European Commission

STANDARD OPERATING PROCEDURE

T-screen assay measuring cell proliferation of GH3 cells using alamar blue/resazurin, 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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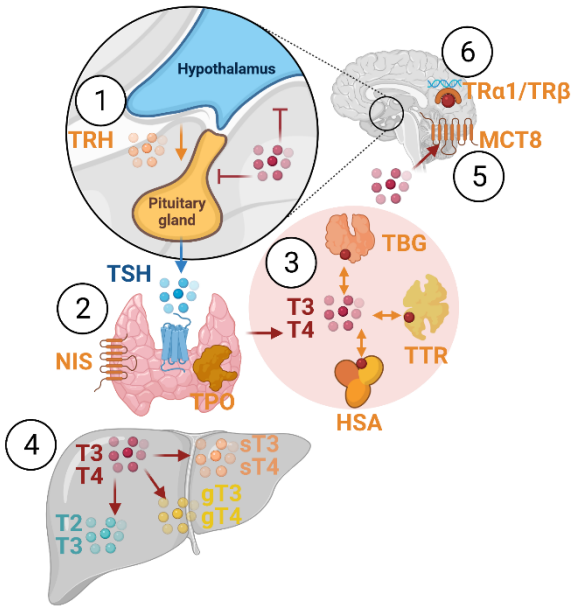

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This Standard Operating Procedure (SOP) has been prepared within the context of a collaboration agreement with the Joint Research Centre (JRC) Directorate for Health, Consumers and Reference Materials (Chemicals Safety and Alternative Methods Unit 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" v1.0** (used in Part1 of the validation study)
2. SOP "Handling, Maintenance and Quality Control of the GH3 cell line " v1.0 (used in Part 1 of the validation study)
3. SOP "Determination of cell proliferation in T screen assay" v1.0 (used in Part 1 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.

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Standard Operating Procedure

T-screen assay measuring cell proliferation of GH3 cells using alamar blue/resazurin

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Contents

1. Description of the method

The T-Screen represents an *in vitro* bioassay based on thyroid hormone (TH) dependent cell proliferation of a rat pituitary tumour cell line (GH3) in serum-free medium. It can be used to study interference of compounds with TH at the cellular level, thus bridging the gap between limitations of assays using either isolated molecules (enzymes, transport proteins) or complex *in vivo* experiments with all the complex feedback mechanisms present. Test items are tested both in the absence and presence of TH (EC_{50} concentration of T3) to test for both agonistic and antagonistic potency.
GH3 cell growth is increased in the presence of TH agonists and decreased in the presence of TH plus TH antagonists. Cell growth is measured with AlamarBlue/Resazurin cell proliferation assay using a standard plate reader. In this method a colorimetric assay is used, where resazurin is reduced from a blue oxidized form into its violet reduced form of resorufin. The change of colour can be detected as a change in absorbance using a microplate reader.

1.1 Scope and Limitations of the Application

The T-screen assay is used for testing of compounds for TH receptor (TR)-mediated effects.

1.2 Safety

The use of endocrine disrupting chemicals can be extremely hazardous, and precautions such as using gloves, protective goggles and masks under a laminar flow hood should always be taken while performing chemical treatments.

1.3 Required Standard Operating Procedures

- *Handling and Maintenance of GH3 cell line*
- *Determination of cell proliferation in T-screen assay*

1.4 Test System

- GH3-cell line – a rat pituitary tumour cell line, (ATCC® CCL-82.1™)

The GH3 cells may be used when the following requirements are met:

1.4-1 Test system must be free of microbial and mycoplasma contamination

1.4-2 The doubling time of the GH3 cells in cDMEM/F12 medium should be 42 ± 5h

1.4-3 Cell number in PCM after 72 ± 1 h and 96 ± 1 h of culture should be at least 40% lower that cell number in cDMEM/F12, when determined simultaneously.

1.4-4 Cells should be used in passage from 3 to 20

1.5 Apparatus and Materials

- Balance (minimal value: 50 mg; the precision requirement: 50 ± ≤0.001 mg) and the appropriate mass standards F1, e.g. 1 - 500 mg ± ≤0.02 mg; 1 g ± ≤0.01 mg
- Clean glass vials for the preparation of stock solutions (e.g., gas chromatography vials or Sarstedt #86.1509)
- 96-well microplates (e.g., Nunc #167008)
- Freezer below -16 °C
- Refrigerator at 2-10 °C
- CO_2 humidified incubator at 37°C +/- 2 °C, 5% CO_2 +/- 0.5%
- 37 °C water bath

- PipetteAid
- Pipettors (p1000, p200, p20) or Micropipettes (1000-100; 100-10; 10-0.5)
- Centrifuge
- Vacuum aspirator
- Microplate Reader; for measuring absorbance
- Laminar Flow Hood
- Culture Flasks (T75, T25; e.g. Nunc, Falcon)
- Serological pipettes (1 mL, 5 mL, 10 mL, and 25 mL)
- Sterile, filter pipette tips (10-20 µL, 200 µL, and 1000 µL)
- Sterile, pipette tips without filter (e.g. 10-20 µL, 200 µL, and 1000 µL)
- Conical tubes (e.g. 5 mL, 15 mL, and 50 mL)
- Polypropylene Cluster Tubes (1.1 mL e.g. Corning #MTS-11-8-C)
- Syringe filters (0.22µm)

1.6 Reagents and Chemicals

- Foetal calf/bovine serum (e.g. Gibco #10270106)
- Dulbecco's modified Eagle's medium/Ham's Nutrient Mixture F-12 (DMEM/F-12; 1:1 mixture with phenol red and HEPES (15 mM) (e.g. Sigma-Aldrich #D6421)
- DMEM/F-12 w/o L-Glutamine, with 15 mM HEPES and w/o phenol red (e.g. Sigma-Aldrich #D6434)
- HEPES 1M solution (e.g. Sigma-Aldrich #H0887)
- Penicillin-Streptomycin Solution (e.g. Sigma-Aldrich #P0781)
- Bovine insulin (e.g. Sigma-Aldrich #I0516)
- Ethanolamine (e.g. Sigma-Aldrich #E0135)
- Sodium selenite (e.g. Sigma-Aldrich #S5261)
- Human apotransferrin (e.g. Sigma-Aldrich #T2036)
- Bovine serum albumin (e.g. Sigma-Aldrich #A9418)
- DMSO 99.9% purity (e.g. Sigma-Aldrich #D8418)
- Ethyl alcohol 99.8% pure p.a. (e.g. POCH #396480111)
- Deionised water (dH_2O)

1.7 Reference items, positive and negative control Item(s)

	Agonism	**Antagonism**
Reference item	3,3'-5-triiodothyronine (T3) [CAS 6893-02-3], ≥ 95% purity	5,5-Diphenylhydantoin (DPH, or Phenytoin) [CAS 57-41-0]
Positive control item	T4, L-Thyroxine (3,3',5,5''-Tetraiodo-L-thyronine) [CAS 51-48-9], ≥ 98%	
Negative control item	Mefenamic acid (MfA) [CAS 61-68-7]	
Cytotoxic positive control item	Sodium Dodecyl Sulfate (SDS) [CAS 151-21-3]	

1.8 Preparations of media and reagents

1.8.1 Cell culture medium (cDMEM/F12; completed cDMEM/F12)

- Dulbecco's modified Eagle's medium/Ham's Nutrient Mixture F-12 (DMEM/F-12; 1:1 mixture; with phenol red and 15 mM HEPES) supplemented with 10% Foetal calf serum, 2.5 mM L-Glutamine and additional amount of HEPES to obtain 25 mM.

To prepare *cDMEM/F-12* the following volume of supplements should be added:

	Final concentration	Volume [mL]
DMEM/F-12 w/o L-Glutamine, with 15 mM HEPES (e.g. Sigma-Aldrich #D6421)*		500
Heat inactivated FCS/FBS**	10%	57.6
200 mM L-Glutamine	2.5 mM	7.2
1 M HEPES	up to 25 mM	5.8
Penicillin-Streptomycin solution (100x)	1%	5.8
Total		**576.4**

*if different DMEM/F-12 is used the final concentration of HEPES and L-Glutamine should be adjusted to 2.5 mM and 25 mM, respectively.

** if heat-inactivated FCS/FBS was purchased, it should be defrost at 2-10^{o}C e.g. during the night and stored in aliquots at below -16^{o}C.
If non-inactivated FCS/FBS was purchased, it should be heat-inactivated in water bath at 56-57°C for 30-35 min and filtered (0.2 µm) before aliquoting.

