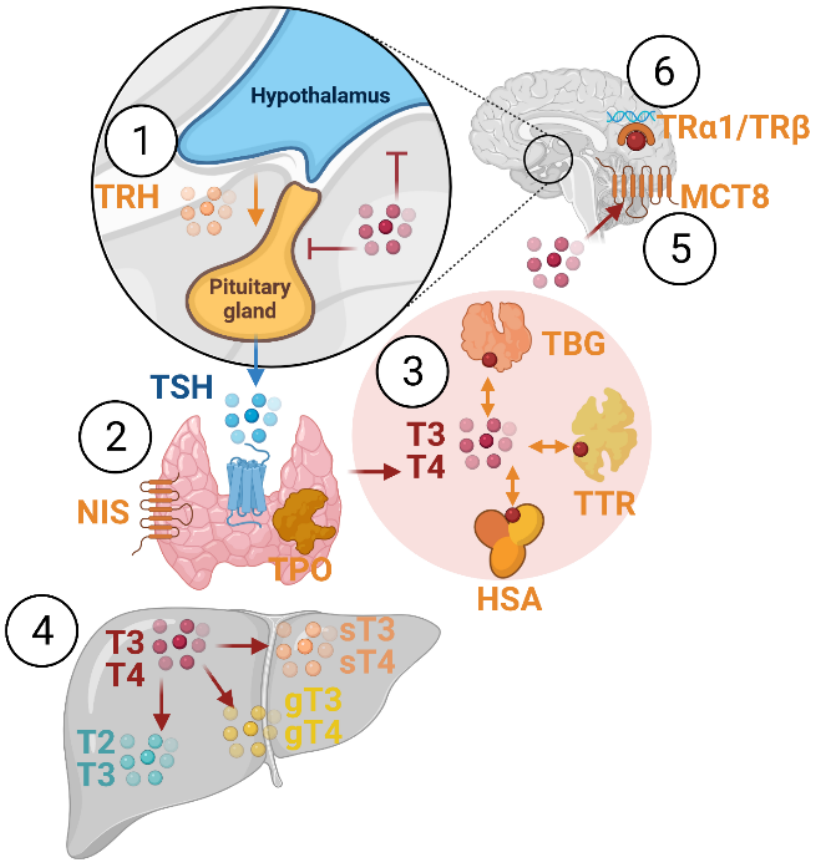
STANDARD OPERATING PROCEDURE

for the non-radioactive sodium/iodide symporter (NIS)-mediated iodide uptake assay based on Sandell-Kolthoff reaction, 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



2023

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

The SOP provided was used during Part 1 of the validation study. It cannot be excluded that later versions exist.

The method was developed by the United States-Environmental Protection Agency (US-EPA) and implemented by EU-NETVAL laboratory LabFit - HPRD Lda, Portugal, for optimisation and experimental assessment.

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1. Purpose

This SOP applies to Part 1 of the validation process of this method: the Non-Radioactive Sodium/Iodide Symporter (NIS)-Mediated Iodide Uptake Assay based on the Sandell-Kolthoff reaction.

2. Application

This method has been developed to identify test substances that alter thyroid function by disrupting the synthesis of thyroid hormones (TH). A known molecular target for TH disruption is the sodium/iodide (Na^+/I^-) symporter (NIS), an intrinsic membrane glycoprotein that is required during the initial step of TH synthesis to actively transport iodide from the blood into thyroid follicular cells (Darrouzet et al. 2014). Through this action, NIS co-transports two Na^+ cations and one I^- anion across the basolateral membrane to concentrate iodide in the thyroid gland 20-40 times greater than that in plasma.

This method is suitable to measure the active transport of extracellular iodide across the cellular membrane of cells expressing a functional sodium/iodide (Na+/I-) symporter (NIS). This non-radioactive approach follows the same biochemistry principles and experimental design of the radioactive method, which is based on the measurement of radioiodine (^{125}I) uptake into NIS-expressing cells and has been used for several decades to elucidate many of the biochemical details of iodide transport. In this method, the NIS expressing cell line Mel624-CMVhNIS-Neo (Imanis LifeScience) is placed in a sodium iodide containing solution in the presence of the substance to be tested. After the treatment period, the cells are washed, lysed, and intracellular iodide is measured with a spectrophotometric readout following the Sandell-Kolthoff (SK) reaction. By this redox reaction between cerium(IV) and arsenic(III), which is catalysed by iodide in acidic solution, the yellow cerium(IV) is reduced to the colourless cerium(III), as indicated (from Waltz et al. 2010):

$$\underset{\text{Yellow}\ \lambda_{max} = 420\ nm}{Ce^{4+}} + \tfrac{1}{2}As^{3+} \xrightarrow{\text{cat. } I^-} \underset{\text{Colorless}}{Ce^{3+}} + \tfrac{1}{2}As^{5+}$$

The extent of discolouration of the solution is proportional to the iodide concentration. The use of the SK reaction to detect NIS activity was originally described in Waltz et al. (2010) and more recently in Wu et al. (2016).

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This method is designed to identify test substances that inhibit NIS-mediated iodide uptake by directly competing with iodide for intracellular transport, or indirectly, through an inhibition of Na+/K+ ATPase that disrupts maintenance of the critical cross-membrane sodium gradient required for NIS functionality. Cells treated with the uptake buffer control containing iodide in the µM - range reflect maximal iodide uptake, while inhibitory activity is evaluated using seven serial dilutions of each test substance. In addition, a cell viability assay is conducted before the NIS assay to support the selection of concentrations that are known not to be cytotoxic for the cell model, during the same exposure period used for NIS assay.

The current protocol is optimized for short (1-2 h) chemical exposure time to detect rapid effects on and direct interaction with NIS function.

Limitations of the method

Substances that interfere with the S/K reaction –*Possible chemical interference with method readout due to contaminant iodide or interaction with S/K reaction (See page 574 & Supplemental Table 2, Hornung et al., 2018 Tox Sci. 162(2):570-581). This group has identified 10-15 additional chemicals of 700 in the ToxCast phase 2 library that may also interfere with S/K reaction, which therefore should be excluded from the analysis.*

We propose to pre-test the substances for possible interferences by placing the highest concentration in contact with the SK reagents prior to the study to check if a direct discoloration of the cerium solution is achieved.

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3. Definitions

Cell lysis – The bursting or rupturing of the cell membrane due to the osmotic movement of water into the cell. This lysis typically occurs 20 minutes to 30 minutes after addition of water to a dry well.

Cell viability assay – Assays used to determine cell survival following treatment with determined compounds.

Concentration – Content of solute in solution. The highest tested concentration is represented as "C1" and the subsequently lower concentrations are represented as "C2", "C3", "C4", etc.

Dosing plate/Treatment plate - Dosing plates are not seeded with cells. Their purpose is to prepare the plate scheme with the adequate dilutions of each substance, simplifying the downstream application step in the seeded plates, given the importance of respecting time delays during application.

IC_{50} - A drug or inhibitor concentration. The concentration of a drug or inhibitor needed to inhibit a biological process or response by 50 %.

Interference tests – Interference tests are used to determine if a test item could hinder a determined method, falsely altering an assay result.

Negative control - A negative control is an item for which the test system should not give a response.

Positive control - A positive control is an item for which the test system should give a response. It can be the same chemical as the reference item.

Reference item – Reference items are used to provide a basis for comparison with the test item or to validate the response of the test system to the test item. The purpose of the reference item(s) is to grade the response of the test system to the test item (in absolute or relative terms – it sets the responsiveness of the test system).

Replicates - A representative number from a group to undergo the same procedure and the same conditions of the assay conditions, performed on the same day.

