European Commission

STUDY REPORT

for the colorimetric assessment of deiodinases activity based on Sandell-Kolthoff reaction with human microsomes: DIO1-SK assay – Part 1

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

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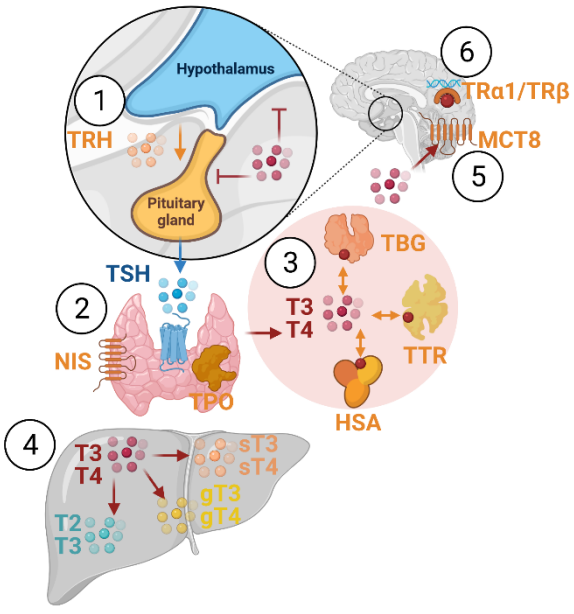

2023

Joint Research Centre

This study report has been prepared within the context of a collaboration agreement 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. It aims to provide evidence-based scientific support to the European policymaking process. The contents of this publication do not necessarily reflect the position or opinion of the European Commission. Neither the European Commission nor any person acting on behalf of the Commission is responsible for the use that might be made of this publication. For information on the methodology and quality underlying the data used in this publication for which the source is neither Eurostat nor other Commission services, users should contact the referenced source. The designations employed and the presentation of material on the maps do not imply the expression of any opinion whatsoever on the part of the European Union concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries.

This study report describes the experimental design and includes data generated in Part 1 of the validation study. The method was developed by Dr. Kostia Renko when working at Charité Universitatsmedizin Berlin (DE) and subsequently implemented by the EU-NETVAL test facility BASF SE (Germany) within the validation study.

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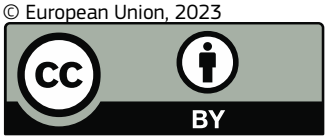

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SUMMARY OF RESULTS

Study report

Part 1: Reproducibility Assessment for method 4a: DIO1-SK assay

Test guideline(s)

Method according to Renko et al., 2015

Author(s)

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Experimental Completion Date

07.10.2020

Test facility

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Test facility project identification

Project No.: 39V0712/00V002

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1 Aim of the study

The objective of this study was the assessment of the activity of chemicals on the reproducibility of the Deiodinase I (DIO1) Sandell-Kolthoff (SK) assay on human liver microsomes via the Sandell-Kolthoff reaction (SK). Six test items, 6-Propyl-2-thiouracil (6PTU), 5-Propy-2-thiouracil (5PTU), Aurothioglucose (ATG), 2-Chloro-N-phenylacetamide (2CPA), Genistein (GEN) and Tetrabromobisphenol A (TBBPA), assumed to interact with DIO1, were tested regarding their inhibition properties in the DIO1- SK assay in at least five independent runs. Three different technicians were generating the data to show the robustness of the method. The generated data were finally used to establish validity criteria of assay runs for the “part 2: validation phase” of the DIO1-SK assay.

2 Introduction

The Deiodinases (DIO), a group of selenocysteine-containing enzymes, consist of three isoforms and regulate thyroid hormone signalling through the deiodination of thyroid hormones, resulting in the formation of thyroid hormone metabolites with differing activity (figure 1). DIO1 plays an important role in systemic T3 production in the thyroid, but also in recycling iodide from thyroid hormone metabolites in excreting organs like the liver and kidney. DIO2 and DIO3 regulate local thyroid hormone signalling in peripheral tissue through activation of T4 to T3 (DIO2) and inactivation (DIO3) of thyroid hormones. The DIO enzymes differ in tissue expression as well as their expression pattern during fetal development (Bianco, Dumitrescu et al. 2019).

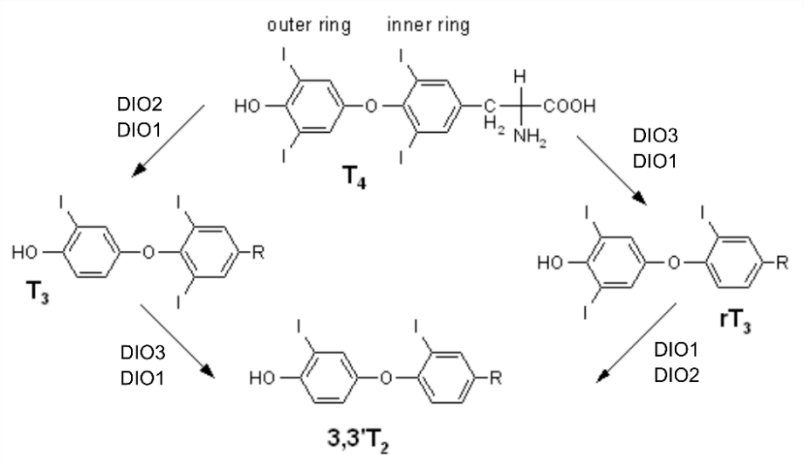

figure 1: Metabolism of thyroid hormone through the Deiodinases (DIO)

This method uses the “Sandell-Kolthoff-reaction”, a non-radioactive, colorimetric reaction which can be used to measure free iodide concentration. The reaction is based on the reduction of yellow-coloured cerium (IV) to colourless cerium (III) and oxidation of arsenite (III) to arsenite (V) depending on the available iodide concentration since iodide functions as a catalysing agent in the reaction(figure 2). The extent of the colour change resulting from

the redox reaction can be quantified through measurement of the optical density (OD) before and after the reaction at 415 nm.

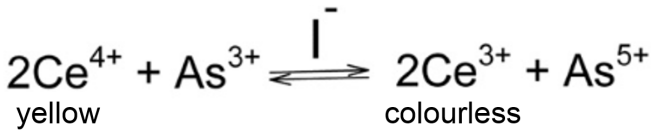

figure 2: Sandell-Kolthoff reaction

Microsomes, broken-down vesicle-like pieces of endoplasmic reticula from hepatocytes are used as enzyme source for DIO, mainly DIO1, in this method. The microsomes possess also other metabolizing enzymes which might influence the test system through metabolization of the test compounds (Knights, Stresser et al. 2016). This might explain differences in the test item inhibition properties compared to assays with purified DIO1 enzymes.

A variety of chemicals are known to inhibit deiodinases under *in vitro* conditions (Renko, Schäche et al. 2015, Olker, Korte et al. 2018) whereas less is known about substance-induced inhibition *in vivo*. Known *in vivo* DIO inhibitors include the pharmaceuticals 6-Propyl-2-thiouracil, iopanoic acid and amiodarone (Leonard and Rosenberg 1978, Leonard, Mellen et al. 1983, Rosene, Wittmann et al. 2010).

The DIO1-SK assay requires an initial iodide release activity test run to determine the batch-specific iodide release activity of the microsome batch. This is needed because suppliers usually do not test for iodide release activity. Based on the measured microsome batch-specific iodide release activity and protein concentration, a microsome batch-specific enzyme concentration is used for the assay runs. Furthermore, to define the appropriate dose range of the test item for the main assay runs, an initial assay run (range finding assay) is performed for unknown test items. In this study, 5 valid assay runs are performed per test items to assess the DIO1-inhibition properties of the test item.

The measurement of endogenous DIO activity via this assay is therefore limited to rich sources of enzymatic activity like DIO1-containing liver microsomes. Furthermore, it is not possible to analyse iodide containing substances since the assay cannot differentiate between released iodide from the test item and the used substrate.

This assay uses the colorimetric Sandell-Kolthoff (SK) reaction as an endpoint of specific iodide release from the substrate reverseT3 (rT3). The known DIO1 inhibitor 6-Propyl-2-thioruacil (6PTU) will be used as reference item and is additionally tested as a test item for this study. The five additional test items 5-Propy-2-thiouracil (5PTU), Aurothioglucose (ATG), 2-Chloro-N-phenylacetamide (2CPA), Genistein (GEN) and Tetrabromobisphenol A (TBBPA) will be tested for their potential to inhibit DIO1 dependent iodide release activity. In addition, ATG is used as the positive control in this assay. A structural analogue of ATG (lacking the gold ligand which is responsible for

DIO1 inhibition), 1-Thio-β-D-glucose sodium salt (TGSS) will be used as a negative control in the assay.

3 Study schedule

Study Start Date: 24.09.2020
Experimental Starting Date: 28.09.2020
Experimental Completion Date: 07.10.2020
Study Completion Date: see date of the Report

4 Guidelines and SOP

No regulatory test guideline is currently available. The method is based on the original non-radioactive deiodinase I inhibition assay from Kostja Renko, Charité, Berlin which used mice liver microsomes or recombinant enzyme (Renko, Hoefig et al. 2012, Renko, Schäche et al. 2015) and was further optimized for the use of human liver microsomes at BASF. A SOP including standardization efforts, controls, initial acceptance criteria etc. originated from mentioned standardization activities.

The following SOP was used for the part 1: “Reproducibility assessment of the DIO1-SK assay” - Colorimetric method for assessing deiodinases activity based on Sandell-Kolthoff reaction with human microsomes: DIO1-SK assay (version: 20200923_SOP DIO1-SK assay; date: 23.09.2020, approved 29.09.2020, see Appendix)

5 Materials and equipment

5.1 Material

table 1: Material that is used in the DIO1-SK assay.

Material:	Requirements Supplier[2]
Volumetric flask	certified with defined volume
Filter plates (96 well format)	UNIFILTER Microplate, 96-well, 800 µl, GF/C, clear polystyrene, filter bottom with long drip director, GE Healthcare Life Sciences[2]
Deep well plates (96 well format)	SPE 96-Deep Square Well Collection Plate, well volume 2 mL, polypropylene, Sigma Aldrich[2]
Assay plates (96 well format)	tissue culture plates, 96 well plate, flat bottom, polystyrene, 0.34 cm^2, sterile, 108/cs, TPP[2]
Gas-tight plate sealers	Sealing tape, polyester, sterile, Sealing tape, polyester, sterile, Nunc[2]
Microcentrifuge tubes 1,5 mL	Eppendorf® Safe-Lock microcentrifuge tubes, volume 1.5 mL, natural, Eppendorf AG[2]
Centrifuge Tubes 15 and 50 mL	centrifuge tubes, volume 50 mL, polypropylene, TPP[2]

	centrifuge tubes, volume 15 mL, polypropylene, TPP[2]

5.2 Technical equipment

table 2: Used technical equipment in the DIO1-SK assay

Apparatus	Requirements Supplier[2]
Analytical balance	capable of accurately weighing up to 30 g with 0.1 mg readability
Incubator	capable of keeping temperatures of 37°C, 5 % CO_2 and ≥90 % humidity
pH meter with electrode and calibration buffers	capable of reading +/- 0.1 pH units
Photometer for absorbance measurement	Sunrise™ Absorbance Reader, INSTSUN-3, Tecan Trading AG[2]
Plate shaker	Thermo Scientific H+P MONOSHAKE VORTEXER microtiter plate, directly controlled, Thermo Fisher Scientific[2]
Centrifuge with swing-out rotor for microtiter plates	Should be high enough to fit a 96-deep well plate with 96-well filter plate on top (at least about 6 cm high)

5.3 Chemicals

table 3: Used chemicals and reagents in the DIO1-SK assay

Chemicals / reagents	Requirements Supplier[2]
1-Thio-β-D-glucose sodium salt CAS: 10593-29-0 MW : 218.20 g/mol	1-Thio-β-D-glucose sodium salt, Sigma-Aldrich[2]
3,3',5'-triiodothyronine (rT3) CAS: 5817-39-0 MW: 650.97 g/mol	3,3′,5′-Triiodo-L-thyronine, Sigma-Aldrich[2]
6-Propyl-2-thiouracil (6PTU) CAS: 51-52-5 MW: 170.23 g/mol	6-Propyl-2-thiouracil, VETRANAL™, analytical standard, Supelco[2]
Acetic acid CAS: 64-19-7 MW: 60.05 g/mol	acetic acid, glacial, ReagentPlus®, ≥99%, Sigma-Aldrich[2]
Arsenic sodium oxide (NaAsO2) CAS: 7784-46-5 MW: 129.91 g/mol	sodium (meta) arsenite, ≥90%, Sigma-Aldrich[2]
Aurothioglucose (ATG) CAS: 12192-57-3 MW: 392.18 g/mol (anhydrous basis)	aurothioglucose hydrate, ≥96% (titration), Sigma-Aldrich[2]
Cerium (IV) ammonium sulphate ($Ce(NH_4)_4(SO_4)_4$) CAS: 10378-47-9 MW: 632.55 g/mol	ammonium cerium (IV) sulphate dihydrate, Sigma-Aldrich[2]
Dimethyl sulfoxide (DMSO) CAS: 67-68-5 MW: 78.13 g/mol	dimethyl sulfoxide (Reag. Ph. Eur.) for analysis, ACS, PanReac AppliChem[2]

Dipotassium hydrogen phosphate (HK_2PO_4) CAS: 7758-11-4 MW: 174.18 g/mol	potassium phosphate dibasic, meets USP testing specifications, Sigma-Aldrich[2]
Dowex 50WX2 CAS: 12612-37-2	Dowex 50WX2 100 200 mesh ion exchange resin, Acros Organics[2]
Dithiothreitol (DTT) CAS: 3483-12-3 MW: 154.25 g/mol	DL-Dithiothreitol solution, BioUltra, for molecular biology, ~1 M in H_2O, Sigma-Aldrich[2]
Ethylenediaminetetraacetic acid (EDTA) CAS: 6381-92-6 MW: 372.24 g/mol	ethylenediaminetetraacetic acid disodium salt dihydrate, Sigma Grade, suitable for plant cell culture, 98.5-101.5 %, Sigma-Aldrich[2]
Iodide (IC standard)	Iodide standard for IC, 1000 mg/L in water, Sigma-Aldrich[2]
Monopotassium phosphate (H_2KPO_4) CAS: 7778-77-0 MW: 136.09 g/mol	potassium phosphate monobasic, powder, suitable for cell culture, suitable for insect cell culture, suitable for plant cell culture, ≥99.0%, Sigma-Aldrich[2]
Sodium chloride (NaCl) CAS: 7647-14-5 MW: 58.44 g/mol	sodium chloride, ACS reagent, ≥99.0%, Sigma-Aldrich[2]
Sulfuric acid (H_2SO_4) CAS: 7664-93-9 MW: 98.08 g/mol	sulfuric acid, Supelco[2]

5.4 Reagents

table 4: Reagents that are prepared before the assay performance

H_2KPO_4 (0.216 M)/ EDTA (2.16 mM) solution	250 mL volumetric flask: 7.34 g H_2KPO_4 and 201 mg Ethylenediaminetetraacetic acid (EDTA) are added to the flask and filled up with ddH_2O to a final volume of 250 mL.
HK_2PO_4 (0.216 M) / EDTA (2.16 mM) solution (250 ml):	250 mL volumetric flask: 9.41 g HK_2PO_4 and 201 mg Ethylenediaminetetraacetic acid (EDTA) are added and filled up with ddH_2O is added to a final volume of 250 mL.
Potassium phosphate / EDTA puffer (2.16 mM EDTA; pH 6.8)	250 mL volumetric flask: H_2KPO_4 / EDTA solution and HK_2PO_4 / EDTA solution are titrated to reach a pH of 6.8 (ratio of HK_2PO_4 / EDTA to H_2KPO_4 / EDTA of about 2:1 ≈ 167 ml of HK_2PO_4 / EDTA and 83 mL of H_2KPO_4 / EDTA solution).
rT3 (15 mM) solution	rT3 is dissolved in an appropriate volume of DMSO to reach a final concentration of 15 mM. Aliquots of 100µL are frozen at -20°C.
Preparation of 15 mL Falcons with aliquoted rT3	4 µL of 15 mM rT3 are added to 15 mL-Falcons and stored at -20°C.
Acidic ammonium cerium solution (25 mM $(NH_4)_4Ce(SO_4)_4{\cdot}2H_2O$, 0,5 M H_2SO_4) (250 mL)	3.95 g of $(NH_4)_4Ce(SO_4)_4{\cdot}2H_2O$ and 125 mL of ddH_2O are added to a 250 mL volumetric flask. Subsequently 125 mL 1 M H_2SO_4 are added to reach a final volume of 250 mL.

Sodium arsenite solution (25 mM $NaAsO_2$, 0,8 M NaCl, 0,5 M H_2SO_4) (250 ml)	0.81 g of $NaAsO_2$, 11.7 g of NaCl and 125 mL of ddH_2O are added to a 250 mL volumetric flask. Subsequently 125 mL 1 M H_2SO_4 are added to reach a final volume of 250 mL.

table 5: Reagents that are prepared on the day of assay performance.

Preparation of the substrate mix (volume enough for 1x96-well plate)	On the day of assay performance, 5.75 mL of potassium phosphate/EDTA buffer (0.216 M KPO_4, pH 6.8) and 0.5 mL DTT is added to the frozen, 4 µl of 15 mM rT3 containing falcon and mixed.

5.5 Software

table 6: Software that is used in the DIO1-SK assay

Software	Requirements[1] Supplier[2]
Statistics software	Able to perform regression analysis that reflect assay characteristics and able to calculate inhibitory concentrations[1]: GraphPad Prims 8[2]

6 Test items and control items

6.1 Information on used Reference and Control items

To control the proper performance of the test system (OECD 2018), the method includes one reference item as well as a positive and negative control. The reference item 6-Propyl-2-thiouracil (6PTU) as well as the positive control Aurothioglucose (ATG) are both well described DIO inhibitors (Visser and Van Overmeeren 1979, Berry, Kieffer et al. 1991) The purpose of a reference item is to control the dose-response of the test system quantitatively and for normalization of the test item data. The purpose of the positive control item was to confirm the inhibitory effect by using a single concentration. A structural analogue of ATG (lacking the gold ligand which responsible for DIO1 inhibition), 1-Thio-β-D-glucose sodium salt (TGSS) was used as a negative control without any inhibitory effect. Moreover, solvent controls were included in all the experiments for normalization and to exclude unspecific activity by the used solvent (preferably DMSO is used). There is historical data available obtained in pretests at BASF SE supporting suitability of the three items acting as reference (6PTU), positive (ATG) and negative control (TGSS) item.

6.1.1 Reference item/ test item

table 7: Information about the used batch of the reference item 6-Propyl-2-thiouracil (6PTU).

Name	6-Propyl-2-thiouracil
Acronym	6PTU
CAS No.	51-52-5
Supplier	Sigma-Aldrich
Batch No.	BCBX0879

Purity [%]	99.6
Expiration date	31.07.2023
Molecular weight [g/mol]	170.23
Storage conditions	RT

6.1.2 Positive control/ test item

table 8: Information about the used batch of the positive control Aurothioglucose (ATG).

Name	Aurothioglucose hydrate
Acronym	ATG
CAS No.	12192-57-3
Supplier	Sigma-Aldrich
Batch No.	0000054738
Purity [%]	96.1
Expiration date	14.05.2024
Molecular weight [g/mol]	392.18
Storage conditions	4°C

6.1.3 Negative control

table 9: Information about the used batch of the negative control 1-Thio-β-D-glucose sodium salt (TGSS).

Name	1-Thio-β-D-glucose sodium salt
Acronym	TGSS
CAS No.	10593-29-0
Supplier	Sigma-Aldrich
Batch No.	0000074213
Purity [%]	99.5
Expiration date	20.03.2025
Molecular weight [g/mol]	218.20
Storage conditions	RT
Solvent	DMSO
Stock solution [M]	10^{-2} M

6.1.4 Solvent control

All used test items were soluble in the solvent of choice, DMSO. The only performed solvent controls were thus DMSO controls (table 10).

table 10: Information on the used solvent control (DMSO).

Solvent:	Dimethyl sulfoxide (DMSO)
Test item preparation:	Solution
Final solvent concentration in the assay	1 %
CAS No.:	67-68-5

Batch No.:	0001429609
Purity /Content:	99.9% p.a.
Molecular weight [g/mol]:	78.13 g/mol
Storage conditions:	ambient (RT)

6.1.5 Test items

In addition to the reference item 6PTU and the positive control ATG that were also used in this study and hence called test item, four additional test items were used in this study (see table 11).

table 11: Information about the used batches of the test items 5-Propyl-2-thiouracil (5PTU), 2-Chloro-N-phenylacetamide (2CPA), Genistein (GEN) and 3,3',5,5'-Tetrabromobisphenol A (TBBPA).

Name:	5-Propyl-2-thiouracil	2-Chloro-N-phenylacetamide	Genistein	3,3',5,5'-Tetrabromobisphenol A	6-Propyl-2-thiouracil	Aurothioglucose hydrate
Acronym	5PTU	2CPA	GEN	TBBPA	6PTU	ATG
CAS No.:	2954-52-1	587-65-5	446-72-0	79-94-7	51-52-5	12192-57-3
Supplier:	Sigma-Aldrich	Sigma-Aldrich	Sigma-Aldrich	Sigma-Aldrich	Sigma-Aldrich	Sigma-Aldrich
Batch No.:	SLBL3627V	MKCC3899	MKCB9769	MKCB9769	BCBX0879	0000054738
Purity [%]	99	99.9	99.3	99.3	99.6	96.1
Expiration date	01.08.2024	04.07.2024	14.08.2024	14.08.2024	31.07.2023	20.03.2025
Molecular weight [g/mol]:	170.23	169.61	543.87	543.87	170.23	218.20
Storage conditions:	RT	RT	-18°C	RT	RT	4°C
Literature regarding DIO inhibition	(Visser, Van Overmeeren et al. 1979)	(Olker, Korte et al. 2018)	(Renko, Schäche et al. 2015, Olker, Korte et al. 2018)	(Butt, Wang et al. 2011)	(Visser and Van Overmeeren 1979, Renko, Hoefig et al. 2012, Renko, Schäche et al. 2015)	(Berry, Kieffer et al. 1991, Renko, Schäche et al. 2015)

7 Test system

The minimum requirements for human liver microsomes are described in table 12. The human microsomes were tested for all known human liver microsomal contaminations in compliance with the Guidance Document on Good In Vitro Method Practices (GIVIMP) (OECD 2018).
Different human microsome batches show differences in their activity to deiodinate rT3 leading to differences in the maximum ΔOD-BG values (≙ enzyme activity). The generation of an enzyme activity curve with the used microsome batch is used in this method to assess the iodide release activity of the microsome batch and to determine an appropriate microsome batch-specific enzyme concentration that will be used for the assay runs. Microsome-batch specific iodide release activity testing is further specified in 8.8. The microsomes should be stored at ≤ -80°C until required for use.

table 12: Information on the used microsome batch

Test system:	liver microsomes
Test species:	human
Supplier:	BIOIVT (Westbury, NY)
Batch:	#QQY
Sex:	mixed gender
Pool:	150 donors
Age:	various
Demonstrated absence of the following contaminations:	Hepatitis B, Hepatitis C, Human Immunodeficiency Virus (HIV)
Storage conditions:	-80°C

The used batch of human liver microsomes was distributed by the European Union Reference Laboratory for alternatives to animal testing (EURL ECVAM, Ispra, Italy) and originally ordered from BioIVT (Westbury, NY). The supplier provides information about the donor demography and characterization data of the microsome batch for common human pathogens including HIV and Hepatitis B/C as well as some metabolizing enzymes. The donor demographics as well as the available lot characterization data for the used human liver microsome batch #QQY are shown in Appendix 14.2.

8 Method

8.1 Casting of DOWEX resin-filled 96-Well filter plates

Before the day of assay performance, a larger quantity of DOWEX resin filled 96-well filter plate was prepared and stored at 4°C.

About 250 g of DOWEX was added to a large beaker and washed with 10 % acetic acid. To mix the DOWEX with the acetic acid a big shaker was used, afterwards it rested for 10 min and the supernatant was removed.

- The supernatant was washed and removed until no more colour was leaking into the solvent (at least 4x in total)
- 100 µL of acetic acid (10 %) were added into each well of 96-well filter plate
- 1 mL tips were cut to widen the opening and 600 µL DOWEX resin were filled into each well of the 96-well filter plate
- Another 150 µL acetic acid (10 %) were added to each well and eluted by centrifuging into the 96-deep well-plate (1 min, 70 g)
- The previous step was repeated if colour was still leaking in any of the wells

The plate was sealed with an impermeable sheet of plastic and stored at 4°C for a maximum of 2 months.

8.2 Solubility testing of test, reference, and control items

The test concentrations for the range finding assay are dependent on the solubility of the test item in pure solvent, the solubility in the following dilutions in water as well as the solubility under assay conditions. The highest tested solubility of a test item in an appropriate solvent in the DIO1-SK assay is 100 mM leading to a highest tested final assay concentration of a test item of 1 mM (1% v/v of solvent).

Prior to the assay, the solubility of the test item in an appropriate solvent was determined to prepare a test item stock solution. The preferred solvent dimethyl sulfoxide (DMSO) was used for dissolving all test items and controls in this study .The starting concentration of the test item for solubility testing was 100 mM to achieve a final maximal concentration of 1 mM in the assay. The solubility was checked by using a microscope. If the test item was not dissolved, heat (up to a maximum of 37°C) and/or ultrasonic was applied to aid solubility.

If the test item was not fully dissolved, subsequent dilution steps of the test item in the appropriate solvent were used, dilutions of 1:3.16 (square root of 10, i.e. dilution steps corresponding to half order of magnitude) until the test item was fully dissolved. The solubility was checked after each dilution step.
Afterwards it was checked whether the highest soluble concentration was still soluble in a 1:10 predilutions with ddH_2O. If this was the case, the 1% final test concentration was prepared with a solution of 50% potassium phosphate / EDTA buffer, 40% ddH_2O and 10% of the 10% test item dilution and the solubility was checked by using a microscope.

If a fully dissolved suspension was not achievable, a lower concentration of the test item was used (dilution with a factor of 3.16 or 10) to prepare the 10 % dilution in ddH_2O and the subsequent 1 % test item dilution. If the test item was not fully dissolved, mentioned steps were repeated with the next lower concentration.

8.3 Preparation of the stock solution and dilutions of the test items

Within this study, 6PTU as the reference item and ATG as the positive control were tested as test items in a dose-response approach while they were also tested as their controlling function in the assay using single-concentration replicates on every assay plate. The preparation of reference item and positive control as single concentration on every plate is described in 8.4 and 8.5, respectively.
On the day of analysis, the stock solution corresponding to the determined highest concentration of the test items was freshly prepared. Based on the amount of needed stock solution, an appropriate amount of substance was weighed into a suitable vessel. By using a pipette, an appropriate amount of the solvent (DMSO) was added. If necessary, the substance was dissolved in the solvent with a vortex or ultrasonication was performed to maintain the solubility of the stock solution.

The “SOP DIO1-SK (29.09.2020) assay” is designed for the testing of test item regarding their inhibition on iodide release activity in the DIO1-SK assay and uses an initial range finding assay to determine a potential iodide release activity of the test item. In case of determined inhibition of iodide release activity in the range finding assay, the used concentrations for the assay runs may be adapted. The preparation of the dilutions (containing 10 % DMSO) for the test item X from the corresponding stock solution for a range finding assay is shown in table 13.

table 13: Preparation of the dilutions for the test item X.

Name of the dilution of substance X	ddH_2O [µL]	10% DMSO/ddH_2O [µL]	Reference item [µL]	Dilution factor compared to prior dilution
X-D1	450	-	50 µL of stock solution of substance X	10
X-D2	-	450	50 µL of X-D1	10
X-D3	-	450	50 µL of X-D2	10
X-D4	-	450	50 µL of X-D3	10
X-D5	-	450	50 µL of X-D4	10
X-D6	-	450	50 µL of X-D5	10
X-D7	-	450	50 µL of X-D6	10
X-D8	-	450	50 µL of X-D7	10

In summary, the used concentrations for an assay run for a test item with an unknown iodide release activity inhibition depend (a) on their highest soluble concentration in their respective solvent and their subsequent dilutions in ddH_2O / assay buffer which defines the highest tested concentration of the test item under assay conditions (but maximal 1 mM under final assay conditions); and (b) on its activity on iodide release inhibition on the range finding assay which has an impact on the choice of concentrations as well as the spacing between the tested concentrations of the test items.
For the used items and controls in this part 1: Reproducibility Assessment, there was comprehensive data on iodide release inhibition of the tested items available at BASF prior to this study which was defined as range finding experiments to determine the final assay concentrations and spacings for each control and test item. This allowed an appropriate dose setting prior to the assay runs and no extra range finding assay was conducted in this study.

8.4 Preparation of the stock solution and dilution of the reference item

On the day of analysis, a 10^{-1} M stock solution of the reference item 6PTU was freshly prepared. Based on the amount of the needed stock solution, an appropriate amount of substance was weighed into a suitable vessel. By using a pipette an appropriate amount of the solvent (DMSO) was added. The substance was dissolved in the solvent with a vortex. Ultrasonication was also performed to maintain the solubility of the stock solution.

According to “SOP DIO1-SK (29.09.2020) assay”, a 6PTU dose response curve must be conducted on the first assay run on each day but not the following assay runs of the same day. Since the reference item 6PTU was also tested as

one of the test items in this study, 6PTU was always tested in a dose-response approach as a test item on the first assay run of each day.

For the single concentration controls on each assay plate the reference item dilution from the 10^{-2} M reference item stock solution was prepared according to table 14 on the day of analysis.

table 14: Preparation of the reference item 6PTU dilution.

Reference item dilution [M]	ddH_2O [µL]	DMSO [µL]	reference item [µL]	Final concentration of positive control in the assay [M]
10^{-2}	450	-	50 µL of 10^{-1} M reference item stock solution	10^{-3}

8.5 Preparation of the stock solution and dilution of the positive control

A 10^{-2} M stock solution for the positive control Aurothioglucose was prepared prior to this study. The stock solution was stored at 4°C and is stable for at least 6 months without loss of activity. Based on the amount of the needed stock solution, an appropriate amount of substance was weighed into a suitable vessel. By using a pipette an appropriate amount of the solvent (DMSO) was added. The substance was dissolved in the solvent with a vortex.

On the day of analysis, the positive control dilution from the 10^{-2} M positive control stock solution was prepared according to table 15.

table 15: Preparation of the positive control Aurothioglucose dilution.

Positive control dilution [M]	ddH_2O [µL]	DMSO [µL]	Positive control [µL]	Final concentration of positive control in the assay [M]
10^{-3}	450	-	50 µL of 10^{-2} M positive control stock solution	10^{-4}

8.6 Preparation of a stock solution and dilution of the negative control

A 10^{-2} M stock solution for the negative control 1-Thio-β-D-glucose sodium salt was freshly prepared on the day of analysis. Based on the amount of the needed stock solution, an appropriate amount of substance was weighed into a suitable vessel. By using a pipette an appropriate amount of the solvent (DMSO) was added. The substance was dissolved in the solvent with a vortex. Ultrasonication was also performed to maintain the solubility of the stock.

