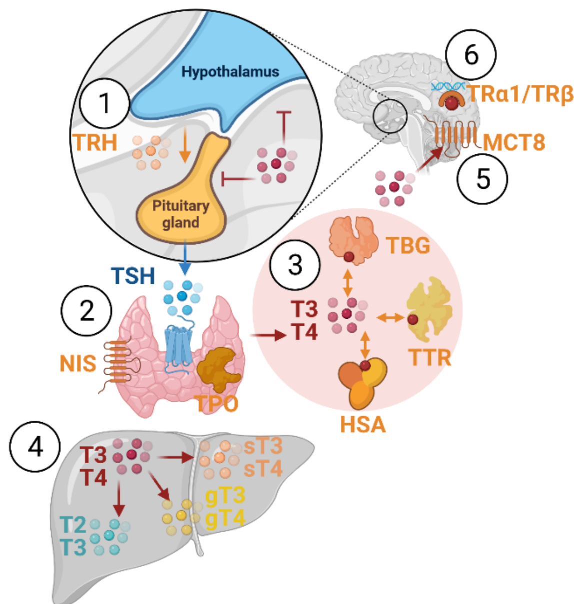
European
Commission

STANDARD OPERATING PROCEDURE

T-screen assay measuring cell proliferation of GH3 cells using alamar blue/resazurin, version 2.0

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

Roszak J.



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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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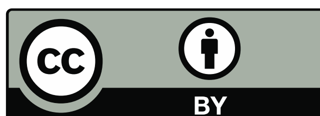

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1 Standard Operating Procedure

2

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

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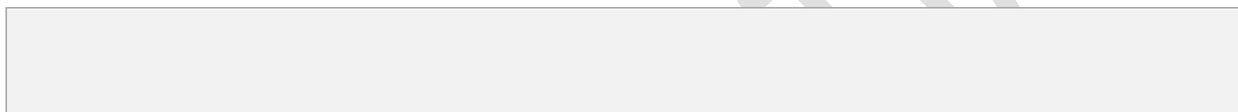
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61 **1. Description of the method**

62 The T-Screen represents an *in vitro* bioassay based on thyroid hormone (TH) dependent cell
63 proliferation of a rat pituitary tumour cell line (GH3) in serum-free medium. It can be used to study
64 interference of compounds with TH at the cellular level, thus bridging the gap between limitations of
65 assays using either isolated molecules (enzymes, transport proteins) or complex *in vivo* experiments
66 with all the complex feedback mechanisms present. Test items are tested in the absence of TH to
67 test for agonistic potency.

68 GH3 cell growth is increased in the presence of TH agonists. Cell growth is measured with
69 AlamarBlue/Resazurin cell proliferation assay using a standard plate reader. In this method a
70 colorimetric assay is used, where resazurin is reduced from a blue oxidized form into its violet
71 reduced form of resorufin. The change of colour can be detected as a change in absorbance using a
72 microplate reader.

73 **1.1 Scope and Limitations of the Application**

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

75 **1.2 Safety**

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

79 **1.3 Required Standard Operating Procedures**

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

82 **1.4 Test System**

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

84

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

86 **1.4-1** Test system must be free of microbial and mycoplasma contamination
87 **1.4-2** The doubling time of the GH3 cells in cDMEM/F12 medium should be 42 ± 5h
88 **1.4-3** Cell number in PCM after 72 ± 1 h and 96 ± 1 h of culture should be at least 35% lower
89 that cell number in cDMEM/F12, when determined simultaneously.
90 **1.4-4** Cells should be used in passage from 3 to 25

91 **1.5 Apparatus and Materials**

92 - Balance (minimal value: 50 mg; the precision requirement: 50 ± ≤0.001 mg) and the
93 appropriate mass standards F1, e.g. 1 - 500 mg ± ≤0.02 mg; 1 g ± ≤0.01 mg
94 - Clean glass vials for the preparation of stock solutions (e.g., gas chromatography vials or
95 Sarstedt #86.1509)
96 - 96-well microplates (e.g., Nunc #167008)
97 - Freezer below -16 °C
98 - Refrigerator at 2-10 °C
99 - CO_2 humidified incubator at 37°C +/- 2 °C, 5% CO_2 +/- 0.5%
100 - 37 °C water bath
101 - PipetteAid
102 - Pipettors (p1000, p200, p20) or Micropipettes (1000-100; 100-10; 10-0.5)
103 - Centrifuge
104 - Vacuum aspirator