Test item - Sample that is undergoing testing.

Untreated control – Wells with Uptake Buffer

Vehicle/solvent control - This control assures a response does not originate from the applied solvent used to dissolve the test item.

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4. Responsibilities

It is the responsibility of each laboratory technician involved in the execution of the test to comply with the procedure. Responsibilities of the Study Director, Quality Assurance and other personnel involved are in accordance with OECD GLP Principles.

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5. Procedure

5.1 . Apparatus

5.1.1 Freezer -20 °C

5.1.2 Freezer -80 °C

5.1.3 Refrigerator at 4 (± 2) °C

5.1.4 Precision balance (0.1 g)

5.1.5 Analytical balance (0.0001 g)

5.1.6 Type 1 Ultrapure Water System

5.1.7 CO_2 incubator at 37 ˚C, 5 % CO_2 and 95 % RH

5.1.8 Class II (laminar flow) biological safety cabinet

5.1.9 37 ˚C water bath

5.1.10 Pipette Aid

5.1.11 Pipettor p1000

5.1.12 Pipettor p200

5.1.13 Pipettor p20

5.1.14 Positive displacement pipette (to be used with viscous test items)

5.1.15 Multi-channel pipettor 8 wells (p300 µL) / *96 well pipettor*

5.1.16 Multi-channel pipettor 8 wells (p10 µL)

5.1.17 pH meter

5.1.18 Vacuum pump

5.1.19 Centrifuge

5.1.20 Stopwatch (for sample application time delay; to control the SK reaction time for reading; to control cytotoxicity incubation periods)

5.1.21 Neubauer chamber

5.1.22 Inverted microscope

5.1.23 Spectrophotometer microplate reader (filter to measure absorbance at 405 nm and 560 ± 10 nm)

5.1.24 Stir Plate

5.1.25 Pipettor p10 000

5.1.26 Pipettor p5 000

5.2 Materials

5.2.1 Flask (T25)

5.2.2 Flask (T75)

5.2.3 Flask (T175)

5.2.4 96-well plates

5.2.5 Serological pipettes 5 mL

5.2.6 Serological pipettes 10 mL

5.2.7 Serological pipettes 25 mL

5.2.8 Serological pipettes 50 mL

5.2.9 Sterile pipette tips 20 µL

5.2.10 Sterile pipette tips 200 µL

5.2.11 Sterile pipette tips 1000 µL

5.2.12 Syringe 6 mL

5.2.13 Syringe 50 mL

5.2.14 Conical tubes 15mL

5.2.15 Conical tubes 50mL

5.2.16 Centrifuge tubes 1.7 mL

5.2.17 Sterilizing 0.22 µm filters

5.2.18 Absorbent paper

5.2.19 Aluminium foil

5.2.20 Usual laboratory equipment

5.3 Reagents

5.3.1 Fetal bovine serum (FBS)

5.3.2 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; CAS No. 7365-45-9)

5.3.3 Hanks' Balanced Salt Solution (HBSS) without phenol red

5.3.4 Sodium iodide (CAS No. 7681-85-5) used as solution

5.3.5 Ammonium cerium (IV) sulfate hydrate (CAS No. 10378-47-9)

5.3.6 Sodium arsenite (III) (CAS No. 1327-53-3)

5.3.7 Sodium chloride (NaCl) (CAS No. 7647-14-5)

5.3.8 Sodium hydroxide (NaOH) (CAS No. 1310-73-2)

5.3.9 Dimethyl sulfoxide (DMSO) (CAS No. 67-68-5)

5.3.10 Sulfuric acid (H_2SO_4) (CAS No. 7664-93-9)

5.3.11 DMEM Low Glucose

5.3.12 Sodium bicarbonate (CAS No. 144-55-8)

5.3.13 Penicillin/Streptomycin

5.3.14 Trypsin-EDTA 0.05%

5.3.15 PBS 1X

5.3.16 Thiazolyl Blue Tetrazolium Bromide (CAS No. 298-93-1)

5.3.17 Sodium Bicarbonate (CAS No. 144-55-8)

5.3.18 G418 (CAS No. 108321-42-2)

5.3.19 Sodium Iodide (NaI) solution

5.3.20 Isopropanol (CAS No. 67-63-0)

5.3.21 Sodium Dodecyl Sulfate (SDS) (CAS No.151-21-3)

5.4 Reference Item

5.4.1. Sodium perchlorate hydrate ($NaClO_4$) (CAS No. CAS 7601-89-0)

5.5 Control items

5.5.1. Positive (i.e., inhibit iodide uptake thorough NIS)

5.5.1.1. Sodium perchlorate hydrate ($NaClO_4$) (CAS No. 7601-89-0)

5.5.2. Negative (i.e., no effects on iodide uptake thorough NIS)

5.5.2.1. Sodium fluoride (NaF) (CAS No. 7681-49-4)

5.5.3. Solvents

5.5.3.1. Ultrapure Type 1 water (for aqueous soluble test substances)

5.5.3.2. Dimethyl sulfoxide (DMSO) (CAS No. 67-68-5) (for non-aqueous soluble test substances)

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5.6 Test System

The test system described for this procedure is the Imanis cell line expressing NIS (Mel624-CMVhNIS-Neo). Mel624-hNIS-Neo is a monoclonal population of the human malignant melanoma cell line Mel624. Parental Mel624 cells were transduced with LV-hNIS-Neo (LV013), selected using antibiotic G418, and amplified from a single cell to achieve stable reporter expression in the monoclonal population. LV-hNIS-Neo encodes the human sodium iodide symporter (hNIS) cDNA linked to the neomycin resistance gene (Neo) via an internal ribosomal entry site (IRES) under the spleen focus-forming virus (SFFV) promoter.

Below, some cell line information and general culturing procedures are provided.

5.6.1. Description

Species	Human (*Homo sapiens*)
Tissue	Skin
Cell type	Melanoma
Parental cells	Mel624
Morphology	Epithelial
Growth mode	Adherent
Reporter gene	Human sodium iodide symporter (hNIS)
Selection gene	Neomycin (Neo)

5.6.2. Biosafety

BSL2

These cells were generated via lentiviral vector transduction. The lentiviral vector used for transduction was a self-inactivating (SIN) vector in which the viral enhancer and promoter have been deleted. Transcription inactivation of the LTR in the SIN provirus increases biosafety by preventing mobilization by replication-competent viruses and enables the regulated expression of the genes from the internal promoters without *cis*-acting effects of the LTR1. Nevertheless, trained personnel should perform all work with these cells under biosafety-level 2 (BSL2) conditions. Institutional requirements may permit handling of these cells under BSL1 conditions if certain criteria are met.

5.6.3. Growth Medium

- Dulbecco's Modified Eagle's Medium (DMEM; 5.3.11)
- Sodium Bicarbonate (5.3.12)
- 10 % Fetal bovine serum (FBS; 5.3.1)
- 1 % Penicillin/Streptomycin (5.3.13)
- 0,4 mg/mL G418 (5.3.18)

Note 1: *G418 should NOT be added to the medium until a culture has been well established from the thawed cells (about 1 week). It is also recommended that a backup frozen cell stock be generated (see below) before adding G418 to the growth medium.*

*Note 2: For Incomplete growth media do not add any FBS (*5.3.1) *nor Penicillin/streptomycin (*5.3.13).