On the day of analysis, the negative control dilution from the 10-2 M negative control stock solution was prepared according to table 16.

table 16: Preparation of the negative control 1-Thio-β-D-glucose sodium salt dilution.

Negative control dilution [M]	ddH_2O [µL]	DMSO [µL]	Negative control [µL]	Final concentration of negative control in the assay [M]
10^{-3}	450	-	50 µL of 10^{-2} M negative control stock solution	10^{-4}

8.7 Preparation of the human microsome dilutions

Varying iodide release activity of different human liver microsome batches have shown the need for standardisation efforts of enzyme concentration the DIO1-SK assay. For that human liver microsome dilutions are tested in the DIO1-SK in different concentrations per assay well as well as different dilutions in 10 % acetic acid prior after the DOWEX separation prior to the Sandell-Kolthoff reaction.

Human liver microsome dilutions were prepared in ddH_2O as shown in table 17. The calculation is based on a stock solution of 20 mg enzyme/mL, as the microsome batch #QQY was supplied from BioIVT in this concentration which was used for this study.

table 17: Preparation of the human liver microsome dilutions for the testing of iodide release activity.

Microsome per well [µg]	ddH_2O [µL]	Microsome dilution [µL]	Final enzyme concentration in the assay [µg/mL]
20	780	20 µL of 20 mg/mL microsome stock solution	200
10	400	400 µL of 20 µg Microsome per well dilution	100
5	400	400 µL of 10 µg Microsome per well dilution	50
2.5	400	400 µL of 5 µg Microsome per well dilution	25
1.25	400	400 µL of 2.5 µg Microsome per well dilution	12.5
0.68	400	400 µL of 1.25 µg Microsome per well dilution	6.8

8.8 Measuring activity of the microsomes

Different human microsome batches show differences in their activity to deiodinate rT3, leading to differences in the maximum ΔOD-BG values (≙ Iodide release activity) of the batches of about ~2 to 3x. The generation of an enzyme activity curve with the used microsome batch was used in this method to assess the iodide release activity of the microsome batch and to determine a microsome batch-specific enzyme concentration as well as the used dilution in 10 % acetic acid (generally 1:2 or 1:4 dilutions are used to assure linear responses in the Sandell-Kolthoff reaction) that was used for the assay runs. After determination of the microsome batch specific enzyme concentration, the microsomes were stored in aliquots sufficient for one or the desired amount of assay plates.

The reference item dilution of 6PTU as described in table 14, the microsome dilutions as described in table 17 and the substrate mix as described in table 5 were prepared on the day of microsome activity testing.

- 10 µL of 10^{-2} M 6PTU as reference item were added to a 96-well plate (see table 18).
- For the solvent controls 10 µL of the 10% solvent dilution in ddH_2O (e.g. 10 % DMSO in ddH_2O) were added. A final assay concentration of 1 % solvent in all samples were prepared.
- 40 µL of different microsome dilutions in ddH_2O (resulting in 20, 10, 5, 2.5, 1.25, 0.68 and 0 µg enzyme per well) were added to the 96- well plate.
- On ice, 50 µL of freshly prepared substrate mix was added to each well
- The plate was sealed with an impermeable sheet of plastic
- The 96-well plate was then placed on a shaker in an incubator (37°C at 600 rpm) and was incubated for 2h

table 18: plate layout for measuring the activity of the microsome batch.

	1	2	3	4	5	6	7	8	9	10	11	12
A	20 µg enzyme per well			20 µg enzyme per well 10^{-3} M 6PTU			10 µg enzyme per well			10 µg enzyme per well 10^{-3} M 6PTU		
B	5 µg enzyme per well			5 µg enzyme per well 10^{-3} M 6PTU			2,5 µg enzyme per well			2,5 µg enzyme per well 10^{-3} M 6PTU		
C	1,25 µg enzyme per well			1,25 µg enzyme per well 10^{-3} M 6PTU			0,68 µg enzyme per well			0,68 µg enzyme per well 10^{-3} M 6PTU		
D	0 µg enzyme per well			0 µg enzyme per well 10^{-3} M 6PTU								
E												
F												
G												
H												

reference item **solvent control** **Empty**

- To stop the reaction, the plates were placed on ice.
- The separation of the substrate and released iodide of the assay is conducted analogous to 8.9.2
- The measurement of the Sandell-Kolthoff reaction is conducted analogous to 8.9.3. The samples were measured in the Sandell-Kolthoff reaction undiluted as well as diluted in 10 % acetic acid (1:2 dilution and 1:4 dilution)

8.9 DIO1-SK assay

8.9.1 Microsome incubation with test items

The reference item stock solution as well as dilution of 6PTU as described in 8.4, the positive and negative control stock solution as well as dilution as described in 8.5 and 8.6. and the test item stock solutions as well as dilutions as described in 8.3 were prepared on the day of analysis. For the solvent control 10 µL of 10 % (v/v) DMSO (in ddH_2O) solution were added. For the positive and negative control dilution as well as reference item dilutions,10 µL of the prepared dilutions were added to the 96-well plate. 10 µL of the test item dilutions were added to the 96-well plate. On the first assay run of a day, the test item and reference item 6PTU was conducted on this assay plate.

The general plate layout for the assay day for the test items X, Y and Z is shown in table 19. The first assay plate on assay day always contained the reference item and test item 6PTU on varying positions (X, Y or Z).

- 40 µL of the protein dilution was added to the wells
- 50 µL of the freshly prepared substrate mix (on ice) (see table 5) were added to the samples
- The plate was sealed with an impermeable sheet of plastic
- The 96-well plate was then placed on a shaker in an incubator (37°C at 600 rpm) and was incubated for 2h
- To stop the reaction the plates were placed on ice

table 19: plate layout for assay runs of the DIO1-SK.

	1	2	3	4	5	6	7	8	9	10	11	12
A	SC			RI			NC			RI		
B	X-C1			X-C2			X-C3			X-C4		
C	X-C5			X-C6			X-C7			X-C8		
D	Y-C1			Y-C2			Y-C3			Y-C4		
E	Y-C5			Y-C6			Y-C7			Y-C8		
F	Z-C1			Z-C2			Z-C3			Z-C4		
G	Z-C5			Z-C6			Z-C7			Z-C8		
H	SC			RI			SC			PC		

SC **solvent control**

PC **positive control** Aurothioglucose 10^{-4} M

RI **reference item** 6-Propyl-2-thiouracil 10^{-3} M

NC **negative control** 1-Thio-β-D-glucose sodium salt 10^{-4} M

TI **test item**

8.9.2 Separation via DOWEX resin-filled 96-well filter plate

- A prepared DOWEX resin-filled 96-well filter plate (as prepared in 8.1) was positioned on top of a used 96-deep well-plate
- 150 µL of 10 % acetic acid were added to each well of the DOWEX resin-filled 96-well filter plate to wet the columns
- The acetic acid was eluted by centrifuging into the used 96-deep well-plate with 70 g in a centrifuge for 1 min
- The used 96-deep well-plate was replaced with a novel, unused 96-deep well plate
- 75 µL of the samples were transferred from the incubated 96-well plate into the DOWEX resin-filled 96-well filter plate maintaining the initial plate layout
- 100 µL of 10 % acetic acid was added to each well of the DOWEX resin-filled 96-well filter plate
- The samples were eluted by centrifuging into the novel 96-deep well-plate with 70 g in a centrifuge for 1 min and the DOWEX resin-filled 96-well filter plate was removed afterwards.

8.9.3 Sandell-Kolthoff reaction

- Depending on the determined dilution factor of the samples in 10 % acetic acid for the used microsome batch (see 8.8), 50 µL of the diluted sample solution were added to a novel 96-well plate. E.g. for a 1:2 dilution, 25 µL of 10 % acetic acid were added to each well. Subsequent, 25 µL of the samples from the 96-deep well-plate were added to the 96-well plate.
- 50 µL of cerium solution [25 mM $(NH_4)_4Ce(SO_4)_4{*}2H_2O$; 0.5 M H_2SO_4] were added to the samples in the 96-well plate
- The reaction was started by adding 50 µL of arsenite solution [25 mM $NaAsO_2$; 0.8 M NaCl; 0.5 M H_2SO_4] to the samples in the 96-well plate. For fast addition of arsenite solution, a multichannel pipette was used.
- Immediately after the application of arsenite solution, the absorption OD in the plate reader with the following settings was determined:
    - Absorption parameters: 415 nm (±2 nm)
    - Initial shaking: medium for 2 s
    - Measurement of the OD every minute for 21 min

8.9.4 Evaluation of the data

The data was processed and analysed using Microsoft Excel. The OD of the 21-minute samples from the initial OD values were subtracted to generate ΔOD values. The background was eliminated by subtracting the ΔOD of the inhibited 10^{-3} M 6PTU samples from the ΔOD values of all samples, thus generating ΔOD-BG values. The calculated ΔOD-BG values were then normalized to their respective solvent controls via division of the ΔOD-BG values of the test item(s) by the mean of the ΔOD-BG values of the respective solvent controls generating values termed as “iodide release activity” in percentage. The tested concentrations of the test item were transformed into logarithmic values. Using statistics software, replicates of iodide release activity” values in percentage on y-axis (linear) and the transformed test item concentration on x-axis (linear) were plotted, and the sigmoidal dose response model “log(Inhibitor) vs. response – Variable slope (four parameters)” was used to generate a function

reflecting assay characteristics, visualize a dose-response relationship and, if possible, calculate the 50% inhibition concentration (IC_{50}) of the test item:

$$Y = Bottom + (Top - Bottom) / (1 + 10^{(LogIC50 - X) * HillSlope}$$

where “Top” represents the maximal response, “Bottom” represents the lowest response, and “HillSlope” describes the steepness of the curve.

8.9.5 Acceptance criteria

One important aim of this study “part 1: Reproducibility assessment” was to generate data that is used to establish robust validity criteria for assay runs of the DIO1-SK assay. This study therefore only uses two acceptance criteria since more criteria will be established based on the data of this study.

An assay run is considered valid and will be accepted when all the acceptance criteria are met. The acceptance criteria that are used to define valid assay runs in this study of the assay are shown in table 20.

table 20: Used acceptance criteria to assess validity in the part 1 of the DIO1-SK assay.

Acceptance criteria	Valid run, if
Shape of reference item (sigmoidal, yes/no?)	curve is sigmoidal
IC_{50} of the reference item 6PTU	10^{-6} – 10^{-5} M

8.10 Safety measures

Safety measures are followed according to the safety data sheets of the used materials and test items. The safety precautions according to the BASF internal risk assessment “Gefährdungsbeurteilung: Umgang mit Sodium Arsenit in der Sandell-Kolthoff-Reaktion (Version 1.0)“ are followed.

9 Results and evaluation

9.1 Solubility of test items

The highest soluble concentration of the reference item 6PTU, the positive control ATG and the negative control TGSS in the solvent DMSO was investigated and determined prior to this study and the used SOP for this study.

The positive control / test item ATG was prepared as a 10^{-2} M stock solution after first opening of the vial due to low amount of the item and was soluble at this concentration after warming the solution in a 37°C water bath. Solubility of the positive control ATG was not tested at higher concentrations (table 21). Data from previous results as well as the data generated in this study show that the highest concentration of ATG tested already causes a comparable, complete iodide release inhibition as the reference item 6PTU.

Solubility of the reference item and negative control was reassessed according to “SOP DIO1-SK (29.09.2020) assay” but did not lead to changes to the used stock solution concentrations (table 21).

table 21: Highest soluble stock solution concentrations of the used reference and control items in the solvent DMSO.

Function	Reference item / test item	Positive control / test item	Negative control
Acronym	6PTU	ATG	TGSS
Solvent	DMSO	DMSO	DMSO
Stock solution concentration [M]	10^{-1}	10^{-2}	10^{-2}
Highest soluble concentration under assay conditions [M]	10^{-3}	10^{-4}	10^{-4}

The further used used test items (see table 11) were tested regarding their limit of solubility in the solvent DMSO, the subsequent dilutions as well as the resulting dilution under final assay conditions resulting in the used stock solution concentrations of the test items (see table 22).

The stock solution for the test item 2CPA was prepared as a 10^{-2} M stock solution after first opening of the vial due to low amount of the test item prior to this study and the SOP. Solubility of the test item 2CPA was not tested at higher concentrations.

Detailed information about the solubility of all test items is available in the Appendix 14.3 in table 35. If a stock solution of a test item concentration was fully dissolved in the stock solution in DMSO, the 10 % dilution of the test item in ddH_2O as well as under final assay conditions consisting of 50% potassium phosphate / EDTA buffer, 40% ddH_2O and 10% of the 10% test item dilution, the stock solution was used for testing in this study.

table 22: Highest soluble stock solution concentrations of the used test items in the solvent DMSO.

Acronym	5PTU	2CPA	GEN	TBBPA
Solvent	DMSO	DMSO	DMSO	DMSO
Stock solution concentration [M]	10^{-1}	10^{-2}	$3.16*10^{-4}$	10^{-3}
Highest concentration under assay conditions [M]	10^{-3}	10^{-4}	$3.16*10^{-6}$	10^{-5}

9.2 Microsome batch-specific iodide release activity

No information about the iodide release activity of the used microsome batch from the supplier was available. According to 8.8, a dose-response curve with differing concentration of the microsome batch #QQY from BioIVT was derived and tested in a 1:2 and 1:4 dilution in 10 % acetic acid in the SK reaction.

A protein concentration of 5 µg protein per 100 µl reaction volume (≙ one well) in a 1:2 dilution with 10 % acetic acid in the Sandell-Kolthoff reaction was determined to be sufficient signal for the derivation of future experiments with this batch (figure 3). Higher amounts of protein per well reach a plateau and do not provide additional resolution between full reaction and solvent controls.The

microsome solution was aliquoted into 5 mL Cryovials in a volume enough for one 96-well assay plate and stored at -80°C until required for use.

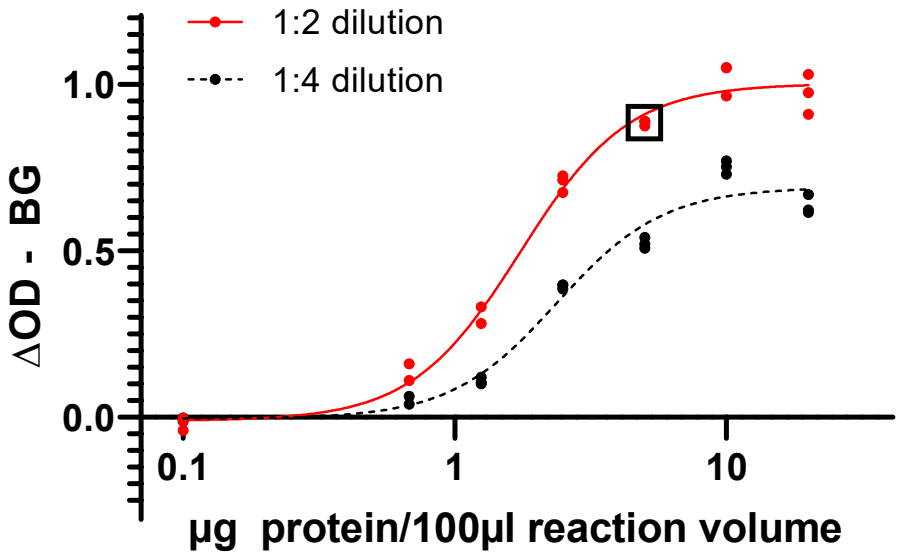

figure 3: iodide release activity of the human liver microsome batch #QQY measured in a 1:2 and 1:4 dilution of 10 % acetic acid in the Sandell-Kolthoff reaction. The black box indicates the determined concentration of the batch-specific microsome and determined dilution for the SK reaction.

9.3 Tested concentrations of the test items in this study

The determined highest soluble concentrations of the test items in 9.1 determined the highest tested concentration in the DIO1-SK assay. Comprehensive data on iodide release inhibition of the test items available at BASF prior to this study was used to determine further final assay concentrations and spacings between the test item concentrations for the test items.

The final assay concentrations of the six test items of this study that were tested in the DIO1-SK assay are shown in table 23.

table 23: Final assay concentrations of the test items in this study

Test item dilution	6PTU [M]	5PTU [M]	ATG [M]	GEN [M]	TBBPA [M]	2CPA [M]
Conc. 1	10^{-3}	10^{-3}	10^{-4}	$3.16*10^{-6}$	10^{-5}	10^{-4}
Conc. 2	10^{-4}	10^{-4}	10^{-5}	10^{-6}	$3.16*10^{-6}$	$6*10^{-5}$
Conc. 3	$3.16*10^{-5}$	$3.16*10^{-5}$	$3.16*10^{-6}$	$3.16*10^{-7}$	10^{-6}	$2*10^{-5}$
Conc. 4	10^{-5}	10^{-5}	10^{-6}	10^{-7}	$3.16*10^{-7}$	10^{-5}
Conc. 5	$3.16*10^{-6}$	$3.16*10^{-6}$	$3.16*10^{-7}$	$3.16*10^{-8}$	10^{-7}	$6*10^{-6}$
Conc. 6	10^{-6}	10^{-6}	10^{-7}	10^{-8}	$3.16*10^{-8}$	$2*10^{-6}$
Conc. 7	10^{-7}	10^{-7}	10^{-8}	$3.16*10^{-9}$	10^{-8}	10^{-6}
Conc. 8	10^{-8}	10^{-8}	10^{-9}	10^{-9}	$3.16*10^{-9}$	10^{-7}

9.4 Iodide release inhibition of the test items in the DIO1-SK assay

In this study (Part 1) at least five independent and valid assay runs were performed to test the robustness as well as validity of the DIO1-SK assay using six known DIO1 inhibitors as test items. In case of invalid assay runs, a maximum of eight runs would have been performed.

9.4.1 Validity of assay runs

In total, two assay runs per day on seven different days were performed, resulting in 14 total assay runs for part 1 of the DIO1-SK assay (table 24). Each test item was tested on each assay day, with different plate assignments of the test items on the first and second plate of a day. Since 6PTU was used as the reference item as well as test item, it was always tested on the first assay plate of the day to check validity of the assay plate.

Six of the seven performed assay runs were defined as valid because they met the acceptance criteria. The validity of the assay runs depend on the achievement of the set acceptance criteria. The results of the achievement of the set acceptance criteria in the performed assay runs are shown in table 25. The assay run on 05.10.2020 was determined to be invalid because dose-response curve of the reference item did not meet the set acceptance criteria. In addition, another assay run was excluded from further evaluation even though it met the acceptance criteria. This assay plate from the 29.09.2020 was inverted at some point during the assay procedure resulting in high standard deviation of the sample. The five final valid assay runs were used for the assessment of reproducibility in this study.

table 24: Used test items for the individual assay runs each day of the part 1: Reproducibility Assessment. The plate layout of the tested test item is analogue to the plate layout in table 19 as “test item X, test item Y, test item Z”.

Date	Tested test items in the 1st assay plate of the day	Tested test items in the 2nd assay plate of the day	Designated name of the valid assay runs
28.09.2020	6PTU, 2CPA, ATG	5PTU, GEN, TBBPA	Assay run 1
~~*29.09.2020**~~	~~*GEN, 2CPA, 6PTU**~~	~~*ATG, 5PTU, TBBPA**~~	-
30.09.2020	ATG, 6PTU, GEN	2CPA, 5PTU, TBBPA	Assay run 2
01.10.2020	6PTU, 5PTU, ATG	GEN, 2CPA, TBBPA	Assay run 3
02.10.2020	TBBPA, 6PTU, ATG	GEN, 2CPA, 5PTU	Assay run 4
~~*05.10.2020**~~	~~*TBBPA, GEN, 6PTU**~~	~~*5PTU, ATG, 2CPA**~~	-
07.10.2020	TBBPA, GEN, 6PTU	5PTU, ATG, 2CPA	Assay run 5

*The assay runs on the 29th of September and 5th of October were determined as invalid

table 25: Acceptance of the performed assay runs as valid assay runs based on the set acceptance criteria

Acceptance criterion	Shape of reference item 6PTU	IC_{50} of the reference item 6PTU [µM]
Requirement / Date	curve is sigmoidal?	1 – 10
28.09.2020	Sigmoidal	2.88
29.09.2020	Sigmoidal	2.23
30.09.2020	Sigmoidal	2.65
01.10.2020	Sigmoidal	4.37

02.10.2020	Sigmoidal	7.61
05.10.2020	**Not sigmoidal**	**11.03**
07.10.2020	Sigmoidal	3.08

9.4.2 6-Propyl-2-thiouracil (6PTU)

Human liver microsome incubated with the reference item as well as test item 6PTU was tested for iodide release inhibition at the final assay concentrations of 0.01, 0.1, 1, 3.16, 10, 31.6, 100 and 1000 µM in five independent assay runs in the DIO1-SK assay. The mean, SD and derived IC_{50} of the triplicates for the individual assay runs for 6PTU are shown in table 26; based on the data, based on the data a curve was fitted which is shown in figure 4.

table 26: mean and standard deviation (SD) of iodide release activity in the five assay runs after incubation with the test and reference item 6PTU. Mean and SD was calculated from the triplicates of the respective assay run.

		Run 1		Run 2		Run 3		Run 4		Run 5	
Concentration [µM]	**Iodide release activity [%]**										
1000	**Mean [%]**	-1.80		-2.42		0.11		-1.72		2.71	
	SD [%]		3.25		1.33		3.69		7.63		1.12
100	**Mean [%]**	-1.41		3.88		7.34		5.30		5.47	
	SD [%]		2.20		2.39		2.41		3.62		1.17
31.6	**Mean [%]**	8.74		13.55		16.91		20.16		20.29	
	SD [%]		1.91		4.25		2.11		5.32		1.99
10	**Mean [%]**	9.67		24.92		23.61		43.36		39.52	
	SD [%]		5.33		3.43		1.09		1.77		3.55
3.16	**Mean [%]**	36.44		43.34		61.62		59.59		52.77	
	SD [%]		3.95		2.18		4.98		16.34		1.85
1	**Mean [%]**	74.92		64.22		65.66		75.32		75.04	
	SD [%]		4.59		4.25		1.38		5.26		8.02
0.1	**Mean [%]**	82.52		86.22		87.71		94.13		105.61	
	SD [%]		3.29		4.54		3.44		2.28		5.69
0.01	**Mean [%]**	78.84		96.32		93.85		94.35		109.31	
	SD [%]		6.23		6.72		10.57		3.83		1.88
IC50	**[µM]**	2.87		2.65		4.37		7.61		3.08	

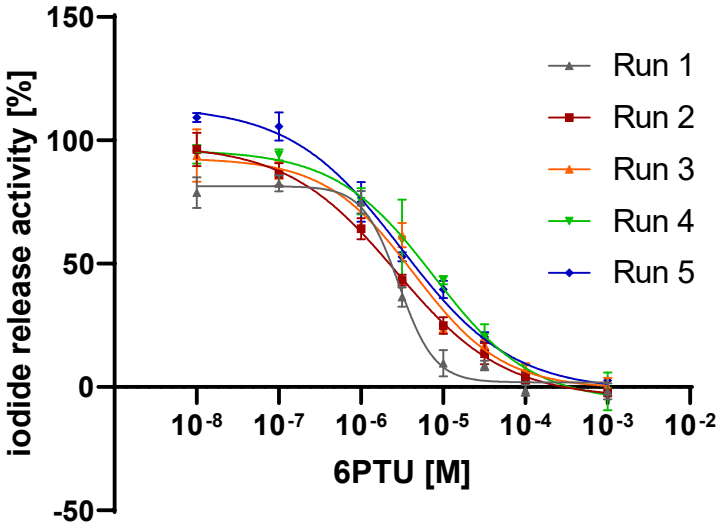

figure 4: Iodide release activity in the DIO1-SK assay after incubation with the test and reference item 6PTU in five independent assay runs. All 5 assay runs, each comprising of the triplicates of its individual valid assay runs, are shown as mean ± SD. A sigmoidal dose-response curve was derived using the "log(inhibitor) vs. response -- Variable slope (four parameters)".

The means of the triplicates of the individual 6PTU assay runs were plotted and curve fit was applied to generate a dose-response curve resulting in a calculated IC_{50} of 3.84 µM for 6PTU (95% Confidence interval (C.I.): 2.63 to 5.66 µM) for the combined assay runs (figure 5).

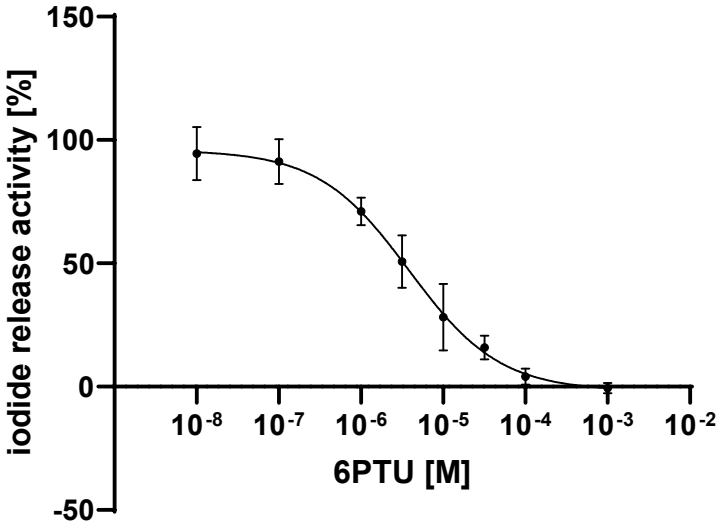

figure 5: iodide release activity in human liver microsomes after incubation with the test item 6PTU. Each data point results from the mean values of the respective assay run. A sigmoidal dose-response curve was derived using the "log(inhibitor) vs. response -- Variable slope (four parameters)".

9.4.3 5-Propyl-2-thiouracil (5PTU)

Human liver microsome incubated with the test item 5PTU was tested for iodide release inhibition at the final assay concentrations of 0.01, 0.1, 1, 3.16, 10, 31.6, 100 and 1000 µM in five independent assay runs in the DIO1-SK assay. The mean, SD and derived IC_{50} of the triplicates for the individual assay runs for 5PTU are shown in table 27; based on the data, based on the data a curve was fitted which is shown in figure 6.

table 27: mean and standard deviation (SD) of iodide release activity in the five assay runs after incubation with the test item 5PTU. Mean and SD was calculated from the triplicates of the respective assay run.

		Run 1		Run 2		Run 3		Run 4		Run 5	
Concentration [µM]	**Iodide release activity [%]**										
1000	**Mean [%]**	-5.16		-5.18		-2.60		7.92		-5.53	
	SD [%]		0.81		3.06		2.15		6.21		2.01
100	**Mean [%]**	-6.15		-1.84		1.88		3.37		-2.44	
	SD [%]		1.74		1.71		2.93		3.34		1.30
31.6	**Mean [%]**	1.46		2.16		7.54		5.65		7.11	
	SD [%]		4.39		1.45		1.63		1.69		4.80
10	**Mean [%]**	0.33		7.29		17.39		14.67		18.01	
	SD [%]		1.69		1.73		3.05		8.34		4.12
3.16	**Mean [%]**	22.52		19.86		31.09		24.24		45.49	
	SD [%]		6.66		1.54		6.73		5.71		8.23
1	**Mean [%]**	42.77		44.58		62.51		49.52		61.63	
	SD [%]		1.15		3.04		2.90		2.22		1.15
0.1	**Mean [%]**	81.65		81.99		89.53		82.78		87.15	
	SD [%]		8.39		7.13		1.75		7.95		5.11
0.01	**Mean [%]**	85.84		91.98		93.93		83.15		100.41	
	SD [%]		7.88		3.09		9.84		6.11		7.67
IC50	**[µM]**	1.16		0.95		1.81		1.22		2.46	

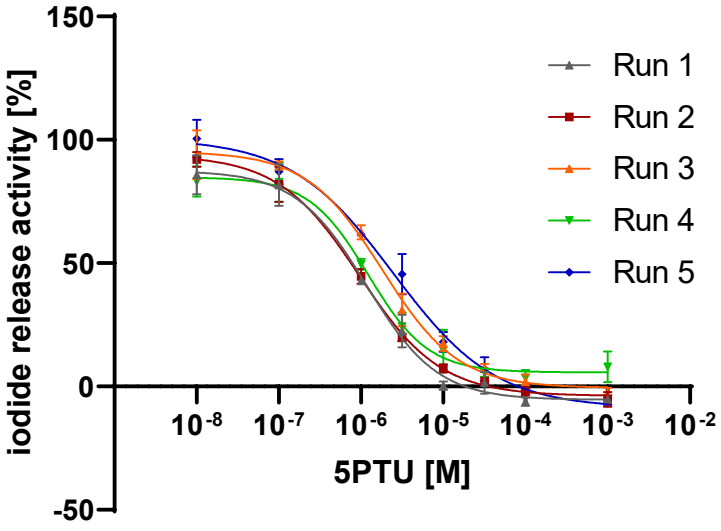

figure 6: Iodide release activity in the DIO1-SK assay after incubation with the test item 5PTU in five independent assay runs. All 5 assay runs, each comprising of the triplicates of its individual valid assay runs, are shown as mean ± SD. A sigmoidal dose-response curve was derived using the "log(inhibitor) vs. response -- Variable slope (four parameters)".

The means of the triplicates of the individual 5PTU assay runs were plotted and curve fit was applied to generate a dose-response curve resulting in a calculated IC_{50} of 1.41 µM for 5PTU (95% C.I.: 1.03 to 1.86 µM) for the combined assay runs (figure 7). 5PTU was described as the DIO1 inhibitor with the highest relative activity of DIO1 inhibition compared to various other 2-thiouracil analogues, also being more potent as 6PTU (Visser, Van Overmeeren et al. 1979). The normalization of the more potent 5PTU data to

the reference item 6PTU might explain inhibition below zero percent for 5PTU as observed in figure 6 and figure 7.

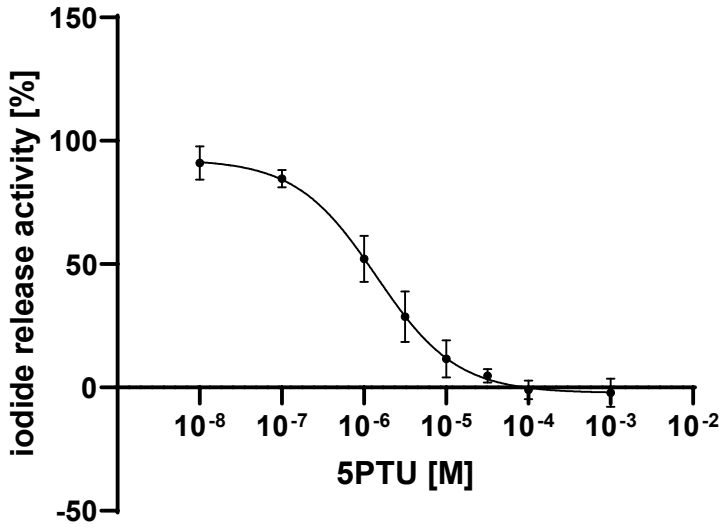

figure 7: iodide release activity in human liver microsomes after incubation with the test item 5PTU. Each data point results from the mean values of the respective assay run. A sigmoidal dose-response curve was derived using the "log(inhibitor) vs. response -- Variable slope (four parameters)".