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- 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
Reference item	3,3’-5-triiodothyronine (T3) [CAS 6893-02-3], ≥ 95% purity
Positive control item	T4, L-Thyroxine (3,3’,5,5’’-Tetraiodo-L-thyronine) [CAS 51-48-9], ≥ 98%
Negative control item	Furosemide (Fs) [54-31-9]
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.

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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°C e.g. during the night and stored in aliquots at below -16°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 1.8.4-1)	10 µM	0.105
50 µg/mL sodium selenite (Section 1.8.4-2)	10 ng/mL	0.105
2 mg/mL human apotransferrin (Section 1.8.4-3)	10 µg/mL	2.6
100 mg/mL bovine serum albumin (Section 1.8.4-4)	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 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 3.1.1 and/or Section 3.1.2.

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1.8.3-1 **3,3'-5-triiodothyronine (T3) [2mM 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: 2 mM → (100x) → 20 µM --(20x)→ 1 µM. Then, use the *concentrated solution* of T3 to prepare *working solution* according to Section 3.2.2

1.8.3-2 **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 3.2.2.

1.8.3-3 **Furosemide (FS) [100 mM stock solution] and [200 nM working solution]**

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

The *exposure concentration* of FS in the T-Screen assay is 100 nM – to prepare the *concentrated solution* of Fs [50 µM], dilute Fs stock solution in the solvent/DMSO as follows: 100 mM –(100x)→ 1 mM –(20x)→ 50 µM. Then, use *the concentrated solution* of Fs to prepare *working solution* according to Section 3.2.2.

1.8.3-4 **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 3.2.2

1.8.4 Stock solutions of reagents

1.8.4-1 **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.

1.8.4-2 **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.

1.8.4-3 **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.

1.8.4-4 **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

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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 ± 2 h at 37 ± 1 °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 of % Dye reduction (%DR) according to formulas given in SOP *Determination of cell proliferation in T-screen assay* in Section 2.
Blank w/o AB - PCM medium without cells (AlamarBlue is not added in the proliferation assay); used for calculations of %DR according to formula given in SOP *Determination of cell proliferation in T-screen assay* in Section 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*".

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234 2.3 - 2. Based on received results (optical density/absorbance), calculate **% Dye reduction**
235 **(%DR)** for each triplicate sample using formulas given in SOP *Determination of cell*
236 *proliferation in T-screen assay* in Section 2.

237 2.3 - 3. Calculate the increase of *Cell proliferation* that is expressed as **the relative proliferative**
238 **effect (RPE)** according to the formula given in Section 3.4.2
239 2.3 - 4. Calculate the **EC50** of T3 according to the formula given in Section 3.4.3.

