European Commission

STUDY REPORT

for the colorimetric assessment of deiodinases activity based on Sandell-Kolthoff reaction with human microsomes: DIO-SK assay - Part 2

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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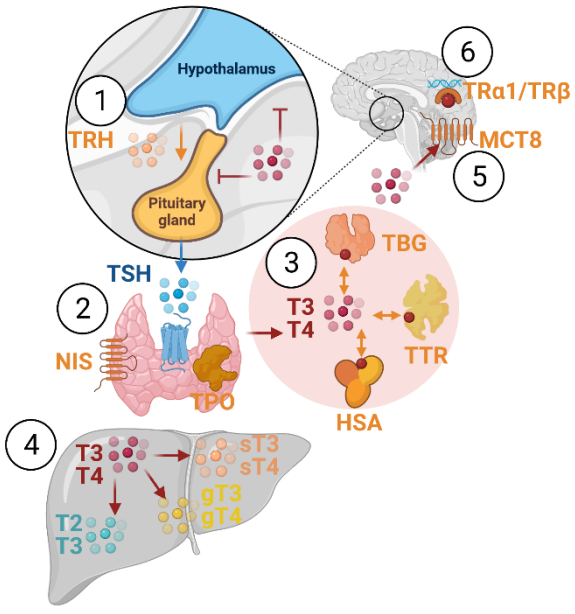

2023

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 (German Federal Institute for Risk Assessment) and subsequently implemented by the EU-NETVAL test facility BASF SE (Germany) within the validation study.

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SUMMARY OF RESULTS

Study report

Part 2: Relevance assessment for method 4a: DIO1-SK assay

Test guideline(s)

Method according to Renko et al., 2015

Author(s)

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Dr. Barbara Birk

Completion Date

Experimental Completion Date 17.08.2021

Study Report Completion Date 26.04.2022

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Table of Contents Page

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1 Aim of the study

The objective of this study was the assessment of the activity of unknown test items in the Deiodinase I (DIO1)-Sandell-Kolthoff (SK) assay using human liver microsomes and the Sandell-Kolthoff reaction as iodide quantification readout. A set of 40 blinded test items including described *in vitro* inhibitors of deiodinases or other thyroid Mode of Actions (MoA) as well as negative substances were tested regarding their inhibition properties in the DIO1-SK assay in at least three independent runs. Additional testing strategies were implemented to investigate the specificity of the results in the DIO1-SK assay. Three different technicians were generating the data to show the robustness of the method.

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 foetal development (Bianco, Dumitrescu et al. 2019).
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 (6PTU), iopanoic acid and amiodarone (Leonard and Rosenberg 1978, Leonard, Mellen et al. 1983, Rosene, Wittmann et al. 2010).

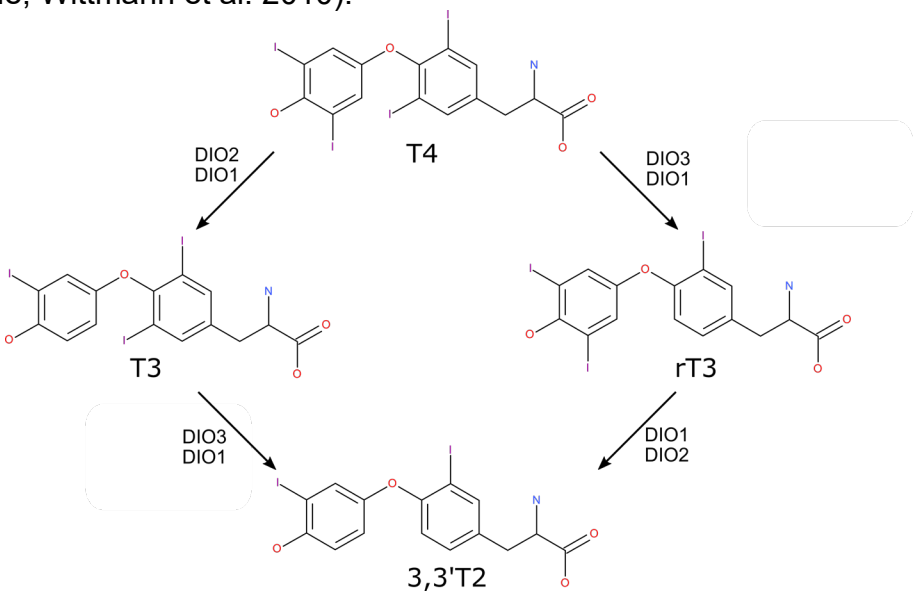

figure 1: Metabolism of thyroid hormone through the Deiodinases (DIO)

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. Microsomes, broken-down vesicle-like pieces of endoplasmic reticula from hepatocytes are used as enzyme source for DIO, mainly DIO1, in this method. Depending on the deiodinase activity of the microsomes, iodide is released from the used substrate, which can be quantified in the SK reaction. The microsomes possess also other metabolizing enzymes which might influence the test system through metabolization of the test items (Knights, Stresser et al. 2016). This might explain differences in the test item inhibition properties compared to assays with purified DIO1 enzymes.

$$\underset{\textit{yellow}}{2Ce^{4+}} + As^{3+} \overset{I^-}{\rightleftharpoons} \underset{\textit{colourless}}{2Ce^{3+}} + As^{5+}$$

figure 2: Sandell-Kolthoff reaction

The DIO1-SK assay requires an initial iodide release activity (IRA) test run to determine the batch-specific IRA of the microsome batch. This is needed because suppliers usually do not test for IRA. Based on the measured microsome batch-specific IRA 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.

The measurement of endogenous DIO activity via this assay is limited to rich sources of enzymatic activity like DIO1-containing liver microsomes since the assay sensitivity is limited, compared to other methods like liquid chromatography-tandem mass spectrometry (LC-MS/MS). 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.

The assay is part of a validation program led by the EU Reference Laboratory for alternatives to animal testing (EU RL ECVAM). In cooperation with the European Union Network of Laboratories for the Validation of Alternative Methods (EU-NETVAL) and the respective method developer of each method, an *in vitro* test battery consisting of different *in vitro* methods covering individual MoAs of thyroid hormone signalling should be generated. The validation effort comprises of two separate parts: 1. Testing a small number of known MoA inhibitors in five independent runs should show the reproducibility of the method and the experience gained should result in a robust standard operation procedure (SOP) (reproducibility assessment, part 1 (Birk and Weber 2020)), 2. Testing a set of blinded test items with known activity in the MoA of interest should show the predictivity of each method (relevance assessment, part 2).

The reproducibility assessment for the DIO1-SK assay has been finished showing reproducibility of generated data and resulted in a SOP that was used for part 2 testing. Additionally, different identified challenges like definition of controls, solubility of test items, standardization efforts or acceptance criteria for the validity of assay runs were addressed and summarized in the SOP.

Here, we report the results of the relevance assessment for the DIO1-SK assay. A set of 40 blinded test item was tested at the testing facility. The set comprises of described *in vitro* inhibitors of deiodinases or other thyroid MoAs as well as negative compounds that are to be tested in all methods of the ECVAM validation study and items consisting of known DIO1 inhibitors that was compiled specifically for the DIO1-SK assay validation to increase the ratio of expected DIO1 inhibiting to non-inhibiting test items. In addition to testing in the DIO1-SK assay, test items were tested in two strategies to assess the specificity of observed DIO1 inhibition in the DIO1-SK assay: (i) Testing of the test item without microsomes present during incubation phase can show test items that lead to spontaneous iodide release or substances that interfere with the SK reaction, provided they reach the SK reaction. (ii) the activity of an additional enzyme, that is present in human liver microsomes, was addressed to investigate potential unspecific, test item-induced, protein interactions and/or modifications that could lead to structural denaturation. The Alkaline Phosphatase (ALP) was chosen as secondary, microsomal enzyme since the common ALP activity readout is colorimetric.

3 Study schedule

Study Start Date:	05.11.2020
Experimental Starting Date:	05.11.2020
Experimental Completion Date:	17.08.2021
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). The method was further optimized for the use of human liver microsomes at BASF and reproducibility was shown in part 1 of the validation effort. Further, the DIO1-SK was used in a case study to investigate gold-induced inhibition of DIO1 in human liver microsomes by testing a set of organic and inorganic gold compounds, their structural analogues lacking the gold moiety and gold nanoparticles (Weber, Birk et al. 2021).

The material and method of part 2 adhered to the SOP that 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 14.1). Changes in the material and method based on material change led to the derivation of

SOP version “20220413_SOP DIO1-SK assay” (date: 13.04.2022, see Appendix 14.2).

Also, the establishment and standardization of ALP activity testing led to the derivation of the SOP “SOP: ALP activity testing” (Appendix 14.3).

5 Materials and equipment

5.1 Material

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

Material:	Requirements[1] Supplier[2]
Volumetric flask	certified with defined volume[1]
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[1] Supplier[2]
Analytical balance	capable of accurately weighing up to 30 g with 0.1 mg readability[1]
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	The photometer used must be able to heat up to 37°C[1], e.g., 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)[1]

5.3 Chemicals

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

Chemicals / reagents	Requirements[1] Supplier[2]
10% (w/w) Hydrogen chloride (HCl) CAS: 7647-01-0 MW: 36.46 g/mol	Hydrochloric acid 10%, EMPROVE® EXPERT Ph Eur,JP,NF, Sigma-Aldrich[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] 3,3′,5′-Triiodo-L-thyronine, Cayman[2] 3,3′,5′-Triiodo-L-thyronine, Santa Cruz Biotechnology[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]
Diethanolamine (DEA) CAS: 111-42-2 MW: 105.14 g/mol	Diethanolamine, reagent grade, ≥98.0%, 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] AmberChrom® 50WX2 hydrogen form, 100-200 mesh, Merck[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	ethylenediaminetetraacetic acid disodium salt dihydrate, Sigma Grade, suitable for plant cell culture, 98.5-101.5 %, Sigma-Aldrich[2]

MW: 372.24 g/mol	
Iodide (IC standard)	Iodide standard for IC, 1000 mg/L in water, Sigma-Aldrich[2]
Magnesium chloride ($MgCl_2$) CAS: 7791-18-6 MW: 203.30 g/mol	Magnesium chloride hexahydrate, ACS reagent, 99.0-102.0%, 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]
para-Nitrophenyl phosphate (PNPP) CAS: 333338-18-4 MW: 371.14 g/mol	Phosphatase substrate, 5 mg tablets, 4-Nitrophenyl phosphate disodium salt hexahydrate, 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]
TNAP inhibitor CAS: 496014-13-2 MW: 344.38 g/mol	e.g. TNAP Inhibitor - 2,5-Dimethoxy-N-(quinolin-3-yl) benzenesulfonamide - CAS 496014-13-2 – Calbiochem, Sigma-Aldrich[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 diH_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 diH_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 diH_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.
Acidic ammonium cerium solution	6.32 g of $(NH_4)_4Ce(SO_4)_4{\cdot}2H_2O$ and 125 mL of diH_2O are added to a 250 mL volumetric flask.

(40 mM $(NH_4)_4Ce(SO_4)_4 \cdot 2H_2O$, 0.5 M H_2SO_4) (250 mL)	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 diH_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] e.g. GraphPad Prims 8, GraphPad[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 uses a reference item as well as a positive and negative control (table 7). 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, Weber, Birk et al. 2021) The purpose of a reference item is to control the concentration-response of the test system quantitatively and to normalization generated test items results. 6PTU is therefore tested in concentration-response testing on the first assay run of each assay as well as in single concentrations on each assay plate. The purpose of the positive control item is to confirm the inhibitory effect by testing in single concentration. A structural analogue of ATG (lacking the gold ligand which is responsible for DIO1 inhibition), 1-Thio-β-D-glucose sodium salt (TGSS), was tested in single concentrations on each assay run as negative control since TGSS shows no inhibitory effect on DIO1 (Weber, Birk et al. 2021). Moreover, solvent controls were included in all the experiments for normalization and to exclude potential inhibitory activity of the used solvent on DIO1 (preferably DMSO is used). Historical data available obtained in over years of assay performance with the control setup at BASF SE, supports the suitability of the three items acting as reference (6PTU), positive (ATG) and negative control (TGSS) item (Weber, Birk et al. 2021).

table 7: Used control setup and control items in the DIO1-SK assay in accordance with GIVIMP.

Controls:	
Reference item (RI)	Quantitative control. The inhibition observed with the test item is normalized to maximum inhibition obtained with the highest concentration of the reference item. The highest concentration of the reference item is tested on each assay plate and a concentration-response curve of the reference item is performed on each assay day. Used in this method: 6-Propyl-2-thiouracil (6PTU) at a maximum assay concentration of 10^{-3} M. Concentration-response curves of 6PTU ranged from 10^{-3} to 10^{-8} M
Positive control (PC)	Qualitative control. The highest concentration of the positive control is tested on each assay plate. Used in this method: Aurothioglucose (ATG) at a maximum assay concentration of 10^{-4} M.
Negative control (NC)	Control not inhibiting DIO1 activity. The highest concentration of the negative control is tested on each assay plate. Used in this method: 1-Thio-β-D-glucose sodium salt (TGSS) at a maximum assay concentration of 10^{-4} M.
Solvent control (SC)	Blank control. The solvent control ensures that the response does not originate from the applied solvent and shows no DIO1 inhibition. Solvent controls are used to normalize the inhibition observed with the test items to the maximum possible DIO1 activity. Used in this method: Dimethyl sulfoxide (DMSO) at a concentration of 1%.

6.1.1 Reference item/ test item

table 8: 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 9: 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 10: 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 11).

table 11: 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

A set of 40 blinded test item was supplied by EURL ECVAM to the testing facility (table 12). The test items were supplied in brown, glass vials and were stored according to their specified storage conditions at room temperature, in the refrigerator or in the freezer. Based on the provided information of the known hazard, safety measurements for handling the substances in laboratory according to BASF safety rules were applied.

table 12: Provided information about the used test items provided by the supplier EURL ECVAM.

Chemical Code	State Storage Inert gas?	Molecular weight (approx.) [g/mol]	Sample Weight [mg]	H hazard
56	Solid RT	325	324	H315,H319,H335
125	Liquid RT	Aqueous solution (approx. 1 Molar)	1236	H302+H332,H314,H351, H360,H362,H372,H411
130	Solid RT	200	514	H302
160	Liquid -20°C inert gas	300	473	none
194	Solid 4°C	550	515	H301,H360F,H373,H400, H410
218	Solid RT	275	539	H315,H319
220	Solid RT inert gas	200	330	H301,H330,H340,H350,H 360fd, H372,H410
227	Solid RT	300	618	H315, H319,H410
229	Solid RT	550	685	H410
279	Solid RT	350	1133	H317, H319,H400
294	Solid RT	175	360	H317, H410
307	Solid RT	1725	320	none
325	Solid RT inert gas	375	315	none
377	Solid 4°C	350	552	H315,H317,H319,H334
437	Solid 4°C	150	506	H302
442	Solid RT	200	585	H302+H332, H318, H335, H341,H361d,H372,H411
506	Solid 4°C	275	571	none
511	Solid RT	475	391	H301,H410
526	Solid RT	275	528	H301+H311,H315,H319, H330,H335,H351,H410
543	Solid RT	175	544	H302,H351
551	Liquid RT	300	540	H360FD,H410
598	Solid RT	125	540	H302,H351,H360D,H372
603	Solid -20°C	750	259	H300, fatal if swallowed
610	Solid RT	325	305	H302,H315,H319,H335
615	Solid 4°C	700	537	H315,H31 H361fd,H362,H373
667	Solid -20°C	350	85	none
680	Solid RT	375	358	H302,H315,H318,H335,H 373
741	Solid 4°C	325	524	H302,H315,H317,H319,H 334,H335
798	Solid RT	250	580	H302,H317,H319
818	Liquid -20°C inert gas	300	501	none
827	Solid RT	125	331	H302,H315,H318,H400
839	Solid -20°C	475	212	none

850	Solid/liquid MP 40°C RT	350	2767	H315,H317,H319
868	Solid RT	250	521	H302
877	Solid RT	125	612	H271,H302,H319,H373
878	Solid RT	325	507	H301,H361d,H372,H411
925	Solid RT	450	421	H301+H311+H331,H319, H372,H410
933	Solid RT inert gas	300	553	H301+H311+H331,H315, H319
940	Solid 4°C	500	100	H302
974	Liquid RT	300	555	none

7 Test system

The details of the human liver microsomes used for the study, meeting the requirements according to the DIO-SK SOP (20220330_SOP DIO1-SK assay), are described in table 13. 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 IRA 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 IRA testing is further specified in 8.1.8. The microsomes were stored at ≤ -80°C until required for use.

table 13: 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 Human Immunodeficiency Virus (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 14.4.

8 Method

8.1 DIO1-SK assay

8.1.1 Casting of ion exchange resin-filled 96-Well filter plates

Before the day of assay performance, a larger quantity of ion exchange resin-filled 96-well filter plates was prepared and stored at 4°C:

About 250 g of resin was added to a large beaker and washed with 10 % acetic acid (v/v). To mix the resin with the acetic acid, a big shaker was used; afterwards the suspension 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 5x in total)
- 100 µL of 10% acetic acid was added into each well of a 96-well filter plate; the 96-well filter plate was placed on top of a 96-well deep well-plate
- 1 mL tips were cut to widen the opening and 600 µL resin suspension was filled into each well of the 96-well filter plate using an automatic multi-channel pipette
- Another 150 µL 10% acetic acid was added to each well and eluted by centrifugation into the 96-deep well-plate (1 min, 70 g)
- The addition of 150 µL 10% acetic acid and centrifugation was repeated if colour was still leaking in any of the wells
- The 96-deep well plates were removed, and the ion exchange resin-filled 96-well filter plates were sealed with gas-impermeable plastic seals and stored at 4°C for a maximum of 2 months.

8.1.2 Solubility testing of test items

The test concentrations for an initial 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 stock solution in an appropriate solvent in the DIO1-SK assay is 100 mM leading to the highest tested assay concentration of a test item of 1 mM (1% v/v of solvent). A highest tested concentration of 1 mM is in line with OECD accepted *in vitro* enzymatic assays like the “Test No. 493: Performance-Based Test Guideline for Human Recombinant Estrogen Receptor (hrER) In Vitro Assays to Detect Chemicals with ER Binding Affinity” (OECD 2015).

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 via microscopical inspection. If the test item was not dissolved, heat (up to a maximum of 37°C) and/or ultrasonic was applied to aid solubility. Homogenous suspensions, characterized by small, homogenously distributes particles, were accepted as dissolved suspensions for testing in the method. If the test item was not fully dissolved, subsequent dilution steps of the test item in the appropriate solvent were used, dilutions of 1:10 (v/v) or 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 in the test tied stock solution was still soluble in a 1:10 (v/v) dilution with diH_2O. If the dilution was fully dissolved, the final assay concentration of the test item was prepared with a solution of 50% potassium phosphate / EDTA buffer, 40% diH_2O and 10% of the 10% test item dilution; solubility was checked by microscopical inspection

If a fully dissolved suspension was not achievable in any of the conditions, a lower concentration of the test item was used (dilution with a factor of 3.16 or 10 of the stock solution) to prepare the 10 % dilution in diH_2O and the subsequent assay concentration of the test item. If the test item was not fully dissolved, dilution steps were repeated leading to the next lower concentration.

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

On the day of analysis, the stock solution corresponding to the determined highest concentration of the test items in the solubility testing 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 volume 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 DIO1-SK assay is designed for the testing of unknown test items regarding their inhibition on IRA in the DIO1-SK assay and uses an initial range finding assay to determine the iodide release inhibition of the test item. If the determined inhibition of IRA in the range finding assay was greater than 20% in any of the tested concentrations, concentrations for future assay runs were adapted including more concentrations in the range of inhibition. The preparation of the dilutions (containing 10 % DMSO) for a test item X from the corresponding stock solution for a range finding assay is shown in table 14.

table 14: Preparation of the dilutions for a test item X.

Name of the dilution of test item X	diH_2O [µL]	10% DMSO/ diH_2O [µL]	Test item [µL]	Dilution factor compared to prior dilution
TIX-D1	450	-	50 µL of stock solution of test item X	10
TIX-D2	-	450	50 µL of X-D1	10
TIX-D3	-	450	50 µL of X-D2	10
TIX-D4	-	450	50 µL of X-D3	10
TIX-D5	-	450	50 µL of X-D4	10
TIX-D6	-	450	50 µL of X-D5	10
TIX-D7	-	450	50 µL of X-D6	10
TIX-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 diH_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 in 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 blinded test items in this part 2: relevance assessment, there was no information on iodide release inhibition available. As such an initial range finding assay run had to be performed for each test item. If the determined iodide release inhibition was lower than 20% in any tested concentration of the test item, concentrations were not adapted and the run was used as one of the three final assay runs for the test item.

8.1.4 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 volume of the solvent (DMSO) was added. The substance was dissolved in the solvent with a vortex. If needed, ultrasonication was performed to maintain the solubility of the stock solution.

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. Additionally, 6PTU is tested in single concentrations on each assay plate to control assay performance and final data normalization. The preparation of the reference item dilutions for the concentration-response testing is shown in table 15; the reference item dilution RI-D1 is used for single concentration testing on each assay plate.

table 15: Preparation of the dilutions for the reference item 6-Propyl-2-thioruacil.

Name of the reference item dilution	Reference item dilution concentration [M]	diH_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	$3.16*10^{-4}$	308	34.2	158 µL of RI-D2	$3.16*10^{-5}$
RI-D4	10^{-4}	405*	45*	50 µL of RI-D2	10^{-5}
RI-D5	$3.16*10^{-5}$	405*	45*	50 µL of RI-D3	$3.16*10^{-6}$
RI-D6	10^{-5}	405*	45*	50 µL of RI-D4	10^{-6}
RI-D7	10^{-6}	405*	45*	50 µL of RI-D6	10^{-7}
RI-D8	10^{-7}	405*	45*	50 µL of RI-D7	10^{-8}

*Alternatively, a 10% (v/v) DMSO / diH_2O solution can be prepared and 450 µL of the 10% solution is added

8.1.5 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 16. The prepared 100 µL are sufficient for three assay runs; adapt the concentrations accordingly if more assay runs are performed.

table 16: Preparation of the positive control Aurothioglucose dilution.

Positive control dilution [M]	diH_2O [µL]	DMSO [µL]	Positive control [µL]	Final concentration of positive control in the assay [M]
10^{-3}	90	-	10 µL of 10^{-2} M positive control stock solution	10^{-4}

8.1.6 Preparation of the 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 each 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 17.

table 17: Preparation of the negative control 1-Thio-β-D-glucose sodium salt dilution.

Negative control dilution [M]	diH_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}

8.1.7 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 in the DIO1-SK assay. As such, 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 ion exchange separation prior to the Sandell-Kolthoff reaction.

The described microsome dilutions only need to be prepared once for each microsome batch since the microsome activity testing is only performed once for each batch (see 8.1.8):
Human liver microsome dilutions were prepared in diH_2O as shown in table 18; perform the microsome dilution preparations on ice. Also, keep the prepared microsome dilutions on ice until needed for activity testing. The calculations in the table are based on a stock solution of 20 mg enzyme/mL, as the microsome batch #QQY was supplied in this concentration from BioIVT, which was used for this study.

table 18: Preparation of the human liver microsome dilutions for the testing of iodide release activity.

Microsome per well [µg]	diH_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

8.1.8 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 a microsome batch-specific activity curve was used in this method to standardize iodide release activity of the microsome batch. As such, a microsome batch-specific enzyme concentration as well as the used dilution in 10 % acetic acid prior to testing in the SK reaction (generally 1:2 or 1:4 dilutions are used to assure linear responses in the Sandell-Kolthoff reaction) was determined. After

determination of the microsome batch specific enzyme concentration, the microsomes are stored in aliquots sufficient for one or the desired amount of assay plates.

The reference item dilution of 6PTU as described in 8.1.4, the microsome dilutions on ice as described in table 18 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 (RI-D1) as reference item were added to the reference item controls on a 96-well plate (see plate layout in table 19).
- For the solvent controls, 10 µL of a 10% (v/v) solvent dilution in diH_2O (e.g., 10 % DMSO in diH_2O) were added to the solvent control on the plate.
- 40 µL of prepared microsome dilutions in diH_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.
- 50 µL of freshly prepared substrate mix was added to each well
- The plate was sealed with a gas-impermeable plastic seal
- The 96-well plate was then placed on a shaker (600 rpm) in an incubator (37°C) and was incubated for 2 h

table 19: 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**

- Plates were placed on ice to stop the reaction
- The separation of the substrate and released iodide of the assay is conducted analogous to 8.1.9.2
- The measurement of the Sandell-Kolthoff reaction is conducted analogous to 8.1.9.3 with the following deviation: 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; if the generated activity

curve in the highest acetic acid dilution is still not in a linear range, higher dilutions in acetic acid can be performed; also see table 20).

table 20: Added sample solution and 10% acetic acid in the SK reaction for different dilutions

Dilution	Sample solution [µL]	10% acetic acid [µL]
Undiluted	50	-
1:2	25	25
1:4	12.5	37.5
1:8	6.3	42.7

- ΔOD was determined by subtracting the OD_{21min} from the initial measured OD_{0min}
- ΔOD-BG was determined by subtracting the inhibited ΔOD of the reference item from each enzyme concentration from the solvent control ΔOD of the respective enzyme concentration
- ΔOD-BG of the enzyme concentration samples were plotted for each dilution in a statistics software with ΔOD-BG on y-axis (linear) and protein concentration on x-axis (logarithmic)
- A curve curve-fit algorithm was used to generate a function optimally reflecting assay characteristics (e.g. “[Inhibitor] vs. response -- Variable slope (four parameters)”)
- The dilution factor for the samples in 10 % acetic acid and enzyme concentration that led to the highest possible ΔOD-BG in the Sandell-Kolthoff reaction without reaching a plateau was determined **The determined dilution and protein concentration was used for all microsomal incubations of the respective microsome batch**
- The supplied microsome stock solution was aliquoted in appropriate amounts, sufficient for the performance of for one or the intended runs per day

8.1.9 DIO1 activity testing

8.1.9.1 Microsome incubation with test items

The reference item stock solution as well as dilution of 6PTU as described in 8.1.4, the positive and negative control stock solution as well as dilution as described in 8.1.5 and 8.1.6., and the test item stock solutions as well as dilutions as described in 8.1.3 were prepared on the day of analysis. The substrate mix was prepared as described in table 5. A microsome solution resulting in the defined concentration per well (see 8.1.8) was prepared by dilution of carefully thawed aliquoted microsome solution in diH_2O and kept on ice until needed for incubation.