9.4.4 Aurothioglucose (ATG)

Human liver microsome incubated with the test item and positive control ATG was tested for iodide release inhibition at the final assay concentrations of 0.001, 0.01, 0.1, 0.316, 1, 3.16, 10 and 100 µM in five independent assay runs in the DIO1-SK assay. The mean, SD and derived IC_{50} of the triplicates for the individual assay runs for ATG are shown in table 28; based on the data, based on the data a curve was fitted which is shown in figure 8.

table 28: mean and standard deviation (SD) of iodide release activity in the five assay runs after incubation with the test item and positive control ATG. Mean and SD was calculated from the triplicates of the respective assay run.

		Run 1		Run 2		Run 3		Run 4		Run 5	
Concentration [µM]	**Iodide release activity [%]**										
100	**Mean [%]**	0.60		-3.65		0.03		-3.86		-0.53	
	SD [%]		0.96		4.06		0.64		1.89		3.59
10	**Mean [%]**	2.91		-0.50		7.18		2.50		3.13	
	SD [%]		2.34		1.07		1.98		1.73		0.21
3.16	**Mean [%]**	8.59		6.87		18.24		15.99		18.82	
	SD [%]		4.38		2.03		2.19		4.38		5.50
1	**Mean [%]**	16.14		22.78		38.68		41.11		36.10	
	SD [%]		5.28		2.27		4.22		4.05		3.87
0.32	**Mean [%]**	38.01		46.76		59.60		62.33		60.98	
	SD [%]		3.36		2.13		4.75		12.84		6.40
0.1	**Mean [%]**	64.43		72.50		87.22		77.35		80.98	
	SD [%]		3.37		1.90		7.96		1.91		4.78
0.01	**Mean [%]**	88.69		95.46		95.50		93.80		93.70	
	SD [%]		1.03		4.58		5.85		2.83		4.06

0.001	**Mean [%]**	87.08		95.73		97.32		99.51		95.57	
	SD [%]		11.77		5.21		11.05		5.70		3.88
IC50	**[µM]**	0.23		0.33		0.6		0.71		0.63	

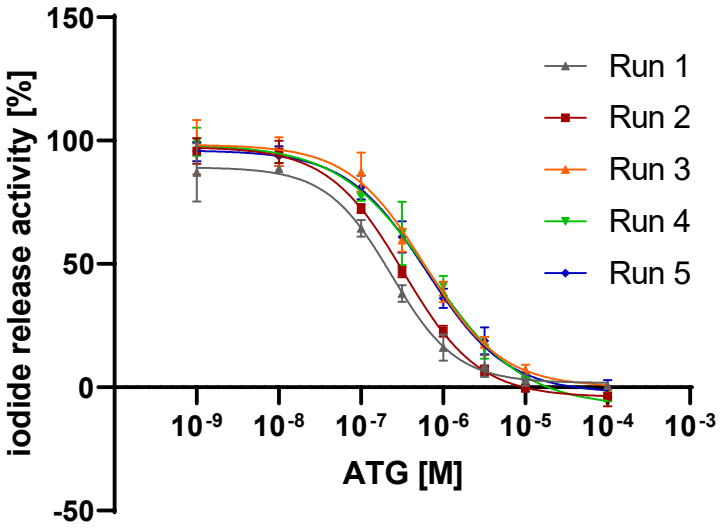

figure 8: Iodide release activity in the DIO1-SK assay after incubation with the test item and positive control ATG in five independent assay runs. All 5 assay runs, each comprising of the triplicates of its individual valid assay runs, are shown as mean ± SD. A sigmoidal dose-response curve was derived using the "log(inhibitor) vs. response -- Variable slope (four parameters)".

The means of the triplicates of the individual ATG assay runs were plotted and curve fit was applied to generate a dose-response curve resulting in a calculated IC_{50} of 0.46 µM (95% C.I.: 0.35 to 0.60 µM) for ATG for the combined assay runs (figure 9).

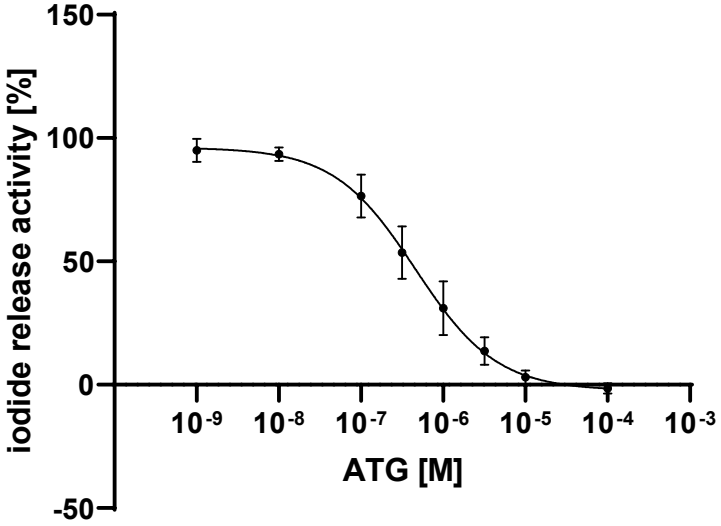

figure 9: iodide release activity in human liver microsomes after incubation with the test item and positive control ATG. Each data point results from the mean values of the respective assay run. A sigmoidal dose-response curve was derived using the "log(inhibitor) vs. response -- Variable slope (four parameters)".

9.4.5 Genistein (GEN)

Human liver microsome incubated with the test item GEN was tested for iodide release inhibition at the final assay concentrations of 0.001, 0.0032, 0.01, 0.032, 0.1, 0.32, 1 and 3.16 µM in five independent assay runs in the DIO1-SK assay. The mean, SD and derived IC_{50} of the triplicates for the individual assay runs for GEN are shown in table 29; based on the data, based on the data a curve was fitted which is shown in figure 10.

table 29: mean and standard deviation (SD) of iodide release activity in the five assay runs after incubation with the test GEN. Mean and SD was calculated from the triplicates of the respective assay run.

		Run 1		Run 2		Run 3		Run 4		Run 5	
Concentration [µM]	**Iodide release activity [%]**										
3.160	**Mean [%]**	46.96		49.80		71.62		49.21		78.03	
	SD [%]		3.74		11.88		5.52		8.03		6.23
1.000	**Mean [%]**	72.25		78.96		91.48		68.23		90.39	
	SD [%]		5.92		6.62		5.21		13.86		2.40
0.316	**Mean [%]**	77.92		90.07		102.68		83.58		102.08	
	SD [%]		2.26		1.39		1.08		5.75		4.16
0.100	**Mean [%]**	92.00		100.05		105.89		84.00		110.47	
	SD [%]		5.99		0.37		0.67		18.07		0.95
0.032	**Mean [%]**	96.68		95.67		98.75		105.54		88.61	
	SD [%]		9.90		7.87		3.11		11.05		0.94
0.010	**Mean [%]**	92.50		96.64		101.37		86.15		91.02	
	SD [%]		8.32		4.58		3.55		5.89		5.29
0.003	**Mean [%]**	100.29		97.49		103.52		86.70		111.32	
	SD [%]		9.12		5.18		1.14		6.15		10.57
0.001	**Mean [%]**	88.54		101.01		106.99		76.89		113.46	
	SD [%]		5.41		1.30		0.77		13.76		3.10
IC50	**[µM]**	6.76		5.43		1.21		1.16		Ambiguous	

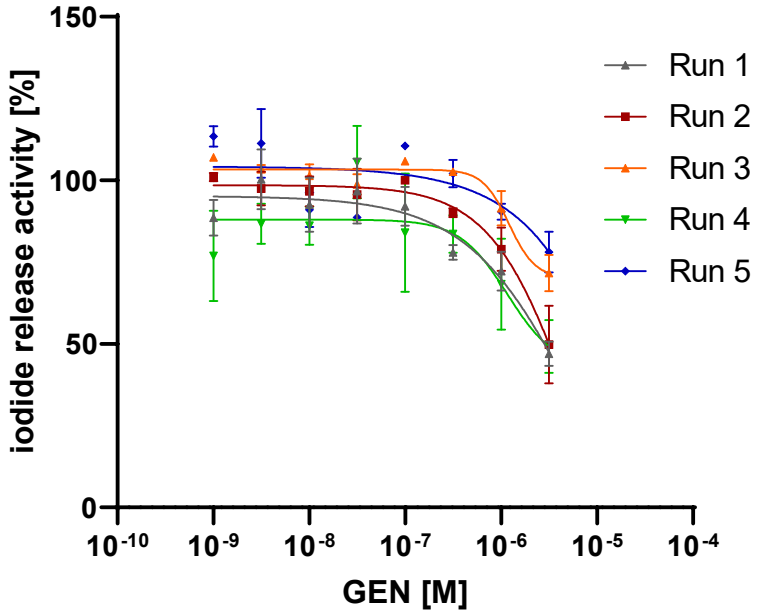

figure 10: Iodide release activity in the DIO1-SK assay after incubation with the test item GEN in five independent assay runs. All 5 assay runs, each comprising of the triplicates of its individual valid assay runs, are shown as mean ± SD. A sigmoidal dose-response curve was derived using the "log(inhibitor) vs. response -- Variable slope (four parameters)".

The means of the triplicates of the individual GEN assay runs were plotted and curve fit was applied to generate a dose-response curve resulting in a calculated IC_{50} of 1.93 µM (95% C.I.: not determinable) for GEN for the combined assay runs (figure 11). The fitted curve for GEN with the tested concentrations did not cover a full dose-response relationship and the true bottom of the curve could not be determined with a high degree of certainty. The derived IC_{50} should therefore also be treated with caution.
Higher concentrations of GEN concentrations might lead to higher iodide release inhibition and more complete dose-response curve but were not tested based on precipitations in higher concentrations of GEN in the predilutions and under final assay conditions (see Appendix 14.3, table 35).

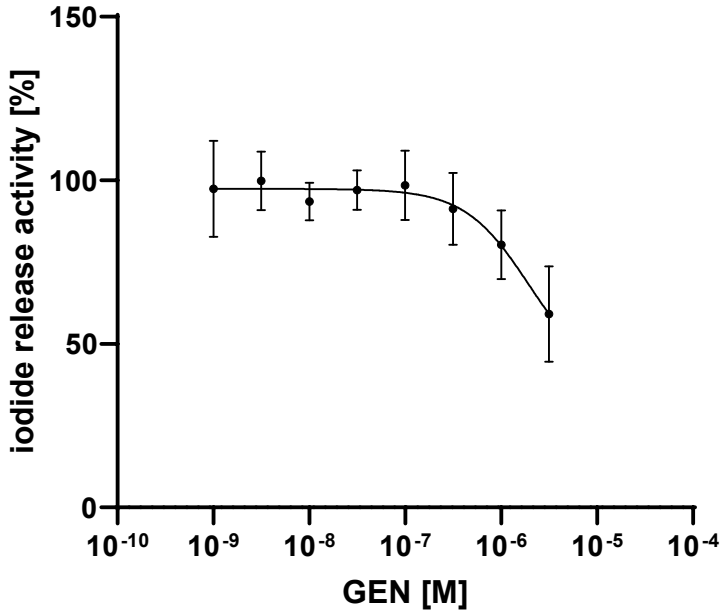

figure 11: iodide release activity in human liver microsomes after incubation with the test item positive control GEN. Each data point results from the mean values of the respective assay run. A sigmoidal dose-response curve was derived using the “log(inhibitor) vs. response -- Variable slope (four parameters)”.

9.4.6 Tetrabromobisphenol A (TBBPA)

Human liver microsome incubated with the test item TBBPA was tested for iodide release inhibition at the final assay concentrations of 0.0032, 0.01, 0.032, 0.1, 0.32, 1, 3.16 and 10 µM in five independent assay runs in the DIO1-SK assay. The mean, SD and derived IC_{50} of the triplicates for the individual assay runs for TBBPA are shown in table 30; based on the data, based on the data a curve was fitted which is shown in figure 12.

table 30: mean and standard deviation (SD) of iodide release activity in the five assay runs after incubation with the test TBBPA. Mean and SD was calculated from the triplicates of the respective assay run.

		Run 1		Run 2		Run 3		Run 4		Run 5	
Concentration [µM]	**Iodide release activity [%]**										
10	**Mean [%]**	38.13		35.25		63.42		40.29		67.37	
	SD [%]		7.05		2.57		1.98		6.09		1.88
3.16	**Mean [%]**	75.67		81.72		91.06		76.91		87.71	
	SD [%]		9.09		3.51		0.55		4.22		3.12
1	**Mean [%]**	81.79		86.63		99.85		88.48		105.79	
	SD [%]		6.38		2.67		2.41		5.02		1.72
0.32	**Mean [%]**	87.01		101.31		108.13		92.32		101.10	
	SD [%]		7.27		1.12		1.90		2.77		6.41
0.1	**Mean [%]**	97.27		94.89		92.11		91.77		91.55	
	SD [%]		2.65		2.33		4.04		8.40		1.74
0.032	**Mean [%]**	97.54		97.10		101.79		88.54		95.12	
	SD [%]		3.58		1.26		0.81		2.47		4.33
0.01	**Mean [%]**	93.40		93.70		103.31		88.38		104.63	
	SD [%]		8.94		3.10		3.39		10.06		4.58
0.0032	**Mean [%]**	83.95		92.73		109.82		95.17		109.53	

	SD [%]		15.22		7.02		1.80		5.67		8.35
IC50	**[µM]**	Ambiguous		Ambiguous		7.71		8.01		Ambiguous	

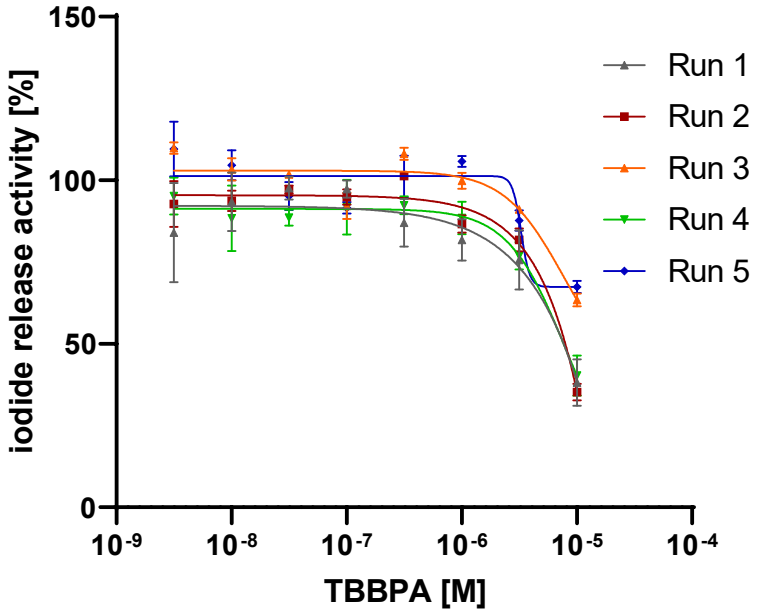

figure 12: Iodide release activity in the DIO1-SK assay after incubation with the test item TBBPA in five independent assay runs. All 5 assay runs, each comprising of the triplicates of its individual valid assay runs, are shown as mean ± SD. A sigmoidal dose-response curve was derived using the “log(inhibitor) vs. response -- Variable slope (four parameters)”.

The means of the triplicates of the individual TBBPA assay runs were plotted and curve fit was applied to generate a dose-response curve resulting in a calculated IC_{50} of 22.09 µM (95% C.I.: not determinable) for TBBPA for the combined assay runs (figure 13).
The fitted curve for TBBPA with the tested concentrations did not cover a full dose-response relationship and the true bottom of the curve could not be determined with a high degree of certainty. The derived IC_{50} should therefore also be treated with caution.
Higher concentrations of TBBPA concentrations might lead to higher iodide release inhibition and more complete dose-response curve but were not tested based on precipitations in higher concentrations of TBBPA in the predilutions and under final assay conditions (see Appendix 14.3, table 35).

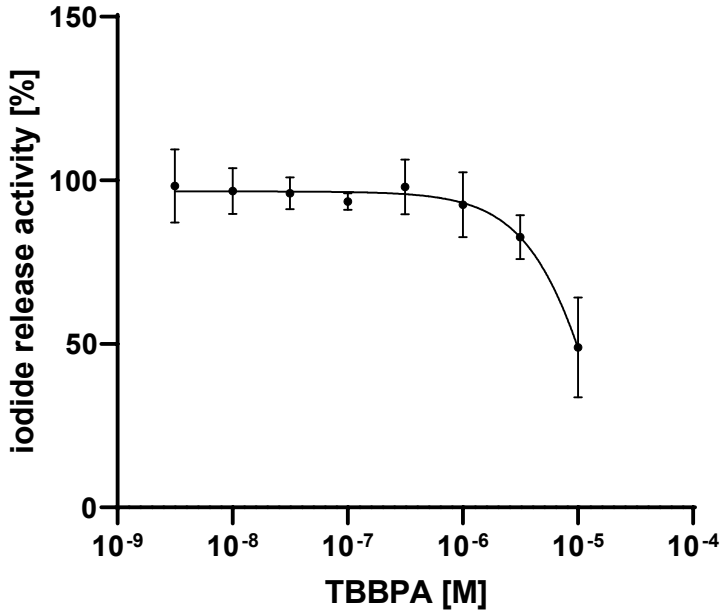

figure 13: iodide release activity in human liver microsomes after incubation with the test item positive control GEN. Each data point results from the mean values of the respective assay run. A sigmoidal dose-response curve was derived using the “log(inhibitor) vs. response -- Variable slope (four parameters)”.

9.4.7 2-Chloro-N-phenylacetamide (2CPA)

Human liver microsome incubated with the test item 2CPA was tested for iodide release inhibition at the final assay concentrations of 0.1, 1, 2, 6, 10, 20, 60 and 100 µM in five independent assay runs in the DIO1-SK assay. The mean, SD and derived IC_{50} of the triplicates for the individual assay runs for 2CPA are shown in table 31; based on the data, based on the data a curve was fitted which is shown in figure 14. In assay run 1 from 28.09.2020 including 2CPA, 2 concentrations of 2CPA were excluded from data evaluation based on technical errors during preparation of the 2CPA dilutions.

table 31: mean and standard deviation (SD) of iodide release activity in the five assay runs after incubation with the test 2CPA. Mean and SD was calculated from the triplicates of the respective assay run. *: indicates concentrations that were excluded from analysis based on a technical error during assay performance.

		Run 1		Run 2		Run 3		Run 4		Run 5	
Concentration [µM]	**Iodide release activity [%]**										
100	**Mean [%]**	-2.88		-0.70		6.63		7.98		6.79	
	SD [%]		5.22		0.98		2.20		1.93		3.08
60	**Mean [%]**	*14.18**		0.81		10.23		5.83		5.08	
	SD [%]		*6.61**		1.35		2.79		2.53		3.44
20	**Mean [%]**	3.50		12.47		26.20		17.73		24.88	
	SD [%]		4.45		2.68		2.17		6.78		3.18
10	**Mean [%]**	20.46		31.63		56.45		31.91		42.11	
	SD [%]		2.92		1.96		5.90		2.59		14.30
6	**Mean [%]**	*81.98**		47.77		64.73		56.39		67.68	
	SD [%]		*5.83**		4.46		5.68		3.80		4.93

2	**Mean [%]**	56.58		71.63		87.00		71.36		79.59	
	SD [%]		4.42		1.30		1.62		10.77		3.84
1.	**Mean [%]**	70.95		82.96		96.04		75.29		86.87	
	SD [%]		3.39		1.47		4.73		4.01		0.99
0.1	**Mean [%]**	81.05		98.78		105.63		78.97		107.36	
	SD [%]		8.99		2.39		2.17		13.04		16.26
IC50	**[µM]**	4.45		5.79		9.8		8.32		8.59	

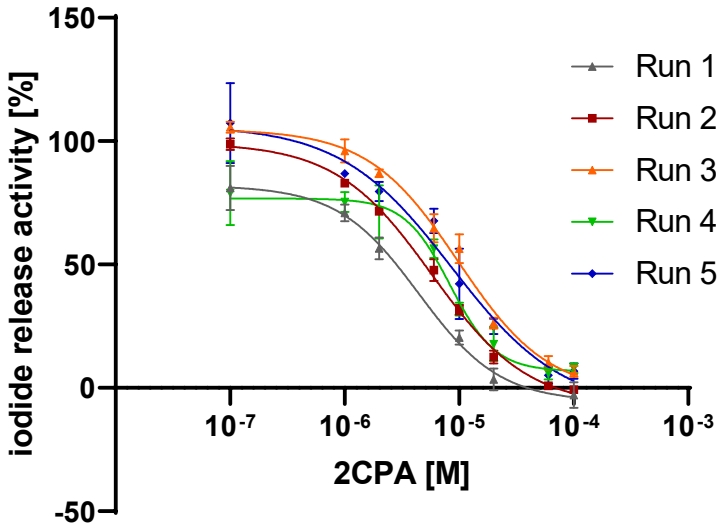

figure 14: Iodide release activity in the DIO1-SK assay after incubation with the test item 2CPA in five independent assay runs. All 5 assay runs, each comprising of the triplicates of its individual valid assay runs, are shown as mean ± SD. A sigmoidal dose-response curve was derived using the “log(inhibitor) vs. response -- Variable slope (four parameters)”.

The means of the triplicates of the individual 2CPA assay runs were plotted and curve fit was applied to generate a dose-response curve resulting in a calculated IC_{50} of 7.62 µM (95% C.I.: 5.24 to 11.62 µM) for 2CPA for the combined assay runs (figure 15).

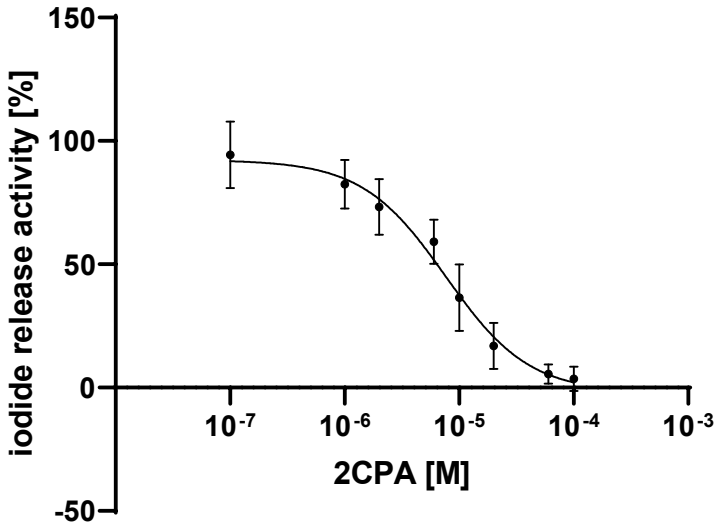

figure 15: iodide release activity in human liver microsomes after incubation with the test item positive control 2CPA. Each data point results from the mean values of the respective assay run. A sigmoidal dose-response curve was derived using the “log(inhibitor) vs. response -- Variable slope (four parameters)”.

9.5 Reproducibility of performed assay runs

The assay runs in this “part 1: Reproducibility Assessment” was performed by three different laboratory staff members to reduce any personal influence on the generation of the results.

In order to assess variability within a performed assay plate, the number of triplicates with a coefficient of variation (CV) over 20, 25 and 30 % over the performed assay plates was monitored (Appendix, table 36). There were hardly any differences between the triplicates of the respective control or test item across the 10 assay plates carried out.

Monitoring the control items of the assay can provide further information about the variability between the different assay plates. The reference item as well solvent, negative, and positive control values of the ten performed assay plates on five different days is shown in figure 16 and indicated reproducibility between the 10 assay plates. The solvent control (final assay concentration: 1 % DMSO) as well as the negative control values usually ranged between 85 and 115 % iodide release activity over the five assay days. The mean values of the negative control values ranged between 90 and 110 %. The iodide release inhibiting control values of 6PTU as the reference item and ATG as the positive control usually ranged between -10 to 10 % iodide release activity. The mean of the positive control did not exceed 10 % of iodide release activity.

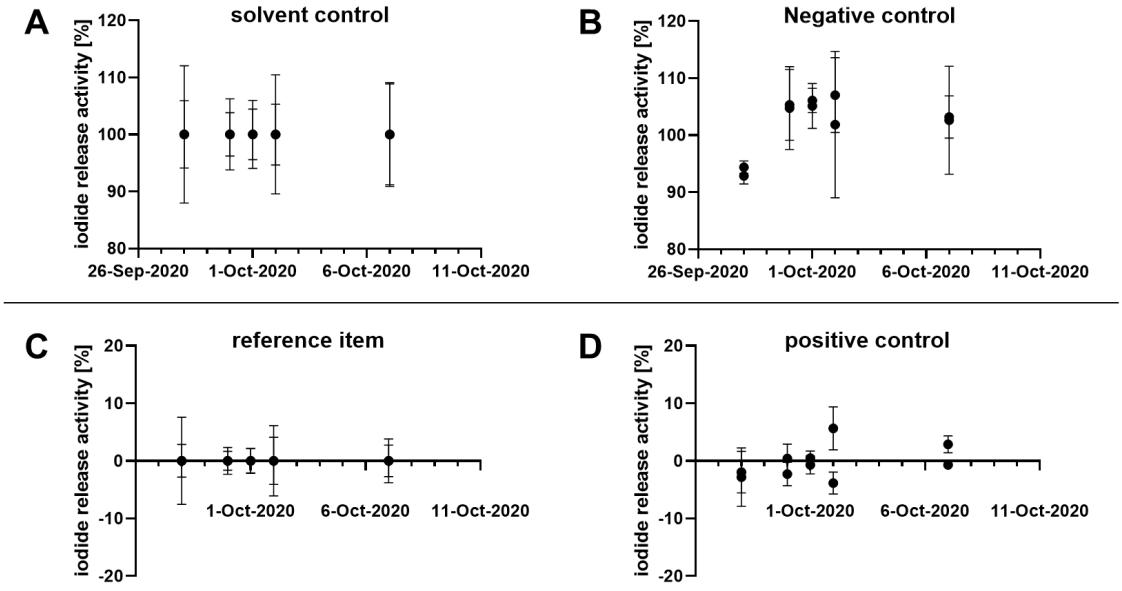

figure 16: Temporal display of the control development of **A** solvent (DMSO) control, **B** negative control (1-Thio-β-D-glucose sodium salt; TGSS), **C** reference item (6-Propyl-2-thioruracil, 6PTU) and **D** positive control (Aurothioglucose, ATG). Shown is the mean and standard deviation of the nine replicates (for reference item and DMSO control) and three replicates (for negative and positive control) of each assay plate on the five assay days. Data was normalized to the reference item 6PTU (0 % iodide release activity) and the solvent control (100 % iodide release activity).

9.6 Threshold limits based on untreated controls in the DIO1-SK assay

As with every *in vitro* method, the control data in the DIO1-SK assay should be considered for the determination of a threshold for substance-induced effects.

To distinguish between a substance-induced effect from variation of the untreated controls that may occur by change, it is important to consider the variability within the study and between the studies

One possibility is the determination of a prediction limit for the group mean which takes only the variance within the groups into account.
The lower limit of the prediction interval is used which is an estimate of an interval in which a future mean of the control data will fall with 95 % possibility based on the values of the current control data. This can be taken as a threshold value below which a substance-induced effect might lie.
The variability between the studies was not considered because the data is highly affected by the study. For the comparison of the data between the studies only the standardized values relative to the solvent control means are examined. Therefore, the variability between the studies cannot be estimated.

$$\mu_{SC} \pm S.D_{SC} * \sqrt{\frac{1}{n} + \frac{1}{n_{group}}} * t_{n-1;0.95}$$

μ_{SC} = mean of the solvent control values
$S.D_{SC}$ = standard deviation of the solvent control values
n = number of the current solvent controls
n_{group} = number of the concentration group
$t_{n-1;0.95}$ = t-quantile with n-1 degrees of freedom

For the control mean of the solvent controls of an assay run, the 95 % lower prediction limit based on nine replicate solvent control values for a new, estimated triplicate mean value was calculated for each of the respective assay runs (table 32). Most of the lower limit prediction intervals per assay run do not fall below 90 % iodide release activity but on some assay plates with higher variation the lower limit prediction interval was lower (down to 88.2 %). Based on the 5 conducted assay runs in each case consisting of 2 assay plates, it is highly likely that a newly derived triplicate of an unknown test item with a mean iodide release activity <88.2 % is a substance-mediated effect since the solvent control values usually distribute less. To account for cases of higher variance in assay plates, a proposed threshold for the definition of a test item-mediated effect might be set to 80 % iodide release activity. This value might change based on upcoming data in the validation process of the method.

table 32: derived prediction limit values for the solvent control replicates of the respective assay runs. The lower limit prediction value was calculated based on the nine replicates of each assay run and was predicted for a mean triplicate value of solvent controls.

Date	Tested test items on the assay plate	Lower limit of the prediction interval per assay run for the mean of an estimated triplicate (expressed as iodide release activity in [%])
28.09.2020	6PTU, 2CPA, ATG	94.2
28.09.2020	5PTU, GEN, TBBPA	88.2
30.09.2020	ATG, 6PTU, GEN	94
30.09.2020	2CPA, 5PTU, TBBPA	96.4

01.10.2020	6PTU, 5PTU, ATG	95.8
01.10.2020	GEN, 2CPA, TBBPA	94.5
02.10.2020	TBBPA, 6PTU, ATG	89.8
02.10.2020	GEN, 2CPA, 5PTU	95.3
07.10.2020	TBBPA, GEN, 6PTU	91.9
07.10.2020	5PTU, ATG, 2CPA	91.7

10 Definition of acceptance criteria for valid assay runs

In coordination with EURL ECVAM, several numeric and binary acceptance criteria covering the potency and variability of the used controls as well as assessing the overall variability in the assay were chosen to be suitable to assess the validity of future assay runs in the DIO1-SK assay. The values of the acceptance criteria for the generated assay data of this study was evaluated and is presented in table 33.

table 33: proposed acceptance criteria for the DIO1-SK assay and their respective values in this study. Mean and standard deviation (SD) was calculated from the corresponding values of the 10 valid assay plates.

Acceptance criteria		
Numeric	**Mean**	**±SD**
IC_{50} of reference item [µM]	4.12	1.94
CV of log IC_{50} estimate of reference item [%]	1.28	0.47
Ratio of 6PTU normalized negative control/solvent control [%]	102.20	4.86
Ratio of 6PTU normalized positive control/solvent control [%]	-0.28	2.83
z-Factor	0.65	0.14
Binary	**Yes / no**	
Shape of reference item (sigmoidal?)	Yes (In all valid assay plates)	
The final dose response curve of the reference item is composed of minimum six concentrations from three replicates	Yes (In all valid assay plates	
The final dose response curve of the test item is composed of minimum six concentrations from three replicates	Yes (In all valid assay plates	

In consultation with EURL ECVAM cut off values were defined for the proposed acceptance criteria on the basis of the data generated in this part 1 study of the DIO1-SK assay (table 34).

table 34: Acceptance criteria for future runs of the DIO1-SK assay.