1.8.2 PCM medium

PCM medium is a Dulbecco's modified Eagle's medium/Ham's Nutrient Mixture F-12 (DMEM/F-12; 1:1 mixture) without phenol red supplemented with 10 µg/mL bovine insulin, 10 µM ethanolamine, 10 ng/mL sodium selenite, 10 µg/mL human apotransferrin, 500 µg/mL bovine serum albumin. Neither T3 nor T4 are added to the PCM medium.
To prepare *PCM medium* the following volume of supplements should be added:

	Final concentration	**Volume [mL]**
DMEM/F-12 w/o L-Glutamine, with 15 mM HEPES (e.g. Sigma-Aldrich # D6434)*		500
200 mM L-Glutamine	2.5 mM	6.5
1 M HEPES	up to 25 mM	5.2
Penicillin-Streptomycin solution (100x)	1%	5.2
10 mg/mL bovine insulin	10 µg/ml	0.523
50 mM ethanolamine (Section 1.8.5-1)	10 µM	0.105
50 µg/mL sodium selenite (Section 1.8.5-2)	10 ng/mL	0.105
2 mg/mL human apotransferrin (Section 1.8.5-3)	10 µg/mL	2.6
100 mg/mL bovine serum albumin (Section 1.8.5-4)	500 µg/mL	2.6
Total		**523.0**

*if different DMEM/F-12 is used the final concentration of HEPES and L-Glutamine should be adjusted to 2.5 mM and 25 mM, respectively.

1.8.3 PCM medium +4x EC_{50} concentration of T3 (PCM-T3 medium)

Once for each working cell bank and for each new batch of T3 the laboratory should verify the EC50 value for reference item T3 as described in Section 2. If the EC50 value of T3 meets the acceptance criteria (Section 2.4) then the T3 concentration of 0.1 nM should be used as the EC50 value, i.e. PCM medium with 0.4 nM T3 will be considered as PCM-T3 medium (4x EC50).

For each test item 2.5 mL of PCM-T3 medium is needed. To this end, prepare 3-step dilution of T3 stock solution (1 mM) in PCM medium to get 0.4 nM of T3 (1 mM→(100x)→10 µM→(100x)→100 nM→(250x)→ 0.4 nM). Since the solvent concentration in PCM-T3 is lower than 0.001% (exactly 0.00004%) and the maximum accepted concentration for DMSO is 0.5%, the effect of the solvent is omitted (the final concentration of DMSO in test or control items during the T-screen test is 0.40004%). PCM-T3 medium will be used for antagonist experiments – Section 3.2-2c and Section 3.3.3-2.

1.8.4 Stock and working solutions of chemicals

To prepare the ***exposure concentration*** of every chemical given below, firstly the ***concentrated solution*** is prepared that is used to prepare **the *working solution*** (the double desired exposure concentration) according to Section 3.2-2b and/or Section 3.2-2c.

1.8.4-1 **3,3'-5-triiodothyronine (T3) [1mM stock solution] and [4 nM working solution]**

Dissolve T3 in DMSO to produce a 1 mM stock solution. Use fresh or store in aliquots below -16 °C.

T3 is used in the T-Screen assay in the full dose range (the first Agonism plate) and in the highest test concentration, i.e. 2 nM – to prepare the *concentrated solution* of T3 [1 µM], dilute T3 stock solution in the solvent/DMSO as follows: 1 mM → (50x) → 20 µM --(20x)→ 1 µM. Then, use the *concentrated solution* of T3 to prepare *working solution* according to Section 3.2-2b (only in Agonism plate). T3 is added at the final concentration EC50 into every test well on Antagonism plates except UC (PCM) and the preparation of sample with addition of T3 EC50 is described in Section 1.8.3.

1.8.4-2 **3,3',5,5'-tetraiodothyroxine (T4) [2 mM stock solution] and [20 nM working solution]**

Dissolve T4 in DMSO to produce a 2 mM stock solution. Use fresh or store in aliquots below -16 °C.

The *exposure concentration* of T4 in the T-Screen assay is 10 nM – to prepare the *concentrated solution* of T4 [5 µM], dilute T4 stock solution in the solvent/DMSO as follows: 2 mM → (400x) → 5 µM. Then, use *the concentrated solution* of T4 to prepare *working solution* according to Section 3.2-2b, for Agonism experiment.

1.8.4-3 **5,5-Diphenylhydantoin (Phenytoin; DPH) [50 mM stock solution] and [100 µM working solution]**

Dissolve DPH in DMSO to produce a 1 mM stock solution. Use fresh or store in aliquots below -16 °C.

The exposure concentration of DPH in the T-Screen assay is 50 µM – to prepare the *concentrated solution* of DPH [25 mM], dilute DPH stock solution in the solvent/DMSO as follows: 50 mM → (2x) → 25 mM . Then, use *the concentrated solution* of DPH to prepare *working solution* according to Section 3.2-2c, for Antagonism experiment.

1.8.4-4 **Mefenamic acid (MfA) [100 mM stock solution] and [200 nM working solution]**

Dissolve MfA in DMSO to produce a 100 mM stock solution. Use fresh or store in aliquots below -16°C.

The exposure concentration of MfA in the T-Screen assay is 100 nM – to prepare the *concentrated solution* of MfA [50 µM], dilute MfA stock solution in the solvent/DMSO as follows: 100 mM → (100x) → 1 mM --(20x)→ 50 µM. Then, use the *concentrated solution* of MfA to prepare *working solution* according to Section 3.2-2b and Section 3.2-2c, for Agonism and Antagonism experiments, respectively.

1.8.4-5 **Sodium Dodecyl Sulfate (SDS) [100 mM stock solution] and [200 µM working solution]**

Prepare the 100 mM stock solution of SDS in DMSO. Use fresh or store in aliquots at room temperature.

The exposure concentration of SDS in the T-Screen assay is 100 µM – to prepare the *concentrated solution* of SDS [50 mM], dilute SDS stock solution in the solvent/DMSO as follows: 100 mM → (2x)→ 50 mM. Then, use the *concentrated solution* of SDS to prepare working solution according to Section 3.2-2b (SDS is used only on Agonism plate).

1.8.5 Stock solutions of reagents

1.8.5-1 **Ethanolamine [50 mM]**

Prepare a 50 mM stock solution of ethanolamine in dH_2O by diluting a pure ethanolamine (16.6 M) 332x, i.e. 10 µL of ethanolamine (16.6 M) added to 3.310 mL of dH_2O, filter (0.22 µm pore size). Use fresh or store at room temperature in closed glass containers.

1.8.5-2 **Sodium selenite [50 µg/mL]**

Prepare a stock solution of sodium selenite (50 µg/mL) in dH_2O, filter (0.22 µm pore size) and store in aliquots below -16 °C.

1.8.5-3 **Human apotransferrin [2 mg/mL]**

Prepare a 2 mg/mL stock solution of human apotransferrin in dH_2O, filter (0.22 µm pore size), and store in aliquots below -16 °C. Stock solutions of apo-transferrin stored at 2-8°C are stable for 5–10 days.

1.8.5-4 **Bovine serum albumin [100 mg/mL]**

Prepare a 100 mg/mL stock solution of bovine serum albumin in dH_2O, filtered (0.22 µm pore size) and store in aliquots at 2-8°C

2. Verification of the EC50 value of T3

Once for each working cell bank and for each new batch of T3 the laboratory should verify the EC50 value for reference item T3 as described below.

2.1 Seeding the GH3 cells onto 96-well plate

Perform the cell seeding as described for the T-screen (Section 3.3.1). Seed cells onto a 96-well microplate in three replicates (e.g. rows B-D; Figure 1).

2.2 Exposure of the cells to the range of T3 concentrations

2.2 - 1. Prepare T3 at the range of double desired concentrations by diluting the concentration of 4 nM using dilution factor (DF) = 3. The final 7 concentration of T3 should be: 2; 0.667; 0.222; 0.074; 0.025; 0.008 and 0.003 nM

2.2 - 2. Add 100 µL T3 in PCM medium at the appropriate concentration into the 100 µL already present (with the cells) in the respective well(s) (to achieve a 1:1 dilution). Test T3 and control items in triplicate (Figure 1).