5.6.4. Thawing Instructions

- Thaw cells by gently swirling in a 37 °C ± 1 °C water bath. To limit contamination, do not submerge the O-ring and cap.
- When cells are approximately 70 % thawed (approximately 1 min), remove the vial and wipe down with 70 % ethanol. Allow tube to dry completely.
- In a biosafety cabinet, transfer the cells into a 15 mL conical tube containing 5 mL of pre-warmed complete growth medium. Centrifuge cells at approximately 250 x g for 3-5 min.
- Remove supernatant and resuspend cells in 1 mL complete growth medium. Transfer cells to a T75 flask containing 10 mL pre-warmed complete growth medium.
- Incubate the culture at 37°C ± 1°C with 5 % CO_2. Cells should reach full confluency 1-2 days after thawing.

5.6.5. Subculturing Instructions

Volumes are given for a T75 flask. Increase or decrease as needed.

- Remove culture medium from cells.
- Carefully wash the cell monolayer with 5-10 mL of phosphate buffered saline.
- Add 5 mL of 0,25 % Trypsin-EDTA solution to the flask and incubate until cells have dissociated (approx. 2-5 min).
- Neutralize the trypsin by adding 5 mL complete growth medium and mix by gently pipetting up and down.
- Add fresh complete growth medium to a new T-flask, about 13 mL, transfer the desired portion of the cells and return cells to 37°C ± 1 °C, 5 % CO_2 incubator.

For maintenance, a sub-cultivation ratio of 1:10 is recommended. At this ratio cells will be ready for passage approximately every 3-4 days.

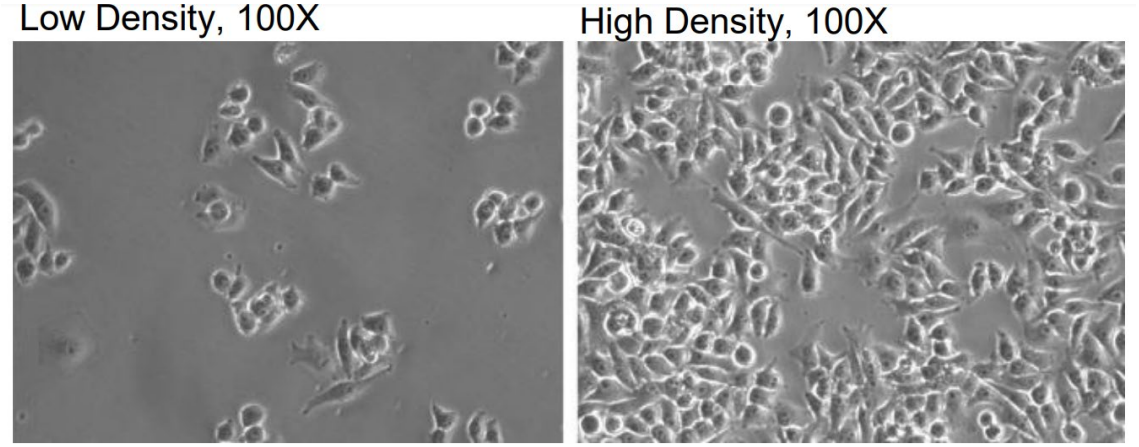
5.6.6. Freezing Medium

Cells can be amplified and used to generate additional frozen stocks. Frozen stocks should be preserved in a designated cryopreservation medium or in complete growth medium without G418 supplemented with 5-10 % DMSO.

5.6.7. Storage temperature

Liquid nitrogen vapor phase.

5.6.8. Morphology



Source : https://imanislife.com/products/mel624-cmv-hnis-neo/#!

5.7 Solutions

5.7.1. Uptake buffer

- In the Biological Safety Cabinet (5.1.8), measure 100 mL (5.2.8 and 5.1.10) of HBSS (5.3.3) into a 1 L container.
- Weight 2,383 g (5.1.5) of HEPES (5.3.2) and allow it to dissolve in the previously measured HBSS, with agitation (5.1.24).
- Add approximately 800 mL of Ultrapure water (5.1.6) and adjust the solution pH (5.1.17) to 7,4.
- Using a volumetric flask (5.2.20) complete the volume with ultrapure water (5.1.6) to make up 1 L.
- Back in the Biological Safety Cabinet (5.1.8), filter the solution through a 0,2 µm filter (5.2.17) using a vacuum pump (5.1.18).

Note 1: This solution should be prepared freshly on the day of the assay.

Note 2: If volumes must be adjusted, prepare no less than 250 mL of uptake buffer for an assay (one control plus one test item plate and one test items plate - two test items), and consider increasing the volume by 150 mL for each two extra test items tested. Please keep in mind that if a viability assay is to be conducted in parallel prepare no less than 500 mL for an assay (one control plus one test item plate and one test items plate - two test items, in duplicate – assay + viability), and consider increasing the volume by 100 mL for each two extra test items tested in duplicate (assay + viability).

5.7.2. Ammonium cerium (IV) sulfate solution 4x (42 mM)

- Dissolve 12,53 g of ammonium cerium (IV) sulfate hydrate (5.3.5) in 200 mL ultrapure water (5.1.6).
- Add 50 mL of concentrated H_2SO_4 (5.3.10) to the solution, which is cooled with an ice bath.
- After cooling, the solution is diluted to 500 mL with ultrapure water (5.1.6).
- Dilute the solution four-fold (final concentration 10,5 mM) with ultrapure water (5.1.6) prior to use.

Note 1: For best results, leave this solution to stand at 4 °C ± 1 °C for 1 week before first use. Store the solution in the dark at 4 °C ± 1 °C (5.1.3) for up to 6 months (with no loss of activity).

Note 2: From the stock solution at 42 mM (5.3.5)

$$Vi = \frac{10{,}5\ mM \times 50\ mL}{42\ mM} \Leftrightarrow$$

$$Vi = \ 12{,}5\ mL\ (5.2.7\ and\ 5.1.10)\ Ammonium\ cerium$$

$$+\ 37{,}5\ mL\ (5.2.8\ and\ 5.1.10) Ultrapure\ water (5.1.6)$$

Enough for the SK reaction in 8 plates (0,1 mL per well / 60 wells per plate).

5.7.3. Sodium arsenite(III) solution 4x (96 mM)

For safety reasons, it is highly recommended to use a commercial standard stock solution: Sodium arsenite solution 0,05 mol/l (0,1 N) Titripur® Reag. USP (this will be the solution used throughout Part 1).

If powder manipulation cannot be avoided, please refer to the safety data sheets of the reagents for handling and storage instructions, as well as personal protective equipment and disposal recommendations.

- Dissolve 4,75 g (5.1.3.) of arsenic(III) oxide (5.3.6) and 24 g (5.1.4) of NaCl (5.3.7) in 50 mL of 2M NaOH (5.3.8).
- Bring the volume to 500 ml with ultrapure water (5.1.6). Centrifuge (5.1.19) to remove insoluble material.
- Store the solution in the dark at room temperature (15 °C – 25 °C) for up to 6 months (with no loss of activity).
- Dilute the solution fourfold (final concentration 24 mM) with ultrapure water (5.1.6) prior to use.

Note 1: Sodium arsenite used in SK reaction could induce cancers in multiple organs and is classified as carcinogenic by International Agency for Research on Cancer of (IARC). Therefore, caution should be taken and relevant regulations should be abided during the assay process.

Note 2: From the commercial solution at 50 mM (5.3.6)

$$Vi = \frac{24\ mM \times 50\ mL}{50\ mM} \Leftrightarrow$$

$$Vi = 24\ mL\ (5.2.7\ and\ 5.1.10)\ Arsenite + 26\ (5.2.8\ and\ 5.1.10)\ mL\ Ultrapure\ water (5.1.6)$$

Enough for the SK reaction in 8 plates (0,1 mL per well / 60 wells per plate).