- For the solvent controls, 10 µL of 10 % (v/v) DMSO in diH_2O solution were added to a clear 96-well plate. 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, concentration-response testing of the reference item 6PTU was conducted and prepared dilutions were added to the plate. The plate layout for the first assay run of the day for the test items 1 and 2 (TI1, TI2) is shown in table 21. The second assay run of the same day with the test items 3, 4 and 5 (TI3, TI4, TI5) is shown in table 22.

- 40 µL of the prepared microsome dilution was added to each well
- 50 µL of the freshly prepared substrate mix was added to the samples to start the reaction
- The plate was sealed with a gas-impermeable plastic seal
- The 96-well plate was then placed on a shaker (600 rpm) in an incubator (37°C) and was incubated for 2 h
- Following the 2 h incubation, the plates were placed on ice to stop the reaction

table 21: 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} to 10^{-8} M

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

TI **test item**

table 22: 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**	**RI-C1**	**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**
PC	**positive control** Aurothioglucose 10^{-4} M						

8.1.9.2 Separation via ion exchange resin-filled 96-well filter plate

- A prepared ion exchange resin-filled 96-well filter plate (as prepared in 8.1.1) was positioned on top of a used 96-deep well-plate
- 150 µL of 10 % acetic acid were added to each well of the resin-filled 96-well filter plate to wet the columns
- The acetic acid was eluted by centrifuging into the used 96-deep well-plate in a centrifuge with 200 g for 1 min
- The used 96-deep well-plate was replaced with a novel, unused 96-deep well plate
- 133 µL 10% (v/v) acetic acid was added to each well of the sample containing 96-well plate and was shaken with 600 rpm for 1 min
- 175 µL of each sample was transferred from the sample containing 96-well plate into the ion exchange resin-filled 96-well filter plate maintaining the initial plate layout
- The samples were eluted by centrifuging into the novel 96-deep well-plate with 200 g in a centrifuge for 1 min and the ion exchange resin-filled 96-well filter plate was discarded afterwards.

8.1.9.3 Sandell-Kolthoff reaction

Due to delivery problems, the ion exchange resin as well as 96-well filter plates had to be changed on the 05.07.2021. The change in material resulted in an increase in background reaction, probably due to increased leakage of the cofactor DTT during ion exchange. This led to z'-factors that often did not meet the acceptance criteria. To increase the dynamic range of the assay and derived z'-factors, the Cerium concentration in the cerium solution was

increased from 25 mM to 40 mM for the runs starting from 05.07.2021. A reference item-response curve derived with 25 mM, 30 mM and 40 mM Cerium produced comparable concentration-response curves while increasing the z -factor at higher Cerium concentrations.

- Depending on the determined dilution factor of the samples in 10% acetic acid for the used microsome batch (see 8.1.8 and table 20), 50 µL of the diluted sample solution were added to a novel 96-well plate. E.g., for a defined 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] or 50 µL of cerium solution [40 mM $(NH_4)_4Ce(SO_4)_4{*}2H_2O$; 0.5 M H_2SO_4] was added to the samples in the 96-well plate
- The reaction was initiated 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, an automatic multichannel pipette was used.
- Immediately after the application of arsenite solution, the absorption OD was determined in the 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

8.1.9.4 Evaluation of the data

The data was processed and analysed using Microsoft Excel. The OD_{21min} were subtracted from the initial OD_{0min} to generate ΔOD_{21min}:

$$\Delta OD_{21min} = OD_{21min,415nm} - OD_{0min,415nm}$$

Present background reaction was removed by subtracting the mean of ΔOD_{21min} values of the inhibited reference item samples from the ΔOD_{21min} of all samples, thus generating ΔOD-BG:

$$\Delta OD\text{-}BG = \Delta OD_{21min} - \overline{\Delta OD_{21min,RI}}$$

Where “RI” represents the reference item 6PTU

The calculated ΔOD-BG were then normalized to their respective solvent control values via division of the item(s) ΔOD-BG by the mean of the ΔOD-BG of the respective solvent control, generating iodide release activity (IRA) in %:

$$iodide\ release\ activity\ (IRA) = \frac{\Delta OD\text{-}BG_{TI}}{\overline{\Delta OD\text{-}BG_{SC}}} * 100$$

where “TI” represents the test item at used concentrations and “SC” the solvent control

Using statistics software, the IRA values of the different test item concentration samples were plotted with IRA values on y-axis (linear) and test item concentration on x-axis (logarithmic) in a scatter plot. A curve-fit algorithm to visualize a concentration-response relationship (e.g. “[Inhibitor] vs. response --

Variable slope (four parameters)" in GraphPad Prism 8) was used to generate a function reflecting assay characteristics, visualize a concentration-response relationship and, if possible, calculate the 50% inhibition concentration (IC_{50}) of the test item:

$$Y = Bottom + (Top - Bottom) / (1 + (IC_{50} / X)^{HillSlope})$$

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

The model used often leads to curves that become sigmoidal early for test items that do not lead to full inhibition. As such, the bottom of the model was constantly set to 0.

8.1.9.5 Acceptance criteria

Based on the data generated in the part 1 study (Birk and Weber 2020), different acceptance criteria for the assessment of valid assay runs were defined which were used for part 2 testing (table 23 and table 24). An assay run was considered as valid and was accepted when all the acceptance criteria were met. If one of the acceptance criteria were not met, the assay run was considered as invalid, was not considered for further evaluation, and had to be repeated.

table 23: Used acceptance criteria for the determination of valid runs in the part 2 study 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
Iodide release activity of negative control [%]	80 < x < 120
Iodide release activity of positive control [%]	x < 20
z'-Factor	x > 0.5
Binary	
Shape of reference item (sigmoidal?)	x = yes
The final concentration-response curve of the reference item is composed of minimum six concentrations from three replicates	x = yes
The final concentration-response curve of the test item is composed of minimum six concentrations from three replicates	x = yes

table 24: Calculation of the numeric acceptance criteria of the part 2 study of the DIO1-SK assay.

Acceptance criteria	**Calculation**
IC_{50} of reference item [µM]	Calculated in 8.1.9.4
CV of log IC_{50} estimate of reference item [%]	$= \left(\frac{\sigma_{log\ IC_{50}}}{log\ IC_{50}}\right) * 100$
Iodide release activity of negative control [%]	$= \left(\frac{\overline{IRA_{NC}}}{\overline{IRA_{SC}}}\right) * 100$
Iodide release activity of positive control [%]	$= \left(\frac{\overline{IRA_{PC}}}{\overline{IRA_{SC}}}\right) * 100$
z'-factor	$= 1 - \frac{3 * \left(\sigma_{\Delta OD_{21min,RI}} + \sigma_{\Delta OD_{21min,SC}}\right)}{\left\lvert \mu_{\Delta OD_{21min,RI}} - \mu_{\Delta OD_{21min,SC}} \right\rvert}$

Where “σ” represents standard deviation, “μ” represents mean, “NC” represents negative control, “SC” represents solvent control, “PC” represents positive control, and “RI” represents reference item.

8.2 Specificity testing

In addition to testing in the DIO1-SK assay, the test items of part 2 were analysed in two specificity testing approaches: 1. Microsome incubation in the DIO1-SK assay with and without microsome present to identify potential unspecific SK interference by the test item and 2. ALP activity testing to verify functionality of used microsomes.

8.2.1 Identification of potential unspecific SK interference (testing without microsome)

The reference item stock solution as well as the RI-C1 dilution as described in 8.1.4, and the test item stock solutions as well as the dilutions with the highest test item concentration (TIX-D1) as described in 8.1.3 were prepared on the day of analysis. The substrate mix was prepared as described in table 5. A microsome solution resulting in the defined concentration per well (see 8.1.8) was prepared by dilution of carefully thawed aliquoted microsome solution in diH_2O and kept on ice until needed for incubation.

1. For the solvent controls, 10 µL of 10 % (v/v) DMSO in diH_2O solution was added to a 96-well plate; for the reference item RI-C1 samples, 10 µL of the prepared dilution was added to the plate. 10 µL of the test item dilutions was added to the 96-well plate. The plate layout for the DIO1-SK testing with and without microsome is shown in table 25.
2. Either 40 µL of the microsome dilution or 40 µL diH_2O was added to the wells, in accordance with the plate layout in table 25
3. 50 µL of the freshly prepared substrate mix were added to the samples
4. The plate was sealed with a gas-impermeable plastic seal
5. The 96-well plate was then placed on a shaker (600 rpm) in an incubator (37°C) and was incubated for 2 h
6. Following the 2 h incubation, the plates were placed on ice to stop the reaction

table 25: plate layout for testing of items with and without microsomes during incubation in the DIO1-SK assay.

	1	2	3	4	5	6	7	8	9	10	11	12
A	**SC**			SC			**RI-C1**			RI-C1		
B	**TI1-C1**			TI1-C1			**TI2-C1**			TI2-C1		
C	**TI3-C1**			TI3-C1			**TI4-C1**			TI4-C1		
D	**TI5-C1**			TI5-C1			**TI6-C1**			TI6-C1		
E	**TI7-C1**			TI7-C1			**TI8-C1**			TI8-C1		
F	**TI9-C1**			TI9-C1			**TI10-C1**			TI10-C1		
G	**TI11-C1**			TI11-C1			**TI12-C1**			TI12-C1		
H	**TI13-C1**			TI13-C1			**TI14-C1**			TI14-C1		
	with microsomes			no microsomes (diH_2O only)			**with microsomes**			no microsomes (diH_2O only)		

SC	**solvent control**	**RI-C1**	**reference item** 6-Propyl-2-thiouracil 10^{-3} M	**TI**	**test item**

7. Ion-exchange separation and iodide quantification via the SK reaction was performed as described in 8.1.9.2 and 8.1.9.3
8. The derivation of IRA values was performed as described in 8.1.4 with few adjustments: generation of ΔOD-BG values for samples with or without microsomes was performed by subtracting the mean of the respective RI-C1 control with or without microsomes; generation of IRA was always performed via division of the item(s) ΔOD-BG by the mean of the ΔOD-BG of the solvent control with microsomes.
9. Using statistics software, the IRA values of each test item for each condition were plotted with IRA values on y-axis (linear) and test item identifier on x-axis in a grouped bar chart.

8.2.2 ALP activity testing

The testing of the Alkaline Phosphatase as a secondary enzyme activity readout is used in this method to assess the non-specific interaction of test items with the microsomal protein, leading to loss of activity. The ALP is used because the readout can also be quantified using a photometer and there is no overlap in necessary cofactors or known substances that inhibit both DIO1 and ALP activity.

8.2.2.1 ALP assay buffer preparation

1. Diethanolamine (DEA) was thawed at 37°C (melting point of DEA: ~28°C): big volumes were thawed overnight; once thawed, small aliquots

of DEA were prepared for future ALP assay buffer preparation and stored at RT
2. A 200 mM $MgCl_2$ stock solution (1000x stock) was prepared by weighing in 40.66 mg $MgCl_2$, dissolving in 1 mL diH_2O and the solution was vortexed
3. 500 mL ALP assay buffer (20 mM DEA / 200 µM $MgCl_2$) was prepared in diH_2O: 1.05 g of thawed DEA was weighed in, dissolved by addition of 500 mL diH_2O and 500 µL of 200 mM $MgCl_2$ was added (1000x stock)
4. pH was adjusted to 9.8 by dropwise addition of 10% (w/w) HCl or NaOH
5. ALP assay buffer was stored at 4°C

8.2.2.2 Control setup in ALP activity testing

For ALP activity testing, a reference item to show ALP inhibition and a negative control without ALP inhibiting properties was used (table 26). The reference item "tissue-nonspecific (TN) alkaline phosphatase (AP) inhibitor" (2,5-Dimethoxy-N-(quinolin-3-yl) benzenesulfonamide, CAS no.: 496014-13-2) is a described specific inhibitor of TNAP (Dahl, Sergienko et al. 2009) and was used as the reference item for ALP activity testing. The DIO1 and TPO inhibitor 6-Propyl-2-thiouracil (6PTU) was used as a negative control in ALP activity testing since 6PTU does not inhibit ALP activity.

table 26: Control setup for ALP activity testing.

Controls:	
Reference item (RI)	Quantitatively controls ALP inhibition in the assay and is used for normalization to maximum inhibition in the assay. In addition to control replicates on each assay plate of the highest concentration, a concentration-response curve of the reference item is performed on each assay day. Used in ALP activity testing: TNAP inhibitor at a maximum assay concentration of $3.16*10^{-5}$. Concentration-response curves of TNAP inhibitor ranged from $3.16*10^{-5}$ to 10^{-9} M.
Negative control (NC)	A substance that leads to no inhibition of ALP activity. Used in ALP activity testing: 6-Propyl-2-thiouracil at a maximum assay concentration of 10^{-3} M.
Solvent control (SC)	Blank control. The solvent control ensures that the response does not originate from the applied solvent and shows no ALP inhibition. Solvent controls are used to normalize the inhibition observed with the test items to the maximum possible ALP activity. Final solvent concentration in the assay: Dimethyl sulfoxide (DMSO) at an assay concentration of 1%.

8.2.2.3 Solubility testing of test items in ALP assay buffer

In the context of ALP activity testing, the solubility of test items needs to be assessed in their stock solutions, in prepared dilutions in water and under assay conditions. The solubility of the test items in solvent (DMSO preferred) as a stock solution as well as the 10% (v/v) dilutions in water was already assessed in the DIO1-SK assay (see 8.1.2) and the defined conditions will be used for the preparation of the stock solution in the ALP activity testing.

1. The stock solution of the test item in DMSO was prepared; the concentration that was determined as fully soluble in DIO1-SK testing was used
2. The tube was gently mixed at room temperature and vortexed if necessary
3. A 10% (v/v) dilution of the test item stock solution was prepared in diH_2O as determined for DIO1-SK testing
4. 100 µL of the prepared dilution, 400 µL diH_2O and 500 µL ALP assay buffer was added to a well of a 24-well plate to test solubility under assay conditions; it was ensured that the added solutions were mixed, either by gentle shaking or pipetting
5. The test item solution was visually checked by using a microscope if the solution was dissolved under ALP assay conditions
6. If the test item wasn't dissolved, water bath sonification was used for up to 5 mins or the solution was warmed to 37°C for up to 60 mins; step 5 to check if the test item is dissolved was repeated
7. If the test item was not soluble under assay conditions, the test item stock solution was diluted (e.g., by reducing the concentration by a factor of 10), the resulting 10% (v/v) DMSO/ diH_2O dilutions of the test item stock solutions in diH_2O as well the solutions under assay conditions as described in step 4 was prepared
8. The test item solution was visually checked by using a microscope if the solution was dissolved under ALP assay conditions
9. If necessary, step 7 was repeated

8.2.2.4 Preparation of the stock solution and dilutions of the reference item in ALP activity testing

The TNAP inhibitor was used as the reference item for ALP activity testing (ALP-RI). On the first plate on each assay day, a full concentration-response curve was performed. Additionally, the highest concentration of the reference item (ALP-RI-D1) was included on all plates in at least 6 replicates and was used to determine the background reaction occurring at full ALP inhibition.
The stock solution of the TNAP inhibitor can be prepared prior to the assay run and was stored at 4°C. The dilutions in diH_2O were prepared fresh on each day of assay performance.

1. A respective amount of reference item was weighed in a brown glass vial and dissolved in DMSO resulting in a concentration of 100 mM (ALP-RI-S); if the amount of the reference item in a supplied vial was ≤5 mg, the amount of DMSO to the vial directly resulting in a concentration of 100 mM (ALP-RI-S) was added
2. ALP-RI-SO was stored at 4°C
   Decrease of activity of TNAP inhibitor stock solutions after longer storage times were observed. For future experiments, small volumes of the TNAP inhibitor stock solution should be aliquoted into opaque glass tubes and be stored at 4°C.
3. On the day of analysis, the reference item dilutions from the 100 mM reference item stock solution (ALP-RI-S0) were prepared according to table 27; ALP-RI-D0 was not used for testing in the assay

table 27: Preparation of the dilutions for the reference item TNAP inhibitor.

Name of the reference item dilution	Reference item dilution concentration [M]	diH_2O [µL]	DMSO [µL]	Reference item [µL]	Final concentration of reference item in the assay [M]
ALP-RI-D0	$3.16*10^{-3}$	450	34.2	15.8 µL of ALP-RI-S	-
ALP-RI-D1	$3.16*10^{-4}$	405*	45*	50 µL of RI-D0	$3.16*10^{-5}$
ALP-RI-D2	$3.16*10^{-5}$	405*	45*	50 µL of RI-D1	$3.16*10^{-6}$
ALP-RI-D3	10^{-5}	308*	34.2*	158 µL of RI-D2	10^{-6}
ALP-RI-D4	$3.16*10^{-6}$	405*	45*	50 µL of RI-D2	$3.16*10^{-7}$
ALP-RI-D5	10^{-6}	405*	45*	50 µL of RI-D3	10^{-7}
ALP-RI-D6	$3.16*10^{-7}$	405*	45*	50 µL of RI-D4	$3.16*10^{-8}$
ALP-RI-D7	10^{-7}	405*	45*	50 µL of RI-D5	10^{-8}
ALP-RI-D8	10^{-8}	405*	45*	50 µL of RI-D7	10^{-9}

*A 10 % DMSO / diH_2O solution can also be prepared, and the needed volume can be added.

8.2.2.5 Preparation of the stock solution and dilution of the negative control

1. On the day of analysis, a 10^{-1} M stock solution of the negative control 6PTU was freshly prepared in DMSO: an appropriate amount of substance was weighed into a brown, glass vessel, the appropriate volume of DMSO was added using a pipette and the solution was vortexed for up to 10 s.
2. The dilution of 6PTU in diH_2O was prepared according to table 28.

table 28: Preparation of the negative control 6-propyl-2-thiouracil (6PTU) dilution for ALP activity testing.

Negative control dilution [M]	diH_2O [µL]	DMSO [µL]	Negative control [µL]	Final concentration of negative control in the assay [M]
10^{-2}	450	-	50 µL of 10^{-1} M negative control stock solution	10^{-3}

8.2.2.6 Preparation of stock solutions and dilutions of the test items

On the day of analysis, the stock solution corresponding to the determined highest concentration of the test item in the solubility testing (as defined in 8.2.2.3) 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 volume 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 preparation of the dilution (containing 10 % DMSO) for a test item X from the corresponding stock solution is shown in table 29.

table 29: Preparation of the dilutions for the test item X.

Name of the dilution of test item X	diH_2O [µL]	Reference item [µL]	Dilution factor compared to prior dilution
ALP-TIX-D1	450	50 µL of stock solution of test item X	10

8.2.2.7 Preparation of the PNPP substrate solution

The substrate solution was prepared fresh on each day. The substrate solution was kept in the dark to prevent loss of activity in the assay.

1. A 5 mg PNPP table was dissolved in 450 µL diH_2O to prepare a 100x PNPP (30 mM) stock solution
2. The 100x PNPP stock solution was diluted 1:100 (v/v) in ALP assay buffer to prepare a 1x PNPP (0.3 mM) solution; 6 mL of 1x PNPP solution was sufficient for one 96-well plate of ALP testing

8.2.2.8 ALP activity testing in human liver microsomes

1. The reference item stock solution as well as dilutions as described in 8.2.2.4, the negative control stock solution as well as dilution as described in 8.2.2.5 and the test item stock solution(s) as well as dilution(s) as described in 8.2.2.6 were prepared on the day of analysis. Also, the 1x PNPP solution was prepared as described in 8.2.2.7.
2. A microsome solution resulting in the same concentration per well as defined for the DIO1-SK assay (see 8.1.8) was prepared by dilution of carefully thawed aliquoted microsome solution in diH_2O and kept on ice until needed for incubation.
3. The absorbance reader was preheated to 37°C
4. On the first plate of an assay day, 10 µL of the reference item dilutions were added to a 96-well plate to perform a full concentration-response testing. 10 µL of the ALP-RI-D1 dilution was added to all plates. For the solvent control, 10 µL of a 10 % (v/v) DMSO/ diH_2O solution was added to all plates. For the negative control, 10 µL of the prepared dilution of the negative control was added to all plates. For the test item(s), 10 µL of the test item dilution(s) were added to the 96-well plate.
   A plate layout for the first run of an assay day is shown in table 30; a plate layout for additional runs on the same assay day is shown in table 31.
5. 40 µL of the defined microsome dilution was added to the wells
6. 50 µL 1x PNPP solution was added to each well using a multichannel pipette
7. As soon as possible after the application of 1x PNPP solution, the optical density (OD) was determined in a plate reader with the following settings:
   - Absorption parameters: 415 nm (±2 nm)
   - Initial shaking: weak for 5 s
   - Target temperature: 37°C, valid temperature range 35 – 39°C
   - Measurement of the OD every minute for 60 min (other time periods are possible, make sure the detection is in linear range), also measuring the initial OD

table 30: plate layout for the first plate of an assay day of the ALP activity testing.

	1	2	3	4	5	6	7	8	9	10	11	12
A	SC			ALP-RI-C1			NC			ALP-RI-C1		
B	ALP-RI-C1			ALP-RI-C2			ALP-RI-C3			ALP-RI-C4		
C	ALP-RI-C5			ALP-RI-C6			ALP-RI-C7			ALP-RI-C8		
D	TI1-C1			TI2-C1			TI3-C1			TI4-C1		
E	TI5-C1			TI6-C1			TI7-C1			TI8-C1		
F	TI9-C1			TI10-C1			TI11-C1			TI12-C1		
G	TI13-C1			TI14-C1			TI15-C1			TI16-C1		
H	SC			ALP-RI-C1			SC			NC		

SC **solvent control** 1% DMSO
RI **reference item** TNAP inhibitor 3.16*10^{-5} to 10^{-9} M
NC **negative control** 6PTU 10^{-3} M
TI **test item**

table 31: plate layout for additional plates of an assay day of the ALP activity testing.

	1	2	3	4	5	6	7	8	9	10	11	12
A	SC			ALP-RI-C1			NC			ALP-RI-C1		
B	TI17-C1			TI18-C1			TI19-C1			TI20-C1		
C	TI21-C1			TI22-C1			TI23-C1			TI24-C1		
D	TI25-C1			TI26-C1			TI27-C1			TI28-C1		
E	TI29-C1			TI30-C1			TI31-C1			TI32-C1		
F	TI33-C1			TI34-C1			TI35-C1			TI36-C1		
G	TI37-C1			TI38-C1			TI39-C1			TI40-C1		
H	SC			ALP-RI-C1			SC			NC		

SC **solvent control** 1% DMSO
RI **reference item** TNAP inhibitor 3.16*10^{-5} M
NC **negative control** 6PTU 10^{-3} M
TI **test item**

8.2.2.9 Data evaluation of ALP activity testing

The following data analysis was performed for an incubation time of 60 min. Other incubation times can also be used, provided the range between the reference item and the solubility control is large enough (e.g., characterized by a z'-factor greater than 0.5).

1. ΔOD_{60min} were determined via subtraction of the $OD_{60min,415nm}$ of all samples from the initial measured $OD_{0min,415nm}$:

   $$\Delta OD_{60min} = OD_{60min,415nm} - OD_{0min,415nm}$$

2. ΔOD-BG were determined by subtracting the mean of ΔOD_{60min} values of the inhibited $3.16*10^{-5}$ M TNAP inhibitor reference item controls from the ΔOD_{60min} values of all samples:

   $$\Delta OD\text{-}BG = \Delta OD_{60min} - \overline{\Delta OD_{60min,RI}}$$

   Where "RI" represents the reference item TNAP inhibitor

3. Samples were normalized to the respective solvent control values via division of the test item(s) ΔOD-BG values by the mean of the ΔOD-BG values of the respective solvent control, generating Alkaline phosphatase (ALP) activity values in %:

   $$ALP\ activity = \frac{\Delta OD\text{-}BG_{TI}}{\Delta OD\text{-}BG_{SC}} * 100$$

   where "TI" represents the test item at used concentrations and "SC" the solvent control

4. Using statistics software, the ALP activity values of the different test item concentration samples were plotted with ALP activity values on y-axis (linear) and test item identifier on x-axis in a bar chart.

8.3 Part 2 testing strategy

All 40 blinded test items of part 2 were tested in the DIO1-SK assay as well as the specificity testing strategies: the DIO1-SK assay without microsomes and ALP activity testing.

