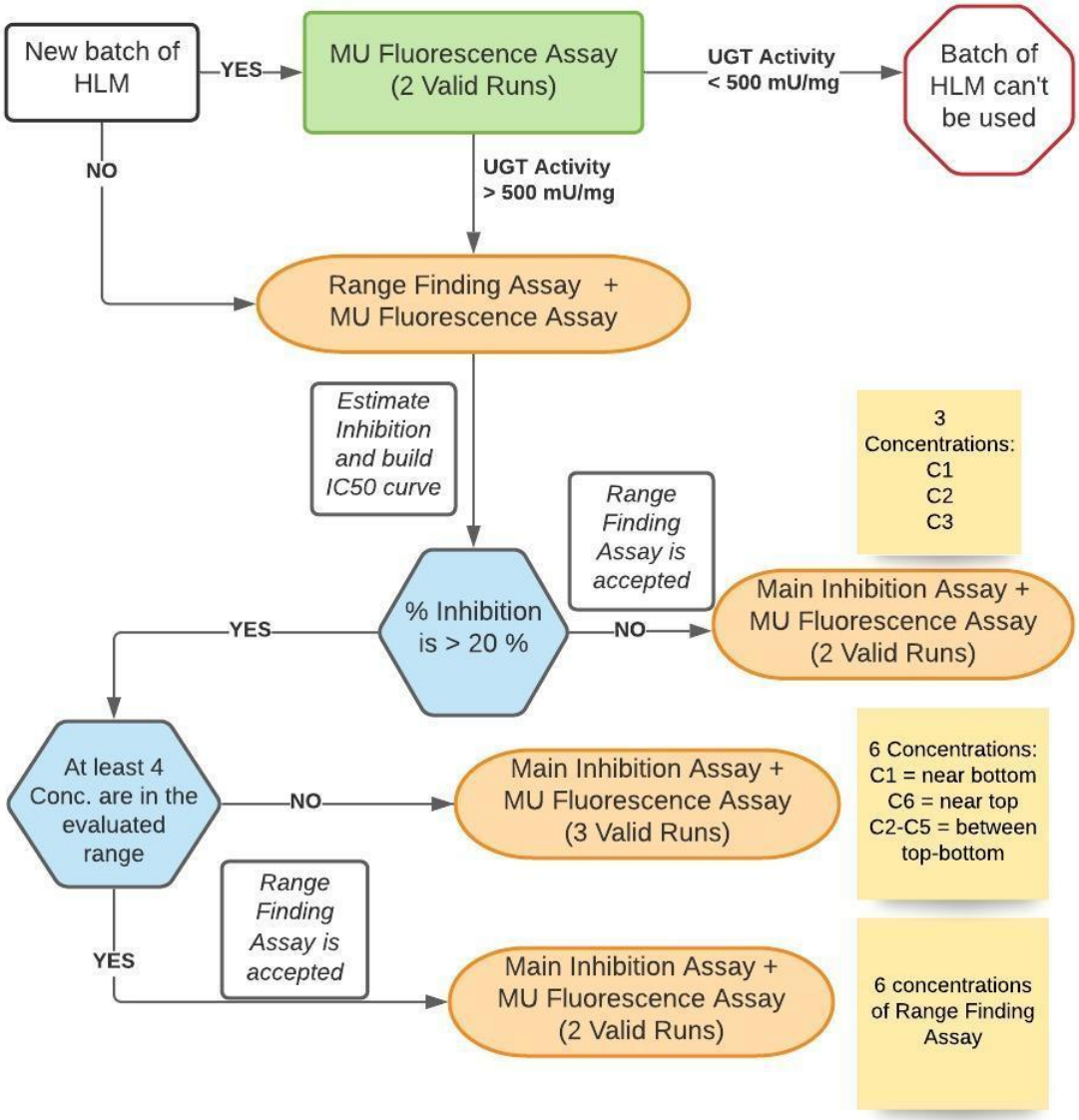


Figure 2: Experimental design of the in vitro test method using human liver microsomes

2.2. Equipment

- Balance (minimum precision 1.000 mg)
- Vials and Plate Centrifuges
- 4 °C Fridge

- -20 ± 5 °C and -80 ± 10 °C Freezer
- Liquid chromatography system coupled with a tandem mass spectrometer (LC-MS/MS)
- Fluorescence spectrometry plate reader
- Pipettes P10, P100, P200, P1000
- Multichannel pipettes and/or Liquid handling robot
- Plate heater
- Plate shaker
- Vortex
- Ultrasonic Sonicator system
- Water bath

2.2.1. Fluorescence spectroscopy and LC-MS method

2.2.1.1. General fluorescence spectroscopy and LC-MS method performance requirements

Each laboratory may use an LC-MS system of its choice for the analysis of glucuronide products (e.g. Triiodo-thyronine glucuronide, T3G, and Thyroxine glucuronide, T4G) and a fluorescence spectroscopy for 4-methylumbelliferone (MU) analysis as long as it meets the performance criteria described in this section.

Prior to initiation of experiments, the laboratory should demonstrate that the metabolites can be measured with sufficient accuracy and precision to meet the Quality Control.

Each laboratory may use) as long as it meets the performance criteria described in this section.

Prior to initiation of experiments, the laboratory should demonstrate that glucuronides and methylumbelliferone can be measured with sufficient accuracy and precision to meet the Quality Control.

Performance criteria

The method developed and implemented for fluorescence spectrometry and LC-MS quantification of the analytes must be validated for accuracy, precision, 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). At least, a "fit-for-purpose" validation has to be performed.

During method development, accuracy of the developed method is evaluated determining "trueness" and "precision" (ISO 5725-Part 1, General Principles and Definitions). "Trueness" refers to the closeness of agreement between the arithmetic mean of a large number of test results and the true or accepted reference value and is normally expressed in terms of bias. "Precision" refers to the closeness of agreement between test results, expressed as percentage of the coefficient of variation (%CV) of triplicates. Values to check and their accepted ranges are:

- Lowest limit of Quantitation (LLOQ) is the lowest concentration of the analyte present in the sample matrix that is measured under the method acceptance criteria at a concentration within:
    - ± 20% of variability (%CV) and 80 – 120% (%Bias) of target concentration for LC-MS/MS analysis (T3G and T4G).
    - ± 25% of variability (%CV) and 75 – 125% (%Bias) of target concentration for fluorescence analysis (MU). The sum between %CV and %Bias should be ≤ 40 %, total analytical error.

  The LLOQ must be ≤ 5 nM for T3 and T4 glucuronides and ≤ 1 µM for MU.
- Low Quality Control (LQC), Mid Quality Control (MQC) and High Quality Control (HQC) are measured under the method acceptance criteria at a concentration within:
    - ± 15% of variability (%CV) and 85 – 115% (%Bias) of target concentration for LC-MS/MS analysis (T3G and T4G).
    - ± 20% of variability (%CV) and 80 – 120 (%Bias) of target concentration for fluorescence analysis (MU). The sum between %CV and %Bias should be ≤ 30 %, total analytical error
- Upper Limit of Quantitation (ULOQ) is the highest concentration of the analyte present in the sample matrix that is measured under the method acceptance criteria at a concentration within:
    - ± 15% of variability (%CV) and 85 – 115% (%Bias) of target concentration for LC-MS/MS analysis (T3G and T4G).
    - ± 25% of variability (%CV) and 75 – 125% (%Bias) of target concentration for LC-MS/MS analysis (T3G and T4G). The sum between %CV and %Bias should be ≤ 40 %, total analytical error.

  The ULOQ must be ≥ 2 µM for T3 and T4 glucuronides ≥ 80 µM for MU.
- The response of interferences/contaminants and of carry-over samples should be ≤ LLOQ calibration standard.

2.2.1.2. LC-MS system

An LC system consisting of a suitable pump and refrigerated autosampler (+ 4 ± 3 °C) is required. Mass spectrometry is performed on a qualified instrument. The use of high-resolution mass spectrometry or of triple quadrupoles is accepted for the analysis of the glucuronide products. Tandem mass spectrometry can be used if the sensitivity of the method is sufficient.

Analytes (e.g. products and internal standard) are separated on a suitable LC column. Chromatographic conditions have to be optimized in a way that adequate chromatographic retention of substrates (T3 and T4), products (T3G and T4G) and internal standard (e.g. Fexofenadine) is ensured.

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 both T3 and T4 glucuronides.

Suggested, but not mandatory ions to be used to quantify the items in the multiple reaction monitoring mode (MRM) are given in *Table 1*.

Compound	ESI Polarity	Molecular weight	Precursor ion (m/z)	Product ion(s) (m/z)
T3	-	650.97	650.0	127.0
T4	-	776.87	775.7	127.0
T3G	-	827.11	825.7	127.0; 650.0
T4G	-	953.00	951.8	127.0; 776.0

Table 1: MS and chromatographic parameters for analysis of specific metabolites

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.

2.2.2. Disposables

- 1.5 mL, 15 mL und 50 mL centrifugation tubes, conically shaped, polypropylene, sterile
- 50 mL polystyrene reservoir
- Sterile serological pipettes individually wrapped, polystyrene
- 96-well plates flat bottom with lid, polypropylene
- 96-well plates deep-well flat bottom with lid (e.g. volume 800 µL), polypropylene
- 384-well plates cone bottom, polypropylene
- 96-well plates flat bottom black with lid, polypropylene with micro-clear bottom (for "MU fluorescence assay")

2.3. Materials and Stock Solutions

This procedure concerns positive and negative controls, UGT substrates, UGT metabolites, internal standard and test items.

Chemical known purity (e.g. analytical standard grade) from reputable commercial sources should be used. The source, lot number, expiration date or re-test date and storage condition should be available from supplier (e.g., on CoA).

2.3.1. Handling of the chemicals

The chemicals should be kept at room temperature before weighing. The weighted quantity and the theoretical quantity should be registered and kept in an appropriate study file. If possible, the weight receipt should be attached.

The vial, where the powder is placed to prepare the stock solution, must be of the appropriate volume and must be previously labelled according to the facility internal procedures.

The stock solutions should be used only if the chemicals are dissolved completely in a suitable solvent at a suitable concentration. The stock solution that is not used on the same day of preparation should be aliquoted in labelled vials. The vials should be put in a container, stored appropriately, and should be recorder.

2.3.1.1. Positive and Negative controls stock solution

- *Mefenamic Acid (positive control)*
  e.g. Merck M4267, CAS 61-68-7
  MW 241.29 g/mol
  Storage -20 °C
  A 100 mM stock solution in DMSO is prepared, aliquoted and stored at -20 °C for up to X days (see *Table 29*).
- *Fluconazole, Analytical standard grade (negative control)*
  e.g. Merck F8929, CAS 86386-73-4
  MW 306.27 g/mol
  Storage -20 °C
  A 100 mM stock solution in DMSO is prepared, aliquoted and stored at -20 °C for up to X days (see *Table 29*).

2.3.1.2. UGT Substrates stock solutions

- *Triiodo-L-thyronine (T3), Analytical standard grade*
  e.g. Merck T6397, CAS 55-06-1
  MW 650.97 g/mol
  Storage -20 °C
  A 10 mM stock solution in NaOH 1.0 N : DMSO (1:1) is prepared, aliquoted and stored at -20 °C for X days (see *Table 29*).
- *Tetraiodo-thyronine (L-Thyroxine) (T4), Analytical standard grade*
  e.g. Merck T2376, CAS 51-48-9
  MW 776.87 g/mol
  Storage RT
  A 10 mM stock solution in NaOH 1.0 N : DMSO (1:1) is prepared, aliquoted and stored at -20 °C for the X days (see *Table 29*).

2.3.1.3. UGT metabolites stock solutions

- *Triiodo-thyronine glucuronide (T3G), Analytical standard grade*
  e.g. TRC/LGC, T796445 CAS: 29919-72-0
  MW 827.10 g/mol
  Storage at -20 °C
  A 100 µM stock solution in MeOH : H_2O is prepared, aliquoted and stored at -20 °C for X days (see *Table 29*).
- *Thyroxine glucuronide (T4G), Analytical standard grade*
  e.g. TRC/LGC, T425630, CAS: 21462-56-6
  MW 952.99 g/mol
  Storage at 4 °C

A 100 µM stock solution in MeOH : H_2O is prepared, aliquoted and stored at -20 °C for X days (see *Table 29*).

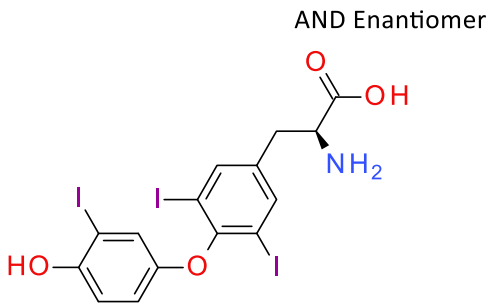
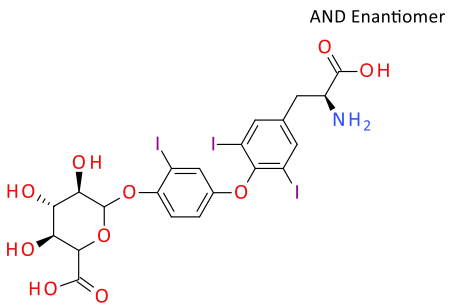
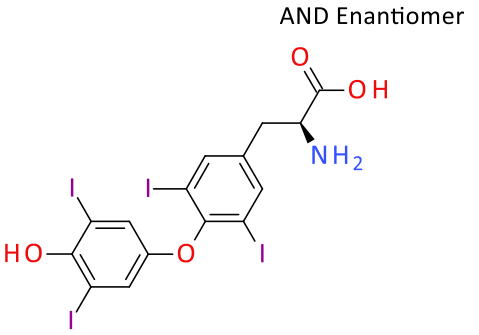
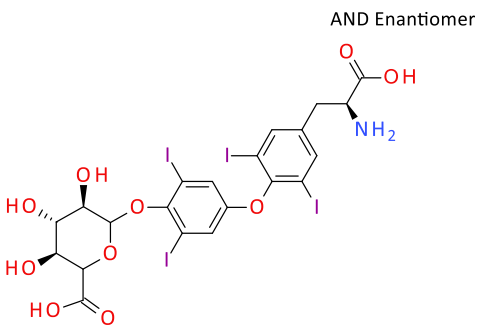
Probe substrate	Metabolite
	
Triiodo-L-thyronine (T3)	Triiodo-L-thyronine 4'-O-β-D-glucuronide (T3G)
	
Thyroxine (T4)	Thyroxine 4'-O-β-D-glucuronide (T4G)

Table 2: UGT Substrates and Metabolites structure

2.3.1.4. 4-Methylumbelliferone stock solutions

- *4-Methylumbelliferone*
  e.g. Merck M1381, CAS 90-33-5
  MW 176.17 g/mol
  Storage RT
  A 16 mM stock solution in DMSO is prepared, aliquoted and stored at 4 °C for X days (see *Table 29*).

