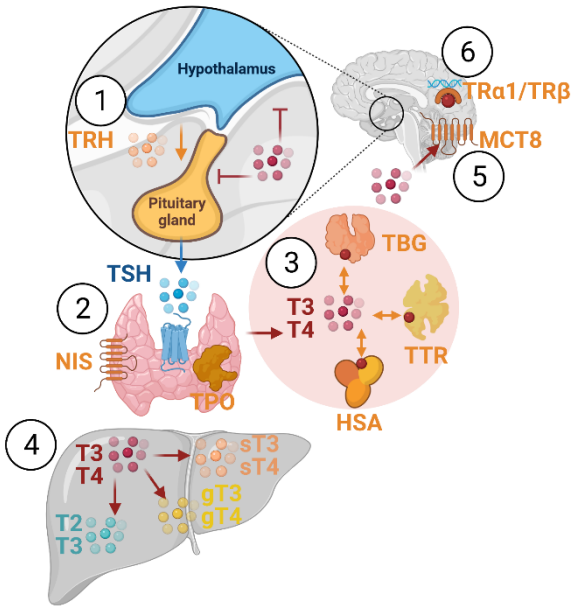
European Commission

STUDY REPORT

for testing of six test items for TH receptor (TR)-mediated cell proliferation to assess the robustness and reliability of the T-screen method and confirm the reference and control items - Part 1

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..



2023

Joint Research Centre

This study report 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.

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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Nofer Institute of Occupational Medicine (NIOM)
Department of Translational Research
ZTM

Report of the study No. ZTM/2021/02/T-screen

„Testing of six test items for TH receptor (TR)-mediated cell proliferation to assess the robustness and reliability of the T-screen method and confirm the reference and control items"

17th November, 2021

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1. GENERAL INFORMATION

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1.1 Information on Reference items

	Agonist Reference item	**Antagonist reference item**
Chemical name	3,3',5-Triiodo-L-thyronine **(T3)**	5,5-Diphenyl Hydantoin; Phenytoin **(DPH)**
Cas No.	6893-02-3	57-41-0
Supplier / Cat. No.	JRC-IRMM Geel	Merck D4007
Lot No.	ERM-AC469	MKCF7191
Purity	98%	≥99%
Physical state	Solid	Solid
Storage	4°C	RT
Hazard statement	H302	H302
Expiry date	Undefined	Undefined

1.2 Information on positive and negative control items

	Agonist positive control item	**Agonist & Antagonist negative control item**	**Cytotoxicity positive control item**
Chemical name	3,3',5,5''-Tetraiodo-L-thyronine; L-Thyroxine **(T4)**	Mefenamic acid **(MfA)**	Sodium dodecyl sulfate; Sodium lauryl sulfate **(SDS)**
Cas No.	51-48-9	61-68-7	151-21-3
Supplier / Cat. No.	Merck T2376	Merck M4267	Merck 436143
Lot No.	BCBV2496	MKCH3607	MKCJ9719
Purity	≥98%	≥98% (HPLC) ≥98% (Titration with NaOH)	≥96% (GC) ≥99% (Titration by NaOH)
Physical state	Solid	Solid	Solid
Storage	RT	RT	RT
Hazard statement	none	H302	H228, H302+H332, H315, H318, H335, H412
Expiry date	02.2022	Undefined	Undefined

1.3 Information on the Test items (with the concentration used in the T-Screen test)

Chemical name	**Test item ***					
	3,3',5,5''-Tetraiodo-L-thyronine; L-Thyroxine **(T4)**	Mefenamic acid **(MfA)**	Sodium dodecyl sulfate; Sodium lauryl sulfate **(SDS)**	3,3',5,5'-Tetraiodo thyroacetic acid **(Tetrac)**	Amiodarone hydrochloride **(AD)**	Furosemide **(FS)**
CAS No.	51-48-9	61-68-7	151-21-3	67-30-1	19774-82-4	54-31-9
Supplier / Cat. No.	Merck T2376	Merck M4267	Merck 436143	TCI T3730	TCI A2530	Merck F4381
Lot No.	BCBV2496	MKCH3607	MKCJ9719	SU3CK	BKKII	MKCJ1412
Purity	≥98%	≥98% (HPLC) ≥98% (Titration with NaOH)	≥96% (GC) ≥99% (Titration by NaOH)	98.9% (HPLC) 98.8% (Neutralization titration)	99.9% 100.4% (nonaqueous titration)	≥98%
Physical state	Solid	Solid	Solid	Solid	Solid	Solid
Storage	RT	RT	RT	-20°C	4°C light sensitive	RT
Hazard statement	none	H302	H228, H302+H332, H315, H318, H335, H412	H300 Fatal if swallowed	H315, H361fd, H362, H319, H373	H360
Expiry date	02.2022	Undefined	Undefined	Undefined	Undefined	12.2024

2. AIM OF THE STUDY

This study is performed for PART 1 of the EURL ECVAM coordinated Thyroid Validation Study. After the full description of the method (the Pre-screen experiments and T-screen assay) in a standard operating procedure, the robustness and reliability of the method was assessed in this study by performing five valid runs with 3,3',5,5'-tetraiodothyroacetic acid (Tetrac), 3,3',5,5"-Tetraiodo-L-thyronine (T4 or L-Thyroxine), Amiodarone (AD), Mefenamic acid (MfA), Furosemide (FS) and Sodium dodecyl sulfate (SDS).
The positive and negative control items currently provided in the T-screen assay SOP were considered as test items in this study, to be tested at 7 concentrations to obtain dose response information.
All results were calculated in two ways – the first way is applicable only for Bio-Rad kit (%AR), whereas the second one is universal for different brands of AlamarBlue (%DR). Based on both calculations the other essential parameters were calculated (e.g. RPE, RIE, EC50, IC50) and compared and after this study it will be decided to keep the general calculation (DR%) or not.
On basis of the results, the positive and negative control items, and their concentrations to be used in the method, will be confirmed.
Also, EC50 value of T3 will be verified according to the SOP but in the conditions without antibiotics. Comparison of T3 EC50 values, obtained using medium with or without antibiotics, was done and after this study it will be decided if the T-screen test may be performed without antibiotics.

3. 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.

4. TIME SCHEDULE OF THE STUDY

Study plan:	30.03.2021
Date of start of the experimental part:	30.03.2021
Date of end the experimental part:	30.06.2021
Report for the Customer:	17.11.2021

Quality assurance: The study was conducted in accordance with the principles of Good Laboratory Practice (GLP), but out of the system.
Study inspections: Magdalena Stanisławska, PhD, Head of Quality Assurance Unit

At the beginning of the study the following area were verified:
- the place of the performing the study (there was used only one cell system in the incubator and only one study in the lab in the same time)

- records on laboratory cleaning monitoring
- records on equipment used in the study
- records on cell system and reagents (the storage place of reagents and cell system)
- records on proceeding with cell system
- records on temperature monitoring in freezers
- documentation of microscopic observations (records and photos) and other documentation

5. DEVIATIONS FROM THE STUDY PLAN (or STANDARD OPERATING PROCEDURE)

The study was performed according to the Standard Operating Procedures as follows:

- Handling and Maintenance of GH3 cell line v01 / 2021-03-03
- T-screen assay using GH3 cell line v01 / 2021-03-03
- Determination of cell proliferation in T-screen assay v01 / 2021-03-03

Seven deviations from the study plan or SOP were recorded during the study:

Deviation 1 from SOP "*Handling and Maintenance of GH3 cell line*" Section 2.3.5 – less cells were put into T25 flask: (1) on 12.04.2021 to multiply cells for Mycoplasma sp. testing ; (2) on 02.06.2021 to set the culture designed for test because the cells were cultured one day longer than usual due to holiday in Poland

Deviation 2 from SOP "*Handling and Maintenance of GH3 cell line*" Section 2.3.4 – every time when the cells were detached for the test (after 48 preincubation in PCM medium) the activity of Accutase was stopped using PCM medium instead of cDMEM/F12 medium containing serum.

Deviation 3 from SOP "*T-screen assay using GH3 cell line*" Section 3.1.2 – for some test items the first Pre-screen test was performed using DF different than 10 (the DF used as was determined in previous experiments)

Deviation 4 from the Study Plan and SOP "*T-screen assay using GH3 cell line*" Section 1.8.4-1 and 1.8.4-2 –in some experiments twice concentrated stock solutions were used (i.e. 2 mM T3; 4 mM T4) due to too large weight /not enough volume capacity of a vial. For these chemicals, good solubility of the chemicals in the solvent at the concentration used was confirmed. Additionally, no impact of the stock concentration on the results was observed.

Deviation 5 from SOP "*T-screen assay using GH3 cell line*" Figure 4 (Plate layout) – on 30.04.2021 during exposure of the cells in the first Pre-screen test the working solutions of Tetrac were wrongly plated on the upper part of the plate. To be able to get any information from the test, the plate was appropriate described immediately after exposure and after receiving results (absorbance) the raw data was rearranged to obtained correct layout – such kind of results were used only to check if the range of concentrations need to be modified or not. The test was repeated in the next Pre-screen test.

Deviation 6 from the Study Plan – as Ref(DPH)/EC50T3 the concentration of 10 µM was used instead of 50 µM due to unexpected results of solubility determination

Deviation 7 from SOP "*Determination of cell proliferation in T-screen assay*" – during the second round of the T-Screen experiment the cells were incubated with AlamarBlue for 4-5 hour instead

of 3.5±0.5 due to unexpected failure of software. All plates were read as soon as the problem was overcome. As the results of the preliminary studies showed that an incubation time of at least 5 hours with AlamarBlue did not affect the results, the measurements from the second experiment were used for further analysis.

Deviation 8 from SOP "*T-screen assay using GH3 cell line*" Section 3.1.1. The concentration of stock solution for each chemical was determined based on previous experiments thus during the study solubility of chemicals was only confirmed in the concentrations of stock solution used.

Briefly results of the preliminary study were as follows:

DPH, SDS, MfA and FS were soluble in DMSO at the concentration of 100 mM.

Solubility of T3, T4 and Tetrac were checked at the concentration up to 10 mM and all these were soluble. Solubility at the concentrations higher than 10 mM were not checked since based on literature (Gutlab et al., 2005) the chemicals were effective at very low concentrations.

The highest stock concentration of AD checked was 10 mM because it was impossible to prepare 100 mM solution of AD in any liquid. AD did not dissolve in DMSO but AD was dissolved in ethanol at 10 mM.

6. RESULTS

6.1. The test system

- GH3-cell line – a rat pituitary tumour cell line, (ATCC® CCL-82.1™; Lot No. 59257317)

Before the T-Screen tests the GH3 cells were verified to meet the acceptance criteria given in the SOP "*Handling and maintenance of GH3 cell line*" (Section 2.5), i.e.:

- Absence of mycoplasma was confirmed
- Absence of microbiological contamination was confirmed
- The doubling time of the GH3 cells in cDMEM/F12 medium was confirmed as 42 ± 5h
- Cell number in PCM in 72h±1h and 96h±1h of culture was at least 40% lower that cell number in cDMEM/F12, when determined simultaneously.

6.1.1. Verification of GH3 cell culture for absence of *Mycoplasma sp.* contamination

After restoring the GH3 cells (frozen in NIOM in 13.11.2019) the cells were cultured 7 days in cDMEM/F12 w/o antibiotics (prepared according to Section 2.2.1 of the SOP "Handling and Maintenance of GH3 cell line", but without adding antibiotics) and the presence of *Mycoplasma sp.* was checked using MycoBlue Mycoplasma Detector (Vazyme #D101; LOT #7E420F0).
Also, after the last passage of GH3 cells needed for the test (the passage +19), cells were cultured in cDMEM/F12 medium without antibiotics for 9 days and after that, the cell culture was checked for absence of microbiological contamination and the presence of *Mycoplasma sp.* was checked as mentioned above.
Both tests gave a negative results confirming that the study was performed on GH3 cells free from *Mycoplasma sp.*.

Additionally, five millions of cells in 2 vials (each at least 2.5 million of cells) were collected and frozen (according to SOP "Handling and Maintenance of GH3 cell line", Section 2.3.3. and 2.3.2, respectively) to be sent back to EURL ECVAM for quality control.

6.1.2. Verification of GH3 cell culture for microbiological contamination

The culture of GH3 cells was regularly checked for signs of microbiological contamination such as fungi, bacteria, yeast by microscopic observation, i.e. before every passage, before each change of medium and before cryopreservation of cells. Also, cells plated on 96-well plates were checked for microbiological contamination at the end of exposure (before the addition of Alamar Blue; according to SOP "*Determination of cell proliferation in T-screen assay*" Section 2.1).

No microbiological contamination was observed in any of the culture flask or plates, confirming that GH3 cells used in the study were free from microbiological contamination.

6.1.3. Determination of the doubling time (Td) of the GH3 cells

Before starting the experiments the GH3 cells were passaged 3 times according to SOP "*Handling and Maintenance of GH3 cell line*" and the cultures were set for the doubling time determination (6 x T25 in cDMEM/F12 and 6x T25 in PCM); passage +4).
Since work time (days off in Poland) did not allow to perform the Td determination starting from Friday as it was recommended by SOP "*Handling and Maintenance of GH3 cell line*" Section 2.4 and there was

a great need to start experiments as soon as possible it was decided to performed Td determination twice: (1) as soon as possible in the schedule from Monday to Tuesday/next week and (2) when it will be possible in the schedule from Friday to Monday/next week (provided that results of the first test are acceptable). Results of both Td determinations are presented in the Figure 1 and Table 1.

Figure 1. Growth curve for GH3 cells grown in two kinds of media (cDMEM/F12 and PCM) in two time variants (from Mo to Tue/next week and from Fri to Mo/next week).

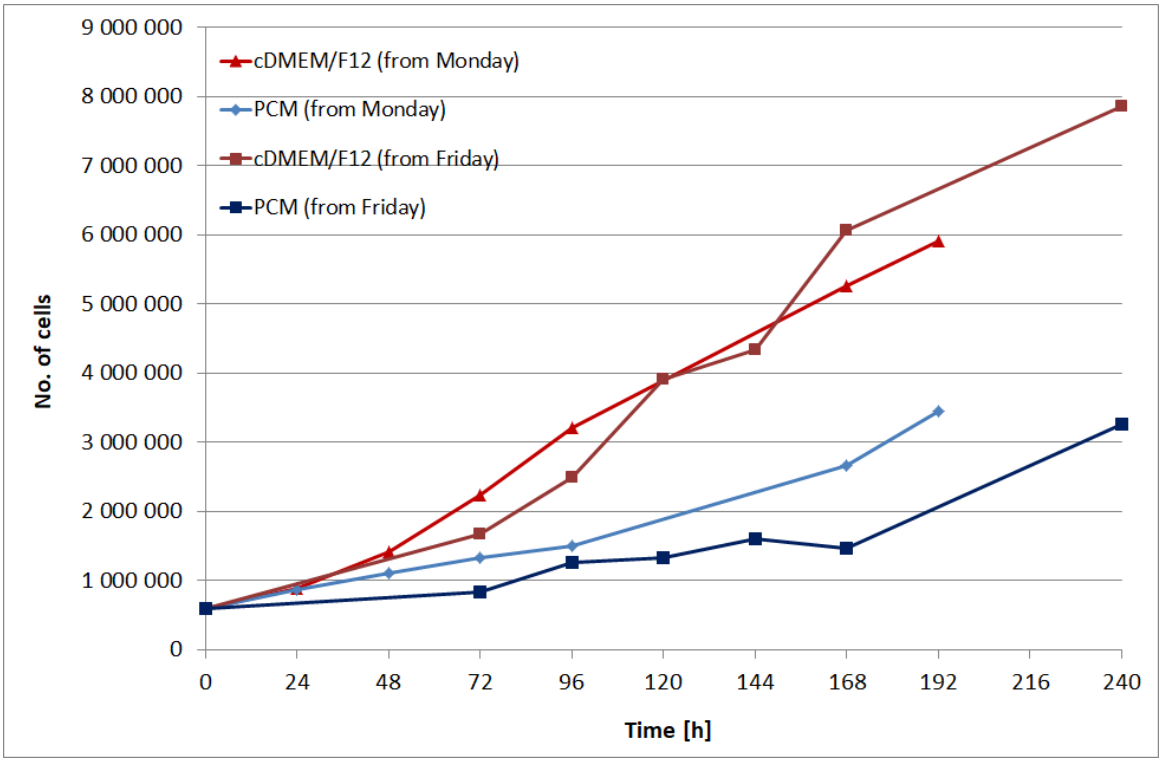


Table 1. Criteria calculated based on results of Td determination for both time variants.