5.7.4. MTT

- Dissolve 50 mg (5.1.5) of MTT (5.3.16) in 10 mL of 1X PBS Solution (5.3.15).
- Sterilize by filtration with a 0,22 µm filter (5.2.17).

Note 1: Please handle it safely, taking into account that it has a mutagenic and carcinogenic potential. Do not expose to light as it is photosensitive, or excessive heat. The tube must be protected with aluminium foil (5.2.19) and work conducted with the lights of the laminar flow chamber turned off.

Note 2: This Solution can be stored frozen at -20 °C (5.1.1) for up to one year.

Note 3: MTT solution in incomplete medium (1 mg/mL): From the stock solution of MTT (5 mg/mL; 5.7.4) in 1X PBS (5.3.15), prepare a solution of MTT in incomplete culture medium with a concentration of 1 mg/mL.

For 10 mL: add 2 mL (5.1.25) of solution (5.7.4) to 8 mL (5.1.25) of incomplete culture medium (5.6.3). This solution is sterilized by filtration using 0,22 µm syringe filters (5.2.17 and must be used in the same day.
Enough for 3 plates (0.05 mL per well / 60 wells per plate). Adjust volumes if necessary for a larger number of test items .

5.7.5. SDS

To be used as a viability control (C+) in the viability assays.
Note: For 5 mL, add 0,5 mL (5.1.11) of SDS at 20 % to 4,5 mL (5.1.26) of sterile ultrapure water (5.1.6). This solution should be used fresh in the day of the assay.

5.7.6. NaI solutions

- Dissolve 55,7 mg (5.1.4) of NaI (5.3.19) in ultrapure water (5.1.6) to make a 40 mM stock solution.

Note 1: for the preparation of this stock solution, please consider the purity of the reagent used, and make any adjustments needed in order to ensure the accuracy of the final concentration.
Note 2: Store this solution in the dark at room temperature (15 °C – 25 °C) for up to 2 months

5.7.6.1. Preparation of NaI at 100 µM

To be used in the addition of NaI to each well (described in 5.8.2 and 5.8.3.2)

2 mM	0,5 mL of **40 mM** + 9,5 mL of ultrapure water
100 µM	0,5 mL of **2 mM** + 9,5 mL of ultrapure water

5.7.6.2. Iodide standards

Prepare a solution of NaI at 20 µM from a solution of 2 mM (preparation previously described).

20 µM	0,1 mL of **2 mM** + 9,9 mL of ultrapure water

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Prepare the solutions for the calibration curve from 100 nM to 700 nM according to the following table:

Table 1 - Iodide standards dilutions.

Final Concentration (nM)	NaI (20 µM) volume (µL)	H_2O type I volume	Final volume (mL)
100	50	9 mL + 950 µL	10
200	100	9 mL + 900 µL	10
300	150	9 mL + 850 µL	10
400	200	9 mL + 800 µL	10
500	250	9 mL + 750 µL	10
600	300	9 mL + 700 µL	10
700	350	9 mL + 650 µL	10

Enough for 100 assays (considering one calibration plate with 0,1 mL per well)

Note 1: Store these solutions in the dark at room temperature (15 °C – 25 °C) for up to 2 months.

5.7.7. Reference and control Items

5.7.7.1. Sodium perchlorate solution – Reference and positive control

Dissolve $NaClO_4$ (5.4.1) in ultrapure water (5.1.6) at a 100 mM stock solution suitable to prepare the following working concentrations: 0,10 µM; 0,32 µM; 1 µM; 3,16 µM; 10 µM; 31,62 µM; 100 µM (full dose-response to be included in one plate of each assay for the evaluation of assay performance).

Note 1: for the preparation of this stock solution, please consider the purity of the reagent used, and make any adjustments needed in order to ensure the accuracy of the final concentration.

Note 2: Store the stock solution in the dark at room temperature (15 °C – 25 °C) for up to 2 months.

Note 3: The positive control corresponds to the highest concentration of the reference.

Note 4: $NaClO_4$ – Preparation of the dilutions from the 100 mM stock

Take into consideration that preparation of intermediate and work solutions takes into account the posterior addition of NaI to the well. Therefore, to prepare the full dose-response mentioned above the real concentrations needs to be 0,11 µM; 0,35 µM; 1,11 µM; 3,51 µM; 11,11 µM; 35,12 µM; 111,10 µM.

Intermediate Solutions (100 % solvent)		Work Solutions (Uptake Buffer + 1 % Solvent)	
20 mM	0,10 mL of **100 mM** + 0,40 mL of solvent	NA	NA
11,11 mM	0,28 mL of **20 mM** + 0,22 mL of solvent	111,10 µM	0,03 mL of **11,11 mM** + 2,97 mL Uptake Buffer
3,51 mM	0,16 mL of **11,11 mM** + 0,34 mL of solvent	35,13 µM	0,03 mL of **3,51 mM** + 2,97 mL Uptake Buffer
1,11 mM	0,16 mL of **3,51 mM** + 0,34 mL of solvent	11,11 µM	0,03 mL of **1,11 mM** + 2,97 mL Uptake Buffer
0,35 mM	0,16 mL of **1,11 mM** + 0,34 mL of solvent	3,51 µM	0,03 mL of **0,35 mM** + 2,97 mL Uptake Buffer
0,11 mM	0,16 mL of **0,35 mM** + 0,34 mL of solvent	1,11 µM	0,03 mL of **0,11 mM** + 2,97 mL Uptake Buffer
0,04 mM	0,16 mL of **0,11 mM** + 0,34 mL of solvent	0,35 µM	0,03 mL of **0,04 mM** + 2,97 mL Uptake Buffer
0,01 mM	0,16 mL of **0,04 mM** + 0,34 mL of solvent	0,11 µM	0,03 mL of **0,01 mM** + 2,97 mL Uptake Buffer

5.7.7.2. Sodium fluoride solution - Negative control

Dissolve 62,4 mg of NaF (5.5.2.1) in 14,86 mL of ultrapure water (5.1.6) in order to obtain a stock solution (100 mM) suitable to prepare the following working concentrations: 0,11 µM; 0,35 µM; 1,11 µM; 3,51 µM; 11,11 µM; 35,12 µM; 111,10 µM; (full dose-response to be included in one plate of each assay for the evaluation of assay performance).

Note 1: for the preparation of this stock solution, please consider the purity of the reagent used, and make any adjustments needed in order to ensure the accuracy of the final concentration.

Note 2: Store the stock solution in the dark at room temperature (15 °C – 25 °C) for up to 2 months.

Note 3: NaF – Preparation of the dilutions from the 100 mM stock

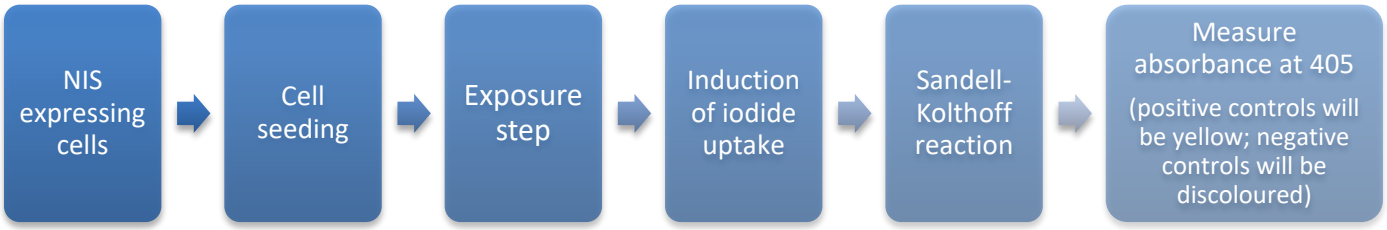
Take into consideration that preparation of intermediate and work solutions takes into account the posterior addition of NaI to the well. Therefore, to prepare the full dose-response mentioned above the real concentrations needs to be 0,11 µM; 0,35 µM; 1,11 µM; 3,51 µM; 11,11 µM; 35,12 µM; 111,10 µM.