2.3.2. Solvents and solutions

- Acetonitrile (HPLC gradient grade)
- DMSO (analytical grade)
- Acetic Acid (purum ≥ 98%)

- NaOH (1.0 N)
- Water (LC-MS Grade)
- Methanol (HPLC gradient grade)

2.3.2.1. Stop solvent and Internal standard (ISTD)

The stop solvent used is acetonitrile (ACN) and it is used to stop the biotransformation activity. The internal standard (Fexofenadine) is used to normalize the data and minimize variance in the analytical assay by compensating for differences in recovery, liquid handling, sample evaporation, ionization efficiency. The Internal Standard is diluted in the stop solvent.

Fexofenadine is used as internal standard:

Fexofenadine hydrochloride, >98% (HPLC)
e.g. Sigma, F9427, CAS 153439-40-8
MW 538.12 g/mol
Storage -20 °C
A 1 mM stock solution is prepared in DMSO and stored at -20 °C. This stock solution can be used for X days (see *Table 29*).

e.g. 50 µL of internal standard stock solution 1 mM is diluted with 450 µL ACN to give a 100 µM working solution (V1). 200 µL working solution (V1) is diluted in 20 mL of ACN to give a 1 µM working solution (V2). This solution is stored at 4° C and used for X days (see *Table 29*).

2.3.2.2. Stop Solution

Stop solution is used to block the glucuronidation reactions. The solution is obtained using the same volume of stop solvent working solution with internal standard V2, see paragraph *2.3.2.1*) with 1% acetic acid in water (1:1, v/v).

Stop solution and 1% acetic acid in water must be freshly prepared on the day of the experiment and keeping at +4 °C until use.

2.3.3. Media and Additives

Please note that media as well as media supplements are available from different suppliers. Powders can be used, but fresh solutions should be used.

The expiration dates of the solutions are indicated in the CoA. Once opened, follow the guidance below.

- *Trizma HCl solution (1 M pH 7.4)*
  e.g. Merck, T2194
  Store at room temperature. To avoid bacterial contamination, store aliquots of bottle solution in a refrigerator at 4 °C once opened.
- *Alamethicin from Trichoderma viride >97%*
  e.g. Merck, A4665, CAS 27061-78-5
  Store at 4 °C. At first use, dissolve in DMSO at 50 mg/mL and split in aliquots. The solution is stable at -20 °C for up to 6 months (frequent freeze and thawing may lead to decrease in activity).
- *Magnesium Chloride Solution (1M), >97%*
  e.g. Merck, M1028, CAS 7786-30-3
  Store at RT. To avoid bacterial contamination, tore aliquots of bottle solution in a refrigerator at 4 °C once opened.

2.3.3.1. Incubation medium preparation

The incubation medium consists of Trizma HCl solution (100 mM), Alamethicin from Trichoderma viride (25 µg/mL) and Magnesium Chloride Solution (2 mM) diluted in ultrapure water. The solutions of the media supplements should be prepared and diluted in water except for Alamethicin which must be dissolved in DMSO at 50 mg/mL to keep the solvent percentage in the incubation within the acceptance range. The solution of Alamethicin must be vortexed and sonicated for 5 minutes upon first use and after each thawing of aliquots.

The media supplements volumes and final concentrations are summarized in the following table. The incubation medium should be freshly prepared on the day of the experiment.

Item	Stock Solution Concentration	Working Solution Concentration	Dilution Factor	Volume
Tris-HCl	1000 mM	100 mM	1:10	10 mL
$MgCl_2$	1 M	2 mM	1:500	0.2 mL
Alamethicin	50 mg/mL	25 µg/mL	1:1000	0.1 mL
Water	N/A	N/A	N/A	90 mL

Table 3: Summary of incubation medium composition

2.3.3.2. Cofactor solution (UDPGA)

The UGTs are involved in the transfer to the substrate of a molecule of glucuronic acid from cofactor uridine-5′-diphospho-α-D-glucuronic acid (UDPGA). The reaction can occur only in the presence of the cofactor.

- *Uridine 5'-diphospho-glucuronic acid trisodium salt (UDPGA)*
  e.g. Merck U6751, CAS 63700-19-6
  MW 646.23 g/mol
  Store at -20 °C.

The solution must be freshly prepared on the day of the experiment and kept at 4 °C; just before the experiment the solution is pre-warmed to 37 °C. UDPGA solution is prepared in water at 100 mM. Initial weight and working solution preparation must be recorded.

2.3.4. Test system

A human liver microsomes pool of at least 50 mixed sex donors (e.g. BioIVT, X008070, or equivalent) and at least 20 mg/mL must be used.

The test system should be delivered with the appropriate documentation (e.g. certificate of analysis). The certificate of analysis of each batch should be shipped with the vials. This document should indicate the protein concentration, the rate of a glucuronide formation (e.g. 7-hydroxycoumarin glucuronide formation or another known specific metabolite of UGT) and the donors' demographic characterization. If any of the above information is missing, it should be requested to the supplier.

The rate of glucuronide formation must be > 900 pmol/min/mg (see UGT1 or UGT1A1 isoform value, if specified).

If the packaging of the test system is intact, the vials should be stored as suggested by the analytical document or as described in the next paragraphs. All documentation relative to the test system should be registered in an appropriate study file.

Upon receipt, technical specifications provided by the supplier for storage should be followed. If not indicated, immediately upon on receipt, the cryovials should be placed in a freezer at 80 ± 10 °C. The temperature should be monitored.

Before using a new batch of microsomes it is necessary to evaluate the UGTs activity of that batch with the "MU fluorescence assay" (see paragraph *2.5*).

2.3.4.1. Test System: Thawing microsomes and dilution

1. Remove the microsomes cryovial from the 80 ± 10 °C.
2. Briefly twist the cap a quarter turn to release the internal pressure and then close it again.
3. Quickly transfer the cryovial to the water bath at +37 °C. Do not submerge it completely, being careful not to allow water to penetrate into the cap. While holding the tip of the cryovial, gently agitate the vial for 1 to 2 minutes.
4. Gently homogenize the microsomes by manual swirling (no vortex).
5. Dilute microsomes to 20 mg/mL with Trizma in a vial kept in ice until it is transferred into the incubation plate.

The microsome vial must be kept on ice. Immediately after use, it can be returned to -80 °C and used again. Only one freeze/thawed cycle can be done. This procedure must be registered.

2.4. Preparation of working solution by dilution stock solutions

2.4.1. General requirements

Working solutions are prepared by dilution of stock solutions in order to have a low percentage of final solvent (0.5 % of DMSO or Methanol). Indeed, in the inhibition plate the percentage of solvent must be (0.6 % of solvent). Table 4 summarized the final concentration of organic solvent in the incubation plate.

Compound	Percentage of organic solvent in the incubation plate
T3 or T4 WS	0.05% DMSO
Alamethicin WS	0.05% DMSO
Test item or PC/NC WS	0.5% DMSO or MeOH
Final	0.6% Organic Solvent

Table 4: Final concentration of organic solvent in the incubation plate

Vials of working solutions must be labelled according to the facility internal procedures and recorded.

Working solutions described in this paragraph will be used for "MU fluorescence assay" for characterized a new batch of HLM and for the inhibition assay.

Working Solution	"MU fluorescence assay" for new HLM batch	Inhibition assay + "MU fluorescence assay"
Positive and Negative controls WS	X	X
Solvent-Control of PC-NC WS	X	X
Test items WS		X
Solvent-Control of Test Item Controls WS		X
No Solvent-Control WS	X	X
UGT Substrates (T3 or T4) WS		X
Methylumbelliferone WS	X	X
Cofactor Solution WS	X	X

Table 5: Summary of working solutions

2.4.2. Positive and Negative controls WS

Positive and negative controls working solutions must be prepared in triplicate in the appropriate volume starting from a single stock solution for each control; stock solutions are diluted 1:200 in incubation medium, (e.g. 1990 µL incubation medium + 10 µL Stock solutions), to obtain a 1.25-fold concentration with respect to the final inhibition concentration.

Fresh and frozen working solutions must be eventually thawed at room temperature and vortexed, heated gently to 37 °C in a water bath and sonicated in an ultrasonic bath for at least 10 minutes.
Working solutions are stable at -20 °C for X days (see *Table 29*).

Positive and Negative Controls	MW	Stock solution Concentration	Working solution Concentration	Incubation solution Concentration
Mefenamic Acid Positive Control	241.29 g/mol	100 mM 100% v/v DMSO	500 µM 0.5% v/v DMSO	400 µM 0.6% v/v DMSO
Fluconazole Negative Control	306.27 g/mol	100 mM 100% v/v DMSO	500 µM 0.5% v/v DMSO	400 µM 0.6% v/v DMSO

Table 6: Summary of positive and negative controls concentrations

2.4.3. UGT Substrates WS

T3 and T4 stock solutions are diluted in water (e.g. 1980 µL water + 20 µL Stock solutions), to obtain working solutions (0.1 mM) at 10-fold higher concentrations than the intended final substrate concentrations in the incubations (10 µM).
T3 and T4 working solutions must not be prepared in incubation medium to avoid precipitation of substrates.

2.4.4. Test items Working Solutions

Test items working solutions have to be prepared freshly every day in triplicate. They are prepared by diluting the stock solutions 200-fold in incubation medium (e.g. 1990 µL incubation medium + 10 µL Stock solutions), to obtain 1.25-fold concentrations with respect to the final concentration for inhibition.

Test items working solutions must be vortexed, heated gently to 37 °C in a water bath and sonicated in an ultrasonic bath for at least 10 minutes.

The concentrations are chosen on the basis of the solubility assay (see paragraph *2.6*).

2.4.5. Solvent-Control of Positive Negative Controls Working Solutions

The solvent-control of positive and negative working solutions must be prepared in triplicate and consists of 0.5% of DMSO in incubation medium, the same percentage of solvent as the positive/negative controls working solutions (e.g. 1990 mL incubation medium + 10 µL DMSO).
These working solutions should be prepared, stored and thaw at the same time and way of the positive and negative controls working solutions.

2.4.6. Solvent-Control of Test Item Working Solutions

The solvent-control of test item working solutions must be prepared in triplicate and consists of the solvent in incubation medium, at the same percentage of solvent as the test item. Each test item has its relative solvent control working solution.
These working solutions should be prepared at the same time and way of test item working solutions.

2.4.7. No Solvent-Control Working Solutions

The no solvent-control working solution must be prepared in triplicate and consists of only incubation medium.

2.4.8. Methylumbelliferone Working Solutions

Methylumbelliferone working solutions must be prepared in duplicate by 40-fold dilution of the stock solution in incubation medium (400 µM). Initial weight and preparation of stock and working solutions must be recorded.
Working solutions are stable at -20 °C for X days (see *Table 29*).

2.5. Determination of UGTs activity: MU fluorescence assay

2.5.1. General requirements

The assay utilizes a highly fluorescent UGT substrate, 4-methylumbelliferone (MU), that allows determination of UGTs activity by tracking the drop in fluorescence emission as the substrate is converted into its non-fluorescent glucuronide conjugate. UGTs specific activity is calculated by comparing the fluorescence loss versus a control performed in the absence of the required cofactor UDPGA. For the evaluation of modulation of UGTs activity the positive and negative controls (Mefenamic Acid and Fluconazole, respectively) must be included.

The UGTs activity determination with the methylumbelliferone quantification must be performed before using a new batch of microsomes and in parallel with each inhibition assay.

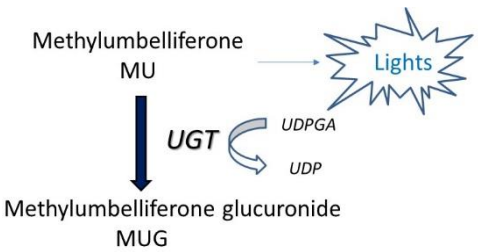


Figure 3. Reaction Mechanism of "MU fluorescence assay"

The UGTs activity with methylumbelliferone fluorescence must be performed to:

- characterize the UGT activity of a new batch of HLM before being used for the inhibition assay (see paragraph 2.5.2).
- in parallel with each inhibition assay (see paragraph 2.7.4).

2.5.2. Assay Procedure for a new batch of microsomes

UGTs activity of a new batch of microsomes must be evaluated before being used in the inhibition study. Two valid runs must be performed and this the batch of microsomes will be used only if the UGT activity exceeds the acceptance criteria (≥ 500 mU/mg, to be confirm in the next experiments with different batches of HLM) (see 3.4, for the elaboration data). If the UGTs activity in two valid runs is lower than 500 mU/mg the microsome batch cannot be used.

Microsomes are incubated in incubation medium with and without UDPGA, in the presence of incubation medium, solvent only (0.5% DMSO in incubation medium) or positive/negative controls. These incubates must be diluted and transferred to a black plate containing the substrate MU to be read by the spectrophotometer in order to reduce HLM concentration and the MU clearance.

This procedure is summarized in the *Table 7* and described in the next paragraphs.

PLATE	Plate Type	INCUBATION			DESCRIPTION
INCUBATION PLATE OF MU	96-well plate flat bottom	- Solvent-control WS - No Solvent-control WS -PC/NC WS	HLM 1 mg/ml	w/ or w/o UDPGA 5 mM	After 15 ± 5 min, incubates are transferred in a reading plate
READING PLATE	96-well plate black with micro-clear and flat ottom	80 µL - Solvent-control WS - No Solvent-control WS -PC/NC WS	20 µL of incubation	20 µL of MU WS	Plate read with the fluorometer

Table 7. Summary of the UGTs activity procedure

2.5.3. UGTs activity assay: Assay set-up

The MU incubation plate is prepared for the UGTs activity evaluation of a new batch of microsomes and in parallel of inhibition assay. An example of an assay set-up is given in *Figure 4*. It is recommended to perform the experiments in triplicate.