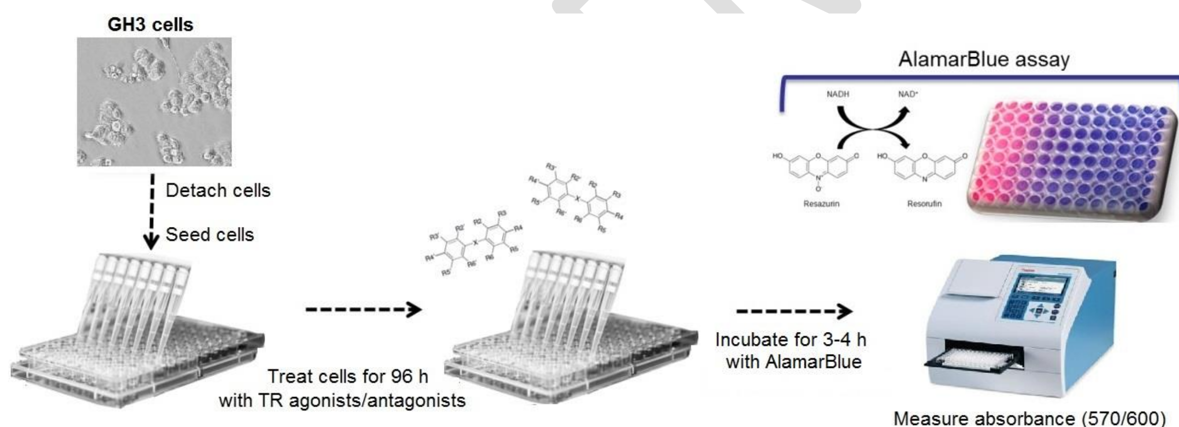
240 **2.4 Acceptance criteria**

241 Based on the obtained results the mean EC50 value of T3 should be calculated. The mean EC50
242 value should be -10±0.4 log10(Molar) units (in the range from -10.4 to -9.6 log10(Molar) units).
243 If acceptance criteria for the mean EC50 value of T3 are met, 0.1 nM T3 should be used as the
244 EC50 value of T3 in the T-screen test.
245 Every result, i.e. the EC50 of T3, should be registered to create the historical data EC50 value of T3.
246 Also, the mean EC50 of T3 calculated from the results obtained during every T-screen test (the first
247 plate, Section 3.4.3) should be included to the historical data.

248 **3. T-Screen experimental procedure**

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

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



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253 **3.1 Preparation of Test Item**

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

256 ***3.1.1 Stock solutions***

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

260 3.1.1-1 Prepare a 100 mM or 10 mg/mL stock solution (may be adapted on basis of data if too low
261 or high) of test item by weighing a nominal amount into a clear glass vial and add the
262 appropriate volume of solvent (use DMSO as a default solvent and if not possible to

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263 dissolve test item in DMSO then other solvents, e.g. water, PCM medium or ethanol
264 should be used. For visual inspection, it is important to have at least 0.5 ml in the vial to be
265 able to observe well.
266 3.1.1-2 Visually check if dissolved. If not dissolved, heat (37°C; up to 30 min) may be applied to aid
267 solubility.
268 3.1.1-3 If not possible to solubilise, prepare a lower (e.g. 1:2) stock solution (or dilute existing
269 stock) and again check solubility. Change solvent if needed.
270 3.1.1-4 Continue until the stock solution is soluble.

271 ***3.1.2 Work solutions to determine solubility***

272 3.1.2-1 Dilute the stock solution of test item in solvent prepared in Section 3.1.1. Prepare the
273 range of 4 concentrations (*the concentrated solutions*) using a dilution factor 2 (50 µL of
274 stock solution of the appropriate *concentrated solution* to 50 µL solvent in a 96-well plate).
275 For test item dissolved in DMSO or ethanol prepare the 500x *concentrated solutions*,
276 whereas for test item dissolved in water or PBS, prepare 100x *concentrated solutions*.
277 3.1.2-2 Prepare the 2x concentrated solutions (*working solutions*) in PCM medium by transferring:
278 2 µL of the appropriate *concentrated solutions* to 498 µL of PCM in a 24-well plate or 1.1
279 mL cluster tubes.
280 Solvent concentration in the *working solution*s of test item should not exceed 0.4% for
281 DMSO and ethanol or 10% for water or PBS (the final concentration 0.2% or 5%,
282 respectively for DMSO/ethanol or water/PBS).
283 3.1.2-3 Visually check(using a microscope) if the working solutions are dissolved.
284 3.1.2-4 Incubate the plate with the working solutions for 24 h at 37 ± 1 °C and 5 ± 0.5% (v/v) CO_2
285 in a humid atmosphere to check if any precipitates appear.
286 3.1.2-5 Identify the highest soluble concentration.