Intermediate Solutions (100 % solvent)		Work Solutions (Uptake Buffer + 1 % Solvent)	
20 mM	0,10 mL of **100 mM** + 0,40 mL of solvent	NA	NA
11,11 mM	0,28 mL of **20 mM** + 0,22 mL of solvent	111,10 µM	0,03 mL of **11,11 mM** + 2,97 mL Uptake Buffer
3,51 mM	0,16 mL of **11,11 mM** + 0,34 mL of solvent	35,13 µM	0,03 mL of **3,51 mM** + 2,97 mL Uptake Buffer
1,11 mM	0,16 mL of **3,51 mM** + 0,34 mL of solvent	11,11 µM	0,03 mL of **1,11 mM** + 2,97 mL Uptake Buffer
0,35 mM	0,16 mL of **1,11 mM** + 0,34 mL of solvent	3,51 µM	0,03 mL of **0,35 mM** + 2,97 mL Uptake Buffer
0,11 mM	0,16 mL of **0,35 mM** + 0,34 mL of solvent	1,11 µM	0,03 mL of **0,11 mM** + 2,97 mL Uptake Buffer
0,04 mM	0,16 mL of **0,11 mM** + 0,34 mL of solvent	0,35 µM	0,03 mL of **0,04 mM** + 2,97 mL Uptake Buffer
0,01 mM	0,16 mL of **0,04 mM** + 0,34 mL of solvent	0,11 µM	0,03 mL of **0,01 mM** + 2,97 mL Uptake Buffer

5.8 Assay description and workflow

$NaClO_4$ is a well-documented competitive inhibitor of NIS-mediated iodide uptake with an IC_{50} value of approximately 0,2 µM in other methods. Therefore, it is used as a reference for the NIS assay (e.g., a full dose-response is included on each assay (0,10 µM; 0,32 µM; 1 µM; 3,16 µM; 10 µM; 31,62 µM; 100 µM), with the highest concentration (100 µM) used as a measurement of background signal, i.e., the signal that corresponds to the level of iodide taken up by the cells when NIS is totally inhibited).

A cell viability assay is to be performed prior to the NIS assay to assure that the tested concentrations are not cytotoxic to the cells (causing a decrease in iodide uptake) in the experimental conditions used for NIS assay. Chemical concentrations and exposure time are to be consistent for both NIS and cell viability assays. Concentrations that produce a reduction in cell viability by > 20% (relative to solvent controls) are typically considered cytotoxic.



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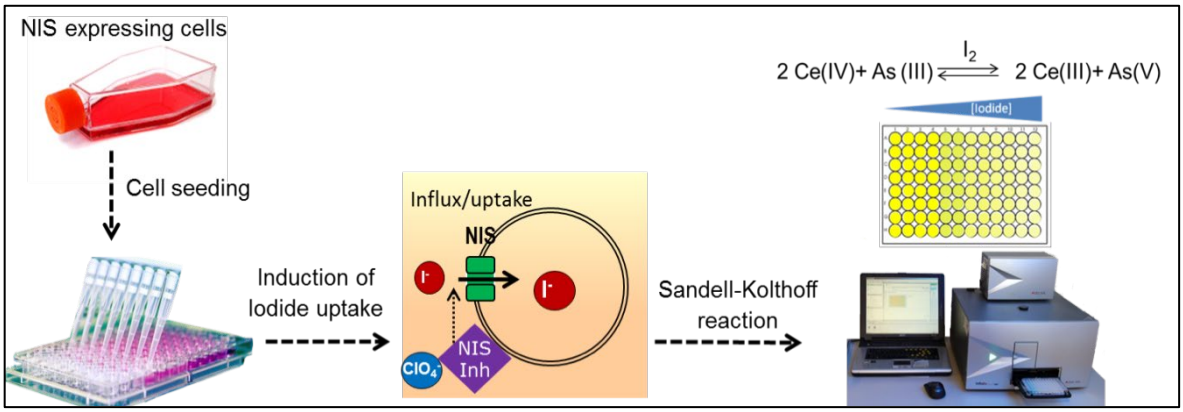

Figure 1. Workflow of sodium Iodide uptake assay.

5.8.1. Pre-tests

5.8.1.1. Solubility determination and range finder

Conduct preliminary tests to determine the limit of solubility for each test substance in water or DMSO based upon the presence of cloudiness, colour or precipitate under a microscope. A reasonable starting concentration for stock solutions is 20 mM (maximum concentration of DMSO in the assay well should be limited to 1 %). If other solvent is used, cytotoxicity shall be previously studied.

Range finder testing consists of a solvent control along with seven, serial dilutions of the test item using dilution factor half-log, starting at 20 mM, or lower, based upon the limit of solubility, and noting the presence of cloudiness, colour or precipitate.

5.8.1.2. Interference test – SK reaction

To determine interference with the SK reaction, 100 µL of the highest concentration of the test substance will be added to a row of wells of a seeded 96 well-plate. Ultrapure water will be used as control. To each of the occupied wells 100 µL of each of the SK reaction reagents will be added and incubated for 20 minutes in the dark. After the incubation period, absorbance at 405 nm will be recorded using a spectrophotometer with a plate reader and results evaluated in comparison with the control.

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5.8.1.3. Interference test – MTT test

To determine interference with the MTT for the viability assay, in a microtube 100 µL of the highest concentration of the test substance will be added to 50 µL of the MTT 1 mg/mL and incubated for 2 hours ± 5 minutes (37 ± 1 °C, 5 ± 1 % CO2). Untreated MTT medium is used as control. If the MTT solution turns blue/purple, the test substance reduces MTT.

5.8.1.4. Viability assay

A viability test must be conducted in order to ensure that the concentrations used in the assay are not cytotoxic to the test system. This way, any inhibitory effect that might be observed can accurately be attributed to the sample's ability to disrupt the NIS transporter, rather than cell loss due to the sample cytotoxicity. Therefore, the viability assay shall be conducted as a pre-test, under the same conditions as the measurement of intracellular iodide content using the Sandell-Kolthoff reaction (adding the NaI solution to the medium and keeping the contact with the cells for 60 min), as a way to determine which concentration should effectively be tested for NIS-mediated iodide uptake inhibition. In case the substance has revealed cytotoxicity in the pre-test a range of concentrations starting from the first that is not cytotoxic shall be tested.

The viability test should then be repeated in parallel with the assay. Viability tests described in this procedure will be performed through MTT but other methods could also be applied (e.g., Cell Titer Glo).

- Seed Imanis hNIS cells (5.6) in a 96-well plate (5.2.4), assay plate, at a cell density (5.1.21) of 10 000/well to reach 80-90 % confluency after 48 h. Observe cell confluency under an inverted microscope (5.1.22).
- After the incubation period, remove the culture medium and replace it with uptake buffer (5.7.1) at 15 – 25 °C, washing 3 times via repeated aspiration/dispense steps (3x200 µl) (5.1.15). From this point on, the frame of the plate (Line A and H, column 1 and column 12) should not be manipulated until mentioned otherwise.
- Prepare concentration dilutions of positive and negative controls (5.7.7) and test items to be studied in uptake buffer (5.7.1).
- *Note 1: Although not required, we suggest the preparation of dosing plates (see 7.1).*
- *Note 2: Take into consideration that preparation of intermediate and work solutions needs to take into account the posterior addition of NaI to the well*

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- Using only 6 channels of the multichannel pipette (5.1.15), remove the content of a single column of the assay plate (seeded and washed with uptake buffer (5.7.1) and immediately replace it with the solutions pre-diluted in the corresponding column of dosing plate A (7.1.1).
- Repeat this process for each column, respecting a time delay (e.g., 30 seconds; 5.1.20) between each column. When all columns have been dosed, apply 10 µL (5.1.16) of the NaI solution (from a single column of the dosing plate B (7.1.2), once again respecting the same time delay (5.1.20) between each column.