Acceptance criteria	**Cut-off value**
Numeric	
IC_{50} of reference item [µM]	1 < x <10
CV of log IC_{50} estimate of reference item [%]	x <3
Ratio of 6PTU normalized negative control/solvent control [%]	80 < x < 120
Ratio of 6PTU normalized positive control/solvent control [%]	x < 20
z-Factor	x > 0.5
Binary	**Yes / no**
Shape of reference item (sigmoidal?)	x = yes
The final dose response curve of the reference item is composed of minimum six concentrations from three replicates	x = yes

The final dose response curve of the test item is composed of minimum six concentrations from three replicates	x = yes

11 Records to be retained

All raw data, the performed data analysis in all software as well as data visualization will be retained for 2 years and sent to EURL ECVAM after completion of the study.

12 Definitions and abbreviations

2CPA:	2-Chloro-N-phenylacetamid
5PTU:	5-Propyl-2-thiouracil
6PTU:	6-Propyl-2-thiouracil
95% C.I.:	95% Confidence interval
ATG:	Aurothioglucose
CV:	coefficient of variation
ddH_2O:	double-distilled water
DIO:	deiodinase
DIO1:	deiodinase I
DIO2:	deiodinase II
DIO3:	deiodinase III
DMSO:	dimethylsulfoxid
DTT:	Dithiothreitol
EDTA:	Ethylenediaminetetraacetic acid
EURL ECVAM:	European Union Reference Laboratory for alternatives to animal testing
GEN:	Genistein
GIVIMP:	Guidance Document on Good In Vitro Method Practices
OD:	optical denstiy
rT3:	reverse T3
SD:	standard deviation
SK:	Sandell-Kolthoff
SOP:	standard operation procedure
TBBPA:	Tetrabromobisphenol A
TGSS:	1-Thio-β-D-glucose sodium salt
IC_{50}:	50% inhibition concentration

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14 Appendix

14.1 SOP DIO1-SK (29.09.2020) assay

Standard Operation Procedure (SOP)

Colorimetric method for assessing deiodinases activity based on Sandell-Kolthoff reaction with human microsomes: DIO1-SK assay

AUTHOR

BASF SE

1. INTRODUCTION

1.1. BACKGROUND AND OBJECTIVE

The deiodination of thyroid hormone plays a fundamental role in the regulation of thyroid hormone concentration. The Deiodinase 1 (DIO1) is thought to possess iodide recycling capacity through the deiodination of the inactive reverse T3 (rT3) but is also capable to deiodinate thyroid hormone substrates towards T3 or 3,3'-T2 (Figure 1). The objective of this assay is to assess the functional capacity of the Deiodinase I (DIO1) enzyme to deiodinate thyroid hormone after application of chemicals.

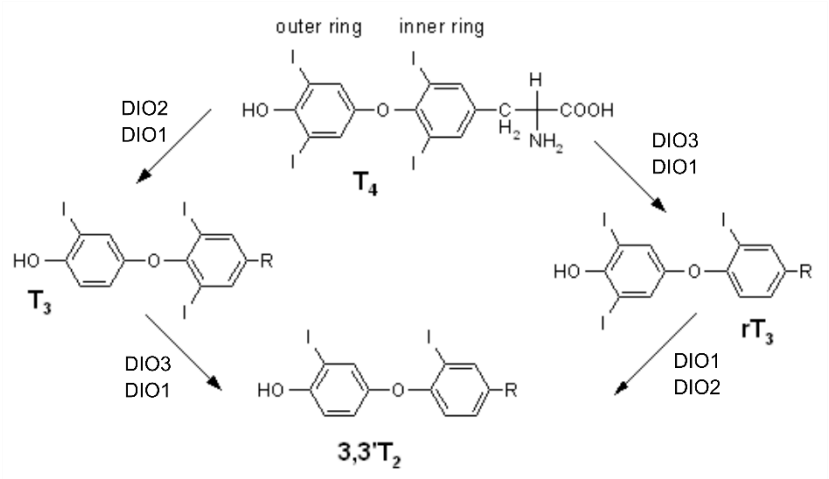

Figure 1: Metabolism of thyroid hormone

This method uses the "Sandell-Kolthoff-reaction", a nonradioactive, colorimetric reaction which can be used to measure free iodide concentration. The reaction is based on the reduction of cerium (IV) to cerium (III) and oxidation of arsenite (III) to arsenite (V) depending on the available iodide concentration. The yellow coloured cerium (IV) loses its colour after the reduction to cerium (III) which can be visualized through measurement of the optical density (OD) before and after the reaction, typically measured at between 405 to 420 nm (Figure 2). The Sandell-Kolthoff reaction can be influenced by several ions and molecules like impurities of different iodide species, metal ions like silver or mercury or substances with strong oxidizing capacities.

Monitoring the performance of the Sandell-Kolthoff reaction over time is an important step to control the quality and functionality of the assay. Regularly performed iodide standard curves in the Sandell-Kolthoff reaction can be used to identify systemic changes and to assure the quality of the assay.

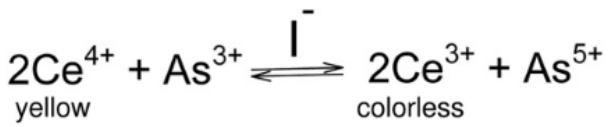

Figure 2: Sandell-Kolthoff-reaction

This method uses microsomes, broken-down, vesicle-like pieces of endoplasmic reticula from hepatocytes, as an enzyme source for DIO, mainly DIO1. The microsomes possess also metabolizing enzymes (e.g. cytochrome P450s, Flavin-containing monooxygenase, uridine 5'-diphospho-glucuronosyltransferases, carboxylesterases) which might influence the test system through metabolism of the test compounds (Knights et al, 2016). This can lead to different inhibition properties compared to assays with purified DIO1 enzymes.

The method requires an initial iodide release activity testing run to determine the batch-specific iodide release activity of the microsome batch since suppliers usually do not test for iodide release activity. By using different microsome concentrations of the specific microsome batch, an enzyme concentration-iodide release activity curve can be derived which will be used to define a microsome batch-specific enzyme concentration for the assay runs. Furthermore, an initial assay run to define the appropriate dose range of the test items for the main assay runs (range finding assay) with the test items is performed.

This *in vitro* method is suitable for high to medium throughput screenings as well as creating mechanistical information for the inhibition of the DIO1 enzyme. It should be noted that based on the available information regarding the used reagents and chemicals, the entire method is animal free.
The measurement of endogenous DIO activities via this assay is therefore limited to rich sources of enzymatic activity. Furthermore, it is not possible to analyse iodide containing substances since the assay cannot differentiate between released iodide from the test substance and the used substrate.

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2. MATERIALS AND METHODS

Table 1: Used apparatus in the DIO1-SK assay

Apparatus	Requirements[1] Suggested type[2]
Analytical balance	capable of accurately weighing up to 30 g with 0.1 mg readability[1]
Pipets capable of delivering 1 to 10 µL	
Pipets capable of delivering 10 to 100 µL	
Pipets capable of delivering 100 to 1000 µL	
Multichannel pipette capable of delivering 10 to 100 µL	
Multichannel dispenser capable of delivering 50 to at least 1000 µL	
Repeater pipette	
Pipets for higher volumes	serological pipettes, e.g. 10, 25, 50 mL[2]
Incubator	capable of keeping temperatures of 37°C, 5 % CO_2 and ≥90 % humidity[1]
pH meter with electrode and calibration buffers	capable of reading +/- 0.1 pH units[1]
Photometer for absorbance measurement	e.g., Sunrise™ Absorbance Reader, INSTSUN-3, Tecan Trading AG[2]
Plate shaker	e.g. Thermo Scientific H+P MONOSHAKE VORTEXER microtiter plate, directly controlled, Thermo Fisher Scientific[2]
Centrifuge with swing-out rotor for microtiter plates	Should be high enough to fit a 96-deep well plate with 96-well filter plate on top (at least about 6 cm high)[1]

Table 2: Used chemicals and reagents in the DIO1-SK assay

Chemicals / reagents	Requirements[1] Suggested type[2]
1-Thio-β-D-glucose sodium salt CAS: 10593-29-0 MW : 218.20 g/mol	e.g. 1-Thio-β-D-glucose sodium salt, S igma-Aldrich[2]
3,3’,5’-triiodothyronine (rT3) CAS: 5817-39-0 MW: 650.97 g/mol	e.g. 3,3′,5′-Triiodo-L-thyronine, Sigma-Aldrich[2]
6-Propyl-2-thiouracil (6PTU)	e.g. 6-Propyl-2-thiouracil, VETRANAL™, analytical standard, Supelco[2]

CAS: 51-52-5 MW: 170.23 g/mol	
Acetic acid CAS: 64-19-7 MW: 60.05 g/mol	e.g. acetic acid, glacial, ReagentPlus®, ≥99%, Sigma-Aldrich[2]
Arsenic sodium oxide (NaAsO2) CAS: 7784-46-5 MW: 129.91 g/mol	e.g. sodium (meta) arsenite, ≥90%, Sigma-Aldrich[2]
Aurothioglucose (ATG) CAS: 12192-57-3 MW: 392.18 g/mol (anhydrous basis)	e.g. aurothioglucose hydrate, ≥96% (titration), Sigma-Aldrich[2]
Cerium (IV) ammonium sulphate ($Ce(NH_4)_4(SO_4)_4$) CAS: 10378-47-9 MW: 632.55 g/mol	e.g. ammonium cerium (IV) sulphate dihydrate, Sigma-Aldrich[2]
Dimethyl sulfoxide (DMSO) CAS: 67-68-5 MW: 78.13 g/mol	e.g. dimethyl sulfoxide (Reag. Ph. Eur.) for analysis, ACS, PanReac AppliChem[2]
Dipotassium hydrogen phosphate (HK_2PO_4) CAS: 7758-11-4 MW: 174.18 g/mol	e.g. potassium phosphate dibasic, meets USP testing specifications, Sigma-Aldrich[2]
Dowex 50WX2 CAS: 12612-37-2	e.g. Dowex 50WX2 100 200 mesh ion exchange resin, Acros Organics[2]
Dithiothreitol (DTT) CAS: 3483-12-3 MW: 154.25 g/mol	e.g. DL-Dithiothreitol solution, BioUltra, for molecular biology, ~1 M in H_2O, Sigma-Aldrich[2]
Ethylenediaminetetraacetic acid (EDTA) CAS: 6381-92-6 MW: 372.24 g/mol	e.g. ethylenediaminetetraacetic acid disodium salt dihydrate, Sigma Grade, suitable for plant cell culture, 98.5-101.5 %, Sigma-Aldrich[2]
Human liver microsomes	e.g. Human Microsomes, 50 Donors, HMMCPL, Gibco[2] or Microsomes from Liver, Pooled, from human, Sigma-Aldrich[2]
Iodide (IC standard)	e.g. Iodide standard for IC, 1000 mg/L in water, Sigma-Aldrich[2]
Monopotassium phosphate (H_2KPO_4) CAS: 7778-77-0 MW: 136.09 g/mol	e.g. potassium phosphate monobasic, powder, suitable for cell culture, suitable for insect cell culture, suitable for plant cell culture, ≥99.0%, Sigma-Aldrich[2]
Sodium chloride (NaCl) CAS: 7647-14-5	e.g. sodium chloride, ACS reagent, ≥99.0%, Sigma-Aldrich[2]

MW: 58.44 g/mol	
Sulfuric acid (H_2SO_4) CAS: 7664-93-9 MW: 98.08 g/mol	e.g. sulfuric acid, Supelco[2]

Table 3: Material that is used in the DIO1-SK assay.

Material:	Requirements[1] Suggested type[2]
Volumetric flask	certified with defined volume[1]
Filter plates (96 well format)	e.g. UNIFILTER Microplate, 96-well, 800 µl, GF/C, clear polystyrene, filter bottom with long drip director, GE Healthcare Life Sciences[2]
Deep well plates (96 well format)	e.g. SPE 96-Deep Square Well Collection Plate, well volume 2 mL, polypropylene, Sigma Aldrich[2]
Assay plates (96 well format)	e.g. tissue culture plates, 96 well plate, flat bottom, polystyrene, 0.34 cm^2, sterile, 108/cs, TPP[2]
Gas-tight plate sealers	e.g. Sealing tape, polyester, sterile, Sealing tape, polyester, sterile, Nunc[2]
Microcentrifuge tubes 1,5 mL	e.g. Eppendorf® Safe-Lock microcentrifuge tubes, volume 1.5 mL, natural, Eppendorf AG[2]
Centrifuge Tubes 15 and 50 mL	e.g. TPP® centrifuge tubes, volume 50 mL, polypropylene, TPP[2] e.g. TPP® centrifuge tubes, volume 15 mL, polypropylene, TPP[2]

Table 4: Software that is used in the DIO1-SK assay

Software	Requirements[1] Suggested type[2]
Statistics software	Able to perform regression analysis that reflect assay characteristics and able to calculate inhibitory concentrations[1] e.g. GraphPad Prims 8, GraphPad[2]

3. CONTROLS AND TEST ITEMS

3.1. CONTROLS

In the DIO1-SK assay one reference item as well as a positive and negative control is used. Also, solvent controls for all solvents that are use to solubilize your controls and test items are done in all the experiments (Table 5).

Table 5: Controls that are used in the DIO1-SK assay.

Controls:	
Reference item (RI)	A substance that causes a known DIO inhibition / decrease in measured ΔOD-BG in the test system. Used in this test system: 6-Propyl-2-thiouracil
Positive control (PC)	A substance that lead to DIO inhibition in the test system Used in this test system: Aurothioglucose
Negative control (NC)	A substance that does not lead to DIO inhibition in the test system Used in this test system: 1-Thio-β-D-glucose sodium salt
Solvent control (SC)	Control without test item/PC/NC but contains the same solvent concentration as in the assay (e.g. DMSO) Final solvent concentration in the assay: 1 % (1 % DMSO does not interfere with the assay)

3.1.1. Reference item

The reference item 6-Propyl-2-thiouracil (6PTU) is a known DIO1 inhibitor and is used in this assay for a first normalization step to subtract background signal from the generated data (Table 6). Additionally, the reference item 6PTU is used to derive an inhibitory concentration of 50 % (IC_{50}) of measured iodide release activity on a day-to-day basis to monitor assay performance. The generation of a 6PTU IC_{50} is always required on the first plate of an assay day; additional runs on the same day do not require further 6PTU dose-response testing. Repeat the generation of a 6PTU IC_{50} through dose-response testing of 6PTU if there are changes in assay conditions between assay runs on the same day (e.g. different microsome batch, new arsenic/cerium solution, …).

Table 6: Information on the reference item 6-Propyl-2-thiouracil

Name:	6-Propyl-2-thiouracil
CAS No.:	51-52-5
Molecular weight [g/mol]:	170.23
Storage conditions:	RT
Solvent	DMSO
Stock solution [mol/L]:	10^{-1} M

3.1.2. Positive control

Table 7: Information on the positive control Aurothioglucose

Name:	Aurothioglucose
CAS No.:	12192-57-3
Molecular weight [g/mol]:	392.18
Storage conditions:	4°C
Solvent	DMSO
Stock solution [mol/L]:	10^{-2} M
Storage conditions of stock solution	4°C
Stability of stock solution	stable for at least 6 months with no observed loss of activity

3.1.3. Negative Control

Table 8: Information on the negative control 1-Thio-β-D-glucose sodium salt

Name:	1-Thio-β-D-glucose sodium salt
CAS No.:	10593-29-0
Molecular weight [g/mol]:	218.20
Storage conditions:	−20°C
Solvent	DMSO
Stock solution [mol/L]:	10^{-2} M

3.2. TEST ITEMS

The plate layout of the DIO1-SK assay is designed for up to three test items per assay plate. If more than one test item is tested in the assay, make sure to name the test items accurately.

4. TEST SYSTEM

The minimum requirements for human liver microsomes are described in Table 9. The human microsomes should be tested for all known human liver microsomal contaminations in compliance with GIVIMP (OECD, 2018).
Before the microsome batch can be used for further experiments, the microsome batch must be tested for their maximum iodide release activity. Microsome-batch specific iodide release activity testing is further specified in 6.4.1.
The microsomes should be stored at ≤ -80°C until required for use.

Table 9: Minimum requirements for the used microsome batch.

Species	human
Tissue	liver
Sex	mixed gender
Pool	≥25 donors
Age	various
Demonstrated absence of the following contaminations	Hepatitis B Hepatitis C Human Immunodeficiency Virus (HIV)
Iodide Release Activity	Microsome-batch specific iodide release activity that is measured in this method (see 6.4.2)

5. TEST CONCENTRATIONS

A total number of three independent assay runs per test item should be performed. The DIO1-SK assay requires an initial range finding run of the assay to estimate the range of inhibition of a test item. For the final assay runs, the concentrations of a test item need to be adapted if the test item shows a dose-response activity in the range finding assay. For test items that result in little to no iodide release activity inhibition, the same concentrations that are used in the range finding assay can be used in the final assay runs. If the concentrations of a test item without iodide release inhibition were not changed between the range finding to the actual assay runs, the initial range finding run can be considered as one of the three final assay runs.

5.1. RANGE FINDING ASSAY

The test concentrations for the range finding assay are dependent on the solubility of the test item in pure solvent as well as the solubility in the following dilutions in water and under assay conditions (covered in section 6.3.2):
Use the highest soluble concentration in an appropriate solvent (preferably DMSO) of the test item to prepare a test item stock solution which is then used to prepare test item dilutions. In brief, the test item stock solution is subsequently diluted with ddH_2O and solvent in a 1:10 ratio to obtain test item dilutions and a solvent concentration of 10 % (v/v) in the test item dilutions as described in 6.3.4. The final solvent concentration in the assay is 1 % which is prepared with a solution of 50 % potassium phosphate / EDTA buffer, 40 % ddH_2O and 10 % of the 10 % test item dilution. The final concentration tested in the assay should not exceed 1 mM.

5.2. ASSAY RUNS

1. If the test item leads to DIO1 inhibition in the range finding assay: The total number of tested concentrations in the assay runs remains at 8. If necessary, vary the concentration range and concentration spaces accordingly to make sure to include at least 4 concentrations in the linear region of the inhibition. 2

concentrations of the test item should result in little to no iodide release activity to ensure that the statistical model recognizes the baseline activity.

2. If there is no measurable DIO1 inhibition in the range finding assay: Repeat the assay runs with the proposed test item dilutions from the range finding assay.

6. METHOD

6.1. INITIAL CONSIDERATIONS FOR THE DIO1-SK ASSAY

- Three valid assay runs per test item are proposed
- The setup is defined for up to 3 test items per assay run
- Testing is performed in triplicates in a 96-well format
- If possible: solvent of choice: DMSO
  - Final solvent concentration in the assay: 1 % (v/v) DMSO

6.2. REAGENTS

Table 10: Reagents that are prepared before the assay performance

H_2KPO_4 (0.216 M)/ EDTA (2.16 mM) solution	Add 7.34 g H_2KPO_4 and 201 mg Ethylenediaminetetraacetic acid (EDTA) to a 250 mL volumetric flask and add ddH_2O to a final volume of 250 mL.
HK_2PO_4 (0.216 M) / EDTA (2.16 mM) solution (250 ml):	Add 9.41 g HK_2PO_4 and 201 mg Ethylenediaminetetraacetic acid (EDTA) to a 250 mL volumetric flask and add ddH_2O to a final volume of 250 mL.
Potassium phosphate / EDTA puffer (2.16 mM EDTA; pH 6.8)	Using a 250 mL volumetric flask, titrate the H_2KPO_4 / EDTA solution and HK_2PO_4 / EDTA solution to reach a pH of 6.8 (ratio of HK_2PO_4 / EDTA to H_2KPO_4 / EDTA of about 2:1 ≈ 167 ml of HK_2PO_4 / EDTA and 83 mL of H_2KPO_4 / EDTA solution).
rT3 (15 mM) solution	Dissolve rT3 in an appropriate volume of DMSO to reach a final concentration of 15 mM and freeze 100 µL aliquots at -20°C.
Preparation of 15 mL Falcons with aliquoted rT3	Add 4 µL of 15 mM rT3 to 15 mL-Falcons and store at -20°C.
Acidic ammonium cerium solution (25 mM $(NH_4)_4Ce(SO_4)_4{*}2H_2O$, 0,5 M H_2SO_4) (250 mL)	Add 3.95 g of $(NH_4)_4Ce(SO_4)_4{*}2H_2O$ and 125 mL of ddH_2O to a 250 mL volumetric flask. Add 125 mL 1 M H_2SO_4 to reach a final volume of 250 mL.
Sodium arsenite solution (25 mM $NaAsO_2$, 0,8 M NaCl, 0,5 M H_2SO_4) (250 ml)	Add 0.81 g of $NaAsO_2$, 11.7 g of NaCl and 125 mL of ddH_2O to a 250 mL volumetric flask. Add 125 mL 1 M H_2SO_4 to reach a final volume of 250 mL.

Table 11: Reagents that are prepared on the day of assay performance.

Preparation of the substrate mix (volume enough for 1x96-well plate)	On the day of assay performance, add 5.75 mL of potassium phosphate/EDTA buffer (0.216 M KPO_4, pH 6.8) and 0.5 mL DTT to the frozen, 4 µl of 15 mM rT3

	containing falcon. Mix and use the mix on the day of preparation.

6.3. PRE-ASSAY WORK

6.3.1. Casting of DOWEX resin-filled 96-well filter plate

It is recommended to prepare a larger quantity of DOWEX resin-filled 96-well filter plate before the day of assay performance which can be stored at 4°C for a maximum of 2 months

1. Approach for a single plate: Add about 30 g of DOWEX resin to a vessel and wash with 10 % acetic acid, letting the resin settle down for 10 min and subsequent removal of the dyed supernatant
   Can also be done in larger scale: Add about 250 g (or the desired quantity) of DOWEX to a large beaker and wash with 10 % acetic acid. Use a big shaker to mix the DOWEX with the acetic acid, let it rest afterwards for 10 min and remove the supernatant
2. Wash and remove the supernatant until no more colour is leaking into the solvent (at least 4x in total)
3. Add 100 µL of acetic acid (10 %) into each well of 96-well filter plate
4. Cut 1 mL tips to widen the opening and cast 600 µL DOWEX resin into each well of the 96-well filter plate
5. Add another 150 µL acetic acid (10 %) to each well and apply vacuum with a vacuum pump to elute the acetic acid into a 96-deep well-plate
6. Repeat the previous step if colour is still leaking in any of the wells
7. Seal the plate with an impermeable sheet of plastic and store at 4°C for a maximum of 2 months

6.3.2. Solubility assessment for test items

Test item stock solution:

Prior to the assay, the limit of solubility of each test item in an appropriate solvent is to be determined to prepare a test item stock solution.
The preferred solvent in the DIO1-SK assay is dimethyl sulfoxide (DMSO). When a substance is not soluble in DMSO, other solvents may be suitable. Using an untested (within the method) solvent would need to be assessed on a need (study) basis. Keep in mind to also carry out solvent controls of the solvent of the reference item 6PTU (solvent: DMSO) as well as the additional solvent(s).
The highest tested solubility of a test item in an appropriate solvent in the DIO1-SK assay is 100 mM since the highest tested final assay concentration of a test item is 1 mM (1 % v/v of solvent).

1. Prepare a 100 mM test item stock solution in an appropriate solvent by weighing an appropriate amount of test item in a vessel and add the needed amount of solvent (test item and solvent should be at room temperature)
2. Gently mix at room temperature. Vortex the tube if necessary
3. Visually check by using a microscope if the test item is dissolved

4. If the test item hasn't dissolved, use water bath sonification for up to 5 mins and repeat step 3 to check if the test item is dissolved
5. If the test item is not dissolved after sonification, warm the solution to 37°C for up to 60 mins in a 37°C water bath or an incubator at 37°C. Repeat step 3 to check if the test item is dissolved
6. If the test item is not dissolved after heating, use subsequent dilution steps of the test item in the appropriate solvent e.g. using subsequent dilutions of 1:10 or 1:3.16 (square root of 10). Return to step 2 after dilution of the insoluble stock solution. If the volume of insoluble test item stock solution becomes too large to work with, start at step 1 again but reduce the concentration of the test item stock solution by weighing less amount of the test item and dissolving in an appropriate volume of solvent to achieve the desired test item stock solution.

10 % test item dilution:

Once the highest soluble concentration of the test item in an appropriate solvent is determined, prepare a 1:10 dilution in ddH_2O and check if they are still fully dissolved.

7. Prepare a 10 % test item dilution in ddH_2O by diluting the highest soluble test item stock solution (generated in step 1 to 6) in ddH_2O by applying a dilution factor of 1:10
8. Gently mix at room temperature. Vortex the tube if necessary
9. Visually check by using a microscope if the test item dilution is dissolved
10. If the test item hasn't dissolved, use water bath sonification for up to 5 mins and repeat step 9 to check if the test item is dissolved
11. If the test item is not dissolved after sonification, warm the solution to 37°C for up to 60 mins in a 37°C water bath or an incubator at 37°C. Repeat step 9 to check if the test item is dissolved
12. If the test item is not dissolved after heating, return to step 6 and prepare a test item stock solution with lower concentration of the test item.

1 % test item dilution under assay conditions:

Once the test item in the 10 % test item dilution is fully dissolved, further check if the test item is also dissolved under assay conditions by preparing the 1 % final assay concentration with a solution of 50 % potassium phosphate / EDTA buffer, 40 % ddH_2O and 10 % of the 10 % test item dilution.

13. Prepare a 1 % test item dilution under final assay conditions by generating a solution of 50 % potassium phosphate / EDTA buffer (as prepared in Table 11, 6.2), 40 % ddH2O and 10 % of the 10 % test item dilution
14. Gently mix at room temperature. Vortex the tube if necessary
15. Visually check by using a microscope if the test item is dissolved
16. If the test item hasn't dissolved, use water bath sonification for up to 5 mins and repeat step 15 to check if the test item is dissolved
17. If the test item is not dissolved after sonification, warm the solution to 37°C for up to 60 mins in a 37°C water bath or an incubator at 37°C. Repeat step 15 to check if the test item is dissolved

18. If the test item is not dissolved after heating, return to step 6 and prepare a test item stock solution with lower concentration of the test item

6.3.3. Preparation of a stock solution and dilution of the reference item

Prepare a 10^{-1} M stock solution for the reference item 6PTU fresh on the day of analysis.

1. Based on the amount of stock solution needed, weigh an appropriate amount of substance into a suitable vessel.
2. Add the appropriate amount of the solvent (DMSO) using a pipette.
3. Dissolve the substance in the solvent with a vortex. If the substance does not dissolve, use ultra-sonication for several minutes (see Table 6).

   Example:
   To prepare 1 mL of a 10^{-1} M stock solution of 6-Propyl-2-thiouracil in DMSO with a molecular weight of 170.23 g/mol, 17 mg of the substance was weighed into a vessel and solved in 1 mL of DMSO.

$$m = c * V * M = 0.1 \frac{mol}{l} * 0.001l * 170.23 \frac{g}{mol} = 0.017g = 17mg$$

4. On the day of analysis, prepare the reference item dilutions from the 10^{-1} M reference item stock solution according to Table 12.
5. Label the subsequent reference item dilutions derived from the reference item stock solution adequately (e.g. RI-D1, RI-D2,…., RI-D8).

Table 12: Preparation of the dilutions for the reference item 6-Propyl-2-thioruacil.

Name of the reference item dilution	Reference item dilution concentration [M]	ddH_2O [µL]	DMSO [µL]	Reference item [µL]	Final concentration of reference item in the assay [M]
RI-D1	10^{-2}	450	-	50 µL of 10^{-1} M reference item stock solution	10^{-3}
RI-D2	10^{-3}	405*	45*	50 µL of RI-D1	10^{-4}
RI-D3	10^{-4}	405*	45*	50 µL of RI-D2	10^{-5}
RI-D4	10^{-5}	405*	45*	50 µL of RI-D3	10^{-6}
RI-D5	10^{-6}	405*	45*	50 µL of RI-D4	10^{-7}
RI-D6	10^{-7}	405*	45*	50 µL of RI-D5	10^{-8}
RI-D7	10^{-8}	405*	45*	50 µL of RI-D6	10^{-9}
RI-D8	10^{-9}	405*	45*	50 µL of RI-D7	10^{-10}

*You can also prepare a 10 % DMSO / ddH_2O solution and add 450 µL of the dilution

6.3.4. Preparation of a stock solution and dilution of the positive control

Prepare a 10^{-2} M stock solution for the positive control Aurothioglucose. The stock solution can be stored at 4°C and is stable for at least 6 months without loss of activity (see Table 7).

1. Based on the amount of stock solution needed, weigh an appropriate amount of substance into a suitable vessel.
2. Add the appropriate of the solvent (DMSO) using a pipette.
3. Dissolve the substance in the solvent with a vortex. If the substance does not dissolve, use ultra-sonication for several minutes.

   Example:
   To prepare 1 mL of a 10^{-2} M stock solution of Aurothioglucose in DMSO with a molecular weight of 392.18 g/mol, 3.9 mg of the substance was weighed into a vessel and solved in 1 mL of DMSO.

$$m = c * V * M = 0.01 \frac{mol}{l} * 0.001l * 392.18 \frac{g}{mol} = 0.039g = 3.9mg$$

4. On the day of analysis, prepare the negative control dilution from the 10^{-2} M negative control stock solution according to Table 13.

Table 13: Preparation of the positive control Aurothioglucose dilution.

Positive control dilution [M]	ddH_2O [µL]	DMSO [µL]	Positive control [µL]	Final concentration of positive control in the assay [M]
10^{-3}	450	-	50 µL of 10^{-2} M positive control stock solution	10^{-4}

6.3.5. Preparation of a stock solution and dilution of the negative control

Prepare a 10^{-2} M stock solution for the negative control 1-Thio-β-D-glucose sodium salt fresh on the day of analysis.

1. Based on the amount of stock solution needed, weigh an appropriate amount of substance into a suitable vessel.
2. Add the appropriate amount of the solvent (DMSO) using a pipette.
3. Dissolve the substance in DMSO with a vortex. If the substance does not dissolve, use ultra-sonication for several minutes (see Table 8).

   Example:
   To prepare 2 mL of a 10^{-2} M stock solution of 1-Thio-β-D-glucose sodium salt in DMSO with a molecular weight of 218.20 g/mol, 4.4 mg of the substance was weighed into a vessel and solved in 2 mL of DMSO.

$$m = c * V * M = 0.01 \frac{mol}{l} * 0.002l * 218.20 \frac{g}{mol} = 0.017g = 4.4mg$$

4. On the day of analysis, prepare the negative control dilution from the 10^{-2} M negative control stock solution according to Table 14.

Table 14: Preparation of the negative control 1-Thio-β-D-glucose sodium salt dilution.