287 **3.2 Pre-screen experiment**

288 During the pre-screen experiment it is assessed if the test item is:
289 • A Thyroid Hormone Agonist
290 • Cytotoxic
291
292 and at which dose-range it increases or decreases cell proliferation (Range finder).
293
294 **Note:** GH3 cells are not able to divide properly in PCM without T3 but keep basal or low
295 activity. Concentrations of test items lower than 15% of the cellular activity of GH3 cells
296 cultured in PCM medium without T3 (as determined with the cell proliferation assay) are
297 considered to be cytotoxic.

298 ***3.2.1 Seeding the GH3 cells onto 96-well plate***

299 Perform the cell seeding as described for the T-screen (Section 3.3.1).
300 - Seed cells into all internal wells, i.e. B2-G11.
301 - Add 100 µL of PCM medium to each well in columns 1 and 12, for *Blank* and *Blank w/o AB*
302 (Figure 4) that are used for calculations in the proliferation assay.
303 - Add 100 µL of PCM medium or PBS to each well in rows A and H (external wells) to ensure
304 proper humidity for the cells.

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305 ***3.2.2 Preparation of test, reference and control item solutions***

306 On the day of treatment, prepare the range of 7 *working solutions*:

307 3.2.2-1 Stage 1: Prepare stock solution of test item at the concentration and solvent determined
308 above (Section 3.1.1). Prepare a dilution series of test item stock in the solvent (the range
309 of 7 *concentrated solutions*) using a dilution factor 10 (10 µL of stock solution of the
310 appropriate concentrated solution to 90 µL solvent in a 96-well plate).
311 For test item dissolved in DMSO or ethanol prepare the 500x concentrated solutions,
312 whereas for test item dissolved in water or PBS, prepare 100x concentrated solutions.

313 In the first experiment a dose range finding is performed to capture the whole dose response range
314 for the next experiments. The highest concentration to be tested is the highest non cytotoxic soluble
315 concentration and dilution factor 10 is applied for the endpoint measurement. In the next
316 experiment a dose range includes 7 concentrations where the highest concentration shows the max
317 effect (the induction of proliferation) and the lowest concentration shows no effect (effect
318 comparable to solvent control). Dilution factor is adjusted to cover 7 concentrations and each test
319 item have its own dilution factor (e.g. DF 1.5, 2, 3, 4 or 5).

320

321 3.2.2-2 Stage 2: Prepare *working solutions* as described in Section 3.1.2
322 In the first experiment/the dose range finding an interference of test item with the
323 assay/AlamarBlue reagent should be assessed. To this end, prepare *working solutions* as described in
324 Section 3.1.2 using double volumes to have enough solutions both for test plate and the additional
325 plate for testing interference of AlamarBlue with test item.
326

327 - Add 498 µL PCM medium into 1.1 mL-cluster tubes, then
328 - Add 2 µL of the appropriate *concentrated solution* to the mixture of PCM medium and PCM-
329 T3 medium already present into 1.1 mL-cluster tubes. Solvent concentration should not
330 exceed 0.5%.
331

332 ***3.2.3 Exposure of the cells***

333 Test all samples in triplicate. Assess one test item or reference item on an upper part of a
334 plate(B2:D11) and another test item on a lower part of a plate (E2:G11). Perform the exposure as
335 follows:

336 3.2.3-1 Test plate:
337 - Add 100 µL *working solutions* (*the double desired exposure concentrations*) of test item
338 (TI) or solvent control (SC) prepared in PCM medium to the 100 µL already present (with
339 the cells) in the respective well(s) (for TI 1/SC 3B:9D/2B-D and 11B-D and for and TI 2/SC
340 3E:9G/2E:G; to achieve a 1:1 dilution).
341 - Add 100 µL PCM medium only to three wells designed “UC (PCM)” (10E:G; untreated
342 control/cell system control).
343 - Add 100 µL 2 x EC50T3 = the concentration 0.2 nM T3 (if it was positively verified; Section
344 2.4)
345 - Add 100 µL 2x Ref(T3)C1 = the highest concentration in the range described in Section
346 3.2.2 (Figure 4).

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- 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.

-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.

3.2.3-2 Additional plate (interference assessment)

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. Also, add extra PCM medium in columns 1 and 12 and in rows A and H, as given in Section 3.2.3-1.