2.2 - 3. Incubate the plate for 96 ± 1 h at 37 ± 2 °C and 5 ± 0.5% (v/v) CO_2 in a humid atmosphere.

Figure 1. **Scheme of the plate layout for verification of the EC50 value of T3**

	1	2	3	4	5	6	7	8	9	10	11	12
A	Z	Z	Z	Z	Z	Z	Z	Z	Z	Z	Z	Z
B	Blank	SC	T3 C7	T3 C6	T3 C5	T3 C4	T3 C3	T3 C2	T3 C1	PCM	SC	Blank w/o AB
C	Blank	SC	T3 C7	T3 C6	T3 C5	T3 C4	T3 C3	T3 C2	T3 C1	PCM	SC	Blank w/o AB
D	Blank	SC	T3 C7	T3 C6	T3 C5	T3 C4	T3 C3	T3 C2	T3 C1	PCM	SC	Blank w/o AB
E	Z	Z	Z	Z	Z	Z	Z	Z	Z	Z	Z	Z
F												
G												
H												

SC (wells 2B-D and 11B-D) **-** solvent control

PCM (wells 10B-D) - test system control

T3 C (wells 3B-9D) - the range of T3 concentrations (0.003 - 2 nM); where C1 is the lowest T3 concentration

Z – external wells (medium or PBS)

Blank - PCM medium without cells (AlamarBlue is added in the proliferation assay); used for calculations both % AlamarBlue reduction (%AR) and % Dye reduction (%DR) according to formulas given in SOP *Determination of cell proliferation in T-screen assay* in Section 2.2.1 or Section 2.2.2, respectively

Blank w/o AB - PCM medium without cells (AlamarBlue is not added in the proliferation assay); used for calculations only %DR according to formula given in SOP *Determination of cell proliferation in T-screen assay* in Section 2.2.2.

2.3 Measurement of cell proliferation

2.3 - 1. Perform the cell proliferation assay according to Section 2: "AlamarBlue assay" described in SOP "*Determination of cell proliferation in T-screen assay*".

2.3 - 2. Based on received results (optical density/absorbance), calculate **% AlamarBlue reduction (%AR)** or **% Dye reduction (%DR)** for each triplicate sample using formulas given in SOP *Determination of cell proliferation in T-screen assay* in Section 2.2.1 or Section 2.2.2, respectively.

2.3 - 3. Calculate the increase of *Cell proliferation* that is expressed as **the relative proliferative effect (RPE)** according to the formula given in Section 3.4.1-2.

2.3 - 4. Calculate the **EC50** of T3 according to the formula given in Section 3.4.1-3.

2.4 Acceptance criteria

Based on the obtained results the mean EC50 value of T3 should be calculated. The mean EC50 value should be -10±0.4 log10(Molar) units (in the range from -10.4 to -9.6 log10(Molar) units).

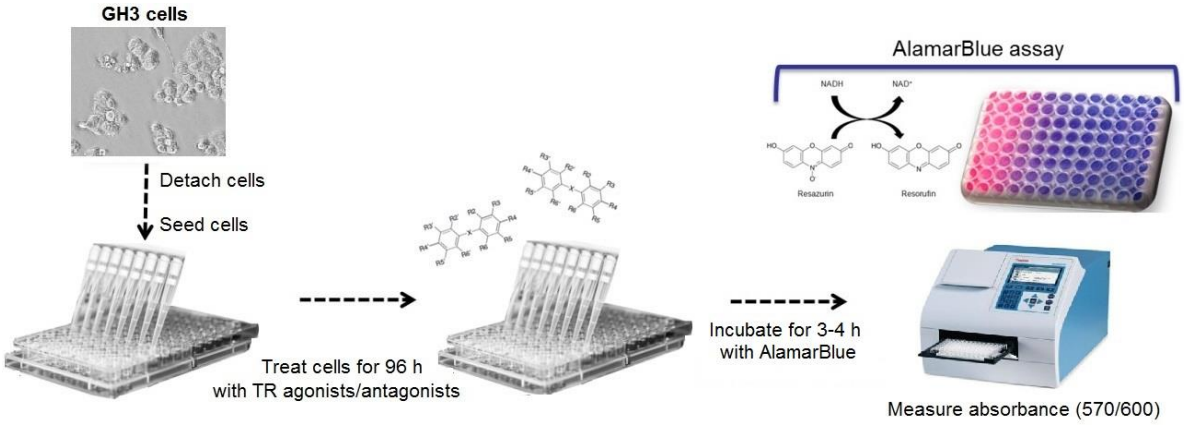
If acceptance criteria for the mean EC50 value of T3 are met, 0.1 nM T3 should be used as the EC50 value of T3 in the T-screen test.

Every result, i.e. the mean EC50 of T3, should be registered to create the historical data EC50 value of T3. Also, the mean EC50 of T3 calculated from the results obtained during every T-screen test (the first agonism plate, Section 3.3.3) should be included to the historical data.

3. T-Screen experimental procedure

Figure 2 summarises the main steps required to run this method.

Figure 2. The T-screen assay workflow with absorbance of AlamarBlue dye detection.



3.1 Preparation of Test Item

The test item's solubility in the solvent and medium will need to be assessed. Both stock solutions and working solutions should be assessed.

3.1.1 Stock solutions

It is recommended to use the same solvent for all reference and control items and to use that solvent for the test items, to ensure all samples are tested under the same conditions. In case another solvent is used for the test item, the impact on the test system and the results must be assessed.

3.1.1-1 Prepare a 100 mM or 10 mg/mL stock solution (may be adapted on basis of data if too low or high) of test item by weighing a nominal amount into a clear glass vial and add the appropriate volume of solvent (use DMSO as a default solvent and if not possible to dissolve test item in DMSO then other solvents, e.g. water, PCM medium or ethanol should be used. For visual inspection, it is important to have at least 0.5 ml in the vial to be able to observe well.

3.1.1-2 Visually check if dissolved. If not dissolved, heat (37^{o}C; up to 30 min) may be applied to aid solubility.

3.1.1-3 If not possible to solubilise, prepare a lower (e.g. 1:2) stock solution (or dilute existing stock) and again check solubility. Change solvent if needed.

3.1.1-4 Continue until the stock solution is soluble.

3.1.2 Work solutions to determine solubility

3.1.2-1 Dilute the stock solution of test item in solvent prepared in Section 3.1.1. Prepare the range of 7 concentrations (*the concentrated solutions*) using a dilution factor 10 (10 µL of stock solution of the appropriate *concentrated solution* to 90 µL solvent in a 96-well plate).

For test item dissolved in DMSO or ethanol prepare the 500x *concentrated solutions*, whereas for test item dissolved in water or PBS, prepare 100x *concentrated solutions*.

3.1.2-2 Prepare the 2x concentrated solutions (*working solutions*) in PCM medium by transferring: 2 µL of the appropriate *concentrated solutions* to 498 µL of PCM in a 24-well plate or 1.1 mL cluster tubes.
Solvent concentration in the *working solution*s of test item should not exceed 0.4% for DMSO and ethanol or 10% for water or PBS (the final concentration 0.2% or 5%, respectively for DMSO/ethanol or water/PBS).

3.1.2-3 Visually check if the working solutions are dissolved.

3.1.2-4 Identify the highest soluble concentration.

3.2 Pre-screen experiment

During the pre-screen experiment it is assessed if the test item is:

- A Thyroid Hormone Agonist
- A Thyroid Hormone Antagonist
- Cytotoxic

and at which dose-range it increases or decreases cell proliferation (Range finder).

Note: GH3 cells are not able to divide properly in PCM without T3 but keep basal or low activity. Concentrations of test items that lower the cellular activity of GH3 cells cultured in PCM medium without T3 (as determined with the cell proliferation assay) are considered to be cytotoxic.

3.2.1 Seeding the GH3 cells onto 96-well plate

Perform the cell seeding as described for the T-screen (Section 3.3.1).

- Seed cells into all internal wells, i.e. B2-G11.
- Add 100 µL of PCM medium to each well in columns 1 and 12, for *Blank* and *Blank w/o AB* (Figure 4) that are used for calculations in the proliferation assay.
- Add 100 µL of PCM medium or PBS to each well in rows A and H (external wells) to ensure proper humidity for the cells.