Criterion	GH3 cell culture		Acceptance criteria
	from Monday to Tuesday/next week	from Friday to Monday/next week	
Td in cDMEM/F12 medium based on results obtained for 72-h and 96-h cultures	46 h	42 h	42 ± 5h
No. of cells in PCM vs cDMEM/F12 in 72-h culture	1 333 333 (PCM) 2 240 000 (cDMEM/F12) (40.5%)	833 333 (PCM) 1 677 778 (cDMEM/F12) (50.3%)	At least 40% lower cell number in PCM than in cDMEM/F12
No. of cells in PCM vs cDMEM/F12 in 96-h culture	1 500 000 (PCM) 3 211 111 (cDMEM/F12) (53.3%)	1 266 667 (PCM) 2 488 889 (cDMEM/F12) (49.1%)	

6.2. Verification of the EC50 value of T3

Before starting the experiments the response of GH3 cells to T3 was verified. Verification of the EC50 value of T3 was performed according to SOP "*T-screen assay using GH3 cell line*" Section 2. Measurements (three independent experiments) were performed in PCM medium without antibiotics to compare effect of antibiotics on the T3 EC50 value. To this end, during the time needed to perform the EC50 of T3 verification GH3 cells were cultured in two variants, i.e. in cDMEM/F12 with and without antibiotics (prepared according to Section 2.2.1 of the SOP "*Handling and Maintenance of GH3 cell line*", but with and without adding antibiotics). Hence, the culture maintained in cDMEM/F12 was verified in PCM and the culture maintained in cDMEM/F12 w/o antibiotics was verified in PCM w/o antibiotics according to SOP "*T-screen assay using GH3 cell line*" Section 2.
The mean EC50 value of T3 was calculated in two ways – the first way, based on % AlamarBlue reduction (%AR; according to the method specified in the BioRad manual) and the second one, based on **% Dye reduction (%DR)** (according to Section 2.2.1 and Section 2.2.2 of the SOP "Determination of cell proliferation in T-screen assay", respectively).
The mean EC50 value of T3 calculated based on %AR was 0.071 ± 0.024 and 0.068 ± 0.003 nM, for conditions with and without antibiotics, respectively, whereas the mean EC50 value of T3 calculated based on %DR was 0.068 ± 0.003 and 0.071 ± 0.024 nM, for conditions with and without antibiotics, respectively (Table 2).
Because the mean EC50 values of T3 for both cell culture variants were in the range from -10.4 to -9.6 log10(Molar) units, the acceptance criteria for the mean EC50 value of T3 were met and 0.1 nM T3 was used as the EC50 value of T3 in the T-screen test.
Additionally, the EC50 of T3 verification was performed using two different seeding and treatment schedule variants (A and B, Figure 2) and no impact of schedule variants on results was observed.

Figure 2. The seeding and treatment schedule variants of the test used in the study

				preculture in PCM					test		
variant A	passage			1 day	2. day	plating	0/exposure	24	48	72	96/results
	Fri	Sat	Sun	Mo	Tue	Wed	Thur	Fri	Sat	Sun	Mo
				preculture in PCM					test		
variant B			passage	1 day	2. day	plating	0/exposure	24	48	72	96/results
			Mo	Tue	Wed	Thur	Fri	Sat	Sun	Mo	Tue

Table 2. Cell proliferation expressed as the relative proliferative effect (RPE) calculated in two ways: (A) based on % AlamarBlue reduction (%AR) and (B) based on % Dye reduction (%DR). The EC50 values of T3 (in nM units) were determined using the Hill curve model in Graphpad Prism v.6.05 and recalculated to log10(Molar) units.

A. Results calculated based on %AR								
T3 [nM]	**RPE [%]**							
	PCM				**PCM w/o antibiotics**			
	Ex 1 [(A)]	Ex 2 [(B)]	Ex 3 [(A)]	**AVG ± SD**	Ex 1 [(A)]	Ex 2 [(B)]	Ex 3 [(A)]	AVG ±SD
0.003	0	6	4	3 ± 3	-1	6	2	2 ± 3
0.008	6	7	18	10 ± 7	-9	23	10	8 ± 16
0.025	20	23	41	28 ± 12	19	22	24	22 ± 3
0.074	47	N/A	74	60 ± 19	53	59	52	55 ± 4
0.222	73	79	102	85 ± 16	103	91	79	91 ± 12
0.667	88	101	125	105 ± 18	109	96	92	99 ± 9
2.000	100	100	100	100 ± 0	100	100	100	100 ± 0
EC50 [nM]	0.0856	0.0851	0.0426	**0.0711 ± 0.0247**	0.0648	0.0689	0.0711	**0.0683 ± 0.0032**
EC50 [log10(Molar)]	-10.0674	-10.0702	-10.3705	**-10.1694 ± 0.1742**	-10.1882	-10.1618	-10.1481	**-10.1660 ± 0.0204**

B. Results calculated based on %DR								
T3 [nM]	**RPE [%]**							
	PCM				**PCM w/o antibiotics**			
	Ex 1 [(A)]	Ex 2 [(B)]	Ex 3 [(A)]	**AVG ± SD**	Ex 1 [(A)]	Ex 2 [(B)]	Ex 3 [(A)]	AVG ±SD
0.003	0	6	4	**3 ± 3**	-1	6	2	**2 ± 3**
0.008	5	7	18	**10 ± 7**	-9	23	10	**8 ± 16**
0.025	20	23	41	**28 ± 12**	19	22	24	**22 ± 2**
0.074	47	N/A	74	**60 ± 19**	53	59	52	**55 ± 4**
0.222	73	79	102	**85 ± 16**	103	91	79	**91 ± 12**
0.667	88	101	125	**105 ± 18**	109	96	92	**99 ± 9**
2.000	100	100	100	**100 ± 0**	100	100	100	**100 ± 0**
EC50 [nM]	0.0848	0.0851	0.0426	**0.0708 ± 0.0244**	0.0648	0.0689	0.0711	**0.0683 ± 0.0032**
EC50 [log10(Molar)]	-10.0718	-10.0702	-10.3705	**-10.1708 ± 0.1729**	-10.1882	-10.1618	-10.1481	**-10.1660 ± 0.0204**

[(A)] Experiment performed in the seeding and treatment schedule variant A
[(B)] Experiment performed in the seeding and treatment schedule variant B

6.3. Determination of solubility of reference, control and test items

Solubility of all items in the solvent and medium was assessed. Both stock solutions and working solutions were assessed.

Solvent, the concentrations of stock solution, the exposure concentrations and dilution factor (DF) for each chemical was determined based on previous experiments.

However, before the Pre-screen experiments solubility of each chemical in the determined solvent as well as in PCM medium were verified according to SOP "*T-screen assay using GH3 cell line*" Section 3.1. (Table 3).

All chemicals, except AD, were dissolved in DMSO, whereas AD was dissolved in EtOH. Previous experiment showed that EtOH at the concentration used (0.4%) had no impact on the test system, and it was confirmed during every experiment by comparing results for SC/AD (i.e. SC for AD) with results for PCM medium as well as results for SC/DMSO.

For two chemicals (DPH and MfA) the concentration of the highest working solution (and thus also the highest concentrated solution) was found to be insoluble and new values were determined for the Pre-screen experiment (Figure 3). The reason for that was that during the previous experiment solubility was assessed in the final (exposure) concentrations instead of in the working concentrations. Results of solubility verification and the working concentration determined for the Pre-screen experiment are presented in Table 3.

Table 3. Results of solubility verification and the working concentration determined for the Pre-screen experiment

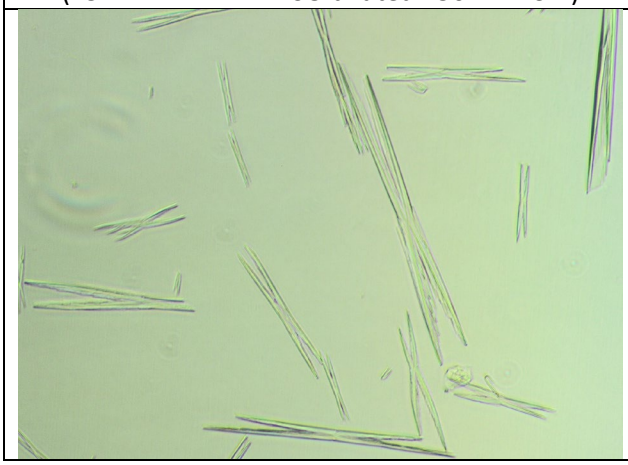
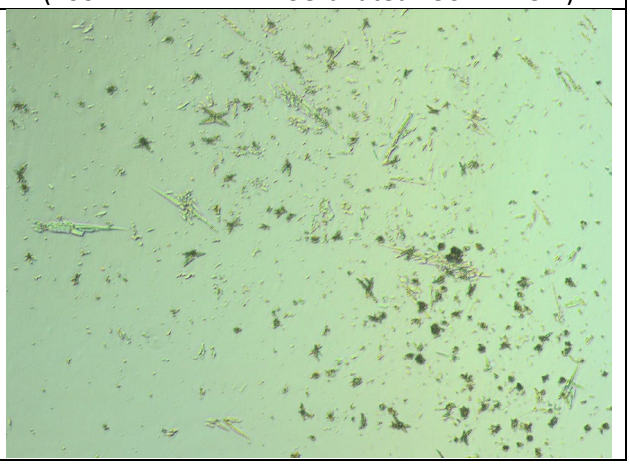
Items	**Solvent**	**Stock concentration**	**DF**	**the highest concentration of working solution**	**Microscopic observation of working solution at the highest concentration**	**pH value of working solution at the highest concentration**
T3	DMSO	1 mM/2 mM (1)	3	4 nM	Clear solution	7.067
DPH	DMSO	50 mM	5	100 µM (2) 80 µM	Big single crystals (Figure 3) No crystals seen	N/A 7.165
Tetrac	DMSO	5 mM	10	20 µM	Clear solution	7.061
T4	DMSO	2 mM/4 mM (1)	10	8 µM	Clear solution	7.185
AD	EtOH	5 mM	5	20 µM	Clear solution	7.096
SDS	DMSO	100 mM	2	400 µM	Clear solution	7.126
MfA	DMSO	100 mM	10	400 µM (2) 100 µM (3) 80 µM (3) 40 µM	A lot of small crystals (Figure 3) Still crystals seen Still crystals seen No crystals seen	N/A N/A N/A 7.171
FS	DMSO	100 mM	10	400 µM	Clear solution	7.049
PCM medium	-	-	-	-	Clear solution	7.100

(1) In some experiments twice concentrated stock solutions were used (i.e. 2 mM T3; 4 mM T4) due to too large weight /not enough volume capacity of a vial. For these chemicals, good solubility of the chemicals in the solvent at the concentration used was confirmed. Additionally, no impact of the stock concentration on the results was observed.

(2) The concentration wrongly identified based on results of previous experiments

(3) unacceptable concentration

Figure 3. Microscopic (phase-contrast) image of the DPH and MfA solutions at the indicated concentrations taken during solubility determination with the Olympus UC30 camera attached to the Olympus IX70 microscope (magnification 100x /10x10).

100 µM DPH (25 mM DPH in DMSO diluted 250x in PCM)	**400 µM MfA** (100 mM MfA in DMSO diluted 250x in PCM)
	

6.4. The Pre-screen test

The Pre-screen experiments were performed according to SOP "*T-screen assay using GH3 cell line*" Section 3.2 using the plate layout presented in Figure 4 of the SOP. In the first Pre-screen experiments the range of concentrations for T-Screen test for three of eight items, i.e. T3, T4 and FS was determined/confirmed. Pre-screen tests for AD, DPH, SDS and MfA were repeated using different range of concentrations due to cytotoxic effect of the highest concentrations of the item used. The Pre-screen test for Tetrac was repeated using the originally designed range of concentration due to an error in applying the working solutions during the first test, but finally the range of concentrations determined for the T-Screen test was modified to exclude too many concentrations giving the response similar to control solvent. Similarly, the range concentrations for T4 and SDS were adjusted based on results of the Preliminary test to capture the whole dose response - 7 concentrations where the highest concentration showing the max effect (the induction of proliferation in Agonist experiment) and the lowest concentration showed no effect (effect comparable to solvent control in Agonist experiment).
During the first Pre-screen test an interference of test items with the assay/AlamarBlue reagent was assessed according to SOP "*T-screen assay using GH3 cell line*" Section 3.2.3-5.