3.2.3-3 Incubate plates for 96 ± 2 h at 37 ± 1 °C and 5 ± 0.5% (v/v) CO_2 in a humid atmosphere.

Plate layout for Pre-screen experiment is presented in Figure 4. It is advised to assess each item in triplicates.

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 **% Dye reduction (%DR)** for each triplicate sample using formulas given in SOP *Determination of cell proliferation in T-screen assay* in Section 2.

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 3.4.2

3.2.5 Acceptance criteria for pre-screen assay

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

3.2.5-2 RPE for EC50 T3 (i.e. 0.1 nM 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 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 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 effect is observed, choose DF to obtain the whole dose response (e.g. DF 1.5, 2, 3, 4 or 5) (if any).

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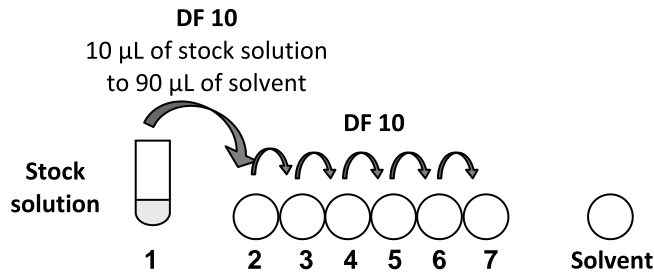
389 ***Figure 3.*** *A schema of test item preparation for pre-screen experiment and the T-Screen test.*

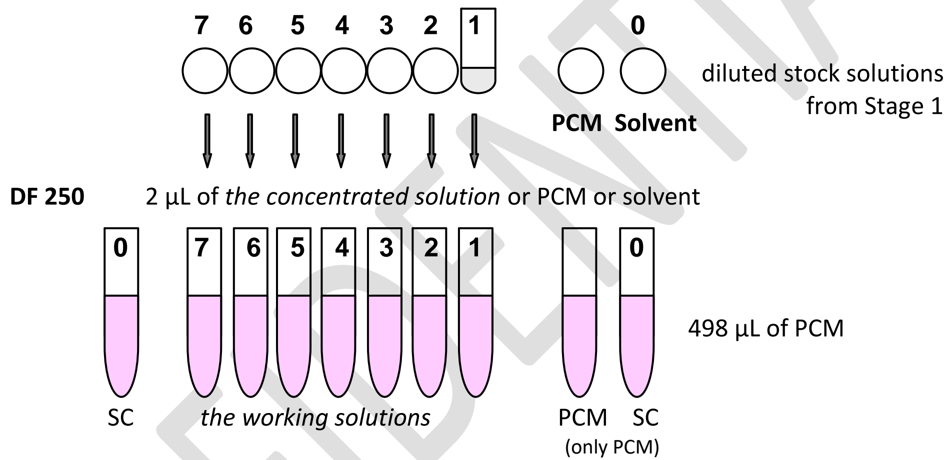
390

Stage 1. Dilution of a stock solution to prepare the range of ***the concentrated solutions***



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

Stage 2. Preparation of the ***working solutions*** (the double desired exposure concentrations) 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 %.

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

		1	2	3	4	5	6	7	8	9	10	11	12
	A	*	*	*	*	*	*	*	*	*	*	*	*
	B	Blank	SC	TI1 C7	TI1 C6	TI1 C5	TI1 C4	TI1 C3	TI1 C2	TI1 C1	UC (PCM)	SC	Blank w/o AB
TI 1	C	Blank	SC	TI1 C7	TI1 C6	TI1 C5	TI1 C4	TI1 C3	TI1 C2	TI1 C1	UC (PCM)	SC	Blank w/o AB
	D	Blank	SC	TI1 C7	TI1 C6	TI1 C5	TI1 C4	TI1 C3	TI1 C2	TI1 C1	UC (PCM)	SC	Blank w/o AB
	E	Blank	SC	TI2 C7	TI2 C6	TI2 C5	TI2 C4	TI2 C3	TI2 C2	TI2 C1	EC50 T3	Ref(T3)C1	Blank w/o AB
TI 2	F	Blank	SC	TI2 C7	TI2 C6	TI2 C5	TI2 C4	TI2 C3	TI2 C2	TI2 C1	EC50 T3	Ref(T3)C1	Blank w/o AB
	G	Blank	SC	TI2 C7	TI2 C6	TI2 C5	TI2 C4	TI2 C3	TI2 C2	TI2 C1	EC50 T3	Ref(T3)C1	Blank w/o AB
	H	*	*	*	*	*	*	*	*	*	*	*	*