In the DIO1-SK assay, three valid assay runs were performed for each test item. The plate layout of the assay is designed to test three test items per assay run. It was ensured that test items were tested in at least two of the three possible positions in the assay runs to avoid a potential influence of the plate position on the results. Three different laboratory co-workers performed the experiments to ensure intra-laboratory reproducibility.
Every test item was initially tested in a range finding assay to estimate the concentration range of inhibition, provided the test item inhibited iodide release activity (IRA). Test concentrations of the range finding assay were based on the highest solubility determined under assay conditions; following test concentrations were prepared by consecutive 10-fold (v/v) dilution steps.
If a test item led to an IRA decrease greater than 20% in any of the tested concentrations in the range finding assay, test concentrations for following

assays were adapted including more test item concentrations in the range of observed inhibition. In addition, it was ensured that the adapted two lowest concentrations showed little or no IRA inhibition, so that the curve fitting function for data evaluation could recognize the top of the curve. If the observed inhibition in the range finding assay was less than 20%, concentrations were not changed and the valid range finding assay run was included in the final set of three valid assay runs that was performed for every test item.

For testing of both specificity readouts, one initial assay run was performed for all test items using the highest concentration only. Two additional, independent assay runs were performed for the test item (i) for DIO1-SK assay without microsomes: if the highest tested concentration without microsomes present, led to a mean IRA greater than 10% and (ii) for ALP testing: if the highest tested concentration led to inhibition greater 15% (corresponds to 85% ALP activity).

8.4 Safety measures

Safety measures were followed according to the safety data sheets of the used materials and test items. Based on the provided information of the known hazard for the blinded test items, safety measurements for handling the substances in laboratory according to BASF safety rules were applied. The safety precautions according to the BASF internal risk assessment “Gefährdungsbeurteilung: Umgang mit Sodium Arsenit in der Sandell-Kolthoff-Reaktion (Version 1.0) “ were followed.

9 Results and evaluation

9.1 DIO1-SK assay

9.1.1 Microsome batch-specific iodide release activity

A concentration-response curve with differing concentrations of the microsome batch #QQY from BioIVT was derived according to 8.1.8, and was 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 for both the DIO1-SK assay as well as ALP activity testing (figure 3). Higher amounts of protein per well reach a plateau and do not provide additional resolution between full reaction and solvent controls as observed for other microsome batches. 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.

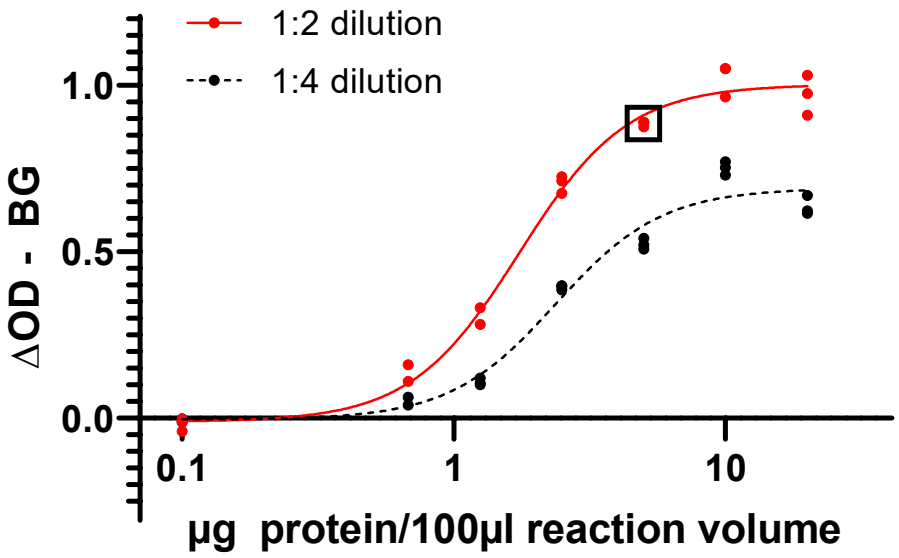

figure 3: iodide release activity of the human liver microsome batch #QQY measured in a 1:2 and 1:4 dilution of 10 % acetic acid in the Sandell-Kolthoff reaction. The black box indicates the determined concentration of the batch-specific microsome and determined dilution factor for the SK reaction.

9.1.2 Solubility of test items in DIO1-SK testing

The solubility of the part 2 test items was assessed in the DMSO stock solution, the 10% (v/v) DMSO / diH_2O dilution and under final assay conditions (1% DMSO), resulting in concentrations that were fully dissolved in all tested conditions (table 32). Extensive information about the solubility assessment is available in Appendix 14.5 with linked photos on insoluble / poorly soluble concentrations of test items in Appendix 14.6. Most of the test items were soluble at final assay concentrations of 10^{-3} and 10^{-4} M with some of the test items being less soluble, up to 10^{-6} M. Test items were often fully soluble in pure DMSO at high concentrations. Once aqueous solutions were prepared, the test item precipitated, and concentrations had to be reduced.

table 32: Results of the solubility assessment of the 40 blinded test items in part 2 testing if the DIO1-SK assay. Solubility was assessed in the DMSO stock solution, the 10% (v/v) DMSO / diH_2O dilution and under final assay conditions.

Chemical Code	Final test item concentration under assay conditions [M]	Chemical Code	Final test item concentration under assay conditions [M]
56	10^{-4}	551	10^{-4}
125	10^{-3}	598	10^{-3}
130	10^{-3}	603	10^{-4}
160	$3.16*10^{-5}$	610	$3.16*10^{-4}$
194	10^{-3}	615	10^{-3}
218	$3.16*10^{-4}$	667	$3.16*10^{-4}$
220	10^{-3}	680	10^{-4}
227	10^{-3}	741	10^{-3}
229	10^{-3}	798	10^{-3}
279	10^{-4}	818	10^{-3}
294	$3.16*10^{-4}$	827	10^{-3}
307	10^{-3}	839	10^{-4}
325	10^{-3}	850	10^{-3}
377	10^{-4}	868	10^{-6}
437	10^{-3}	877	10^{-3}
442	$3.16*10^{-5}$	878	10^{-3}
506	$3.16*10^{-5}$	925	10^{-5}
511	$3.16*10^{-4}$	933	10^{-4}
526	10^{-5}	940	10^{-3}
543	10^{-3}	974	$3.16*10^{-4}$

9.1.3 Assay performance

The performance of the DIO1-SK assay was monitored for each performed valid assay run during performance of part 2 testing. The iodide release activity values of the control items and the reference item as well as the IC_{50} values of the reference item and the z'-factor over time are shown in figure 4; the mean and SD of the control parameters are shown in table 33. The results of the 33 performed concentration-response curves of the reference item 6PTU are shown in figure 5.

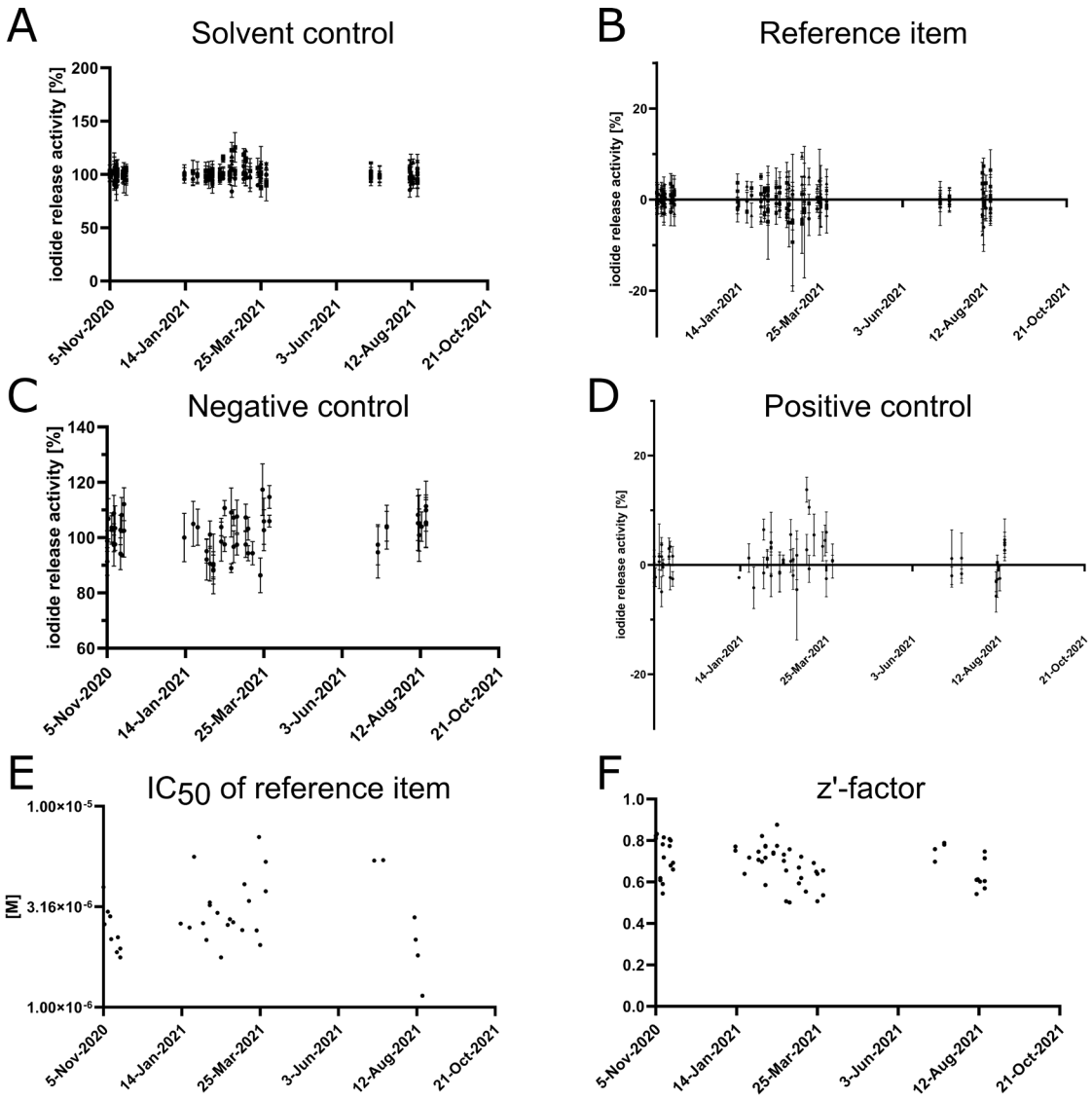

figure 4: Temporal display of the control development of solvent (DMSO) control (A), negative control (1-Thio-β-D-glucose sodium salt; TGSS) (B), reference item (6-Propyl-2-thioruracil, 6PTU) (C) and positive control (Aurothioglucose, ATG) (D) as well as the derived 50% inhibition concentration (IC_{50}) of the reference item 6PTU (E) and the z'-factor that was derived for each assay run (F). A-D: Shown is the mean and standard deviation of the iodide release activity over the nine replicates (for reference item and DMSO control) and three replicates (for negative and positive control) of each assay plate. Data was normalized to the reference item 6PTU (0 % iodide release activity) and the solvent control (100 % iodide release activity). E: The IC_{50} of a concentration-response curve of the reference item 6PTU was derived on the first assay run of each assay day by using the function "[Inhibitor] vs. response -- Variable slope (four parameters)". F: The z'-factor was derived for each assay plate using the mean and standard deviation of the nine solvent control and reference item replicates.

table 33: Iodide release activity (IRA) of the used control and reference items, the derived 50% inhibition concentration (IC_{50}) of the reference item 6PTU and z'-factor of each assay run in the part 2 of the DIO1-SK assay over all 61 performed assay runs.

Control	Mean	SD
IRA of solvent control [%]	100	6.92
IRA of reference item [%]	0	3.13
IRA of negative control [%]	99.99	9.09
IRA of positive control [%]	1.04	4.06
IC_{50} of the reference item [µM]	3.04	1.34
Z'-factor	0.69	0.09

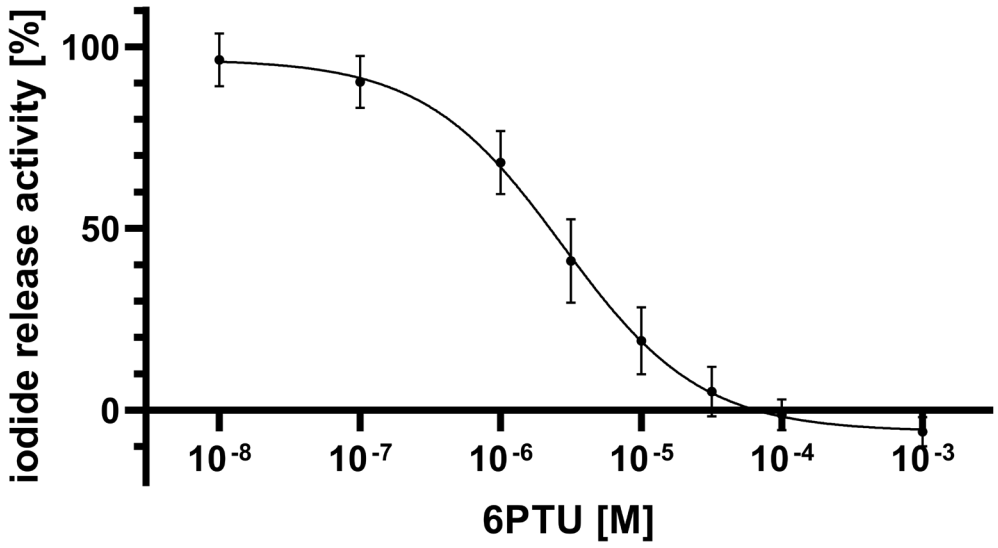

figure 5: results of the reference item 6-Propyl-2-thiouracil in the concentration-response testing of the DIO1-SK assay for part 2 testing. 33 runs on individual days were performed by three different technicians. Data is shown as mean iodide release ± SD of three technical replicates per concentration. A sigmoidal concentration-response curve was derived using the function "[Inhibitor] vs. response -- Variable slope (four parameters)".

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

In each assay run, up to three test items could be tested; on the first assay run on an assay day, the reference item-response was performed and hence, only two test items could be tested. As such, the 40 blinded test items were tested in the DIO1-SK assay in 61 valid assay runs from 05.11.2020 until 17.08.2021 in at least three valid, independent assay runs according to 8.1. For 24 of the 40 test items concentrations were adapted after the initial range finding assay run based on an IRA decrease greater than 20% in any of the tested concentrations. Three assay runs were performed for 14 test items, four assay runs were performed for 24 test items and five assay runs were performed for two test items.

During data generation the filter plates and the ion exchange resin had to be changed due to supply chain issues. This resulted in changed assay performance and the assay protocol had to be adapted. Centrifugation speed of ion of ion exchange resin-filled 96-well filter plates to elute acetic acid or sample solution had to be increased up to 200xg to fully elute the solutions. The cerium concentration in the SK reaction was increased from 25 to 40 mM to increase the reducible range and increase the resolution of the assay. The change in cerium concentration does not affect test item-induced inhibition testing in the assay. The mean inhibition of the highest tested concentration of each test item over all performed assay runs is shown in table 34.

table 34: Maximum inhibition of the test items in the DIO1-SK assay of part 2 testing. Performed runs also include performed range finding assay runs.

Identifier	Highest tested concentration [µM]	Mean maximum inhibition [%]	Standard deviation of maximum inhibition [%]	Performed runs [#]
6PTU	1000	105.95	3.43	33
056	100	97.02	4.36	4
125	1000	98.59	7.77	5
130	1000	6.27	7.89	3
160	31.6	48.90	36.92	4
194	1000	27.96	20.01	3
218	316	19.04	2.64	4
220	1000	69.51	21.06	3
227	1000	105.64	4.93	4
229	1000	102.75	1.27	4
279	100	47.53	8.22	4
294	316	68.95	12.00	4
307	1000	104.61	2.79	4
325	1000	51.21	10.61	4
377	100	12.29	5.22	3
437	1000	8.71	2.79	3
442	31.6	-1.00	3.94	3
506	31.6	50.40	18.02	4
511	316	12.79	8.69	3
526	10	42.81	6.42	5
543	1000	105.75	5.39	4
551	100	8.85	2.53	3
598	1000	19.64	1.76	4
603	100	84.64	3.34	4
610	316	105.50	2.98	4
615	1000	36.75	24.50	4
667	316	41.99	14.27	3
680	100	97.21	4.19	3
741	1000	71.88	5.26	4
798	1000	95.38	2.08	4
818	1000	103.98	8.10	4
827	1000	16.18	3.19	3
839	100	31.53	13.68	4
850	1000	101.31	0.99	3
868	1	6.18	2.08	3
877	1000	4.43	2.41	4
878	1000	57.16	4.00	4
925	1	11.22	5.42	3
933	100	58.03	11.85	4
940	1000	62.11	18.12	4
974	316	11.89	4.69	4

The results of the concentration-response testing of the blinded test items were structured into four different groups, depending on the mean maximum inhibition of the highest test item concentration over all assay runs:

1. Test items leading to inhibition of IRA ≤ 25%
2. Test items leading to inhibition of IRA between 25 and 50%
3. Test items leading to inhibition of IRA between 50 and 75%
4. Test items leading to inhibition of IRA ≥75%

The grouped test item results of the concentration-response screening of part 2 testing in the DIO1-SK assay is shown in figure 6, figure 7, figure 8 and figure 9.

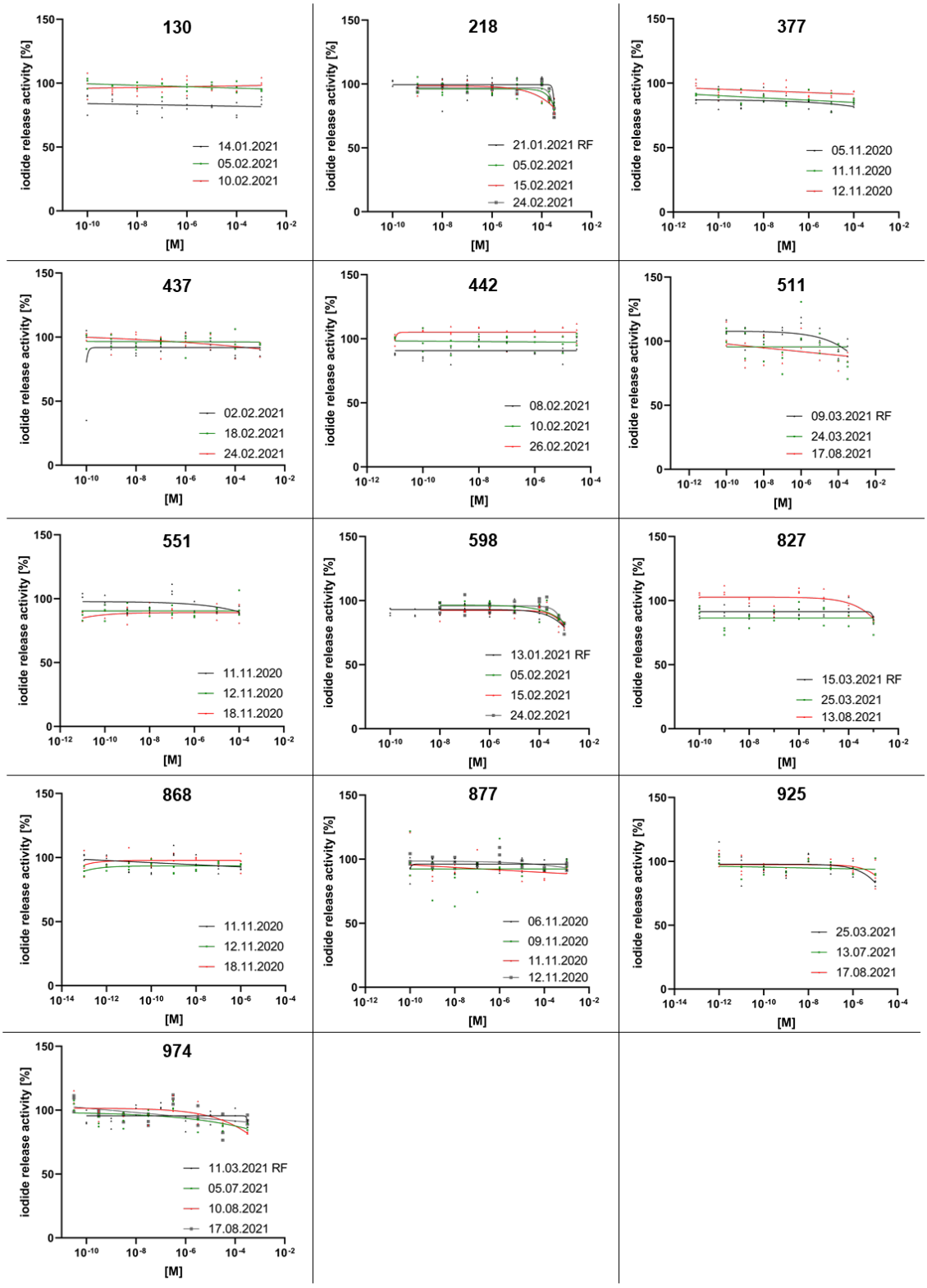

figure 6: results of the concentration-response testing in the DIO1-SK assay of part 2 testing for test items that lead to inhibition of iodide release activity (IRA) ≤ 25%. At least three independent assay runs with three technical replicates per concentration and test were performed by three different laboratory staff members. Data is shown as mean iodide release ± SD of three technical replicates per concentration. A sigmoidal concentration-response curve was derived using the function "[Inhibitor] vs. response -- Variable slope (four parameters)" and constraining the bottom of the model to 0.

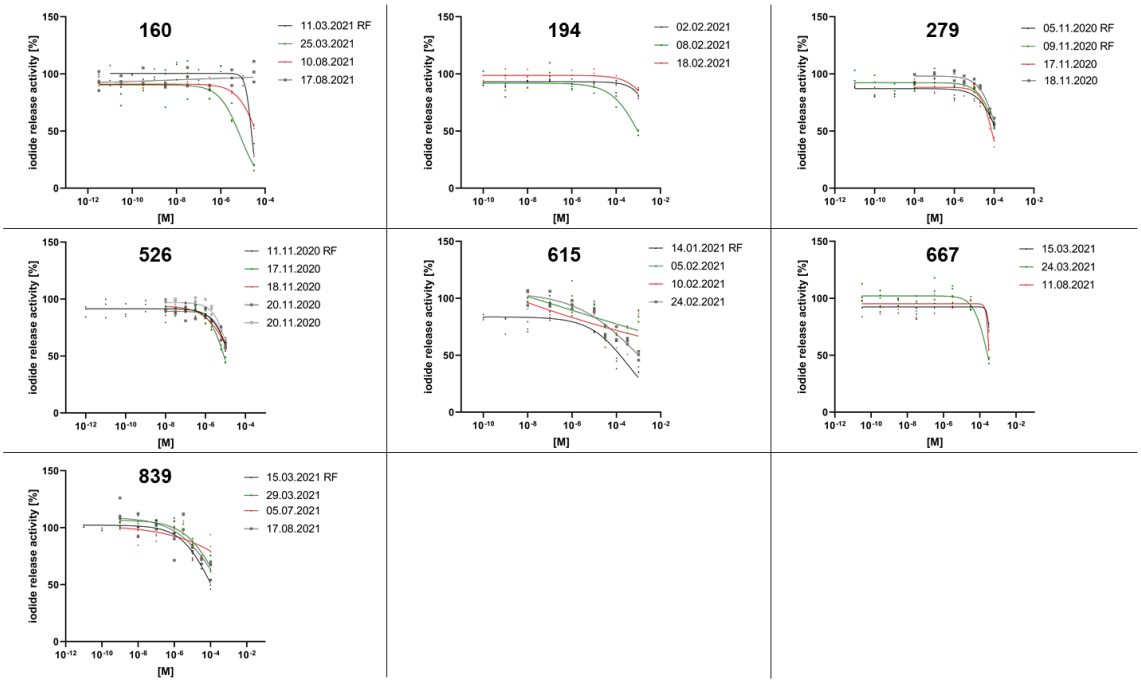

figure 7: results of the concentration-response testing in the DIO1-SK assay of part 2 testing for test items that lead to inhibition of iodide release activity (IRA) between 25 and 50%. At least three independent assay runs with three technical replicates per concentration and test were performed by three different laboratory staff members. Data is shown as mean iodide release ± SD of three technical replicates per concentration. A sigmoidal concentration-response curve was derived using the function "[Inhibitor] vs. response -- Variable slope (four parameters)" and constraining the bottom of the model to 0.