The following treatment groups are included in each incubation plate (see *Figure 4*).

- No solvent-control consists of incubation medium, HLM 1 mg/mL with or without UDPGA (columns 9 and 11, respectively). These controls are evaluated to ensure, with the addition of the substrate MU, that the UGTs reactions are taking place.
- Solvent-controls of positive and negative controls consist of all incubation components except for positive/negative controls: incubation medium, HLM 1 mg/mL, 0.5% DMSO with or without UDPGA (column 9 and 11, respectively). These controls are used to control the effect of the solvent on the UGTs reactions.
- A positive and a negative control at one specific concentration with or without UDPGA (columns 10 and 12, respectively).

In the reading plate these solutions are used to evaluate the following items (*Figure 5*):

- The disappearance of MU with or without solvent and positive/negative controls, (columns 5-8, with UDPGA, columns 9-12, without UDPGA)
- The calibration curve of MU in duplicate (column 1-2)
- The quality controls (LQC, MQC and HQC) in duplicate (column 3-4) and the blank control that consists of incubation matrix only. Each solution is analyzed in two wells generally proximal; the mean of the instrumental response is used to generate one result for that sample.

	1	2	3	4	5	6	7	8	9	10	11	12
A	w	w	w	w	w	w	w	w	w	w	w	w
B									Solvent P/NC	PC	Solvent P/NC No UDPGA	PC No UDPGA
C									Solvent P/NC	PC	Solvent P/NC No UDPGA	PC No UDPGA
D									Solvent P/NC	PC	Solvent P/NC No UDPGA	PC No UDPGA
E									No Solvent	NC	No Solvent No UDPGA	NC No UDPGA
F									No Solvent	NC	No Solvent No UDPGA	NC No UDPGA
G									No Solvent	NC	No Solvent No UDPGA	NC No UDPGA
H	w	w	w	w	w	w	w	w	w	w	w	w

Figure 4: Incubation plates for the "MU fluorescence assay"
Abbreviations: Solvent= Solvent-control of Positive and Negative controls; PC = Positive Control; NC = Negative Control; w= water.

	1	2	3	4	5	6	7	8	9	10	11	12
A	STD 1	STD 1	BLK	BLK								
B	STD 2	STD 2	BLK	BLK					Solvent P/NC	PC	Solvent P/NC No UDPGA	PC No UDPGA
C	STD 3	STD 3	LQC	LQC					Solvent P/NC	PC	Solvent P/NC No UDPGA	PC No UDPGA
D	STD 4	STD 4	*LQC*	LQC					Solvent P/NC	PC	Solvent P/NC No UDPGA	PC No UDPGA
E	STD 5	STD 5	*MQC*	MQC					No Solvent	NC	No Solvent No UDPGA	NC No UDPGA
F	STD 6	STD 6	MQC	MQC					No Solvent	NC	No Solvent No UDPGA	NC No UDPGA
G	STD 7	STD 7	*HQC*	HQC					No Solvent	NC	No Solvent No UDPGA	NC No UDPGA
H	STD 8	STD 8	*HQC*	HQC								

Figure 5: Reading Plate for the "MU fluorescence assay"
Abbreviations: Solvent P/NC= Solvent-control of Positive and Negative controls; PC = Positive Control; NC = Negative Control; BLK =control blank; STD =calibration curve.

2.5.4. Procedure: Determination of MU fluorescence assay

Incubation plate of MU

1. Pre-warm the incubation medium in a water bath at 37 °C.
2. Pre-warm the shaking heating plate.
3. Prepare the "positive and negative controls working solutions", "solvent-controls of positive/negative controls working solution" and "no solvent-control working solution" as described in paragraph *2.4*. Warm at 37 °C.
4. Weigh and dissolve UDPGA with water at 100 mM as described in paragraph *2.3.3.2*.
5. Dispense 96 µL of no solvent-control WS, solvent-control positive/negative controls WS, positive/negative controls WS in the 96-well incubation plate (columns 9-10). In columns 11 and 12 dispense 95 µL of incubation medium (see *Figure 4*).
6. Thaw microsomes and dilute to 20 mg/mL as described in paragraph *2.3.4.1*. Dispense 6 µL/well in incubation plate.
7. Dispense 12 µL of no solvent-control WS in each well (columns 9-12)
8. Pre-incubate for 15 ± 5 min to aid pore-forming activity of Alamethicin. Pre-incubation time starts with the addition of the microsomes to the first well. Gently shake.

9. Add 6 µL UDPGA in the columns 9 and 10 using an 8-channel pipette (column by column) and proceed in the other columns quickly. Document starting time (addition of the substrates to the first well).
10. Incubate for 15 minutes at 37 °C.

Reading plate:

11. Transfer 120 µl of the calibration curve solutions, quality controls samples and blank solution (see 3.2.2 and 3.2.3) in columns 1-4 of the reading plate shown in *Figure 5*.
12. Dispense 72 µL of no solvent-control WS, the solvent-control of positive and negative controls WS and the positive and negative controls WS in the "reading plate" as shown in the *Figure 4*. Warm at 37 °C.
13. Dispense 24 µL of methylumbelliferone working solution (400 µM, see paragraph 2.4.8) in columns 9-12 (*Figure 5*). Warm at 37 °C.
14. After 15 ± 3 minutes of incubation, transfer 24 µL of "incubation plate" solutions to the respective wells using an 8-channel pipette to minimize lag time among wells as described in *Table 8.* Document the starting time of MU fluorescence assay (addition of solutions to the first column).

"MU fluorescence assay"	Column of Incubation plate → Columns of reading plate
New batch of HLM characterization	9 → 9, 10 → 10, 11 → 11, 12 → 12

Table 8: Transfer from incubation plate into reading plate

15. Immediately read the plate (see paragraph 3.1).

2.6. Determination of solubility of test items

2.6.1. General requirements

The test item must be dissolved in DMSO, methanol or water at a suitable concentration.

The intended concentration for UGT inhibition depends on the solubility of the test item. The content of solvent should be kept as low as possible. As the concentration of organic solvents must not exceed 0.6% v/v in the incubation plate, the percentage of solvent contribution from the test item working solution must be 0.5% or lower (see *Table 4*). Therefore, the stock solution of the test item in DMSO or methanol has to be at least 250-fold, e.g. for an inhibitor to be tested at a starting concentration of 4 mM, the concentration of the stock solution in pure DMSO, methanol or water has to be 1000 mM. The procedure for solubility testing should be documented.

2.6.2. Preparation of stock solutions

By default, test items are dissolved at 1000 mM 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.

Weigh about 20 mg test item into a screw cap glass vial. Add DMSO according to the following equation to prepare a starting concentration of 1000 mM:

$$Volumesolvent[\mu l] = \frac{initialweight[mg] * \frac{\%purity}{100} * 1000 * 1000}{desiredconcentration[mM] * molecular\ weight\ [\frac{g}{mol}]}$$

1. Vortex-mix or shake for 1 min.
2. Visually inspect the solubilisation of the compound. In case of any undissolved particles, repeat vortex-mixing.
3. Visually inspect the solubilisation of the compound. In case of any undissolved particles, place the tightly closed vial into an ultrasonic bath and apply ultrasonic for 2 min.
4. Visually inspect the solubilisation of the compound. In case of any undissolved particles, vortex-mix for 10 sec and apply ultrasonic for 5 min.
5. 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.
6. Visually inspect the solubilisation of the compound. In case of any undissolved particles, the intended concentration cannot be applied.
7. Continue to add DMSO to obtain a solution of two-fold lower strength and repeat steps 1-10. The concentrations are: (mM) 1000 → 500 → 250 → 125 → 62.5 → 31.25 → 15.62 → 7.81→ 3.90→ 1.95 → 0.975
8. If the test item does not dissolve at any of the highest concentrations (e.g. ≤ 125 mM) it is advisable to evaluate the solubility also in methanol, proceeding by following steps 1-10. Record the soluble concentrations in the evaluated solvents.
9. In case of any undissolved particles at 0.975 mM, the test item cannot be submitted to the inhibition assay (to be evaluated if 0.975 mM is the last concentration to be tested).

2.6.3. 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 inhibition assays by diluting the test item stock solution in incubation medium in a

1:200 ratio for DMSO or methanol as solvent. This pre-test should evaluate the solubility of test item. Record the soluble concentration.

1. Use the test item stock solution at the highest concentration chosen as described above. Add 20 µL of this stock solution in 4 mL of incubation medium (1:200 ratio in case of DMSO and methanol)
2. The resulting incubation solution (highest concentration of intended testing range) is visually inspected for compound precipitation.
3. Vortex-mix or shake for 1 min and visually inspect for solubilisation of the compound. Record the soluble concentration of test item before the incubation.
4. The solution is transferred to 1.5 mL reaction tubes (500 µL, n=3).
5. One additional reaction tube is prepared using incubation medium without test item for comparison.
6. Tubes are incubated at 37 °C for 180 ± 10 min.
7. At the end of the incubation, the reaction tubes are centrifuged (4,400 - 4,700 g, 10 min, RT). The tubes are visually inspected for compound precipitation (solubility after incubation).
8. In case of compound precipitation, steps 1-8 must be repeated using stock solutions of two-fold lower strength.

2.7. Inhibition Assay

2.7.1. General requirements

The inhibition assay must be performed as summarized in *Figure 6*. The inhibition of UGTs activity should be evaluated in an “range finding assay” where the test items are tested at six concentrations in a wide range in order to identify the testing range of “main inhibition assay” where the IC50 value of test items are calculated in 3 valid runs.

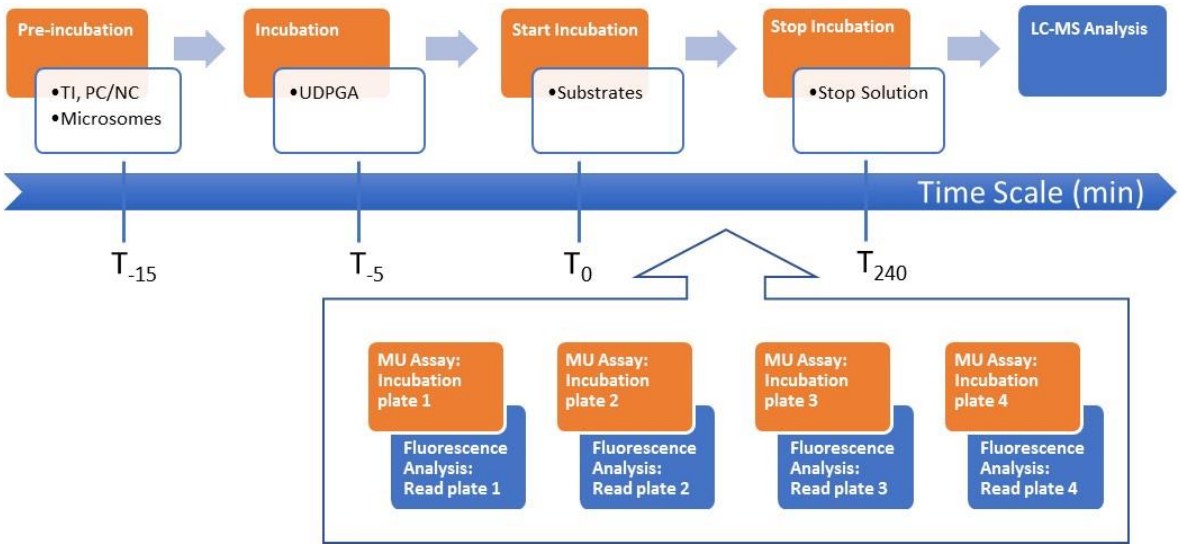
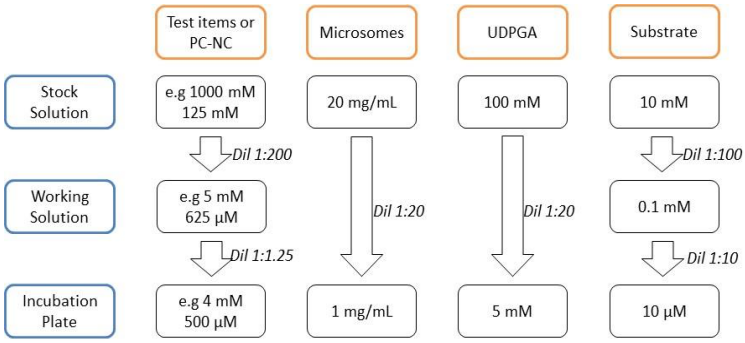


Figure 6: Experimental design of the in vitro test using human liver microsomes.

The incubation assay should be performed in a 96-well plate using the concentrations and the volumes described in *Figure 7*.



Incubation Plate	Dilution	Volume
Test Item, PC, NC or solvent control WS	1:1.25	200 µL
Microsomes	1:20	12.5 µL
UDPGA	1:20	12.5 µL
Substrates	1:10	25 µL
Total Volume		250 µL

Figure 7: Set-up for inhibition assay

2.7.1.1. Range finding assay

The range finding assay should be performed in order to estimate the inhibition behaviour of test items. An estimated IC50 curve should be determined.

Each test item is tested at six concentrations. Stock solutions and working solutions must be prepared freshly every day. The following concentrations represent a generic concentration set:

C1 – C2 – C3 – C4 – C5 – C6 (1:10 dilution)

Test items are dissolved in solvent (DMSO or Methanol) or water at the concentrations chosen in the solubility assay (described in paragraph *2.6*). Depending on the solubility properties of the test items the concentrations in the incubation solutions may vary (see *Figure 8*).