Negative control dilution [M]	ddH_2O [µL]	DMSO [µL]	Negative control [µL]	Final concentration of negative control in the assay [M]
10^{-3}	450	-	50 µL of 10^{-2} M negative control stock solution	10^{-4}

6.3.6. Preparation of stock solutions and dilutions of the test item

Use the highest soluble test item concentration in an appropriate solvent (preferably DMSO) determined in 6.3.2. to prepare the test item stock solution on the day of analysis. If more than one test item is tested in the assay, make sure to name the test item stock solutions appropriately. Label the test item stock solutions adequately.

For the preparation of the test item dilutions, the test item stock solution is subsequently diluted seven times with ddH_2O and the appropriate solvent in a 1:10 ratio to obtain eight test item dilutions with a solvent concentration of 10 % (v/v) as shown in Table 15. Final test item concentrations in the assay medium will be 1 % (v/v) of the solvent. Label the subsequent test item dilutions derived from the test item stock solution adequately.

Table 15: Preparation of the test item dilutions using test item stock solutions.

Name of the test item dilution	ddH_2O [µL]	solvent [µL]	Test item	Dilution factor
TI1-C1	450	-	50 µL of test item 1 stock solution	1:10
TI1-C2	405*	45*	50 µL of TI1-C1	1:10
TI1-C3	405*	45*	50 µL of TI1-C2	1:10
TI1-C4	405*	45*	50 µL of TI1-C3	1:10
TI1-C5	405*	45*	50 µL of TI1-C4	1:10
TI1-C6	405*	45*	50 µL of TI1-C5	1:10
TI1-C7	405*	45*	50 µL of TI1-C6	1:10
TI1-C8	405*	45*	50 µL of TI1-C7	1:10

*You can also prepare a 10 % solvent / ddH_2O solution and add 450 µL of the dilution

6.3.7. Preparation of the human microsome dilutions

Varying iodide release activity of different human liver microsome batches have shown the need for standardisation of enzyme concentration the DIO1-SK assay (see 6.4.2 for further explanation).

Prepare human liver microsome dilutions in ddH_2O as shown in Table 16. The calculation assumes a stock solution of 20 mg enzyme/mL, as most microsome batches are supplied from the manufacturers in this concentration. If the supplied microsome batch enzyme concentration differs, vary the preparation of the microsome solutions accordingly. Once the batch-specific microsome activity testing according to 6.4.1 is concluded and an enzyme concentration for further testing is derived, aliquoting the manufacturers microsome stock solution in appropriate amounts is proposed, depending on the intended amount of assay runs per day.

Table 16: Preparation of the human liver microsome dilutions for the testing of iodide release activity.

Microsome per well [µg]	ddH_2O [µL]	Microsome dilution [µL]	Final enzyme concentration in the assay [µg/mL]
20	780	20 µL of 20 mg/mL microsome stock solution	200
10	400	400 µL of 20 µg Microsome per well dilution	100
5	400	400 µL of 10 µg Microsome per well dilution	50
2.5	400	400 µL of 5 µg Microsome per well dilution	25
1.25	400	400 µL of 2.5 µg Microsome per well dilution	12.5
0.68	400	400 µL of 1.25 µg Microsome per well dilution	6.8

6.4. STANDARDISATION OF THE TEST SYSTEM

6.4.1. Standardization of the Sandell-Kolthoff reaction

A respective standard curve should be run on a regular basis (e.g. monthly or prior to a large experimental setting) to monitor systematic changes (e.g. by contamination) within the Sandell-Kolthoff setup. This can be checked by using an iodide standard curve in the Sandell-Kolthoff reaction. Long-term records can be used for quality control. In case of major changes within the Sandell-Kolthoff setting (e.g. change of photometer, used chemicals (LOT), plate type, …), this test setup assures their direct applicability and prevents systematic errors in the assay setup. Furthermore, the use of a certified iodide standard allows inter-lab comparison.

Time flow of the assay:	
Prepared beforehand:	preparation of ammonium cerium and sodium arsenite solution
Day 1:	preparation of iodide dilutions measurement via Sandell-Kolthoff reaction

1. Prepare iodide dilutions from a respective iodide source (e.g. iodide standard solution) with recommended concentrations of 1500, 1000, 750, 500, 400, 300, 200, 100, 50, 25, 10, 5 and 1 nM iodide of a respective iodide standard in ddH_2O. The used concentrations can be varied if needed.

2. Add 50 µL of the prepared iodide dilutions to a 96-well plate. Preparing three replicates per concentration is recommended. Also add 50 µL of pure ddH_2O with three replicates to the plate. A recommended plate layout is shown in Table 17.

Table 17: plate layout for standardizing the Sandell-Kolthoff reaction

	1	2	3	4	5	6	7	8	9	10	11	12
A	1500 nM I^-			1000 nM I^-			750 nM I^-			500 nM I^-		
B	400 nM I^-			300 nM I^-			200 nM I^-			100 nM I^-		
C	50 nM I^-			25 nM I^-			10 nM I^-			5 nM I^-		
D	1 nM I^-			ddH_2O only								
E												
F												
G												
H												

iodide dilutions **ddH_2O only** **Empty**

3. Add 50 µL of cerium solution [25 mM $(NH_4)_4Ce(SO_4)_4{*}2H_2O$; 0.5 M H_2SO_4] to the iodide dilutions in the 96-well plate
4. Start the reaction by adding 50 µL of arsenite solution [25 mM $NaAsO_2$; 0.8 M NaCl; 0.5 M H_2SO_4] to the samples in the 96-well plate. The use of a multichannel pipette for fast addition of arsenite solution is recommended.
5. As soon as possible after the application of arsenite solution, determine the absorption in a plate reader with the following settings:
    - Absorption parameters: 415 nm (±2 nm)
    - Initial shaking: medium for 2 seconds
    - Measurement of the OD every minute for 21 min (other time periods are possible, make sure the detection is in linear range), also measuring the initial OD

6. Evaluate the data by subtracting the OD of the 21-minute samples from the initial OD values to generate ΔOD values
7. Plot the ΔOD values of the iodide concentration samples in a statistics software with ΔOD on y-axis (linear) and iodide concentration on x-axis (linear)
8. Use a curve-fit algorithm to generate a function to optimally reflect assay characteristics (e.g. “exponential plateau” in GraphPad Prism)
9. Monitor the ΔOD values of the used iodide dilutions as well as the background ΔOD values of the pure ddH_2O samples in the Sandell-Kolthoff reaction

Comments on obtained ΔOD values:
The following indications of usually observed values can vary, depending on the used laboratory setup and should be handled with care.
Used laboratory setup: Plate reader: Tecan Sunrise INSTSUN-3, measured at 415 nm after 21 min of incubation.

Pure ddH_2O sample: Usually a background of ΔOD > 0.3 in the pure ddH_2O control would need attention and further investigation of underlying causes (e.g. contamination, low quality water, As or Ce-batch)
Iodide dilutions: The overall dynamic range of the reaction is usually found in the range of 50 to 700 nM of the used iodide dilutions. The highest ΔOD is usually found in the range of 500-700 nM (higher iodide concentrations only lead to marginal ΔOD increases) of the used iodide dilution and should be ΔOD > 1.3.

6.4.2. Measuring activity of the microsomes

Different human microsome batches show differences in their activity to deiodinate rT3 leading to differences in the maximum ΔOD-BG values (≙iodide release activity) of the batches about ~2 to 3x. The generation of an enzyme activity curve with the used microsome batch is used in this method to assess the iodide release activity of the microsome batch and to determine a microsome batch-specific enzyme concentration that will be used for the assay runs.
After determination of the microsome batch specific enzyme concentration, the microsomes can be stored in aliquots sufficient for one or the desired amount of assay plates.

Careful: The measurement of the microsome activity must be carried out for every differing batch of microsomes!

Time flow of the assay:	
Prepared beforehand:	preparation of potassium phosphate buffer, substrate mix falcons, ammonium cerium solution, sodium arsenite solution casting of DOWEX resin-filled 96-well filter plate
Day 1:	preparation of reference item 6PTU, solvent control and microsome dilutions preparation of assay plates measurement of assay plates via Sandell-Kolthoff reaction

1. Prepare the reference item dilution of 6PTU as described in 6.3.3, the microsome dilutions as described in 6.3.7 as well as the substrate mix (see 6.2, Table 11: “preparation of the substrate mix”)
2. Add 10 µL of 10^{-2} M 6PTU as reference item to a 96-well plate. For the solvent controls add 10 µL of the 10% solvent dilution in ddH_2O (e.g. 10 % DMSO in ddH_2O). Keeping a final assay concentration of 1 % solvent in all samples is recommended. A proposed plate layout is shown in Table 18
3. Add 40 µL of different microsome dilutions in ddH_2O (resulting in 20, 10, 5, 2.5, 1.25, 0.68 and 0 µg enzyme per well) to the 96- well plate
4. On ice, add 50 µL of freshly prepared substrate mix to each well
5. Seal the plate with an impermeable sheet of plastic

6. The 96-well plate is then placed on a shaker in an incubator (37°C at 600 rpm) and is incubated for 2 h

Table 18: plate layout for measuring the activity of the microsome batch.

	1	2	3	4	5	6	7	8	9	10	11	12
A	20 µg enzyme per well			20 µg enzyme per well 10^{-3} M 6PTU			10 µg enzyme per well			10 µg enzyme per well 10^{-3} M 6PTU		
B	5 µg enzyme per well			5 µg enzyme per well 10^{-3} M 6PTU			2,5 µg enzyme per well			2,5 µg enzyme per well 10^{-3} M 6PTU		
C	1,25 µg enzyme per well			1,25 µg enzyme per well 10^{-3} M 6PTU			0,68 µg enzyme per well			0,68 µg enzyme per well 10^{-3} M 6PTU		
D	0 µg enzyme per well			0 µg enzyme per well 10^{-3} M 6PTU								
E												
F												
G												
H												

reference item **solvent control** **Empty**

7. Place on ice to stop the reaction
8. Conduct the separation of the substrate and released iodide of the assay analogous to 6.5.2.
9. Conduct the measurement of the Sandell-Kolthoff reaction analogous to 6.5.3 with the following deviations:
   Measure the samples in the Sandell-Kolthoff reaction undiluted as well as diluted in 10 % acetic acid (1:2 and 1:4 are recommended; depending on the microsome activity, higher dilutions might be beneficial)

10. Determine the ΔOD via subtracting the 21-minute values from the initial measured values
11. To determine the ΔOD-BG values, subtract the inhibited ΔOD values of the reference item from the respective enzyme concentration ΔOD values of the solvent control
12. Plot the ΔOD-BG values of the different enzyme concentration samples in a statistics software with ΔOD-BG on y-axis (linear) and inhibitor concentration on x-axis (logarithmic)
13. Use a curve-fit algorithm to generate a function to optimally reflect assay characteristics (e.g. “[Inhibitor] vs. response -- Variable slope (four parameters)”)
14. Determine the dilution factor for the samples in 10 % acetic acid that still leads to the highest possible ΔOD-BG values in the Sandell-Kolthoff reaction.

Also determine the enzyme concentration that leads to the highest possible ΔOD-BG values.

Use these values for every following measurement of the same microsome batch in the assay runs

15. Aliquot the manufacturers microsome stock solution in appropriate amounts, depending on the intended amount of runs per day.

Comments on obtained ΔOD-BG values:

The following indications of usually observed values can vary, depending on the used laboratory setup and should be handled with care.
Used laboratory setup: Plate reader: Tecan Sunrise INSTSUN-3, measured at 415 nm after 21 min of incubation.

Usually an iodide release of ΔOD > 0.5 is easily achievable in the 1:4 or 1:2 acetic acid dilutions of the 20 µg enzyme per well sample and usually ranges around a ΔOD of 1. Higher iodide release activities increase the range of the reaction and can help to consistently meet the acceptance criteria for a valid assay run. If enzyme concentrations <20 µg enzyme per well lead to comparable ΔOD values, a reduced concentration of enzyme per well may be used (5 to 20 µg enzyme per well are typically used values). Enzyme concentrations >20 µg enzyme per well tend to increase the background of the method and are not recommended for use.

6.5. TESTING OF RANGE FINDING AND ASSAY RUNS

The testing of test items in the DIO1-SK assay require an initial range finding assay. The range finding assay is conducted with the proposed test item dilutions according to 6.3.4. The test item concentrations for the actual assay run depend on the results of the range finding assay and may have to be modified; the derivation procedure is described in 5.2.
On the first plate of an assay day, the generation of reference item (6PTU) dose response curve is required.

Time flow of the assay:	
Prepared beforehand:	preparation of potassium phosphate buffer, substrate mix falcons, ammonium cerium solution, sodium arsenite solution casting of DOWEX resin-filled 96-well filter plate
Day 1:	preparation of test item(s), reference item, negative control and positive control preparation of assay plates measurement of assay plates via Sandell-Kolthoff reaction

6.5.1. Microsome incubation with test items

1. Prepare the reference item stock solution as well as dilutions of 6PTU as described in 6.3.3, the positive and negative control stock solution as well as dilution as described in 6.3.4 and 6.3.5 and the test item stock solutions as well as dilutions as described in 6.3.6.
2. On the first plate of an assay day, add 10 µL of the reference item dilutions to a 96-well plate. For the solvent control add 10 µL of 10 % (v/v) DMSO (in purified

water) solution. For the positive and negative control dilution, add 10 µL of the prepared dilutions. Add 10 µL of the test item dilutions to the 96-well plate. Keeping a final concentration of 1 % DMSO in all samples is recommended.
A proposed plate layout for the first run of an assay day is shown in Table 19; a proposed plate layout for additional runs on the same assay day is shown in Table 20.

3. Add 40 µL of a defined protein dilution (resulting in the calculated amount of enzyme per sample well calculated in 6.4.2) to the wells
4. On ice, add 50 µL of the freshly prepared substrate mix (see 6.2) to the samples
5. Seal the plate with an impermeable sheet of plastic
6. The 96-well plate is then placed on a shaker in an incubator (37°C at 600 rpm) and is incubated for 2 h
7. Place on ice to stop the reaction

Table 19: plate layout for the first plate of an assay day for a range finding / assay run of the DIO1-SK.

	1	2	3	4	5	6	7	8	9	10	11	12
A	SC			RI-C1			NC			RI-C1		
B	RI-C1			RI-C2			RI-C3			RI-C4		
C	RI-C5			RI-C6			RI-C7			RI-C8		
D	TI1-C1			TI1-C2			TI1-C3			TI1-C4		
E	TI1-C5			TI1-C6			TI1-C7			TI1-C8		
F	TI2-C1			TI2-C2			TI2-C3			TI2-C4		
G	TI2-C5			TI2-C6			TI2-C7			TI2-C8		
H	SC			RI-C1			SC			PC		

SC **solvent control**

PC **positive control** Aurothioglucose 10^{-4} M

RI **reference item** 6-Propyl-2-thiouracil 10^{-3} M

NC **negative control** 1-Thio-β-D-glucose sodium salt 10^{-4} M

TI **test item**

This plate layout is designed to test up to 2 test-items in parallel. Each test item concentration is tested in triplicates. The plate contains further 3 replicates of the negative control, 3 replicates of the positive control, 9 replicates of the reference item 6PTU and 9 replicates of the solvent control.

Table 20: plate layout for additional plates of an assay day for a range finding / assay run of the DIO1-SK.

	1	2	3	4	5	6	7	8	9	10	11	12
A	SC			RI-C1			NC			RI-C1		
B	TI3-C1			TI3-C2			TI3-C3			TI3-C4		

C	TI3-C5	TI3-C6	TI3-C7	TI3-C8
D	TI4-C1	TI4-C2	TI4-C3	TI4-C4
E	TI4-C5	TI4-C6	TI4-C7	TI4-C8
F	TI5-C1	TI5-C2	TI5-C3	TI5-C4
G	TI5-C5	TI5-C6	TI5-C7	TI5-C8
H	SC	RI-C1	SC	PC

SC **solvent control**

PC **positive control** Aurothioglucose 10^{-4} M

RI **reference item** 6-Propyl-2-thiouracil 10^{-3} M

NC **negative control** 1-Thio-β-D-glucose sodium salt 10^{-4} M

TI **test item**

This plate layout is designed to test up to 3 test-items in parallel. Each test item concentration is tested in triplicates. The plate contains further 3 replicates of the negative control, 3 replicates of the positive control, 9 replicates of the reference item 6PTU and 9 replicates of the solvent control.

6.5.2. Separation via DOWEX resin-filled 96-well filter plate

1. Put a prepared DOWEX resin-filled 96-well filter plate (as prepared in 6.3.1) on top of a used 96-deep well-plate
2. Add 150 µL of 10 % acetic acid to each well of the DOWEX resin-filled 96-well filter plate to wet the columns
3. Elute the acetic acid by centrifuging into the used 96-deep well-plate with 70 g in a centrifuge with swing-out rotor for microtiter plates for 1 min
4. Replace the used 96-deep well-plate with a novel 96-deep well plate
5. Transfer 75 µL of the samples from the incubated 96-well plate into the DOWEX resin-filled 96-well filter plate maintaining the initial plate layout
6. Add 100 µL of 10 % acetic acid to each well of the DOWEX resin-filled 96-well filter plate
7. Elute the samples by centrifuging into the 96-deep well-plate with 70 g in a centrifuge with swing-out rotor for microtiter plates for 1 min and remove the DOWEX resin-filled 96-well filter plate
   The 96-deep well-plate can be sealed with an impermeable sheet of plastic and stored at 4°C. This allows additional measurements in case of manual / technical errors or changes of the dilution factor in the Sandell-Kolthoff reaction.

6.5.3. Sandell-Kolthoff reaction

Careful: Sodium arsenite is classified as **carcinogenic** to humans (Hazard class 1) by the International Agency for Research on Cancer (IARC).
Extra safety instructions to ensure conformity with laboratory and/or country specific safety regulations are recommended. Potential measures are explained below:

The handling of the pure substance should be done under a fume hood while wearing the appropriate personal protective equipment (safety glasses and safety gloves). This also applies to work with the resulting solutions.
For work in the fume hood, a shallow drip tray can be used for disposal, an extra container can be created and labelled with “Sandell-Kolthoff”. The waste to be disposed can be collected separately from other waste and disposed according to the Safety Data Sheet.

1. Depending on the determined dilution factor of the samples in 10 % acetic acid for the used microsome batch (see 6.4.1), add 50 µL of the diluted sample solution to a novel 96-well plate. E.g. for a 1:4 dilution, add 37.5 µL of 10 % acetic acid to each well. Subsequent, add 12.5 µL of the samples from the 96-deep well-plate to the 96-well plate.
2. Add 50 µL of cerium solution [25 mM $(NH_4)_4Ce(SO_4)_4*2H_2O$; 0.5 M H_2SO_4] to the samples in the 96-well plate
3. Start the reaction by adding 50 µL of arsenite solution [25 mM $NaAsO_2$; 0.8 M NaCl; 0.5 M H_2SO_4] to the samples in the 96-well plate. The use of a multichannel pipette for fast addition of arsenite solution is recommended
4. As soon as possible after the application of arsenite solution, determine the absorption OD in a plate reader with the following settings:
    - Absorption parameters: 415 nm (±2 nm)
    - Initial shaking: medium for 2 s
    - Measurement of the OD every minute for 21 min (other time periods are possible, make sure the detection is in linear range), also measuring the initial OD

6.6. EVALUATION OF THE DATA

1. Determine the ΔOD values via subtraction of the 21-minute values of all samples from the initial measured values
2. To determine the ΔOD-BG values, subtract the mean of the inhibited ΔOD values of the reference item (10^{-4} M 6PTU) from the ΔOD of all samples
3. Normalize the values of the test item to the respective solvent control values via division of the test item ΔOD-BG values by the mean of the solvent control ΔOD-BG values. State the normalized ΔOD-BG values in %. Keep in mind that test items with differing solvents need to be normalized to their respective solvent controls.
4. Plot the normalized ΔOD-BG values of the different test item concentration samples in a statistics software with normalized ΔOD-BG values on y-axis (linear) and test item concentration on x-axis (logarithmic)
5. Use a curve-fit algorithm to generate a function to optimally reflect assay characteristics (e.g. “[Inhibitor] vs. response -- Variable slope (four parameters)” in GraphPad Prism 8)
6. If applicable, determine the IC_{50} of the active test item

6.7. ASSESSING VALIDITY OF RUNS

6.7.1. Assessment criteria

Different assessment criteria covering performance of the reference item will be used to assess the validity of an assay run. An assay run is considered valid and will be accepted when all the acceptance criteria are met (Table 21).

Table 21: Used assessment criteria in the DIO1-SK assay.

Acceptance criteria	Valid run, if
Shape of reference item (sigmoidal, yes/no?)	curve is sigmoidal
IC_{50} of the reference item 6PTU	10^{-6} – 10^{-5} M

If an assay run is classified as non-valid, the assay run would have to be repeated.

14.2 Donor demographics as well as the available lot characterization data for the batch #QQY

BioreclamationIVT
The complete resource for all biologicals

*InVitro*CYP™ 150-donor Mixed Gender Pooled Human Liver Microsomes, 10 mg

Product Number: X008070

Lot Number: QQY* **Storage Conditions** -70ºC

Test Results

Specification	Result
20-26 mg/mL protein concentration	24.1 mg/mL
nmol/mg total P450 concentration	0.425 nmol/mg

Lot Characterization Results

Assay		Result
		Rate at K_m concentration
ECOD:	total rate of formation of 7-HC and metabolites	501 pmol/min/mg
UGT:	rate of formation of 7-hydroxycoumarin glucuronide	1617 pmol/min/mg
CYP1A2:	rate of formation of acetaminophen	299 pmol/min/mg
CYP2A6:	total rate of formation of 7-HC and metabolites	267 pmol/min/mg
CYP2B6:	rate of formation of hydroxybupropion	278 pmol/min/mg
CYP2C8:	rate of formation of desethylamodiaquine	1189 pmol/min/mg
CYP2C9:	rate of formation of 4'-methylhydroxytolbutamide	173 pmol/min/mg
CYP2C19:	rate of formation of 4'-hydroxymephenytoin	64.0 pmol/min/mg
CYP2D6:	rate of formation of dextrorphan	48.3 pmol/min/mg
CYP2E1:	rate of formation of 6-hydroxychlorzoxazone	386 pmol/min/mg
CYP3A4:	rate of formation of 6β-hydroxytestosterone	920 pmol/min/mg
	rate of formation of 1-hydroxymidazolam	362 pmol/min/mg

**Updated to include Vmax metabolic data and donor demographic information*

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Donor Demographics, as reported to BioreclamationIVT

Gender	Age	Race	Cause of death	Height	Weight	Social history	Medical history	Serology testing EBV	RPR	CMV	Hepatitis B	Hepatitis C	HIV
F	51	C	CVA	67"	99 Kg	ETOH: 1-2 drinks on occasion; Tobacco: 1/2 ppd since teens - quit 18yrs ago; Drugs: THC >30yrs ago - occasionally/ rarely since pregnancy. No IVDA	Adult onset asthma, anxiety. Meds: inhaler, antianxiety meds	Not reported	Neg	Pos	Neg	Neg	Neg
F	48	B	CVA	170cm	88 Kg	ETOH: 1 glass of gin occassionally for 16 yrs; Tobacco: 1 ppd x 35 yrs; no drug use.	Asthma, HTN - unk duration, Diabetes - insulin dependent last 6 mos, suspected renal cell carcinoma.	IgG+	Neg	Pos	Neg	Neg	Neg
F	41	H	Stroke	66"	79 Kg	No ETOH, Tobacco or drug use	Appendectomy 16 yrs ago.	Not reported	Neg	Pos	Neg	Neg	Neg
F	37	C	CVA	71"	96 Kg	ETOH: approx 10 drinks/day x 10yrs; No tobacco use; Drugs: remote cocaine use	No history reported	Pos	Neg	Pos	Neg	Neg	Neg
F	62	A	CVA	152 cm	53.1Kg	No ETOH, Tobacco or drug use	HTN x 5 yrs	IgG+	Neg	Pos	Neg	Neg	Neg
F	42	C	Anoxia; 2nd to Cardio-vascular	5'10"	102Kg	ETOH: (Liquor, Wine, Beer) 2-3 drinks at most socially on weekends. No tobacco or drug use.	Sleep apnea, HTN x 15yrs: non-compliant, ADD, Depression, skin cancer w/in last 4-5yrs:- removed/treated - no f/u, gastric bypass, Anemia, Fibromyalgia, obesity, Rhinoplasty. Meds: Adderral, MVI.	IgG +	Neg	Pos	Neg	Neg	Neg
F	68	B	Anoxic Injury	64"	80 Kg	No ETOH, Tobacco or drug use	NIDDM, HTN, Hyperlipodemia, Hysterectomy, thyroid disease, RA x 10yrs, cardiac history. Meds: Lipitor, glucophage	Not reported	Neg	Pos	Neg	Neg	Neg

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F	38	C	Head Trauma; 2nd to Blunt Injury	5'4"	164lb	ETOH: 4-5 drinks/day x 23yrs; Tobacco: 1 ppd since teen; no drug use.	Circulation problems suggesting signs of MS	Not reported	Neg	Pos	Neg	Neg	Neg
F	45	C	Overdose	5'7"	92.3Kg	ETOH: 1 pint of liquor every 4 mos ; Tobacco: 1/2 ppd x 13yrs, quit 1 yr ago.; Drugs: IVDA x 2 yrs, cocaine and marijuana x 6 mos.	HTN x 5 yrs, Diabetes x 10 yrs.	IgG+	Neg	Neg	Neg	Neg	Neg
F	75	C	Head Trauma 2nd to Fall	67"	132lb	ETOH: 1 -2 glasses of wine per month; Tobacco: 1 cigarette every 2 months, quit 20 years ago; No drug use.	Parkinson's, CHF, CABG x 3, Pacemaker, fungi on large toe, HTN x 12 years - compliant, osteoporosis	Pos	Neg	Neg	Neg	Neg	Neg
F	12	C	Anoxia 2nd to Cardiovascular	5'1"	55 Kg	No ETOH, Tobacco or drug use	No history reported	Not reported	Neg	Neg	Neg	Neg	Neg
F	64	C	CVA	5'9"	90.7Kg	ETOH: Not often/very little - quit 20yrs ago; tobacco: 1ppd x 12yrs - quit 20yrs ago; no drug use	Diabetes 10-12yrs	IgG+	Neg	Pos	Neg	Neg	Neg
F	68	A	SOH and SAH	62"	72 Kg	ETOH: None in 28 yrs, Champagne before; No tobacco or drug use	Uterine Cancer x 25yrs - no chemo or rad but total Hysterectomy, Allergic to dust and mold, HTN x 10yrs, NIDDM x 10 yrs, Asthma x 20yrs, Shingles; Meds: Nexium, KCl, Plavix, Clordiazepoxide, Norvasc, Gylburide, Lipitor	Not reported	Neg	Pos	Neg	Neg	Neg
F	49	C	Anoxia	5'7"	160lb	ETOH : 4 -6 beers/day x 36 yrs; Drug: Marijuana (inhaled) min 3 x/wk x 36 yrs; Hydrocodone (ingested) unknown amount x 10yrs; Tobacco: 1 -2 ppd x 36 yrs, last 10 mos began smoking only 1 ppd.	HTN newly diagnosed (last 2 days), Seizures x 3-4 yrs, Anxiety, Liver Disease, Alcoholism, seizure disorder, Hepatitis, uncontrolled HTN; Meds. Celexa, Klonopin, Soma, Benadryl, Aspirin, Vitamin B, Omega 3s, Fish Oil	IgG +	Neg	Pos	Neg	Neg	Neg

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F	49	C	ICH	5'4"	140lb	ETOH: 1-3 drinks per day x 10yrs; Tobacco: 1ppd x 20yrs; Drugs: marijuana during teens	Hypertension 6-10 yrs	Neg	Neg	Neg	Neg	Neg	Neg
F	65	C	ICH	68"	260lb	No ETOH; Tobacco - quit 30 yrs ago; no drug use	Colitis, had high blood pressure 15yrs ago.	Not reported	Neg	Neg	Neg	Neg	Neg
F	53	C	Anoxia	4'11"	118lb	1/5 liquor & beer daily x 25 yrs; smoked 2 ppd cigarettes x 25 yrs; no drug use	No history reported	IgG +	Neg	Neg	Neg	Neg	Neg
F	53	H	Anoxia; 2nd to Cardiovascular	61"	81 Kg	No ETOH, Tobacco or Drug use	HTN, ALS, Cholecyst-ectomy	IgG +	Neg	Pos	Neg	Neg	Neg
F	63	C	Head Trauma 2nd to Blunt Injury	165 cm	94 Kg	Tobacco: 1 pack/week, quit 40+ yrs ago.; no ETOH or drug use.	Breast cancer 1 yr ago - did not spread, radiation done and considered cured.	IgG+	Neg	Pos	Neg	Neg	Neg
F	58	C	CVA	65"	88 Kg	ETOH: 1-2 drinks/yr; tobacco: 1-2ppd x 30yrs - quit 10 yrs ago; no drug use	Heterozygous leiden factor 5 deficiency, HTN x 5yrs, multiple fractures and sxs - cervical, clipped nerve, shoulder, thumb, lumbar infusion, knee replacement, pituitary adenoma 1 yr ago, Pos TB skin test - not sure when cleared. Some type of hepatitis after meno., cellulitis. Meds: vitamins, neurontin, lynca, vicodin, docazosin, deflucan, coumadin, DVT's, fluoxtine, zocov, zetia.	Not reported	Neg	Pos	Neg	Neg	Neg
F	52	C	Cardiac Arrest; 2nd to Head trauma/Fall	64"	79 Kg	No ETOH, Tobacco or drug use	High cholesterol, diabetes NIDDM x 10yrs - oral meds	Not reported	Neg	Pos	Neg	Neg	Neg
F	58	C	S/P Cardiac Arrest	5'9"	74.1Kg	No ETOH, Tobacco or drug use	Lymes Disease, previous liver lac from horse kick to abd	IgG+	Neg	Pos	Neg	Neg	Neg