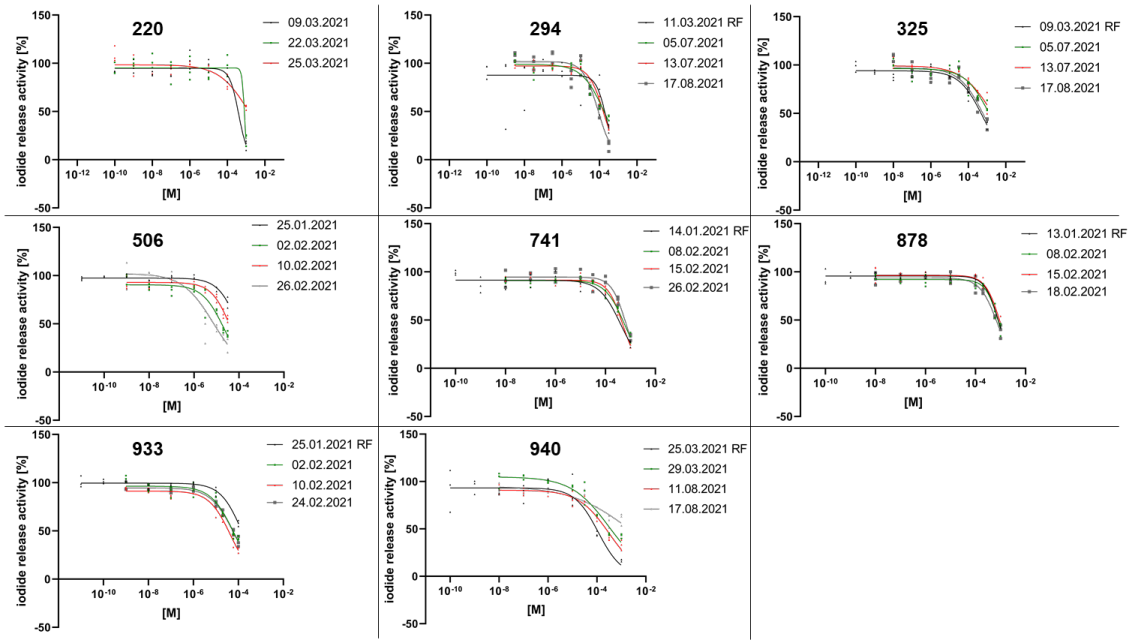

figure 8: results of the concentration-response testing in the DIO1-SK assay of part 2 testing for test items that lead to inhibition of iodide release activity (IRA) between 50 and 75%. At least three independent assay runs with three technical replicates per concentration and test were performed by three different laboratory staff members. Data is shown as mean iodide release ± SD of three technical replicates per concentration. A sigmoidal concentration-response curve was derived using the function "[Inhibitor] vs. response -- Variable slope (four parameters)" and constraining the bottom of the model to 0.

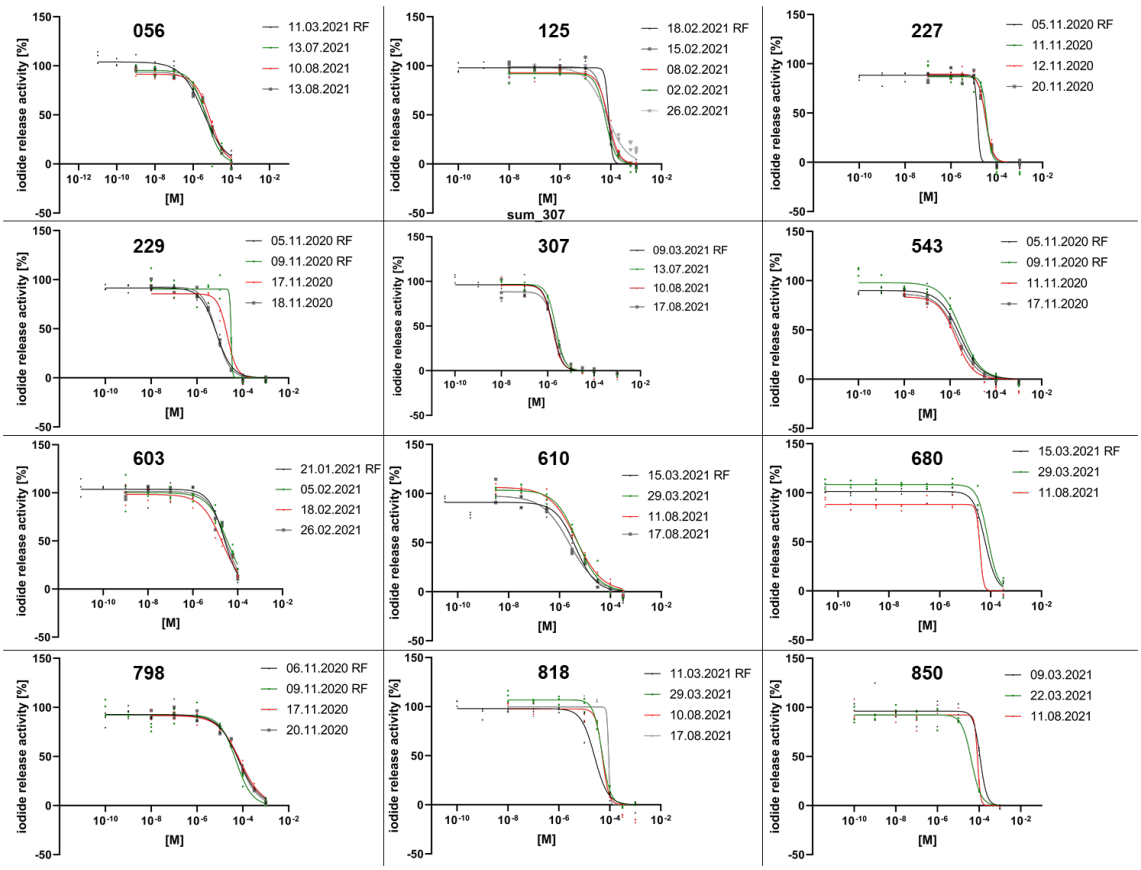

figure 9: results of the concentration-response testing in the DIO1-SK assay of part 2 testing for test items that lead to inhibition of iodide release activity (IRA) ≥75%. At least three independent assay runs with three technical replicates per concentration and test were performed by three different laboratory staff members. Data is shown as mean iodide release ± SD of three technical replicates per concentration. A sigmoidal concentration-response curve was derived using the function "[Inhibitor] vs. response -- Variable slope (four parameters)" and constraining the bottom of the model to 0.

Inhibition values varied heavily for test items leading to less than 50% inhibition. It is likely that the models can only derive imprecise parameters due to the missing data. As such, curve parameters and IC_{50} values were only derived for test items leading to at least 50% inhibition of IRA (table 35).

table 35: Summary of the concentration-response testing for the test items leading to a maximum inhibition ≥50% in the DIO1-SK assay of part 2 testing. The model "[Inhibitor] vs. response -- Variable slope (four parameters)" was used, the bottom of the model was constrained to 0, and curve parameters as well as concentrations causing 50% inhibition (IC_{50}) values were derived.

Identifier	mean IC_{50} [µM]	SD IC_{50} [µM]	median IC_{50} [µM]	mean Hill Slope	Max inhibition [%]	Highest tested conc [µM]
Test items leading to a max. inhibition greater 75%						
56	5.45	1.80	5.25	-0.97	97.02	100
125	72.41	8.47	69.24	-1.90	98.59	1000
227	29.09	9.86	32.76	-3.24	105.64	1000
229	16.60	11.67	14.22	-1.60	102.75	1000
307	1.89	0.29	1.85	-2.19	104.61	1000
543	2.42	0.58	2.39	-1.03	105.75	1000
603	26.91	6.08	26.78	-1.08	84.64	100
610	4.07	1.08	4.41	-0.94	105.50	316
680	58.65	19.62	60.44	-2.02	97.21	100
798	64.00	10.72	65.53	-1.03	95.38	1000
818	53.98	26.72	50.30	-2.53	103.98	1000
850	79.89	35.13	84.66	-2.29	101.31	1000
Test items leading to a max. inhibition between 50 and 75%						
220	897.67	538.00	820.70	-1.17	69.51	1000
294	165.33	59.73	174.30	-1.14	68.95	316
325	1161.00	669.73	1043.40	-0.65	51.21	1000
506	40.98	37.31	32.17	-0.88	50.40	31.6
741	533.28	106.66	537.70	-1.28	71.88	1000
878	864.68	88.19	872.40	-1.48	57.16	1000
933	79.14	44.07	63.80	-0.88	58.03	100
940	854.10	1222.81	310.20	-0.63	62.11	1000

9.2 DIO1-SK assay without microsome

Many substance classes can interfere with the SK reaction and need to be identified. Substances that lead to spontaneous iodide release or show activity in the SK reaction can be identified by testing in the DIO1-SK without microsomes present during incubation. The highest concentration of each test items (see table 32) was tested in the DIO1-SK assay according to 8.2.1. The control substances and the reference item were also included in this testing approach. One initial assay run was performed for all test items (figure 10, A). Every test item that led to an IRA increase over 10% without microsomes present, was retested until three independent, assay runs were performed. In addition, multiple runs were performed for substances that showed abnormalities during testing (figure 10, B). A threshold of 20% IRA increase without microsomes present was chosen to classify test items that are interfering with the SK reaction. These test items are termed "not applicable in the DIO1-SK assay". Of the 40 test items, three substances, 603, 615 and 940, were determined as active in the SK reaction itself and were termed as "not applicable in the DIO1-SK assay-

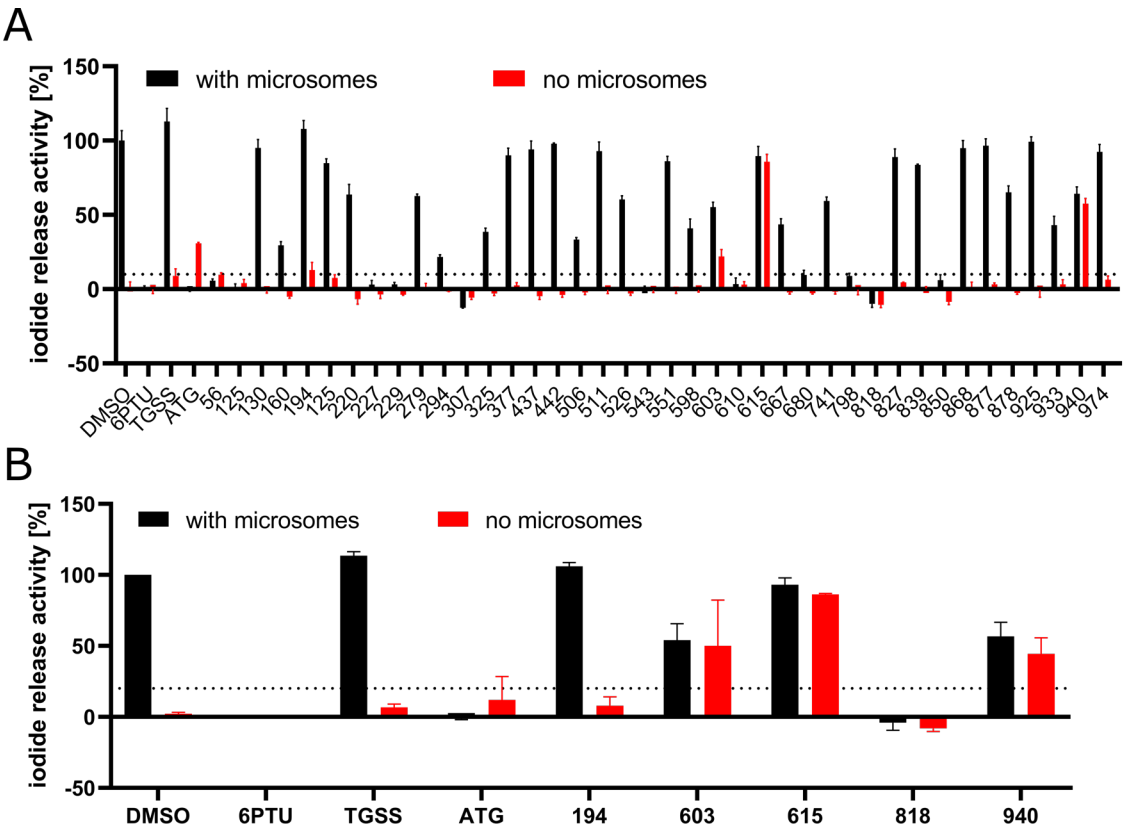

figure 10: DIO1-SK testing of part 2 test items with and without human liver microsomes. (A) All used test and control items of part 2 DIO1-SK testing were incubated with and without human liver microsome during microsome incubation, released iodide was separated using ion-exchange separation and quantified in the SK reaction. Data is shown as mean iodide release activity (IRA) values ± SD of three technical replicates per test item and positive (ATG) as well as negative control (TGSS), nine replicates for the reference item (PTU) and the solvent control (DMSO). The dashed line represents the 10% IRA threshold above which further repetitions must be made if the test item sample without microsomes exceeds the threshold. (B) Three independent assay runs with and without microsome were performed for each test item that exceeded the 10% threshold. Data is shown as the mean of the three assay run IRA values ± SD. The dashed line represents the 20% IRA threshold above which the test item was defined as “not applicable in DIO1-SK assay” since the test item itself was active in the Sandell-Kolthoff reaction.

DMSO: Dimethyl sulfoxide, 6PTU: 6-Propyl-2-thiouracil, TGSS: 1-Thio-β-D-glucose sodium salt; ATG: Aurothioglucose

9.3 ALP activity testing

Another potential unspecific effect that would lead to a virtual decrease in iodide release activity is unspecific binding or modification of the protein by the test item, ultimately leading to structural denaturation of the protein. Therefore, the activity of a secondary enzyme present in human microsomes was investigated as an indicator for unspecific protein interactions. The Alkaline Phosphatase (ALP) was chosen as secondary microsomal enzyme since ALP activity can be displayed by the formation of a yellow dye that can be quantified using a photometer.

The solubility of the 40 blinded test items under ALP testing conditions (10% of 10% test item dilution in water, 40% diH_2O and 50% ALP assay buffer) was assessed according to 8.2.2.3. No differences in the solubility were observed as compared to DIO1-SK solubility testing and the tested concentrations for ALP testing are the same as the defined highest tested concentrations in the DIO1-SK assay (see table 32).

All test items were tested regarding potential ALP inhibition as described in 8.2.2.8 and ALP activity values were derived according to 0, additionally deriving ALP activity values of 15- and 30-minute timepoints. Some test items (56, 307, 325, 511, 615, 741, 798, 839, 850, 974) led to an increase of the initial measured OD ($OD_{0min,415nm}$) that was higher than the solvent control. For example, the $OD_{0min,415nm}$ of test item 798 was 1.33, up to 20-fold higher than the initially measured OD of the solvent control. The ALP inhibition values of these test items often differ over time, indicating interference in the assay. One explanation could be the spontaneous formation of the coloured *para*-Nitrophenol (PNP) from *para*-Nitrophenol phosphate (PNPP) after addition of these test items. This would lead to a decreased availability of PNPP for the ALP reaction, that would impact the kinetics of the ALP reaction. An $OD_{0min,415nm} \geq 0.15$ was defined as a threshold above which ALP testing is not applicable for such test item. The value of 0.15 was chosen since it is twice the mean value of the $OD_{0min,415nm}$ of the solvent control and correlates with the observed differences in the ALP activity values as a function of time.

Every test item that led to inhibition greater 15% (corresponds to 85% ALP activity) in any of the three timepoints, was assessed in two additional, independent assay runs. Also, for test items with unusual kinetics a total of three independent assay runs was performed. The results of the ALP activity testing for all 40 test items are shown in table 36. The results of the test items that led to an inhibition greater 15% in any on the three timepoints, and whose $OD_{0min,415nm}$ was not greater or equal to 0.15, are shown in figure 11. Still, the test items 220 and 610 appear to lead to assay interference based on high variation of the ALP activity values as a function over time. Careful interpretation of the data is still necessary.

table 36: Alkaline Phosphatase (ALP) activity of the 40 test items of part 2 testing. ALP activity is shown as the mean of three replicates for test items with one performed assay run. For test items with two or more assay runs, data is shown as the mean of the mean of each assay run. The data was marked by colouring if the initially measured OD was above 0.15, and ALP testing is not applicable for such test item.

Test item	Tested concentration [M]	ALP activity [%]			# of independent assay runs
		15 min	30 min	60 min	
56	1.00E-04	55.76	61.71	64.65	3
125	1.00E-03	-3.44	-3.08	-2.94	2
130	1.00E-03	81.08	81.96	82.69	3
160	3.16E-05	104.60	107.19	109.32	1
194	1.00E-03	49.77	41.09	41.31	3
218	3.16E-04	83.84	85.20	86.44	3
220	1.00E-03	54.39	37.46	7.14	3
227	1.00E-03	103.89	102.48	103.36	1
229	1.00E-03	59.25	61.52	63.03	3
279	1.00E-04	95.78	96.90	96.44	1
294	3.16E-04	95.97	99.41	104.78	1
307	1.00E-03	215.60	154.07	116.86	3
325	1.00E-03	21.26	8.70	2.22	3
377	1.00E-04	94.26	94.99	94.73	1
437	1.00E-03	98.42	100.27	101.81	1
442	3.16E-05	4.86	2.88	2.64	3
506	3.16E-05	97.76	98.74	99.33	1
511	3.16E-04	42.88	69.41	90.44	1
526	1.00E-05	94.42	94.72	94.44	1
543	1.00E-03	85.93	85.19	86.37	1
551	1.00E-04	94.36	95.18	96.27	1
598	1.00E-03	105.47	106.74	107.11	1
603	1.00E-04	107.19	108.07	108.34	1
610	3.16E-04	163.86	112.41	67.66	3
615	1.00E-03	74.39	94.30	102.82	1
667	3.16E-04	55.81	58.17	59.23	3
680	1.00E-04	112.37	112.74	112.14	1
741	1.00E-03	116.69	111.63	109.93	1
798	1.00E-03	55.43	30.10	20.40	3
818	1.00E-03	91.65	94.74	99.02	1
827	1.00E-03	98.13	100.52	101.96	1
839	1.00E-04	114.53	117.63	118.02	1
850	1.00E-03	95.87	82.67	72.99	3
868	1.00E-06	95.23	95.70	96.11	1
877	1.00E-03	97.96	98.43	98.71	1
878	1.00E-03	82.60	83.79	84.77	3
925	1.00E-06	105.04	106.07	106.38	1
933	1.00E-04	104.60	106.30	106.87	1
940	1.00E-03	66.35	67.59	68.90	3
974	3.16E-04	44.44	52.90	58.13	3

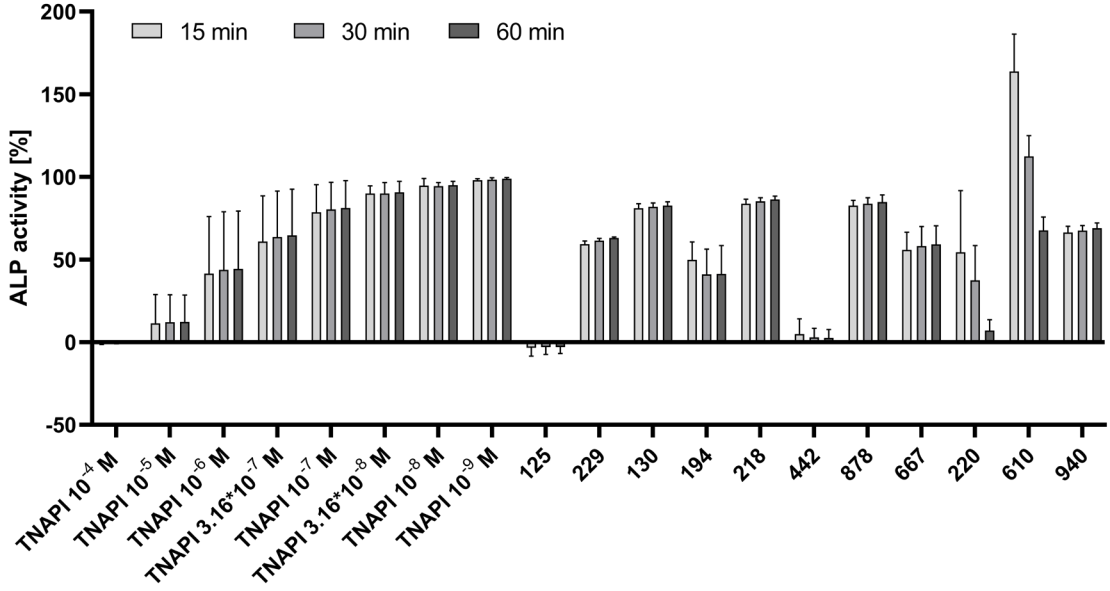

figure 11: Alkaline Phosphatase (ALP) activity after 15, 30 and 60 minutes of the ALP inhibiting test items as well as the reference item of ALP testing, the tissue-nonspecific alkaline phosphatase inhibitor (TNAPI). Only the data of the test items is shown that led to inhibition greater 15% in any on the three timepoints, and whose $OD_{0min,415nm}$ was not greater or equal to 0.15. Data is shown as the mean of all performed assay runs for each timepoint.

9.4 Summary of results

The results of the DIO1-SK assay and the two specificity testing approaches, testing with and without microsome and the ALP activity testing, are summarized for the set of 40 blinded test items of part 2 testing in table 37.

table 37: Summary of results of the DIO1-SK assay and testing for interference, including testing with and without microsome and Alkaline Phosphatase (ALP) activity testing, for the test items of part 2 and the reference item 6-Propyl-2-thiouracil (6PTU).

		DIO1-SK assay			**Assay interference in …?**	
Identifier	Highest tested concentration [µM]	Mean maximum inhibition [%]	Mean IC_{50} [µM]	Mean HillSlope	testing +/- microsome	ALP activity testing[a]
6PTU	1000	106.0	3.0	-0.92	-	-
056	100	97.0	5.5	-0.97	-	-[b]
125	1000	98.6	72.4	-1.90	-	yes
130	1000	6.3	-	-	-	-
160	31.6	48.9	-	-	-	-
194	1000	28.0	-	-	-	yes
218	316	19.0	-	-	-	-
220	1000	69.5	897.7	-1.17	-	-[b]
227	1000	105.6	29.1	-3.24	-	-
229	1000	102.8	16.6	-1.60	-	yes
279	100	47.5	-	-	-	-
294	316	69.0	165.3	-1.14	-	-
307	1000	104.6	1.9	-2.19	-	-[b]
325	1000	51.2	1161.0	-0.65	-	-[b]
377	100	12.3	-	-	-	-
437	1000	8.7	-	-	-	-
442	31.6	-1.0	-	-	-	yes
506	31.6	50.4	41.0	-0.88	-	-
511	316	12.8	-	-	-	-[b]
526	10	42.8	-	-	-	-
543	1000	105.8	2.4	-1.03	-	-
551	100	8.9	-	-	-	-
598	1000	19.6	-	-	-	-
603	100	84.6	26.9	-1.08	yes	-
610	316	105.5	4.1	-0.94	-	-[b]
615	1000	36.8	-	-	yes	-[b]
667	316	42.0	-	-	-	yes
680	100	97.2	58.7	-2.02	-	-
741	1000	71.9	533.3	-1.28	-	-[b]
798	1000	95.4	64.0	-1.03	-	-[b]
818	1000	104.0	54.0	-2.53	-	-
827	1000	16.2	-	-	-	-
839	100	31.5	-	-	-	-[b]
850	1000	101.3	79.9	-2.29	-	-[b]
868	1	6.2	-	-	-	-
877	1000	4.4	-	-	-	-
878	1000	57.2	864.7	-1.48	-	-
925	1	11.2	-	-	-	-
933	100	58.0	79.1	-0.88	-	-
940	1000	62.1	854.1	-0.63	yes	yes
974	316	11.9	-	-	-	-[b]

[a] A threshold of 20% inhibition of ALP activity after 60 min of incubation time was used to classify test item as ALP interfering

[b] These test items interfered with ALP activity testing and results were not used for evaluation

10 Conclusion

In this study, 40 blinded test items were tested in the DIO1-SK assay as well as two independent specificity testing strategies targeting different modes of potential interference in the method. The assay performed comparable to the part 1 testing and acceptance criteria were met consistently.
In the DIO1-SK assay, most of the test items produced reproducible concentration-response curves. Maximum inhibition values ranged from full inhibition to no inhibition.
The readout of the assay is based on a redox reaction. Many substances are known to interfere. As such, iodide containing substances cannot be tested in the method. Other ions like nitrite and ferrous (Sandell and Kolthoff 1937) but also copper, Hg^{2+} or thiocyanates (Shelor and Dasgupta 2011) are known to interfere with the SK reaction. The specificity testing using samples with and without microsomes investigates test-item induced interference with the method and revealed three test items that interfered with the method. The test item alone, without the presence of DIO1 (in the microsomes), was able to drive the SK reaction. The exact mechanism of these interferences was not elucidated in this study. These test items should be excluded from testing in the method since potential DIO1 inhibition cannot be differentiated from assay interference.
ALP activity testing was used as a secondary microsomal activity test to investigate potential unspecific test item/protein binding that could lead to structural and/or functional denaturation. Multiple test items led to constant inhibition values over time that were greater than 20%. Two test items that fully inhibited DIO1 activity, 125 and 229, also inhibited ALP activity by 102.9% and 37.0% after 60 minutes, respectively. These test items might bind to microsomes, leading to loss of activity in both readouts by an unspecific effect that is not specific to DIO1 inhibition. Careful interpretation of ALP results is still necessary.

Based on the results from this study, a testing strategy for the testing of unknown test items initially require a range finding assay run. The tested concentrations depend on the determined solubility of the test item under assay conditions. If the test item does not lead to inhibition greater 20% in the range finding assay run, two additional assay runs should be performed since the range finding assay run is counted towards the final set of three assay runs. If any of the concentrations lead to inhibition greater 20% in the range finding assay run of the DIO1-SK run, the following tests need to be performed: (i) three additional assay runs with adapted concentrations of the DIO1-SK assay have to be performed, (ii) the highest concentration of the test item is tested for assay interference by testing in the DIO1-SK assay with and without microsome present, and (iii) the highest concentration of the test item is tested in ALP activity testing. If the test item leads to activity in the SK reaction without microsomes present (ii), this test item is not applicable for this readout and defined as “not applicable in the DIO1-SK assay”. Inhibition in ALP activity testing might hint towards a nonspecific inhibition of microsomal activity that is not specific for the DIO1 MoA. Nevertheless, the interpretation of the ALP results remains difficult, since many test items react non-specifically and the impact on the microsome activity should be further elucidated.