Note: The delay time can be adjusted according to each operator's expertise and comfort, but it must be coherent throughout the whole assay. 96-Well pipettors can also be used.

- Leave the assay plate to stand in the dark (covered in aluminium foil; 5.2.19) at 37 °C ± 1 °C for 60 min (5.1.20).
- After the incubation period, using only 6 channels of the multichannel pipette (5.1.15), wash the assay plate 2 times with 200 µl (5.1.15) uptake buffer (5.3.3) to remove extracellular NaI (5.3.19) not taken up into the cells, following the same time delay (5.1.20) as described before.
- Discard medium of the frame of plate with a multichannel pipette.
- Discard residual supernatant by inverting the assay plate on absorbent paper (5.2.18). and taping the plate twice onto the paper.
- Turn the plates back up and apply 50 µL (5.1.15) of MTT (1 mg/mL;5.7.4).
- Again, cover the plates in aluminium foil (5.2.19) and incubate for 2 hours (5.1.20) at 37 °C, 5 % CO_2 (5.1.7).
- Using only 6 channels of the multichannel pipette (5.1.15), remove all the MTT 1 mg/mL (5.7.4) and add 100 µL (5.1.15) of isopropanol (5.3.20) to each well. Mix thoroughly by pipetting up & down.
- Record absorbance at 560 ± 10 nm using a spectrophotometer with plate reader (5.1.23)

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5.8.2. NaI calibration curve without cells

- In a 96-well plate without cells, add 100 µL of each concentration of NaI (prepared in 5.7.6.2), following the scheme of Table 2.
- Add 100 µL (5.1.15) of ammonium cerium (IV) sulfate solution at 10.5 mM (5.7.2.) and 100 µl (5.1.15) of the sodium arsenite solution at 24 mM (5.7.3) to attain a final volume of 300 µl/well.
- Incubate in the dark (covered in aluminium foil (5.2.19) at room temperature (15 °C – 25 °C) for 20 minutes (5.1.20), record absorbance at 405 nm using a spectrophotometer with plate reader (5.2.4).

Note: It is suggested to perform this calibration at the beginning of the assay, as it will confirm all reagents of the SK reaction are responding as expected, as well as the spectrophotometer. The incubation period can be used to prepare the dilutions and dosing plates for the assay with study items.

Table 2 - Calibration Plate - Calibration curve of NaI without cells (7 concentrations, 3 replicates)

	1	2	3	4	5	6	7	8	9	10	11	12
A	NaI 700 nM	NaI 700 nM	NaI 700 nM									
B	NaI 600 nM	NaI 600 nM	NaI 600 nM									
C	NaI 500 nM	NaI 500 nM	NaI 500 nM									
D	NaI 400 nM	NaI 400 nM	NaI 400 nM									
E	NaI 300 nM	NaI 300 nM	NaI 300 nM									
F	NaI 200 nM	NaI 200 nM	NaI 200 nM									
G	NaI 100 nM	NaI 100 nM	NaI 100 nM									
H	H_2O	H_2O	H_2O									

The Calibration plate (Table 2) is used to assess the calibration curve without cells. It is recommended to perform this plate in the same day as the main assay, as it is a quality control of the preparation of the reagents and/ or the equipment.

5.8.3. Induction of Iodide uptake

5.8.3.1. Plate Layouts

In the end, if it is intended to test two test items, the main assay will generate 4 plates (one for SK reaction of control and its respective cytotoxicity determination; and for test item plate - one for SK reaction and one for cytotoxicity determination). The plates represented below are those already seeded with cells.

Table 3 - Control Items (SK reaction)

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		$NaClO_4$ C1	$NaClO_4$ C2	$NaClO_4$ C3	$NaClO_4$ C4	$NaClO_4$ C5	$NaClO_4$ C6	$NaClO_4$ C7				
C		$NaClO_4$ C1	$NaClO_4$ C2	$NaClO_4$ C3	$NaClO_4$ C4	$NaClO_4$ C5	$NaClO_4$ C6	$NaClO_4$ C7				
D		$NaClO_4$ C1	$NaClO_4$ C2	$NaClO_4$ C3	$NaClO_4$ C4	$NaClO_4$ C5	$NaClO_4$ C6	$NaClO_4$ C7	UC	UC	UC	
E		NaF C1	NaF C2	NaF C3	NaF C4	NaF C5	NaF C6	NaF C7				
F		NaF C1	NaF C2	NaF C3	NaF C4	NaF C5	NaF C6	NaF C7				
G		NaF C1	NaF C2	NaF C3	NaF C4	NaF C5	NaF C6	NaF C7				
H												

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Table 4 - Control Items (cytotoxicity assay)

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		$NaClO_4$ C1	$NaClO_4$ C2	$NaClO_4$ C3	$NaClO_4$ C4	$NaClO_4$ C5	$NaClO_4$ C6	$NaClO_4$ C7				
C		$NaClO_4$ C1	$NaClO_4$ C2	$NaClO_4$ C3	$NaClO_4$ C4	$NaClO_4$ C5	$NaClO_4$ C6	$NaClO_4$ C7				
D		$NaClO_4$ C1	$NaClO_4$ C2	$NaClO_4$ C3	$NaClO_4$ C4	$NaClO_4$ C5	$NaClO_4$ C6	$NaClO_4$ C7	UC	UC	UC	
E		NaF C1	NaF C2	NaF C3	NaF C4	NaF C5	NaF C6	NaF C7				
F		NaF C1	NaF C2	NaF C3	NaF C4	NaF C5	NaF C6	NaF C7				
G		NaF C1	NaF C2	NaF C3	NaF C4	NaF C5	NaF C6	NaF C7	**SDS 2%**	**SDS 2%**	**SDS 2%**	
H												

Note: Cytotoxicity of controls items will only be performed in Part 1. Afterwards only the test items cytotoxicity will be performed with the highest concentration of controls in that plate (see Table 6).

Table 5 - Test items plate (SK reaction)

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		Test item 1 C1	Test item 1 C2	Test item 1 C3	Test item 1 C4	Test item 1 C5	Test item 1 C6	Test item 1 C7	SC 1	SC 1	SC 1	
C		Test item 1 C1	Test item 1 C2	Test item 1 C3	Test item 1 C4	Test item 1 C5	Test item 1 C6	Test item 1 C7	SC 2	SC2	SC2	
D		Test item 1 C1	Test item 1 C2	Test item 1 C3	Test item 1 C4	Test item 1 C5	Test item 1 C6	Test item 1 C7	UC	UC	UC	
E		Test item 2 C1	Test item 2 C2	Test item 2 C3	Test item 2 C4	Test item 2 C5	Test item 2 C6	Test item 2 C7	$NaClO_4$ C1	$NaClO_4$ C1	$NaClO_4$ C1	
F		Test item 2 C1	Test item 2 C2	Test item 2 C3	Test item 2 C4	Test item 2 C5	Test item 2 C6	Test item 2 C7	NaF C1	NaF C1	NaF C1	
G		Test item 2 C1	Test item 2 C2	Test item 2 C3	Test item 2 C4	Test item 2 C5	Test item 2 C6	Test item 2 C7				
H												