Since GH3 cells are not able to divide properly in PCM without T3, but keep basal or low activity, according to SOP "*T-screen assay using GH3 cell line*" Section 3.2, concentrations of test items that reduce the cellular activity of GH3 cells cultured in PCM medium without T3 (as determined with the cell proliferation assay in Agonist experiment) are considered to be cytotoxic. Because one of acceptance criteria for the Pre-Screen test (Section 3.2.5-1 of the SOP) is that %DR or %AR for UC(PCM) should not be more than 15% different from TI SC, it was decided to apply the same criterion for cytotoxic effect to precisely confirm the results for the highest exposure concentrations. The new criterion was determined as follows: **%DR or %AR for C1 of TI should not be less than 15% of %DR or %AR for TI SC.**

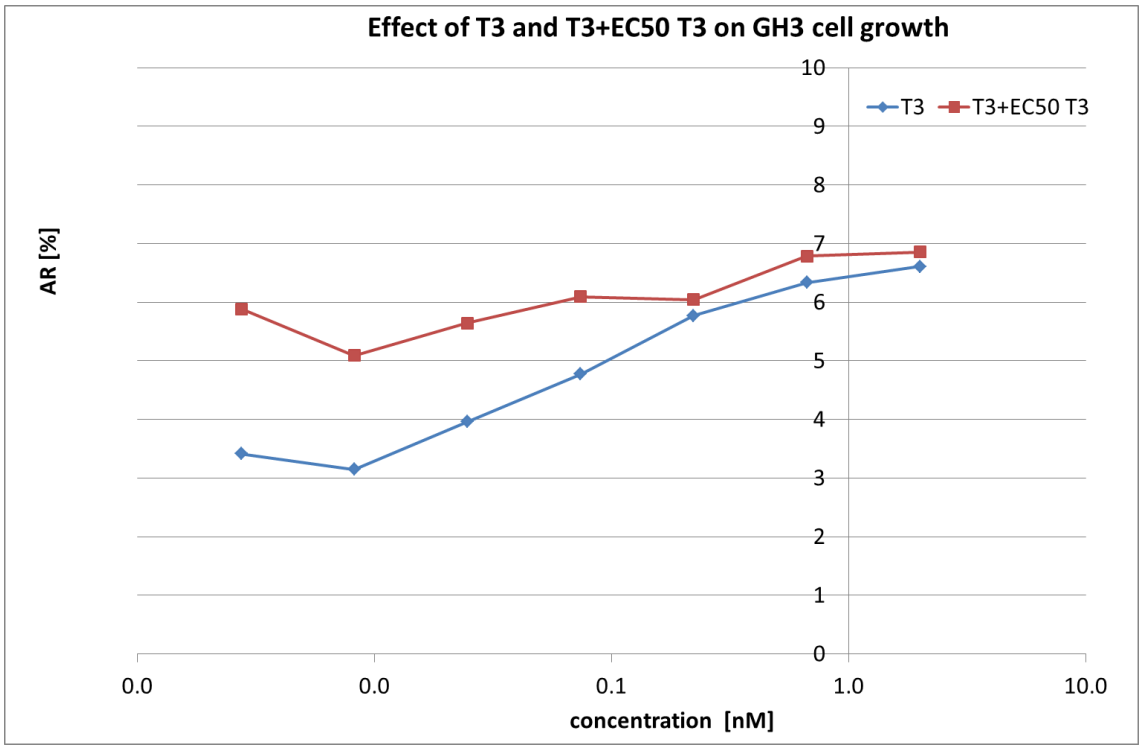
The highest concentrations of the concentrated and working solutions and DF chosen for T-Screen test are presented in Table 4. Results of Pre-screen test and testing the interference chemicals with AlamarBlue determined according to the method specified in the BioRad manual (%AR) are presented in Figure 4 A-H. The chosen ranges of concentrations were non-cytotoxic. For some items agonistic effect was observed, i.e. for T3, T4 and Tetrac, or antagonistic effect, i.e. AD and the highest concentrations of SDS and MfA. Effect of DPH was ambiguous.
Results of absorbance measurements performed for solutions of test items preincubated without cells (96 h) and then incubated with AlamarBlue (3-4 h) according to SOP "*T-screen assay using GH3 cell line*" Section 3.2.3-5. indicated that there was no interference of any of the test items with the AlamarBlue and thereby that AlamarBlue can be used for determination of the GH3 cell proliferation following of exposure to these chemicals.

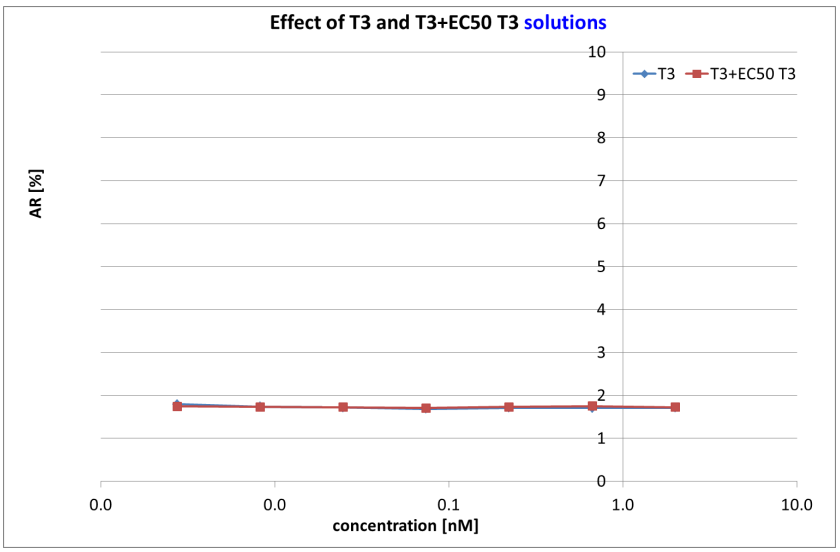
Table 4. The highest concentrations of the concentrated and working solutions, DF and the highest final/exposure concentrations determined for the T-Screen test

Items	**the highest concentration of *concentrated solution* (500x)**	**DF**	**the highest concentration of *working solution* (2x)**	**the highest final concentration (1x)**
T3	1 µM	3	4 nM	2 nM
DPH	5 mM	5	20 µM	10 µM
Tetrac	500 µM	5	2 µM	1 µM
T4	250 µM	5	1 µM	500 nM
AD	250 µM	2	1 µM	500 nM
SDS	20 mM	2	80 µM	40 µM
MfA	5 mM	5	20 µM	10 µM
FS	100 mM	10	400 µM	200 µM

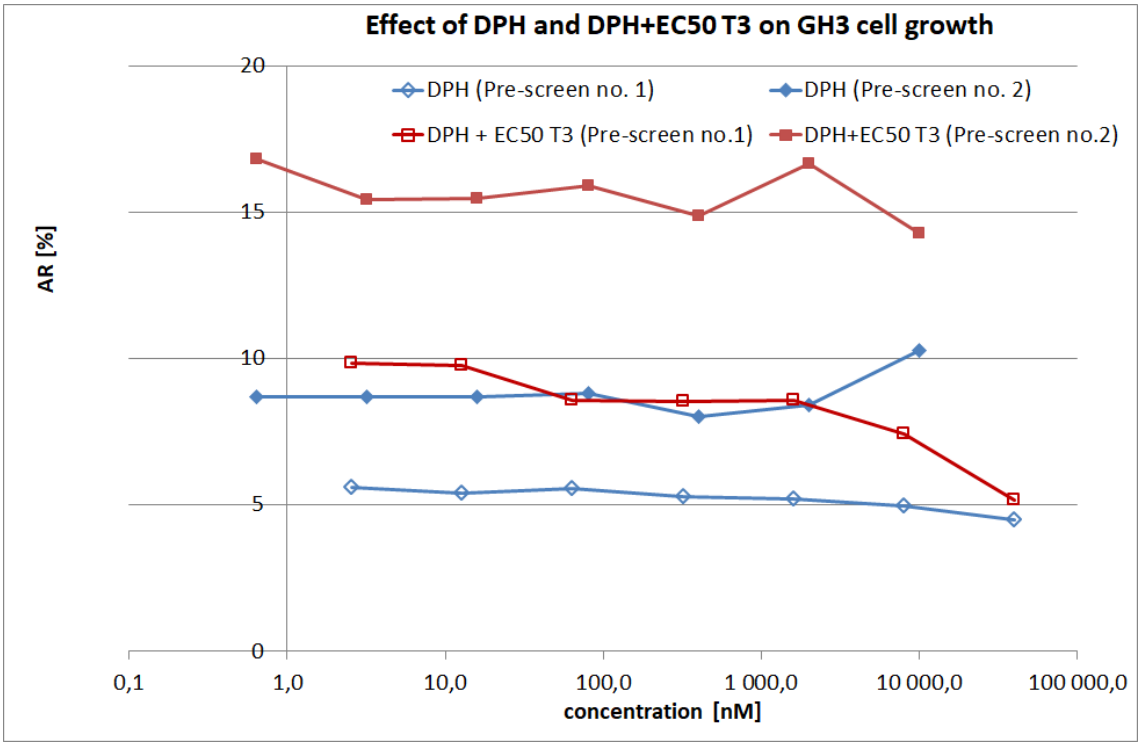
Figure 4. Results of Pre-screen test performed for (A) T3, (B) DPH, (C) Tetrac, (D) T4, (E) AD, (F) SDS, (G) MfA and (H) FS as well as results of testing the interference chemicals with AlamarBlue

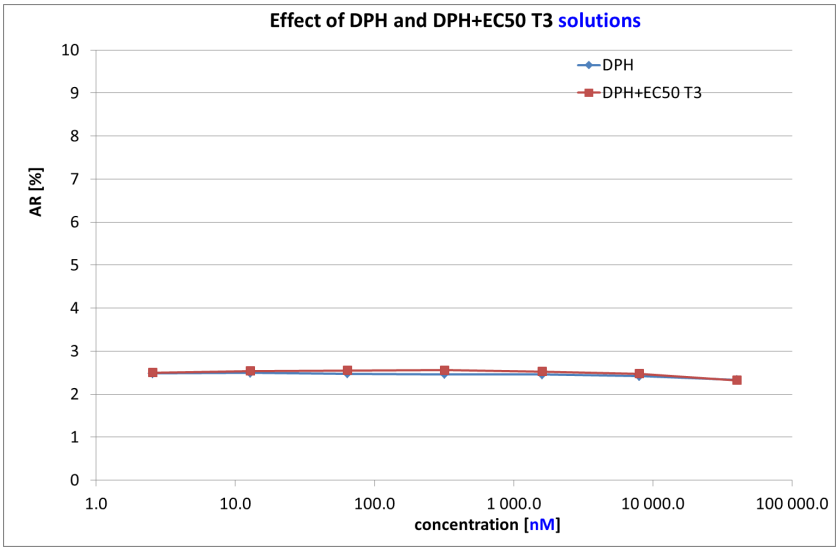
A. T3



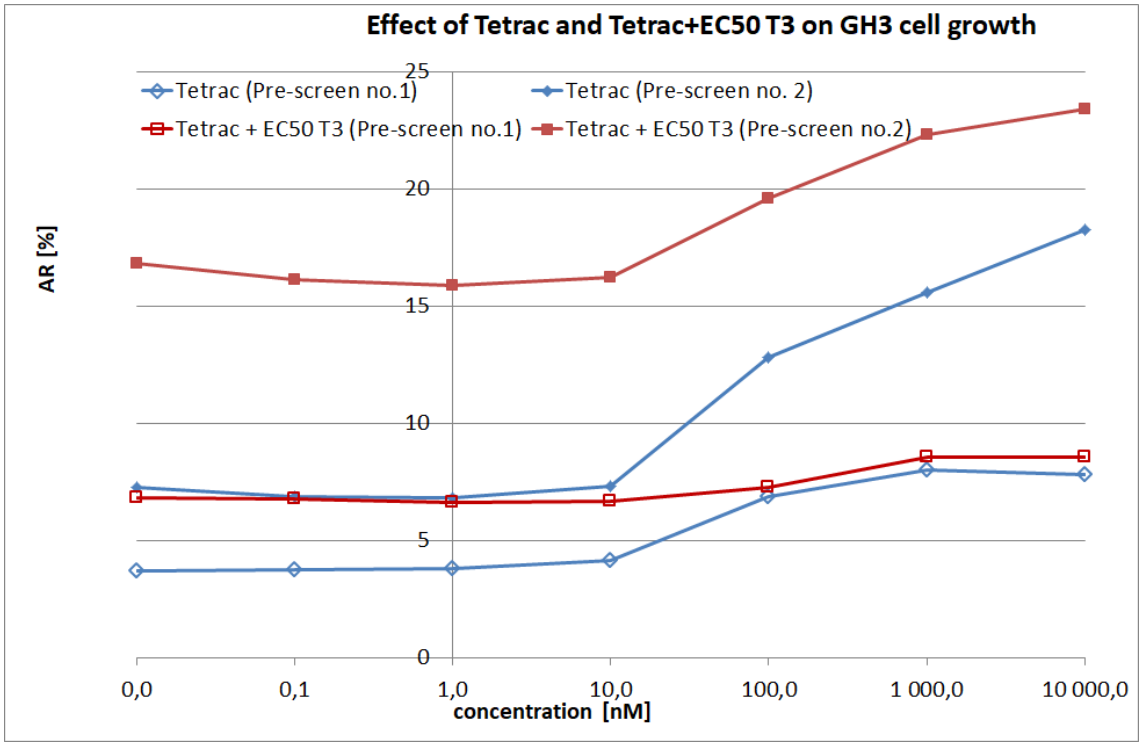


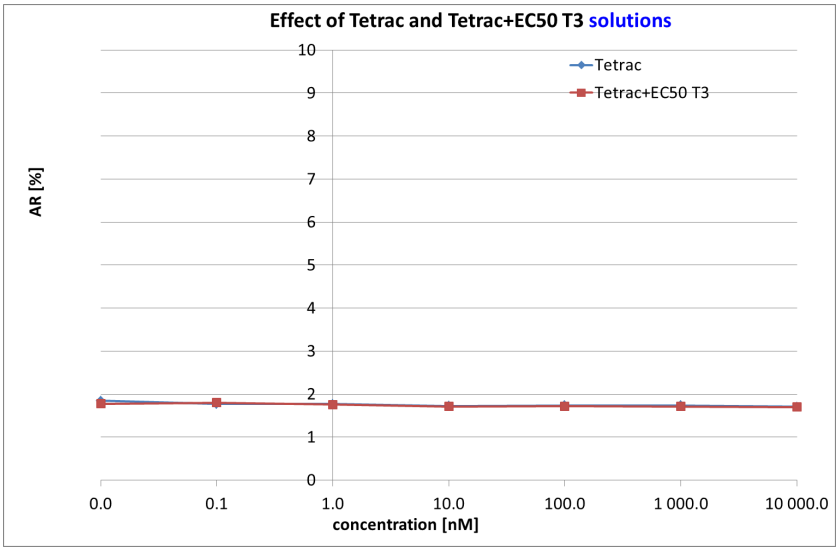
B. DPH


Effect of DPH and DPH+EC50 T3 on GH3 cell growth
DPH (Pre-screen no. 1)
DPH (Pre-screen no. 2)
DPH + EC50 T3 (Pre-screen no.1)
DPH+EC50 T3 (Pre-screen no.2)
AR [%]
20
15
10
5
0
0,1
1,0
10,0
100,0
1 000,0
10 000,0
100 000,0
concentration [nM]

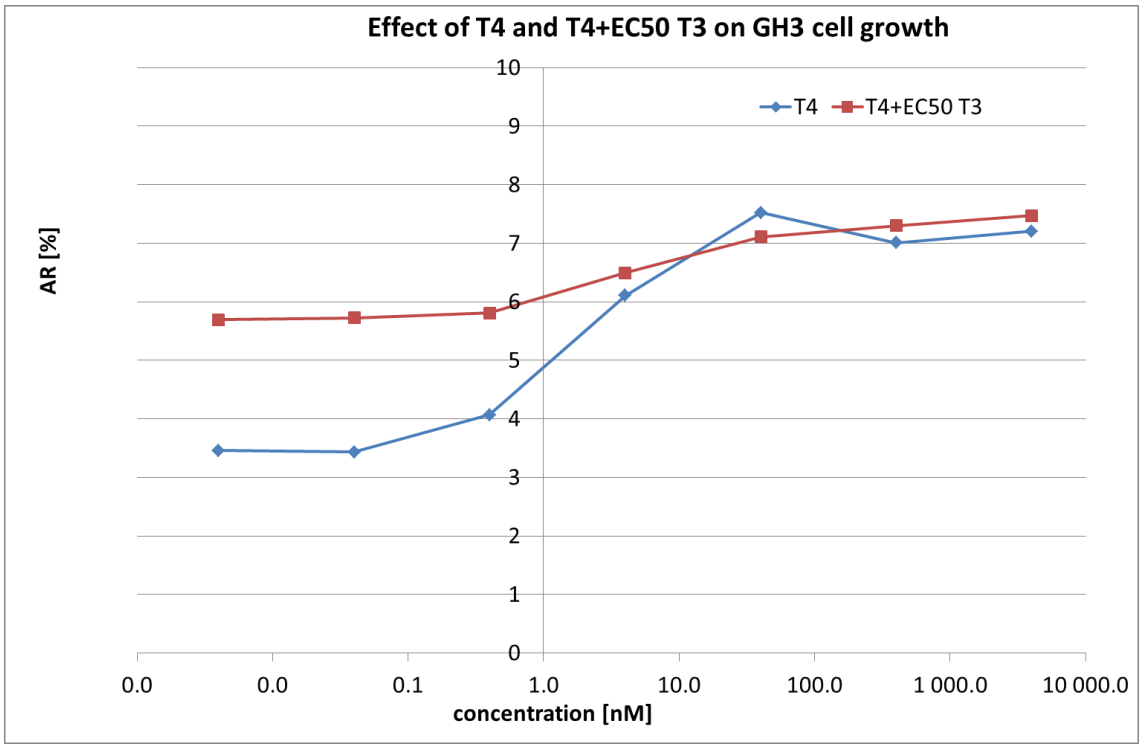

Effect of DPH and DPH+EC50 T3 solutions
DPH
DPH+EC50 T3
AR [%]
10
9
8
7
6
5
4
3
2
1
0
1.0
10.0
100.0
1 000.0
10 000.0
100 000.0
concentration [nM]