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F	51	B	Cerebro-vascular Stroke; 2nd to ICH	68"	91 Kg	ETOH: Cognac (1-3 glasses/mo) and beer (2-4/mo) for 15yrs - quit 9 yrs ago; Tobacco: 1ppd x 30+ yrs; Drugs: Cocaine (specifics unknown).	Diabetes - started w/ pills and progressed to insulin shot past 6yrs, HTN x 6yrs, anxiety, sleeping problems and acid reflux, diarrhea on and off past few years, allergic to PCN. Dx COPD 4 yrs ago - rec'd "pump" for treatment. Meds: Nexium, tenormin, lantus insulin, lisinopril, hydrocodone, Norco, ibuprofen, HCTZ, xanax, seroquel, ambien, fibromyalgia meds.	IgG+	Neg	Pos	Neg	Neg	Neg
F	52	C	ICH; 2nd to Head Trauma	165cm	85.7Kg	ETOH: 2 drinks on weekends; Tobacco: 1/2 to 1 ppd x 33yrs; No drug use.	Hypertention 10-15 yrs, controlled w/ low NA diet	IgG+	Neg	Neg	HBV NAT Neg	Neg	Neg
F	64	C	SAH	66"	75 Kg	No ETOH, Tobacco - quit 20 yrs ago; no drug use	HTN (uncontrolled), rheumatic fever when young	Not reported	Neg	Pos	Neg	Neg	Neg
F	48	H	CVA; 2nd to ICH	61"	90 Kg	No ETOH, Tobacco or drug use	Hypertension x 5 yrs - compliant.	IgG+	Neg	Pos	Neg	Neg	Neg
F	46	C	Anoxia; 2nd to Cardio-vascular	66"	153lb	ETOH: Occasional; Tobacco: none in past 25yrs; no drug use	Anxiety, breast augmentation, liposuction 10yrs ago, c-section 15yrs ago, peri-menopausal, night sweats. Meds: Zoloft	IgG+	Neg	Neg	Neg	Neg	Neg
F	58	C	Anoxia; 2nd to pulm embolism	64"	78 Kg	ETOH: 6yrs extensively - 1 gallon of vodka QD, quit 1-1/2 yrs ago; Tobacco: 40 pack yrs; Drugs: Marijuana 1 x 1 month	Positive Tox screen (ETOH/ anti-depressants), bipolar. Meds: antidepressant	Not reported	Neg	Not reported	Neg	Neg	Neg
F	40	B	ICH; 2nd to SAH & cerebral edema	70"	115 Kg	ETOH: social, Tobacco: 1ppd x 20yrs, Drugs: possible marijuana	Diabetes - inconsistent w/meds, HTN x 10yrs - non-compliant, bipolar, pituitary tumor 8 yrs ago, renal cell carcinoma - kidney; Meds: Coreg, effexor, ferrous sulfate, novolog-insulin, lipitor, lisinopril, norvasc, clonidine.	Not reported	Neg	Pos	Neg	Neg	Neg

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Gender	Age	Race	Cause of death	Height	Weight	Social history	Medical history	EBV	RPR	CMV	Hepatitis B	Hepatitis C	HIV
F	39	C	Anoxia; 2nd to Cardio-vascular	66"	170lb	ETOH: 1 glass wine 4x/yr x 10yrs; Tobacco: 1/2ppd x 5yrs; no drug use	HTN crisis 14 mos ago; C-section/ hysterectomy due to fibroids, HTN x 10yrs - compliant, Hx seizures - unk etiology, broken L foot. Meds: Keppra, HCTZ	IgG +	Neg	Pos	Neg	Neg	Neg
F	54	C	Anoxia; 2nd to CPA	66"	76 Kg	ETOH: 1/2 gallon vodka/day on and off x30yrs; No tobacco use; Drugs: prescription drug abuse - percocet, benzos, oxycodone, darvaset, did cocaine 15yrs ago	Cardiac arrest, hernia repair 3 mo ago, rhinoplasty x3, botox, bipolar, breast implants removed, drug rehab, flu shot, small ox vacc as child, asthma-nebulizing treatments, MD in eyes 2yrs ago	Pos	Neg	Neg	Neg	Neg	Neg
F	27	C	Anoxia; 2nd to Drug Overdose	66"	66 Kg	ETOH: 1/5 vodka every 3 months; Tobacco: 1ppd x 12yrs; Drugs: Heroine 3yrs, snorts cocaine, marijuana	NIDDM x 16yrs - insulin dependent; knee replacement 6yrs ago, 3 excisions from breast (fibra adenomas and melanocytic nevus), Pos HPB test few years ago. Meds: Insulin, Clonazepam, Hydroxyzine, Ziprasidone, Ambien, Depacote, Viibrid	Not reported	Neg	Pos	Neg	Neg	Neg
F	67	H	Anoxia	152.4 cm	74 Kg	No ETOH, Tobacco or drug use	CAD, CHF, CABG, HTN x 19yrs, Diabetes x 25yrs	IgG+	Neg	Pos	Neg	Neg	Neg
F	62	C	Head Trauma-ICH; 2nd to Fall	64"	64 Kg	No ETOH or drug use; Tobacco: 1ppd x 15yrs ago, quit multiple times (smoke few yrs, quit few years, then smoke again)	Hysterecomy 33yrs ago for uterine cancer, routine follow-up - no recurrence of cancer; femoral bypass; CVA 5 yrs ago, blind right eye from CVA; diagnosed 2 months ago with borderline NIDDM (no meds); shoulder fracture 1yr ago; positive for MRSA on nasal swab	Not reported	Neg	Pos	Neg	Neg	Neg
F	61	H	ICH/ Stroke	170cm	96 Kg	ETOH: Wine once/month x 45yrs; no Tobacco or drug use	HTN x 30yrs; Diabetes x 5yrs	IgG+	Neg	Pos	Neg	Neg	Neg
F	49	C	CVA/ICH	5'4"	179lb	ETOH: 1xmonth; no Tobacco or drug use	No history reported	IgG+	Neg	Pos	Neg	Neg	Neg

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Gender	Age	Race	Cause of death	Height	Weight	Social history	Medical history	EBV	RPR	CMV	Hepatitis B	Hepatitis C	HIV
F	77	C	ICH	61"	48 Kg	ETOH: couple of drinks a few time/yr ; Tobacco: 2 cigarettes daily, quit 10 yrs ago.; no drug use.	HTN x 12 yrs, Cancer - Oral 1 yr ago (surgically removed).	IgG+	Neg	Neg	Neg	Neg	Neg
F	27	C	Interstitial lung disease; 2nd to anoxia-DCD	63"	86 Kg	No ETOH, Tobacco or drug use	Cerebral palsy, lupus, Interstitial lung disease, MRSA , migraine & seizure history, knee/hip sx, bladder infection. Meds: atovaquone-an	Not reported	Neg	Pos	Neg	Neg	Neg
F	59	C	CVA	68"	204lb	ETOH: Socially - quit 20 yrs ago; Tobacco: 1 ppd x 8yrs - quit 30yrs ago; no drug use	HTN x >15yrs, potential lupus 10yrs ago, arthritis, tubal ligation, cholecystectomy. Meds: Toprol, Hisar	Pos	Neg	Neg	Neg	Neg	Neg
F	43	C	Anoxia	5'10"	240lb	No ETOH, Tobacco or drug use	Liver disease	IgG+	Neg	Neg	Neg	Neg	Neg
F	51	C	Anoxia due to Cardio-vascular	48"	50.9Kg	No ETOH, Tobacco or drug use	Hypertension x 5yrs, no Diabetes - A1C 6.6	Neg	Neg	Neg	Neg	Neg	Neg
F	59	C	CVA/ICH	5'3"	138lb	ETOH: 6-18 beers/day x 30yrs; Tobacco: 1-2 ppd x 45yrs; no drug use	No history reported	IgG +	Neg	Pos	Neg	Neg	Neg
F	64	C	Head Trauma, Blunt Injury	5'8"	71.7Kg	ETOH: 1 hard liquor 2x/yr x 20yrs - quit 20yrs ago; Tobacco: 1/2 ppd x 40yrs - quit 4 months ago; no drug use	COPD 10-12yrs, CAD, Rheumatic Heart Disease, V-tach, HTN x 20+ yrs	IgG+	Neg	Pos	Neg	Neg	Neg
F	50	C	Anoxia 2nd to Cardiac Arrest	4'11"	81 Kg	ETOH: 1-2 glasses a wine a night ; Tobacco: 5-6 cigs/day x 4 yrs.; no drug use.	No history reported	Not reported	Neg	Pos	Neg	Neg	Neg
F	53	C	Anoxia	64"	79.1Kg	ETOH: 1 bottle of wine a day; Tobacco: 1ppd; no drug use	Asthma	Not reported	Neg	Neg	Neg	Neg	Neg
F	48	C	CVA; 2nd to ICH	5'4"	169lb	ETOH: (Wine, Liquor) unk amts daily >25yrs; Tobacco: 1ppd >25yrs; no drug use reported	COPD, HTN x 10yrs non-compliant, ovarian cancer - hysterectomy 7yrs ago - last check up 2 yrs, DM type II x 1yr - poor compliance. Meds: Simvastin, Verapamil, Aspirin, HCTZ, Clonidine, Trillpine.	IgG +	Neg	Pos	Neg	Neg	Neg

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F	77	C	Head Trauma; 2nd to Blunt Injury, non-MVA	63"	190lb	ETOH: 1-2 beers per mo x 47 yrs; No tobacco or drug use	HTN 10-15 yrs - compliant, CHF w/pacemaker, COPD x 4-5 yrs, periperal neuropathy, fibromyalgia, flu shot last year, knee & hip replacement. Meds: nebulizer, omeprazole, metoprolol, pregablin, losartan, KCl, furosemide, fluoxetine, gigoxin, warfarin, singular, fentanyl, ipr.	Not reported	Neg	Pos	Neg	Neg	Neg
F	26	C	Trauma	5'11"	256lb	ETOH: heavy use 1/5 a day/4 x wk; Drugs: IV DA - herion and meth X 1.5 yrs- current; no tobacco use.	No history reported	IgG+	Neg	Pos	Neg	Neg	Neg
F	50	C	Head Trauma	5'4"	68.1Kg	Tobacco: quit 3 yrs ago; no ETOH and drug use.	No history reported	Not reported	Neg	Neg	Neg	Neg	Neg
F	39	C	CVA/Stroke 2nd to ICH	67"	211lb	ETOH: 6 pack/beer on weekends x 4 yrs, quit 20 yrs ago; Tobacco: 1/2 ppd x 20 yrs.; Drugs: MET, Benzos, Opiates	HTN x 2 yrs, Lupus x 2 yrs,d/t hermatoma on kidney 1 yr ago (unknown which kidney), hx of pneumonia. Meds: hydroxyl-chloroquine, furosemide, lisinopril, carvedilol, spironolactone, unspecified diet pills.	IgG+	Neg	Neg	Neg	Neg	Neg
F	40	C	Anoxia	63"	95.3Kg	ETOH: 2-3 drinks/month; no tobacco or drug use	Asthma	Neg	Neg	Neg	Neg	Neg	Neg
F	35	C	Stroke	65"	198lb	No ETOH, Tobacco or drug use	Kidney stone 2 yrs ago, tubal ligation 12 yrs ago.	Not reported	Neg	Pos	Neg	Neg	Neg
F	32	C	Natural Causes	5'5"	237lb	ETOH: 1 drink/week; no tobacco or drug use.	No history reported	IgG+	Neg	Pos	Neg	Neg	Neg
F	20	C	CVA/ Stroke	5'4"	57.8Kg	ETOH: Occasional beer/wine cooler; Tobacco: 1ppd x 5yrs - quit past 2 months; no drug use	No history reported	Not reported	Not reported	Neg	Neg	Neg	Neg

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F	45	C	Anoxia	5'4"	124 lbs	ETOH: heavy; no tobacco or drug use	No history reported	IgG+	Neg	Pos	Neg	Neg	Neg
F	47	C	Abdominal aortic aneurysm	65"	80 Kg	ETOH: heavy drinker - quit 7yrs ago; Tobacco: 1ppd x 20yrs - quit 7yrs ago; no drug use.	HTN	Not reported	Neg	Pos	Neg	Neg	Neg
F	52	C	Blunt Injury, MVA	162 cm	82 Kg	No tobacco use. ETOH and drug use not reported.	Diabetes, HTN	IgG+	Neg	Pos	Neg	Neg	Neg
F	61	C	Anoxia, Cardiac Arrest	64"	47.7Kg	ETOH: rare beer; no tobacco or drug use	Paraxismal A-Fib, Squamous Basal Cell Skin CA	IgG+	Neg	Neg	Neg	Neg	Neg
F	48	C	Cardiac Arrest; 2nd to Seizure Activity	60"	159lb	No ETOH, Tobacco or drug use	Lobectomy d/t hx of seizures from epilepsy dx at 9 months. Meds: Lamictal	Not reported	Neg	IgG +	Neg	Neg	Neg
F	39	C	Anoxia; 2nd to Cardiac arrest	63"	90 Kg	ETOH: occasionally; Tobacco: 1/2 ppd x 10yrs; Drugs: abused prescription narcotics, smoked marijuana	HTN x 3yrs: non-compliant	Pos	Neg	Pos	Neg	Neg	Neg
F	59	C	ICH; 2nd to Stroke	68"	73 Kg	No ETOH, Tobacco or drug use	Double mastectomy/ chemo for breast cancer 15 yrs ago, migraines. Meds: keflin, levophed, calcium, crestor	Pos	Neg	Pos	Neg	Neg	Neg
F	44	C	Stroke	62"	261lb	No ETOH, Tobacco or drug use	HTN, Type 2 diabetes (NIDDM), obstructive pulmonary disease, possible renal cell carcinoma	Not reported	Neg	Pos	Neg	Neg	Neg
F	55	C	OD Heroin	173cm	109.8Kg	ETOH: Vodka - unknown amount; Tobacco: 1ppd x 15yrs; Drugs: Heroin, Marijuana, Cocaine	No history reported	IgG+	Neg	Neg	Neg	Neg	Neg
F	74	C	Stroke	5'5"	146lb	ETOH and Tobacco use - unknown amt; no drug use	Hypertension	IgG+	Neg	Pos	Neg	Neg	Neg
F	57	Black/ Hispanic	Head trauma; 2nd to MVA	65"	153lb	ETOH: Light drinker; No tobacco or drug use	HTN x 10yrs - compliant, hysterectomy 20yrs ago, genital herpes 6 mos ago - no active lesions. Meds: HTN meds.	IgG +	Neg	IgG +	Neg	Neg	Neg

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F	72	C	CVA	5'4"	78.9Kg	No ETOH, Tobacco or drug use	HTN	IgG+	Neg	Neg	Neg	Neg	Neg
F	74	C	Head Trauma	65"	53 Kg	ETOH: 3-4 glasses of wine/day x 30yrs; No tobacco or drug use	No history reported	Pos	Neg	Pos	Neg	Neg	Neg
F	57	AA	Anoxia 2nd to Cardiovascular	5'9"	113.5Kg	ETOH: previously, quit 3 yrs ago ; Tobacco: 2.5 ppd x 10 yrs.; Drugs: snorted heroin, crack, cocaine.	COPD/ Asthma, CHF, HTN x 10 yrs, Diabetes Type 2	Not reported	Neg	Pos	Neg	Neg	Neg
F	45	C	GSWH; 2nd to homicide	66"	190lb	ETOH: Whiskey, beer weekends socially; Tobacco: 1 ppd x 31yrs; Drugs: smoke meth, used xanex - both in past 3-5yrs. Marijuana - last time 1 mo ago	No HTN	Neg	Neg	Pos	Neg	Neg	Neg
F	38	C	Drug over dose	5'6"	86.2Kg	ETOH: binge drinks 2/wk since age 14; Tobacco: 1/2 ppd x 20 yrs; Drug: current IVDA.	No history reported	IgG +	Neg	Neg	Neg	Neg	Neg
F	71	C	ICH	67"	132Kg	ETOH: 3 glasses wine QDx50 yrs; Tobacco - quit 25 yrs ago ; no drug use	Knee replacement, hysterectomy, 5 yrs ago long plane flt-DVT in leg - progressed to PE. Meds: Coumadin, Paxil	Not reported	Neg	Pos	Neg	Neg	Neg
F	70	C	ICH/ Stroke	66"	89 Kg	No ETOH; Tobacco: 1 ppd x 25-30yrs - quit 3 months ago; no drug use	Hypothyroidism, HTN, Hyperlipidemia, knee surgery 8 mos. ago, extremely dry skin. Meds: cortisone, synthroid, aspirin, lipitor	Not reported	Neg	Pos	Neg	Neg	Neg
F	71	C	Anoxia/ Cardio-vascular	5'1"	152lb	Tobacco: amt/type not reported; no ETOH and drug use.	Asthma/ Bronchitis, HTN x 20 yrs, Basal cell Squamous	IgG+	Neg	Pos	Neg	Neg	Neg
F	51	A	CVA	160 cm	44.1Kg	No ETOH, Tobacco or drug use	Cardio-myopathy	IgG+	Neg	Neg	Neg	Neg	Neg
M	49	C	CVA	70"	256lb	No ETOH, Tobacco or drug use	HTN x 10yrs, Diabetes 6-10yrs, CAD. Meds: anti-HTN, diabetes	Not reported	Neg	Neg	Neg	Neg	Neg
M	48	H	Head Trauma; 2nd to Homicide	66"	73 Kg	ETOH: unknown quantity and duration; no tobacco or drug use	Heart problems	Not reported	Neg	Pos	Neg	Neg	Neg

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M	62	C	CVA	5'10"	168lb	ETOH: 1 beer/yr x 40 yrs; Tobacco: 1 pack x 40 year, quit 12 years ago; No drug use.	COPD, MI, AICD, Stents, Type 2 Diabetes, HTN	Not reported	Neg	Not reported	Neg	Neg	Neg
M	29	C	GSW	69"	94 Kg	ETOH: Alcoholic; Tobacco: 1 ppd x 8 yrs; no drug use	Depression	Not reported	Neg	Neg	Neg	Neg	Neg
M	53	C	Head Trauma	170 cm	80 Kg	Tobacco: 1ppd smoker x 30 yrs; no ETOH and drug use.	Type 2 IDDM x for 5-7 yrs. Meds: Insulin 4-5 yrs.	Not reported	Neg	Pos	Neg	Neg	Neg
M	85	C	Head Trauma; 2nd to Fall from roof	68"	251lb	No ETOH or Tobacco use. No drug use reported, but tox screen positive for benzos and opiates	NIDDM >10yrs, HTN >10yrs - compliant, ruptured bladder - 35 yrs ago, ruptured gallbladder 3 yrs ago, Cataracts-bilat. Meds: Oral meds for diabetes, HTN	IgG+	Neg	Pos	Neg	Neg	Neg
M	56	B	CVA	71"	103Kg	No ETOH, Tobacco or drug use	Tooth extraction, CABG, end stage renal disease x 7 yrs, CAD, pneumonia 1 yr ago, HTN x 25 yrs, enlarged heart >15 yrs, IDDM, heart bypass 2 yrs ago, high blood pressure > 25 yrs, pos skin test for TB 6 yrs ago	Not reported	Neg	Pos	Neg	Neg	Neg
M	28	C	ICH-Stroke	5'10"	218lb	ETOH: Beer/Wine - once/month x 1yr; Tobacco: 15yrs ago; Drugs: marijuana smoked once 15yrs ago	Paraplegia from cervical spine injury, frequent kidney/bladder infections due to catheter 2nd to accident 14yrs ago. Meds: Prilosec	IgG +	Neg	Neg	Neg	Neg	Neg
M	53	C	CVA	71"	121Kg	ETOH: Rarely; Tobacco: 1/2ppd x 12yrs - quit 25 yrs; Drug use reported but unknown type/duration	CABG, saph vein graft, HTN x 8yrs w/meds, IDDM x 2 wks, NIDDM x 5 yrs. Meds: Coumadin, HTN meds, insulin, ameradane, Vakadin	Not reported	Neg	Pos	Neg	Neg	Neg
M`	44	C	MVA	71"	75 Kg	ETOH: Heavy, Tobacco: 1ppd x 20yrs; Drugs: marijuana & cocaine	Positive on admit for marijuana and cocaine	Not reported	Neg	Pos	Neg	Neg	Neg

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M	64	C	CVA	74"	150 Kg	ETOH: 2 beer/year; Tobacco: 4ppd x 15 yrs previously - quit 20 yrs ago; no drug use	Sq cell CA removed on hands 6 mos. ago, NIDDM x 10 yrs, HTN 15-20 yrs, PVD, Osteoarthritis, Bilat knee repl., kidney stones removed 33 yrs ago; Meds: Glucoside, quananine, isosorbine, metaprolol, aspirin	Not reported	Neg	Neg	Neg	Neg	Neg
M	43	C	Stroke	62"	46 Kg	No ETOH use or drug use; Tobacco: smoked for 20 yrs.	Schizophrenia, cerebal palsy, GERD, hypothyroidism, HTN, endocarditis, anemia, testicular cancer last year stage 2, unknown treatment, open heart surgery as child for valve repair.	Pos	Neg	Neg	Neg	Neg	Neg
M	27	C	Head Trauma	66"	76 Kg	ETOH and Tobacco use - unk amt, duration; No drug use.	No history reported	IgG +	Neg	Pos	Neg	Neg	Neg
M	38	C	Anoxia (Suicide)	177 cm	77.4Kg	ETOH: 3 - 6 cans/day; Tobacco: daily smoker; Drugs: Daily THC, cocaine 5 yrs ago, Meth prior to admit - IVDA and smoked.	No history reported	Not reported	Neg	Neg	Neg	Neg	Neg
M	53	C	ICH	180 cm	121Kg	No ETOH, Tobacco or drug use	HTN, Diabetes	IgG+	Neg	Neg	Neg	Neg	Neg
M	66	C	Stroke	72"	240lb	No ETOH, Tobacco or drug use	T2 Diabetes and HTN x 13 yrs - compliant with meds., 2 previous CVA.	Not reported	Neg	Pos	Neg	Neg	Neg
M	41	C	Anoxia due to drug intoxication	5'5"	170lb	ETOH: up to one case daily beer and malt liquor; Tobacco: 1ppd x adult life; Drugs: heroin, cocaine, marijuana, prescription meds	Hypertension, Respiratory disease - active influenza A	Not reported	Neg	Neg	Neg	Neg	Neg
M	48	AA	CVA; 2nd to ICH	168cm	83 Kg	ETOH: 3 beers & 1 liquor drink 1x/week x 30yrs; No tobacco or drug use	CHF & cardiomyopathy, HTN x 10yrs, Diabetes x 10yrs	Not reported	Neg	Pos	Neg	Neg	Neg
M	45	C	Anoxia	170 cm	102.4Kg	Tobacco: 1ppd; no ETOH or drug use.	No history reported	IgG+	Neg	Pos	Neg	Neg	Neg

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M	47	C	Head Trauma, Blunt injury	5'8"	124.4Kg	No ETOH, Tobacco or drug use	No history reported	Not reported	Not reported	Pos	Neg	Neg	Neg
M	10mos	C	Anoxia	2'7"	8.2 Kg	No ETOH, Tobacco or drug use	No history reported	Neg	Neg	Neg	Neg	Neg	Neg
M	48	C	Anoxia	6'0"	291lb	Tobacco: 1ppd x 30 yrs; no ETOH or drug use.	CAD w/ stent placement 1 yrs ago, HTN - unk duration (non-compliant with meds), Diabetes - possible type 2 (undiagnosed)	Not reported	Neg	Neg	Neg	Neg	Neg
M	64	C	CVA	6'0"	229lb	ETOH: 1 beer a year; Tobacco: 1ppd x 30yrs - quit 18yrs ago; no drug use.	COPD, CAD, CHF, LVH, MI, HTN x 15yrs, Diabetes x 15yrs.	IgG +	Neg	Pos	Neg	Neg	Neg
M	52	C	Head Trauma; 2nd to Blunt injury (non-MVA)	69"	77 Kg	ETOH: Heavy drinker for 33 yrs (liquor); No tobacco or drug use	High BP x 10 yrs (non-compliant), periods of confusion 3 yrs ago, back surgery 27 yrs ago for slip disc. Meds: BP meds (non-compliant), aspirin, meds for neck pain	IgG+	Neg	Pos	Neg	Neg	Neg
M	59	C	Anoxia	5'8"	230lb	No ETOH last 18yrs; Tobacco: 1/2ppd x 8 yrs - quit 27yrs ago; no drug use.	Hypertention 0-5yrs	IgG+	Neg	Pos	Neg	Neg	Neg
M	74	C	CVA	72"	218lb	ETOH: 1/2 case beer/day x 33yrs; Tobacco: 1.5ppd x 50yrs; No drug use	HTN, depression, COPD 5-6yrs, MI 17yrs ago, OA, prostate cancer 15yrs ago cured, bilateral hip replacement, splenectomy, bladder cancer 4yrs ago w/chemo, kidney stones 6mo ago. Meds: ASA, metoprolol, symbostatin, spiriva, albuterol, doxizosin, amitriptyline, bupropion.	Not reported	Neg	Pos	Neg	Neg	Neg
M	57	C	Resp. Distress	69"	220Kg	ETOH: 8 oz brandy/day x 20 yrs - quit 10 yrs ago; Tobacco: 1ppd x 20yrs, quit 10 yrs ago; Cocaine x 8yrs – quit 20yrs ago.	HTN x 10yrs, GSW abdomen (long ago), PVD, Asthma 2 yrs ago (maybe COPD), Appendectomy	Not reported	Neg	Pos	Neg	Neg	Neg

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M	51	C	IC Bleed	70"	298lb	ETOH: as a teen; no tobacco use; Drugs: Marijuana as a teen	Diabetes Type II, HTN x 6yrs, noncaseating granuloma	Not reported	Neg	Pos	Neg	Neg	Neg
M	38	C	Blunt Head Trauma; 2nd to MVA	74"	124Kg	ETOH: 3-5 beers socially on weekends; Tobacco: 1-2 ppw x 15 yrs; Drugs: none reported but positive for THC on Admit and benzos (after ACLS meds)	Hernia repair - 1 yr ago, eyelid laceration - 16 yrs ago, c/o stiff knees	Not reported	Neg	Neg	Neg	Neg	Neg
M	29	C	Anoxia; 2nd to Cardiovascular	73"	86 Kg	ETOH: unknown amt; no Tobacco use; Drugs: Cocaine (snorted) unknown amt x 3 mos, Marijuana (smoked) unknown amt x 2yrs	No history reported	IgG+	Neg	Neg	Neg	Neg	Neg
M	56	C	Anoxia; 2nd to Cardiac Arrest	70"	220lb	ETOH: social 2 drinks/QD - quit 7 yrs ago; Tobacco: 1ppd x 5 yrs; no drug use.	HTN x 5yrs - compliant, CAD, COPD, CABG x 4, AICD, pacer, eczema, bronchial asthma as child.	Not reported	Neg	Pos	Neg	Neg	Neg
M	38	C	Cardiac/ Anoxic	6'1"	176lb	ETOH: 12pk/day since 18yrs old; no Tobacco or drug use	HTN - compliant	IgG+	Neg	Neg	Neg	Neg	Neg
M	11	H	Anoxia 2nd to Respiratory distress/ Arrest	42"	29 lb	No ETOH, Tobacco or drug use	Born w/ Werdnig-Hoffman disease, pegged and trached at 9 mos, bed ridden all his life, Osteogenesis imperfecta, seizures, hernia repair, sinusitis, UTI, blood transfusion. Meds: Luminial, Prevacid, Singulair, Albuterol	Neg	Neg	Neg	Neg	Neg	Neg
M	50	C	CVA	69"	97 Kg	ETOH: beer on weekends; tobacco: 1ppwk x 30yrs; Drugs: Marijuana since age 15 - current	HTN and NIDDM x 3yrs - non-compliant for both, dx w/lyme's disease upon admission. Meds: Allopurinol, Metformin (non-compliant with both)	IgG +	Neg	Neg	Neg	Neg	Neg

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M	46	C	CVA/ICH	70"	195 lb	ETOH: 6 pack per day; Tobacco: 8 cigars x 20 yrs; Drugs: Marijuana	Back sx, Rheumatic fever as child	Not reported	Neg	Pos	Neg	Neg	Neg
M	37	C	Anoxia; 2nd to Cardio-vascular	70"	Not reported	ETOH: Socially on weekends - quit 1.5 yrs ago; Tobacco: socially for unknown duration - quit 7-9 yrs ago; Drugs: smoked marijuana since college - last use 1 week ago.	Jaundice at birth - resolved shortly after, laser eye surgery for vision problems, vasectomy, flu vaccine last year.	IgG+	Neg	Neg	Neg	Neg	Neg
M	47	C	Anoxia; 2nd cardio pulmonary arrest	74"	427lb	Tobacco: 0.5ppd x 15 yrs - quit 4 months ago; no ETOH or drug use.	Cardiac arrest, NIDDM. Meds: diabetes medications	Not reported	Neg	Pos	Neg	Neg	Neg
M	38	H	CVA	70"	204lb	ETOH: social 1-2 beers weekends/special occasions; no tobacco or drug use	No history reported	Not reported	Neg	Neg	Neg	Neg	Neg
M	37	H	HT; 2nd SIGSW	5'10"	199lb	ETOH: Past year 12 pack/day of beer, prior drank socially; No tobacco or drug use reported.	No history reported	Not reported	Neg	Pos	Neg	Neg	Neg
M	53	C	Anoxia, Cardiovascular	68"	64 kg	ETOH: several beers/day since age 16, hard liquor for past 2 years; Tobacco: 1/2 ppd x 12 yrs, 1-2 cigs/day past 8 yrs; Drugs: nonprescribed pain medication, cocaine and marijuana many years ago.	No history provided	Neg	Neg	Pos	Neg	Neg	Neg
M	54	C	CVA	69"	100Kg	Tobacco: 1ppd since age 16; no ETOH or drug use	IBS 9-10 yrs, HTN x 10 yrs , Diabetes (NIDDM) and Marfan's Syndrome dx 7 yrs ago, Open heart sx for Marfans-aneurism in heart, Kidney stones 5 yrs ago, 3 stents placed in heart, right decompression hemicraniotomy; Meds: High blood pressure and oral meds for NIDDM	Not reported	Neg	Pos	Neg	Neg	Neg
M	21	C	Gunshot Wound	178cm	69 Kg	ETOH: Occasional, Tobacco: 1ppw x 4 yrs; no drug use	No history reported	Not reported	Neg	Pos	Neg	Neg	Neg

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M	50	H	CVA/Stroke	168cm	236lb	ETOH: 10-12 packs of beer/week and Tequila; Drugs: snorted cocaine 30 yrs ago; no tobacco use.	Hypertension x 2yrs with meds	IgG+	Neg	Pos	Neg	Neg	Neg
M	45	C	Anoxia, Cardio-vascular	6'4"	243lb	ETOH: 3-4 vodka/wine per day x 20 yrs; Tobacco: 1/4 ppd x 20 yrs; Drugs: marijiuana 2 x day x 20 yrs.	HTN	IgG +	Neg	Pos	Neg	Neg	Neg
M	63	C	CVA	65"	61 Kg	ETOH: beer social; Tobacco: chewed tobacco daily x 30yrs; no drug use	HTN x 1yr, Heart Arythmia, CHE, Afib, dilated cardiomyopathy, diverticulitis - sx 12yrs ago	Not reported	Neg	Pos	Neg	Neg	Neg
M	53	C	ICH	6'1"	98 Kg	ETOH: 2-3 beers/week; Tobacco: previous tobacco chewer; no drug use.	Hypertension	IgG+	Neg	Pos	Neg	Neg	Neg
M	57	C	ICH/Stroke	6'1"	231lb	ETOH: Daily at least 1, beer, bourbon, wine since teens; Tobacco: Smoked for 8yrs - quit 14yrs ago, smoked cigars 1 time a year; Drugs: Experimented with cocaine, mushrooms, acid and marijuana in high school	Asthma - inhaler rarely used, Cardiac ablation for SVT 7 yrs ago, HTN 7 yrs ago - unknown treatment/ compliance	IgG+	Neg	Pos	HBsAb+	Neg	Neg
M	35	AA	CVA	66"	106Kg	ETOH: 3-4 6-packs per day x 21 yrs; Tobacco: 1 ppd x 21 yrs; No drug use	Asthma-outgrew in adolescence; HTN x 4 yrs, no meds; borderline diabetes	Not reported	Neg	Neg	HBcAb and NAT Neg	Neg	Neg
M	49	C	Head trauma; 2nd to MVA	66"	91 Kg	ETOH: occasional; Tobacco: 1 -2 ppd x 20 yrs; no drug use.	Depression, bipolar disorder, chronic back pain, back surgery pain, high CDC, UTOX pos. for opiates and benzos; Meds: oxycodone.	IgG+	Neg	Pos	Neg	Neg	Neg
M	60	C	CVA/ICH	6'2"	153lb	No ETOH or Tobacco use; Drugs: THC use	No history reported	IgG +	Neg	Neg	Neg	Neg	Neg