Dil. 1:200 → Dil. 1:1.25 →

Stock solution Concentration (mM)					
C1	C2	C3	C4	C5	C6
1000	200	40	8	1.6	0.32
500	100	20	4	0.8	0.16
250	50	10	2	0.4	0.08
125	25	5	1	0.2	0.04
62.5	12.5	2.5	0.5	0.1	0.02
31.25	6.25	1.25	0.25	0.05	0.01
15.625	3.125	0.625	0.125	0.025	0.005
7.8125	1.5625	0.3125	0.0625	0.0125	0.0025
3.9063	0.7813	0.1563	0.0313	0.0063	0.00125

Working solution Concentration (µM)					
C1	C2	C3	C4	C5	C6
5000	1000	200	40	8	1.6
2500	500	100	20	4	0.8
1250	250	50	10	2	0.4
625	125	25	5	1	0.2
312.5	62.5	12.5	2.5	0.5	0.1
156.25	31.25	6.25	1.25	0.25	0.05
78.125	15.625	3.125	0.625	0.125	0.025
39.063	7.8125	1.5625	0.3125	0.0625	0.0125
19.531	3.9063	0.7813	0.1563	0.0313	0.0063

Incubation solution Concentration (µM)					
C1	C2	C3	C4	C5	C6
4000	800	160	32	6.4	1.28
2000	400	80	16	3.2	0.64
1000	200	40	8	1.6	0.32
500	100	20	4	0.8	0.16
250	50	10	2	0.4	0.08
125	25	5	1	0.2	0.04
62.5	12.5	2.5	0.5	0.1	0.02
31.25	6.25	1.25	0.25	0.05	0.01
15.625	3.125	0.625	0.125	0.025	0.005

Figure 8: Possible concentrations of stock, working and incubation solutions

2.7.1.2. Main Inhibition Assay

The estimated IC50 curve evaluated in the range finding assay should be used to know the inhibition activity of test item; *Table 9* describes how to proceed after the range finding assay.

At the end three valid runs of inhibition assay must be performed.

Main Inhibition Assay		
% activity remaining of TI	**Estimated IC50 curve**	**Description**
≥ 20 %	At least 4 concentrations between top and bottom	The range finding assay is accepted as one valid run. Other 2 valid runs are performed in the same conditions (same 6 concentrations)
≥ 20 %	Less of 4 concentrations between top and bottom	The range finding assay is not accepted. Three main inhibition assays must be performed with C1 near the bottom of the estimated curve, C6 near the top and other concentrations between C1 and C6
< 20 %	It's not evaluable	The range finding assay is accepted as one valid run. Two main inhibition assays must be performed with 3 different concentrations (e.g. C1 – C2 – C3 of range finding assay)

Table 9: Choose concentrations of the main inhibition assay

2.7.2. Set-up of inhibition assay

Inhibition experiments are performed in a 96-well format with flat bottom (incubation plate). The substrates (T3 and T4) are incubated in two different plates. An example of an assay set-up is given in *Figure 9* and *Figure 10*. The experiments have to be performed in triplicate.

Each experiment can include a maximum of 4 test items: test items A and B are tested in incubation plates 1 and 2, where T3 and T4 are respectively incubated, and test items C and D are tested in incubation plates 3 and 4, under the same conditions. Test items must be tested at six concentrations.

For each study, the positive and negative controls (Mefenamic Acid and Fluconazole) are tested in the same conditions as for the test items. Positive and negative controls are included in every run and in every plate. Microsomes are exposed to the controls at one specific concentration for the incubation time in parallel to the exposure to the test items.

The reactions are stopped in two 384-well plates with cone bottom, called "analytical plate" and "backup plate" containing the stop solution in a ratio 1:1 with respect to the incubates' volume. Following centrifugation, the "analytical plate" is immediately analysed, whereas the "backup plate" is immediately frozen.

During analysis it is important that the withdrawal of the samples does not take place at the bottom of the well in order to avoid pellets. Eventually, after centrifugation particle-free supernatants are transferred into a 384-well plate keeping the samples in the same positions (pay attention to cross-contamination). The layouts of analytical and backup plate are shown in *Table 10*.

Plate	Volume
Analytical plate	e.g. 80 µL incubation plate + 80 µL stop solution. Centrifuge. (Optional: transfer samples in a new 384 well plate). Analyse immediately.
Backup plate	e.g. 80 µL incubation plate + 80 µL stop solution. Centrifuge. Freeze immediately. Before analysis: keep plate at least 2 hours at RT. Centrifuge. (Optional: transfer samples in a new 384 well plate). Analyse.

Table 10: Transfer to 384-well plates: examples of volumes

All plates should be labelled according to the facility internal procedures.

The following treatment groups are included on each incubation plate and represented in *Figure 9* and *Figure 10*:

- "Blank with test item" consisting of human liver microsomes, incubation medium, substrates (T3 or T4) and the test item at the highest concentration (UDPGA cofactor is

not included). These controls are evaluated to ensure that the test item in the system does not cause analytical interferences (column 1).

- Test items at six different concentrations (columns 2-7, where C1 is the highest concentration).
- Solvent-controls of test items consisting of all incubation components and the solvent percentage of the test item. Each test item control has its own solvent-treated control containing the solvent used to solubilise it (column 8).
- Solvent-control of positive/negative controls consisting of all incubation components and 0.5% DMSO (column 9).
- No solvent-control consisting of incubation medium, HLM 1 mg/mL and UDPGA. This control is used to check the effect of the solvent on the enzymatic reaction (columns 9).
- A positive and a negative control (PC/NC) at one specific concentration (column 10)

Columns 9-12 contain the treatment groups which are used for the methylumbelliferone assay (see chapter 2.5).

Two wells of rows A and H are filled with test item A and B WS, respectively, at high concentration (C1) and UDPGA (5 mM) in order to check the lack of precipitate at the end of the incubation. The other wells are filled with water and are not used for analysis.

	1	2	3	4	5	6	7	8	9	10	11	12
A	TI_A C1 no HLM	TI_A C1 no HLM	w	w	w	w	w	w	w	w	w	w
B	*TI_A C1* No UDPGA T3	TI_A C1 T3	TI_A C2 T3	TI_A C3 T3	TI_A C4 T3	TI_A C5 T3	TI_A C6 T3	Solvent TI_A T3	Solvent P/NC T3	PC T3	Solvent P/NC No UDPGA T3	PC No UDPGA T3
C	*TI_A C1* No UDPGA T3	TI_A C1 T3	TI_A C2 T3	TI_A C3 T3	TI_A C4 T3	TI_A C5 T3	TI_A C6 T3	Solvent TI_A T3	Solvent P/NC T3	PC T3	Solvent P/NC No UDPGA T3	PC No UDPGA T3
D	*TI_A C1* No UDPGA T3	TI_A C1 T3	TI_A C2 T3	TI_A C3 T3	TI_A C4 T3	TI_A C5 T3	TI_A C6 T3	Solvent TI_A T3	Solvent P/NC T3	PC T3	Solvent P/NC No UDPGA T3	No UDPGA T3
E	*TI_B C1* No UDPGA T3	TI_B C1 T3	TI_B C2 T3	TI_B C3 T3	TI_B C4 T3	TI_B C5 T3	TI_B C6 T3	Solvent TI_B T3	No Solvent T3	NC T3	No Solvent No UDPGA T3	NC No UDPGA T3
F	*TI_B C1* No UDPGA T3	TI_B C1 T3	TI_B C2 T3	TI_B C3 T3	TI_B C4 T3	TI_B C5 T3	TI_B C6 T3	Solvent TI_B T3	No Solvent T3	NC T3	No Solvent No UDPGA T3	NC No UDPGA T3
G	*TI_B C1* No UDPGA T3	TI_B C1 T3	TI_B C2 T3	TI_B C3 T3	TI_B C4 T3	TI_B C5 T3	TI_B C6 T3	Solvent TI_B T3	No Solvent T3	NC T3	No Solvent No UDPGA T3	NC No UDPGA T3
H	TI_B C1 no HLM	TI_B C1 no HLM	w	w	w	w	w	w	w	w	w	w

Figure 9: Incubation Plate 1 layout (for Test Items A and B) or Incubation 3 layout

For Incubation plate 3: Test Item C is in the place of Test Item A and Test item D is in the place of Test Item B.
Abbreviations: TI A = Test Item A; TI B = Test Item B; C1-C6= Concentrations; Solvent = Solvent control; PC = Positive Control; NC = Negative Control; P/NC = Positive and Negative Controls; w = water

	1	2	3	4	5	6	7	8	9	10	11	12
A	TI_A C1 no HLM	TI_A C1 no HLM	w	w	w	w	w	w	w	w	w	w
B	*TI_A C1* No UDPGA T4	TI_A C1 T4	TI_A C2 T4	TI_A C3 T4	TI_A C4 T4	TI_A C5 T4	TI_A C6 T4	Solvent TI_A T4	Solvent P/NC T4	PC T4	Solvent P/NC No UDPGA T4	PC No UDPGA T4
C	*TI_A C1* No UDPGA T4	TI_A C1 T4	TI_A C2 T4	TI_A C3 T4	TI_A C4 T4	TI_A C5 T4	TI_A C6 T4	Solvent TI_A T4	Solvent P/NC T4	PC T4	Solvent P/NC No UDPGA T4	PC No UDPGA T4
D	*TI_A C1* No UDPGA T4	TI_A C1 T4	TI_A C2 T4	TI_A C3 T4	TI_A C4 T4	TI_A C5 T4	TI_A C6 T4	Solvent TI_A T4	Solvent P/NC T4	PC T4	Solvent P/NC No UDPGA T4	No UDPGA T4
E	*TI_B C1* No UDPGA T4	TI_B C1 T4	TI_B C2 T4	TI_B C3 T4	TI_B C4 T4	TI_B C5 T4	TI_B C6 T4	Solvent TI_B T4	No Solvent T4	NC T4	No Solvent No UDPGA T4	NC No UDPGA T4
F	*TI_B C1* No UDPGA T4	TI_B C1 T4	TI_B C2 T4	TI_B C3 T4	TI_B C4 T4	TI_B C5 T4	TI_B C6 T4	Solvent TI_B T4	No Solvent T4	NC T4	No Solvent No UDPGA T4	NC No UDPGA T4
G	*TI_B C1* No UDPGA T4	TI_B C1 T4	TI_B C2 T4	TI_B C3 T4	TI_B C4 T4	TI_B C5 T4	TI_B C6 T4	Solvent TI_B T4	No Solvent T4	NC T4	No Solvent No UDPGA T4	NC No UDPGA T4
H	TI_B C1 no HLM	TI_B C1 no HLM	w	w	w	w	w	w	w	w	w	w

Figure 10: Incubation Plate 2 layout (for Test Items A and B) or Incubation 4 layout

For Incubation plate 3: Test Item C is in the place of Test Item A and Test item D is in the place of Test Item B
Abbreviations: TI A = Test Item A; TI B = Test Item B; C1-C6= Concentrations; Solvent = Solvent control; PC = Positive Control; NC = Negative Control; P/NC = Positive and Negative Controls; w = water

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w
B	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w
C	w	TI A - C1 T3 no UDPGA	TI A - C1 T3	TI A - C2 T3	TI A – C3 T3	TI A – C4 T3	TI A – C5 T3	TI A – C6 T3	Solvent TI A T3	Solvent P/NC T3	PC T3		TI A - C1 T4 no UDPGA	TI A - C1 T4	TI A - C2 T4	TI A – C3 T4	TI A – C4 T4	TI A – C5 T4	TI A – C6 T4	Solvent TI A T4	Solvent P/NC T4	PC T4	w	w
D	w																						w	w
E	w	TI A - C1 T3 no UDPGA	TI A - C1 T3	TI A - C2 T3	TI A – C3 T3	TI A – C4 T3	TI A – C5 T3	TI A – C6 T3	Solvent TI A T3	Solvent P/NC T3	PC T3		TI A - C1 T4 no UDPGA	TI A - C1 T4	TI A - C2 T4	TI A – C3 T4	TI A – C4 T4	TI A – C5 T4	TI A – C6 T4	Solvent TI A T4	Solvent P/NC T4	PC T4	w	w
F	w																						w	w
G	w	TI A - C1 T3 no UDPGA	TI A - C1 T3	TI A - C2 T3	TI A – C3 T3	TI A – C4 T3	TI A – C5 T3	TI A – C6 T3	Solvent TI A T3	Solvent P/NC T3	PC T3		TI A - C1 T4 no UDPGA	TI A - C1 T4	TI A - C2 T4	TI A – C3 T4	TI A – C4 T4	TI A – C5 T4	TI A – C6 T4	Solvent TI A T4	Solvent P/NC T4	PC T4	w	w
H	w																						w	w
I	w	TI B - C1 T3 no UDPGA	TI B - C1 T3	TI B - C2 T3	TI B – C3 T3	TI B – C4 T3	TI B – C5 T3	TI B – C6 T3	Solvent TI B T3	No Solvent T3	NC T3		TI B - C1 T4 no UDPGA	TI B - C1 T4	TI B - C2 T4	TI B – C3 T4	TI B – C4 T4	TI B – C5 T4	TI B – C6 T4	Solvent TI B T4	No Solvent T4	NC T4	w	w
J	w																						w	w
K	w	TI B - C1 T3 no UDPGA	TI B - C1 T3	TI B - C2 T3	TI B – C3 T3	TI B – C4 T3	TI B – C5 T3	TI B – C6 T3	Solvent TI B T3	No Solvent T3	NC T3		TI B - C1 T4 no UDPGA	TI B - C1 T4	TI B - C2 T4	TI B – C3 T4	TI B – C4 T4	TI B – C5 T4	TI B – C6 T4	Solvent TI B T4	No Solvent T4	NC T4	w	w
L	w																						w	w
M	w	TI B - C1 T3 no UDPGA	TI B - C1 T3	TI B - C2 T3	TI B – C3 T3	TI B – C4 T3	TI B – C5 T3	TI B – C6 T3	Solvent TI B T3	No Solvent T3	NC T3		TI B - C1 T4 no UDPGA	TI B - C1 T4	TI B - C2 T4	TI B – C3 T4	TI B – C4 T4	TI B – C5 T4	TI B – C6 T4	Solvent TI B T4	No Solvent T4	NC T4	w	w
N	w																						w	w
O	w	wash	wash	wash	wash	wash	wash	wash	wash	wash	wash	wash	wash	wash	wash	wash	wash	wash	wash	wash	wash	wash	w	w
P	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w

Figure 11: Example layout of analysis plate or backup plate (for 2 test items A and B)