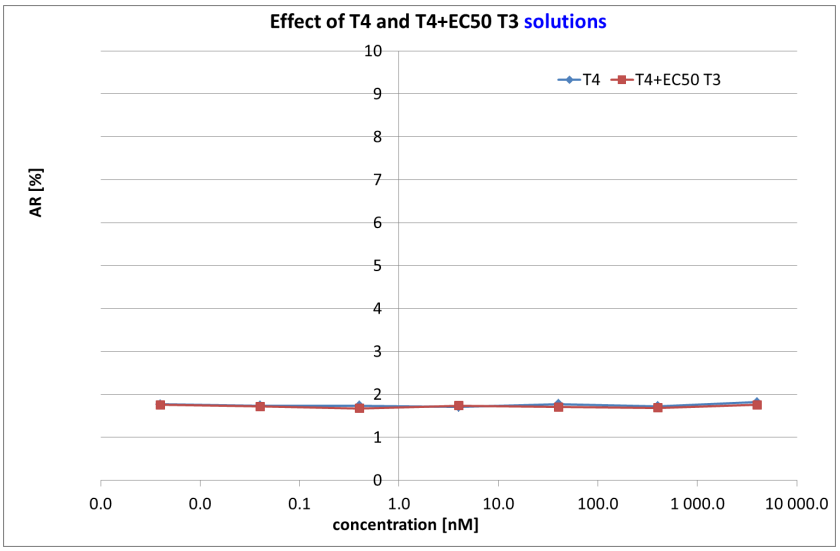
C. Tetrac


Effect of Tetrac and Tetrac+EC50 T3 on GH3 cell growth
Tetrac (Pre-screen no.1)
Tetrac (Pre-screen no. 2)
Tetrac + EC50 T3 (Pre-screen no.1)
Tetrac + EC50 T3 (Pre-screen no.2)
AR [%]
25
20
15
10
5
0
0,0
0,1
1,0
10,0
100,0
1 000,0
10 000,0
concentration [nM]

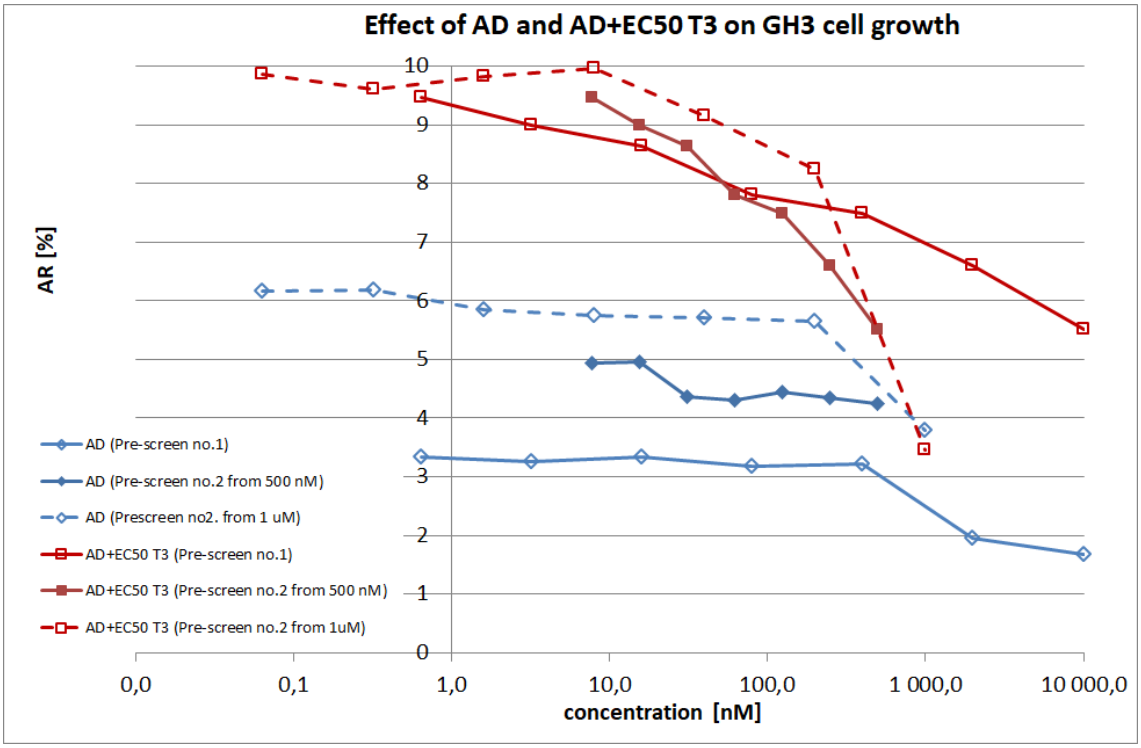

Effect of Tetrac and Tetrac+EC50 T3 solutions
Tetrac
Tetrac+EC50 T3
AR [%]
10
9
8
7
6
5
4
3
2
1
0
0.0
0.1
1.0
10.0
100.0
1 000.0
10 000.0
concentration [nM]

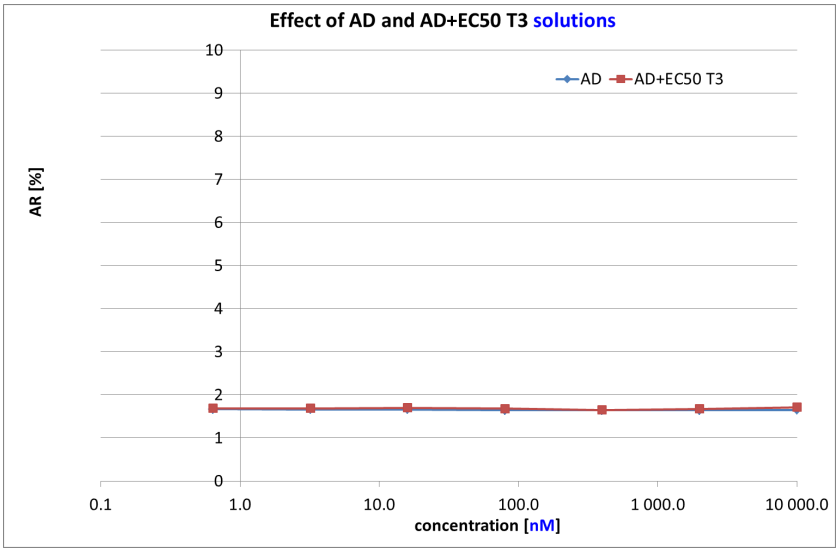
D. T4


Effect of T4 and T4+EC50 T3 on GH3 cell growth
T4
T4+EC50 T3
AR [%]
0
1
2
3
4
5
6
7
8
9
10
0.0
0.0
0.1
1.0
10.0
100.0
1 000.0
10 000.0
concentration [nM]

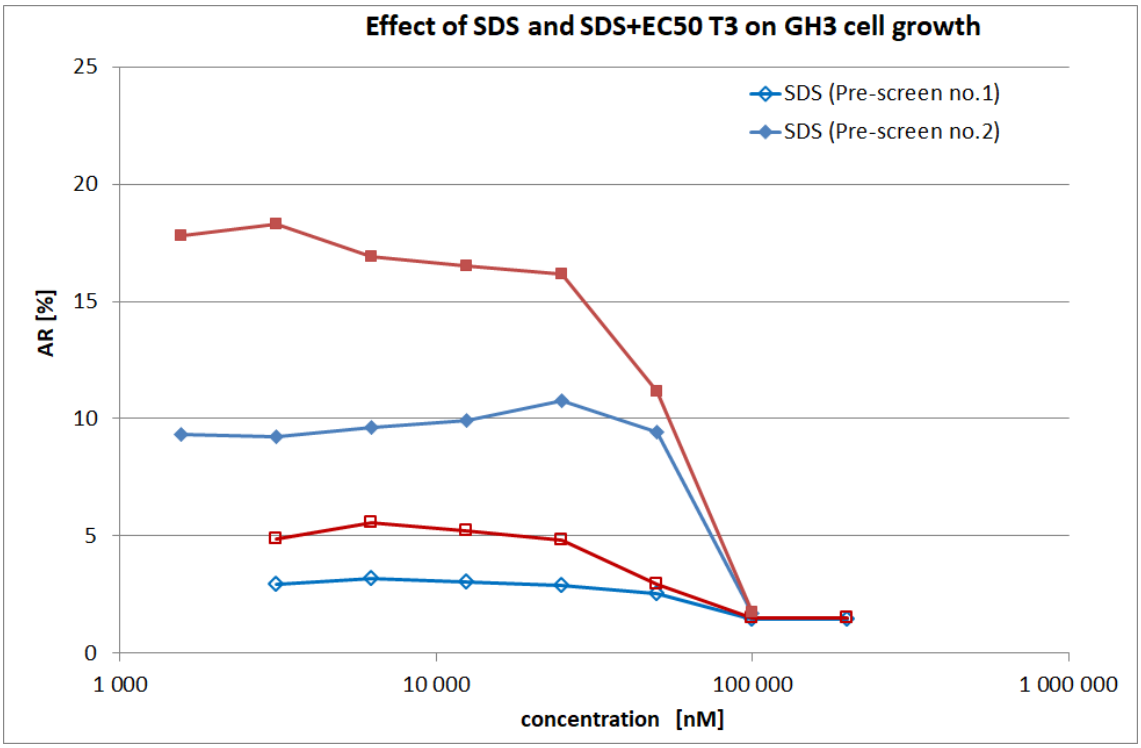

Effect of T4 and T4+EC50 T3 solutions
T4
T4+EC50 T3
AR [%]
0
1
2
3
4
5
6
7
8
9
10
0.0
0.0
0.1
1.0
10.0
100.0
1 000.0
10 000.0
concentration [nM]

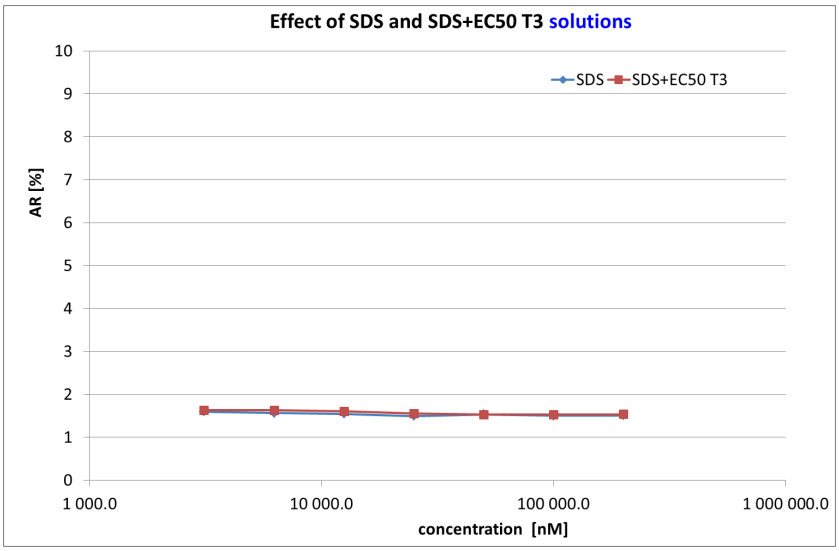
E. AD


Effect of AD and AD+EC50 T3 on GH3 cell growth
AR [%]
concentration [nM]
0,0
0,1
1,0
10,0
100,0
1 000,0
10 000,0
AD (Pre-screen no.1)
AD (Pre-screen no.2 from 500 nM)
AD (Prescreen no2. from 1 uM)
AD+EC50 T3 (Pre-screen no.1)
AD+EC50 T3 (Pre-screen no.2 from 500 nM)
AD+EC50 T3 (Pre-screen no.2 from 1uM)

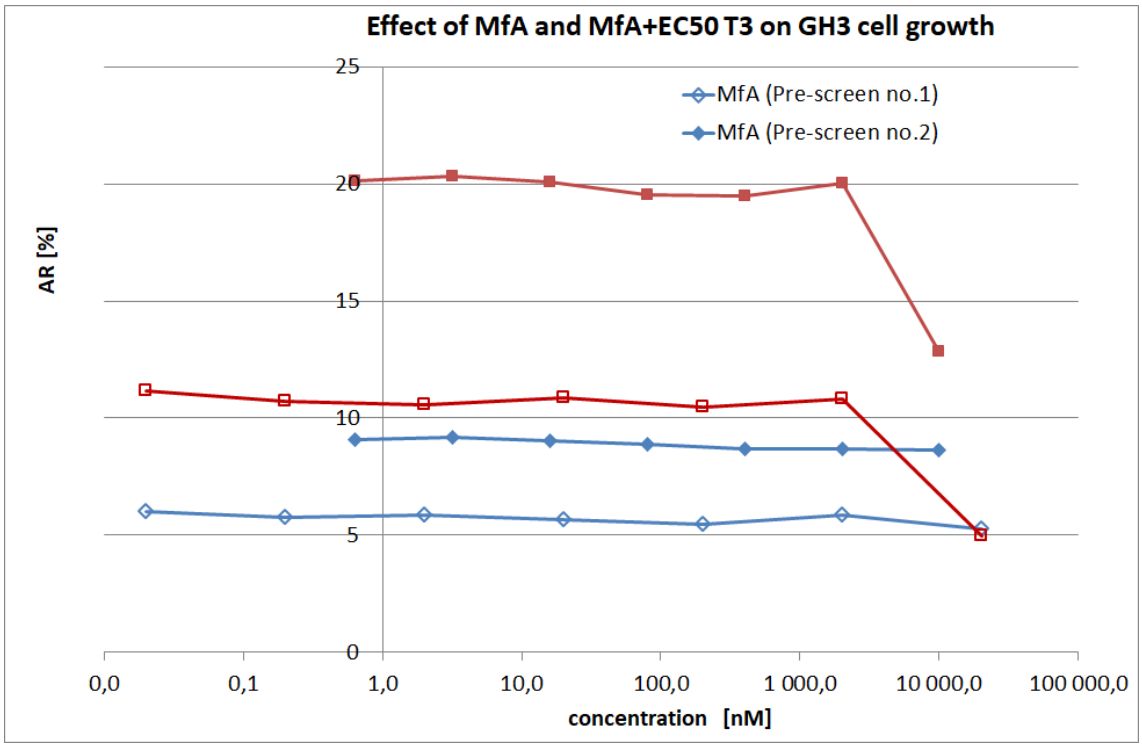

Effect of AD and AD+EC50 T3 solutions
AD
AD+EC50 T3
AR [%]
concentration [nM]
0.1
1.0
10.0
100.0
1 000.0
10 000.0