392

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
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
TI1 C [7-1]	Test item in the range of concentration (C) from C7/Cmin to C1/Cmax (C7 is the lowest concentration tested)
TI2 C [7-1]	Another test item in the range of concentration (C) 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 of % DR according to formulas given in SOP *Determination of cell proliferation in T-screen assay* in Section 2.
Blank w/o AB	PCM medium without cells (AlamarBlue is not added in the proliferation assay); used for calculations of %DR according to formula given in SOP *Determination of cell proliferation in T-screen assay* in Section 2.

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393 **3.3 T-Screen assay**

394 IMPORTANT! Complete Reference item standard curve (Ref(T3)) in the should be included in each
395 assay.

396 ***3.3.1 Seeding the GH3 cells onto 96-well plate***

397 Number of plates depends on number of test items. Because complete Reference item standard
398 curve should be included in each assay thus the first plate is used to assess one TI together with REF
399 (T3). Another plate is used to assess the next two TI (Figure 5).

400 3.3.1-1 48 ± 2 hours prior to plating the cells onto 96-well microplates for the experiment, change
401 the standard culture medium to PCM medium (Section 1.8.2).
402 3.3.1-2 Release cells as described in SOP "*Handling and Maintenance of GH3 cell line*", Section
403 2.3.4 "Detachment of GH3 cells". Pipet cell suspension very carefully several times (cells
404 easily detach but are sensitive to shaking as well as the effects of trypsin). During
405 detachment use only PCM medium (do not use culture medium with serum).
406 3.3.1-3 Determine density of cell suspension taking into account viable cells as described in SOP
407 "*Handling and Maintenance of GH3 cell line*", Section 2.3.6.
408 *3.3.1-4* Calculate cell viability as described in SOP "*Handling and Maintenance of GH3 cell line*",
409 Section 2.3.6.
410 3.3.1-5 Only if viability of GH3 is more than 90%, dilute cell suspension in PCM medium and seed
411 cells onto a 96-well microplate at a density of 2500 viable cells/well in 100 µL PCM
412 medium.
413 3.3.1-6 Pre-incubate for 24 hours ± 2 hours at 37 ± 1 °C and 5 ± 0.5% (v/v) CO_2 in a humid
414 atmosphere to allow cells to attach to bottom of wells before the treatment.
415 3.3.1-7 Before the treatment, based on microscopic observation, the following acceptance
416 criterion should be met (otherwise, the plate is rejected): not more than 50% of cells in the
417 well are floating

418 ***3.3.2 Preparation of test, reference and control item solutions***

419 Prepare test item *working solutions* as for the pre-screen experiment, using the dilution factor (DF)
420 identified in the range finder pre-screen experiment that will capture the whole dose response. If no
421 agonistic effect is observed the same range of concentrations (and the same DF) should be used in T-
422 screen assay. If any agonistic effect is observed adjust DF to obtain the whole dose response (e.g. DF
423 2, 3, 4 or 5) (if any) and then repeat the experiment.
424 Reference and control items are used in T-Screen assay using the fixed concentration – the
425 preparation of *working solutions* of reference and control items (*the double desired exposure*
426 *concentrations*) are described in Section 1.8.3.