The integration of the DIO1 inhibition data into a classification system is not part of this report and will be proposed in a scientific publication in a peer-review journal.

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

6PTU:	6-Propyl-2-thiouracil
95% C.I.:	95% Confidence interval
ALP	Alkaline phosphatase
ATG:	aurothioglucose
CV:	coefficient of variation
DEA	diethanolamine
diH_2O:	deionized water
DIO:	deiodinase
DIO1:	deiodinase I
DIO2:	deiodinase II
DIO3:	deiodinase III
DMSO:	dimethyl sulfoxide
DTT:	dithiothreitol
EDTA:	ethylenediaminetetraacetic acid
EURL ECVAM:	European Union Reference Laboratory for alternatives to animal testing
EU-NETVAL	European Union Network of Laboratories for the Validation of Alternative Methods
GIVIMP:	Guidance Document on Good In Vitro Method Practices
HCl	Hydrogen chloride
IC_{50}:	50% inhibition concentration
IRA:	iodide release activity
MoA	mode of action
NC	negative control
OD:	optical density
PC:	positive control
RI	reference item
rT3:	reverse T3, 3,3',5'-triiodothyronine
SC	solvent control
SD:	standard deviation
SK:	Sandell-Kolthoff
SOP:	standard operation procedure
TGSS:	1-Thio-β-D-glucose sodium salt
TNAP	tissue-nonspecific alkaline phosphatase

13 Literature

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14 Appendix

14.1 SOP DIO1-SK assay (29.09.2020)

Standard Operation Procedure (SOP)

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

AUTHOR

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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.

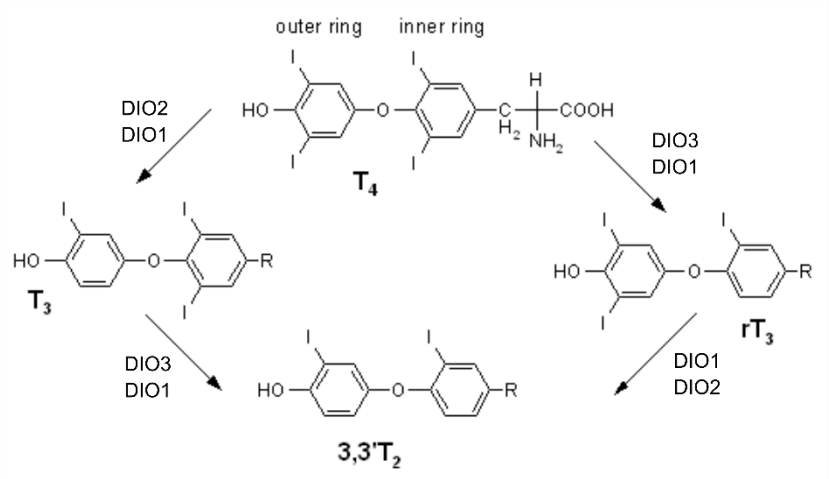

Figure 1: Metabolism of thyroid hormone

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.

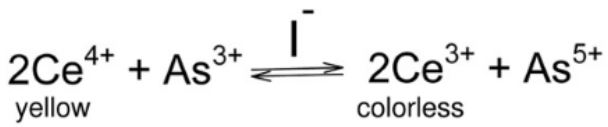

Figure 2: Sandell-Kolthoff-reaction

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.

1.2. REFERENCES

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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 SOP DIO1-SK assay (13.04.2022)

Standard Operation Procedure (SOP)

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

Date: 13.04.2022

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.

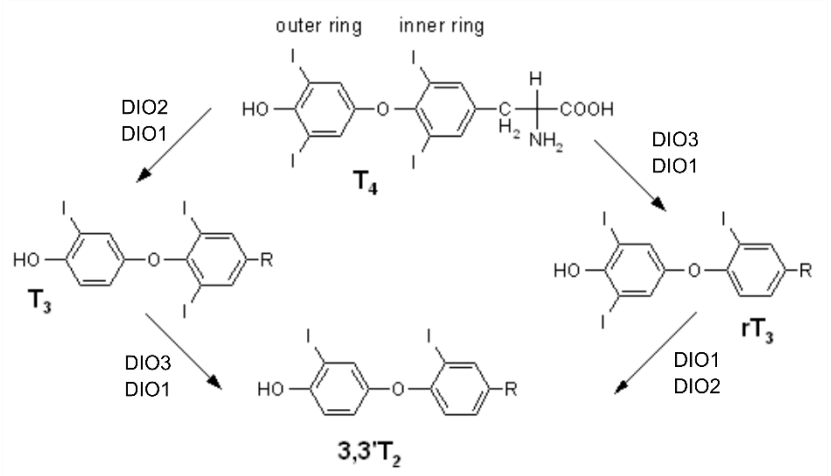

Figure 1: Metabolism of thyroid hormone

This method is based on the method originally published by Renko et al. using murine liver microsomes as DIO1 enzyme source (Renko, Hoefig et al. 2012). It uses the "Sandell-Kolthoff-reaction", a nonradioactive, colorimetric reaction, which can be used to measure free iodide concentration (Sandell and Kolthoff 1937). 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.

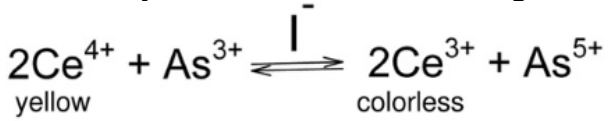

Figure 2: Sandell-Kolthoff-reaction

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.

Here, microsomes, broken-down, vesicle-like pieces of endoplasmic reticula from human hepatocytes are used as an enzyme source for DIO, mainly DIO1. The microsomes possess also other 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, Stresser et al. 2016). This might lead to different inhibition properties compared to assays using purified DIO1 enzyme.

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. Generated samples need to be diluted in 10% acetic acid to fit in the linear quantification range of the SK-reaction. 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.

Several ions and molecules are known to directly interact with the reaction like nitrite and ferrous ions (Sandell and Kolthoff 1937) as well as copper, chromium, Ni^{2+}, Hg^{2+}, Al^{2+} or thiocyanate ions (Shelor and Dasgupta 2011). Also, iodide containing compounds can drive the SK reaction itself (Baudry, Mallet et al. 1997). As such, test items that lead to inhibition ≥20% in the range finding assay run of the DIO1-SK assay, are tested for potential interference in the SK reaction. These test items are tested in a similar setup compared to the DIO1-SK assay, but without microsome during incubation phase. Test items that still lead to activity in the SK reaction without microsome present, interfere with the assay and cannot be tested in the DIO1-SK assay.

The testing of a secondary protein present in human liver microsomes that is independent from DIO1 activity can provide information about the functionality of microsomes. This testing follows the consideration of cytotoxicity testing in cell-based assays as an indicator for interaction that are not based on the specific MoA of interest. If the secondary analysed enzyme is affected in addition to the DIO1 readout, this hints towards unspecific interaction of the test item with the microsomal proteins, ultimately

leading to loss of activity. Here, the Alkaline Phosphatase (ALP) is used to test for unspecific protein interaction. Analog to testing for SK interference, inhibition of DIO1 activity ≥20% in the range finding assay of the DIO1-SK assay triggers the testing for ALP inhibition. Testing for ALP activity is performed according to SOP: ALP activity testing".

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.

1.2. REFERENCES

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1.3. SOP CHANGES

The following changes were integrated into this SOP version (20220330_SOP DIO1-SK assay) compared to the previous version (20200923_SOP DIO1-SK assay).

table 1: major changes to previous SOP version of the DIO1-SK assay.

Section	Topic	Changes
1.1. Background and Objective	Adapted “introduction”	Added information about range finding procedure, assay interference and specificity testing
1.3. SOP changes	Added “SOP changes”	Includes information about changes in the SOP compared to previous versions
3.1. Controls	Expanded section “controls”	Information about the background of control choices and overall information about the controls were added
5.1. Range finding assay	Expanded section “range finding assay”	Information about the procedure of concentration selection for the range finding assay was added/clarified.
6.3.3. Preparation of a stock solution and dilution of the reference item	Adapted concentrations for the reference item-response curve	The concentrations for the reference item-response curved were adapted to include more concentrations in the dynamic range of inhibition
6.6. Evaluation of the data	Data evaluation was expanded	The evaluation of the data was changed to include formulas for the derivation of descriptive values
6.7.1. Acceptance criteria	Set of acceptance criteria was expanded	The acceptance criteria that were defined in part 1 (Birk and Weber 2020) and the respective calculations were added to the SOP.
7. Identification of potential unspecific assay interferencea	Added chapter about testing for potential unspecific assay interference	A chapter about specificity testing was added to the SOP including testing for interference with the SK reaction which is tested in the DIO1-SK assay with and without microsomes. A secondary enzyme activity test using the Alkaline Phosphatase (ALP) is performed according to SOP “SOP: ALP activity testing” and was referenced.
-	Changed elution by applying vacuum to centrifugation	All elution protocols that included the application of vacuum was changed to elution by centrifugation.
-	Added multiple comments on usual observed performance of the method	Sections with practical advice on method procedure were added (e.g., 6.3.1 Casting of ion exchange resin-filled 96-well filter plate)
-	Allowed the use of different cerium concentrations for the SK reaction	The usage of different concentrated cerium solutions for the SK reaction was added since higher cerium concentrations increase the reducible OD

2. MATERIALS AND METHODS

Table 2: 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 3: 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, Sigma-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] or 3,3',5'-Triiodo-L-thyronine, Cayman[2] or 3,3′,5′-Triiodo-L-thyronine, Santa Cruz Biotechnology[2]
6-Propyl-2-thiouracil (6PTU) CAS: 51-52-5 MW: 170.23 g/mol	e.g. 6-Propyl-2-thiouracil, VETRANAL™, analytical standard, Supelco[2]

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]
Ion exchange resin like Dowex 50WX2 CAS: 12612-37-2	e.g. Dowex 50WX2 100 200 mesh ion exchange resin, Acros Organics[2] or AmberChrom® 50WX2 hydrogen form, 100-200 mesh, Supelco[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] or INVITROCYP 150-Donor Human Liver Microsomes, BIOIVT[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	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]

MW: 136.09 g/mol	
Sodium chloride (NaCl) CAS: 7647-14-5 MW: 58.44 g/mol	e.g. sodium chloride, ACS reagent, ≥99.0%, Sigma-Aldrich[2]
Sulfuric acid (H_2SO_4) CAS: 7664-93-9 MW: 98.08 g/mol	e.g. sulfuric acid, Supelco[2]

Table 4: 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] or Micro-Plates, 96-well, clear polystyrene, 800 µL, DNA Binding, Whatman[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 5: 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, a reference item as well as a positive and negative control is used. Also, solvent controls for all solvents that are used to solubilize tested controls and test items are performed in all the experiments (Table 6).

Table 6: Used control setup and control items in the DIO1-SK assay in accordance with GIVIMP.

Control item	**Function**
Reference item (RI)	Quantitative control. The inhibition observed with the test item is normalized to maximum inhibition obtained with the highest concentration of the reference item. The highest concentration of the reference item is tested on each assay plate and a concentration-response curve of the reference item is performed on each assay day. Used in this method: 6-Propyl-2-thiouracil (6PTU) at a maximum assay concentration of 10^{-3} M. Concentration-response curves of 6PTU ranged from 10^{-3} to 10^{-8} M.
Positive control (PC)	Qualitative control. The highest concentration of the positive control is tested on each assay plate. Used in this method: Aurothioglucose (ATG) at a maximum assay concentration of 10^{-4} M.
Negative control (NC)	Control not inhibiting DIO1 activity. The highest concentration of the negative control is tested on each assay plate. Used in this method: 1-Thio-β-D-glucose sodium salt (TGSS) at a maximum assay concentration of 10^{-4} M.
Solvent control (SC)	Blank control. The solvent control ensures that the response does not originate from the applied solvent and shows no DIO1 inhibition. Solvent controls are used to normalize the inhibition observed with the test items to the maximum possible DIO1 activity. Used in this method: Dimethyl sulfoxide (DMSO) at a concentration of 1%.

3.1.1. Reference item

The reference item 6-Propyl-2-thiouracil (6PTU) is a known and well described DIO1 inhibitor (Visser, Van Overmeeren et al. 1979, Renko, Hoefig et al. 2012, Olker, Korte et al. 2018) and is used in this assay as a normalization step to subtract background signal from the generated data (Table 7). 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 using concentration-response testing 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 concentration-response testing. Repeat the generation of a 6PTU IC_{50} through concentration-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 7: 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

The gold-containing Aurothioglucose (ATG) inhibits all three DIO isoforms (Renko, Schäche et al. 2015, Weber, Birk et al. 2021) based on the affinity of its gold ligand to the selenocysteine-containing catalytic center of DIO (Kuiper, Kester et al. 2005). ATG is used as a positive control in this method (see Table 8), qualitatively controlling DIO1 inhibition and is performed in replicates as a single concentration on each assay plate.

Table 8: 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

1-Thio-β-D-glucose sodium salt (TGSS) is a structural analogue of ATG lacking the DIO1 inhibiting gold ligand showing no DIO1 inhibition in the method (Berry, Banu et al. 1991, Weber, Birk et al. 2021). TGSS is used as a negative control in the method (see Table 9), controlling maximum performance of the method. Replicates are performed in singular concentrations on each assay plate.

Table 9: 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 10. 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 10: 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 concentration-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 used as one of the three final assay runs.

5.1. RANGE FINDING ASSAY

Test concentrations of the range finding assay were based on the highest solubility determined under assay conditions; following test concentrations were prepared by consecutive 10-fold (v/v) dilution steps (covered in section 6.3.2).

5.2. ASSAY RUNS

1. If the test item leads to DIO1 inhibition greater or equal to 20% in any of the tested concentrations 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 DIO1 inhibition of the test item is less than 20% in any of the tested concentration in the range finding assay: Repeat the assay runs with the proposed test item dilutions from the range finding assay.

6. DIO1-SK ASSAY

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 11: 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 diH_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 diH_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).
Aliquoting of 1 M DTT	Aliquot a prepared or supplied 1 M DTT solution in H_2O as 0.5 mL aliquots into 1.5 mL microcentrifuge tubes and store at -20°C.
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 centrifuge tubes (“substrate mix tubes”) with aliquoted rT3	Carefully thaw one 100 µL 15 mM rT3 aliquot on ice. Add 4 µL of 15 mM rT3 to 15 mL centrifuge tubes and store 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)	Add 3.95 g of $(NH_4)_4Ce(SO_4)_4{\cdot}2H_2O$ and 125 mL of diH_2O to a 250 mL volumetric flask. Add 125 mL 1 M H_2SO_4 to reach a final volume of 250 mL.
Acidic ammonium cerium solution (40 mM $(NH_4)_4Ce(SO_4)_4{\cdot}2H_2O$, 0.5 M H_2SO_4) (250 mL)	Add 6.32 g of $(NH_4)_4Ce(SO_4)_4{\cdot}2H_2O$ and 125 mL of diH_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 diH_2O to a 250 mL volumetric flask. Add 125 mL 1 M H_2SO_4 to reach a final volume of 250 mL.

Table 12: 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 thawed 1 M DTT aliquot to the 4 µl of 15 mM rT3 containing substrate mix tubes. Mix and use the substrate mix on the day of preparation.

6.3. PRE-ASSAY WORK

6.3.1. Casting of ion exchange resin-filled 96-well filter plate

It is recommended to prepare a larger quantity of ion exchange resin-filled 96-well filter plates 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 ion exchange 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 ion exchange resin to a large beaker and wash with 10% acetic acid. Use a big shaker to mix the ion exchange resin with the acetic acid, let it rest afterwards for 10 min and remove the supernatant
2. Wash by addition of 10% acetic acid, let the resin suspension rest for 10 min and remove the supernatant until no more colour is leaking into the solvent (at least 5x in total)[a]
3. Place a 96-well filter plate on top of a used 96-deep well plate
4. Add 100 µL of acetic acid (10%) into each well of the 96-well filter plate
5. Cut 1 mL tips to widen the opening and cast 600 µL ion exchange resin into each well of the 96-well filter plate
6. Add another 150 µL acetic acid (10%) to each well of the 96-well filter plate and elute the acetic acid by centrifuging into the used 96-deep well-plate with 70xg in a centrifuge with swing-out rotor for microtiter plates for 1 min[b]
7. Repeat the step 6 if colour is still leaking in any of the wells
8. Seal the plate with an impermeable sheet of plastic and store at 4°C for a maximum of 2 months

[a] Ion exchange resin constituents are known to affect the SK reaction if not washed out properly. The removed supernatant can be used directly in the SK reaction (see 6.3.3; use 50 µl supernatant sample, add 50 µl cerium and finally 50 µl arsenite solution) and can be compared against a 10% acetic acid sample (blank control) to ensure complete washing out of the components.

[b] Some 96-well filter plate require stronger centrifugation to elute all liquid. Generally, 200xg was sufficient for full elution in all used 96-well filter plates, before and after addition of ion exchange resin.

Comments on filling of filter plates with ion exchange resin:

Different 96-well filter plates allow different volumes of ion exchange resin filling. In principle, the highest possible filling volume of resin suspension should be used since higher resin filling volume help to minimize potential background increases in the SK reaction; keep in mind that in the ion exchange separation step 175 µl liquid (75 µl microsomal incubation sample + 100 µl 10% acetic acid) is added, which must be prevented from spilling over into the neighbouring wells. With the usually used 96-well filter plates (UNIFILTER), 600 µl resin suspension was the maximum possible filling volume that filled up the wells while not spilling over into neighbouring wells.

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). If 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. In this case, 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 diH_2O and check if they are still fully dissolved.

7. Prepare a 10% test item dilution in diH_2O by diluting the highest soluble test item stock solution (generated in step 1 to 6) in diH_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 % diH_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 (2.16 mM EDTA; pH 6.8; as prepared in 6.2, Table 12), 40 % diH_2O 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 7).

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 13.
5. Label the subsequent reference item dilutions derived from the reference item stock solution adequately (e.g. RI-D1, RI-D2,…., RI-D8).

Table 13: Preparation of the dilutions for the reference item 6-Propyl-2-thioruacil.

Name of the reference item dilution	Reference item dilution concentration [M]	diH_2O [µL]	DMSO [µL]	Reference item	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	$3.16*10^{-5}$	307.8*	34.2*	158 µL of RI-D3	$3.16*10^{-6}$
RI-D5	10^{-5}	405*	45*	50 µL of RI-D3	10^{-6}
RI-D6	$3.16*10^{-6}$	405*	45*	50 µL of RI-D4	$3.16*10^{-7}$
RI-D7	10^{-6}	405*	45*	50 µL of RI-D5	10^{-7}
RI-D8	10^{-7}	405*	45*	50 µL of RI-D7	10^{-8}

*You can also prepare a 10% DMSO / diH_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 8).

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 2 mL of a 10^{-2} M stock solution of Aurothioglucose in DMSO with a molecular weight of 392.18 g/mol, 7.8 mg of the substance was weighed into a vessel and solved in 2 mL of DMSO.

$$m = c * V * M = 0.01 \frac{mol}{l} * 0.002l * 392.18 \frac{g}{mol} = 0.078g = 7.8mg$$

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

Table 14: Preparation of the positive control Aurothioglucose dilution.

Positive control dilution [M]	diH_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 9).

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 15.

Table 15: Preparation of the negative control 1-Thio-β-D-glucose sodium salt dilution.

Negative control dilution [M]	diH_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 for a range finding assay, the test item stock solution is subsequently diluted seven times with diH_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 16. 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. If the concentrations were adapted based on an observed response in the range finding assay, prepare test item dilutions accordingly.

Table 16: Preparation of the test item dilutions for a range finding assay using test item stock solutions of the test item 1.

Name of the test item dilution	diH_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 / diH_2O solution and add 450 µL of the dilution

6.3.7. Preparation of the human microsome dilutions

Careful: The preparation of microsome dilutions must only be carried out if the iodide release activity of a novel microsome has to be determined!

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 diH_2O as shown in Table 17. 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, modify 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 17: Preparation of the human liver microsome dilutions for the testing of iodide release activity.

Microsome per well [µg]	diH_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 using a respective iodide standard in diH_2O. The iodide 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 diH_2O with three replicates to the plate. A recommended plate layout is shown in Table 18.

Table 18: 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^-			diH_2O only								
E												
F												
G												
H												

iodide dilutions **diH_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 to 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_{21min} from the initially measured OD_{0min} 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 (logarithmic) *Some of the high iodide concentrations might need to be excluded from analysis since ΔOD is virtually decreasing based on reaction that occurs before the initial measurement step. This can be outlined by the OD_{0min} which is then greatly reduced compared to samples with lower iodide concentration.*
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 diH_2O samples in the Sandell-Kolthoff reaction in a historical database

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 0 and 21 min of SK reaction, 25 mM Ce solution [25 mM $(NH_4)_4Ce(SO_4)_4{*}2H_2O$; 0.5 M H_2SO_4]

Pure diH_2O sample: Usually a background of ΔOD > 0.3 in the pure diH_2O control would need attention and further investigation of underlying causes (e.g., contamination in the As or Ce-batch, low water quality).

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

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 ion exchange 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 first reference item dilution of 6PTU (RI-D1) 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 12: “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 a 10% (v/v) solvent dilution in diH_2O (e.g., 10% DMSO in diH_2O). Keeping a final assay concentration of 1 % solvent in all samples is recommended. A proposed plate layout is shown in Table 19
3. Add 40 µL of microsome dilutions in diH_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 19: 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 ion exchange analogous to 6.5.2.
9. Conduct the measurement of the Sandell-Kolthoff reaction analogous to 6.5.3 with the following deviations:
   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; if the generated activity curve in the highest 10% acetic acid dilution is still not in a linear range, higher dilutions in acetic acid can be performed; also see Table 20)

Table 20: Added sample solution and 10% acetic acid in the SK reaction for different dilutions

Dilution	**Sample solution [µL]**	**10% acetic acid [µL]**
Undiluted	50	-
1:2	25	25
1:4	12.5	37.5
1:8	6.3	42.7

10. Determine the ΔOD by subtracting the OD_{21min} from the initial measured OD_{0min}
11. To determine the ΔOD-BG values, subtract the inhibited ΔOD of the reference item from each enzyme concentration from the solvent control ΔOD of the respective enzyme concentration
12. Plot the ΔOD-BG values of the different enzyme concentration samples in a statistics software with ΔOD-BG on y-axis (linear) and protein 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 as well as the enzyme concentration that still leads to the highest possible ΔOD-BG values in the Sandell-Kolthoff reaction without reaching a plateau .
    Use these values for every following measurement of the same microsome batch in the DIO1-SK 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 0 and 21 min in the SK reaction.

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 requires an initial range finding assay. The range finding assay is conducted with the proposed test item dilutions according to6.3.6. 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 a reference item (6PTU) concentration-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 ion exchange 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. Prepare the substrate mix as described in Table 11. Prepare a microsome suspension in the defined concentration per well (see 6.4.2) by diluting carefully thawed, aliquoted microsome solution in diH_2O. Keep the microsome suspension on ice until needed for incubation.
3. Add 10 µL of the reference item dilutions to a 96-well plate; on the first plate of an assay day also add the reference item dilutions for the concentration-response. For the solvent control add 10 µL of 10% (v/v) DMSO (in diH_2O) 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 21; a proposed plate layout for additional runs on the same assay day is shown in Table 22.
4. Add 40 µL of the defined microsome suspension to the wells
5. On ice, add 50 µL of the freshly prepared substrate mix to the samples
6. Seal the plate with an impermeable sheet of plastic
7. The 96-well plate is then placed on a shaker in an incubator (37°C at 600 rpm) and is incubated for 2 h
8. Place on ice to stop the reaction

Table 21: plate layout for the first plate of an assay day for a range finding / assay run of the DIO1-SK. The identifiers of the prepared dilutions of the reference item in 6.3.3 and the test item(s) in 6.3.6 correspond to the identifier in the plate layout (RI-D1 -> RI-C1, TI1-D1 -> TI1-C1).

	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} to 10^{-8} 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 22: plate layout for additional plates of an assay day for a range finding / assay run of the DIO1-SK. The identifiers of the prepared dilutions of the reference item in 6.3.3 and the test item(s) in 6.3.6 correspond to the identifier in the plate layout (RI-D1 -> RI-C1, TI1-D1 -> TI1-C1).

	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 ion exchange resin-filled 96-well filter plate

1. Put a prepared ion exchange 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 ion exchange 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 70xg in a centrifuge with swing-out rotor for microtiter plates for 1 min[a]
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 ion exchange resin-filled 96-well filter plate maintaining the initial plate layout
6. Add 100 µL of 10% acetic acid to each well of the ion exchange resin-filled 96-well filter plate
7. Elute the samples by centrifuging into the 96-deep well-plate with 70xg in a centrifuge with swing-out rotor for microtiter plates for 1 min and remove the ion exchange resin-filled 96-well filter plate[a]
   The 96-deep well-plate with samples can be sealed with an impermeable sheet of plastic and stored at 4°C for at least 3 months. This allows additional measurements in case of manual / technical errors or changes

of the dilution factor in the Sandell-Kolthoff reaction or measurement on the following days.