3.2.2 Preparation of test, reference and control item solutions

On the day of treatment, prepare the range of 7 *working solutions,* both for **Agonist experiments** and **Antagonist assessment,** as follows:

3.2.2-1 Stage 1: Prepare stock solution of test item at the concentration and solvent determined above (Section 3.1.1). Prepare a dilution series of test item stock in the solvent (the range of 7 *concentrated solutions*) as described in Section 3.1.2-1 (Figure 3; Stage 1).

In the first experiment a dose range finding is performed to capture the whole dose response range for the next experiments. The highest concentration to be tested is the highest non cytotoxic soluble concentration and dilution factor 10 is applied for the endpoint measurement. In the next experiment a dose range includes 7 concentrations where the highest concentration shows the max effect (the induction of proliferation in Agonist experiment) and the lowest concentration shows no

effect (effect comparable to solvent control in Agonist experiment). Dilution factor is adjusted to cover 7 concentrations and each test item have its own dilution factor (e.g. DF 1.5, 2, 3, 4 or 5).

Use the same range of the 7 *concentrated solutions* to prepare *working solutions*, both for **Agonist** (Stage 2a) and **Antagonist assessment** (Stage 2b)**.**

3.2.2-2 For **Agonist assessment (Treatment without T3)** (Figure 3; Stage 2a)

Stage 2a: Prepare *working solutions* as described in Section 3.1.2-2.

In the first experiment/the dose range finding an interference of test item with the assay/AlamarBlue reagent should be assessed. To this end, prepare *working solutions* as described in Section 3.1.2-2 using double volumes to have enough solutions both for test plate and the additional plate for testing interference of AlamarBlue with test item.

3.2.2-3 For **Antagonist assessment – Treatment with EC_{50} of T3** (Figure 3; Stage 2b)

Stage 2b: Prepare *working solutions* containing 2xEC50 of T3 (dilute *concentrated solutions* prepared in Stage 1 in the appropriate mixture of PCM medium and PCM-T3 medium), as follows:

- Add 248 µL PCM medium into 1.1 mL-cluster tubes, then
- Add 250 µL PCM-T3 medium (4x EC50 of T3; prepared as described in Section 1.8.3) into 1.1 mL-cluster tubes, then
- Add 2 µL of the appropriate *concentrated solution* to the mixture of PCM medium and PCM-T3 medium already present into 1.1 mL-cluster tubes. Solvent concentration (both for test item and T3) should not exceed 0.5%.

3.2.3 Exposure of the cells

Perform the exposure as follows:

3.2.3-1 Upper part of the plate/B2:D11 (agonism assessment):

- Add 100 µL *working solutions* (*the double desired exposure concentrations*) of test item (TI) or solvent control (SC) prepared in PCM medium to the 100 µL already present (with the cells) in the respective well(s) (to achieve a 1:1 dilution). Test all samples in triplicate.
- Add 100 µL PCM medium only to three wells designed UC (PCM) (untreated control/cell system control; Figure 4)).
- Add the same samples in the same order and volume (100 µL) into the additional plate prepared for testing interference of AlamarBlue with test item (Section 3.2-3e).

3.2.3-2 Lower part of the plate/E2:G11 (antagonism assessment):

- Add 100 µL test item or solvent control (S/T3) *working solutions* (*the double desired exposure concentrations*) prepared in PCM medium with addition of 2x EC50 value of T3 to the 100 µL already present (with the cells) in the respective well(s) (to achieve a 1:1 dilution). Test all samples in triplicate.
- Add 100 µL PCM medium with addition of 2x EC50 value of T3 to three wells designed (EC50 T3; Figure 4).
- Add 100 µL 2x Ref(T3)C1 = the highest concentration in the range described in Section 3.1.2.
- Add the same samples in the same order and volume (100 µL) into the additional plate prepared for testing interference of AlamarBlue with test item (Section 3.2.3-5).

3.2.3-3 Add 100 µL of PCM medium to the 100 µL already present (without the cells) in each well in columns 1 and 12 (Blank and Blank w/o AB; Figure 4) that are used for calculations in the proliferation assay.

3.2.3-4 Add 100 µL of PCM medium or PBS to each well in rows A and H (external wells) to ensure proper humidity for the cells.

3.2.3-5 Prepare the additional plate for testing interference of AlamarBlue reagent with test items (samples incubated in PCM medium without cells) – add 100 µL of PCM medium into every well needed (triplicates/sample). Then, add all prepared samples (100 µL) in the same order as indicated in Section 3.2.3-1 and Section 3.2.3-2. Also, add extra PCM medium as given in Section 3.2.3-3 and Section 3.2.3-4.

3.2.3-6 Incubate plates for 96 ± 1 h at 37 ± 2 °C and 5 ± 0.5% (v/v) CO2 in a humid atmosphere.

Plate layout for Pre-screen experiment is presented in Figure 4. It is advised to assess agonism and antagonism effect on the same plate (both in triplicates). For antagonism all treatments are performed in presence of T3.

3.2.4 Measurement of cell proliferation and data analysis

3.2.4-1 Perform the AlamarBlue assay according to Section 2 in SOP "*Determination of cell proliferation in T-screen assay*" to assess cell proliferation or cytotoxic effects.

3.2.4-2 Based on received results (optical density/absorbance), calculate % AlamarBlue reduction (%AR) or **% Dye reduction (%DR)** for each triplicate sample using formulas given in SOP *Determination of cell proliferation in T-screen assay* in Section 2.2.1 or Section 2.2.2, respectively.

3.2.4-3 Calculate the **Cell proliferation (%CP)** for each test item (the concentration range) based on values %AR and %DR calculated above (Section 3.2.4-2) according to the following formulas, respectively for calculated %DR or %AR:

$$\%\mathrm{CP} = (\%DR_{x/T3} - \%DR_{x}) \times 100$$

OR*

$$\%\mathrm{CP} = (\%AR_{x/T3} - \%AR_{x}) \times 100$$

where:

x - the effect of TI, PC, NC or REF T3 at the concentration analysed; tested without EC50 of T3

x/T3 - the effect of TI, PC, NC or REF T3 at the concentration analysed; tested in the presence of EC50 of T3

* - %AR or %DR is used depending on the method choose for calculation results of the proliferation assay (SOP *Determination of cell proliferation in T-screen assay* in Section 2.2.1 or Section 2.2.2, respectively)

3.2.4-4 Calculate the increase of cell proliferation that is expressed as **the relative proliferative effect (RPE)** for "EC50 T3" according to Section 3.4.1-2.

3.2.5 Acceptance criteria for pre-screen assay

3.2.5-1 %DR or %AR for UC(PCM) should not be more than 15% different from TI SC

3.2.5-2 RPE for EC50 T3 should be in the range of 30-70%

3.2.6 Identification of the range concentrations of TI for the next experiments

Identify the range concentrations of TI for the next experiments that captures the whole dose response range (from no effect to the highest agonistic/antagonistic effect). The range of concentration should have not more than two concentrations given the maximal response and not more than two concentrations given the minimal response (near background/ not more than 5% of SC).

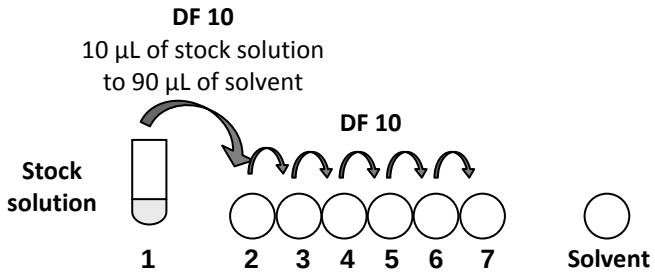
3.2.6-1 If a strong cytotoxic effect is detected, i.e. three or more concentrations are found to be cytotoxic, repeat the test using the changed range of concentrations (start from the highest non-cytotoxic concentration and adjust DF to get 7 concentrations) to better identify range of test concentrations of test item. The highest concentration for the T-screen assay should be the highest noncytotoxic concentration of test item.

3.2.6-2 If no cytotoxic effect is observed, but the agonistic or antagonistic effect is not observed choose for the next experiment the same range of concentration and DF 10

3.2.6-3 If no cytotoxic effect is observed, but any agonistic or antagonistic effect is observed, choose DF to obtain the whole dose response (e.g. DF 1.5, 2, 3, 4 or 5) (if any).