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Table 6 - Test items plate (cytotoxicity assay)

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		Test item 1 C1	Test item 1 C2	Test item 1 C3	Test item 1 C4	Test item 1 C5	Test item 1 C6	Test item 1 C7	SC 1	SC 1	SC 1	
C		Test item 1 C1	Test item 1 C2	Test item 1 C3	Test item 1 C4	Test item 1 C5	Test item 1 C6	Test item 1 C7	SC 2	SC2	SC2	
D		Test item 1 C1	Test item 1 C2	Test item 1 C3	Test item 1 C4	Test item 1 C5	Test item 1 C6	Test item 1 C7	UC	UC	UC	
E		Test item 2 C1	Test item 2 C2	Test item 2 C3	Test item 2 C4	Test item 2 C5	Test item 2 C6	Test item 2 C7	$NaClO_4$ C1	$NaClO_4$ C1	$NaClO_4$ C1	
F		Test item 2 C1	Test item 2 C2	Test item 2 C3	Test item 2 C4	Test item 2 C5	Test item 2 C6	Test item 2 C7	NaF C1	NaF C1	NaF C1	
G		Test item 2 C1	Test item 2 C2	Test item 2 C3	Test item 2 C4	Test item 2 C5	Test item 2 C6	Test item 2 C7	**SDS 2%**	**SDS 2%**	**SDS 2%**	
H												

5.8.3.2. Method

- Seed Imanis hNIS cells (5.6) in a 96-well plate (5.2.4), assay plate, at a cell density (5.1.21) of 10 000/well to reach 80-90 % confluency after 48 h. Observe cell confluency under an inverted microscope (5.1.22).
- After the incubation period, remove the culture medium and replace it with uptake buffer (5.7.1) at 15 – 25 °C, washing 3 times via repeated aspiration/dispense steps (3x200 µl) (5.1.15). From this point on, the frame of the plate (Line A and H, column 1 and column 12) should not be manipulated until mentioned otherwise.
- Prepare concentration dilutions of positive and negative controls (5.7.7) and test items to be studied in uptake buffer (5.7.1), previously determined in the range finding assay.

Note 1: Although not required, we suggest the preparation of dosing plates (see 7.1) following the scheme of the final plate (5.8.3.1). Each well should have at least 250 µL of the substance. This procedure will decrease the risk of dosing errors.

Note 2: Take into consideration that preparation of intermediate and work solutions needs to take into account the posterior addition of NaI to the well

Note 3: Although cytotoxicity is performed previously to identify the concentration range to be tested, a viability assay should be performed in parallel (independent plates as minimum).

- Using only 6 channels of the multichannel pipette (5.1.15), remove the content of a single column of the assay plate (seeded and washed with uptake buffer (5.7.1) and immediately replace it with the solutions pre-diluted in the corresponding column of dosing plate A (7.1.1).
- Repeat this process for each column, respecting a time delay (e.g., 30 seconds; 5.1.20) between each column. When all columns have been dosed, apply 10 µL (5.1.16) of the NaI solution (from a single column of the dosing plate B (7.1.2), once again respecting the same time delay (5.1.20) between each column.

Note: The delay time can be adjusted according to each operator's expertise and comfort, but it must be coherent throughout the whole assay. 96-Well pipettors can also be used.

- Leave the assay plate to stand in the dark (covered in aluminium foil; 5.2.19) at 37 °C ± 1°C for 60 min (5.1.20).
- Using only 6 channels of the multichannel pipette (5.1.15), wash the assay plate 2 times with 200 µl (5.1.15) ice-cold uptake buffer (5.3.3) to remove extracellular NaI not taken up into the cells, following the same time delay (5.1.20) as described before.
- Discard medium of the frame of plate with a multichannel pipette.
- Discard residual supernatant by inverting the assay plate on absorbent paper (5.2.18) and tapping twice into the paper.
- Turn the plates back up and let them dry for 20 minutes (5.1.20) in the biological safety cabinet (5.1.8).
- Determine iodide taken up into the cells using the Sandell-Kolthoff (SK) reaction

5.8.4. Measurement of intracellular iodide content using the Sandell-Kolthoff reaction

- In order to lyse the cells, add 100 µL (5.1.15) of ultrapure water (5.1.6) to each well of the assay plates (Columns 2-11, lines A-G) (5.1.6).
- Add 100 µL of ammonium cerium (IV) sulfate solution at 10,5 mM (5.3.5) to the same wells.
- Add 100 µL of the sodium arsenite solution at 24 mM (5.3.6) to attain a final volume of 300 µl/well in the same wells.
- Immediately after, record absorbance at t0 (time zero) at 405 nm using a spectrophotometer with plate reader (5.1.23)
- Close the plate and incubate in the dark (covered in aluminium foil (5.2.19)) for 20 minutes (5.1.20), at room temperature (15 °C – 25 °C).

- Record absorbance at t20 (time 20 minutes) at 405 nm using a spectrophotometer with plate reader (5.1.23)

Note 1: After the first experiment, the dose response obtained should be evaluated. If the test item is not showing a response, the next experiment should be performed with the same concentrations. If the test item shows a response, when possible, the highest, lowest and dilution factor should be determined in order to obtain a full dose-response curve.

5.9 Calculations and interpretation of results

For this assay, the border wells were not used. Due to this, all the calculations described below and performed in the calculation workbook do not consider those wells.

5.9.1. Viability assay through MTT

Reduction in metabolic activity of cells treated with the test, reference or control item is measured by the amount of formazan converted from MTT reagent (the less formazan formed, the greater the decrease in viability, lower colour intensity, lower optical density).

The steps for data analysis and calculation reported in the calculation sheet are in accordance with the following structure:

- Calculate the mean of the absorbances of the triplicate test items for each concentration of the tested substance.
- Cell viability is calculated for each well as % of the mean of the solvent control of the test item:

$$Viab\ (\%) = \frac{100 \times DO_{560e}}{DO_{560n}}$$

Where:

DO_{560e}: mean value of the optical density of the triplicate test items

DO_{560n}: average value of the optical density of the solvent control

The lower the Viab (%) value, the higher the cytotoxic potential. Cytotoxic potential is considered if cellular viability is < 80%.

5.9.2. SK reaction

There is no need to convert the readouts of the assay (Absorbance measured at 405 nm) in concentrations.

The absorbances of the plate (test items, reference and control items) should be measured at t0 and t20 minutes.

The steps for data analysis and calculation reported in the calculation sheet are in accordance with the following structure:

- Calculate the average value of Solvent Control (SC) at t0 and t20 separately. These values will be used as evidence of **0 % inhibition (Ref 0 %)**.
- To each value of condition, the deduction of Ref 0 % for each timepoint is performed.

$\boldsymbol{Abs_{405}t0} - \boldsymbol{Abs_{405}t0_{UC}}$ and $\boldsymbol{Abs_{405}t20} - \boldsymbol{Abs_{405}t20_{UC}}$ are calculated

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- Afterwards, perform **t20 - t0** to deduct any background or interference in OD with no translation to SK reaction.

$$\boldsymbol{Abs_{405}t20 - Abs_{405}t0} \text{ is calculated}$$

- Calculate the average value of C1 of $NaClO_4$ (reference value for positive response). This value will be used as evidence of **100 % inhibition (Ref 100 %)**.
- To each value of condition, the normalization with Ref 100 % is performed.

$$(\boldsymbol{Abs_{405}t20 - Abs_{405}t0}) \times \frac{\boldsymbol{100}}{\boldsymbol{Ref\ 100\ \%}} \text{ is calculated.}$$

- Calculate the average and standard deviation of each tested condition.
- Plot the logarithmic concentrations (XX) versus the % value of inhibition of the hNIS transporter (YY) to determine the IC50 value on Graphpad Software through the "Nonlinear regression (curvefit)" analysis, situated under **XY analyses** and selecting the equation "Sigmoidal, 4PL, X is log (concentration)" (under **Standard curves to interpolate**) with the confidence interval of 95 %.