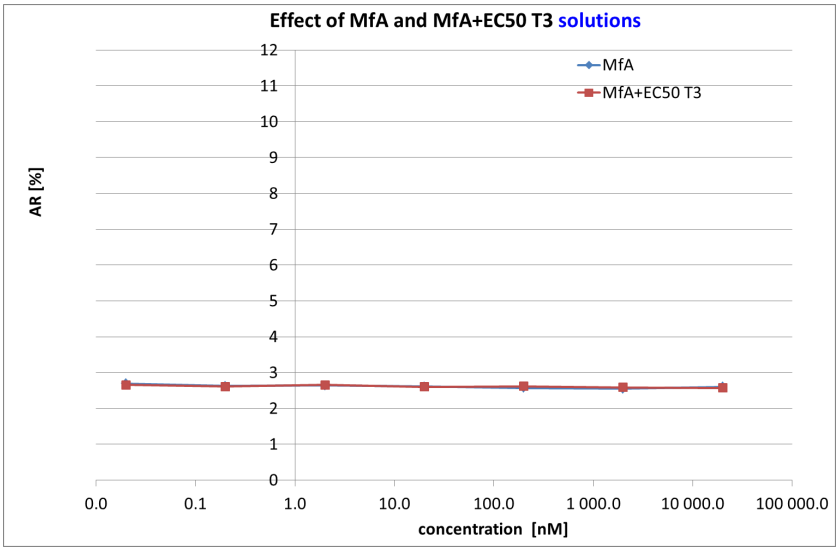
F. SDS


Effect of SDS and SDS+EC50 T3 on GH3 cell growth
SDS (Pre-screen no.1)
SDS (Pre-screen no.2)
AR [%]
25
20
15
10
5
0
1 000
10 000
100 000
1 000 000
concentration [nM]

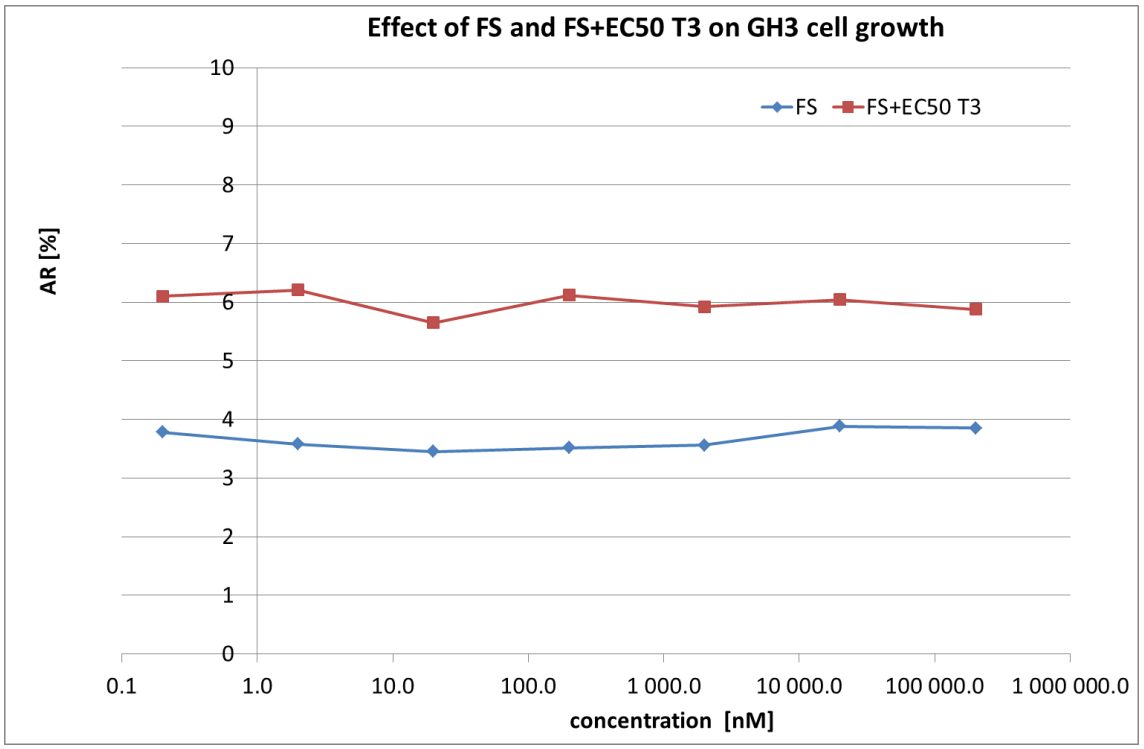

Effect of SDS and SDS+EC50 T3 solutions
SDS
SDS+EC50 T3
AR [%]
10
9
8
7
6
5
4
3
2
1
0
1 000.0
10 000.0
100 000.0
1 000 000.0
concentration [nM]

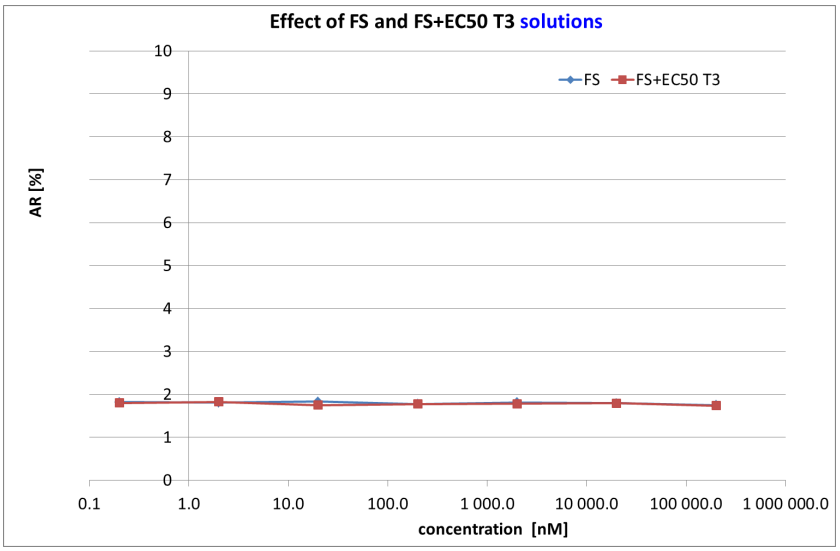
G. MfA


Effect of MfA and MfA+EC50 T3 on GH3 cell growth
MfA (Pre-screen no.1)
MfA (Pre-screen no.2)
AR [%]
25
20
15
10
5
0
0,0
0,1
1,0
10,0
100,0
1 000,0
10 000,0
100 000,0
concentration [nM]


Effect of MfA and MfA+EC50 T3 solutions
MfA
MfA+EC50 T3
AR [%]
12
11
10
9
8
7
6
5
4
3
2
1
0
0.0
0.1
1.0
10.0
100.0
1 000.0
10 000.0
100 000.0
concentration [nM]

H. FS


Effect of FS and FS+EC50 T3 on GH3 cell growth
FS
FS+EC50 T3
AR [%]
10
9
8
7
6
5
4
3
2
1
0
0.1
1.0
10.0
100.0
1 000.0
10 000.0
100 000.0
1 000 000.0
concentration [nM]


Effect of FS and FS+EC50 T3 solutions
FS
FS+EC50 T3
AR [%]
10
9
8
7
6
5
4
3
2
1
0
0.1
1.0
10.0
100.0
1 000.0
10 000.0
100 000.0
1 000 000.0
concentration [nM]

6.5. The T-Screen test

The T-Screen tests were performed according to SOP "*T-screen assay using GH3 cell line*" Section 3.3 using the plate layout presented in Figure 5 of the SOP. Taking into account two rules: (1) agonistic and antagonistic potential of test item should be assessed simultaneously but on separate plates and (2) complete Reference item standard curves (for Ref(T3) and Ref(DPH) in the agonism and antagonism plates, respectively) it was noticed that half of plate is not used but still control wells need to be included. In such situation it was decided that in each round every test item will be test only once but Reference item standard curves will be performed twice. Thus, finally four plates had layout as described in the Figure 5A of the SOP (two agonism plates and two antagonism plates) and six plates had layout as described in the Figure 5B of the SOP (three agonism plates and three antagonism plates). The following design of plates was used for the T-Screen assay:

Plate (Layout acc. to the SOP)	**AGONISM**	**ANTAGONISM**
1 (Figure 5A)	Upper part: Ref(T3) Lower part: T3	Upper part: Ref(DPH)/EC50T3 Lower part: T3/EC50T3
1a (Figure 5A)	Upper part: Ref(T3) Lower part: FS	Upper part: Ref(DPH) /EC50T3 Lower part: FS/EC50T3
next-2 (Figure 5B)	Upper part: DPH Lower part: Tetrac	Upper part: DPH/EC50T3 Lower part: Tetrac/EC50T3
next-3 (Figure 5B)	Upper part: T4 Lower part: AD	Upper part: T4/EC50T3 Lower part: AD/EC50T3
next-4 (Figure 5B)	Upper part: SDS Lower part: MfA	Upper part: SDS/EC50T3 Lower part: MfA/EC50T3

For each test item the range of test concentrations was designed based on the highest concentration of working solution and DF determined in the Pre-screen experiments (Table 4).

For some unknown reasons the effect observed for AD in the T-Screen test was a bit different than this observed in the Pre-Screen test. Although the range of working solutions in the T-Screen test was prepared in the same way as in the Pre-Screen test and all acceptance criteria for the Pre-screen test were met (SOP "*T-screen assay using GH3 cell line*" Section 3.2.5 and also the new acceptance criterion mention on the page 12 of the report/Section 5.4 that precisely defines if the effect is cytotoxic or not cytotoxic) the highest exposure concentration (C1) of AD turned out to be cytotoxic in the T-Screen assay. Because after the first round it was unknown if the results would be repetitive it was decided to continue the study (the T-Screen assay) in the range of concentration determined in the Pre-Screen test. Finally, AD seemed to be cytotoxic in C1 in all five rounds of the T-Screen assay, which resulted in the exclusion of the results for C1.

All results were calculated in two ways – the first way was applicable only for Bio-Rad kit (%AR), whereas the second one was universal for different brands of AlamarBlue (%DR). Based on both calculations the other essential parameters were calculated (e.g. RPE, RIE, EC50, IC50) and they are presented in Tables 5 A-J and 6 A-J as well as in Figures 5 and 6. Because the antagonist reference item DPH was given too variable results and the DPH response did not reach UC level, it has been decided (together with EURL ECVAM) to repeat data analysis of the antagonist experiments using the UC values

in the calculations instead of the highest DPH concentration C1. Results of the alternative analysis is given in Table 6 A-J and in Figure 6.

Table 5. The relative proliferative effect (RPE [%]) calculated based on % AlamarBlue reduction (%AR) and % Dye reduction (%DR). The results are presented as the average value of n scores ± standard deviation SD), where n is a number of accepted rounds.

A

Ref(T3)[nM] plate 1	**RPE [%] (AVG ± SD)**	
	based on %AR (n=5)	**based on %DR (n=5)**
0.003	0 ± 2.2	0 ± 2.2
0.008	5 ± 1.8	5 ± 1.8
0.025	17 ± 4.4	17 ± 4.4
0.074	50 ± 5.2	50 ± 5.2
0.222	75 ± 3.0	75 ± 3.0
0.667	89 ± 3.5	89 ± 3.5
2	100 ± 0.0	100 ± 0.0
EC50 for Ref(T3) [nM]	0.078 ± 0.02	0.078 ± 0.02
EC50 for Ref(T3) [log10(Molar)]	-10.112 ± 0.08	-10.112 ± 0.08

B

Ref(T3)[nM] plate 1a	**RPE [%] (AVG ± SD)**	
	based on %AR (n=5)	**based on %DR (n=5)**
0.003	1 ± 4.1	1 ± 4.6
0.008	5 ± 5.5	5 ± 3.7
0.025	16 ± 2.0	16 ± 1.9
0.074	51 ± 12.8	51 ± 11.5
0.222	74 ± 8.2	74 ± 9.5
0.667	91 ± 13.9	91 ± 15.9
2	100 ± 0.0	100 ± 0.0
EC50 for Ref(T3) [nM]	0.090 ± 0.03	0.090 ± 0.03
EC50 for Ref(T3) [log10(Molar)]	-10.066 ± 0.16	-10.070 ± 0.13

C

T3 [nM]	**RPE [%] (AVG ± SD)**	
	based on %AR (n=5)	**based on %DR (n=5)**
0.003	2 ± 3.7	2 ± 3.7
0.008	7 ± 1.6	7 ± 1.6
0.025	18 ± 3.3	18 ± 3.3
0.074	54 ± 9.8	54 ± 9.8
0.222	71 ± 6.2	71 ± 6.2
0.667	95 ± 18.3	95 ± 18.3
2	110 ± 21.6	110 ± 21.6
EC50 for T3 [nM]	0.114 ± 0.05	0.114 ± 0.05
EC50 for T3 [log10(Molar)]	-9.977 ± 0.18	-9.977 ± 0.18

Table 5. cd

D

DPH [nM]	RPE [%] (AVG ± SD)	
	based on %AR (n=6)	based on %DR (n=5)
0.64	0 ± 1.3	0 ± 0.9
3.2	-1 ± 3.6	-1 ± 3.5
16	0 ± 3.5	-1 ± 2.6
80	-2 ± 3.9	-4 ± 3.1
400	-3 ± 3.9	-4 ± 2.8
2 000	-2 ± 4.6	-3 ± 4.1
10 000	-4 ± 1.4	-4 ± 1.5
EC50 for DPH [nM]	Impossible to determine	Impossible to determine
EC50 for DPH [log10(Molar)]		

E

Tetrac [nM]	RPE [%] (AVG ± SD)	
	based on %AR (n=5)	based on %DR (n=5)
0.06	-3 ± 1.5	-3 ± 1.6
0.3	0 ± 2.9	0 ± 2.9
2	15 ± 5.8	15 ± 5.8
8	56 ± 10.0	56 ± 9.9
40	77 ± 10.2	77 ± 10.3
200	86 ± 9.3	87 ± 9.3
1 000	104 ± 10.1	104 ± 10.1
EC50 for Tetrac [nM]	10.223 ± 8.55	10.6 ± 9.3
EC50 for Tetrac [log10(Molar)]	-8.078 ± 0.28	-8.1 ± 0.3

F

T4 [nM]	RPE [%] (AVG ± SD)	
	based on %AR (n=5)	based on %DR (n=5)
0.032	1 ± 1.4	1 ± 1.4
0.16	4 ± 1.9	4 ± 1.9
0.8	17 ± 9.9	17 ± 9.9
4	49 ± 18.0	49 ± 18.0
20	78 ± 13.8	78 ± 13.8
100	91 ± 8.8	91 ± 8.7
500	97 ± 10.0	97 ± 10.0
EC50 for T4 [nM]	5.184 ± 4.93	5.2 ± 4.9
EC50 for T4 [log10(Molar)]	-8.392 ± 0.30	-8.4 ± 0.3

Table 5. cd

G

AD [nM]	**RPE [%] (AVG ± SD)**	
	based on %AR (n=5)	**based on %DR (n=5)**
7.8	0 ± 0.6	0 ± 0.6
15.6	-1 ± 1.8	-1 ± 1.8
31	-4 ± 1.0	-4 ± 1.0
63	-4 ± 1.9	-4 ± 2.0
125	-5 ± 1.7	-5 ± 1.7
250	-5 ± 2.7	-5 ± 2.7
500	-8 ± 2.3 cytotoxic	-8 ± 2.2 cytotoxic
EC50 for AD [nM]	Impossible to determine	Impossible to determine
EC50 for AD [log10(Molar)]		