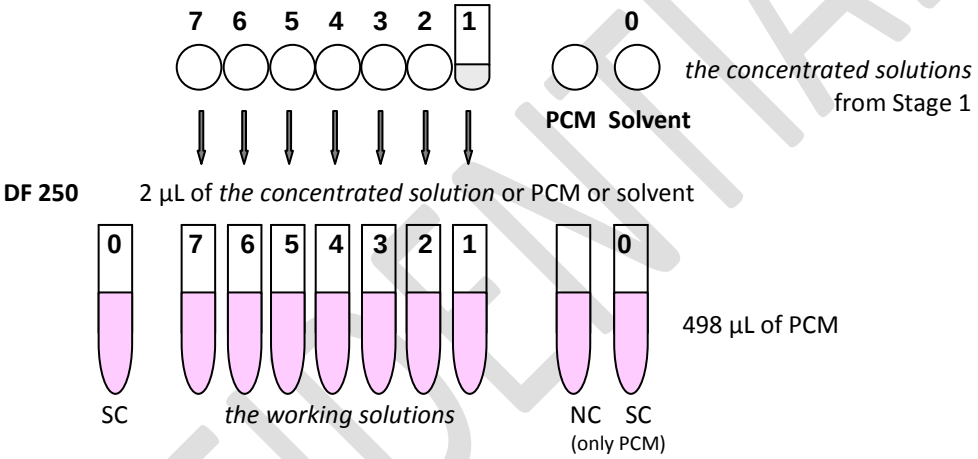
439 ***Figure 3.*** A schema of test item preparation for pre-screen experiment and the T-Screen test.
440

Stage 1. Dilution of a stock solution



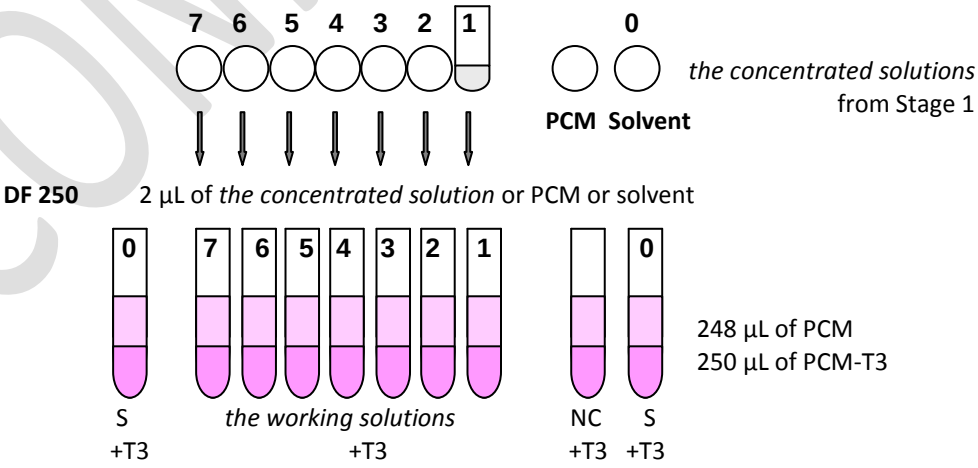
The solvent concentration in the diluted stock solutions/*concentrate solutions* is considered as 100%.

Stage 2a. Preparation of the *working solutions* (double desired exposure concentrations) for AGONIST experiments.
Cells should be exposed to a test item from minimal to maximal concentration.



The solvent concentration in the *working solutions* is considered as 0.4 %.

Stage 2b. Preparation of the *working solutions* (double desired exposure concentrations) for ANTAGONIST experiments.
Cells should be exposed to a test item from minimal to maximal concentration.



Since the solvent concentration in PCM-T3 medium is only 0.00004%, the effect of the solvent is omitted. The total solvent concentration in *the working solutions* can be considered as 0.4% - regardless of whether solvent of test item is the same as solvent of T3 (i.e. DMSO) or different e.g. ethanol.

441 ***Figure 4.*** Plate layout for pre-screen experiment

	1	2	3	4	5	6	7	8	9	10	11	12
A	*	*	*	*	*	*	*	*	*	*	*	*
B	Blank	SC	TI C7	TI C6	TI C5	TI C4	TI C3	TI C2	TI C1	UC (PCM)	SC	Blank w/o AB
agonism C	Blank	SC	TI C7	TI C6	TI C5	TI C4	TI C3	TI C2	TI C1	UC (PCM)	SC	Blank w/o AB
D	Blank	SC	TI C7	TI C6	TI C5	TI C4	TI C3	TI C2	TI C1	UC (PCM)	SC	Blank w/o AB
E	Blank	S/T3	TI C7/ T3	TI C6/ T3	TI C5/ T3	TI C4/ T3	TI C3/ T3	TI C2/ T3	TI C1/ T3	EC50 T3	Ref(T3)C1	Blank w/o AB
antagonism G	Blank	S/T3	TI C7/ T3	TI C6/ T3	TI C5/ T3	TI C4/ T3	TI C3/ T3	TI C2/ T3	TI C1/ T3	EC50 T3	Ref(T3)C1	Blank w/o AB
G	Blank	S/T3	TI C7/ T3	TI C6/ T3	TI C5/ T3	TI C4/ T3	TI C3/ T3	TI C2/ T3	TI C1/ T3	EC50 T3	Ref(T3)C1	Blank w/o AB
H	*	*	*	*	*	*	*	*	*	*	*	*

442

UC (PCM)	Untreated Control; Tested as triplicate sample on each plate, PCM Medium Only
EC50 T3	PCM medium + EC50 T3; Tested as triplicate sample on each plate
SC	PCM medium + solvent; Tested as double triplicate sample on each side of the plate
S/T3	PCM medium + solvent + EC50 T3; Tested as double triplicate sample on each side of the plate
Ref(T3)C1	the max. concentration of T3 used in the study/the concentration no. 1; 2 nM); it will be used for calculation of RPE for EC50
*	Outer wells with PBS or PCM medium only; Outer wells may only be used only when the plate has the additional collar/space to fill it with liquid to ensure the cells proper humidity
TI C [7-1]	Test item in the range of concentration (C) from C7/Cmin to C1/Cmax (C7 is the lowest concentration tested) TI tested without addition of T3 (agonism experiment)
TI C [7-1] / T3	Test item in the range of concentration (C) from C7/Cmin to C1/Cmax (C7 is the lowest concentration tested); TI tested in the presence of EC50 T3 (antagonism experiment)
Blank	PCM medium without cells (AlamarBlue is added in the proliferation assay); used for calculations both % AR and % DR according to formulas given in SOP *Determination of cell proliferation in T-screen assay* in Section 2.2.1 or Section 2.2.2, respectively
Blank w/o AB	PCM medium without cells (AlamarBlue is not added in the proliferation assay); used for calculations only %DR according to formula given in SOP *Determination of cell proliferation in T-screen assay* in Section 2.2.2

3.3 T-Screen assay

IMPORTANT! Agonistic and antagonistic potential of test item should be assessed simultaneously but on separate plates. Complete Reference item standard curves (for Ref(T3) and Ref(DPH) in the agonism and antagonism plates, respectively) should be included in each assay.

3.3.1 Seeding the GH3 cells onto 96-well plate

Number of plates depends on number of test items. Because agonistic and antagonistic potential of TI should be assessed on separate plates, to test one TI two plates are needed. Because complete Reference item standard curves should be included in each assay thus the first set of two plates is used to assess one TI together with REF (T3) or REF (DPH) on the agonism or antagonism plates, respectively. The second set of two plates is used to assess the next two TI (Figure 5).

3.3.1-1 Forty-eight hours prior to plating the cells onto 96-well microplates for the experiment, change the standard culture medium to PCM medium (Section 1.8.2).

3.3.1-2 Release cells as described in SOP "*Handling and Maintenance of GH3 cell line*", Section 2.3.4 "Detachment of GH3 cells". Pipet cell suspension very carefully several times (cells easily detach but are sensitive to shaking as well as the effects of trypsin).

3.3.1-3 Determine density of cell suspension taking into account viable cells as described in SOP "*Handling and Maintenance of GH3 cell line*", Section 2.3.6.

3.3.1-4 Calculate cell viability as described in SOP "*Handling and Maintenance of GH3 cell line*", Section 2.3.6.

3.3.1-5 Only if viability of GH3 is more than 90%, dilute cell suspension in PCM medium and seed cells onto a 96-well microplate at a density of 2500 viable cells/well in 100 µL PCM medium.

3.3.1-6 Pre-incubate for 24 hours +/- 2 hours at 37 ± 2 ^{o}C and 5 ± 0.5% (v/v) CO_2 in a humid atmosphere to allow cells to attach to bottom of wells before the treatment.