Abbreviations: TI A = Test Item A; TI B = Test Item B; C1-C6= Concentrations; Solvent = Solvent-control; No Solvent = No Solvent-control PC = Positive Control; NC = Negative Control; P/NC = Positive and Negative Controls; w = water.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w
B	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w
C	w	TI A - C1 T3 no UDPGA	TI A - C1 T3	TI A - C2 T3	TI A – C3 T3	TI A – C4 T3	TI A – C5 T3	TI A – C6 T3	Solvent TI A T3	Solvent P/NC T3	PC T3		TI A - C1 T4 no UDPGA	TI A - C1 T4	TI A - C2 T4	TI A – C3 T4	TI A – C4 T4	TI A – C5 T4	TI A – C6 T4	Solvent TI A T4	Solvent P/NC T4	PC T4	w	w
D	w	TI C - C1 T3 no UDPGA	TI C - C1 T3	TI C - C2 T3	TI C – C3 T3	TI C – C4 T3	TI C – C5 T3	TI C – C6 T3	Solvent TI C T3	Solvent P/NC T3	PC T3		TI C - C1 T4 no UDPGA	TI C - C1 T4	TI C - C2 T4	TI C – C3 T4	TI C – C4 T4	TI C – C5 T4	TI C – C6 T4	Solvent TI C T4	Solvent P/NC T4	PC T4	w	w
E	w	TI A - C1 T3 no UDPGA	TI A - C1 T3	TI A - C2 T3	TI A – C3 T3	TI A – C4 T3	TI A – C5 T3	TI A – C6 T3	Solvent TI A T3	Solvent P/NC T3	PC T3		TI A - C1 T4 no UDPGA	TI A - C1 T4	TI A - C2 T4	TI A – C3 T4	TI A – C4 T4	TI A – C5 T4	TI A – C6 T4	Solvent TI A T4	Solvent P/NC T4	PC T4	w	w
F	w	TI C - C1 T3 no UDPGA	TI C - C1 T3	TI C - C2 T3	TI C – C3 T3	TI C – C4 T3	TI C – C5 T3	TI C – C6 T3	Solvent TI C T3	Solvent P/NC T3	PC T3		TI C - C1 T4 no UDPGA	TI C - C1 T4	TI C - C2 T4	TI C – C3 T4	TI C – C4 T4	TI C – C5 T4	TI C – C6 T4	Solvent TI C T4	Solvent P/NC T4	PC T4	w	w
G	w	TI A - C1 T3 no UDPGA	TI A - C1 T3	TI A - C2 T3	TI A – C3 T3	TI A – C4 T3	TI A – C5 T3	TI A – C6 T3	Solvent TI A T3	Solvent P/NC T3	PC T3		TI A - C1 T4 no UDPGA	TI A - C1 T4	TI A - C2 T4	TI A – C3 T4	TI A – C4 T4	TI A – C5 T4	TI A – C6 T4	Solvent TI A T4	Solvent P/NC T4	PC T4	w	w
H	w	TI C - C1 T3 no UDPGA	TI C - C1 T3	TI C - C2 T3	TI C – C3 T3	TI C – C4 T3	TI C – C5 T3	TI C – C6 T3	Solvent TI C T3	Solvent P/NC T3	PC T3		TI C - C1 T4 no UDPGA	TI C - C1 T4	TI C - C2 T4	TI C – C3 T4	TI C – C4 T4	TI C – C5 T4	TI C – C6 T4	Solvent TI C T4	Solvent P/NC T4	PC T4	w	w
I	w	TI B - C1 T3 no UDPGA	TI B - C1 T3	TI B - C2 T3	TI B – C3 T3	TI B – C4 T3	TI B – C5 T3	TI B – C6 T3	Solvent TI B T3	No Solvent T3	NC T3		TI B - C1 T4 no UDPGA	TI B - C1 T4	TI B - C2 T4	TI B – C3 T4	TI B – C4 T4	TI B – C5 T4	TI B – C6 T4	Solvent TI B T4	No Solvent T4	NC T4	w	w
J	w	TI D - C1 T3 no UDPGA	TI D - C1 T3	TI D - C2 T3	TI D – C3 T3	TI D – C4 T3	TI D – C5 T3	TI D – C6 T3	Solvent TI D T3	Solvent P/NC T3	PC T3		TI D - C1 T4 no UDPGA	TI D - C1 T4	TI D - C2 T4	TI D – C3 T4	TI D – C4 T4	TI D – C5 T4	TI D – C6 T4	Solvent TI D T4	Solvent P/NC T4	PC T4	w	w
K	w	TI B - C1 T3 no UDPGA	TI B - C1 T3	TI B - C2 T3	TI B – C3 T3	TI B – C4 T3	TI B – C5 T3	TI B – C6 T3	Solvent TI B T3	No Solvent T3	NC T3		TI B - C1 T4 no UDPGA	TI B - C1 T4	TI B - C2 T4	TI B – C3 T4	TI B – C4 T4	TI B – C5 T4	TI B – C6 T4	Solvent TI B T4	No Solvent T4	NC T4	w	w
L	w	TI D - C1 T3 no UDPGA	TI D - C1 T3	TI D - C2 T3	TI D – C3 T3	TI D – C4 T3	TI D – C5 T3	TI D – C6 T3	Solvent TI D T3	Solvent P/NC T3	PC T3		TI D - C1 T4 no UDPGA	TI D - C1 T4	TI D - C2 T4	TI D – C3 T4	TI D – C4 T4	TI D – C5 T4	TI D – C6 T4	Solvent TI D T4	Solvent P/NC T4	PC T4	w	w
M	w	TI B - C1 T3 no UDPGA	TI B - C1 T3	TI B - C2 T3	TI B – C3 T3	TI B – C4 T3	TI B – C5 T3	TI B – C6 T3	Solvent TI B T3	No Solvent T3	NC T3		TI B - C1 T4 no UDPGA	TI B - C1 T4	TI B - C2 T4	TI B – C3 T4	TI B – C4 T4	TI B – C5 T4	TI B – C6 T4	Solvent TI B T4	No Solvent T4	NC T4	w	w
N	w	TI D - C1 T3 no UDPGA	TI D - C1 T3	TI D - C2 T3	TI D – C3 T3	TI D – C4 T3	TI D – C5 T3	TI D – C6 T3	Solvent TI D T3	Solvent P/NC T3	PC T3		TI D - C1 T4 no UDPGA	TI D - C1 T4	TI D - C2 T4	TI D – C3 T4	TI D – C4 T4	TI D – C5 T4	TI D – C6 T4	Solvent TI D T4	Solvent P/NC T4	PC T4	w	w
O	w	wash	wash	wash	wash	wash	wash	wash	wash	wash	wash	wash	wash	wash	wash	wash	wash	wash	wash	wash	wash	wash	w	w
P	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w

Figure 12: Example layout of analysis plate or backup plate (for 4 test items A, B, C and D)

Abbreviations: TI A = Test Item A; TI B = Test Item B; TI C = Test Item C; TI D = Test Item D; C1-C6= Concentrations; Solvent = Solvent-control; No Solvent = No Solvent-control PC = Positive Control; NC = Negative Control; P/NC = Positive and Negative Controls; w = water.

2.7.3. Inhibition enzyme activity assay: Assay Procedure

T3 and T4 glucuronide formation is determined after 180 ± 10 minutes exposure to the microsomes with or without inhibitors. At the end of the incubation time, the reaction is quenched by the addition of a stop solution and the samples are analysed for the specific metabolites shown in *Table 1*.

1. Pre-warm the incubation medium in a water bath at +37 °C.
2. Pre-warm the shaking heating plate.
3. Prepare the test items at six concentrations, positive/negative controls, solvent-control of test items and positive/negative controls, no solvent-control and substrate working solutions by diluting the stock solutions as described in paragraph *2.4*. Warm at +37 °C.
4. Weigh and dissolve the UDPGA with incubation medium as described in paragraph *2.3.3.2*.

   Dispense 200 µL of the working solutions as described in *Table 11*. See *Figure 9* and *Figure 10* for the plates' positions.

Working solution	Conc.	Column n°
Test Item WS	C1	1 and 2
	C2	3
	C3	4
	C4	5
	C5	6
	C6	7

Working solution	Column n°
Solvent Control TI	8
Solvent Control PC/NC WS	9 and 11
No-Solvent Control WS	9 and 11
PC WS	10 and 12
NC WS	10 and 12

Table 11: summary of working solution position in incubation plates

5. Thaw microsomes and dilute to 20 mg/mL as described in paragraph 2.3.4.1. Dispense 12.5 µL/well in the incubation plates in rows B-C-D-E-F-G.
6. Pre-incubate for 15 ± 5 min to aid pore-forming activity of Alamethicin (pre-incubation time starts with the addition of the microsomes to the first well). Gently shake.
7. After 10 minutes, add 12.5 µL of UDPGA in each well of the incubation plates except for column 1, 11 and 12. Gently shake.
8. At the end of the pre-incubation, add 25 µL of substrates working solutions to the respective wells using an 8-channel pipette or liquid handling robot (column by column): dispense T3 working solution into plates 1 and 3 and T4 working solution into plates 2 and 4. Document starting time of inhibition assay (addition of the substrates to the first column). Perform this step for all rows in timed intervals (e.g. start every row after 20 or 30 sec).
9. Incubate at 37 °C under shaking. All incubation plates should be covered with a lid to avoid evaporation.

10. During the incubation the "determination UGTs activity assay" must be performed. See paragraph 2.7.4.

10. Prepare the stop solution (see paragraph 2.1.4.1) and dispense 80 µL/well in the 384-well plates (analytical and backup plates). Place plate on ice.

11. After 180 ± 10 minutes, centrifuge the incubation plates for 1 min at ~1000 x g at room temperature, immediately put them back in the shaking heating and remove the lid. Transfer 80 µL of the incubate solution from the incubation plates into the analytical and backup plates, correspondingly labelled: transfer the incubation plate 1 or 3 into columns 2 to 11 of the 384-well plates, containing T3 incubations and the incubation plate 2 or 4 into columns 13 to 22, containing T4 incubations (see *Figure 11* and *Figure 12*). The transfer of the samples from the two 96-well incubation plates to the 384-well plates follows the following scheme:

Incubation Plates 1 and 2 96-Well Plates	Analytical and Backup Plates 384-Well Plates
Row B	Row C
Row C	Row E
Row D	Row G
Row E	Row I
Row F	Row K
Row G	Row M

Incubation Plates 3 and 4 96-Well Plates	Analytical and Backup Plates 384-Well Plates
Row B	Row D
Row C	Row F
Row D	Row H
Row E	Row J
Row F	Row L
Row G	Row N

Table 12: Transfer into 384-well plates

The positions of the samples must remain unchanged in the analysis and backup plates.

12. The 384-well plates are subsequently centrifuged (15 min at ≥ 2,200 g).

13. The analytical plate is analysed, and the backup plate is immediately frozen at – 20 °C.

2.7.4. UGTs activity assay in parallel with inhibition assay

The MU incubation plate in parallel to the inhibition assay in order to confirm the good outcome of the UGT reactions in all incubation plates. The treatment groups evaluated are in columns 8-12 of the incubation plates (*Figure 9* and *Figure 10*) as described in paragraph

2.5.3 and consist of the no solvent-control, the solvent-controls of positive and negative controls and the positive and a negative controls.

In the reading plate, the solutions are analogous to the one described in paragraph 2.5.3:

- The disappearance of MU with or without solvent and positive/negative controls, (columns 5-8, incubation plate 1 or 2 solutioons, columns 9-12, incubation plate 3 or 4 solutions)
- The calibration curve of MU in duplicate (columns 1-2)
- The quality controls (LQC, MQC and HQC) in duplicate (columns 3-4) and the blank control that consists of incubation matrix only. Each solution is analyzed in two wells generally proximal; the mean of the instrumental response is used to generate one result for that sample.

	1	2	3	4	5	6	7	8	9	10	11	12
A	STD 1	STD 1	BLK	BLK								
B	STD 2	STD 2	BLK	BLK	Solvent P/NC Inc Plate 1	PC Inc Plate 1	Solvent P/NC No UDPGA Inc Plate 1	PC No UDPGA Inc Plate 1	Solvent P/NC Inc Plate 2	PC Inc Plate 2	Solvent P/NC No UDPGA Inc Plate 2	PC No UDPGA Inc Plate 2
C	STD 3	STD 3	LQC	LQC	Solvent P/NC Inc Plate 1	PC Inc Plate 1	Solvent P/NC No UDPGA Inc Plate 1	PC No UDPGA Inc Plate 1	Solvent P/NC Inc Plate 2	PC Inc Plate 2	Solvent P/NC No UDPGA Inc Plate 2	PC No UDPGA Inc Plate 2
D	STD 4	STD 4	*LQC*	LQC	Solvent P/NC Inc Plate 1	PC Inc Plate 1	Solvent P/NC No UDPGA Inc Plate 1	PC No UDPGA Inc Plate 1	Solvent P/NC Inc Plate 2	PC Inc Plate 2	Solvent P/NC No UDPGA Inc Plate 2	PC No UDPGA Inc Plate 2
E	STD 5	STD 5	*MQC*	MQC	No Solvent Inc Plate 1	NC Inc Plate 1	No Solvent No UDPGA Inc Plate 1	NC No UDPGA Inc Plate 1	No Solvent Inc Plate 2	NC Inc Plate 2	No Solvent No UDPGA Inc Plate 2	NC No UDPGA Inc Plate 2
F	STD 6	STD 6	MQC	MQC	No Solvent Inc Plate 1	NC Inc Plate 1	No Solvent No UDPGA Inc Plate 1	NC No UDPGA Inc Plate 1	No Solvent Inc Plate 2	NC Inc Plate 2	No Solvent No UDPGA Inc Plate 2	NC No UDPGA Inc Plate 2
G	STD 7	STD 7	*HQC*	HQC	No Solvent Inc Plate 1	NC Inc Plate 1	No Solvent No UDPGA Inc Plate 1	NC No UDPGA Inc Plate 1	No Solvent Inc Plate 2	NC Inc Plate 2	No Solvent No UDPGA Inc Plate 2	NC No UDPGA Inc Plate 2
H	STD 8	STD 8	*HQC*	HQC								