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M	25	C	Anoxia, 2nd to Drug Intox.	6'0"	262 lbs	ETOH: Beer & Hard Liquor (6 per yr x last 2 yrs; prior to that drank hard liquor daily x 3 yrs); Tobacco: cigarettes (1 ppd x 9 yrs); Drugs: smoke marijuana, snorted cocaine & meth as a teen; methadone x last 1-2 wks	Jaundice at birth. Meds: antidepressants, ambien, percocet, hydrocodone	Not reported	Neg	Pos	Neg	Neg	Neg
M	63	AA	Head Trama	6'	109Kg	ETOH: 2 drinks/wk (beer/liquor) x 30 yrs; Tobacco: 1 PPW x 15 yrs, quit 6 mos. ago; No drug use	HTN: controlled by meds.	Neg	Neg	Pos	Neg	Neg	Neg
M	17	C	Anoxia 2nd to Hanging	71"	66 kg	No ETOH, Tobacco or drug use	Exercise induced asthma w/inhaler.	Neg	Neg	Neg	Neg	Neg	Neg
M	35	H	GSWH 2nd to alleged homicide	68"	103 Kg	ETOH: socially, occassionally whiskey; Tobacco: 1 ppd x many yrs; no drug use.	Asthma since age 2, bx left lung 1 month ago, recent tx for lung infection (unk). Meds: proventil, diet pills, naprosyn.	Pos	Neg	Neg	Neg	Neg	Neg
M	13	C	SIGSW to the head	5'10"	100Kg	No ETOH, Tobacco or drug use	No history reported	IgG +	Neg	Pos	Neg	Neg	Neg
M	33	C	Anoxia	74"	85.2Kg	ETOH: Social drinker - unknown amounts; Tobacco: 1/2ppd x 15yrs; Drugs: Marijuana - unknown amount or duration.	Possible Hep A in childhood	IgG+	Neg	Neg	Neg	Neg	Neg
M	48	C	Anoxia 2nd to S/P Hanging	71"	76 Kg	ETOH: Wine and beer on wkend; Tobacco: closet smoker for 15 yrs, unk amt; no drug use.	HTN x 2 yrs ago - non compliant, BKA 20 yrs ago, pos TB test yrs ago; possible Hepatitis B vaccination; Meds: sybostatin, insomnia and depression meds	Not reported	Neg	Not reported	HBsAb +	Neg	Neg
M	63	C	Head Trauma	74"	124.5Kg	ETOH: 15-30 beers a day x 37 yrs; no Tobacco or Drug use	Cardiac Disease: MI 30+ yrs ago	Not reported	Neg	Neg	Neg	Neg	Neg
M	53	C	Anoxia	72"	90.9Kg	ETOH: a couple of drinks 2-3 nights a week; Tobacco: quit 10 yrs ago; Drugs: marijuana and cocaine past 3 yrs	Asthma	IgG+	Neg	Pos	Neg	Neg	Neg

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M	55	C	CVA	70"	79 Kg	ETOH: Less than 4 drinks/week since teen ; tobacco: 1/2 ppd x 10-15 yrs, quit 20 yrs ago; no drug use.	AVR, AAA & repair, anxiety. Meds: blood thinners, BP meds (not dx), anxiety meds, plavix, aspirin	IgG+	Neg	Neg	Neg	Neg	Neg
M	60	C	Cerebro-vascular Stroke; 2nd to ICH	70"	167lb	ETOH: several beers per month; Tobacco: 1ppd x 37yrs, 2ppd in past 3 yrs; no drug use.	Left carotid artery stenosis with previous endarterectomy 7 yrs ago - left carotid artery stent placed; right renal artery stent, treated for renal artery stenosis 5 months ago - left renal artery stent placed; HTN, CAD, PVD - all dx 7yrs ago - non-compliant with all rx meds.	IgG +	Neg	Pos	Neg	Neg	Neg
M	51	C	CVA/Stroke; 2nd to ICH	70"	108Kg	ETOH: 2-3 beers/wine 2-3x/wk x 30yrs; tobacco: cigars last 5 years; Drugs: Cocaine abuse	HTN x 10yrs - non-compliant w/meds last few months. Meds: HTN meds	IgG +	Neg	IgG +	Neg	Neg	Neg
M	36	C	Head Trauma 2nd to self-inflicted GSW head	76"	278lb	ETOH: 6 pk, 4 per week; Tobacco: 1 PPD x 22 yrs; Drugs: cocaine x 20 yrs, pain killers x 20 yrs (last used 2 days ago).	Asthma dx as a child - 1 -2 episodes/yr, back injury 9 mos ago, vasectomy 2 yrs ago, peanut allergy, hep B vaccine. Meds: Clindamycin.	IgG +	Neg	Pos	Neg	Neg	Neg
M	78	C	Anoxia	73"	244lb	No ETOH, Tobacco or drug use	HTN x 10yrs, CAD, GI disease, black lungs.	Not reported	Neg	Pos	Neg	Neg	Neg
M	50	C	CVA	5'9"	200lb	ETOH: 1 drink - 1x/week; No tobacco or drug use.	Hypertension x 10yrs	IgG+	Neg	Pos	Neg	Neg	Neg
M	41	H	Head Trauma	6'	97 Kg	ETOH: 5 drinks/day 4 times per week - Liquor/tequila 10 oz once per month; Tobacco: 1ppd x 10 yrs - quit 10 yrs ago; no drug use.	Hypertension - unknown duration	Not reported	Not reported	Pos	Neg	Neg	Neg
M	33	C	Drug Intoxication	70"	200lb	ETOH: Binge drinker 24 beers/day when drinking; Tobacco: 1pk per 2weeks x 10yrs; Drugs: smoked marijuana; snorted cocaine, orally took speed, ecstacy mushrooms and acid between 18-20y/o	No history reported	IgM+	Neg	Pos	Neg	Neg	Neg

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M	64	C	CVA/ICH	6'1"	279lb	No ETOH; Tobacco - quit 15yrs ago ; Drugs: THC 23 yrs ago	COPD, Stents 10 & 15yrs ago, HTN x 15 yrs	Not reported	Neg	Pos	Neg	Neg	Neg
M	24	C	Anoxia; 2nd to Stroke	72"	129Kg	ETOH: socially; Tobacco: 1-2 cig occasionally; no drug use.	Valve replacement 8 yrs ago. Meds: Coumadin	Not reported	Neg	Pos	Neg	Neg	Neg
M	56	C	CVA/Stroke	5'11"	81 Kg	ETOH: unknown; No tobacco or drug use.	No history reported	IgG+	Neg	Neg	Neg	Neg	Neg
M	56	C	Head Trauma; 2nd to fell down stairs	70"	160lb	ETOH: 2-3 beers/day; Tobacco: 1ppd x 26 yrs; no drug use	High Cholesterol, High Blood Pressure, recent Bypass-Groin; Meds: Plavix, Fish Oil, Cholesterol meds	Not reported	Neg	Neg	Neg	Neg	Neg
M	64	C	CVA/Stroke	71"	215lb	ETOH: Beer or Liquor 1-2 drinks per mo. x 10yrs; Tobacco: 2ppd x >10yrs; no drug use	Groin surgery 3 months ago, AAA repair 6 yrs ago, HTN x 8yrs, IDDM x 8yrs - compliant with meds. Meds: Proscar, Lipitor, Norvasc, Zestril, Glucophage	IgG+	Neg	Not reported	Neg	Neg	Neg
M	49	C	CVA	70"	251lb	ETOH: 1 -2 beers/month; Tobacco: 1 ppd x 35 yrs; Drugs: marijuana in 20s.	Cardiac arrest, MVA hip fractures 32 yrs ago, depression, infertility treatments 10yrs ago, chest pain 2 yrs ago - hemopexis, infection from smoking, cold sore on lips. Meds: Lexapro.	Not reported	Neg	Pos	Neg	Neg	Neg
M	71	C	Head Trauma; 2nd to fall	5'6"	94 Kg	No ETOH, Tobacco or drug use	Diabetes - unknown duration	IgG+	Neg	Pos	Neg	Neg	Neg
M	43	C	Anoxia	5'11"	251lb	ETOH: rare; no tobacco or drug use.	Heart bypass 4 yrs ago, HTN - compliant with meds unk duration, non-alcholic fatty liver	Not reported	Neg	Neg	Neg	Neg	Neg
F	77	C	Stroke/ ICH	4'11"	150 lb	ETOH: socially 1 glass of wine/month; no tobacco or drug use	No history reported	Not reported	Neg	Pos	Neg	Neg	Neg

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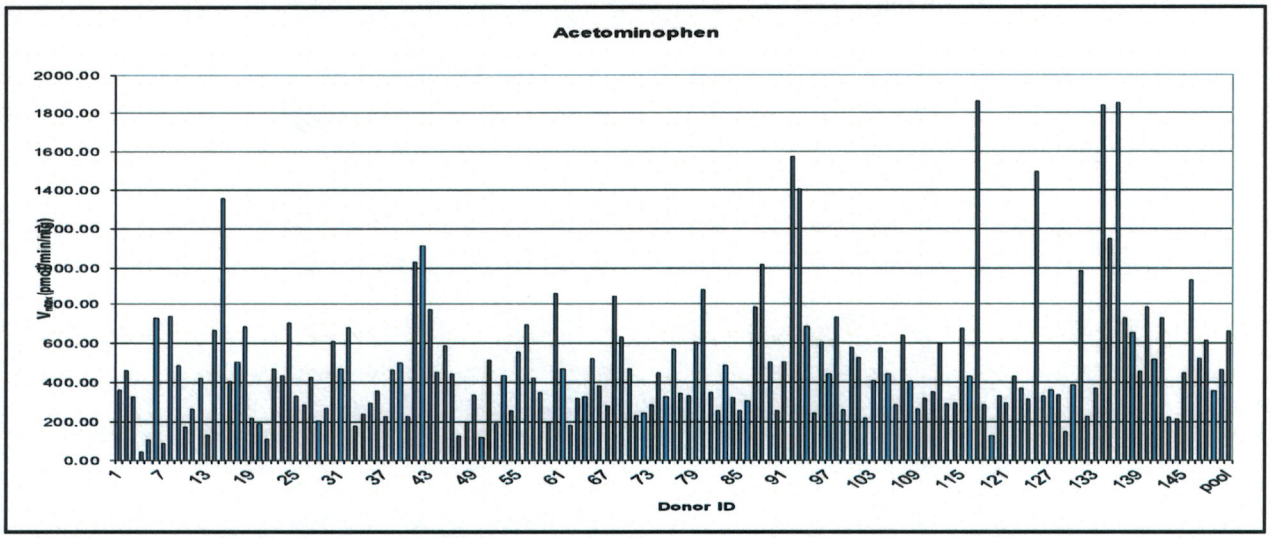

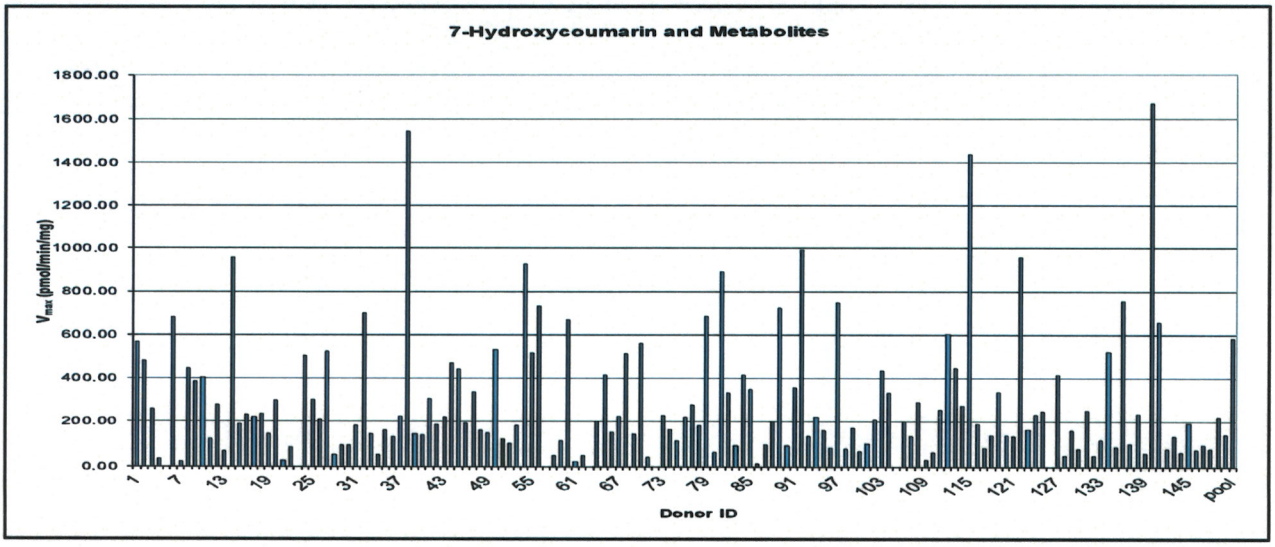

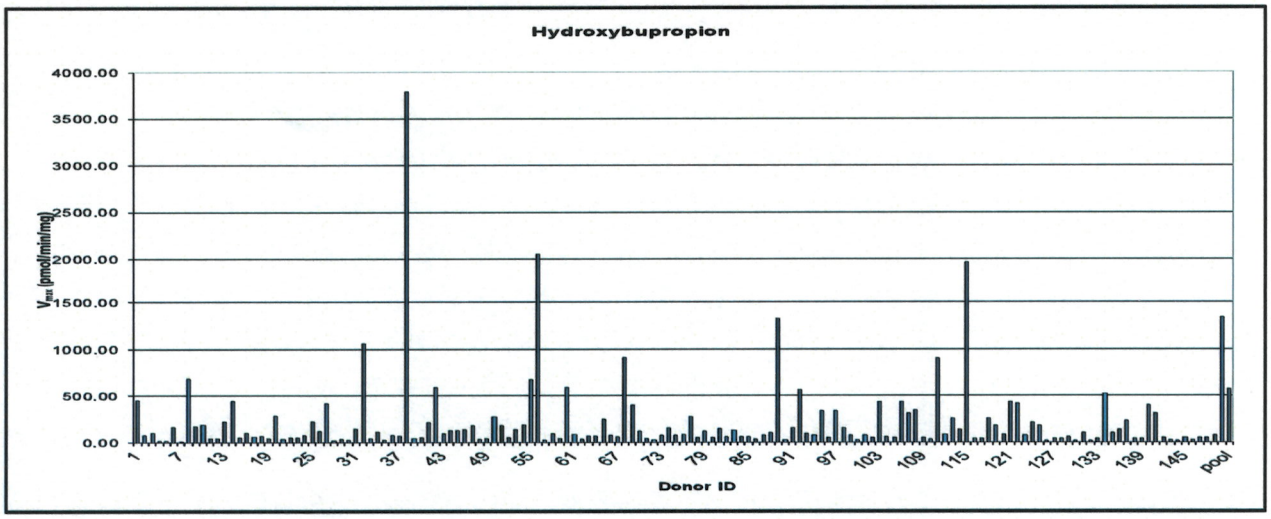

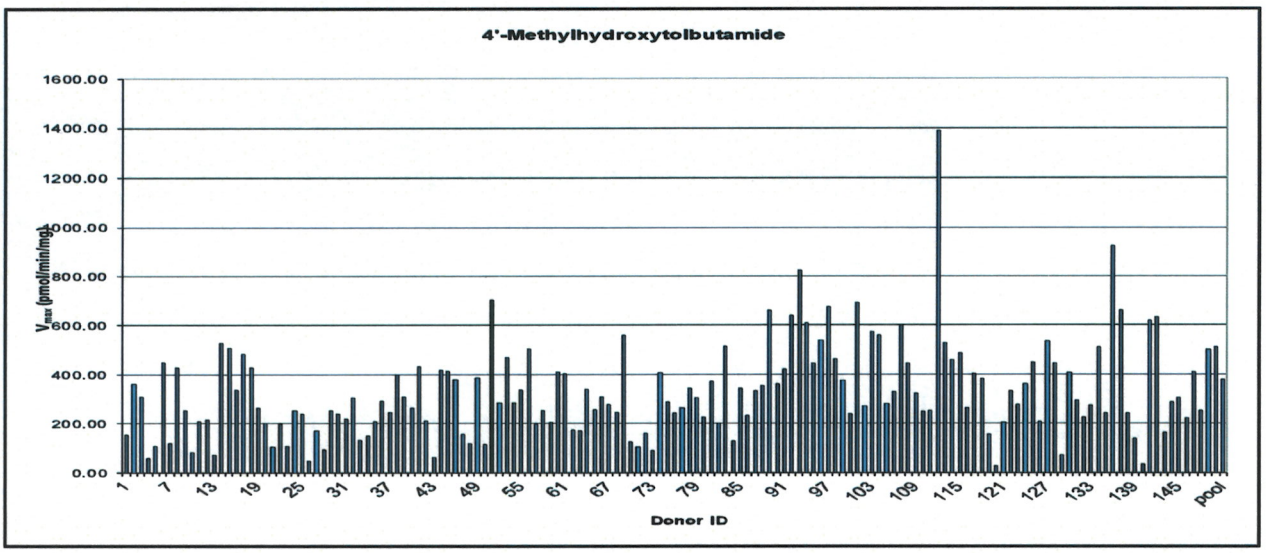

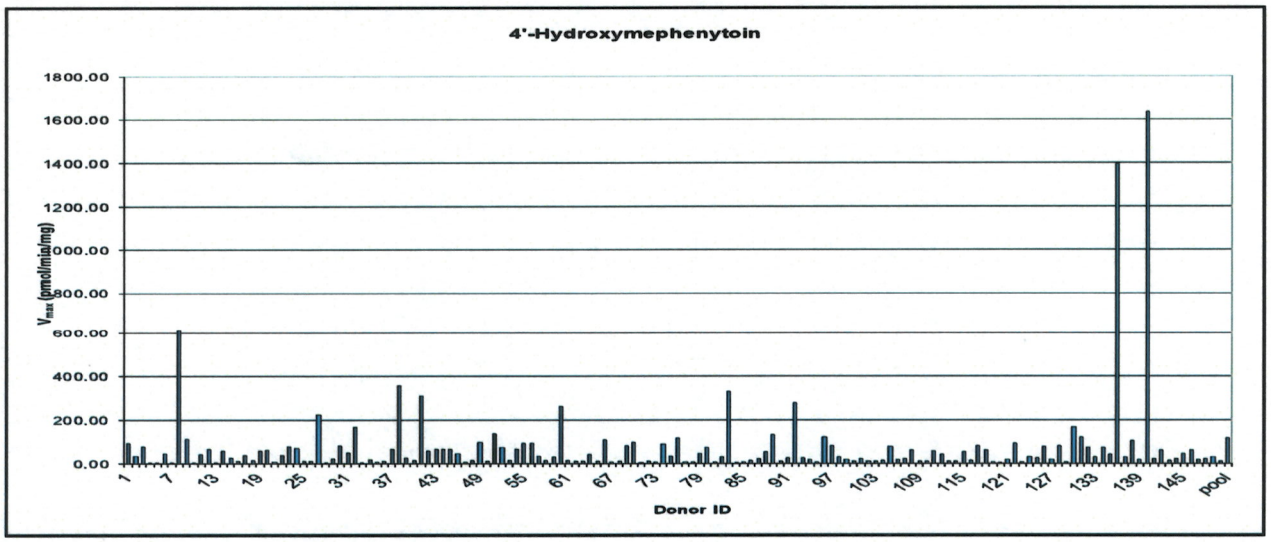

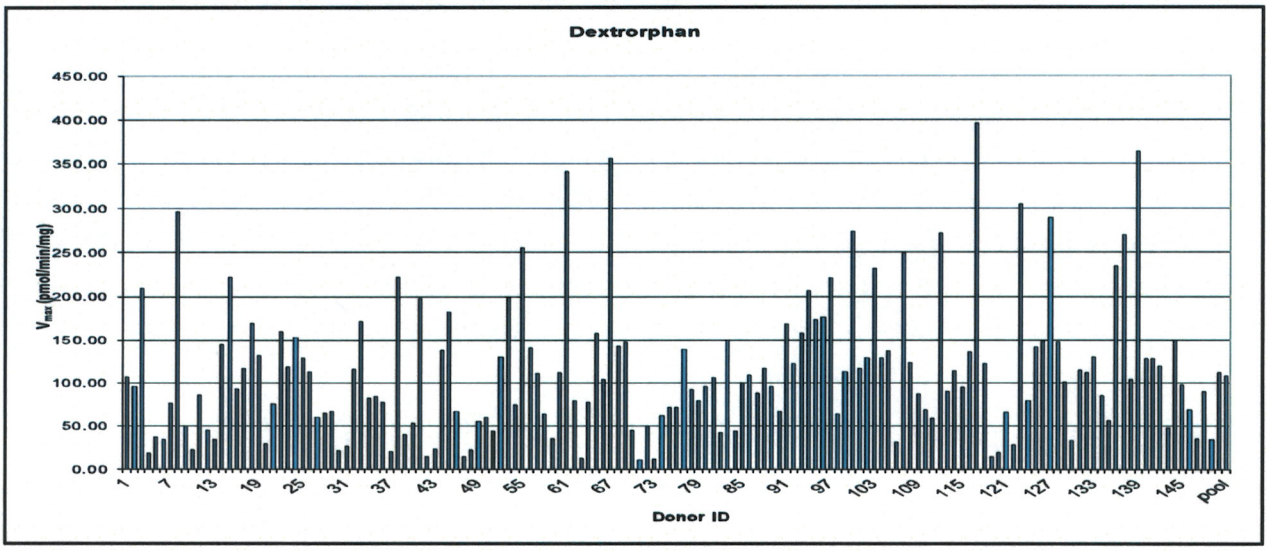

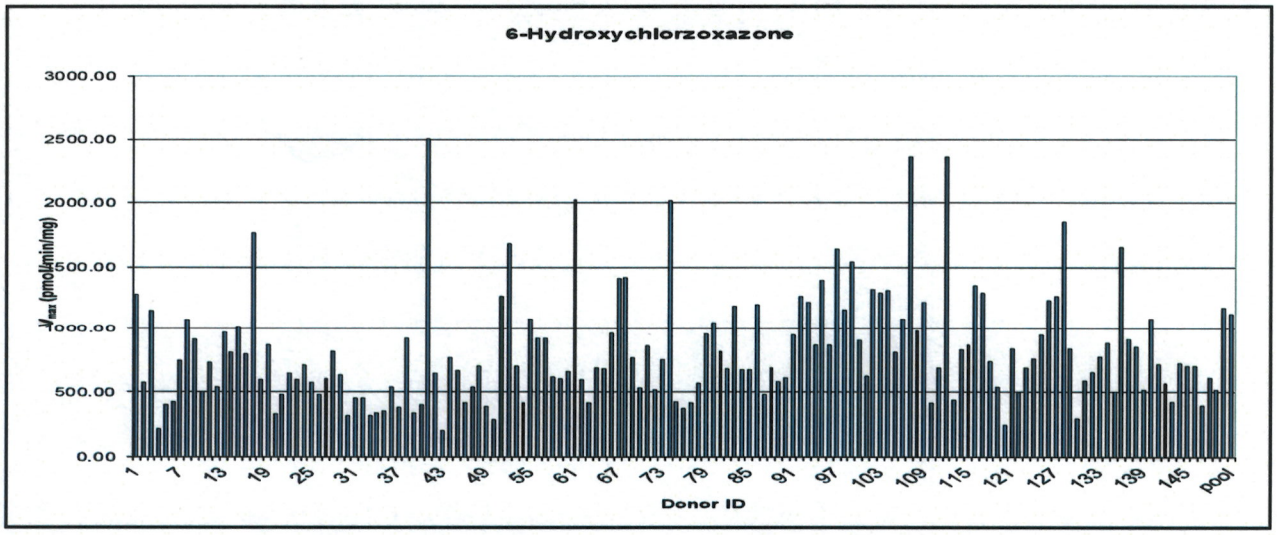

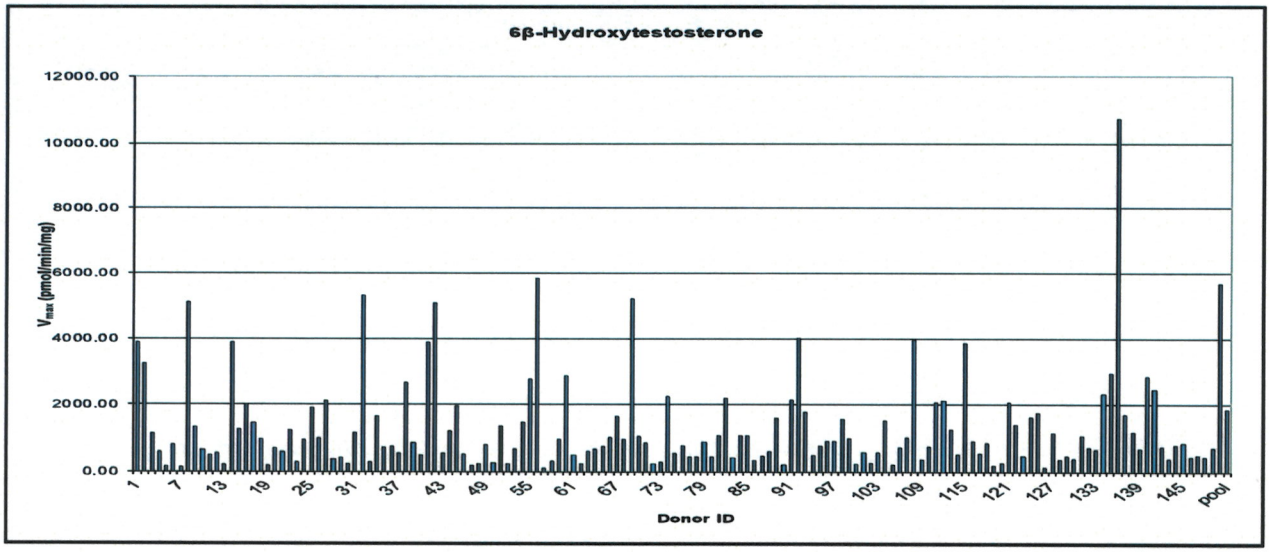

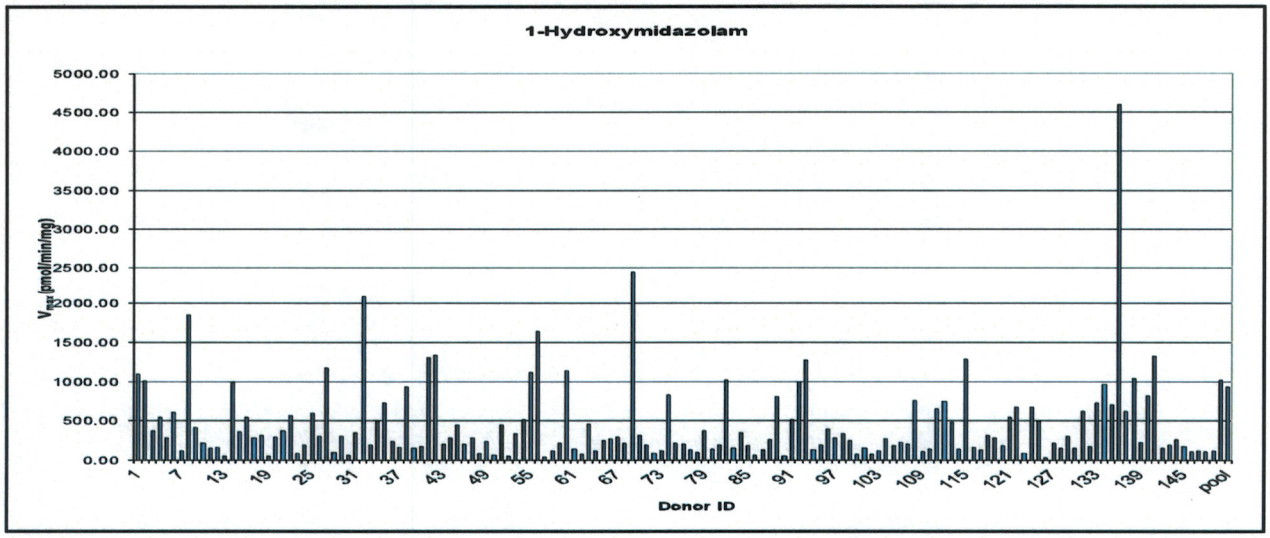

14.3 Solubility of the used test items

table 35: Additional information about the tested solubility of the used test items.

Test item [M]	6-Propyl-2-thiouracil		Aurothioglucose		5-Propyl-2-thiouracil		2-Chloro-N-phenylacetamid		Genistein		Tetrabromobisphenol A	
100% DMSO	10^{-1}	Fully dissolved	10^{-2}	Fully dissolved	10^{-1}	Fully dissolved	10^{-2}	Fully dissolved	10^{-2}	Fully dissolved	10^{-2}	Fully dissolved
10% DMSO in ddH_2O	10^{-2}	Fully dissolved	10^{-3}	Fully dissolved	10^{-2}	Fully dissolved	10^{-3}	Fully dissolved	10^{-3}	Precipitations, black flakes	10^{-3}	cloudy,, very small white particles
1% DMSO under final assay conditions	10^{-3}	Fully dissolved	10^{-4}	Fully dissolved	10^{-3}	Fully dissolved	10^{-4}	Fully dissolved	10^{-4}	Little precipitation, black flakes	10^{-4}	Little cloudy, very small white particles
100% DMSO									10^{-3}	Fully dissolved	10^{-3}	Fully dissolved
10% DMSO in ddH_2O									10^{-4}	Very little precipitations, fragments	10^{-4}	Few white particles
1% DMSO under final assay conditions									10^{-5}	Fully dissolved	10^{-5}	Fully dissolved
100% DMSO									$3.16*10^{-4}$	Fully dissolved		
10% DMSO in ddH_2O									$3.16*10^{-5}$	Fully dissolved		
1% DMSO under final assay conditions									$3.16*10^{-6}$	Fully dissolved		

14.4 Intraassay variability of the performed assay runs

table 36: Number of triplicates of the tested control or test items with a coefficient of variation (CV) over 20, 25 or 30 %. The coefficient of variation was calculated from the ΔOD values of the respective control or test item triplicate.
S.C.: solvent control; R.I.: reference item; P.C.: positive control; N.C.: negative control; T.I.: test item.