[a] Some 96-well filter plate require stronger centrifugation to elute all liquid. Generally, 200xg was sufficient for full elution in all used 96-well filter plates.

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 to the samples in the 96-well plate
    a. 25 mM Cerium solution [25 mM $(NH_4)_4Ce(SO_4)_4{*}2H_2O$; 0.5 M H_2SO_4] : 25 mM Cerium is generally sufficient
    b. 40 mM Cerium solution [40 mM $(NH_4)_4Ce(SO_4)_4{*}2H_2O$; 0.5 M H_2SO_4]: higher cerium concentrations can be used to increase the reducible colorimetric range, e.g. in case of high background activity in the SK reaction; inhibition values are not affected by increased cerium concentration
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_{21min} values via subtraction of the 21-minute values of all samples from the initial measured values:

   $$\Delta OD_{21min} = OD_{21min,415nm} - OD_{0min,415nm}$$

2. Determine the ΔOD-BG values, by subtracting the mean of ΔOD_{21min} values of the inhibited 10^{-3} M 6PTU controls from the ΔOD_{21min} values of all samples:

   $$\Delta OD\text{-}BG = \Delta OD_{21min} - \overline{\Delta OD_{21min,RI}}$$

   Where “RI” represents the reference item 6PTU

3. Normalize the values of the test item to the respective solvent control values via division of the test item(s) ΔOD-BG values by the mean of the ΔOD-BG values of the respective solvent control, generating iodide release activity (IRA) values. State the IRA values in %. Keep in mind that test items with differing solvents need to be normalized to their respective solvent controls:

   $$iodide\ release\ activity\ (IRA) = \frac{\Delta OD\text{-}BG_{TI}}{\Delta OD\text{-}BG_{SC}} * 100$$

   where “TI” represents the test item at used concentrations and “SC” the solvent control

4. Plot the IRA values of the different test item concentration samples in a statistics software with IRA values on y-axis (linear) and test item concentration on x-axis (logarithmic)
5. Use a curve-fit algorithm to visualize a concentration-response relationship (e.g. “[Inhibitor] vs. response -- Variable slope (four parameters)” in GraphPad Prism 8):

   $Y = Bottom + (Top - Bottom) / (1 + (IC_{50} / X)^{HillSlope})$

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

6. If applicable, determine the IC_{50} of the test item

6.7. ASSESSING VALIDITY OF RUNS

6.7.1. Acceptance criteria

Different acceptance 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 acceptance criteria are met (Table 23). Calculation of the acceptance criteria is described in Table 24. If an assay run is classified as non-valid, the assay run would have to be repeated.

Table 23: Used acceptance criteria in the DIO1-SK assay to determine valid assay runs.

Acceptance criteria	**Suggested 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	
Shape of reference item (sigmoidal?)	x = yes
The final concentration-response curve of the reference item is composed of minimum six concentrations from three replicates	x = yes
The final concentration-response curve of the test item is composed of minimum six concentrations from three replicates	x = yes

Table 24: Calculation of derived acceptance criteria in the DIO1-SK assay

Acceptance criteria	Calculation
IC_{50} of reference item [µM]	Derived in 6.6
CV of log IC_{50} estimate of reference item [%]	$= \left(\frac{\sigma_{log\ IC_{50}}}{log\ IC_{50}}\right) * 100$
Iodide release activity of negative control [%]	$= \left(\frac{\overline{IRA_{NC}}}{\overline{IRA_{SC}}}\right) * 100$
Iodide release activity of positive control [%]	$= \left(\frac{\overline{IRA_{PC}}}{\overline{IRA_{SC}}}\right) * 100$
z'-factor	$= 1 - \frac{3 * \left(\sigma_{\Delta OD_{21min,RI}} + \sigma_{\Delta OD_{21min,SC}}\right)}{\left\lvert \mu_{\Delta OD_{21min,RI}} - \mu_{\Delta OD_{21min,SC}} \right\rvert}$

Where "σ" represents standard deviation, "µ" represents mean, "NC" represents negative control, "SC" represents solvent control, "PC" represents positive control, and "RI" represents reference item.

7. IDENTIFICATION OF POTENTIAL UNSPECIFIC ASSAY INTERFERENCE

Testing of test items in the DIO1-SK assay in incubations without microsome present is used in this method to investigate for SK interference. If the test item is active in the SK reaction without microsomes present, the test item cannot be tested in the DIO1-SK assay and is termed as "non-applicable for DIO1-SK assay".

Test items must only be tested in the identification of unspecific SK interference, if the range finding assay run in the DIO1-SK assay led to an IRA reduction of more or equal than 20%.

The test item is initially tested in an initial assay run using only the highest concentration of the test item. Two additional, independent assay runs with the highest test item concentration are performed if the highest tested concentration without microsomes leads to a mean IRA greater than 10% in the initial assay run. A threshold of 20% mean IRA increase without microsomes over all three assay runs is used to classify test items that are interfering with the SK reaction. These test items are termed "not applicable in the DIO1-SK assay".

A secondary strategy to investigate unspecific interaction with the used microsomal protein uses activity testing of a secondary enzyme that is present in human liver microsomes. Here, activity testing of the Alkaline Phosphatase (ALP) was used to check for unspecific protein interactions. This would lead to structural denaturation that would be outlined by inhibition of DIO1 as well as the ALP. The performance of ALP activity testing is described in the SOP “SOP: ALP activity testing”.

7.1. INCUBATIONS WITH AND WITHOUT MICROSOME

1. Prepare the reference item stock solution as well as the RI-C1 dilution as described in 6.3.3, and the test item stock solutions as well as dilutions with the highest test item concentration (TIX-D1) described in 6.3.6.
2. Prepare the substrate mix as described in Table 11. Prepare a microsome suspension in the defined concentration per well (see 6.4.2) by diluting carefully thawed, aliquoted microsome solution in diH_2O. Keep the microsome suspension on ice until needed for incubation.
3. Add 10 µL of the reference item dilution RI-C1 to a 96-well plate. For the solvent control, add 10 µL of 10% (v/v) DMSO (in diH_2O) solution. Add 10 µL of the test item dilution(s) to the 96-well plate. The plate layout for the testing for unspecific SK interference is shown in Table 25.
4. Add 40 µL of the defined microsome suspension to the wells
5. On ice, add 50 µL of the freshly prepared substrate mix to the samples
6. Seal the plate with an impermeable sheet of plastic
7. The 96-well plate is then placed on a shaker in an incubator (37°C at 600 rpm) and is incubated for 2 h
8. Place on ice to stop the reaction

Table 25: plate layout for the identification of unspecific SK interference by test items in the DIO1-SK assay.

	1	2	3	4	5	6	7	8	9	10	11	12
A	**SC**			SC			**RI-C1**			RI-C1		
B	**TI1-C1**			TI1-C1			**TI2-C1**			TI2-C1		
C	**TI3-C1**			TI3-C1			**TI4-C1**			TI4-C1		
D	**TI5-C1**			TI5-C1			**TI6-C1**			TI6-C1		
E	**TI7-C1**			TI7-C1			**TI8-C1**			TI8-C1		
F	**TI9-C1**			TI9-C1			**TI10-C1**			TI10-C1		
G	**TI11-C1**			TI11-C1			**TI12-C1**			TI12-C1		
H	**TI13-C1**			TI13-C1			**TI14-C1**			TI14-C1		
	with microsomes			no microsomes (diH_2O only)			**with microsomes**			no microsomes (diH_2O only)		

SC **solvent control** | **RI-C1** **reference item** 6-Propyl-2-thiouracil 10^{-3} M | **TI** **test item**

9. Perform the ion-exchange separation and iodide quantification via the SK reaction as described in 6.5.2 and 6.5.3.
10. Derive IRA values as described in 6.6 with few adjustments: generate ΔOD-BG values for samples with or without microsomes by subtracting the mean of the respective RI-C1 control with or without microsomes; generate the IRA values via division of the item(s) ΔOD-BG by the mean of the ΔOD-BG of the solvent control with microsomes.
11. Using statistics software, plot the IRA values of each test item for each condition with IRA values on y-axis (linear) and test item identifier on x-axis in a grouped bar chart.
12. A threshold of IRA increases ≥ 20% in the samples without microsome present during incubation in the highest test item concentration, is used to classify test items that are interfering in the SK reaction. These test items are termed “not applicable in the DIO1-SK assay” and need to be excluded from analysis.

14.3 SOP ALP activity testing

Standard Operation Procedure (SOP)

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

-

Assessing specificity of DIO1 interaction using Alkaline phosphatase (ALP) testing as secondary readout

AUTHOR

BASF SE

1. INTRODUCTION

1.1. BACKGROUND AND OBJECTIVE

The alkaline phosphatase (ALP) is a homodimeric enzyme, containing two Zn and one Mg ion on each catalytic site, which are crucial for catalytic function. ALP catalyses the dephosphorylation of compounds and has important functions in bone mineralization and intestinal absorption. Important human isoforms are the tissue-nonspecific alkaline phosphatase (TNAP), the placental alkaline phosphatase, Germ cell alkaline phosphatase, and the germ cell alkaline phosphatase (GCALP). TNAP is expressed in the developing nervous system, skeletal tissues, kidney, and the liver (Millán 2006).

Alkaline phosphatase activity is usually monitored by the formation of yellow *para*-nitrophenol from *para*-nitrophenylphosphate (PNPP) as a function of alkaline phosphatase-mediated dephosphorylation activity with an absorption maximum of 405 nm.

TNAP activity is also present in human liver microsomes and is used in this method as a secondary enzyme activity testing for the DIO1-SK assay. Inhibition in both the DIO1-SK assay as well as on ALP activity might indicate towards unspecific inhibition, for example due to unspecific protein binding or modification.

1.2. REFERENCES

Dahl, R., E. A. Sergienko, Y. Su, Y. S. Mostofi, L. Yang, A. M. Simao, S. Narisawa, B. Brown, A. Mangravita-Novo and M. Vicchiarelli (2009). "Discovery and validation of a series of aryl sulfonamides as selective inhibitors of tissue-nonspecific alkaline phosphatase (TNAP)." Journal of medicinal chemistry **52**(21): 6919-6925.

Millán, J. L. (2006). "Alkaline phosphatases." Purinergic signalling **2**(2): 335-341.

2. MATERIAL

Table 1: Used apparatus

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	The photometer used must be able to heat up to 37°C[1] e.g., Sunrise™ Absorbance Reader, INSTSUN-3, Tecan Trading AG[2]

Table 2: Used chemicals and reagents in ALP activity testing.

Chemicals / reagents	Requirements[1] Suggested type[2]
6-Propyl-2-thiouracil (6PTU) CAS: 51-52-5 MW: 170.23 g/mol	e.g. 6-Propyl-2-thiouracil, VETRANAL™, analytical standard, Supelco[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[2o]
Human liver microsomes	e.g. Human Microsomes, 50 Donors, HMMCPL, Gibco[2] or Microsomes from Liver, Pooled, from human, Sigma-Aldrich[2]
Diethanolamine (DEA) CAS: 111-42-2 MW: 105.14 g/mol	e.g. Diethanolamine, reagent grade, ≥98.0%, Sigma-Aldrich[2]
Magnesium chloride ($MgCl_2$) CAS: 7791-18-6 MW: 203.30 g/mol	e.g. Magnesium chloride hexahydrate, ACS reagent, 99.0-102.0%, Sigma-Aldrich[2]
Para-Nitrophenyl phosphate (PNPP) CAS: 333338-18-4 MW: 371.14 g/mol	e.g. Phosphatase substrate, 5 mg tablets, 4-Nitrophenyl phosphate disodium salt hexahydrate, Sigma-Aldrich[2]
10% (w/w) Hydrogen chloride (HCl) CAS: 7647-01-0	e.g. Hydrochloric acid 10%, EMPROVE® EXPERT Ph Eur,JP,NF, Sigma-Aldrich[2]

MW: 36.46 g/mol	
TNAP inhibitor CAS: 496014-13-2 MW: 344.38 g/mol	e.g. TNAP Inhibitor - CAS 496014-13-2 – Calbiochem, Sigma-Aldrich[2]

Table 3: Material that is used in ALP activity testing.

Material:	Requirements[1] Suggested type[2]
Volumetric flask	certified with defined volume[1]
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]
Microcentrifuge tubes 1.5 mL	e.g. Eppendorf® Safe-Lock microcentrifuge tubes, volume 1.5 mL, natural, Eppendorf AG[2]

Table 4: Software that is used in ALP activity testing.

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 Prism 8, GraphPad[2]

3. TEST SYSTEM

The minimum requirements for human liver microsomes are described in Table 5. The human microsomes should be tested for all known human liver microsomal contaminations in compliance with GIVIMP (OECD, 2018).
The used microsome concentration per well for the ALP reactions is the same than the determined microsome batch-specific concentration for DIO1 testing. Microsome batch-specific DIO activity testing is further specified in SOP: DIO1-SK assay.
The microsomes should be stored at ≤ -80°C until required for use.

Table 5: 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)

4. CONTROLS

The reference item “tissue-nonspecific (TN) alkaline phosphatase (AP) inhibitor” (2,5-Dimethoxy-N-(quinolin-3-yl)benzenesulfonamide, CAS no.: 496014-13-2) is a described specific inhibitor of TNAP (Dahl, Sergienko et al. 2009) and was used as the reference item for ALP testing. The DIO1 and TPO inhibitor 6-Propyl-2-thiouracil (6PTU) was used as negative control in ALP testing since 6PTU does not inhibit ALP activity.

Table 6: Overview of the used controls in ALP activity testing.

Controls:	
Reference item (RI)	Quantitatively controls ALP inhibition in the assay and is used for normalization to maximum inhibition in the assay. In addition to control replicates on each assay plate of the highest concentration, a concentration-response curve of the reference item is performed on each assay day. Used in ALP activity testing: TNAP inhibitor at a maximum assay concentration of $3.16*10^{-5}$. Concentration-response curves of TNAP inhibitor ranged from $3.16*10^{-5}$ to 10^{-9} M.
Negative control (NC)	A substance that leads to no inhibition of ALP activity. Used in ALP activity testing: 6-Propyl-2-thiouracil at a maximum assay concentration of 10^{-3} M.
Solvent control (SC)	Blank control. The solvent control ensures that the response does not originate from the applied solvent and shows no ALP inhibition. Solvent controls are used to normalize the inhibition observed with the test items to the maximum possible ALP activity. Final solvent concentration in the assay: Dimethyl sulfoxide (DMSO) at an assay concentration of 1%.

Table 7: Information on the reference item TNAP

Name:	2,5-Dimethoxy-N-(quinolin-3-yl)benzenesulfonamide
CAS No.:	496014-13-2
Molecular weight [g/mol]:	344.38
Storage conditions:	2-8°C
Solvent	DMSO
Stock solution [mol/L]:	10^{-1}
Storage conditions of stock solution	2-8°C

Table 8: Information on the negative control 6PTU

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}

5. METHOD

5.1. PRE-ASSAY

5.1.1. ALP assay buffer preparation

1. Thaw Diethanolamine (DEA) at 37°C (melting point of DEA: 28°C): big volumes need to be thawed overnight; once thawed, small aliquots of DEA can be prepared for future ALP assay buffer preparation
2. Prepare a 200 mM $MgCl_2$ stock solution (1000x stock) by weighing in 40.66 mg $MgCl_2$ and dissolving in 1 ml diH_2O; vortex to aid dissolution
3. Prepare 500 ml ALP assay buffer (20 mM DEA / 200 µM $MgCl_2$) in diH_2O: weigh in 1.05 g of thawed DEA and dissolve by addition of 500 ml diH_2O; add 500 µl of 200 mM $MgCl_2$ (1000x stock) to the DEA solution
4. Adjust pH to 9.8 by dropwise addition of 10% (w/w) HCl
5. Store the ALP assay buffer at 4°C

5.1.2. Solubility testing of a test item in ALP assay buffer

In the context of ALP testing, the solubility of test items needs to be assessed in their stock solutions, in prepared dilutions and under assay conditions. The solubility of the test item in solvent (DMSO preferred) as a stock solution as well as the dilution in water was already assessed in the DIO1-SK assay (see SOP: DIO1-SK assay) and the defined conditions will be used for the preparation of the stock solution in the ALP activity testing.

1. Prepare the stock solution of the test item in DMSO; use the concentration that was determined in DIO1-SK testing
2. Gently mix at room temperature. Vortex the tube if necessary
3. Prepare a 10% (v/v) dilution of the test item stock solution in diH_2O as determined in DIO1-SK testing
4. Add 100 µl of the prepared dilution, 400 µl diH_2O and 500 µl ALP assay buffer to a 24-well plate to test solubility under assay conditions; make sure the added solutions are mixed, either by shaking or pipetting
5. Visually check by using a microscope if the test item solution is dissolved under assay conditions
6. If the test item hasn't dissolved, use water bath sonification for up to 5 mins or warm the solution to 37°C for up to 60 mins; repeat step 5 to check if the test item is dissolved
7. If the test item is not soluble under assay conditions, dilute the test item stock solution in DMSO (e.g., by reducing the concentration by a factor of 10), prepare the resulting 10% (v/v) dilutions of the test item stock solutions in diH_2O as well the solutions under assay conditions as described in step 4

8. Visually check by using a microscope if the test item solution is dissolved under assay conditions
9. Repeat step 7 until the test item is fully dissolved

5.1.3. Preparation of the reference item TNAP inhibitor stock solution

The TNAP inhibitor is used as the reference item on the first plate on each assay day with a full concentration-response curve. Additionally, the highest concentration of the reference item is included on all plates in at least 6 replicates and is used to determine the background reaction occurring at full ALP inhibition.
The stock solution of the TNAP inhibitor can be prepared prior to the assay run and can be stored at 4°C. The dilutions in DMSO for the concentration-response testing are prepared fresh on each day of assay performance.

Stock solution

1. Weigh in a respective amount of reference item in a brown glass vial and dissolve in DMSO resulting in a concentration of 100 mM (ALP-RI-S0*); if the amount of the reference item in a supplied vial is ≤5 mg, add the amount of DMSO to the vial directly resulting in a concentration of 100 mM (ALP-RI-S0)
2. Store ALP-RI-SO at 4°C

*The stock solution is labelled with “ALP” here to prevent mix-ups of long-termed stored solutions

5.2. ASSAY RUN

5.2.1. Preparation of dilutions

5.2.1.1. Preparation of stock solutions and dilutions of the test items

Use the test item concentration that was fully dissolved in the stock solution and the resulting 10% (v/v) dilution diH_2O as well as solution under assay conditions to prepare the stock solution of the test item:

1. Weigh the appropriate amount of test item into a glass vessel and dissolve in DMSO to prepare the stock solution for test item 1 (TI1-S0)
2. For the preparation of the test item dilutions, the test item stock solution is subsequently diluted seven times with diH_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 9.

Table 9: Preparation of the test item dilutions using test item stock solutions

Name of the test item dilution	diH_2O [µL]	DMSO [µL]	Test item	Dilution factor
TI1-C1	450	-	50 µL TI1-S0	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

*A 10 % (v/v) solvent / diH2O solution can be prepared and 450 µL of the dilution can be added instead

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

The stock solution of the reference item TNAP inhibitor was prepared prior to the assay and was stored at 4°C.

1. On the day of analysis, prepare the reference item dilutions from the 100 mM reference item stock solution (ALP-RI-S0) according to Table 10; RI-D0 is used as a predilution and will not be used for testing in the assay
2. Label the subsequent reference item dilutions derived from the reference item stock solution adequately (e.g. RI-D1, RI-D2,…., RI-D8)

Table 10: Preparation of the dilutions for the reference item TNAP inhibitor.

Name of the reference item dilution	Reference item dilution concentration [M]	diH_2O [µL]	DMSO [µL]	Reference item [µL]	Final concentration of reference item in the assay [M]
RI-D0	$3.16*10^{-3}$	450	34.2	15.8 µL of ALP-RI-S0	-
RI-D1	$3.16*10^{-4}$	405*	45*	50 µL of RI-D0	$3.16*10^{-5}$
RI-D2	$3.16*10^{-5}$	405*	45*	50 µL of RI-D1	$3.16*10^{-6}$
RI-D3	10^{-5}	405*	45*	50 µL of RI-D2	10^{-6}
RI-D4	$3.16*10^{-6}$	405*	45*	50 µL of RI-D3	$3.16*10^{-7}$
RI-D5	10^{-6}	405*	45*	50 µL of RI-D4	10^{-7}
RI-D6	$3.16*10^{-7}$	405*	45*	50 µL of RI-D5	$3.16*10^{-8}$
RI-D7	10^{-7}	405*	45*	50 µL of RI-D6	10^{-8}
RI-D8	10^{-8}	405*	45*	50 µL of RI-D7	10^{-9}

*You can also prepare a 10 % DMSO / diH_2O solution and add 450 µL of the dilution

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

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

1. Based on the amount of stock solution needed, weigh an appropriate amount of 6PTU into a suitable vessel.
2. Add the appropriate amount of DMSO and vortex the vessel

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.

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

3. Prepare the negative control dilution from the 10^{-1} M negative control stock solution according to Table 11.

Table 11: Preparation of the negative control 6-propyl-2-thiouracil (6PTU) dilution.

Negative control dilution [M]	diH_2O [µL]	DMSO [µL]	Negative control [µL]	Final concentration of negative control in the assay [M]

10^{-2}	450	-	50 µL of 10^{-1} M negative control stock solution	10^{-3}

5.2.1.4. Preparation of the PNPP substrate solution

The substrate solution must be prepared fresh on each day. Keep substrate in the dark to prevent loss of activity in the assay.