Figure 13: Reading Plate 1 for the "MU fluorescence assay" in parallel with inhibition assay
Abbreviations: Solvent P/NC= Solvent-control of Positive and Negative controls; PC = Positive Control; NC = Negative Control; BLK =control blank; STD =calibration curve; Inc Plate = Incubation Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	STD 1	STD 1	BLK	BLK								
B	STD 2	STD 2	BLK	BLK	Solvent P/NC Inc Plate 3	PC Inc Plate 3	Solvent P/NC No UDPGA Inc Plate 3	PC No UDPGA Inc Plate 3	Solvent P/NC Inc Plate 4	PC Inc Plate 4	Solvent P/NC No UDPGA Inc Plate 4	PC No UDPGA Inc Plate 4
C	STD 3	STD 3	LQC	LQC	Solvent P/NC Inc Plate 3	PC Inc Plate 3	Solvent P/NC No UDPGA Inc Plate 3	PC No UDPGA Inc Plate 3	Solvent P/NC Inc Plate 4	PC Inc Plate 4	Solvent P/NC No UDPGA Inc Plate 4	PC No UDPGA Inc Plate 4
D	STD 4	STD 4	*LQC*	LQC	Solvent P/NC Inc Plate 3	PC Inc Plate 3	Solvent P/NC No UDPGA Inc Plate 3	PC No UDPGA Inc Plate 3	Solvent P/NC Inc Plate 4	PC Inc Plate 4	Solvent P/NC No UDPGA Inc Plate 4	PC No UDPGA Inc Plate 4
E	STD 5	STD 5	*MQC*	MQC	No Solvent Inc Plate 3	NC Inc Plate 3	No Solvent No UDPGA Inc Plate 3	NC No UDPGA Inc Plate 3	No Solvent Inc Plate 4	NC Inc Plate 4	No Solvent No UDPGA Inc Plate 4	NC No UDPGA Inc Plate 4
F	STD 6	STD 6	MQC	MQC	No Solvent Inc Plate 3	NC Inc Plate 3	No Solvent No UDPGA Inc Plate 3	NC No UDPGA Inc Plate 3	No Solvent Inc Plate 4	NC Inc Plate 4	No Solvent No UDPGA Inc Plate 4	NC No UDPGA Inc Plate 4
G	STD 7	STD 7	*HQC*	HQC	No Solvent Inc Plate 3	NC Inc Plate 3	No Solvent No UDPGA Inc Plate 3	NC No UDPGA Inc Plate 3	No Solvent Inc Plate 4	NC Inc Plate 4	No Solvent No UDPGA Inc Plate 4	NC No UDPGA Inc Plate 4
H	STD 8	STD 8	*HQC*	HQC								

Figure 14: Reading Plate 2 for the "MU fluorescence assay" in parallel with inhibition assay
Abbreviations: Solvent P/NC= Solvent-control of Positive and Negative controls; PC = Positive Control; NC = Negative Control; BLK =control blank; STD =calibration curve; Inc Plate = Incubation Plate

2.7.4.1. Procedure: Determination of MU fluorescence assay

The solutions in the column 8-12 of all incubation plates are used for the MU fluorescence assay.

Incubation Plate 1 and 2 → Reading plate 1:

1. Transfer 120 µl of the calibration curve solutions, quality controls samples and blank solution (see 3.2.2 and 3.2.3) in columns 1-4 of the reading plate 2 shown *Figure 13*.
2. Dispense 74 µL of no solvent-control WS, the solvent-control of positive and negative controls WS and the positive and negative controls WS in the "reading plates" as shown in the *Figure 13* (column 5-12).
3. Dispense 24 µL of methylumbelliferone working solution (400 µM, see paragraph 2.4.8) in columns 5-12 (*Figure 13*). Warm at 37 °C.

4. After 15 ± 3 minutes of incubation (see starting time of inhibition assay), transfer 24 µL of "incubation plate 1" and "incubation plate 2" solutions to the respective wells of "reading plate 1" as described in *Table 13*. Use an 8-channel pipette to minimize lag time among wells and document the "starting time of MU fluorescence assay 1" (addition of solutions to the first column).
5. Immediately read the plate (see paragraph 3.1).

Incubation plate 1 → Reading plate 1	Incubation plate 2 → Reading plate 1
Column 9 → 5	Column 9 → 9
Column 10 → 6	Column 10 → 10
Column 11 → 7	Column 11 → 11
Column 12 → 8	Column 12 → 12

Table 13: Transfer from incubation plates 1 and 2 into reading plate 1

Incubation Plate 3 and 4 → Reading plate 2:

6. After reading the "reading plate 1", transfer 120 µl of the calibration curve solutions, quality controls samples and blank solution (see 3.2.2 and 3.2.3) in columns 1-4 of the reading plate 1 show *Figure 14*.
7. Dispense 74 µL of no solvent-control WS, the solvent-control of positive and negative controls WS and the positive and negative controls WS in the "reading plates" as shown in the *Figure 14* (column 5-12).
8. Dispense 24 µL of methylumbelliferone working solution (400 µM, see paragraph 2.4.8) in columns 5-12 (*Figure 14*). Warm at 37 °C.
9. After 15 ± 3 minutes of incubation (see starting time of inhibition assay), transfer 24 µL of "incubation plate 3" and "incubation plate 4" solutions to the respective wells of "reading plate 1" as described in *Table 14*. Use an 8-channel pipette to minimize lag time among wells and document the "starting time of MU fluorescence assay 1" (addition of solutions to the first column).
10. Immediately read the plate (see paragraph 3.1).

Incubation plate 3 → Reading plate 2	Incubation plate 4 → Reading plate 2
Column 9 → 5	Column 9 → 9
Column 10 → 6	Column 10 → 10
Column 11 → 7	Column 11 → 11
Column 12 → 8	Column 12 → 12

Table 14: Transfer from incubation plates 3 and 4 into reading plate 2

3. ANALYSIS OF MU FLUORESCENCE ASSAY

3.1. Setup of the fluorescence microplate reader: MU fluorescence assay

UGTs activity is quantified by measuring the disappearance of methylumbelliferone, a fluorescent substance that is transformed into non-fluorescent methylumbelliferone glucuronide. A fluorescence microplate reader is used for the quantification of methylumbelliferone. Fluorescence is measured at Ex/Em = 372/445 nm in kinetic mode every 5 minutes for 30 min at 37 °C. It is recommended to preconfigure the fluorescence microplate reader settings before the assay.

While the assay can be performed in either endpoint or kinetic mode, it's strongly recommended reading in kinetic mode in order to ensure that the measurements recorded are within the linear range of the reaction. Ideal measurement time for the linear range may vary depending upon the amount and variety of active UGT isozymes in the sample.

Temperature	37 °C
Excitation wavelength	360 – 372 nm
Emmision wavelength	445 – 460 nm
Total assay time	30 minutes
Kinetic time interval	5 minutes
Shaking	If possible

Table 15: Microplate reader settings

3.2. Analytical samples of "MU fluorescence assay"

An analytical run consists of a blank sample extract (without analyte and internal standard) containing internal standards, calibration standards (STD), quality control samples (QC), System Suitability Testing (SST).

The reading plate (*Figure 5*) is prepared with the incubation samples and the analytical samples, that consist of control blank, calibration standards (STD) and quality control samples (QC). Calibration standards and quality control samples should be prepared independently using separately prepared stock solutions.

The reading plate is analyzed using the fluorescence microplate reader as described in paragraph 3.1.

3.2.1. Control Blank

Control blank must be included in the batch at least in duplicate. The incubation matrix is prepared by dilution of microsomes (see paragraph 2.3.4.1) in incubation medium (see paragraph 2.3.3.1). An example of volumes is represented in *Table 17*.

Incubation Matrix for MU fluorescence assay	Initial Concentration	Final Concentration	e.g. Volume (mL)*	e.g. Volume (mL)**
Microsomes	20 mg/mL	0.2 mg/mL	0.180	0.600
DMSO	100 %	0.5 %	0.100	0.300
Incubation Medium	-	-	18	60

Table 16: Example of Volume for the incubation matrix preparation for "MU fluorescence assay"

**Example of Volume for the preparation of Control blank and Calibration curve solution*
**Example of Volume for the preparation of Control blank, Calibration curve solution and Quality controls*

Background subtraction of the incubation matrix (blank) is not applied.

Each run should contain also the control blank with positive and negative controls in order to check the interference of these compounds. These samples, containing incubation matrix, positive or negative control in absence of methylumbelliferone, are prepared as described in paragraph *2.5.4* and located in column 8 of reading plates (*Figure 5*)

3.2.2. Calibration curve

During the incubation, prepare the MU calibration curve in a 96 well plate as described below:

- Prepare the primary solution (PS, 400 µM) by diluting the methylumbelliferone stock solution in incubation matrix (100 µL MU Stock Solution + 4900 µL Incubation Matrix).
- Prepare STD 1 in duplicate, MU at 80 µM with incubation matrix (e.g. 600 µL PS MU 400 µM + 2400 µL incubation matrix)
- Prepare STD 2 in duplicate, MU 64 µM by dilution of 80 µM (e.g. 1600 µL PS MU 80 µM + 400 µL incubation matrix)
- Prepare the calibration standards in duplicate with 2-fold serial dilution with a starting concentration of 64 µM (e.g. 1000 µL STD + 1000 µL incubation matrix). See *Table 18*.
- Cover the plate and keep at room temperature.

Sample Name	STD 1 MU	STD 2 MU	STD 3 MU	STD 4 MU	STD 5 MU	STD 6 MU	STD 7 MU	STD 8 MU
Concentration µM of MU	80.0	64.0	32.0	16.0	8.0	4.0	2.0	1.0

Table 17: Calibration curve of MU

120 µL of calibration curve solutions are transferred into the reding plate as described in paragraph 2.5.4 step 12 in columns 1 and 2 (see *Figure 5*).

3.2.3. Quality Control Samples

Quality controls samples are usually prepared in bulk and aliquots stored at -20 °C for up to X days (see *Table 29*).

Each run should contain at least 3 different concentrations of quality control samples (QC) assayed in duplicate (total of 6). The QC samples concentrations should be near the Lower Limit of Quantification (LLOQ, 1.0 µM) (within 3 times the LLOQ; low QC), near the mid-range or geometric mean of the CS curve (50% of the calibration curve range, middle QC) and near the Upper Limit of Quantification (75-80% of ULOQ, 80 µM; high QC).

Prepare the MU quality controls in tubes as described below:

- Prepare the primary solution (PS, 400 µM) by diluting the methylumbelliferone stock solution in incubation matrix (100 µL MU Stock Solution + 4900 µL Incubation Matrix).

- Prepare the quality controls in duplicate by diluting the PS in incubation matrix according the following table:

Sample Name	Final Conc. MU (µM)	Solutions			Incubation matrix
		Name	Conc. (µM)	Volume (µL)	Volume (µL)
PS	400	SS	16000	100	3900
HQC	60	PS	400	1200	6800
MQC	10	PS	400	200	7800
LQC	2.5	PS	400	50	7950

Table 18: Methylumbelliferone Quality Control samples Preparation

- Transfer 120 µL of QC into reading plate as described in point 12 (columns 3 and 4, *Figure 5*)
- Prepare aliquots (600 µL) of remaining solutions to store at -20 °C.

3.3. Criteria for accepting or rejecting a run

The run (analytical batch) should be assessed according to the criteria described in this section. Data from rejected analytical runs will be kept but does not need to be reported. However, the fact that the run was rejected and the reason for failure should be documented.

Each sample is analysed in two wells that generate one result. To consider the result acceptable, the CV% of the two registered responses must be less than 20%. When a duplicate analysis is required (e.g. double QCs or calibration standards), 4 wells are used, and 2 results are generated.

$$\%CV(duplicate\ analysis) = \frac{Standard\ Deviation\ of\ Duplicate}{Mean\ of\ Duplicate} x100$$

3.3.1. Controls Blank

The response of interferences/contaminants should be ≤ LLOQ calibration standard.

Eventually the analytical system and the solutions should be checked. If the issue is solved the reading plates can be prepared again transfer solution from incubation plate (starting from point 10) or prepared again the incubation plate (starting from point 1).

Otherwise the experiment is rejected and repeated using new materials and solutions

3.3.2. Calibration curve

For acceptance of a run, the back-calculated concentrations of a minimum of 75% of the calibration standards (12 of 16) should be ≤ ±20.0% (%Bias) of target values except at the LLOQ (STD 8) and ULOQ (STD 1) where ≤ ±25.0% (%Bias) is acceptable. Calibration standards outside these limits should be rejected.

To be accepted, at least one calibration point at the ULOQ (STD 1), and one at the LLOQ (STD 8), must be within the limits stated above for the entire range of the curve.

The calibration curve must be recalculated after one or more calibration standards are deleted. Excluded calibration standards should be indicated in the results tables, but back-calculated values, if available, are excluded from summary statistics.

$$\%BIAS = \frac{Measured\ Value - True\ Value}{True\ Value} x100$$

The linear regression and the R square should be calculated. R square must be greater than 0.990.

3.3.3. Quality Control Samples

Each solution is analyzed in two wells generally proximal; the mean of the instrumental response is used to generate one result for that sample. For acceptance of an analytical run at least 67% (4 of 6) of the QCs must be ≤ ±20% of their respective nominal values; 33% (2 of 6) can be outside the ±20% (%Bias) of nominal values, but not both replicates at the same concentrations. All QC data must be reported unless a known or visible error has occurred and is documented.

$$\%BIAS = \frac{Measured\ Value - True\ Value}{True\ Value} x100$$

3.3.4. Aborted and re-injected runs

In case of the analytical run acquisition stops due to instrument failure or delay in reading plate (> 15 minutes after starting time), it is acceptable to restart the acquisition. The new acquisition doesn't have to be after 45 minutes from starting time (to be verified). The reason for a stopped run or re-analysis should be documented.

It must be checked that the fluorescence values (RFU_1 and RFU_2) are in the linear phase of the reaction progress curves (see paragraph *3.4*).

3.4. Elaboration data: MU fluorescence assay

For each reaction well, choose two time points (T_1 and T_2, e.g. 15 and 25 minutes by "starting time") (step 15) in the linear phase of the reaction progress curves. The linear phase should be evaluated in the no solvent-control with UDPGA as represented in the *Figure 14*. At least six time points should be considered and the linear regression should be calculated: R square must be > 0.990.