5.9.3. Measures of Assay Performance

To monitor the performance of the assay, quality control measures including Z' scores, coefficients of variation (CV) of the test item's solvent control (eg. DMSO), and IC_{50} of positive controls ($NaClO_4$ for iodide uptake) are calculated for each experiment.

- Calculate the CV of solvent control (SC):

$$CV_{SC} = \frac{SD_{SC}}{\mu_{SC}}$$

Where

SD_{SC} is the standard deviation of the raw response value for solvent control wells;

μ_{SC} is the mean of the raw response value for solvent control wells.

- Calculate the Z' scores with the following formula:

$$Z' = 1 - \frac{3(\sigma_{pos}+\sigma_{SC})}{|\mu_{pos}-\mu_{SC}|},$$

Where

σ_{pos} is the standard deviation of normalized positive control ($NaClO_4$ for NIS assay) values at the highest concentration (100 µM) tested;

μ_{pos} is the mean of normalized positive control ($NaClO_4$ for NIS assay) values at the highest concentration (100 µM) tested;

σ_{CS} is the standard deviation of normalized solvent control values;

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μ_{CS} is the mean of normalized solvent control values.

Acceptable test results for each plate should meet the following criteria:

- CV of solvent control of the corresponding plate is ≤ 15%,
- CV of triplicate test items is < 18%
- Z' > 0.5,
- OD_{405} of the reference (positive) at the highest concentration between 1.5 and 2 (after 20min)
- OD_{405} of the reference (negative) at the highest concentration µM between 0.1 and 0.5 (after 20min)
- Linearity is obtained for the standard curve performed with KI

Therefore, test results would be rejected if one or more of the following criteria are observed:

- CV of solvent control of the corresponding plate was >15%,
- Z' < 0.5, and
- OD_{405} obtained for references outside of the specified range

Acceptance criteria for the cytotoxicity assay are:

- CV of solvent control of the corresponding plate is ≤ 15 %,
- CV of triplicate test items is < 18 %
- Viability % of the viability control (C+) < 10 %
- OD_{560} obtained for the reference (untreated cells) is between 0.5 and 1.0

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7. Attachments

7.1 Preparation of dosing plates for the main assay

Plates for **main assay**, after the pre-tests:

Untreated control	5.7.1 Uptake Buffer
	5.7.6 Sodium iodide (NaI)
Reference and Positive item	5.7.7.1 Sodium perchlorate hydrate ($NaClO_4$)
Negative item	5.7.7.2 Sodium fluoride (NaF)
Test substances	Test Item 1
	Test Item 2

7.1.1. Dosing Plate A

InTable 7, a scheme of dosing plate A is described and is to be used in the **main assay**. This dosing plate does not contain cells. Dosing plate A represents a plate of positive/reference control at full range (7 concentrations, 3 replicates each), negative control at full range (7 concentrations, 3 replicates each), untreated control (UC, uptake buffer, 3 replicates). For the cytotoxicity assay performed in parallel with the main assay, a further control should be included: SDS 2 % (viability control, C+, 3 replicates). After the preparation of the dosing plates, their contents should be transferred to a plate seeded with cells, 90 µL per well, following the method described in 5.8.3.2.

Table 7 – Dosing Plate A - Control Items

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		$NaClO_4$ C1	$NaClO_4$ C2	$NaClO_4$ C3	$NaClO_4$ C4	$NaClO_4$ C5	$NaClO_4$ C6	$NaClO_4$ C7				
C		$NaClO_4$ C1	$NaClO_4$ C2	$NaClO_4$ C3	$NaClO_4$ C4	$NaClO_4$ C5	$NaClO_4$ C6	$NaClO_4$ C7				
D		$NaClO_4$ C1	$NaClO_4$ C2	$NaClO_4$ C3	$NaClO_4$ C4	$NaClO_4$ C5	$NaClO_4$ C6	$NaClO_4$ C7	UC	UC	UC	
E		NaF C1	NaF C2	NaF C3	NaF C4	NaF C5	NaF C6	NaF C7				
F		NaF C1	NaF C2	NaF C3	NaF C4	NaF C5	NaF C6	NaF C7				
G		NaF C1	NaF C2	NaF C3	NaF C4	NaF C5	NaF C6	NaF C7	SDS 2%	SDS 2%	SDS 2%	

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7.1.2. Dosing Plate B

InTable 8, a scheme of dosing plate B is shown and is to be used in the **main assay**. This dosing plate does not contain cells. Dosing plate B represents a plate of only NaI at 100 µM and is to be used to easily apply 10 µL of NaI on the plate assay, respecting the time delay necessary. Prepare two columns of NaI 100 µM for each assay plate to be dosed. Pipette at least 150 µL in each well of this plate.

Table 8 - Dosing Plate B – NaI at 100 µM.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		NaI 100µM	NaI 100µM									
C		NaI 100µM	NaI 100µM									
D		NaI 100µM	NaI 100µM									
E		NaI 100µM	NaI 100µM									
F		NaI 100µM	NaI 100µM									
G		NaI 100µM	NaI 100µM									
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7.1.3. Dosing Plate C

In Table 9, a scheme of dosing plate C is shown and is to be used in the **main assay**. This dosing plate does not contain cells. Dosing plate C represents a plate of two test items (test items 1 and 2, 7 concentrations, 3 replicates each), solvent control (SC) for each of the test items (Solvent control 1 and solvent control 2, 3 replicates each), untreated control (uptake buffer, UC, 3 replicates), positive controls (used as reference, maximum concentration used in plate A, Table 7, 3 replicates) and negative controls (maximum concentration used in plate A, Table 7, 3 replicates). For the cytotoxicity assay performed in parallel with the main assay, a further control should be included: SDS 2 % (viability control, C+, 3 replicates). After the preparation of all dosing plates, their contents should be transferred to a plate seeded with cells, 90 µL per well, following the method described in5.8.3.2.

Table 9 - Dosing Plate C – Test items plate

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		Test item 1 C1	Test item 1 C2	Test item 1 C3	Test item 1 C4	Test item 1 C5	Test item 1 C6	Test item 1 C7	SC 1	SC 1	SC 1	
C		Test item 1 C1	Test item 1 C2	Test item 1 C3	Test item 1 C4	Test item 1 C5	Test item 1 C6	Test item 1 C7	SC 2	SC2	SC2	
D		Test item 1 C1	Test item 1 C2	Test item 1 C3	Test item 1 C4	Test item 1 C5	Test item 1 C6	Test item 1 C7	UC	UC	UC	
E		Test item 2 C1	Test item 2 C2	Test item 2 C3	Test item 2 C4	Test item 2 C5	Test item 2 C6	Test item 2 C7	$NaClO_4$ C1	$NaClO_4$ C1	$NaClO_4$ C1	
F		Test item 2 C1	Test item 2 C2	Test item 2 C3	Test item 2 C4	Test item 2 C5	Test item 2 C6	Test item 2 C7	NaF C1	NaF C1	NaF C1	
G		Test item 2 C1	Test item 2 C2	Test item 2 C3	Test item 2 C4	Test item 2 C5	Test item 2 C6	Test item 2 C7	**SDS 2%**	**SDS 2%**	**SDS 2%**	
H												

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