H

SDS [nM]	**RPE [%] (AVG ± SD)**	
	based on %AR (n=6)	**based on %DR (n=5)**
625	3 ± 2.6	3 ± 2.5
1 250	2 ± 2.2	2 ± 2.3
2 500	1 ± 2.0	2 ± 1.9
5 000	2 ± 3.8	3 ± 3.6
10 000	1 ± 1.7	1 ± 1.9
20 000	2 ± 2.2	2 ± 2.3
40 000	2 ± 5.1	2 ± 5.5
EC50 for SDS [nM]	Impossible to determine	Impossible to determine
EC50 for SDS [log10(Molar)]		

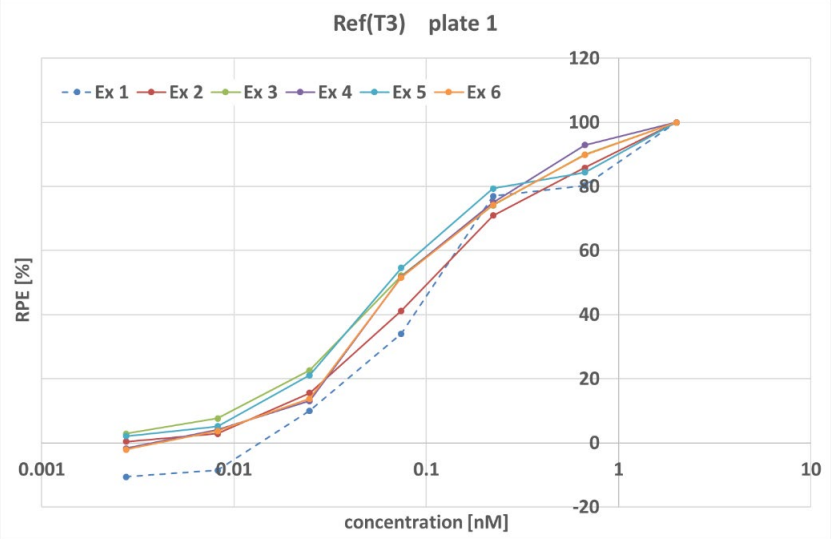
I

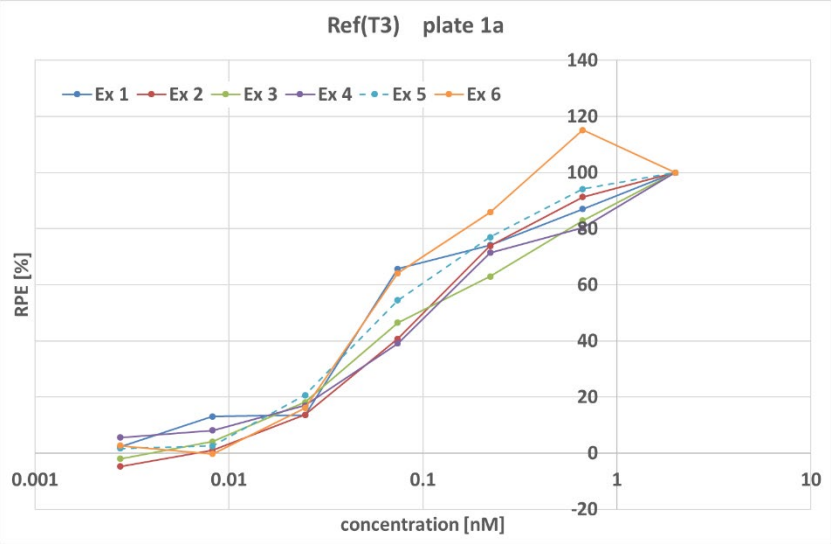
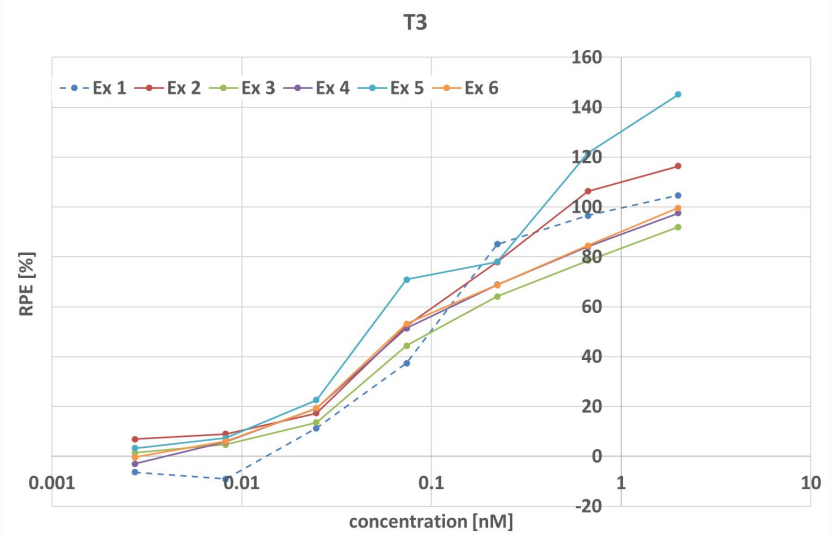
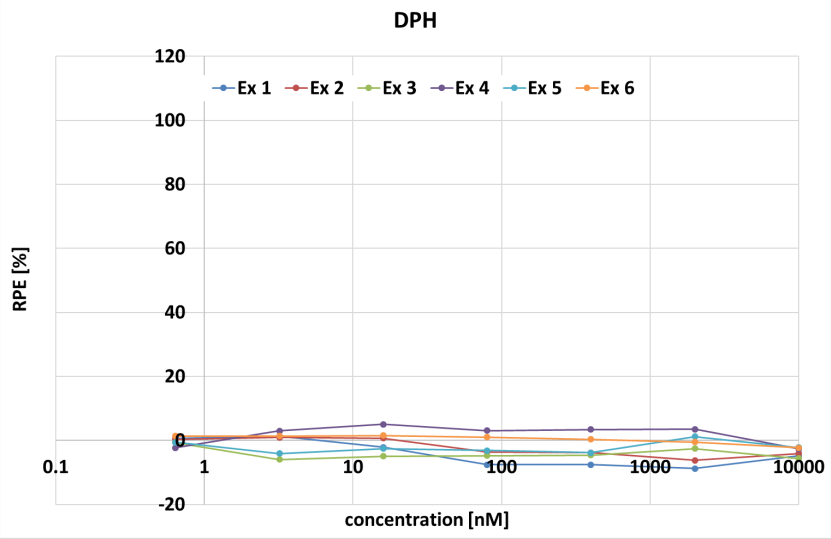
MfA [nM]	**RPE [%] (AVG ± SD)**	
	based on %AR (n=5)	**based on %DR (n=5)**
0.6	-1 ± 2.1	-1 ± 2.1
3.2	0 ± 1.7	0 ± 1.8
16	-2 ± 2.9	-2 ± 3.0
80	-3 ± 1.7	-2 ± 1.6
400	-5 ± 3.1	-5 ± 3.1
2 000	-2 ± 2.4	-2 ± 2.5
10 000	-2 ± 2.2	-2 ± 2.3
EC50 for MfA [nM]	Impossible to determine	Impossible to determine
EC50 for MfA [log10(Molar)]		

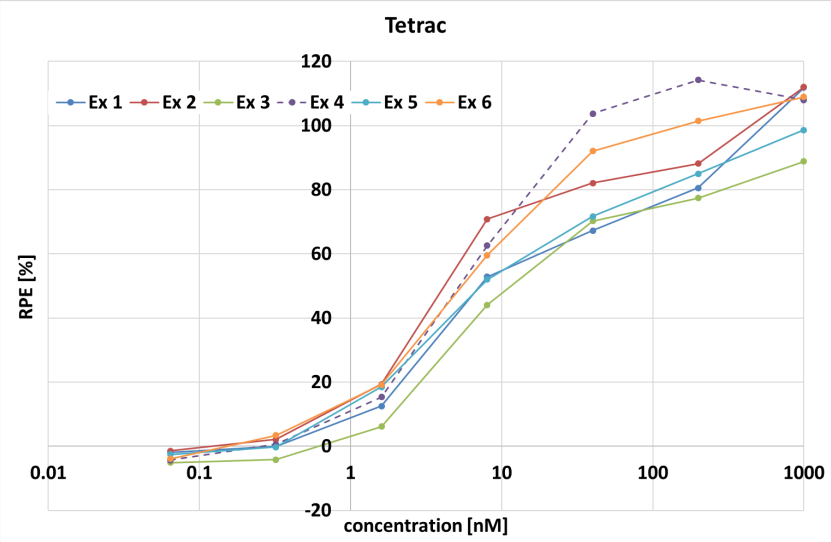
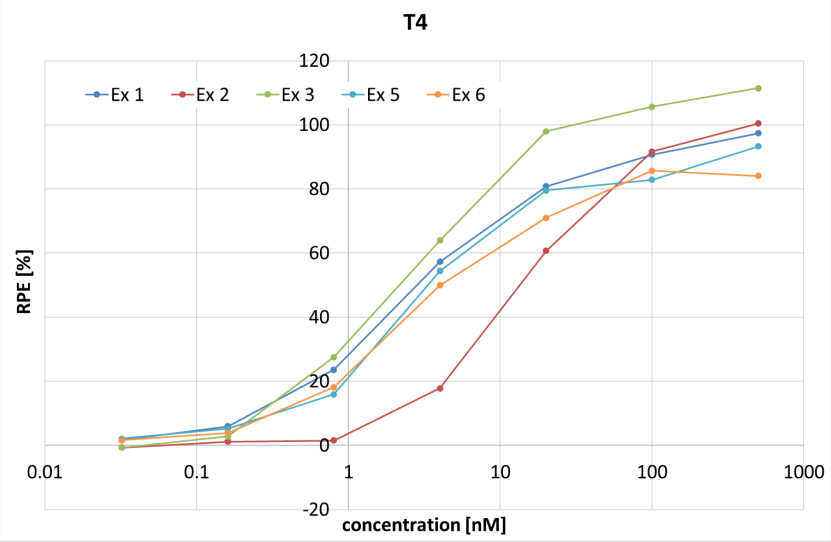
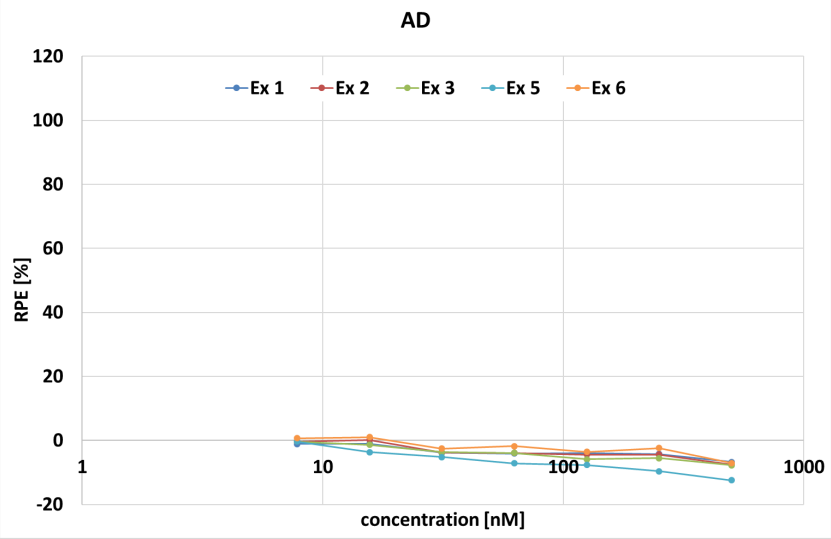
Table 5. cd
J

FS [nM]	RPE [%] (AVG ± SD)	
	based on %AR (n=5)	based on %DR (n=5)
0.2	0 ± 2.8	0 ± 2.8
2	-2 ± 2.3	-2 ± 2.3
20	-4 ± 2.1	-4 ± 2.1
200	-3 ± 2.6	-3 ± 2.6
2 000	-5 ± 3.8	-5 ± 3.8
20 000	-4 ± 3.5	-4 ± 3.5
200 000	-2 ± 5.7	-2 ± 5.7
EC50 for FS [nM]	Impossible to determine	Impossible to determine
EC50 for FS [log10(Molar)]		

Figure 5. The relative proliferation effect (RPE [%]; AGONISM plates) calculated based on % AlamarBlue reduction (%AR) for every accepted round.

Test Item	%RPE (AGONISM plates)
Reference Item	

Ref(T3) plate 1a	
T3	
DPH	

Tetrac	 Tetrac Ex 1 Ex 2 Ex 3 Ex 4 Ex 5 Ex 6 RPE [%] 120 100 80 60 40 20 0 -20 0.01 0.1 1 10 100 1000 concentration [nM]
T4	 T4 Ex 1 Ex 2 Ex 3 Ex 5 Ex 6 RPE [%] 120 100 80 60 40 20 0 -20 0.01 0.1 1 10 100 1000 concentration [nM]
AD	 AD Ex 1 Ex 2 Ex 3 Ex 5 Ex 6 RPE [%] 120 100 80 60 40 20 0 -20 1 10 100 1000 concentration [nM]