At two time points (T_1 and T_2), evaluate the corresponding fluorescence values (RFU_1 and RFU_2) and determine the absolute value of the change in fluorescence over the time interval:

$$\Delta F = \left| RFU_2 - RFU_1 \right|$$

Calculate the specific fluorescence lost due to substrate glucuronidation (denoted by G) by subtracting the ΔF value of the no UDPGA samples from those of the test samples (S).

$$G_S = \Delta F_S - \Delta F_{\text{no UDPGA}}$$

UGTs activity is obtained by applying the G_S values to the substrate standard curve to get B nmole of substrate glucuronidated by sample UGT enzymes during the selected time period.

$$\text{UGTs Activity} = \frac{B}{\Delta T \times P} = \text{nmole/min/mg} = \text{mU/mg}$$

Where:

- B is the amount of MU consumed, calculated from the standard curve (in nmole)
- ΔT is the linear phase reaction time $T_2 - T_1$ (in minutes)
- P is the amount of protein in the well (in mg). The final protein concentration is 0.0156 mg.

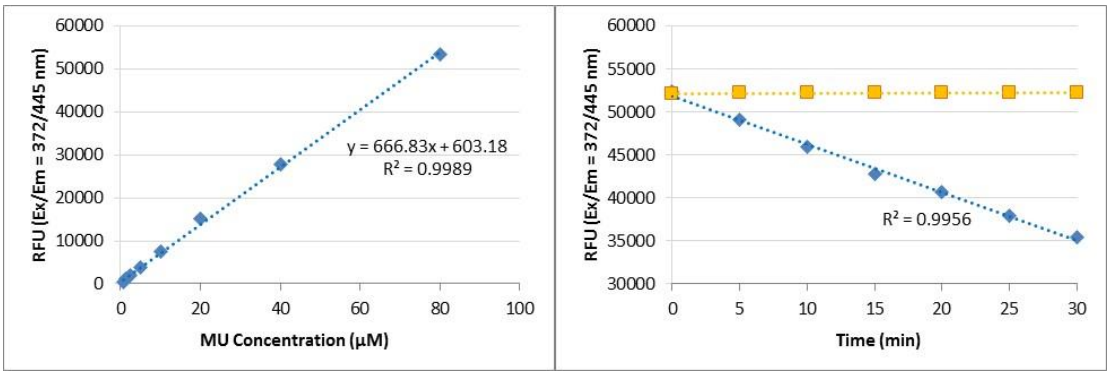


Figure 14: Example of standard curve of UGT substrate fluorescence (on the left) and of reaction kinetics of MU (on the right)

For each condition (no solvent-control, solvent-control of positive/negative controls and positive/negative controls) the UGTs activity is calculated. The intra-plate, inter-plate and the inter-run are calculated as described below:

Intra-plate precision:

In each plate the mean and the percentage of the coefficient of variation (%CV) of triplicates should be calculated using the following formula:

$$\%CV(intra-plate) = \frac{Standard\ Deviation\ of\ Triplicate}{Mean\ of\ Triplicate}\ x100$$

The %CV should be ≤ 20%. In case of triplicates, one outlier can be rejected, and the mean should be recalculated with two values. The outlier does not need to be reported in the calculation tables, however the fact that the value was rejected and the reason for the failure should be documented.

Inter –plate precision:

In a run with more reading plates (e.g. when at least three test items are incubating in inhibition assay and in "MU fluorescence assay" is performed in parallel), the inter-plate variation of UGT activity should be evaluated. The inter-plate %CV should be calculated by comparing the average of UGT activity in each plate. The inter-plate %CV should be ≤20%.

$$\%\ CV(inter\ plate) = \frac{Standard\ Deviation\ of\ mean\ intra\ plate}{Mean\ intra\ plate}\ x100$$

Inter-run precision:

In different runs, the inter-run variation of UGT activity should be evaluated. The inter-run %CV plate should be calculated by comparing the averages of UGT activity in each run. The average UGTs activity calculated in parallel with inhibition assay should be compared with the UGTs activity values obtained in the characterization of new batch. The inter-run %CV should be ≤ 20%.

$$\%\ CV(inter\ run)\ = \frac{Standard\ Deviation\ of\ mean\ inter\ plate}{Mean\ inter\ plate}\ x100$$

4. ANALYSIS OF INHIBITION ASSAY

4.1. Analytical samples of "Inhibition assay"

An analytical run consists of a blank sample extract (without analyte and internal standard) containing internal standards, calibration standards (STD), quality control samples (QC), System Suitability Testing (SST).

Calibration standards and quality control samples should be prepared independently using separately prepared stock solutions.

Calibration standard and quality controls must be prepared separately for each metabolite (T3G and T4G) containing a fixed concentration of relative substrate (T3 or T4). Samples must be freshly prepared, or aliquots of the solutions can be used if still stable (see *Table 29*).

Samples for the analytical run should be dispensed in a 384-well plate ("calibration plate") which must be centrifuged before analyses. The sequence, for each substrate, has to be structured as follows:

- SST
- 3x wash samples
- 1x blank sample
- 1x blank ISTD sample
- 1x Calibration Curve - Set 1 (from lowest to highest)
- 3x blank sample (carry over test)
- 3x matrix sample (selectivity test)
- Unknown samples (Samples without UDPGA – Positive controls samples)
- Unknown samples (Test item A samples, high concentration -> low concentration)

- 1x LQC
- 1x MQC
- 2x blank sample
- Unknown samples (Test item B samples, high concentration -> low concentration)
- 2x blank sample
- 1x MQC
- 1x HQC
- 2x blank sample
- Unknown samples (Test item C samples, high concentration -> low concentration)
- 2x blank sample
- 1x LQC
- 1x HQC
- 2x blank sample
- Unknown samples (Test item D samples, high concentration -> low concentration)
- 2x blank sample
- Unknown samples (Solvent control samples - Negative control samples)
- 2x blank sample
- 1x blank ISTD sample
- 1x calibration Curve - Set 2 (from lowest to highest)
- 1x blank sample
- SST

4.1.1. System Suitability Test (SST)

The SST solution is used to check instrument performance (e.g. sensitivity and chromatographic retention) at the beginning and at the end of each analytical run. It consists of T3, T4, T3G and T4G at approximately the expected LLOQ concentrations. The SST solution is prepared in Tris-HCl 100 mM at the concentration of 10 nM for T3, T4, T3G, T4G (see *Table 20*). This solution is then diluted with stop solution (1:1, v:v).

Sample name	Compound	Final Conc. (µM)	Solution using			Dilute to (mL)
			Name	Conc. (µM)	Volume (mL)	
V1_SST	T3	100	SS T3	10000	0.020	2.00
	T4	100	SS T4	10000	0.020	
V2_SST	T3 and T4	1	V1_SST	100	0.020	2.00
	T3G	1	SS T3G	100	0.020	
	T4G	1	SS T4G	100	0.020	
V3_SST	T3, T4, T3G, T4G	0.01	V2_SST	1	0.200	20.0

Table 19: Example of system suitability test preparation

4.1.2. Blank control with or without internal standard, carry over, selectivity and wash

Control blank without and with internal standard should be included in the batch. These samples consist of incubation matrix extracted with solvent containing and not containing the internal standard. Incubation matrix is prepared by dilution of microsomes to 1 mg/mL in incubation medium and UDPGA 5 mM (see *Table 21*):

Incubation Matrix	Initial Concentration	Final Concentration	Volume (mL)
Microsomes	20 mg/mL	1 mg/mL	2
UDPGA	100 mM	5 mM	2
DMSO	100 %	0.5 %	0.2
Incubation Medium	-	-	36

Table 20: Example of Volume for the incubation matrix preparation

Control blank without internal standard should be placed after each high concentration calibrant to assess carryover.

A matrix sample should be included to evaluate the batch selectivity to verify the interference of substrate or other contaminants in the incubation matrix.

Name	Solution	Extraction solution	Ratio (v:v)
Wash	Water	Acetonitrile	50:50
Control Blank	Incubation matrix	Acetonitrile	75:25
Control Blank with ISTD	Incubation matrix	Stop solution	50:50
Matrix sample	Incubation matrix	Stop solution with T3 or T4 10 µM	50:50

Table 21: Wash, control blank with or without internal standard

4.1.2.1. Calibration curve

Each analytical run should contain at least 6 non-zero concentrations in duplicate for the calibration standards (STD, at least 12 in total), prepared in the appropriate blank matrix. For the duplicate calibration standards, one set should be placed at the beginning of the run and the other at the end. Each point is treated individually in terms of the regression analysis and acceptance criteria.

Eight calibration standards for each metabolite (T3G and T4G) must be prepared with a fixed concentration of relative substrate (T3 and T4 10 µM). The concentration ranges should be to 5 nM and 2 µM for T3G and T4G. The solutions should be freshly prepared or aliquots used if still in the stability range. Preparation of the standards should be documented.

Working solutions (WS_STD) are prepared in the incubation matrix (incubation medium with test system and cofactor) containing 0.6% DMSO.

To obtain the calibration standards (STD), each working solution is diluted to two different wells with the same volume of stop solution, as described for the incubation samples. Calibration standard curves for T3-T3G and T4-T4G are prepared separately.

- T3 and T4 stock solutions are prepared by dissolving approximately 5 mg (accurately weighed) in polypropylene tubes in an appropriate volume of NaOH 1.0 N: DMSO (1:1) in order to obtain the exact final concentration of 10 mM. Correction for potency is performed. The stock solutions are aliquoted and stored at -20 °C.
- T3G and T4G stock solution are prepared by dissolving approximately 2 mg (accurately weighed) in polypropylene tubes in an appropriate volume of H_2O: MeOH (1:1) in order to obtain the exact final concentration of 100 µM. Correction for potency is performed. The stock solutions are aliquoted and stored at -20 °C
- T3G and T4G working solutions (STD 1-8_WS) are prepared by dilution of the stock solutions with incubation matrix (see *Table 23*) according the following table:

Sample Name	Final Conc. T3G or T4G (µM)	Solutions			Incubation matrix
		Name	Conc. (µM)	Volume (µL)	Volume (µL)
STD 1_WS	2	SS	100	20	980
STD 2_WS	1	STD 1_WS	2	150	150
STD 3_WS	0.5	STD 1_WS	2	100	300
STD 4_WS	0.1	STD 1_WS	2	20	380
STD 5_WS	0.05	STD 4_WS	0.1	150	150
STD 6_WS	0.025	STD 4_WS	0.1	100	300
STD 7_WS	0.01	STD 4_WS	0.1	50	450
STD 8_WS	0.005	STD 7_WS	0.005	150	150

Table 22: Calibration Standards Preparation

11. Vortex-mix all solutions for 10 seconds.
12. Prepare the calibration solutions (STD 1-STD 8) with 200 µL of working solution (STD 1_WS-STD 8_WS) and 200 µL of stop solution containing T3 or T4 10 µM in duplicate in the calibration plate.

13. Centrifuge for 5 min at ~4800 x g at room temperature.
14. Transfer 150 µL of the particle-free supernatant to the calibration plate.

4.1.2.2. Quality control samples

Quality controls samples are usually prepared in bulk and frozen under the same storage condition (see *Table 29*).

Each run should contain at least 3 different concentrations of quality control samples (QC) assayed in duplicate (total of 6). The QC samples concentrations should be near the Lower Limit of Quantification (LLOQ) (within 3 times the LLOQ; low QC), near the mid-range or geometric mean of the CS curve (50% of the calibration curve range, middle QC) and near the Upper Limit of Quantification (75-80% of ULOQ; high QC).

- T3 and T4 stock solutions are prepared by dissolving approximately 5 mg (accurately weighed) in polypropylene tubes in an appropriate volume of NaOH 1.0 N: DMSO (1:1) in order to obtain the exact final concentration of 10 mM. Correction for potency is performed. The stock solutions are aliquoted and stored at -20 °C.
- T3G and T4G stock solution are prepared by dissolving approximately 2 mg (accurately weighed) in polypropylene tubes in an appropriate volume of H_2O:MeOH (1:1) in order to obtain the exact final concentration of 100 µM. Correction for potency is performed. The stocks solutions are aliquoted and stored at -20 °C
- T3G and T4G working solutions (QC_WS) are prepared by dilution of the stock solutions with incubation matrix (see *Table 21*) according the following table:

Sample Name	Final Conc. T3G or T4G (µM)	Solutions			Incubation matrix
		Name	Conc. (µM)	Volume (mL)	Volume (mL)
ULOQ_WS	2	SS	100	0.2	9.8
HQC_WS	1.6	ULOQ_WS	2	8.0	2.0
MQC_WS	0.1	ULOQ_WS	2	0.5	9.5
LQC_WS	0.015	ULOQ_WS	2	0.075	10.0

Table 23: Quality Control samples Preparation

15. Vortex-mix all solutions for 10 seconds.
16. Prepare the quality control (HQC, MQC and LQC) with 4.5 mL of working solution (HQC_WS, MQC_WS and LQC_WS) and 4.5 mL of stop solution containing T3 or T4 10 µM in duplicate in the calibration plate.
17. Centrifuge for 5 min at ~4800 x g at room temperature.
18. Transfer 150 µL of the particle-free supernatant to the calibration plate. Aliquots other solutions (500 µL) and stored at -20 °C.

4.1.3. Criteria for accepting or rejecting a run

The run (analytical batch) should be assessed according to the criteria described in this section. Data from rejected analytical runs will be kept but do not need to be reported. However, the fact that the run was rejected and the reason for failure should be documented.

4.1.3.1. Peak Shape, Resolution and Retention

The peak tailing factors (PTF) of the analyte and internal standard are determined in each analytical run according to the method applied by the respective test facility. If not specified, the PTF is determined at 5% peak height. The peak shape, resolution and retention time of the peaks of interest must be adequate and consistent.

4.1.3.2. Calibration curve

For acceptance of a run, the back-calculated concentrations of a minimum of 75% of the calibration standards (12 of 16) should be ≤ ±15.0% (%Bias) and of target values except at the LLOQ where ≤ ±20.0% is acceptable. Calibration standards outside these limits should be rejected.

To be accepted, at least one calibration point at the LLOQ, and one at the ULOQ, must be within the limits stated above for the entire range of the curve.

The calibration curve must be recalculated after one or more calibration standards are deleted. Excluded calibration standards should be indicated in the results tables, but back-calculated values, if available, are excluded from summary statistics.