427 ***3.3.3 Exposure of the cells***

428 *Plate layout for the* 8a T-Screen assay *is presented in Figure 5.*
429 For every test item the half plate is prepared, as follows:
430 - Add 100 µL *working solutions* (*the double desired exposure concentrations*) of test item (TI) or
431 the appropriate controls prepared <u>in PCM medium</u> to the 100 µL already present (with the
432 cells) in the respective well(s) (to achieve a 1:1 dilution). Test all samples in triplicate with

433 exception of solvent control (SC) that is tested in 6-12 repetitions spread out each side of the
434 plate.
435 - Add 100 µL 2x Ref(T3)C1 = the highest concentration in the range described in Section
436 3.2.2Add 100 µL PCM medium to three wells designed UC (PCM) (untreated control/cell
437 system control; Figure 5).
438 - Add 100 µL of PCM medium to the 100 µL already present (without the cells) in each well in
439 columns 1 and 12 (*Blank* and *Blank* w/o AB; Figure 5) that are used in the proliferation assay
440 for calculations.
441 - Add 100 µL of PCM medium or PBS to each well in rows A and H (external wells) to ensure
442 proper humidity for the cells.
443 - Incubate plates for 96 ± 2 h at 37 ± 1 °C and 5 ± 0.5% (v/v) CO_2 in a humid atmosphere.

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

445 (A) the first plate 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
446 appropriate controls.

447

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
PC (A)	Positive control; 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
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
*	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 of % DR according to formulas given in SOP *Determination of cell proliferation in T-screen assay* in Section 2.
Blank w/o AB	PCM medium without cells (AlamarBlue is not added in the proliferation assay); used for calculations of %DR according to formula given in SOP *Determination of cell proliferation in T-screen assay* in Section 2.

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449 **Figure 5. continued**
450
451 **(A) the first plate:**

	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	*	*	*	*	*	*	*	*	*	*	*	*

452
453
454 **(B) the next plates:**
455

	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	*	*	*	*	*	*	*	*	*	*	*	*

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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. **% 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.) are used to calculate the agonistic effect according to formulas given below (Section 3.4).

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

3.4.2 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.

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

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

the proliferation assay (SOP *Determination of cell proliferation in T-screen assay* in Section 2.)

3.4.3 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:

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494 $$y = RPE_{\min} + \frac{(RPE_{\max} - RPE_{\min})}{(1 + 10^{((LogEC_{50} - x) * HillSlope)})}$$

495 where:

496 x - Log of concentration

497 y - Relative induction (%)

498 RPE_{max} - Maximum relative induction (%)

499 RPE_{min} - Minimum relative induction (%)

500 $LogEC_{50}$ - Log of concentration at which 50% of maximum relative induction is observed

501 HillSlope - Slope factor of the Hill curve

502 3.4.4 Calculate the Z-factor for each plate tested according the following formula:

503

504 $$\textbf{\textit{Z-factor}}_{\text{plate no.}} = 1 - 3 \times \frac{(SD_{\text{plate no.}}[SC] + SD_{\text{plate no.}}[Ref(T3)C1])}{abs(\%DR_{\text{plate no.}}[SC] - \%DR_{\text{plate no.}}[Ref(T3)C1])}$$

505 where:

506 abs – absolute value;