3.3.1-7 Before the treatment, based on microscopic observation, the following acceptance criterion should be met (otherwise, the plate is rejected): not more than 50% of cells in the well are floating

3.3.2 Preparation of test, reference and control item solutions

Prepare test item *working solutions* as for the pre-screen experiment, using the dilution factor (DF) identified in the range finder pre-screen experiment that will capture the whole dose response. If no agonistic/antagonistic effect is observed the same range of concentrations (and the same DF) should be used in T-screen assay. If any agonistic/antagonistic effect is observed adjust DF to obtain the whole dose response (e.g. DF 2, 3, 4 or 5) (if any) and then repeat the experiment.
Reference and control items are used in T-Screen assay using the fixed concentration – the preparation of *working solutions* of reference and control items (*the double desired exposure concentrations*) are described in Section 1.8.4.

3.3.3 Exposure of the cells

Plate layout for the 8a T-Screen assay *is presented in Figure 5.*

For every test item the set of two plates is prepared, as follows:

3.3.3-1 **Agonist plates:**

- Add 100 µL *working solutions* (*the double desired exposure concentrations*) of test item (TI) or the appropriate controls prepared <u>in PCM medium</u> to the 100 µL already present (with the cells) in the respective well(s) (to achieve a 1:1 dilution). Test all samples in triplicate with exception of solvent control (SC) that is tested in 6-12 repetitions spread out each side of the plate.
- Add 100 µL 2x Ref(T3)C1 = the highest concentration in the range described in Section 3.1.2.
- Add 100 µL PCM medium to three wells designed UC (PCM) (untreated control/cell system control; Figure 5).
- Add 100 µL of PCM medium to the 100 µL already present (without the cells) in each well in columns 1 and 12 (*Blank* and *Blank* w/o AB; Figure 5) that are used in the proliferation assay for calculations.
- Add 100 µL of PCM medium or PBS to each well in rows A and H (external wells) to ensure proper humidity for the cells.
- Incubate plates for 96 ± 1 h at 37 ± 2 ^{o}C and 5 ± 0.5% (v/v) CO_2 in a humid atmosphere.

3.3.3-2 **Antagonism plates:**

- Add 100 µL *working solutions* (*the double desired exposure concentrations*) of test item (TI/T3) or the appropriate controls prepared <u>in PCM medium with addition of 2x EC50 value of T3</u> to the 100 µL already present (with the cells) in the respective well(s) (to achieve a 1:1 dilution). Test all samples in triplicate with exception of solvent control (S/T3) that is tested in 6-9 repetitions spread out each side of the plate.
- Add 100 µL 2x Ref(T3)C1 = the highest concentration in the range described in Section 3.1.2.
- Add 100 µL PCM medium (without EC50 T3!) to three wells designed UC (PCM) (untreated control/cell system control; Figure 5).
- Add 100 µL of PCM medium to the 100 µL already present (without the cells) in each well in columns 1 and 12 (*Blank* and *Blank* w/o AB; Figure 5) that are used in the proliferation assay for calculations.
- Add 100 µL of PCM medium or PBS to each well in rows A and H (external wells) to ensure proper humidity for the cells.
- Incubate plates for 96 ± 1 h at 37 ± 2 ^{o}C and 5 ± 0.5% (v/v) CO_2 in a humid atmosphere.

Figure 5. Plate layout for the 8a T-Screen assay.

(A) the first set of plates consists of ***the agonism plate 1*** that is used to assess the range of Ref (T3) concentrations (upper part of the plate) and test item 1 (lower part of the plate) together with all appropriate controls and ***the antagonism plate 1*** that is used to assess the range of Ref (DPH) concentrations (upper part of the plate) and test item 1 (lower part of the plate) in the presence of EC50 T3 together with all appropriate controls

REF (T3)	Reference item T3 for AGONISM; Tested as full dose response curve once per series of plates. Each plate should contain the triplicate samples used in data analysis on the plate
REF (DPH)	Reference item Amiodarone for ANTAGONISM; Tested as full dose response curve once per series of plates. Each plate should contain the triplicate samples used in data analysis on the plate
…/T3	Sample tested in the presence of EC50 T3
PC (A)	Positive control for AGONISM; C max; Tested as triplicate sample once per series of plates
PC (ANT)	Positive control for ANTAGONISM; C max; Tested as triplicate sample once per series of plates
NC	Negative Control; C max; Tested as triplicate sample once per series of plates; the same chemical for AGONISM and ATAGONISM
UC (PCM)	Untreated Control; Tested as triplicate sample on each plate, PCM Medium Only,
SC	PCM medium + solvent; Tested as triplicate sample on each plate. **It is recommended to use the same solvent for all reference and control items and to use that solvent for the test items, to ensure all samples are tested under the same conditions. In case another solvent is used for the test item, the impact on the test system and the results must be assessed.**
SDS	Cytotox control; Tested as triplicate sample once per series of plates; only tested on AGONISM plate
*	Outer wells with PBS or PCM medium only; Outer wells may only be used only when the plate has the additional collar/space to fill it with liquid to ensure the cells proper humidity.
TI	Test item; Tested as full dose response curve
C	Concentrations of TI or REF (from C7/Cmin to C1/Cmax; C7 is the lowest concentration tested)
Blank	PCM medium without cells (AlamarBlue is added in the proliferation assay); used for calculations both % AR and % DR according to formulas given in SOP *Determination of cell proliferation in T-screen assay* in Section 2.2.1 or Section 2.2.2, respectively
Blank w/o AB	PCM medium without cells (AlamarBlue is not added in the proliferation assay); used for calculations only %DR according to formula given in SOP *Determination of cell proliferation in T-screen assay* in Section 2.2.2

Figure 5. continued

(A) the first set of plates:

Agonism plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	*	*	*	*	*	*	*	*	*	*	*	*
B	Blank	SC	Ref(T3) C7	Ref(T3) C6	Ref(T3) C5	Ref(T3) C4	Ref(T3) C3	Ref(T3) C2	Ref(T3) C1	UC (PCM)	SDS	Blank w/o AB
C	Blank	SC	Ref(T3) C7	Ref(T3) C6	Ref(T3) C5	Ref(T3) C4	Ref(T3) C3	Ref(T3) C2	Ref(T3) C1	UC (PCM)	SDS	Blank w/o AB
D	Blank	SC	Ref(T3) C7	Ref(T3) C6	Ref(T3) C5	Ref(T3) C4	Ref(T3) C3	Ref(T3) C2	Ref(T3) C1	UC (PCM)	SDS	Blank w/o AB
E	Blank	NC	TI 1 C7	TI 1 C6	TI 1 C5	TI 1 C4	TI 1 C3	TI 1 C2	TI 1 C1	PC(A)	SC	Blank w/o AB
G	Blank	NC	TI 1 C7	TI 1 C6	TI 1 C5	TI 1 C4	TI 1 C3	TI 1 C2	TI 1 C1	PC(A)	SC	Blank w/o AB
G	Blank	NC	TI 1 C7	TI 1 C6	TI 1 C5	TI 1 C4	TI 1 C3	TI 1 C2	TI 1 C1	PC(A)	SC	Blank w/o AB
H	*	*	*	*	*	*	*	*	*	*	*	*

Antagonism plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	*	*	*	*	*	*	*	*	*	*	*	*
B	Blank	S/T3	Ref(DPH)C7/T	Ref(DPH)C6/T	Ref(DPH)C5/T	Ref(DPH)C4/T	Ref(DPH)C3/T	Ref(DPH)C2/T	Ref(DPH)C1/T3	UC (PCM)	REF(T3) C1	Blank w/o AB
C	Blank	S/T3	Ref(DPH)C7/T	Ref(DPH)C6/T	Ref(DPH)C5/T	Ref(DPH)C4/T	Ref(DPH)C3/T	Ref(DPH)C2/T	Ref(DPH)C1/T3	UC (PCM)	REF(T3) C1	Blank w/o AB
D	Blank	S/T3	Ref(DPH)C7/T	Ref(DPH)C6/T	Ref(DPH)C5/T	Ref(DPH)C4/T	Ref(DPH)C3/T	Ref(DPH)C2/T	Ref(DPH)C1/T3	UC (PCM)	REF(T3) C1	Blank w/o AB
E	Blank	NC/T3	TI 1 C7/T3	TI 1 C6/T3	TI 1 C5/T3	TI 1 C4/T3	TI 1 C3/T3	TI 1 C2/T3	TI 1 C1/T3	PC(ANT)/T3	S/T3	Blank w/o AB
G	Blank	NC/T3	TI 1 C7/T3	TI 1 C6/T3	TI 1 C5/T3	TI 1 C4/T3	TI 1 C3/T3	TI 1 C2/T3	TI 1 C1/T3	PC(ANT)/T3	S/T3	Blank w/o AB
G	Blank	NC/T3	TI 1 C7/T3	TI 1 C6/T3	TI 1 C5/T3	TI 1 C4/T3	TI 1 C3/T3	TI 1 C2/T3	TI 1 C1/T3	PC(ANT)/T3	S/T3	Blank w/o AB
H	*	*	*	*	*	*	*	*	*	*	*	*