Samples above the ULOQ should be re-analysed after appropriate dilution. Samples below the lowest calibration standard (LLOQ) can be accepted. In the raw data these data should be reported using an appropriate flag (e.g. BLQ), or as “less than” the nominal concentration of the lowest calibration standard (e.g. < 5 nM). In this case, the LLOQ value should be used for the IC50 calculation.

$$\%BIAS = \frac{Measured\ Value - True\ Value}{True\ Value} x100$$

4.1.3.3. Quality Control Samples

For acceptance of an analytical run at least 67% (four out of six) of the QCs must be ≤ ±15% (%Bias) of their respective nominal values; 33% (two out of six) can be outside the ±15% of nominal values, but not both replicates at the same concentration. All QC data must be reported unless a known or visible error has occurred and is documented.

$$\%BIAS = \frac{Measured\ Value - True\ Value}{True\ Value} x100$$

4.1.3.4. Matrix (Selectivity) and Carry Over

The response of interferences/contaminants and of carry-over samples should be ≤ LLOQ calibration standard.

Eventually the analytical system and solvents/additives should be checked. If the issue is solved the samples are analysed using the "backup plate". Even if the issue is not solved, samples can be analysed using the "backup plate", as it may not exhibit the same interference as the main plate.

Otherwise the experiment is rejected and repeated using new materials and solutions.

4.1.3.5. System suitability test (SST)

No acceptance criteria are set, unless they have been previously established during validation.

4.1.3.6. Aborted and re-injected runs

In case of the analytical run acquisition stops due to instrument failure or it is stopped by the analyst due to other issues (e.g. leak in the fluidic system), it is acceptable to restart the acquisition from the first affected sample or, in alternative, reinject the whole batch. In case of reinjection of the whole batch, the aborted original run should be kept, and all chromatograms printed. The reason for a stopped run should be documented.

An analytical batch acquired correctly can be reinjected only in case of identified instrument issues. The reason for reinjection should be documented and both original and reinjected runs should be saved. Reinjection of a full analytical run or individual samples (e.g. calibration standards, QCs) of a run, simply because the calibration or QCs failed, without any identified analytical cause, is not accepted.

The reinjection is allowed provided that the stability of the final extract or at least of the processed sample viability has been demonstrated.

4.2. Elaboration of data: Inhibition assay

When all the samples are analyzed, the area values should be normalized (ratio between area of analyte and area of the ISTD) and then the triiodothyronine glucuronide and thyroxine glucuronide concentrations should be calculated against the calibration curves. If possible, the software of the analytical instrument should be used for these calculations. The unit of measurement used should be micromolar (µM).

Background subtraction of the solvent control is not applied, as the matrix effect is already assessed as described in paragraph *4.1.3.4*.

Glucuronide formation:

Triiodothyronine glucuronide and thyroxine glucuronide formation rates are determined in the no solvent control and used to confirm positive outcome of the UGT reaction.
Metabolite formation rate expressed in pmol/min/mg should be calculated according to the following formulae: By plotting the area of the glucuronide in the no control solvent condition against the time, the slope is calculated, and its value converted into

$$Formation\ Rate\ = \frac{\mu M\ product\ \ x\ \ \mu L\ incubation}{minute\ of\ incubation\ \ x\ \ mg\ protein}$$

Where:

- µM product is the glucuronide concentration in the no solvent-control calculated with calibration curve
- µL incubation is the total volume of each well in the incubation plate (= 250 µL)
- minutes of incubation is the time of the T3 and T4 incubations (= 180 min)
- mg protein is the amount of microsomes in each well (= 5 mg)

Positive and negative control inhibition:

For the positive and negative control, the percentage of inhibition should be calculated comparing the concentration of glucuronide formed in the presence of the positive or negative control with the concentration of glucuronide in the solvent control of positive and negative controls, according to the following formula:

$$\%\ inhibition\ = 100 - \frac{\mu M\ product\ in\ presence\ of PC\ or\ NC}{\mu M\ product\ in\ solvent\ control\ of\ PC/NC}\ x100$$

The average and the %CV of positive and negative control in each plate should be evaluated.

Test Item inhibition:

The glucuronide concentration is used to calculate the percentage of UGT-mediated enzyme activity remaining given by the ratio between the quantities of metabolite formed in the presence of the inhibitor compared to the solvent control of test item:

$$\%\ activity\ remaining\ = \frac{\mu M\ product\ in\ presence\ of\ TI}{\mu M\ product\ in\ solvent\ control\ of\ TI}\ x100$$

The average of the percentage of activity remaining and %CV are calculated and used to obtain the IC50 values, the concentration of inhibitor needed to inhibit 50% of the glucuronidation reaction.
IC50 values are determined from a plot of the logarithm of the inhibitor concentration (µM) versus the percentage of the enzyme activity relative to the control:

$$Y\ = \frac{Bottom + (Top - Bottom)}{1 + 10\text{^}((logIC50 - X)\ x\ HillSlope)}$$

where X is the logarithm of the inhibitor concentration and Y the % of activity remaining.

The IC50 can be calculated when the percentage of remaining activity is less than 80 % at the higher test items concentrations.

IC50 values should be calculated appropriate software (e.g. GraphPad) by non-linear regression analysis according to the Hill equation (variable slope, four parameters) and setting the bottom equal to zero.

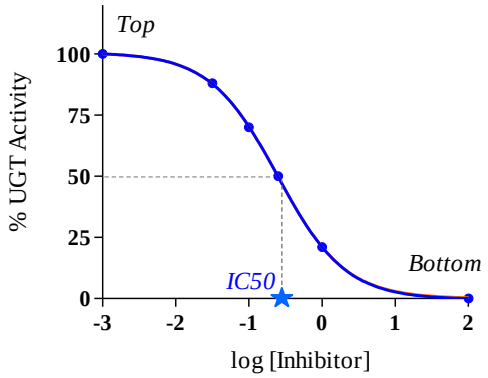


Figure 15. IC50 Graphical Representation

Intra-plate precision:

The calculated concentrations are processed with statistical analysis: for each condition (solvent-control, positive/negative controls and all test item incubations) the mean and the percentage of the coefficient of variation (%CV) of triplicates should be calculated using the following formula:

$$\%CV(intra-plate) = \frac{Standard\ Deviation\ of\ Triplicate}{Mean\ of\ Triplicate}\ x100$$

The %CV should be < 20%. In case of triplicates, one outlier can be rejected, and the mean should be recalculated with two values. The outlier does not need to be reported in the calculation tables, however the fact that the value was rejected and the reason for the failure should be documented.

Inter –plate precision:

In a run with more plates, the inter-plate variation of glucuronide formation and glucuronide inhibition should be evaluated. The inter-plate %CV should be calculated by comparing the average of formation rate in each plate and the average of positive and negative control inhibition percentage in each plate. The inter-plate %CV should be < 20%.

$$\%\ CV(inter\ plate)\ = \frac{Standard\ Deviation\ of\ mean\ intra\ plate}{Mean\ intra\ plate}\ x100$$

Inter-run precision:

In two runs, the inter-run variation of glucuronide formation and glucuronide inhibition should be evaluated. The inter-run %CV plate should be calculated by comparing the average of formation rate in each run and the average of positive and negative control inhibition percentage in each run. The average of IC50 values of two runs should be compared as well by calculating the %CV. The inter-run %CV should be < 20%.

$$\%\ CV(inter\ run)\ = \frac{Standard\ Deviation\ of\ mean\ inter\ plate}{Mean\ inter\ plate}\ x100$$

5. ACCEPTANCE CRITERIA SUMMARY

The acceptance criteria described in this SOP are summarized in the following tables. These criteria must be fulfilled in order to accept an experiment a valid run.

The characteristic of new batch of microsomes should be described and checked (*Table 25).*

Validation Item	Minimum experiments	Default Acceptance Criteria
HLM	Protein concentration Donors	≥ 20 mg/mL ≥ 50 donors
Rate of glucuronide formation of HLM	In the CoA: UGT1 or UGT1A1 rate of formation	> 900 pmol/min/mg

Table 25: Acceptance Criteria of the Batch of Microsomes

The acceptance criteria of the solubility of test item are summarized in the *Table 26*:

Validation Item	Minimum experiments	Default Acceptance Criteria
Test item	In the solubility Assay	No precipitate in the stock solutions and working solutions
Test item	In the incubation plate of the inhibition assay	No precipitate of test item incubated with UDPGA without HLM

Table 26: Acceptance Criteria of the solubility of test item

The acceptance criteria of the "MU fluorescence assay"are summarized in the *Table 27*:

Validation Item	Minimum experiments	Default Acceptance Criteria
Blank control and Blank control with PC/NC	Signal-to-noise of incubation matrix or PC/NC without MU	≤ LLOQ
Calibration Curve	At least 6 non-zero concentrations in duplicate	Minimum 75% of the STD should be considered $R^2 \geq 0.990$
QC Samples: Accuracy and precision	3 concentrations: low QC (LQC), mid QC (MQC), high QC (HQC), n=2 in each run	Mean intra- accuracy 85.0-115.0%, and CV ≤ +15.0%
Linear regression of MU disappearance	In the no solvent controls at least six time points should be considered	$R^2 \geq 0.990$
UGT activity	In no solvent-control	≥ 500 mU/mg To be confirm with different batch of HLM
Effect of solvent	UGT activity in the solvent control of PC/NC vs no solvent control	≤ ±20% To be confirm with different batch of HLM
Positive Control Inhibition	Percentage of Mefenamic Acid inhibition	> 60% To be confirm with different batch of HLM
Negative Control Inhibition	Percentage of Fluconazole inhibition	< 20% To be confirm with different batch of HLM
Precision intra and inter-plates	3 replicates for each sample: Average of each run of Solvent-control, No Solvent-control, PC, NC (%CV)	≤ 20% intra-plate ≤ 20% inter-plate (if there are multiple plates)
Precision inter-run	Average of each run of Solvent-control, no solvent-control, PC, NC, (%CV)	≤ 20% inter-run

Table 26: Acceptance Criteria of the "MU fluorescence assay"

The acceptance criteria of the analytical runs are summarized in the *Table 28*.

Validation Item	**Minimum experiments**	**Default Acceptance Criteria**
Matrix (Background)	Signal-to-noise co-eluting with-metabolite Signal-to-noise co-eluting with-ISTD	≤20% vs LLOQ ≤5% vs ISTD
Calibration Standards:	At least 6 non-zero concentrations in duplicate	Minimum 75% of the CS should be within ±15.0% of target concentrations except at LLOQ ±20.0%.
QC Samples: Accuracy and precision	3 concentrations: low QC (LQC), mid QC (MQC), high QC (HQC), n=2 in each run	Mean intra- accuracy 85.0-115.0%, and CV ≤ +15.0%
Precision intra and inter-plates	3 replicates for each sample: Average of each run of Solvent, PC, NC, TI (%CV)	≤ 20% intra-plate ≤ 20% inter-plate (if there are multiple plates)
Precision inter-run (3 runs)	Average of each run of Solvent, PC, NC, TI (%CV)	≤ 20% inter-run
Amount of glucuronide conjugates in no solvent control (T180)	Range concentration of glucuronide conjugates without inhibitor after 180 minutes of incubation	> 0.05 µM (T3G) > 0.30 µM (T4G) To be confirm with different batch of HLM
Effect of solvent	Amount of glucuronide in the solvent control of TI or PC/NC vs no solvent control	≤ ± 20% (T3G) ≤ ± 20% (T4G) To be confirm with different batch of HLM
Amount of glucuronide conjugates without cofactor	Signal-to-noise co-eluting with-metabolite Signal-to-noise co-eluting with-ISTD	≤ LLOQ ≤5% vs ISTD
Positive Control Inhibition	Percentage of Mefenamic Acid inhibition	> 60% (T3G) > 60% (T4G) To be confirm with different batch of HLM
Negative Control Inhibition	Percentage of Fluconazole inhibition	< 20% (T3G) < 20% (T4G) To be confirm with different batch of HLM

Table 27: Acceptance Criteria of the inhibition assay

6. SOLUTIONS STABILITY

The solution stability has been investigated and in the *Table 29* the storage conditions and times are summarized (the long-term stability is on-going).

Compounds	Solutions	Description	Storage
T3 and T4	Stock Sol.	10 mM NaOH 1.0 N : DMSO (1:1)	At least 1 week at -20 °C - 3 f/t At least 6 h at RT
	Working Sol.	100 µM in water	At least 6 h at RT
	Incubation Sol.	10 µM in incubation matrix	At least 6 h at 37 °C
	Analytical Sol.	5 µM in stopping sol.	At least 48 h in autosampler At least 1 week at -20 °C - 3 f/t
T3G and T4G	Stock Sol.	100 µM in MeOH : H_2O (1:1)	At least 1 week at -20 °C - 3 f/t At least 6 h at RT
	Incubation Sol.	LQC = 0.015 µM and HQC = 1.6 µM incubation matrix	At least 6 h at 37 °C
	Analytical Sol.	LQC and HQC in stopping sol.	At least 48 h in autosampler At least 1 week at -20 °C - 3 f/t
Fexofenadine	Stock Sol.	1 mM in DMSO	At least 1 week at -20 °C - 3 f/t At least 6 h at RT
	Working Sol. = Stop Solvent	1 µM in acetonitrile	At least 1 week at 4 °C At least 6 h at RT
	Analytical Sol.	0.25 µM in stopping sol.	At least 48 h in autosampler At least 1 week at -20 °C - 3 f/t
Stop Solution	-	Stop solvent: 1% acetic acid in water (1:1)	Prepare the day of experiment
UDPGA	Cofactor Sol	100 mM in water	Prepare the day of experiment
Test Items	Stock Sol.	In DMSO, MeOH or water	Prepare the day of experiment
	Working Sol.	≤ 0.5 % DMSO or MeOH in incubation medium	Prepare the day of experiment
Mefenamic Acid and Fluconazole	Stock Sol.	100 mM in DMSO	At least 1 week at -20 °C - 3 f/t At least 6 h at RT
	Working Sol.	500 µM in incubation medium	At least 1 week at -20 °C - 3 f/t At least 6 h at RT
	Incubation Sol.	400 µM in incubation matrix	At least 6 h at 37 °C
MU	Stock Sol.	200 mM in DMSO	To be evaluated
	Working Sol.	160 µM in incubation medium	To be evaluated
	Incubation Sol.	80 µM in incubation matrix	To be evaluated

Table 28: Summary of solutions stability

Abbreviations: Sol. = solution; h= hours; RT = room temperature; in autosampler = sample injected and stability in 384-well plate storage in autosampler (4 ± 3 °C); f/t = freeze/thawed cycles

7. REFERENCES

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