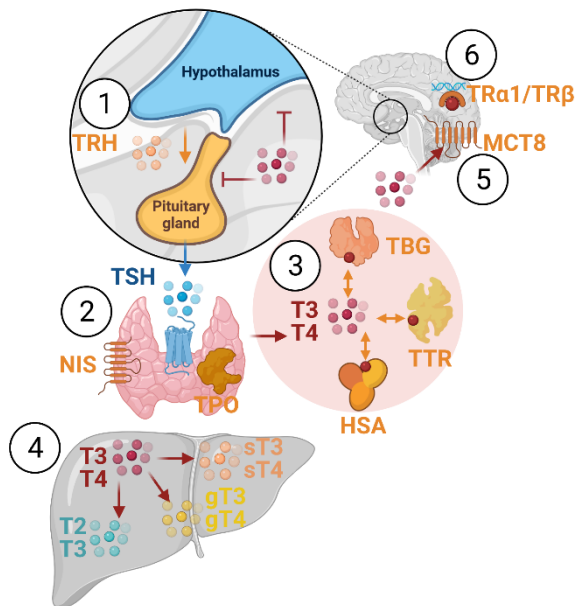


STANDARD OPERATING PROCEDURE

T-Screen assay using a QUANTITATIVE HIGH THROUGHPUT screening approach, 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

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This Standard Operating Procedure (SOP) has been prepared within the context of a collaboration agreement with the Joint Research Centre (JRC) Directorate for Health, Consumers and Reference Materials (Chemicals Safety and Alternative Methods Unit F3 / EURL ECVAM), for the validation of mechanistic methods to identify potential modulators of thyroid hormone signalling. It aims to provide evidence-based scientific support to the European policymaking process. The contents of this publication do not necessarily reflect the position or opinion of the European Commission. Neither the European Commission nor any person acting on behalf of the Commission is responsible for the use that might be made of this publication. For information on the methodology and quality underlying the data used in this publication for which the source is neither Eurostat nor other Commission services, users should contact the referenced source. The designations employed and the presentation of material on the maps do not imply the expression of any opinion whatsoever on the part of the European Union concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries.

This SOP is part of a series of 3 SOPs used to perform the "T-screen assay measuring cell proliferation of GH3 cells using alamar blue/resazurin":

1. SOP "T-screen assay measuring cell proliferation of GH3 cells using alamar blue/resazurin" v2.0 (used in Part 2 of the validation study)
2. SOP "Handling, Maintenance and Quality Control of the GH3 cell line " v2.0 (used in Part 2 of the validation study)
3. SOP "Determination of cell proliferation in T screen assay" v2.0 (used in Part 2 of the validation study)

The method was developed by Arno Gutleb, LIST (Luxembourg) and subsequently implemented by the EU-NETVAL test facility NIOM (Poland) within the validation study. For the purpose of running the method on a robotic platform during part 2 of the validation study, EURL ECVAM prepared a 4th SOP.

4. **SOP "T-Screen assay using a QUANTITATIVE HIGH THROUGHPUT screening approach" v1.0** (used in Part 2 of the validation study)

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Abbreviations and specifications

HT T-Screen	T-Screen method applied in a high-throughput mode
T4	3,3',5,5"-tetraiodo-L-thyronine <i>Synonym:</i> L-thyroxine
T3	3,3',5-Triiodo-L-thyronine
Fs	Furosemide
SDS	Sodium Dodecyl Sulfate
RI	Reference Item
PC	Positive Control
NC	Negative Control
TC	Test Chemical
SC	Solvent Control
qHTS	quantitative high throughput screening
RPE	Relative Proliferative Effect
Test plate	transparent 96-well plate where the assay is prepared
Compound plate	96-well plate where dilution series is prepared
Mother plate	96x1mL glass tubes matrix with 1000x C1 concentration of all test items and controls

1 INTRODUCTION

The T-Screen assay can be performed with an automated liquid handling platform utilising a quantitative high throughput screening (qHTS) approach with 96 well plates. In qHTS, each test plate contains a single concentration of the test Items, positive and negative controls and the reference Item. These Items are tested simultaneously on a series of plates (e.g. 10 to 15) with an increasing dilution per plate. Hence, the dose response is generated over the entire series of plates. A dilution factor of 4 is applied and 10 plates are used. The reference, control and test items are placed in a random fashion over the series of the plates allowing consideration of positioning interference. All wells of each 96 well are utilised and possible edge effects are taken into consideration within the data processing and analysis. The positive, negative and cytotoxicity control Items will also be diluted over the series of the 10 plates.

The HT T-Screen assay is carried out according to the SOPs of the manual approach

- T-screen assay measuring cell proliferation of GH3 cells using alamar blue/resazurin, Version 2 of 25/01/2022
- Determination of cell proliferation in T-screen assay, Version 2 of 27/12/2021
- Handling, Maintenance and Quality Control of the 3 GH3 cell line, Version 2 of 27/12/2021

with the following modifications to allow application on the robotic platform.

2 MODIFICATIONS

2.1 Materials

2.1.1 Equipment

Automated liquid handling platform, volume range 5 - 1000 μ L (yearly calibration performed according to manufacturer's instructions) e.g. Microlab STARLET.