		Triplicates with CV<20%					Triplicates with CV<25%					Triplicates with CV<30%				
Date	Run	S.C.	R.I.	P.C.	N.C.	T.I.	S.C.	R.I.	P.C.	N.C.	T.I.	S.C.	R.I.	P.C.	N.C.	T.I.
28.09.2020	1					1										
28.09.2020	2			1												
30.09.2020	1															
30.09.2020	2															
01.10.2020	1															
01.10.2020	2															
02.10.2020	1		1			1		1					1			
02.10.2020	2															
07.10.2020	1															
07.10.2020	2															

14.5 Representative raw data for the valid assay run from 30.09.2020 (Assay run 1) containing ATG, 6PTU and GEN on the plate

Plate layout for the valid assay run from 30.09.2020 (Assay run 1) containing ATG, 6PTU and GEN on the plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	solvent (DMSO)			PTU			negative control			PTU		
B	A1			A2			A3			A4		
C	A5			A6			A7			A8		
D	R1			R2			R3			R4		
E	R5			R6			R7			R8		
F	B1			B2			B3			B4		
G	B5			B6			B7			B8		
H	solvent (DMSO)			PTU			solvent (DMSO)			positive control		

reference item | negative control | vehcile control | test item | positive control

	A	R	B
	Aurothioglucose	6-Propyl-2-thiouracil	Genistein
Stock	1,00E-02	1,00E-01	3,16E-04
Solvent	DMSO	DMSO	DMSO
conc 1	1,00E-04	1,00E-03	3,16E-06
conc 2	1,00E-05	1,00E-04	1,00E-06
conc 3	3,16E-06	3,16E-05	3,16E-07
conc 4	1,00E-06	1,00E-05	1,00E-07
conc 5	3,16E-07	3,16E-06	3,16E-08
conc 6	1,00E-07	1,00E-06	1,00E-08
conc 7	1,00E-08	1,00E-07	3,16E-09
conc 8	1,00E-09	1,00E-08	1,00E-09

Representative raw data for the valid assay run from 30.09.2020 (Assay run 1) containing ATG, 6PTU and GEN on the plate

SUNRISE; Serial number: 1709003862; Firmware: V 3.51 17/02/14; XFLUOR4 Version: V 4.51

Date:	30.9.20
Time:	11:27
Measurement mode:	Absorbance
Measurement wavelength:	415 nm
Read mode:	Normal
Number of kinetic cycles:	22
Kinetic interval:	60 s
Shake duration (Inside Normal):	2 s

Cycle Number: 1

Rawdata

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	1,4930	1,5480	1,5730	1,6360	1,6340	1,6800	1,5780	1,6720	1,6270	1,7100	1,6150	1,6180
B	1,2280	1,5090	1,6000	1,6000	1,6920	1,6030	1,6920	1,6460	1,7360	1,8990	1,6740	2,2190
C	1,5040	1,5180	1,6080	1,6070	1,7060	1,6260	1,6890	1,7130	1,6220	1,6860	1,6030	1,5870
D	1,5650	1,5020	1,5960	1,6150	1,7300	1,6640	1,7000	1,6900	1,6240	1,6870	1,6970	1,6370
E	1,5480	1,5760	1,6190	1,5910	1,7220	1,6650	1,6390	1,7000	1,6080	1,6320	1,6440	1,6200
F	1,5280	1,7400	1,5770	1,5940	1,6610	1,6780	1,6500	1,6620	1,6770	1,6160	1,6860	1,6400
G	1,5320	1,5490	1,6030	1,6600	1,6640	1,7020	1,6220	1,7150	1,6430	1,6290	1,7040	1,6770
H	1,5620	1,6240	1,6740	1,6240	1,7290	1,6860	1,6890	1,6430	1,7270	1,6320	1,7540	1,6980

Cycle Number: 2 Elapsed time after first cycle: 60 seconds

Rawdata

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	1,4380	1,4840	1,5110	1,6180	1,6250	1,6670	1,5260	1,5980	1,5730	1,6940	1,6080	1,6050
B	1,2270	1,4940	1,6030	1,5980	1,6980	1,6230	1,6830	1,6530	1,7220	1,8990	1,6620	2,2150
C	1,4430	1,4810	1,5870	1,5570	1,6760	1,5800	1,6290	1,6560	1,5600	1,6330	1,5480	1,5290
D	1,5380	1,4800	1,5710	1,5880	1,7010	1,6280	1,6680	1,6620	1,5860	1,6510	1,6630	1,5840
E	1,5230	1,5500	1,5880	1,5570	1,6810	1,6320	1,5980	1,6440	1,5660	1,5810	1,5850	1,5910
F	1,4900	1,7280	1,5370	1,5340	1,6040	1,6220	1,5860	1,5980	1,6050	1,5450	1,6130	1,5650
G	1,4850	1,4810	1,5400	1,5970	1,6070	1,6480	1,5760	1,6530	1,5880	1,5710	1,6460	1,6320
H	1,4740	1,5450	1,5930	1,5830	1,6960	1,6540	1,6150	1,5710	1,6510	1,6060	1,7240	1,6590

Cycle Number: 3 Elapsed time after first cycle: 120 seconds

Rawdata

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	1,3670	1,4140	1,4430	1,6000	1,6040	1,6480	1,4480	1,5260	1,4990	1,6750	1,5810	1,5860
B	1,2020	1,4730	1,5650	1,5690	1,6580	1,5700	1,6510	1,6050	1,6960	1,8420	1,6260	2,1590

C	1,4340	1,4540	1,5460	1,5200	1,6170	1,5410	1,5710	1,5990	1,5150	1,5650	1,4950	1,4660
D	1,5410	1,4740	1,5650	1,5850	1,6980	1,6290	1,6650	1,6540	1,5820	1,6350	1,6480	1,5810
E	1,4850	1,5050	1,5490	1,5050	1,6340	1,5800	1,5360	1,5890	1,4990	1,5110	1,5150	1,5100
F	1,4710	1,6720	1,5120	1,4860	1,5700	1,5850	1,5410	1,5500	1,5660	1,4900	1,5620	1,5140
G	1,4170	1,4110	1,4770	1,5320	1,5430	1,5870	1,5000	1,5860	1,5260	1,5010	1,5790	1,5480
H	1,4350	1,5030	1,5390	1,5930	1,6990	1,6590	1,5600	1,5230	1,5910	1,6040	1,7290	1,6650

Cycle Number: 4 Elapsed time after first cycle: 180 seconds

Rawdata

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	1,3260	1,3560	1,3910	1,5860	1,5970	1,6370	1,4040	1,4600	1,4510	1,6610	1,5760	1,5750
B	1,2170	1,4730	1,5710	1,5680	1,6610	1,5820	1,6510	1,6210	1,6960	1,8490	1,6200	2,1730
C	1,3900	1,4190	1,5120	1,4750	1,5740	1,4890	1,5130	1,5460	1,4560	1,5090	1,4410	1,4100
D	1,5280	1,4630	1,5520	1,5650	1,6780	1,6190	1,6410	1,6330	1,5560	1,6060	1,6290	1,5550
E	1,4630	1,4890	1,5310	1,4880	1,6070	1,5550	1,5030	1,5520	1,4630	1,4720	1,4710	1,4820
F	1,4270	1,6160	1,4680	1,4250	1,5120	1,5290	1,4810	1,4880	1,5050	1,4260	1,4960	1,4500
G	1,3730	1,3510	1,4220	1,4740	1,4920	1,5350	1,4610	1,5310	1,4770	1,4460	1,5280	1,5070
H	1,3590	1,4360	1,4720	1,5590	1,6730	1,6290	1,4950	1,4620	1,5310	1,5880	1,7060	1,6350

Cycle Number: 5 Elapsed time after first cycle: 240 seconds

Rawdata

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	1,2740	1,3140	1,3430	1,5890	1,5920	1,6370	1,3380	1,4120	1,3980	1,6640	1,5620	1,5820
B	1,1840	1,4440	1,5370	1,5390	1,6280	1,5430	1,6150	1,5750	1,6620	1,7980	1,5810	2,1150
C	1,3700	1,3860	1,4810	1,4320	1,5270	1,4580	1,4580	1,4890	1,4090	1,4490	1,3890	1,3580
D	1,5200	1,4530	1,5420	1,5600	1,6700	1,6050	1,6350	1,6250	1,5490	1,5940	1,6070	1,5410
E	1,4330	1,4540	1,4990	1,4430	1,5620	1,5110	1,4490	1,4970	1,4020	1,4070	1,4070	1,4080
F	1,4200	1,5830	1,4570	1,3910	1,4870	1,5020	1,4460	1,4510	1,4710	1,3830	1,4540	1,4160
G	1,3160	1,2900	1,3660	1,4160	1,4370	1,4800	1,3890	1,4710	1,4200	1,3850	1,4690	1,4290
H	1,3220	1,3980	1,4240	1,5680	1,6750	1,6350	1,4470	1,4150	1,4750	1,5800	1,7080	1,6330

Cycle Number: 6 Elapsed time after first cycle: 300 seconds

Rawdata

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	1,2170	1,2430	1,2800	1,5540	1,5670	1,6050	1,2860	1,3350	1,3360	1,6290	1,5420	1,5450
B	1,1970	1,4570	1,5420	1,5380	1,6360	1,5680	1,6130	1,5870	1,6530	1,8010	1,5710	2,1140
C	1,3310	1,3690	1,4660	1,4070	1,5010	1,4280	1,4230	1,4510	1,3720	1,4100	1,3580	1,3380
D	1,5010	1,4360	1,5270	1,5400	1,6540	1,5860	1,6120	1,6010	1,5240	1,5670	1,5900	1,5120
E	1,4070	1,4310	1,4750	1,4140	1,5320	1,4800	1,4120	1,4590	1,3640	1,3680	1,3610	1,3710
F	1,3730	1,5290	1,4050	1,3280	1,4270	1,4400	1,3820	1,3880	1,4090	1,3170	1,3900	1,3470

G	1,2820	1,2430	1,3220	1,3670	1,3930	1,4360	1,3560	1,4200	1,3760	1,3340	1,4190	1,3910
H	1,2600	1,3400	1,3630	1,5410	1,6560	1,6090	1,3890	1,3620	1,4170	1,5670	1,6890	1,6180

Cycle Number: 7 Elapsed time after first cycle: 360 seconds

Rawdata

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	1,1750	1,2090	1,2400	1,5610	1,5690	1,6110	1,2360	1,2970	1,2940	1,6400	1,5380	1,5590
B	1,1670	1,4260	1,5180	1,5200	1,6100	1,5170	1,5900	1,5520	1,6390	1,7560	1,5490	2,0730
C	1,3300	1,3290	1,4270	1,3580	1,4510	1,3800	1,3630	1,3960	1,3200	1,3490	1,3020	1,2600
D	1,5130	1,4420	1,5300	1,5430	1,6470	1,5890	1,6110	1,6040	1,5180	1,5510	1,5820	1,5130
E	1,3700	1,3890	1,4360	1,3680	1,4800	1,4300	1,3580	1,4010	1,3040	1,3050	1,2980	1,3000
F	1,3780	1,5200	1,4080	1,3110	1,4200	1,4310	1,3630	1,3660	1,3910	1,2890	1,3620	1,3230
G	1,2280	1,1920	1,2770	1,3210	1,3510	1,3940	1,2970	1,3760	1,3330	1,2890	1,3760	1,3320
H	1,2230	1,3030	1,3180	1,5470	1,6550	1,6160	1,3460	1,3210	1,3690	1,5630	1,6910	1,6180

Cycle Number: 8 Elapsed time after first cycle: 420 seconds

Rawdata

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	1,1290	1,1550	1,1910	1,5380	1,5500	1,5900	1,1930	1,2360	1,2420	1,6160	1,5210	1,5320
B	1,1780	1,4270	1,5220	1,5160	1,6070	1,5290	1,5820	1,5570	1,6280	1,7580	1,5340	2,0780
C	1,2850	1,3100	1,4080	1,3300	1,4220	1,3490	1,3250	1,3630	1,2800	1,3140	1,2670	1,2320
D	1,4900	1,4240	1,5130	1,5210	1,6300	1,5710	1,5860	1,5800	1,4960	1,5310	1,5570	1,4810
E	1,3580	1,3760	1,4220	1,3500	1,4580	1,4110	1,3320	1,3720	1,2780	1,2760	1,2640	1,2830
F	1,3350	1,4730	1,3660	1,2560	1,3650	1,3780	1,3080	1,3100	1,3350	1,2330	1,3050	1,2590
G	1,1960	1,1500	1,2370	1,2780	1,3110	1,3540	1,2700	1,3340	1,2960	1,2470	1,3350	1,3060
H	1,1660	1,2540	1,2680	1,5200	1,6370	1,5890	1,2970	1,2720	1,3220	1,5490	1,6740	1,5940

Cycle Number: 9 Elapsed time after first cycle: 481 seconds

Rawdata

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	1,0930	1,1230	1,1540	1,5450	1,5490	1,5930	1,1470	1,1990	1,2080	1,6230	1,5220	1,5390
B	1,1530	1,4080	1,5000	1,5000	1,5890	1,5000	1,5640	1,5260	1,6140	1,7190	1,5140	2,0380
C	1,2770	1,2810	1,3790	1,2930	1,3830	1,3220	1,2780	1,3140	1,2420	1,2630	1,2220	1,1830
D	1,4890	1,4180	1,5050	1,5160	1,6180	1,5620	1,5790	1,5730	1,4850	1,5120	1,5410	1,4730
E	1,3270	1,3430	1,3900	1,3100	1,4150	1,3700	1,2850	1,3190	1,2250	1,2220	1,2080	1,2200
F	1,3240	1,4500	1,3530	1,2270	1,3440	1,3540	1,2790	1,2810	1,3080	1,1990	1,2720	1,2280
G	1,1460	1,1000	1,1890	1,2290	1,2650	1,3070	1,2090	1,2830	1,2470	1,1970	1,2860	1,2370
H	1,1320	1,2190	1,2250	1,5270	1,6350	1,5940	1,2540	1,2330	1,2730	1,5440	1,6720	1,5930

Cycle Number: 10 Elapsed time after first cycle: 540 seconds

Representative raw data for the valid assay run from 30.09.2020 (Assay run 1) containing ATG, 6PTU and GEN on the plate

Rawdata

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	1,0560	1,0790	1,1130	1,5270	1,5380	1,5790	1,1110	1,1490	1,1640	1,6060	1,5100	1,5190
B	1,1630	1,4060	1,5010	1,4960	1,5890	1,5120	1,5600	1,5380	1,6100	1,7260	1,5080	2,0500
C	1,2490	1,2450	1,3420	1,2490	1,3400	1,2810	1,2280	1,2650	1,1940	1,2140	1,1770	1,1390
D	1,4810	1,4150	1,5000	1,5060	1,6070	1,5600	1,5620	1,5610	1,4640	1,4880	1,5320	1,4500
E	1,3180	1,3340	1,3810	1,2990	1,4000	1,3590	1,2680	1,2960	1,2060	1,2010	1,1850	1,2190
F	1,2900	1,4110	1,3180	1,1820	1,3010	1,3130	1,2350	1,2350	1,2640	1,1540	1,2270	1,1790
G	1,1150	1,0640	1,1550	1,1930	1,2320	1,2730	1,1880	1,2500	1,2140	1,1630	1,2530	1,2220
H	1,0780	1,1690	1,1780	1,4990	1,6110	1,5690	1,2060	1,1890	1,2280	1,5310	1,6470	1,5730

Cycle Number: 11 Elapsed time after first cycle: 600 seconds

Rawdata

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	1,0240	1,0480	1,0810	1,5310	1,5370	1,5820	1,0670	1,1160	1,1320	1,6110	1,5040	1,5300
B	1,1370	1,3870	1,4800	1,4790	1,5670	1,4800	1,5360	1,5020	1,5870	1,6820	1,4800	1,9990
C	1,2320	1,2340	1,3320	1,2320	1,3200	1,2600	1,1990	1,2380	1,1680	1,1840	1,1490	1,1040
D	1,4780	1,4080	1,4920	1,5000	1,6010	1,5500	1,5570	1,5530	1,4590	1,4810	1,5160	1,4460
E	1,2790	1,2950	1,3440	1,2540	1,3530	1,3110	1,2150	1,2460	1,1510	1,1440	1,1270	1,1400
F	1,2850	1,3960	1,3100	1,1590	1,2840	1,2930	1,2090	1,2080	1,2370	1,1210	1,1940	1,1530
G	1,0780	1,0220	1,1140	1,1510	1,1910	1,2340	1,1330	1,2040	1,1730	1,1190	1,2090	1,1580
H	1,0550	1,1460	1,1450	1,5120	1,6210	1,5810	1,1770	1,1590	1,1910	1,5290	1,6580	1,5770

Cycle Number: 12 Elapsed time after first cycle: 661 seconds

Rawdata

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	0,9850	1,0040	1,0410	1,5090	1,5200	1,5600	1,0360	1,0660	1,0890	1,5890	1,4920	1,5030
B	1,1550	1,3950	1,4940	1,4850	1,5790	1,4990	1,5440	1,5230	1,5950	1,6970	1,4840	2,0210
C	1,2100	1,2140	1,3120	1,2050	1,2950	1,2260	1,1640	1,2080	1,1340	1,1530	1,1180	1,0730
D	1,4610	1,3950	1,4800	1,4830	1,5840	1,5370	1,5360	1,5330	1,4370	1,4580	1,4960	1,4150
E	1,2760	1,2870	1,3360	1,2410	1,3380	1,2990	1,2000	1,2240	1,1360	1,1260	1,1020	1,1440
F	1,2480	1,3580	1,2740	1,1160	1,2400	1,2520	1,1670	1,1660	1,1950	1,0800	1,1510	1,1070
G	1,0490	0,9900	1,0840	1,1180	1,1610	1,2040	1,1160	1,1720	1,1450	1,0870	1,1790	1,1410
H	1,0100	1,1050	1,1060	1,4870	1,6040	1,5580	1,1360	1,1220	1,1530	1,5220	1,6400	1,5620

Cycle Number: 13 Elapsed time after first cycle: 720 seconds

Rawdata

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	0,9580	0,9770	1,0100	1,5150	1,5150	1,5630	0,9970	1,0380	1,0620	1,5940	1,4950	1,5120
B	1,1270	1,3750	1,4690	1,4650	1,5520	1,4670	1,5170	1,4830	1,5690	1,6540	1,4540	1,9750

C	1,1990	1,1950	1,2930	1,1790	1,2670	1,2080	1,1310	1,1730	1,1060	1,1170	1,0850	1,0460
D	1,4630	1,3880	1,4760	1,4840	1,5830	1,5250	1,5340	1,5270	1,4340	1,4510	1,4820	1,4110
E	1,2480	1,2590	1,3090	1,2070	1,3030	1,2630	1,1590	1,1810	1,0900	1,0800	1,0570	1,0860
F	1,2410	1,3410	1,2650	1,0930	1,2240	1,2320	1,1410	1,1410	1,1710	1,0510	1,1230	1,0810
G	1,0140	0,9550	1,0490	1,0830	1,1270	1,1690	1,0670	1,1350	1,1070	1,0500	1,1400	1,0870
H	0,9860	1,0800	1,0740	1,4970	1,6040	1,5650	1,1050	1,0890	1,1170	1,5140	1,6440	1,5600

Cycle Number: 14 Elapsed time after first cycle: 780 seconds

Rawdata

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	0,9210	0,9380	0,9740	1,4920	1,4990	1,5400	0,9620	0,9950	1,0200	1,5730	1,4700	1,4890
B	1,1350	1,3730	1,4680	1,4590	1,5500	1,4700	1,5110	1,4880	1,5600	1,6550	1,4410	1,9740
C	1,1620	1,1720	1,2700	1,1510	1,2370	1,1800	1,1010	1,1410	1,0700	1,0840	1,0590	1,0200
D	1,4470	1,3770	1,4640	1,4660	1,5670	1,5150	1,5150	1,5100	1,4140	1,4290	1,4670	1,3870
E	1,2370	1,2480	1,2980	1,1930	1,2870	1,2490	1,1410	1,1620	1,0730	1,0600	1,0350	1,0700
F	1,2150	1,3120	1,2350	1,0570	1,1890	1,1960	1,1040	1,1030	1,1340	1,0130	1,0840	1,0440
G	0,9910	0,9250	1,0190	1,0510	1,0970	1,1400	1,0480	1,1030	1,0800	1,0200	1,1090	1,0710
H	0,9470	1,0450	1,0370	1,4740	1,5880	1,5430	1,0690	1,0560	1,0840	1,5060	1,6280	1,5480

Cycle Number: 15 Elapsed time after first cycle: 841 seconds

Rawdata

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	0,9010	0,9220	0,9560	1,5060	1,5080	1,5550	0,9290	0,9750	0,9990	1,5850	1,4750	1,5030
B	1,1160	1,3590	1,4520	1,4460	1,5340	1,4520	1,4950	1,4640	1,5480	1,6250	1,4260	1,9480
C	1,1600	1,1520	1,2500	1,1260	1,2110	1,1560	1,0660	1,1080	1,0430	1,0510	1,0240	0,9780
D	1,4490	1,3750	1,4610	1,4650	1,5610	1,5120	1,5110	1,5070	1,4070	1,4200	1,4580	1,3810
E	1,2070	1,2190	1,2710	1,1610	1,2510	1,2140	1,1010	1,1220	1,0290	1,0150	0,9900	1,0170
F	1,2140	1,3040	1,2350	1,0420	1,1770	1,1850	1,0870	1,0840	1,1160	0,9910	1,0620	1,0260
G	0,9570	0,8910	0,9860	1,0170	1,0640	1,1050	1,0040	1,0670	1,0450	0,9840	1,0740	1,0210
H	0,9210	1,0180	1,0050	1,4800	1,5870	1,5480	1,0390	1,0260	1,0470	1,4990	1,6280	1,5420

Cycle Number: 16 Elapsed time after first cycle: 900 seconds

Rawdata

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	0,8720	0,8840	0,9230	1,4770	1,4880	1,5260	0,9060	0,9310	0,9640	1,5560	1,4630	1,4710
B	1,1340	1,3940	1,4720	1,4570	1,5620	1,4810	1,5140	1,4930	1,5600	1,6520	1,4340	1,9830
C	1,1300	1,1380	1,2360	1,1080	1,1960	1,1280	1,0410	1,0890	1,0180	1,0310	1,0030	0,9570
D	1,4350	1,3640	1,4530	1,4510	1,5520	1,4980	1,4950	1,4890	1,3890	1,4040	1,4440	1,3620
E	1,1940	1,2080	1,2580	1,1470	1,2360	1,1990	1,0820	1,1020	1,0170	1,0000	0,9660	1,0150
F	1,1730	1,2660	1,1930	0,9990	1,1330	1,1410	1,0430	1,0430	1,0730	0,9510	1,0210	0,9830

G	0,9310	0,8640	0,9560	0,9860	1,0340	1,0760	0,9870	1,0360	1,0180	0,9560	1,0450	1,0030
H	0,8820	0,9810	0,9720	1,4610	1,5740	1,5290	1,0050	0,9930	1,0160	1,4930	1,6110	1,5310

Cycle Number: 17 Elapsed time after first cycle: 960 seconds

Rawdata

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	0,8500	0,8670	0,9020	1,4890	1,4900	1,5370	0,8720	0,9150	0,9420	1,5720	1,4630	1,4890
B	1,1030	1,3460	1,4400	1,4330	1,5200	1,4380	1,4770	1,4460	1,5290	1,6010	1,4020	1,9200
C	1,1220	1,1140	1,2120	1,0780	1,1630	1,1080	1,0070	1,0520	0,9890	0,9940	0,9690	0,9210
D	1,4370	1,3610	1,4490	1,4520	1,5480	1,4940	1,4930	1,4870	1,3870	1,3950	1,4300	1,3590
E	1,1730	1,1820	1,2320	1,1160	1,2040	1,1690	1,0500	1,0650	0,9780	0,9610	0,9310	0,9650
F	1,1770	1,2580	1,1960	0,9870	1,1260	1,1320	1,0300	1,0270	1,0580	0,9320	1,0020	0,9630
G	0,9020	0,8320	0,9260	0,9560	1,0050	1,0450	0,9450	1,0050	0,9850	0,9250	1,0130	0,9560
H	0,8660	0,9650	0,9480	1,4680	1,5750	1,5350	0,9820	0,9700	0,9870	1,4860	1,6160	1,5280

Cycle Number: 18 Elapsed time after first cycle: 1020 seconds

Rawdata

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	0,8310	0,8390	0,8810	1,4780	1,4870	1,5310	0,8490	0,8860	0,9170	1,5590	1,4570	1,4760
B	1,1150	1,3550	1,4520	1,4390	1,5300	1,4520	1,4840	1,4610	1,5360	1,6110	1,4050	1,9340
C	1,1000	1,1040	1,2030	1,0640	1,1480	1,0840	0,9850	1,0350	0,9690	0,9750	0,9490	0,9010
D	1,4180	1,3510	1,4350	1,4330	1,5300	1,4870	1,4720	1,4710	1,3640	1,3750	1,4200	1,3320
E	1,1660	1,1720	1,2220	1,1040	1,1890	1,1560	1,0360	1,0480	0,9650	0,9480	0,9120	0,9650
F	1,1520	1,2340	1,1720	0,9590	1,0960	1,1040	1,0000	0,9980	1,0280	0,9030	0,9710	0,9290
G	0,8850	0,8130	0,9080	0,9350	0,9860	1,0280	0,9360	0,9850	0,9690	0,9040	0,9950	0,9530
H	0,8360	0,9350	0,9200	1,4480	1,5630	1,5180	0,9530	0,9440	0,9610	1,4790	1,6010	1,5260

Cycle Number: 19 Elapsed time after first cycle: 1080 seconds

Rawdata

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	0,8090	0,8150	0,8540	1,4770	1,4780	1,5230	0,8220	0,8630	0,8920	1,5560	1,4540	1,4770
B	1,0900	1,3330	1,4260	1,4190	1,5070	1,4250	1,4590	1,4270	1,5120	1,5750	1,3790	1,8870
C	1,0860	1,0820	1,1800	1,0380	1,1190	1,0680	0,9570	1,0010	0,9420	0,9410	0,9230	0,8740
D	1,4220	1,3480	1,4350	1,4360	1,5300	1,4790	1,4720	1,4680	1,3630	1,3690	1,4090	1,3290
E	1,1400	1,1470	1,1990	1,0770	1,1590	1,1260	1,0010	1,0160	0,9280	0,9090	0,8760	0,9110
F	1,1480	1,2240	1,1660	0,9430	1,0860	1,0900	0,9840	0,9800	1,0120	0,8830	0,9510	0,9150
G	0,8600	0,7850	0,8790	0,9060	0,9570	0,9970	0,8970	0,9540	0,9370	0,8750	0,9630	0,9130
H	0,8170	0,9160	0,8930	1,4580	1,5640	1,5250	0,9270	0,9190	0,9300	1,4740	1,6030	1,5190

Cycle Number: 20 Elapsed time after first cycle: 1140 seconds

Representative raw data for the valid assay run from 30.09.2020 (Assay run 1) containing ATG, 6PTU and GEN on the plate

Rawdata

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	0,7870	0,7870	0,8320	1,4580	1,4660	1,5070	0,7990	0,8290	0,8630	1,5330	1,4380	1,4530
B	1,1070	1,3380	1,4380	1,4240	1,5140	1,4390	1,4630	1,4450	1,5130	1,5900	1,3800	1,9030
C	1,0720	1,0680	1,1660	1,0200	1,1010	1,0420	0,9340	0,9830	0,9180	0,9230	0,9030	0,8540
D	1,4110	1,3430	1,4280	1,4240	1,5210	1,4750	1,4580	1,4570	1,3480	1,3560	1,3980	1,3150
E	1,1320	1,1390	1,1900	1,0660	1,1470	1,1160	0,9890	1,0020	0,9180	0,8970	0,8590	0,9140
F	1,1160	1,1910	1,1330	0,9090	1,0490	1,0550	0,9490	0,9470	0,9770	0,8510	0,9180	0,8780
G	0,8390	0,7630	0,8580	0,8820	0,9340	0,9740	0,8860	0,9290	0,9170	0,8510	0,9400	0,9030
H	0,7870	0,8880	0,8660	1,4380	1,5500	1,5040	0,9010	0,8940	0,9060	1,4680	1,5880	1,5070

Cycle Number: 21 Elapsed time after first cycle: 1200 seconds

Rawdata

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	0,7670	0,7690	0,8100	1,4670	1,4610	1,5050	0,7750	0,8100	0,8430	1,5390	1,4380	1,4620
B	1,0870	1,3230	1,4180	1,4090	1,4960	1,4160	1,4450	1,4180	1,4990	1,5580	1,3600	1,8690
C	1,0650	1,0460	1,1440	0,9950	1,0750	1,0250	0,9060	0,9520	0,8950	0,8930	0,8760	0,8270
D	1,4110	1,3370	1,4240	1,4250	1,5190	1,4660	1,4560	1,4530	1,3450	1,3480	1,3870	1,3100
E	1,1160	1,1190	1,1700	1,0410	1,1220	1,0910	0,9600	0,9700	0,8860	0,8650	0,8280	0,8730
F	1,1180	1,1860	1,1330	0,8970	1,0430	1,0460	0,9350	0,9320	0,9640	0,8340	0,9010	0,8630
G	0,8170	0,7420	0,8370	0,8610	0,9120	0,9520	0,8510	0,9060	0,8920	0,8290	0,9170	0,8610
H	0,7720	0,8710	0,8440	1,4460	1,5520	1,5130	0,8790	0,8720	0,8800	1,4630	1,5910	1,5060

Cycle Number: 22 Elapsed time after first cycle: 1260 seconds

Rawdata

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	0,7510	0,7460	0,7930	1,4560	1,4620	1,5030	0,7580	0,7840	0,8250	1,5290	1,4330	1,4500
B	1,0970	1,3310	1,4290	1,4170	1,5080	1,4310	1,4550	1,4340	1,5080	1,5690	1,3650	1,8830
C	1,0440	1,0320	1,1300	0,9780	1,0570	0,9980	0,8830	0,9320	0,8730	0,8730	0,8550	0,8070
D	1,4040	1,3370	1,4190	1,4140	1,5060	1,4680	1,4410	1,4440	1,3270	1,3300	1,3820	1,2940
E	1,1100	1,1120	1,1610	1,0330	1,1110	1,0830	0,9500	0,9570	0,8770	0,8550	0,8150	0,8740
F	1,0930	1,1630	1,1080	0,8710	1,0140	1,0210	0,9100	0,9070	0,9370	0,8100	0,8760	0,8340
G	0,8030	0,7220	0,8190	0,8410	0,8930	0,9340	0,8430	0,8870	0,8760	0,8100	0,9000	0,8600
H	0,7470	0,8440	0,8220	1,4250	1,5360	1,4930	0,8550	0,8510	0,8610	1,4590	1,5740	1,4950

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