Figure 5. continued

(B) the next set of plates:

Agonism plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	*	*	*	*	*	*	*	*	*	*	*	*
B	Blank	SC	TI 2 C7	TI 2 C6	TI 2 C5	TI 2 C4	TI 2 C3	TI 2 C2	TI 2 C1	UC (PCM)	SC	Blank w/o AB
C	Blank	SC	TI 2 C7	TI 2 C6	TI 2 C5	TI 2 C4	TI 2 C3	TI 2 C2	TI 2 C1	UC (PCM)	SC	Blank w/o AB
D	Blank	SC	TI 2 C7	TI 2 C6	TI 2 C5	TI 2 C4	TI 2 C3	TI 2 C2	TI 2 C1	UC (PCM)	SC	Blank w/o AB
E	Blank	SC	TI 3 C7	TI 3 C6	TI 3 C5	TI 3 C4	TI 3 C3	TI 3 C2	TI 3 C1	REF(T3) C1	SC	Blank w/o AB
G	Blank	SC	TI 3 C7	TI 3 C6	TI 3 C5	TI 3 C4	TI 3 C3	TI 3 C2	TI 3 C1	REF(T3) C1	SC	Blank w/o AB
G	Blank	SC	TI 3 C7	TI 3 C6	TI 3 C5	TI 3 C4	TI 3 C3	TI 3 C2	TI 3 C1	REF(T3) C1	SC	Blank w/o AB
H	*	*	*	*	*	*	*	*	*	*	*	*

Antagonism plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	*	*	*	*	*	*	*	*	*	*	*	*
B	Blank	S/T3	TI 2 C7/T3	TI 2 C6/T3	TI 2 C5/T3	TI 2 C4/T3	TI 2 C3/T3	TI 2 C2/T3	TI 2 C1/T3	UC (PCM)	REF(T3) C1	Blank w/o AB
C	Blank	S/T3	TI 2 C7/T3	TI 2 C6/T3	TI 2 C5/T3	TI 2 C4/T3	TI 2 C3/T3	TI 2 C2/T3	TI 2 C1/T3	UC (PCM)	REF(T3) C1	Blank w/o AB
D	Blank	S/T3	TI 2 C7/T3	TI 2 C6/T3	TI 2 C5/T3	TI 2 C4/T3	TI 2 C3/T3	TI 2 C2/T3	TI 2 C1/T3	UC (PCM)	REF(T3) C1	Blank w/o AB
E	Blank	S/T3	TI 3 C7/T3	TI 3 C6/T3	TI 3 C5/T3	TI 3 C4/T3	TI 3 C3/T3	TI 3 C2/T3	TI 3 C1/T3	Ref(DPH)C1/T3	S/T3	Blank w/o AB
G	Blank	S/T3	TI 3 C7/T3	TI 3 C6/T3	TI 3 C5/T3	TI 3 C4/T3	TI 3 C3/T3	TI 3 C2/T3	TI 3 C1/T3	Ref(DPH)C1/T3	S/T3	Blank w/o AB
G	Blank	S/T3	TI 3 C7/T3	TI 3 C6/T3	TI 3 C5/T3	TI 3 C4/T3	TI 3 C3/T3	TI 3 C2/T3	TI 3 C1/T3	Ref(DPH)C1/T3	S/T3	Blank w/o AB
H	*	*	*	*	*	*	*	*	*	*	*	*

3.3.4 Measurement of cell proliferation

Perform the cell proliferation assay according to Section 2: "AlamarBlue assay" in SOP "*Determination of cell proliferation in T-screen assay*". Before performing the assay, observe the cells under the microscope to record cytotoxic effect or precipitates (if any) as described in in SOP *Determination of cell proliferation in T-screen assay* in Section 2.1. Wells where precipitates were observed are excluded from calculation as recommended in SOP "*Determination of cell proliferation in T-screen assay*", Section 2.1.1 .

Observation of GH3 cell morphology after the exposure (focus on cytotoxic features, e.g. destroyed cells, destruction of the cell layers; intracytoplasmatic granules) is essential to confirm cytotoxic effect (the lower cellular activity of GH3 cells cultured in PCM medium without T3 as determined with the cell proliferation assay) and distinguish it from lack of agonistic effect (basal or low activity in PCM without T3).

3.4 Data Analysis and Calculations

Results of the cell proliferation assay, i.e. **% AlamarBlue reduction (%AR) or % Dye reduction (%DR),** calculated based on optical density (OD; absorbance) for each triplicate sample using formulas given in SOP *Determination of cell proliferation in T-screen assay* (Section 2.2.1 or Section 2.2.2, respectively) are used to calculate the agonistic and antagonistic effect according to formulas given below (Section 3.4.1 and Section 3.4.2, respectively).

3.4.1 Data analysis for AGONISM plate

3.4.1-1 Based on received results (optical density/absorbance), calculate **% AlamarBlue reduction (%AR)** or **% Dye reduction (%DR)** for each triplicate sample using formulas given in SOP *Determination of cell proliferation in T-screen assay* in Section 2.2.1 or Section 2.2.2, respectively.

3.4.1-2 Calculate the increase of cell proliferation that is expressed as **the relative proliferative effect (RPE)** for all samples. The response observed at 2 nM T3 [Ref(T3) C1] is considered as the maximum response and set as 100%. The response for the solvent control [SC] is set at 0%. Exemplary results are presented in Figure 6A.

$$\mathrm{RPE} = \frac{\%DR_x - \%DR_{SC}}{\%DR_{Ref(T3)C1} - \%DR_{SC}} \times 100$$

OR*

$$\mathrm{RPE} = \frac{\%AR_x - \%AR_{SC}}{\%AR_{Ref(T3)C1} - \%AR_{SC}} \times 100$$

where:

SC - solvent control;

Ref(T3) C1 - the max. response observed for T3 (the concentration no. 1; 2 nM);

x - the effect of TI, PC, NC or REF T3 at the concentration analysed

* - %AR or %DR is used depending on the method choose for calculation results of the proliferation assay (SOP *Determination of cell proliferation in T-screen assay* in Section 2.2.1 or Section 2.2.2, respectively)

3.4.1-3 Determine **the EC50 value of the reference item (Ref(T3))** and **the EC50 value of the test item (TI)** (for dose response) or the concentration giving highest RPE value above specific limit (e.g. > 10% increase or significantly different from SC). The EC50 value can be determined using the Hill curve model in a statistic programme e.g. GraphPad. The Hill curve model is a logistic regression model (variable slope, 4 parameters) that uses the following function:

$$y = RPE_{\min} + \frac{\left(RPE_{\max} - RPE_{\min}\right)}{\left(1+10^{\left((LogEC_{50}-x)*HillSlope\right)}\right)}$$

where:

- x - Log of concentration
- y - Relative induction (%)
- RPE_{max} - Maximum relative induction (%)
- RPE_{min} - Minimum relative induction (%)
- $LogEC_{50}$ - Log of concentration at which 50% of maximum relative induction is observed
- HillSlope - Slope factor of the Hill curve

3.4.1-4 Calculate the Z-factor for each plate tested according the following formula:

$$\textbf{\textit{Z-factor}}\ \textit{plate no.} = 1 - 3 \times \frac{(\text{SD } \textit{plate no...}\ [\text{SC}] + \text{SD } \textit{plate no....}\ [\text{Ref(T3)C1}])}{\text{abs } (\%\text{AR}^{*}\textit{plate no.}\ [\text{SC}] - \%\text{AR}^{*}\ \textit{plate no.}\ [\text{Ref(T3)C1}])}$$

where:

abs – absolute value;
SC – solvent control;
Ref (T3) C1 – the max. response observed for T3 (the concentration no. 1; 2 nM);
* – %AR or %DR is used depending on the method choose for calculation results of the proliferation assay (SOP *Determination of cell proliferation in T-screen assay* in Section 2.2.1 or Section 2.2.2, respectively)

3.4.2 Data analysis for ANTAGONISM plate

3.4.2-1 Based on received results (optical density/absorbance), calculate **% AlamarBlue reduction (%AR)** or **% Dye reduction (%DR)** for each triplicate sample using formulas given in SOP *Determination of cell proliferation in T-screen assay*; Section 2.2.1 or Section 2.2.2, respectively.