507 SC – solvent control;

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

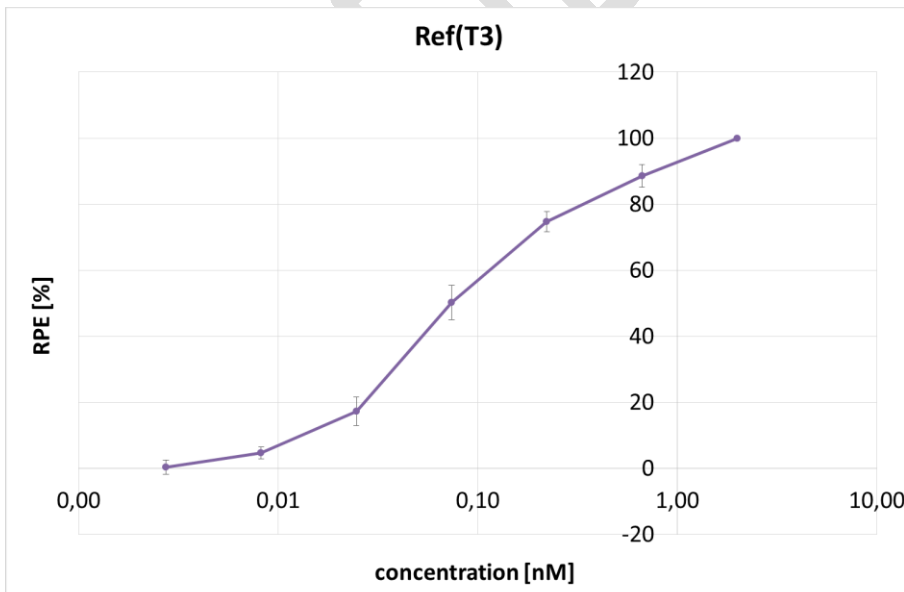
509

510 3.4.5 Relative potency of test items

511 ***The relative potency of test items*** is calculated by dividing the EC_{50} of T3 by the EC_{50} of the test item

512 (or the concentration giving the highest RPE value that is above 10% increase as compared to SC).

513 ***Figure 6.*** Exemplary results obtained for reference item with agonistic potential



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517 **3.5 Acceptance criteria**

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

519 3.5.1. Mean EC_{50} value of T3 should be -10±0.4 log10(Molar) units (in the range from -10.4 to -9.6
520 log10(Molar) units).

521 3.5.2. %DR for TI SC and for Ref SC should not differ more than 15% from UC and REF SC

522 3.5.3. Z-factor > 0.5

523

524 **4. Reference(s)**

525 • Ghisari, M., & Bonefeld-Jorgensen, E. C. (2005). Impact of environmental chemicals on the
526 thyroid hormone function in pituitary rat GH3 cells. *Molecular and Cellular Endocrinology*, 244:
527 31–41. http://doi.org/https://doi.org/10.1016/j.mce.2005.01.013

528 • Gutleb, A. C., Meerts, I. A. T. M., Bergsma, J. H., Schriks, M., & Murk, A. J. (2005). T-Screen as a
529 tool to identify thyroid hormone receptor active compounds. *Environmental Toxicology and*
530 *Pharmacology*, 19: 231–238. http://doi.org/https://doi.org/10.1016/j.etap.2004.06.003

531 • Sirbasku, D.A., Pakala, R., Sato, H., Eby, J.E. (1991). Thyroid hormone dependent pituitary tumor
532 cell growth in serum-free chemically defined culture. A new regulatory role for apotransferrin.
533 *Biochem.* 30: 7466–7477.

534 • Schriks M, Vrabie CM, Gutleb AC, Faassen EJ, Rietjens IM, Murk AJ. (2006) T-screen to quantify
535 functional potentiating, antagonistic and thyroid hormone-like activities of poly halogenated
536 aromatic hydrocarbons (PHAHs). *Toxicol In Vitro.* 20: 490-498.

537 • Hohenwarter O, Waltenberger A, Katinger H. (1990) An in vitro test system for thyroid hormone
538 action. Anal. Biochem. 234: 56-59.

539 • Koyanagi M, Kawakabe S, Arimura Y. (2016). A comparative study of colorimetric cell proliferation
540 assays in immune cells. Cytotechnology, 68, 1489-1498. doi:10.1007/s10616-015-9909-2.

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542 **5. A change log**

543

544 Standard Operating Procedure:
545 T-screen assay measuring cell proliferation of GH3 cells using alamar blue/resazurin
546

ID	Version	Status	Date	List of changes in version 02 with respect to version 01:
1.	v. 01	Approved	03.03.2021	not applicable
2.	v. 02	Draft	09.12.2021	- Antagonist part of the method was removed throughout the SOP - %AR calculation was removed as the results were the same as for the %DR calculation
3.	v. 02	Draft	27.12.2021	- Adding a section: A change log
4.	v. 02	Final	25.01.2022	- updated incubation time from 48h ± 1h to 48 ± 2h, and updated exposure time from 96 ± 1h to 96 ± 2h as it is not critical and allows for more flexibility'

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