1. Dissolve 5 mg PNPP in 449.8 µl diH_2O to a final concentration of 30 mM to prepare a 100x PNPP stock solution
2. Dilute the 100x PNPP stock solution 1:100 (v/v) in ALP assay buffer to prepare a 1x PNPP solution with a final concentration of 0.3 mM of PNPP; 6 ml of 1x PNPP solution is needed for one 96-well plate of ALP testing

5.2.2. Microsome incubation with test items

1. Prepare the reference item stock solution as well as dilutions as described in 5.2.1.2, the negative control stock solution as well as dilution as described in 5.2.1.3 and the test item stock solutions as well as dilutions as described in 5.2.1.1.
2. Preheat the absorbance reader to 37°C
1. On the first plate of an assay day, add 10 µL of the reference item dilutions to a 96-well plate to perform a full concentration-response testing. For the solvent control, add 10 µl of a 10 % (v/v) DMSO in diH_2O solution. For the negative control, add 10 µl of the prepared dilution of the negative control. Add 10 µl of the test item dilutions to the 96-well plate.
   A proposed plate layout for the first run of an assay day is shown in Table 12: plate layout for the first plate of an assay day of the ALP activity testing; a proposed plate layout for additional runs on the same assay day is shown in Table 13.
3. Add 40 µL of a defined protein dilution (resulting in the calculated amount of enzyme per sample well calculated in SOP: DIO1-SK assay that is specific for the used microsome batch) to the wells
4. Add 50 µl 1x PNPP solution to each well using a multichannel pipette
1. As soon as possible after the application of 1x PNPP solution, determine the optical density (OD) in a plate reader with the following settings:
   - Absorption parameters: 415 nm (±2 nm)
   - Initial shaking: weak for 5 s
   - Target temperature: 37°C, valid temperature range 35 – 39°C
   - Measurement of the OD every minute for 60 min (other time periods are possible, make sure the detection is in linear range), also measuring the initial OD

Table 12: plate layout for the first plate of an assay day of the ALP activity testing

	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	NC

SC **solvent control** 1% DMSO — **RI** **reference item** TNAP inhibitor $3.16*10^{-5}$ M — **NC** **negative control** 6PTU 10^{-3} M — **TI** **test item**

Table 13: plate layout for additional plates of an assay day of the ALP activity testing

	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			NC		

SC **solvent control** 1% DMSO — **RI** **reference item** TNAP inhibitor $3.16*10^{-5}$ M — **NC** **negative control** 6PTU 10^{-3} M — **TI** **test item**

5.2.3. Evaluation of the data

1. Determine the ΔOD_{21min} values via subtraction of the 60-minute values of all samples from the initial measured values:

   $$\Delta OD_{60min} = OD_{60min,415nm} - OD_{0min,415nm}$$

2. Determine the ΔOD-BG values, by subtracting the mean of ΔOD_{60min} values of the inhibited $3.16*10^{-5}$ M TNAP inhibitor reference item controls from the ΔOD_{60min} values of all samples:

   $$\Delta OD\text{-}BG = \Delta OD_{60min} - \overline{\Delta OD_{60min,RI}}$$

Where “RI” represents the reference item TNAP inhibitor

3. Normalize the values of the test item to the respective solvent control values via division of the test item(s) ΔOD-BG values by the mean of the ΔOD-BG values of the respective solvent control, generating Alkaline phosphatase (ALP) activity values. State ALP activity values in %. Keep in mind that test items with differing solvents need to be normalized to their respective solvent controls:

$$ALP\ activity = \frac{\Delta OD\text{-}BG_{TI}}{\Delta OD\text{-}BG_{SC}} * 100$$

where “TI” represents the test item at used concentrations and “SC” the solvent control

4. Plot the ALP activity values of the different test item concentration samples in a statistics software with ALP activity values on y-axis (linear) and test item concentrations on x-axis (logarithmic)

5. Use a curve-fit algorithm to visualize a concentration-response relationship (e.g. “[Inhibitor] vs. response -- Variable slope (four parameters)” in GraphPad Prism 8):

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

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

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

Appendix 4: Donor demographics as well as the available lot characterization data for the batch #QQY

BioreclamationIVT
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QUALITY TESTED BioreclamationIVT QUALITY ASSURED

*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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Appendix 4: Donor demographics as well as the available lot characterization data for the batch #QQY

BioreclamationIVT
The complete resource for all biologicals

Donor Demographics, as reported to BioreclamationIVT

								Serology testing					
Gender	Age	Race	Cause of death	Height	Weight	Social history	Medical history	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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Appendix 4: Donor demographics as well as the available lot characterization data for the batch #QQY

BioreclamationIVT
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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	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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BioreclamationIVT
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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	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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Appendix 4: Donor demographics as well as the available lot characterization data for the batch #QQY

BioreclamationIVT
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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	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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Appendix 4: Donor demographics as well as the available lot characterization data for the batch #QQY

BioreclamationIVT
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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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Appendix 4: Donor demographics as well as the available lot characterization data for the batch #QQY

BioreclamationIVT
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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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Appendix 4: Donor demographics as well as the available lot characterization data for the batch #QQY

BioreclamationIVT
The complete resource for all biologicals

QUALITY TESTED BioreclamationIVT QUALITY ASSURED

Gender	Age	Race	Cause of death	Height	Weight	Social history	Medical history	EBV	RPR	CMV	Hepatitis B	Hepatitis C	HIV
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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BioreclamationIVT
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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	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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BioreclamationIVT
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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	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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BioreclamationIVT
The complete resource for all biologicals

Gender	Age	Race	Cause of death	Height	Weight	Social history	Medical history	EBV	RPR	CMV	Hepatitis B	Hepatitis C	HIV
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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Appendix 4: Donor demographics as well as the available lot characterization data for the batch #QQY

BioreclamationIVT
The complete resource for all biologicals

Gender	Age	Race	Cause of death	Height	Weight	Social history	Medical history	EBV	RPR	CMV	Hepatitis B	Hepatitis C	HIV
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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BioreclamationIVT
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Gender	Age	Race	Cause of death	Height	Weight	Social history	Medical history	EBV	RPR	CMV	Hepatitis B	Hepatitis C	HIV
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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BioreclamationIVT
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Gender	Age	Race	Cause of death	Height	Weight	Social history	Medical history	EBV	RPR	CMV	Hepatitis B	Hepatitis C	HIV
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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Appendix 4: Donor demographics as well as the available lot characterization data for the batch #QQY

BioreclamationIVT
The complete resource for all biologicals

Gender	Age	Race	Cause of death	Height	Weight	Social history	Medical history	EBV	RPR	CMV	Hepatitis B	Hepatitis C	HIV
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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BioreclamationIVT
The complete resource for all biologicals

Gender	Age	Race	Cause of death	Height	Weight	Social history	Medical history	EBV	RPR	CMV	Hepatitis B	Hepatitis C	HIV
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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BioreclamationIVT
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Gender	Age	Race	Cause of death	Height	Weight	Social history	Medical history	EBV	RPR	CMV	Hepatitis B	Hepatitis C	HIV
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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BioreclamationIVT
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Gender	Age	Race	Cause of death	Height	Weight	Social history	Medical history	EBV	RPR	CMV	Hepatitis B	Hepatitis C	HIV
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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BioreclamationIVT
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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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BioreclamationIVT
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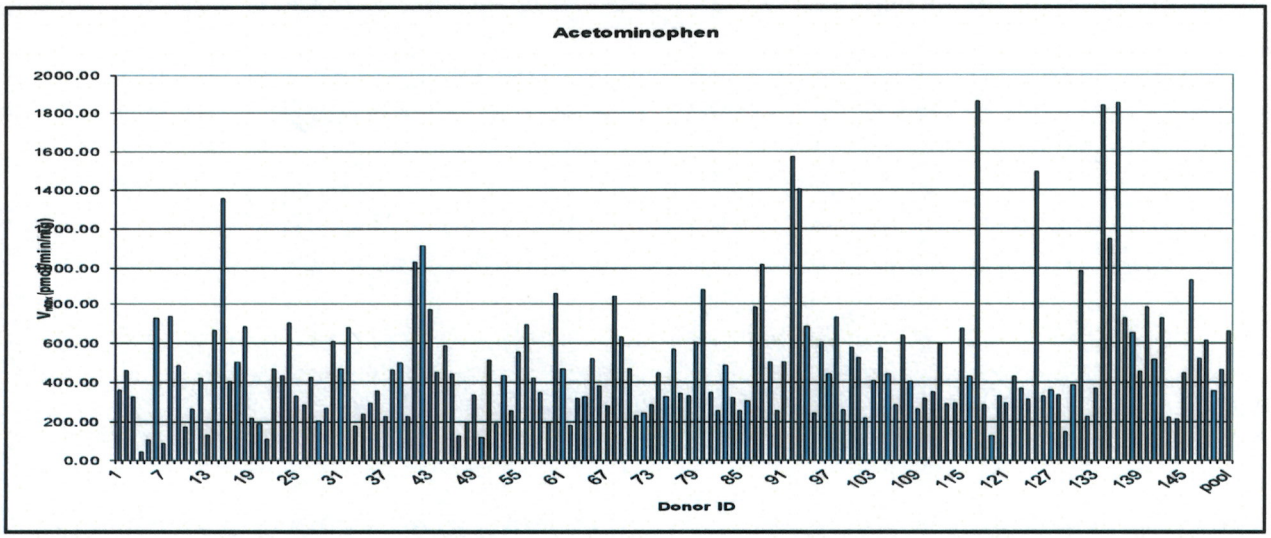

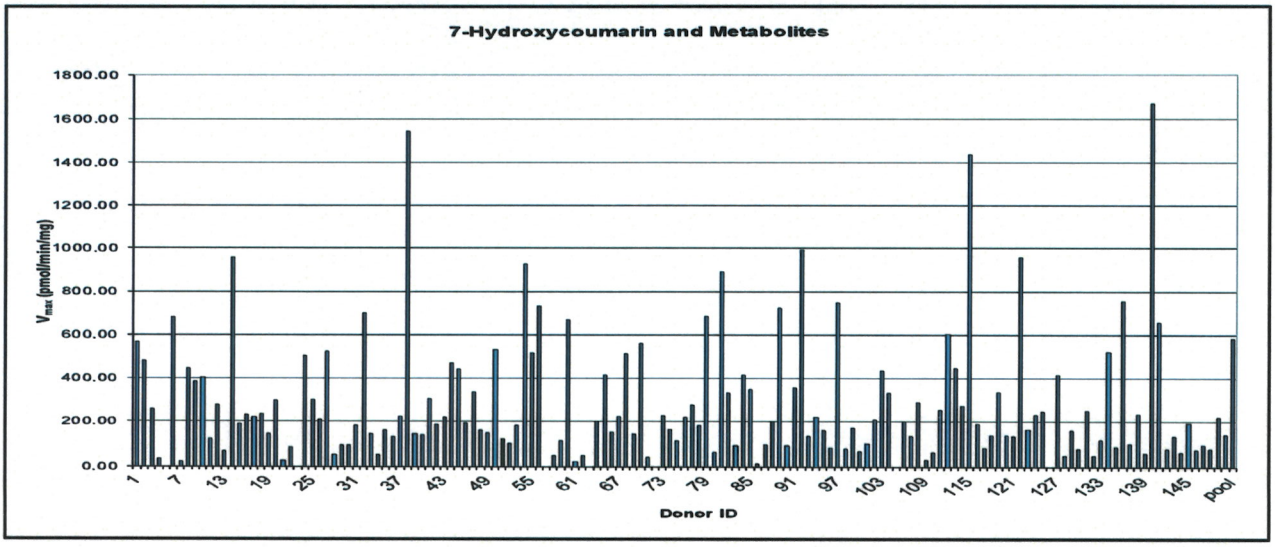

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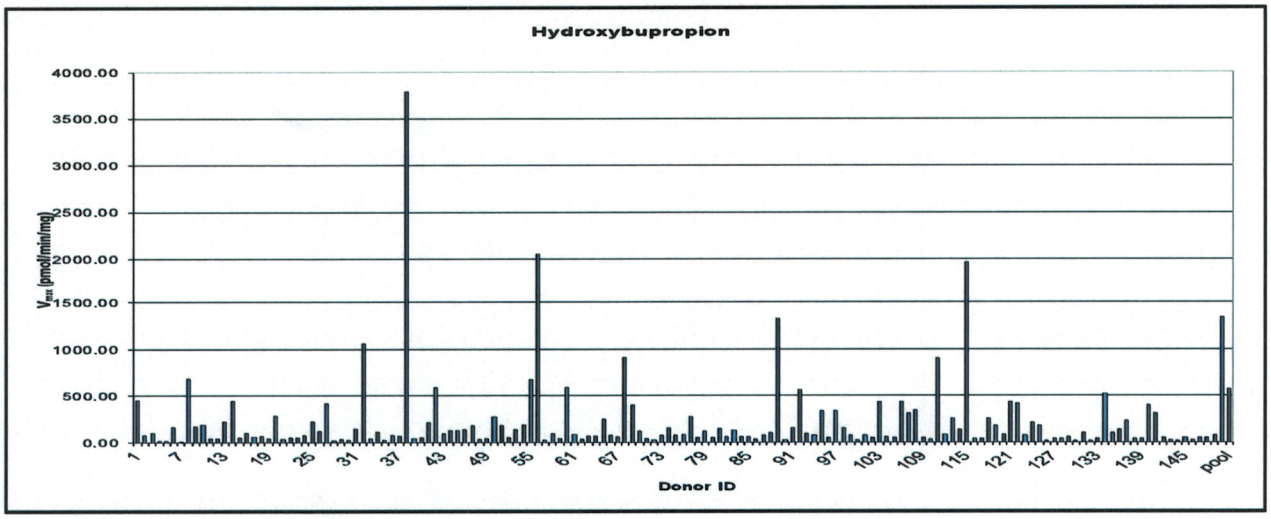
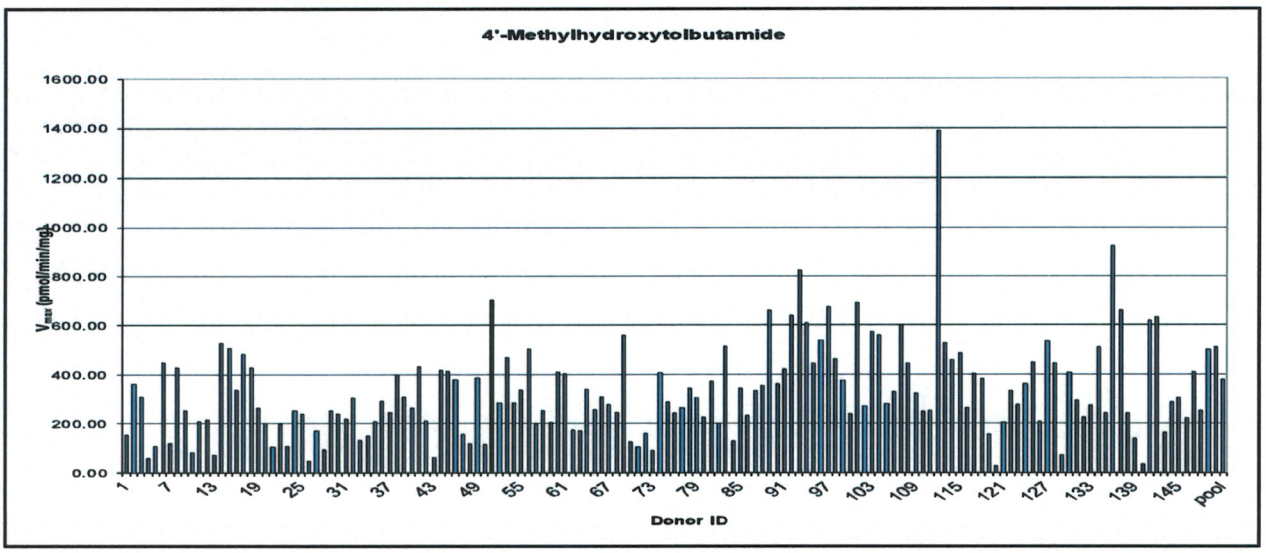
Appendix 4: Donor demographics as well as the available lot characterization data for the batch #QQY



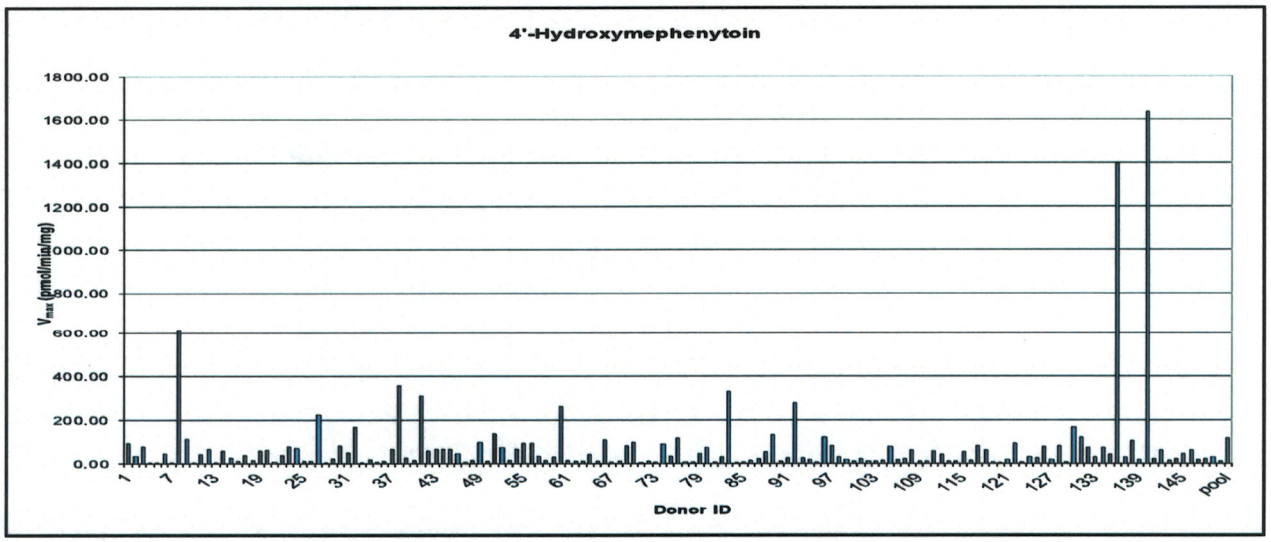


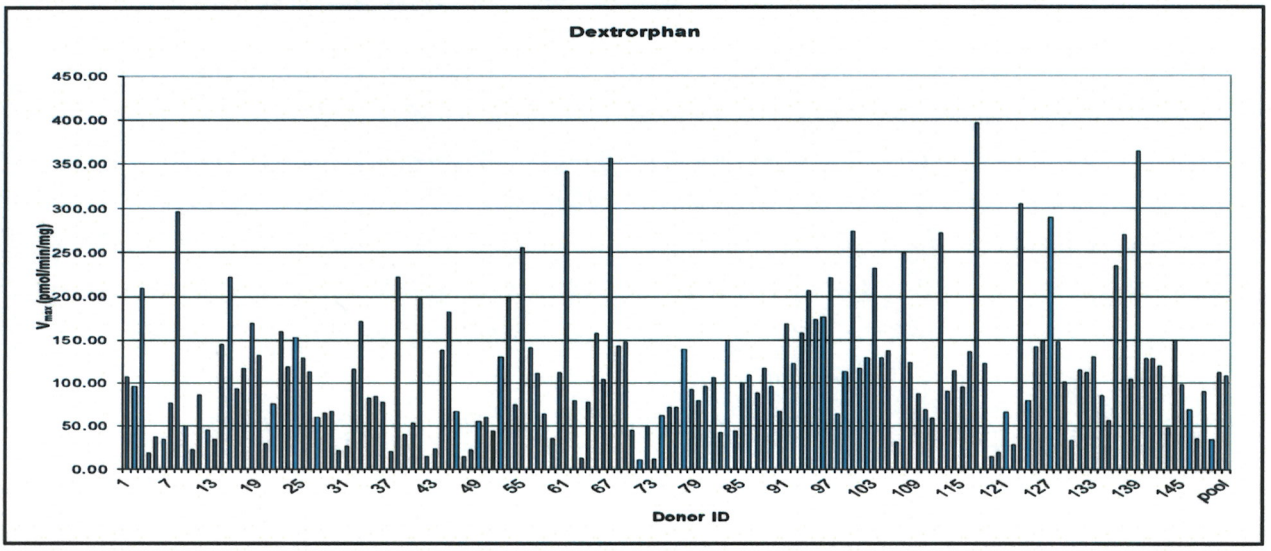
Caution: This product was prepared from fresh human tissue. Treat all products containing human-derived materials as potentially infectious, as no known test methods can offer assurance that products derived from human tissues will not transmit infectious agents.

These products are for research use only. Do not use in animals or humans. These products have not been approved for any diagnostic or clinical procedures.

Appendix 4: Donor demographics as well as the available lot characterization data for the batch #QQY

BioreclamationIVT
The complete resource for all biologicals





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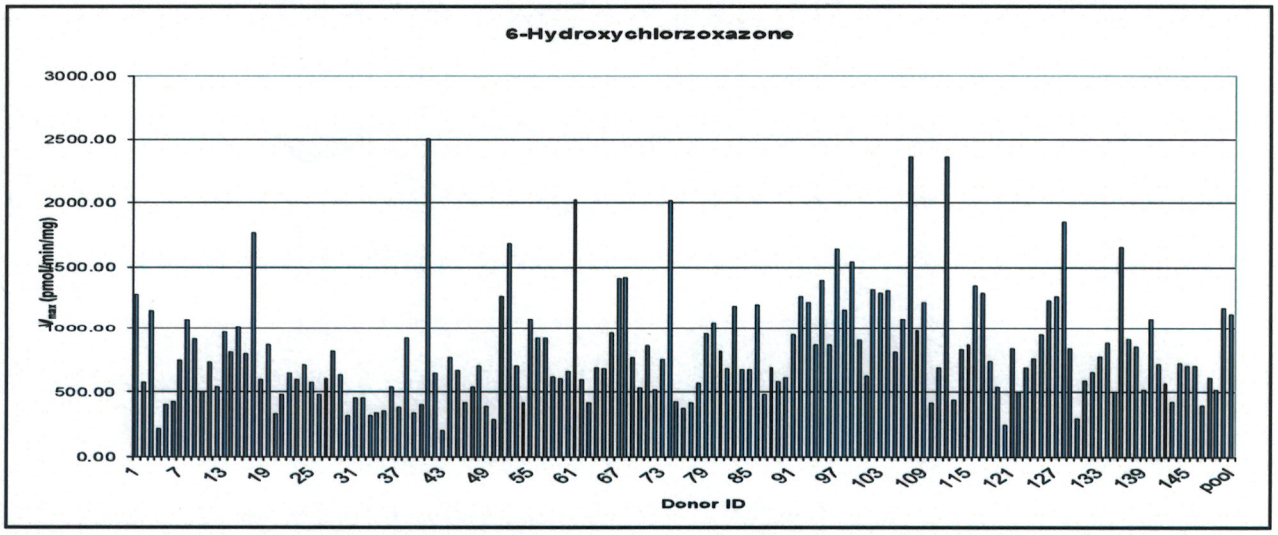

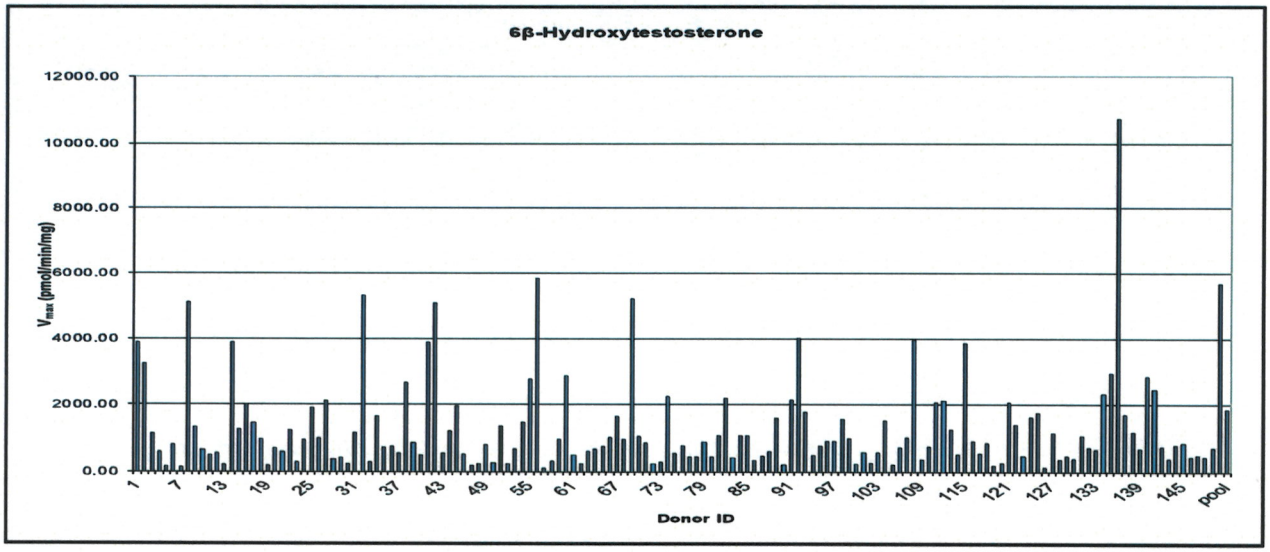

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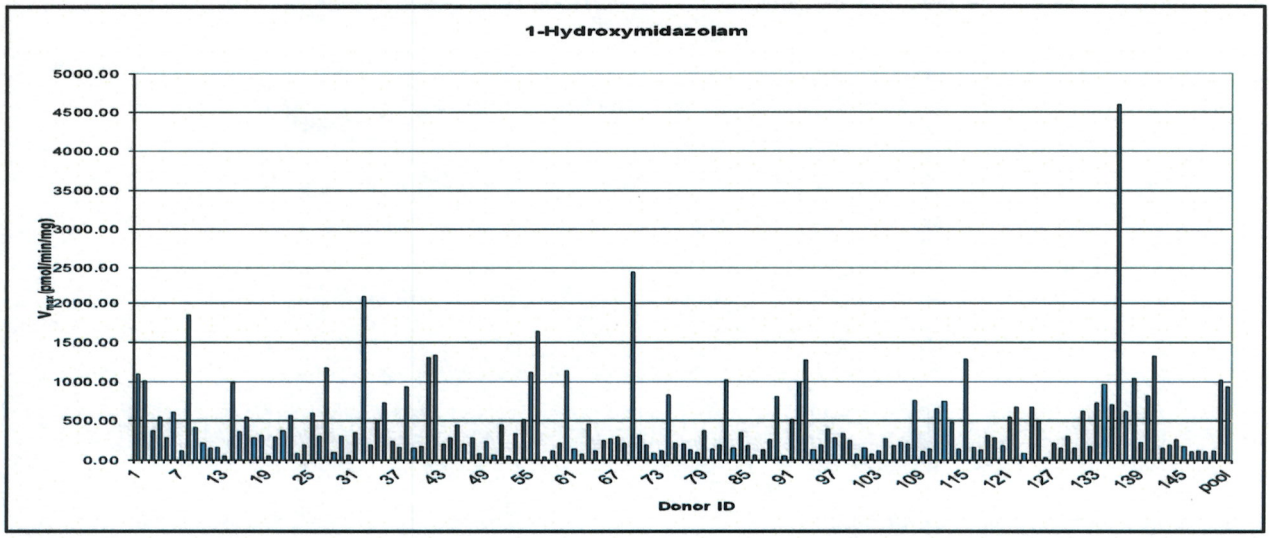

Caution: This product was prepared from fresh human tissue. Treat all products containing human-derived materials as potentially infectious, as no known test methods can offer assurance that products derived from human tissues will not transmit infectious agents.