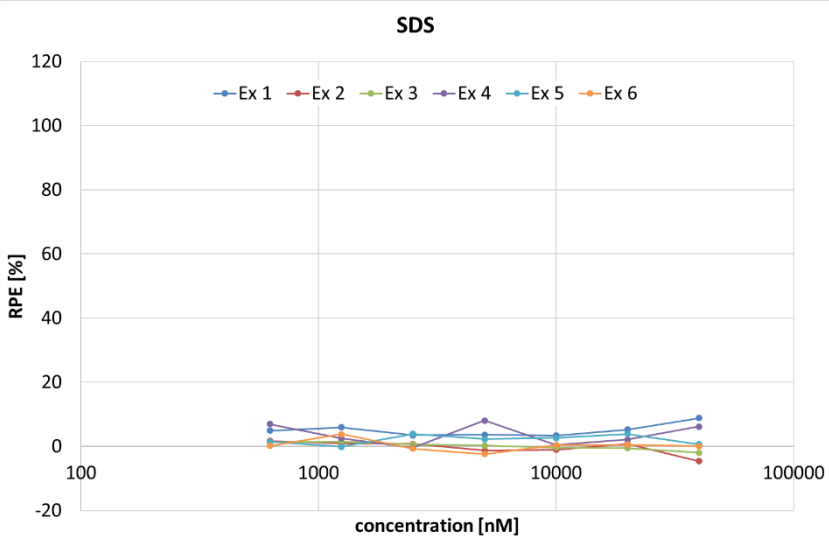
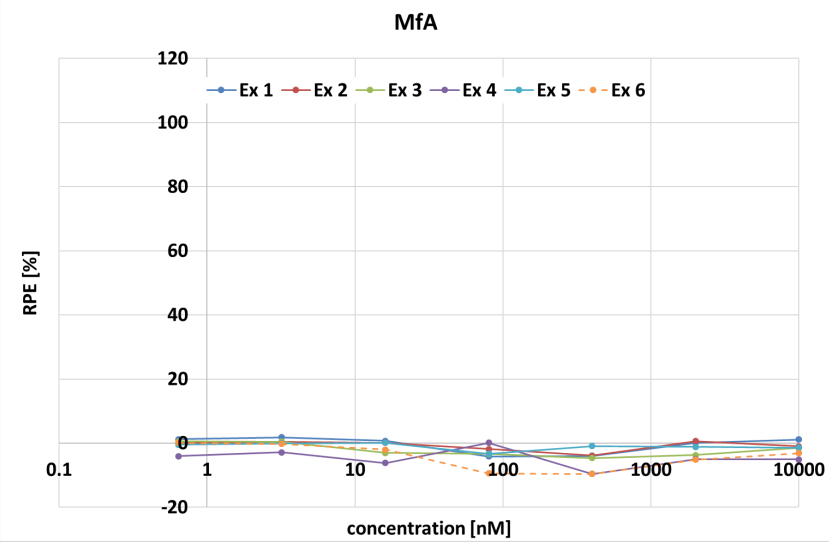
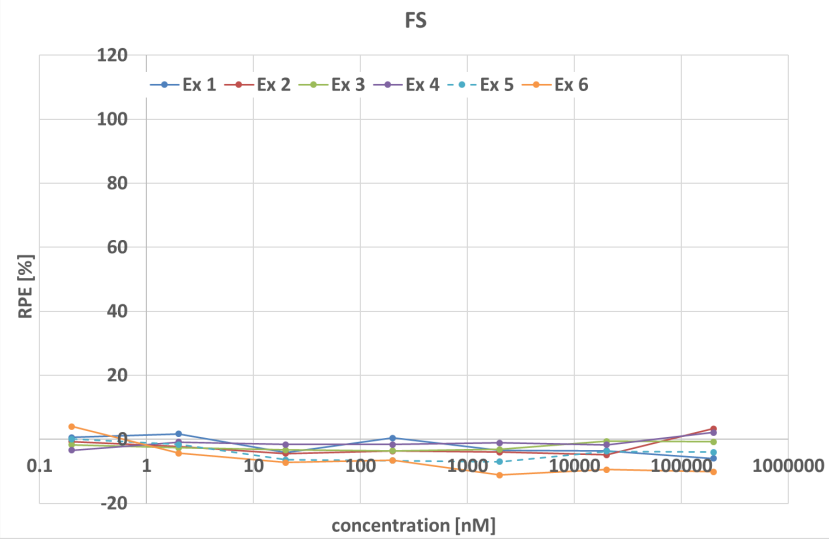
SDS	 SDS Ex 1 Ex 2 Ex 3 Ex 4 Ex 5 Ex 6 RPE [%] 120 100 80 60 40 20 0 -20 100 1000 10000 100000 concentration [nM]
MfA	 MfA Ex 1 Ex 2 Ex 3 Ex 4 Ex 5 Ex 6 RPE [%] 120 100 80 60 40 20 0 -20 0.1 1 10 100 1000 10000 concentration [nM]
FS	 FS Ex 1 Ex 2 Ex 3 Ex 4 Ex 5 Ex 6 RPE [%] 120 100 80 60 40 20 0 -20 0.1 1 10 100 1000 10000 100000 1000000 concentration [nM]

Table 6. The relative inhibitory effect (RIE [%]) for reference, control and test chemicals tested in the presence of EC50 of T3, calculated based on % AlamarBlue reduction (%AR) and % Dye reduction (%DR). The results are presented as the average value of n scores ± standard deviation SD), where n is a number of accepted rounds. I – results calculated using the highest DPH concentration C1 (calculations according to the SOP "*T-screen assay using GH3 cell line*" Section 3.4.2-2 ; II – results calculated using the UC values instead of the highest DPH concentration C1 (the alternative calculations)

A

Ref(DPH)[nM] /EC50T3 plate 1	**I (calculations acc.to SOP)**		**II (the alternative calculations)**	
	RIE [%] (AVG ± SD)		**RIE [%] (AVG ± SD)**	
	based on %AR (n=6)	**based on %DR (n=6)**	**based on %AR (n=6)**	**based on %DR (n=6)**
0.64	77 ± 43.1	77 ± 43.2	91 ± 13.6	91 ± 13.6
3.2	54 ± 28.2	53 ± 28.3	76 ± 14.6	76 ± 14.7
16	52 ± 19.9	52 ± 20.0	76 ± 9.1	76 ± 9.1
80	36 ± 41.7	36 ± 41.7	68 ± 20.1	68 ± 20.0
400	62 ± 30.5	61 ± 30.7	78 ± 19.2	78 ± 19.3
2 000	49 ± 24.5	49 ± 24.6	74 ± 13.3	74 ± 13.3
10 000	0 ± 0.0	0 ± 0.0	48 ± 12.0	48 ± 11.9
IC50 for Ref(DPH) [nM]	Impossible to determine	Impossible to determine	Impossible to determine	Impossible to determine
IC50 for Ref(DPH) [log10(Molar)]				

Table 6. cd.

B

Ref(DPH)[nM] /EC50T3 plate 1a	**I (calculations acc.to SOP)**		**II (the alternative calculations)**	
	RIE [%] (AVG ± SD)		**RIE [%] (AVG ± SD)**	
	based on %AR (n=6)	**based on %DR (n=6)**	**based on %AR (n=6)**	**based on %DR (n=6)**
0.64	94 ± 32.1	94 ± 32.2	96 ± 17.8	96 ± 17.8
3.2	95 ± 17.6	95 ± 17.6	96 ± 12.0	96 ± 12.0
16	76 ± 35.6	76 ± 35.5	84 ± 18.2	84 ± 18.2
80	95 ± 23.5	95 ± 23.5	95 ± 14.3	95 ± 14.4
400	82 ± 31.9	82 ± 31.9	88 ± 16.6	88 ± 16.6
2 000	67 ± 14.2	67 ± 14.3	81 ± 10.9	81 ± 10.9
10 000	0 ± 0.0	0 ± 0.0	45 ± 13.0	45 ± 13.0
IC50 for Ref(DPH) [nM]	Impossible to determine	Impossible to determine	Impossible to determine	Impossible to determine
IC50 for Ref(DPH) [log10(Molar)]				

C

T3 [nM]/EC50T3	**I (calculations acc.to SOP)**		**II (the alternative calculations)**	
	RIE [%] (AVG ± SD)		**RIE [%] (AVG ± SD)**	
	based on %AR (n=6)	**based on %DR (n=6)**	**based on %AR (n=6)**	**based on %DR (n=6)**
0.003	95 ± 27.6	95 ± 27.6	99 ± 11.3	99 ± 11.3
0.008	89 ± 23.7	89 ± 23.9	94 ± 14.3	94 ± 14.3
0.025	95 ± 35.3	95 ± 35.4	95 ± 19.2	95 ± 19.2
0.074	127 ± 43.4	127 ± 43.4	110 ± 20.1	110 ± 20.0
0.222	179 ± 75.1	179 ± 75.3	135 ± 28.1	135 ± 28.2
0.667	237 ± 109.6	237 ± 109.8	162 ± 34.6	162 ± 34.7
2	281 ± 134.0	281 ± 134.0	182 ± 44.5	182 ± 44.5
IC50 for T3 [nM]	0.317 ± 0.07	0.32 ± 0.07	0,317 ± 0.08	0.31 ± 0.07
IC50 forT3 [log10(Molar)]	-9.507 ± 0.09	-9.51 ± 0.10	-9,509 ± 0.10	-9.51 ± 0.09

Table 6. cd.

D

DPH [nM]/EC50T3	I (calculations acc.to SOP)		II (the alternative calculations)	
	RIE [%] (AVG ± SD)		RIE [%] (AVG ± SD)	
	based on %AR (n=6)	based on %DR (n=6)	based on %AR (n=6)	based on %DR (n=6)
0.64	63 ± 42.1	70 ± 48.9	87 ± 15.5	91 ± 13.9
3.2	143 ± 81.1	201 ± 165.8	111 ± 26.1	119 ± 35.4
16	117 ± 83.9	167 ± 155.4	101 ± 30.0	108 ± 37.9
80	83 ± 73.5	117 ± 130.2	89 ± 28.9	94 ± 34.3
400	91 ± 72.6	130 ± 131.0	91 ± 26.0	97 ± 32.4
2 000	33 ± 49.0	33 ± 60.3	77 ± 15.5	81 ± 15.6
10 000	36 ± 61.3	61 ± 103.0	70 ± 27.3	76 ± 34.2
IC50 for DPH [nM]	Impossible to determine	Impossible to determine	Impossible to determine	Impossible to determine
IC50 for DPH [log10(Molar)]				

E

Tetrac [nM] /EC50T3	I (calculations acc.to SOP)		II (the alternative calculations)	
	RIE [%] (AVG ± SD)		RIE [%] (AVG ± SD)	
	based on %AR (n=6)	based on %DR (n=6)	based on %AR (n=6)	based on %DR (n=6)
0.064	47 ± 62.0	42 ± 84.3	83 ± 18.9	86 ± 15.9
0.32	93 ± 32.5	121 ± 66.1	95 ± 14.2	100 ± 17.5
1.6	144 ± 78.9	201 ± 159.6	112 ± 24.4	119 ± 33.6
8	236 ± 138.2	329 ± 281.0	144 ± 35.9	153 ± 47.2
40	320 ± 210.3	447 ± 399.1	171 ± 51.5	181 ± 63.9
200	308 ± 115.9	406 ± 231.7	175 ± 25.5	186 ± 33.6
1 000	361 ± 129.7	486 ± 308.8	196 ± 30.1	208 ± 44.0
IC50 for Tetrac [nM]	31.7 ± 53.0	31.5 ± 52.6	29.8 ± 48.9	36 ± 62
IC50 for Tetrac [log10(Molar)]	-8.1 ± 0.9	-8.1 ± 0.9	-8.1 ± 0.9	-8 ± 0.9

Table 6. cd.

F

T4 [nM] /EC50T3	**I (calculations acc.to SOP)**		**II (the alternative calculations)**	
	RIE [%] (AVG ± SD)		**RIE [%] (AVG ± SD)**	
	based on %AR (n=6)	**based on %DR (n=6)**	**based on %AR (n=6)**	**based on %DR (n=6)**
0.032	78 ± 22.9	76 ± 27.8	93 ± 6.2	92 ± 8.9
0.16	81 ± 40.0	80 ± 43.8	95 ± 13.1	94 ± 15.2
0.8	80 ± 67.5	77 ± 64.5	93 ± 24.0	93 ± 24.3
4	157 ± 62.4	149 ± 49.9	123 ± 19.6	122 ± 18.7
20	242 ± 86.6	228 ± 56.7	155 ± 24.1	154 ± 22.8
100	323 ± 121.2	303 ± 71.4	185 ± 30.4	183 ± 26.4
500	340 ± 105.9	322 ± 69.7	189 ± 15.8	187 ± 14.8
IC50 for T4 [nM]	41 ± 79.1	40.2 ± 77.9	41.8 ± 82.1	38.2 ± 72.6
IC50 for T4 [log10(Molar)]	-7.9 ± 0.6	-7.9 ± 0.6	-7.9 ± 0.6	-7.9 ± 0.6

G

AD [nM] /EC50T3	**I (calculations acc.to SOP)**		**II (the alternative calculations)**	
	RIE [%] (AVG ± SD)		**RIE [%] (AVG ± SD)**	
	based on %AR (n=5)	**based on %DR (n=5)**	**based on %AR (n=6)**	**based on %DR (n=6)**
7.8	67 ± 76.4	71 ± 69.1	67 ± 76.6	91 ± 22.5
15.6	54 ± 59.5	56 ± 59.0	55 ± 60.3	86 ± 19.4
31	24 ± 50.6	27 ± 49.4	28 ± 54.8	73 ± 13.8
63	-1 ± 54.2	3 ± 52.0	6 ± 61.0	63 ± 14.2
125	-16 ± 54.4	-12 ± 52.2	-8 ± 63.4	57 ± 14.2
250	-35 ± 38.4	-32 ± 37.3	-23 ± 51.9	48 ± 5.4
500	-112 ± 62.6 cytotoxic	-104 ± 51.3 cytotoxic	cytotoxic	cytotoxic
IC50 for AD [nM]	Impossible to determine	Impossible to determine	Impossible to determine	Impossible to determine
IC50 for AD [log10(Molar)]				

Table 6. cd.

H

SDS [nM] /EC50T3	**I (calculations acc.to SOP)**		**II (the alternative calculations)**	
	RIE [%] (AVG ± SD)		**RIE [%] (AVG ± SD)**	
	based on %AR (n=6)	**based on %DR (n=6)**	**based on %AR (n=6)**	**based on %DR (n=6)**
625	91 ± 21.8	96 ± 23.3	96 ± 9.5	98 ± 9.6
1 250	101 ± 14.3	107 ± 17.4	100 ± 6.3	103 ± 7.0
2 500	94 ± 23.7	99 ± 25.3	98 ± 10.3	100 ± 10.7
5 000	107 ± 33.5	113 ± 36.3	103 ± 14.0	105 ± 14.7
10 000	103 ± 33.5	109 ± 35.4	101 ± 15.6	103 ± 15.8
20 000	88 ± 30.6	93 ± 31.6	96 ± 12.8	98 ± 12.9
40 000	-6 ± 98.1	-8 ± 104.8	60 ± 34.4	61 ± 35.0
IC50 for SDS [nM]	Impossible to determine	Impossible to determine	Impossible to determine	Impossible to determine
IC50 for SDS [log10(Molar)]				

I

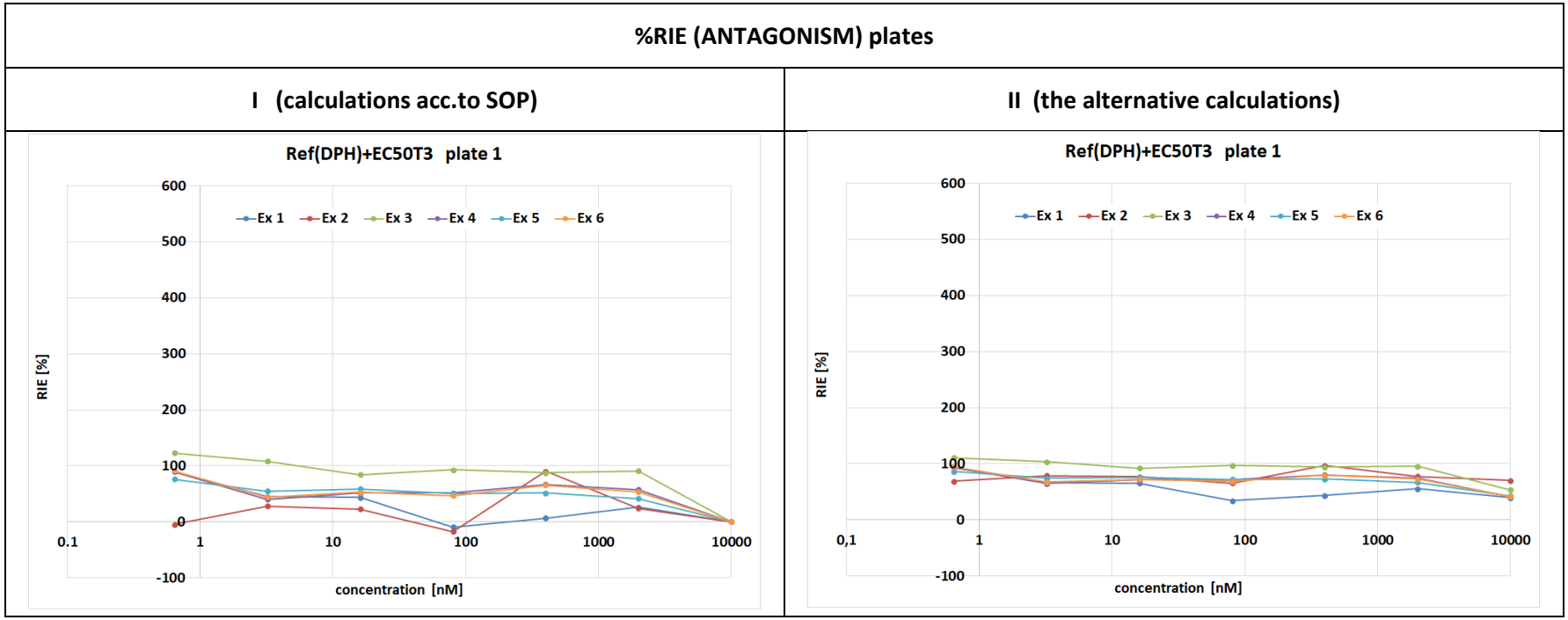
MfA [nM] /EC50T3	**I (calculations acc.to SOP)**		**II (the alternative calculations)**	
	RIE [%] (AVG ± SD)		**RIE [%] (AVG ± SD)**	
	based on %AR (n=6)	**based on %DR (n=6)**	**based on %AR (n=6)**	**based on %DR (n=6)**
0.6	99 ± 32.4	104 ± 34.3	100 ± 13.0	102 ± 13.0
3.2	114 ± 29.7	120 ± 30.6	105 ± 12.4	108 ± 12.1
16	88 ± 29.7	92 ± 30.3	95 ± 11.9	97 ± 11.4
80	73 ± 33.2	76 ± 34.8	88 ± 13.4	90 ± 13.0
400	87 ± 15.8	91 ± 16.1	94 ± 6.9	96 ± 6.4
2 000	79 ± 42.8	82 ± 44.8	91 ± 16.9	92 ± 16.5
10 000	-56 ± 61.0	-60 ± 66.3	39 ± 17.3	39 ± 17.6
IC50 for MfA [nM]	Impossible to determine	Impossible to determine	Impossible to determine	Impossible to determine
IC50 for MfA [log10(Molar)]				

