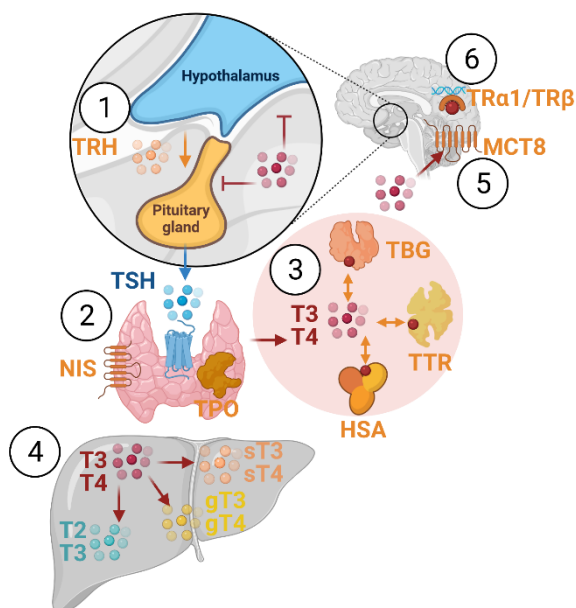


STANDARD OPERATING PROCEDURE

for colorimetric assessment of deiodinases activity based on Sandell-Kolthoff reaction with human microsomes: DIO1-SK assay, version 1.0

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

Weber A., Birk B.



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 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 1.0 (used in Part1 of the validation study)

The “SOP “Colorimetric assessment of deiodinases activity based on Sandell-Kolthoff reaction with human” was updated after Part 1.

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

Contact information

Method developer:

German Federal Institute for Risk Assessment
Department Experimental Toxicology and ZEBET
German Centre for the Protection of Laboratory Animals (Bf3R)
Dr. Kostja Renko
Max-Dohrn-Str. 8-10
10589 Berlin
Germany
Kostja.Renko@bfr.bund.de

EU-NETVAL facility

BASF SE
Experimental Toxicology and Ecology, RG/TB
Dr. Nina Hambruch
Carl-Bosch-Strasse 38
67056 Ludwigshafen am Rhein, Germany
Nina.Hambruch@basf.com

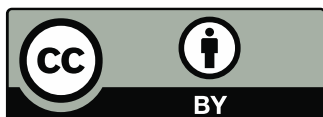
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JRC134705

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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

AUTHOR

BASF SE

1. INTRODUCTION

1.1. BACKGROUND AND OBJECTIVE

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

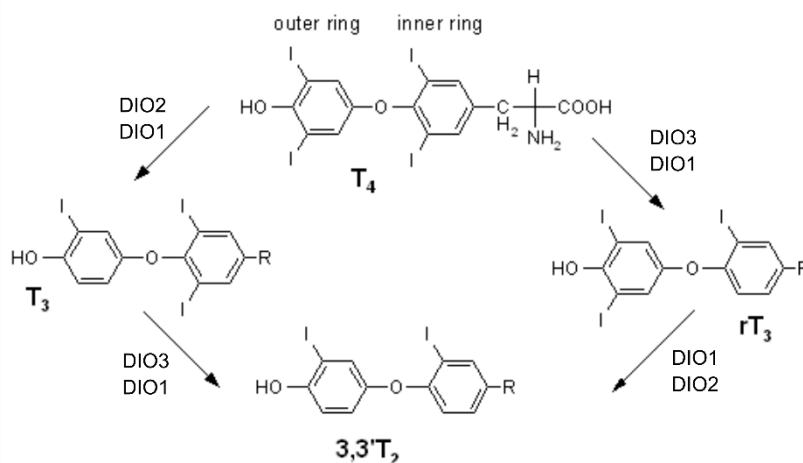


Figure 1: Metabolism of thyroid hormone

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

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

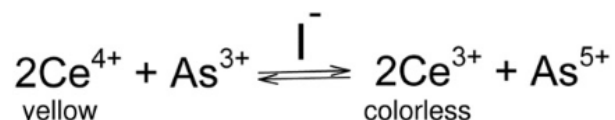


Figure 2: Sandell-Kolthoff-reaction

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

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

This *in vitro* method is suitable for high to medium throughput screenings as well as creating mechanistical information for the inhibition of the DIO1 enzyme. It should be noted that based on the available information regarding the used reagents and chemicals, the entire method is animal free.