2.2.2 Consumables

Reagent / Consumable
96-well sterile plates (clear flat-bottomed with lid) (Test plates) e.g. Corning 3628
96-well plates (round-bottomed) (Compound plates) e.g. Costar 3795
96 Deepwell containers e.g. Thermo 1064-15-6
Automation compatible pipette tips e.g. Hamilton 50 μ l (235979), 300 μ l (235937) and 1000 μ l (235940)
96 Matrix plates (Mother plate) e.g. Thermo Matrix 1.0 ml 3850
Autoclaved reservoirs (for assay medium)
Reservoirs e.g. Thermo 1064-15-8

2.2 Plate layout and randomisation of the Items.

All Items (reference, controls and test) are placed in no particular order on the plate (randomisation). The plate layout of the test plates is the same for every plate. The maximum number of Test Items (TI) is 19 in one experiment. Each TI has three technical replicates on every plate. There are six control Items on the plate: positive (PC), negative (NC), solvent control (SC), cytotoxic control (CC), blank, blank w/o Alamar Blue and in addition also the Reference Item (RI).

All of them are serially diluted.

The following rules are applied to position TI, RI and controls on the plate:

- TI, PC, CC and NC replicates are positioned randomly in the plate
- 5 RI and SC replicates are positioned randomly in inner wells of the plate
- 5 RI and SC replicates are positioned randomly in outer wells of the plate

Plate layout

An indicative plate layout can be found in Annex 1. Final plate layouts are reported in the Study plan.

2.3 Preparation of Reference Item, control Items and Test items

The Reference, controls and test Items are prepared in the same way as described for the manual assay.

In addition, Sodium Dodecyl Sulfate (CAS 151-21-3) is included as the cytotoxicity positive control item, in concentration range 1.907×10^{-4} uM to 200 uM.

2.3.1 Preparation of the mother plate and dilution series

1. Thaw the frozen glass vials of the stock solutions for all items at room temperature
2. Prepare few mother plates with the stock solutions of all items (controls and test chemicals) according to the plate layout for the experiment. Store the plates and remainder of the stock solutions at -20 C
3. Thaw one mother plate at room temperature just before the dilution is planned
4. Prepare a dilution series from the mother plate with the liquid handler (dilution factor 4; e.g., 30 μ L + 90 μ L DMSO) to provide 10 serial concentrations [C1 (highest) – C10 (lowest)],)

Acceptance criteria

Number	Acceptance criteria	
1	The doubling time of the GH3 cells in cDMEM/F12 medium	42 ± 5h
2	Cell number in PCM in 72h±1h and 96h±1h of culture should be at least 40% lower than cell number in cDMEM/F12, when determined simultaneously.	
3	Sigmoidal curve of reference item T3	Yes
4	EC ₅₀ range reference item T3	-10.4 to -9.6 log ₁₀ (Molar) units
5	RPE for EC ₅₀ T3 (i.e. 0.1 nM T3)	30% - 70%
6	Z-factor	> 0.5
7	RPE of SDS at 100 µM	< RPE of SC

2.4 Results

2.4.1 Data processing and analysis

The data are processed with a statistical software e.g. MatLab. The formulas are given in the SOPs 'T-screen assay measuring cell proliferation of GH3 cells using alamar blue/resazurin' and 'Determination of cell proliferation in T-screen assay'.

3 ANNEX 1

3.1 Plate Layout for T-Screen experiment

- The plate layout is indicative only. The final plate layouts are shown in the Study plan.
- There will be 10 test plates, one concentration in each plate (C1- C10). The layout will be the same in each plate.
- Reference Item (RI): 10 replicates (Red).
- Test Item (TI): up to 19, 3 replicates (White).
- Positive Control (PC): 3 replicates (Blue).
- Negative Control (NC): 3 replicates (Yellow).
- Solvent Control (SC): 10 replicates (Green).
- Cytotoxicity control (CC): 3 replicates (Purple)
- Blank: 4 replicates (Peach)
- Blank w/o AB: 4 replicates (Light blue)
- Empty wells will be filled with SC

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	RI	TI 5 r2	TI 5 r1	SC	TI 10 r1	NC	TI 18 r1	TI 15 r3	RI	TI 19 r1	TI 19 r2
B	Blank		SC	TI 8 r2	TI 9 r2	TI 11 r1	CC	TI 12 r3	TI 14 r2	PC	TI 19 r3	SC
C	Blank	RI	TI 3 r1	TI r2	TI 5 r3	TI 12 r1	TI 10 r3	TI 13 r3	TI 16 r3	SC	TI 1 r1	SC
D	Blank	TI 3 r2	TI 4 r3	NC	TI 6 r3	TI 13 r1	TI 14 r1	TI 14 r3	SC	RI	RI	TI 1 r3
E	Blank w/o AB	TI 4 r2	TI 4 r1	CC	TI 9 r1	TI 10 r2	TI 15 r1	TI 17 r1	TI 15 r2	TI 17 r3	RI	TI 1 r2
F	Blank w/o AB	PC	TI 6 r2	RI	TI 7 r3	NC	TI 11 r3	TI 12 r2	TI 16 r2	TI 18 r3	SC	SC
G	Blank w/o AB	TI 3 r3	TI 7 r2	TI 6 r1	TI 8 r1	TI 9 r3	TI 11 r2	TI 13 r2	SC	PC	TI 18 r2	TI 2 r3
H	Blank w/o AB	RI	RI	TI 7 r1	TI 8 r3	RI	TI 16 r1	CC	SC	TI 17 r2	TI 2 r1	TI 2 r2

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