3.4.2-2 Calculate the decrease of cell proliferation expressed as ***the relative inhibitory effect (RIE)*** for all samples. The response for solvent control in the presence of EC50 T3 [S/T3]) is 100%; the max. response observed for REF in the ANTAGONISM plates in the presence of EC50 T3 ([Ref(DPH) Cmax/T3]) is set at 0%. Exemplary results are presented in Figure 6B.

$$\text{RIE} = \frac{\%DR_{x/T3} - \%DR_{\ Ref(DPH)Cmax/T3}}{\%DR_{S/T3} - \%DR_{Ref(DPH)Cmax/T3}} \times 100$$

OR*

$$\mathrm{RIE} = \frac{\%AR_{x/T3} - \%AR_{Ref(DPH)Cmax/T3}}{\%AR_{S/T3} - \%AR_{Ref(DPH)Cmax/T3}} \times 100$$

where:

S/T3 - solvent control in the presence of EC50 T3;

Ref(DPH)C_{max}/T3 - the max. response observed for the reference item (the concentration no. 1; 2 nM) in the presence of EC50 T3;

x/T3 - the effect of TI, PC, NC or Ref(DPH) at the concentration analysed in the presence of EC50 T3

* - %AR or %DR is used depending on the method choose for calculation results of the proliferation assay (SOP *Determination of cell proliferation in T-screen assay* in Section 2.2.1 or Section 2.2.2, respectively)

3.4.2-3 Determine **the IC50 value of the reference item (Ref(DPH))** and **the IC50 value of the test item (TI)** (for dose response) according to the formula given below or the concentration giving highest RIE value above specific limit (e.g. > 20% decrease). The IC50 value can be determined using the Hill curve model in a statistic programme e.g. GraphPad. The Hill curve model is a logistic regression model (variable slope, 4 parameters) that uses the following function:

$$y = RIEmin + \frac{(RIEmax - RIEmin)}{\left(1 + 10^{((LogIC_{50} - x) * HillSlope)}\right)}$$

x = Log of concentration

y = Relative inhibition (%)

RIE_{max} = Maximum relative inhibition (%)

RIE_{min} = Minimum relative inhibition (%)

$LogIC_{50}$ = Log of concentration at which 50% of maximum relative inhibition is observed

HillSlope = Slope factor of the Hill curve

3.4.2-4 Calculate the **Z-factor** for each plate tested according the following formula:.

$$\textbf{\textit{Z-factor}}\ \textit{plate no.} = 1 - 3 \times \frac{(\mathrm{SD}\ \textit{plate no...}\ [\mathrm{S/T3}] + \mathrm{SD}\ \textit{plate no....}\ [\mathrm{Ref(DPH)}\ C_{max}/\mathrm{T3}])}{\mathrm{abs}\ (\%\mathrm{DR}^*\ \textit{plate no.}\ [\mathrm{S/T3}] - \%\mathrm{DR}^*\ \textit{plate no.}\ [\mathrm{Ref(DPH)}C_{max}/\mathrm{T3})}$$

where: abs – absolute value;

SD – Standard deviation

S/T3 – solvent control;

Ref(DPH)C_{max}/T3 – the max. response observed for the reference item (the concentration no. 1; 2 nM) in the presence of EC50 T3;

* – %AR or %DR is used depending on the method choose for calculation results of the proliferation assay (SOP *Determination of cell proliferation in T-screen assay* in Section 2.2.1 or Section 2.2.2, respectively).

3.4.3 Relative potency of test items

Agonism:

The relative potency of test items is calculated by dividing the EC_{50} of T3 by the EC_{50} of the test item (or the concentration giving the highest RPE value that is above 10% increase as compared to SC).

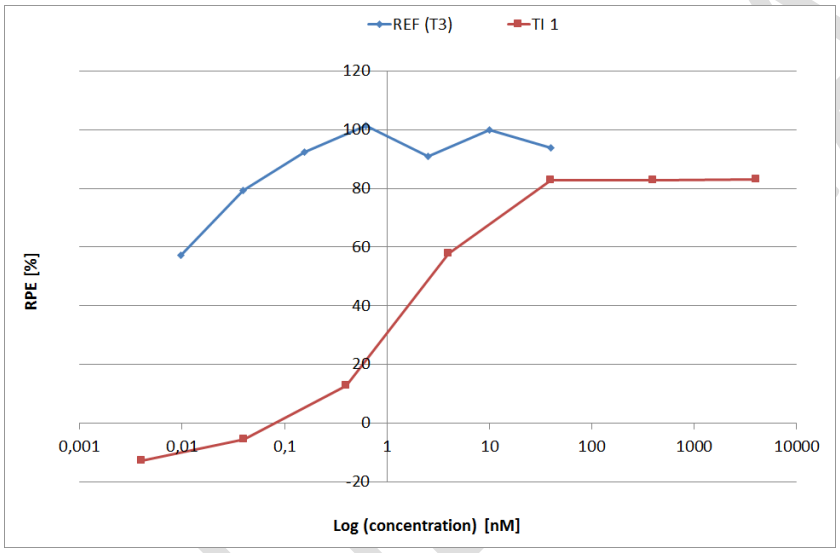
Antagonism:

The relative potency of test items is calculated by dividing the IC_{50} of REF by the IC_{50} of the test item (or the concentration giving the highest RIE value that is above > 10% decrease as compared to SC).

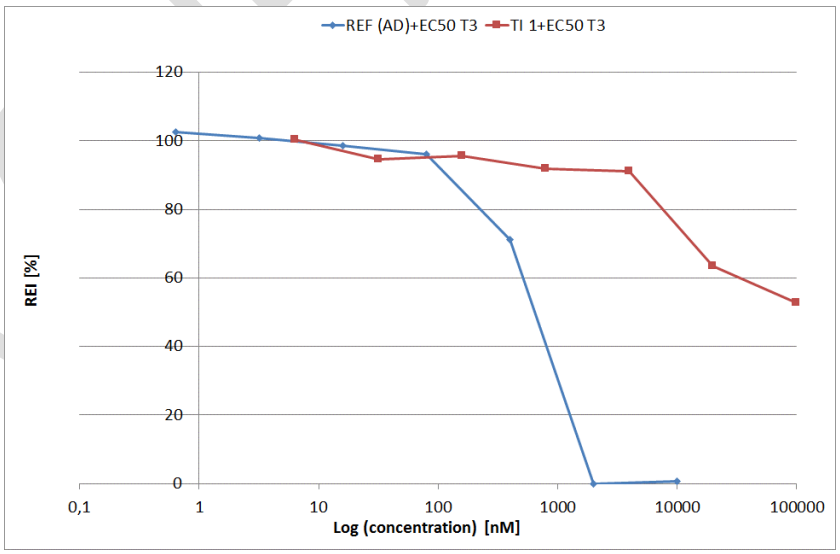
Figure 6. Exemplary results obtained for (A) AGONISM plate 1 and (B) ANTAGONISM plate 1

(corrected graphs will be added after PART 1/receiving new data)

A



B



3.5 Acceptance criteria

To be developed on basis of historical data with the reference and control items.

3.5.1 Acceptance criteria for AGONISM plate

3.5.1-1 Mean EC_{50} value of T3 should be -10±0.4 log10(Molar) units (in the range from -10.4 to -9.6 log10(Molar) units).

3.5.1-2 %DR or %AR for UC should not be more than 15% different from TI SC and REF SC

3.5.1-3 Z-factor > 0.5

3.5.2 Acceptance criteria for ANTAGONISM plate

3.5.2-1 RPE for EC50 T3 should be in the range of 30-70% = will be confirmed after PART 1

3.5.2-2 Z-factor > 0.5

4. Reference(s)

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