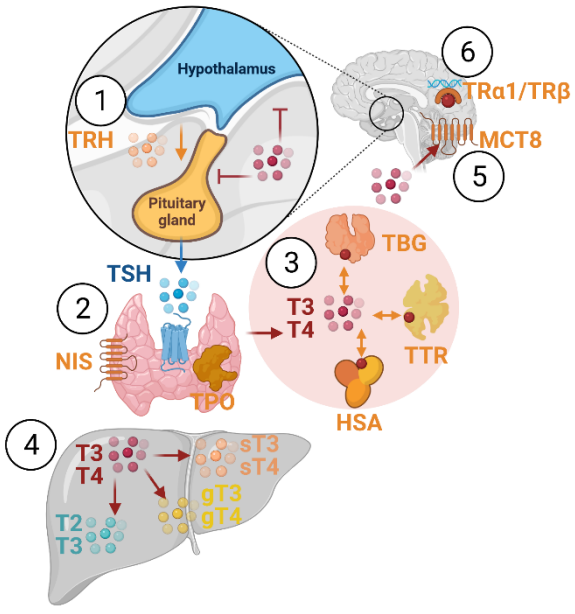
European Commission

STANDARD OPERATING PROCEDURE

for colorimetric assessment of deiodinases activity based on Sandell-Kolthoff reaction with human microsomes: DIO1-SK assay, version 2.0

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

Weber A., Birk B.



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This Standard Operating Procedure (SOP) 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 SOP is part of a series of 2 SOPs used to measure the " "Deiodinase 1 activity based on Sandell-Kolthoff reaction":

1. **SOP "Colorimetric assessment of deiodinases activity based on Sandell-Kolthoff reaction with human microsomes: DIO1-SK assay" version 2.0 (used in Part2 of the validation study)**
2. SOP "Assessing specificity of DIO1 interaction using Alkaline phosphatase (ALP) testing as secondary readout" version 1.0 (used in Part 2 of the validation study)

The method was developed by Kostja Renko when working at Charité Universitatsmedizin Berlin (DE), and subsequently implemented by the EU-NETVAL test facility BASF SE within the validation study.

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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.

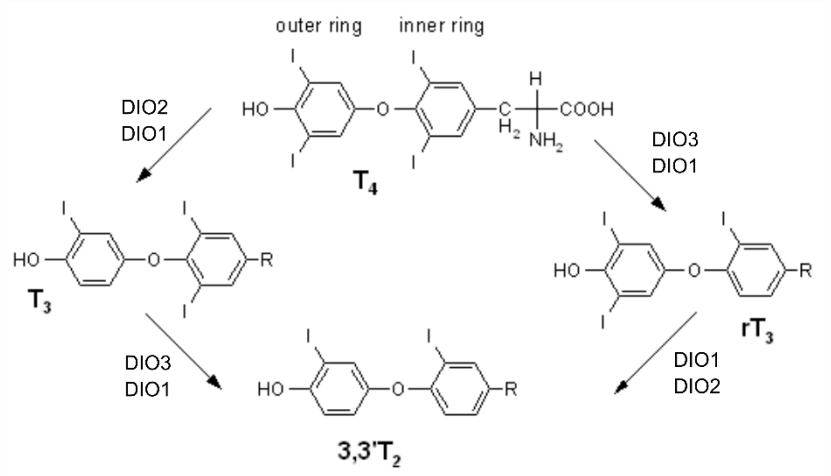

Figure 1: Metabolism of thyroid hormone

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.

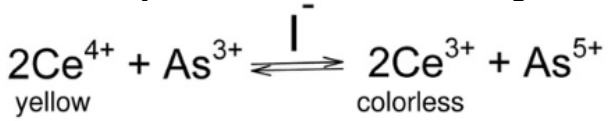

Figure 2: Sandell-Kolthoff-reaction

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.

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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.

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