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

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2. MATERIALS AND METHODS

Table 1: Used apparatus in the DIO1-SK assay

Apparatus	Requirements ¹ Suggested type ²
Analytical balance	capable of accurately weighing up to 30 g with 0.1 mg readability ¹
Pipets capable of delivering 1 to 10 μ L	
Pipets capable of delivering 10 to 100 μ L	
Pipets capable of delivering 100 to 1000 μ L	
Multichannel pipette capable of delivering 10 to 100 μ L	
Multichannel dispenser capable of delivering 50 to at least 1000 μ L	
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 \geq 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, 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
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 (NaAsO ₂) 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 ₂ PO 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 ₂ O, 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 ₂ KPO 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 ₂ SO 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 $\Delta OD-BG$ 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 ddH₂O 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 % ddH₂O 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 ₂ KPO ₄ (0.216 M)/ EDTA (2.16 mM) solution	Add 7.34 g H ₂ KPO ₄ and 201 mg Ethylenediaminetetraacetic acid (EDTA) to a 250 mL volumetric flask and add ddH ₂ O to a final volume of 250 mL.
HK ₂ PO ₄ (0.216 M) / EDTA (2.16 mM) solution (250 ml):	Add 9.41 g HK ₂ PO ₄ and 201 mg Ethylenediaminetetraacetic acid (EDTA) to a 250 mL volumetric flask and add ddH ₂ O 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 ₂ KPO ₄ / EDTA solution and HK ₂ PO ₄ / EDTA solution to reach a pH of 6.8 (ratio of HK ₂ PO ₄ / EDTA to H ₂ KPO ₄ / EDTA of about 2:1 ≈ 167 ml of HK ₂ PO ₄ / EDTA and 83 mL of H ₂ KPO 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) ₄ Ce(SO ₄) ₄ ·2H ₂ O, 0,5 M H ₂ SO ₄) (250 mL)	Add 3.95 g of (NH ₄) ₄ Ce(SO ₄) ₄ ·2H ₂ O and 125 mL of ddH ₂ O to a 250 mL volumetric flask. Add 125 mL 1 M H ₂ SO ₄ to reach a final volume of 250 mL.
Sodium arsenite solution (25 mM NaAsO ₂ , 0,8 M NaCl, 0,5 M H ₂ SO ₄) (250 ml)	Add 0.81 g of NaAsO ₂ , 11.7 g of NaCl and 125 mL of ddH ₂ O to a 250 mL volumetric flask. Add 125 mL 1 M H ₂ SO 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
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	rT3 containing falcon. Mix and use the mix on the day of preparation.
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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 ddH₂O and check if they are still fully dissolved.

7. Prepare a 10 % test item dilution in ddH₂O by diluting the highest soluble test item stock solution (generated in step 1 to 6) in ddH₂O 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 % ddH₂O 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.

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

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-thiouracil.

Name of the reference item dilution	Reference item dilution concentration [M]	ddH ₂ O [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 / ddH₂O 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.

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

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 ₂ O [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.

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

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 ₂ O [μ L]	DMSO [μ L]	Negative control [μ L]	Final concentration of negative control in the assay [M]
10^{-3}	450	-	50 μ L 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 ddH₂O 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 ₂ O [μ L]	solvent [μ L]	Test item	Dilution factor
TI1-C1	450	-	50 μ L of test item 1 stock solution	1:10
TI1-C2	405*	45*	50 μ L of TI1-C1	1:10
TI1-C3	405*	45*	50 μ L of TI1-C2	1:10
TI1-C4	405*	45*	50 μ L of TI1-C3	1:10
TI1-C5	405*	45*	50 μ L of TI1-C4	1:10
TI1-C6	405*	45*	50 μ L of TI1-C5	1:10
TI1-C7	405*	45*	50 μ L of TI1-C6	1:10
TI1-C8	405*	45*	50 μ L of TI1-C7	1:10

*You can also prepare a 10 % solvent / ddH₂O solution and add 450 μ L 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 ddH₂O 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 ₂ O [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.

<u>Time flow of the assay:</u>	
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 ddH₂O. 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 ddH₂O 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 ₂ O only									
E												
F												
G												
H												

	iodide dilutions		ddH ₂ O only		Empty
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3. Add 50 μL of cerium solution [25 mM (NH₄)₄Ce(SO₄)₄·2H₂O; 0.5 M H₂SO₄] to the iodide dilutions in the 96-well plate
4. Start the reaction by adding 50 μL of arsenite solution [25 mM NaAsO₂; 0.8 M NaCl; 0.5 M H₂SO₄] 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 ddH₂O 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 ddH₂O sample: Usually a background of $\Delta OD > 0.3$ in the pure ddH₂O 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 $\Delta OD > 1.3$.

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 (\pm 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
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Day 1:	preparation of reference item 6PTU, solvent control and microsome dilutions preparation of assay plates measurement of assay plates via Sandell-Kolthoff reaction
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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 ddH₂O (e.g. 10 % DMSO in ddH₂O). 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 ddH₂O (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

- 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
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- Place on ice to stop the reaction
- Conduct the separation of the substrate and released iodide of the assay analogous to 6.5.2.
- 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)
- Determine the ΔOD via subtracting the 21-minute values from the initial measured values
- To determine the $\Delta OD-BG$ values, subtract the inhibited ΔOD values of the reference item from the respective enzyme concentration ΔOD values of the solvent control
- Plot the $\Delta OD-BG$ values of the different enzyme concentration samples in a statistics software with $\Delta OD-BG$ on y-axis (linear) and inhibitor concentration on x-axis (logarithmic)
- Use a curve-fit algorithm to generate a function to optimally reflect assay characteristics (e.g. “[Inhibitor] vs. response -- Variable slope (four parameters)”))
- Determine the dilution factor for the samples in 10 % acetic acid that still leads to the highest possible $\Delta OD-BG$ 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 $\Delta OD > 0.5$ 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 $< 20 \mu\text{g}$ 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 $> 20 \mu\text{g}$ 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.

<u>Time flow of the assay:</u>	
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	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
PC	positive control Aurothioglucose 10^{-4} M						

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	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
PC	positive control Aurothioglucose 10^{-4} M						

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 μ L 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 μ L 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 μ L 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 $(\text{NH}_4)_4\text{Ce}(\text{SO}_4)_4 \cdot 2\text{H}_2\text{O}$; 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:
 - o Absorption parameters: 415 nm (± 2 nm)
 - o Initial shaking: medium for 2 s
 - o 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 $\Delta\text{OD-BG}$ 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 $\Delta\text{OD-BG}$ values by the mean of the solvent control $\Delta\text{OD-BG}$ values. State the normalized $\Delta\text{OD-BG}$ values in %. Keep in mind that test items with differing solvents need to be normalized to their respective solvent controls.
4. Plot the normalized $\Delta\text{OD-BG}$ values of the different test item concentration samples in a statistics software with normalized $\Delta\text{OD-BG}$ 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.

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