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14.5 Extensive information about the solubility of part 2 test items in the DIO1-SK assay

Appendix 5: Extensive information about the solubility of part 2 test items in the DIO1-SK assay

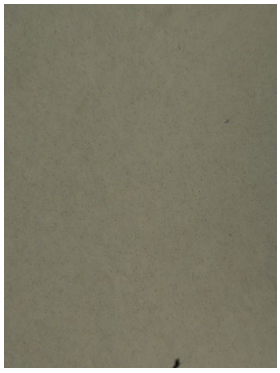
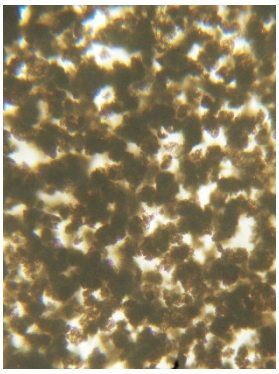
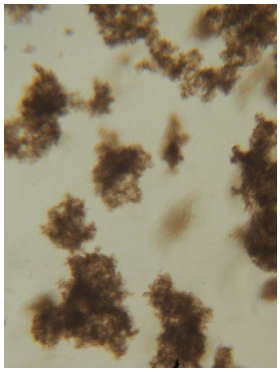
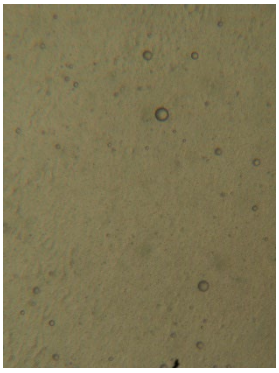
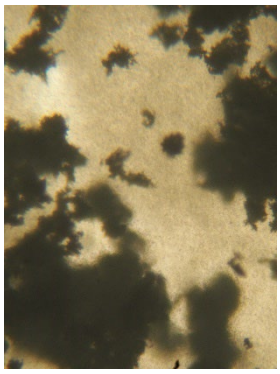
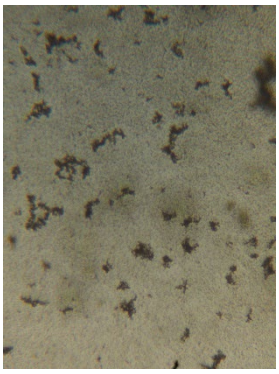
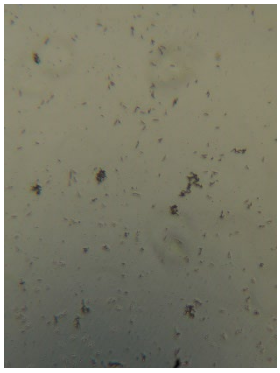
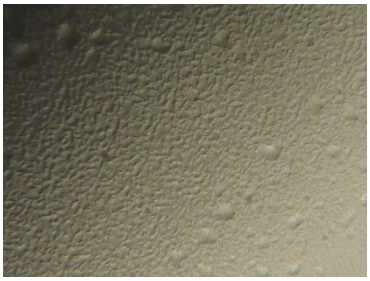
photo	chemical code	stock solution (DMSO)	soluble?	comments	solubility in ddH_2O (10% test item)	soluble?	comments	solubility under assay conditions (1% test item)	soluble?	comments
1) 2)	227	**10^{-1}**	**yes**	**vortex, clear solution**	**10^{-2}**	**yes**	**cloudy, opaque - homogenous**	**10^{-3}**	**yes**	**1) 2) few, homogenously distributed, black particles**
3)	229	**10^{-1}**	**yes**	**vortex, clear solution**	**10^{-2}**	**yes**	**cloudy, opaque - homogenous**	**10^{-3}**	**yes**	**3) many homogenously distributed, black particles**
	279	10^{-1}	yes	vortex, clear solution	10^{-2}	yes	cloudy, opaque, becomes solid			
		$3{,}16 \times 10^{-2}$	yes	vortex, clear solution	$3{,}16 \times 10^{-3}$	no	cloudy, opaque, becomes solid			
		10^{-2}	**yes**	**vortex, clear solution**	**10^{-3}**	**yes**	**vortex, clear solution**	**10^{-4}**	**yes**	**vortex, clear solution**
	377	10^{-1}	no	opaque, white						
		10^{-1}	no	5 min ultrasound						
		10^{-1}	yes	5 min, 37°C ultrasound	10^{-2}	no	clumping			
		10^{-2}	**yes**	**vortex, clear solution**	**10^{-3}**	**yes**	**vortex, clear solution**	**10^{-4}**	**yes**	**vortex, clear solution**
4) 5)	526	10^{-1}	yes	vortex, clear solution	10^{-2}	no	4) black clouds, slimey			
		10^{-2}	yes	vortex, clear solution	10^{-3}	no	5) black clouds			
		10^{-3}	**yes**	**vortex, clear solution**	**10^{-4}**	**yes**	**vortex, clear solution**	**10^{-5}**	**yes**	**vortex, clear solution**
	543	**10^{-1}**	**yes**	**vortex, clear solution**	**10^{-2}**	**yes**	**vortex, clear solution**	**10^{-3}**	**yes**	**vortex, clear solution**
6)	551	10^{-1}	yes	vortex, clear solution	10^{-2}	no	6) opaque, droplet-like particles			
		10^{-2}	**yes**	**vortex, clear solution**	**10^{-3}**	**yes**	**vortex, clear solution**	**10^{-4}**	**yes**	**vortex, clear solution**
	798	**10^{-1}**	**yes**	**bright yellow, clear**	**10^{-2}**	**yes**	**vortex, clear solution**	**10^{-3}**	**yes**	**vortex, clear solution**
7) 8) 9)	868	10^{-1}	yes	slightly yellow, clear	10^{-2}	no	7) black, slimey clouds			
		10^{-2}	yes	vortex, clear solution	10^{-3}	no	8) black particles/clouds			
		10^{-3}	yes	vortex, clear solution	10^{-4}	no	9) many small threads			
		10^{-4}	**yes**	**vortex, clear solution**	**10^{-5}**	**yes**	**vortex, clear solution**	**10^{-6}**	**yes**	**vortex, clear solution**
	877	10^{-1}	no	few, black fragments						
		10^{-1}	**yes**	**5 min ultrasound**	**10^{-2}**	**yes**	**vortex, clear solution**	**10^{-3}**	**yes**	**vortex, clear solution**
	125	**10^{-1}**	**yes**	**vortex, viscous**	**10^{-2}**	**yes**	**vortex, clear solution**	**10^{-3}**	**yes**	**vortex, clear solution**
	130	**10^{-1}**	**yes**	**vortex, clear solution**	**10^{-2}**	**yes**	**vortex, clear solution**	**10^{-3}**	**yes**	**vortex, clear solution**
10) 11)	194	10^{-1}	no	gel-like precipitation						
		10^{-1}	**yes**	**15 min, 37°C ultrasonic**	**10^{-2}**	**yes**	**10) resinous, gel-like, viscous, clear**	**10^{-3}**	**yes**	**11) distributed vesicles, homogeneous**
12) 13)	218	10^{-1}	yes	vortex	10^{-2}	no	12) 13) precipitated; many black particles			
		$3{,}16 \times 10^{-2}$	**yes**	**vortex**	**$3{,}16 \times 10^{-3}$**	**yes**	**clear solution**	**$3{,}16 \times 10^{-4}$**	**yes**	**clear solution**
	437	**10^{-1}**	**yes**	**vortex**	**10^{-2}**	**yes**	**clear solution**	**10^{-3}**	**yes**	**clear solution**
14)	442	10^{-1}	no	vortex, behaves like sand in water						
		$3{,}16 \times 10^{-2}$	no	vortex, behaves like sand in water						
		10^{-2}	no	vortex, behaves like sand in water						
		$3{,}16 \times 10^{-3}$	**yes**	**40 min, 37°C ultrasonic homogeneously distributed particles, orange**	**$3{,}16 \times 10^{-4}$**	**yes**	**14) few, homogeneously distributed particles, orange**	**$3{,}16 \times 10^{-5}$**	**yes**	**clear solution**
15) 16) 17)	506	10^{-1}	no	vortex, light yellow solution, crystals						
		10^{-1}	yes	5', 37°C, ultrasonic	10^{-2}	no	15) 16) brownish, slimy clouds			
		$3{,}16 \times 10^{-2}$	yes	5', 37°C, ultrasonic	$3{,}16 \times 10^{-3}$	no	brownish, slimy clouds			
		10^{-2}	yes	vortex	10^{-3}	no	17) black, spider-like flakes, inhomogeneous			
		$3{,}16 \times 10^{-3}$	**yes**	**vortex**	**$3{,}16 \times 10^{-4}$**	**yes**	**clear solution**	**$3{,}16 \times 10^{-5}$**	**yes**	**clear solution**
	598	**10^{-1}**	**yes**	**light yellow, clear**	**10^{-2}**	**yes**	**clear solution**	**10^{-3}**	**yes**	**clear solution**
18)	603	10^{-1}	yes	vortex, light yellowish	10^{-2}	no	18) inhomogeneous, brownish, cloudy substance			
		$3{,}16 \times 10^{-2}$	yes	vortex	$3{,}16 \times 10^{-3}$	no	inhomogeneous, brownish, cloudy substance			
		10^{-2}	**yes**	**vortex**	**10^{-3}**	**yes**	**macroscopic: whitish**	**10^{-4}**	**yes**	**clear solution**
19)	615	10^{-1}	no	vortex						
		10^{-1}	**yes**	**15 min, 37°C ultrasonic**	**10^{-2}**	**yes**	**clear solution**	**10^{-3}**	**yes**	**19) black, evenly distributed particles, homogeneous**
	741	**10^{-1}**	**yes**	**vortex**	**10^{-2}**	**yes**	**clear solution**	**10^{-3}**	**yes**	**clear solution**
	878	**10^{-1}**	**yes**	**vortex**	**10^{-2}**	**yes**	**clear solution**	**10^{-3}**	**yes**	**clear solution**
20) 21)	933	10^{-1}	yes	vortex	10^{-2}	no	20) macr.: white precipitates; micr.: black, spider-like threads, flakes			
		$3{,}16 \times 10^{-2}$	yes	vortex	$3{,}16 \times 10^{-3}$	no	21) black, spider-like threads, flakes			
		10^{-2}	**yes**	**vortex**	**10^{-3}**	**yes**	**clear solution**	**10^{-4}**	**yes**	**clear solution**
22) 23) 24)	667	10^{-1}	yes	vortex, clear solution	10^{-2}	no	macr.: milky - white; 22) micr.: vesicles of different sizes, inhomogeneous			
		3.16×10^{-2}	**yes**	**vortex, clear solution**	**3.16×10^{-3}**	**yes**	**23) homogeneously distributed particles**	**3.16×10^{-4}**	**yes**	**24) clear solution**
25) 26) 27) 28)	839	10^{-1}	yes	vortex, clear solution	10^{-2}	no	macr.: milky - white; 25) 26) micr.: bright precipitates, streaks	10^{-3}	no	27) macr.: white, cloudy, precipitated, micr.: brown slime, black precip.
		3.16×10^{-2}	yes	vortex, clear solution	3.16×10^{-3}	no	clear solution	3.16×10^{-4}	no	28) macr.: white, milky; micr.: very small black precipitates
		10^{-2}	**yes**	**vortex, clear solution**	**10^{-3}**	**yes**	**clear solution**	**10^{-4}**	**yes**	**clear solution**
	220	**10^{-1}**	**yes**	**2 min vortex, clear solution**	**10^{-2}**	**yes**	**clear solution**	**10^{-3}**	**yes**	**clear solution**
29) 30) 31)	294	10^{-1}	yes	vortex, clear solution	10^{-2}	no	29) 30) black spider-like precipitates			
		3.16×10^{-2}	**yes**	**vortex, clear solution**	**3.16×10^{-3}**	**yes**	**31) clear solution**	**3.16×10^{-4}**	**yes**	**clear solution**
	827	**10^{-1}**	**yes**	**vortex, clear solution**	**10^{-2}**	**yes**	**clear solution**	**10^{-3}**	**yes**	**clear solution**
	325	**10^{-1}**	**yes**	**5min. vortex, clear solution, yellowish**	**10^{-2}**	**yes**	**clear solution**	**10^{-3}**	**yes**	**clear solution**
32) 33) 34) 35) 36)	925	10^{-1}	yes	vortex, clear solution	10^{-2}	no	32) black precipitates, spider-like, inhomogeneous			
		3.16×10^{-2}	yes	vortex, clear solution	3.16×10^{-3}	no	33) black precipitates, inhomogeneous			
		10^{-2}	yes	vortex, clear solution	10^{-3}	no	34) black precipitates, inhomogeneous			
		3.16×10^{-3}	yes	vortex, clear solution	3.16×10^{-4}	no	35) 36) black precipitates, inhomogeneous			
		10^{-3}	**yes**	**vortex, clear solution**	**10^{-4}**	**yes**	**clear solution**	**10^{-5}**	**yes**	**clear solution**
37)	511	10^{-1}	yes	vortex, clear solution	10^{-2}	no	37) flakes, white crystals			
		3.16×10^{-2}	**yes**	**vortex, clear solution**	**3.16×10^{-3}**	**yes**	**clear solution**	**3.16×10^{-4}**	**yes**	**clear solution**
38) 39)	056	10^{-1}	no	38) crystals						
		3.16×10^{-2}	no	39) crystals						
		10^{-2}	**yes**	**vortex, clear solution**	**10^{-3}**	**yes**	**clear solution**	**10^{-4}**	**yes**	**clear solution**
40) 41)	974	10^{-1}	yes	vortex, clear solution	10^{-2}	no	40) emulsion, inhomogeneous			
		3.16×10^{-2}	**yes**	**vortex, clear solution**	**3.16×10^{-3}**	**yes**	**41) emulsion, homogeneous distributed vesicles**	**3.16×10^{-4}**	**yes**	**22) clear solution**
42)	818	**10^{-1}**	**yes**	**vortex, clear solution**	**10^{-2}**	**yes**	**42) white, homogeneous distributed vesicles**	**10^{-3}**	**yes**	**clear solution**
	307	**10^{-1}**	**yes**	**vortex, clear solution**	**10^{-2}**	**yes**	**clear solution**	**10^{-3}**	**yes**	**clear solution**
		10^{-1}	yes	vortex, clear solution	10^{-2}	no	43) emulsion, irregularly distributed drops			

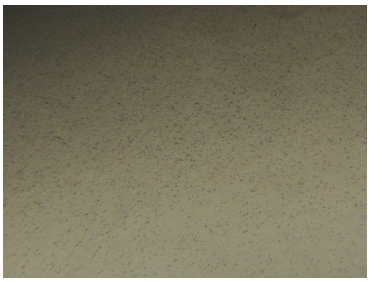
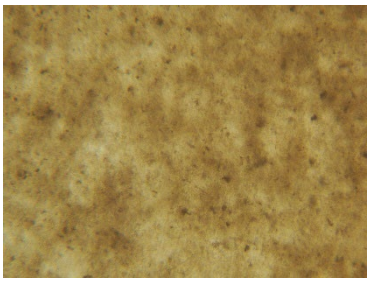
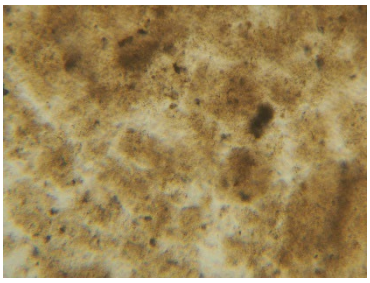
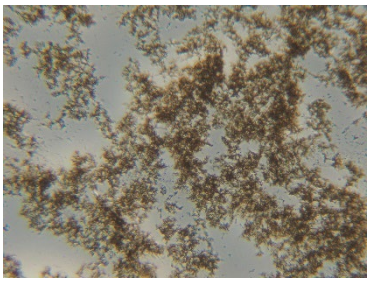
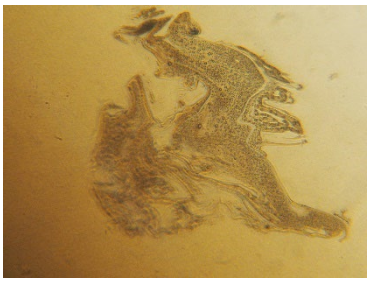
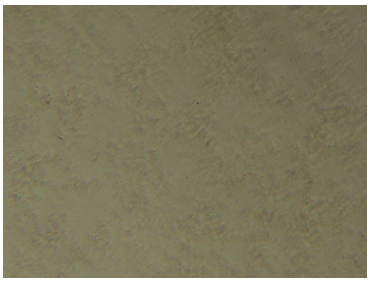
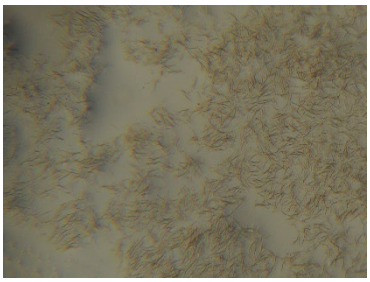
Appendix 5: Extensive information about the solubility of part 2 test items in the DIO1-SK assay

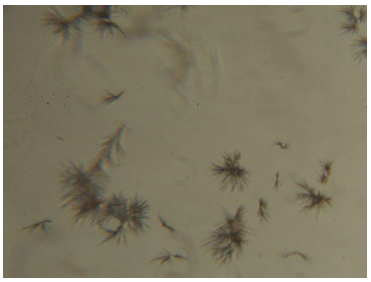
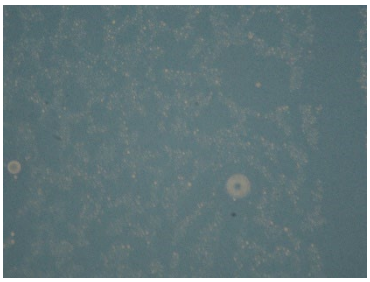
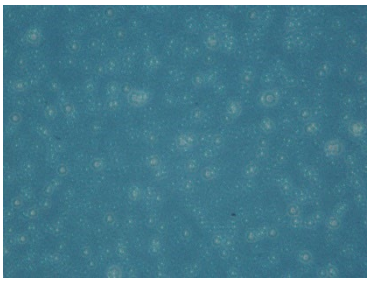
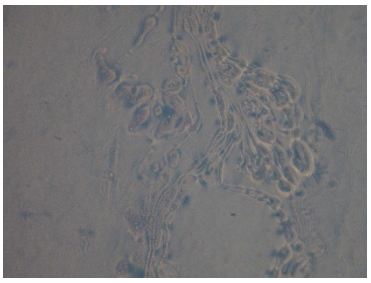
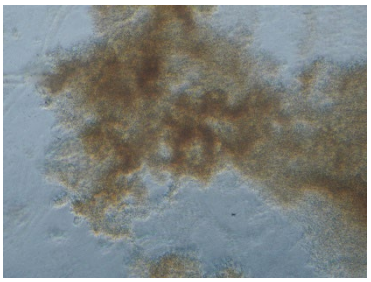
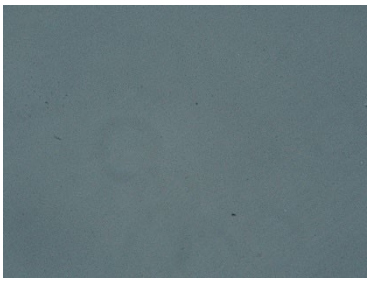
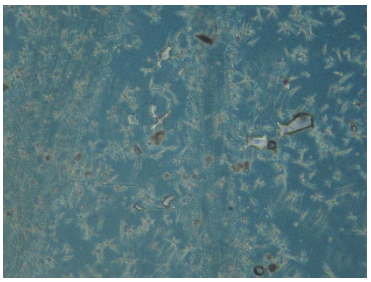
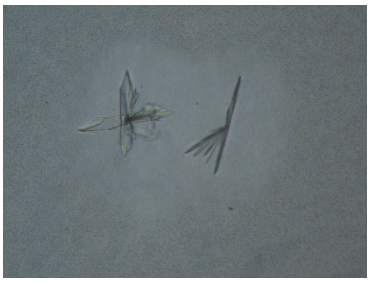
photo	chemical code	stock solution (DMSO)	soluble?	comments	solubility in ddH_2O (10% test item)	soluble?	comments	solubility under assay conditions (1% test item)	soluble?	comments
43) 44) 45)	160	3.16×10^{-2}	yes	vortex, clear solution	3.16×10^{-3}	no	44) emulsion, irregularly distributed drops			
		10^{-2}	yes	vortex, clear solution	10^{-3}	no	45) emulsion, irregularly distributed drops			
		3.16×10^{-3}	**yes**	**vortex, clear solution**	**3.16×10^{-4}**	**yes**	**clear solution**	**3.16×10^{-5}**	**no**	**clear solution**
	850	**10^{-1}**	**yes**	**vortex, clear solution**	**10^{-2}**	**yes**	**macr.: whitish, micr.: cloudy, but homogeneous**	**10^{-3}**	**yes**	**macr.: whitish, micr.: cloudy, but homogeneous**
	610	10^{-1}	yes	vortex, clear solution	10^{-2}	no	crystals			
		3.16×10^{-2}	**yes**	**vortex, clear solution**	**3.16×10^{-3}**	**yes**	**clear solution**	**3.16×10^{-4}**	**yes**	**clear solution**
	680	10^{-1}	no	vortex, ultrasonic - crystals						
		3.16×10^{-2}	no	vortex, ultrasonic - crystals						
		10^{-2}	**yes**	**15min, ultrasonic - clear solution**	**10^{-3}**	**yes**	**clear solution**	**10^{-4}**	**yes**	**clear solution**
	940	**10^{-1}**	**yes**	**vortex, clear solution**	**10^{-2}**	**yes**	**clear solution**	**10^{-3}**	**yes**	**clear solution**

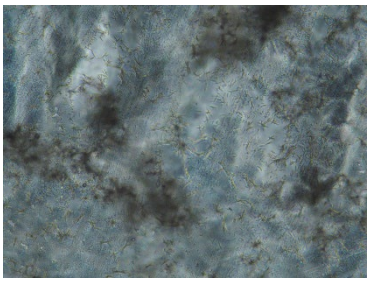
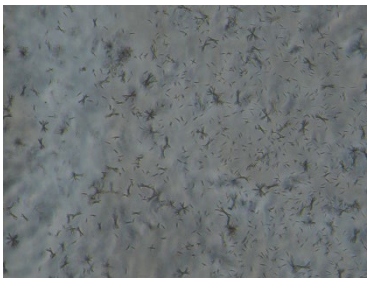
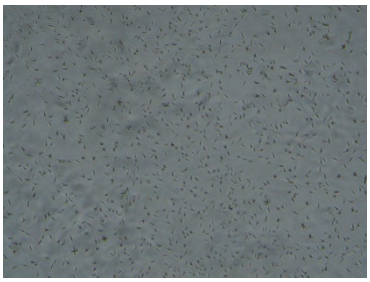
14.6 Pictures of insoluble / hardly soluble test items in the part 2 testing of the DIO1-SK assay

Appendix 6: Pictures of insoluble / hardly soluble test items in the part 2 testing of the DIO1-SK assay

1		2	
3		4	
5		6	
7		8	
9		10	

11		12	
13		14	
15		16	
17		18	
19		20	

21		22	
23		24	
25		26	
27		28	
29		30	

31		32	
33		34	
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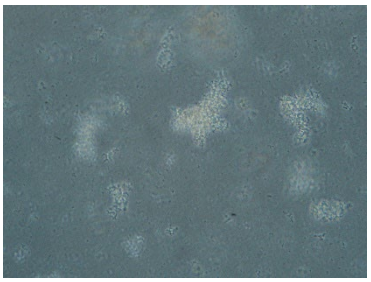
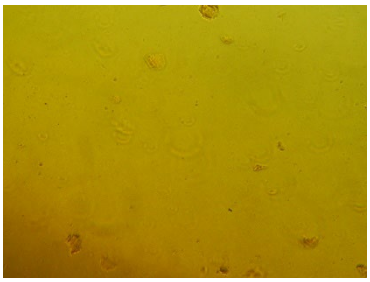
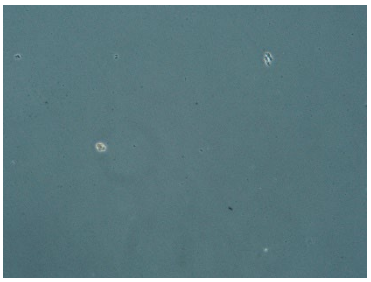
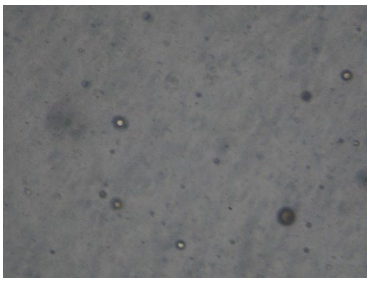
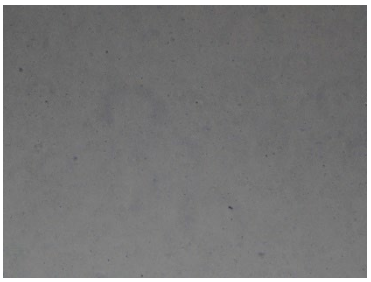
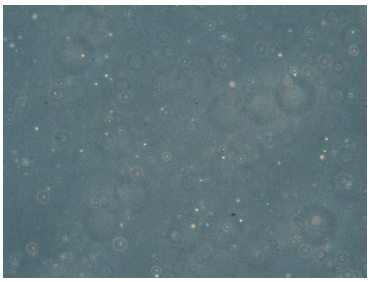
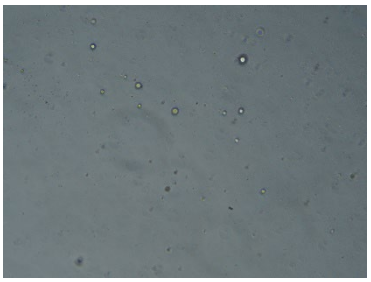
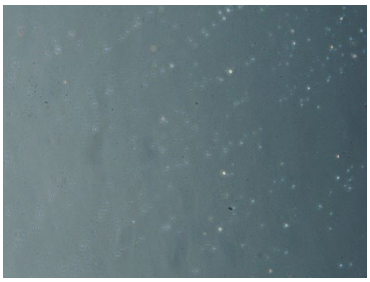
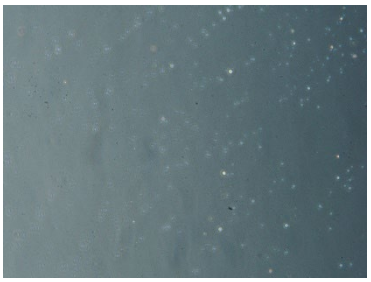
37		38	
39		40	

41		42	
43		44	
45			

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