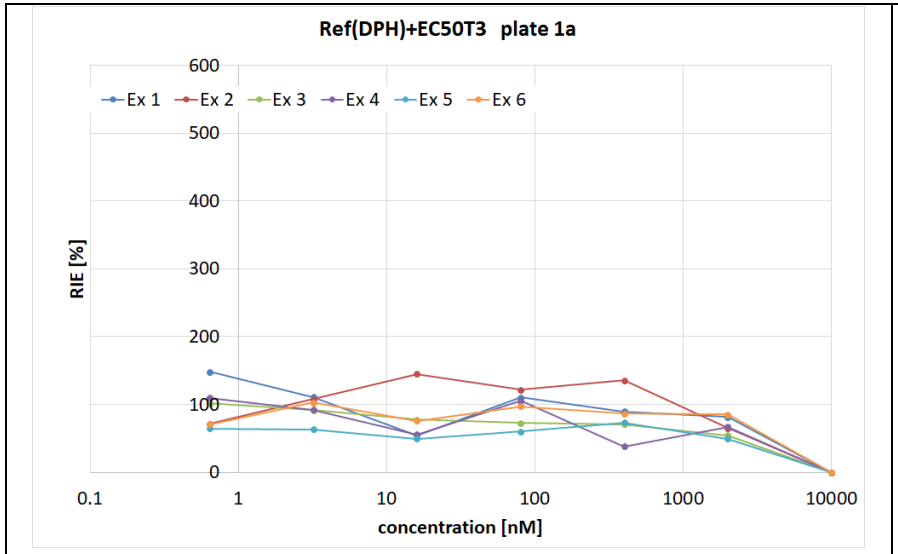
Table 6. cd.

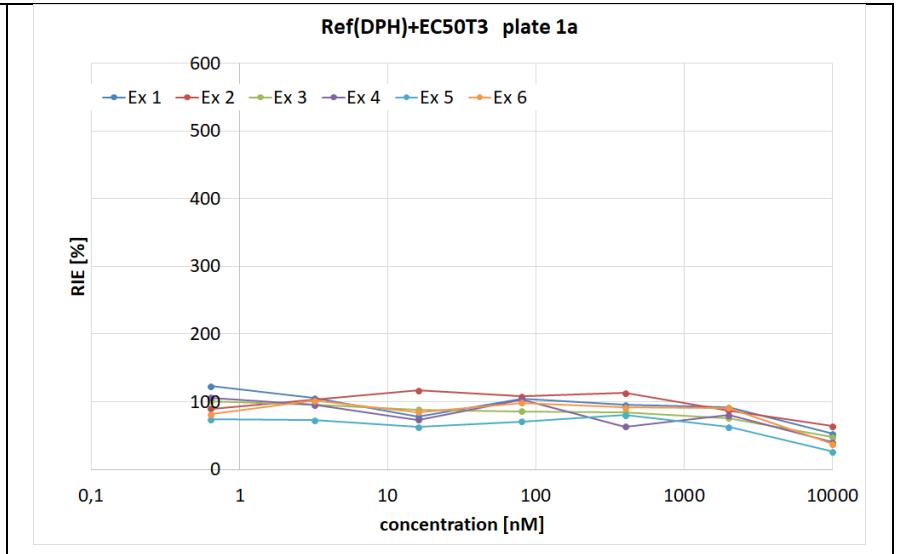
J

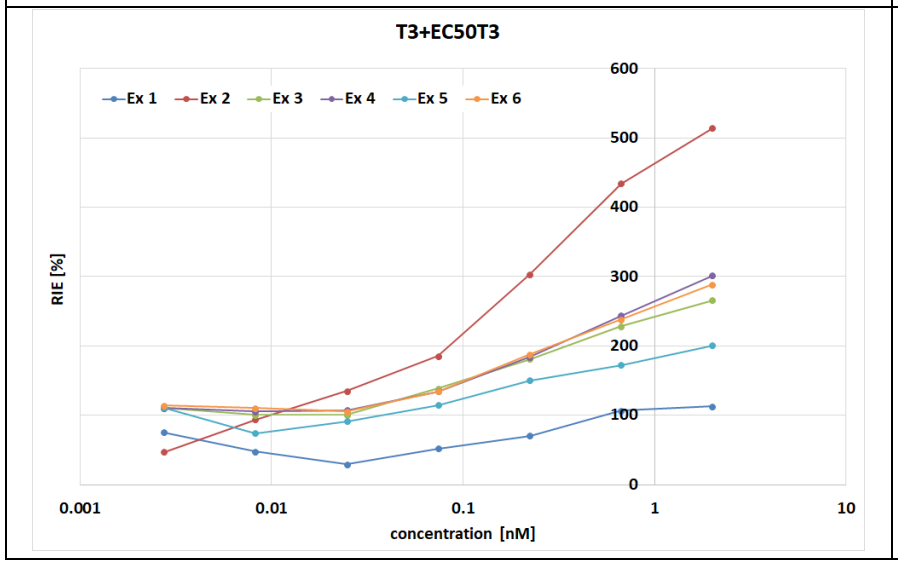
FS [nM] /EC50T3	**I (calculations acc.to SOP)**		**II (the alternative calculations)**	
	RIE [%] (AVG ± SD)		**RIE [%] (AVG ± SD)**	
	based on %AR (n=6)	**based on %DR (n=6)**	**based on %AR (n=6)**	**based on %DR (n=6)**
0.2	106 ± 34.5	106 ± 34.6	101 ± 17.1	101 ± 17.1
2	101 ± 30.1	101 ± 30.0	98 ± 15.6	98 ± 15.6
20	63 ± 40.1	63 ± 40.0	76 ± 20.6	76 ± 20.5
200	84 ± 36.5	84 ± 36.5	90 ± 19.6	90 ± 19.6
2 000	73 ± 47.3	73 ± 47.3	82 ± 23.5	82 ± 23.5
20 000	88 ± 35.8	88 ± 35.8	91 ± 15.5	91 ± 15.5
200 000	71 ± 41.2	71 ± 41.2	83 ± 22.0	83 ± 21.9
IC50 for FS [nM]	Impossible to determine	Impossible to determine	Impossible to determine	Impossible to determine
IC50 for FS [log10(Molar)]				

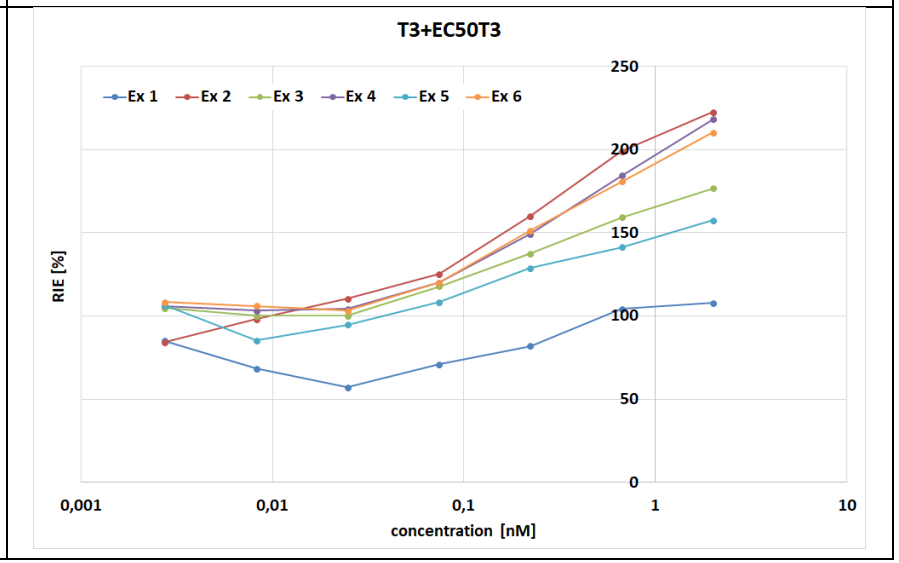
Figure 6. The relative proliferation effect (RPE [%]; AGONISM plates) and the relative inhibitory effect (RIE [%]; ANTAGONISM plates) calculated based on % AlamarBlue reduction (%AR) for every accepted round. I – results calculated using the highest DPH concentration C1 (calculations according to the SOP "*T-screen assay using GH3 cell line*" Section 3.4.2-2 ; II – results calculated using the UC values instead of the highest DPH concentration C1 (the alternative calculations)

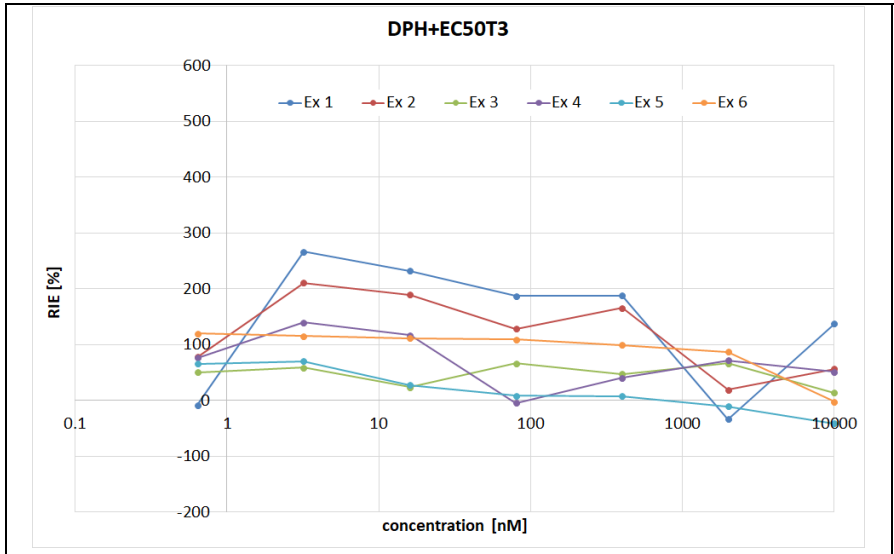


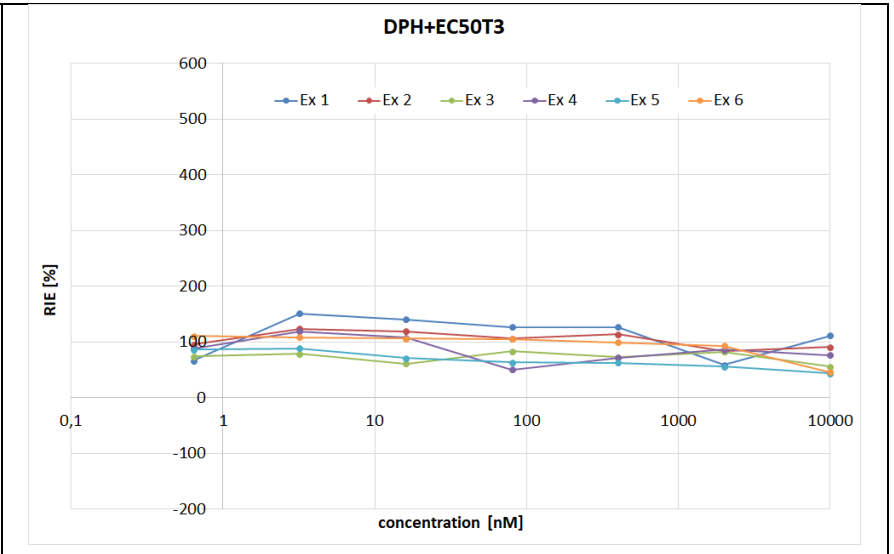

Ref(DPH)+EC50T3 plate 1a
Ex 1
Ex 2
Ex 3
Ex 4
Ex 5
Ex 6
RIE [%]
concentration [nM]

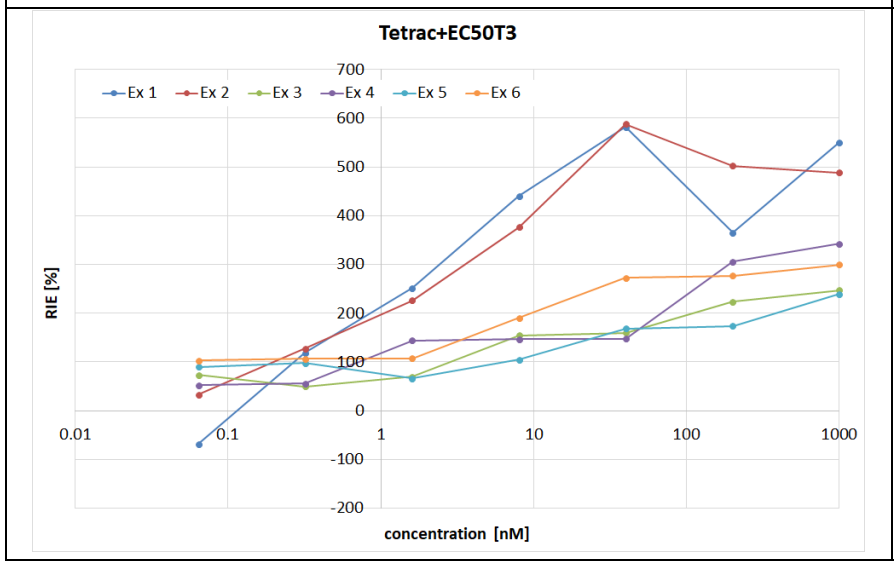

Ref(DPH)+EC50T3 plate 1a
Ex 1
Ex 2
Ex 3
Ex 4
Ex 5
Ex 6
RIE [%]
concentration [nM]

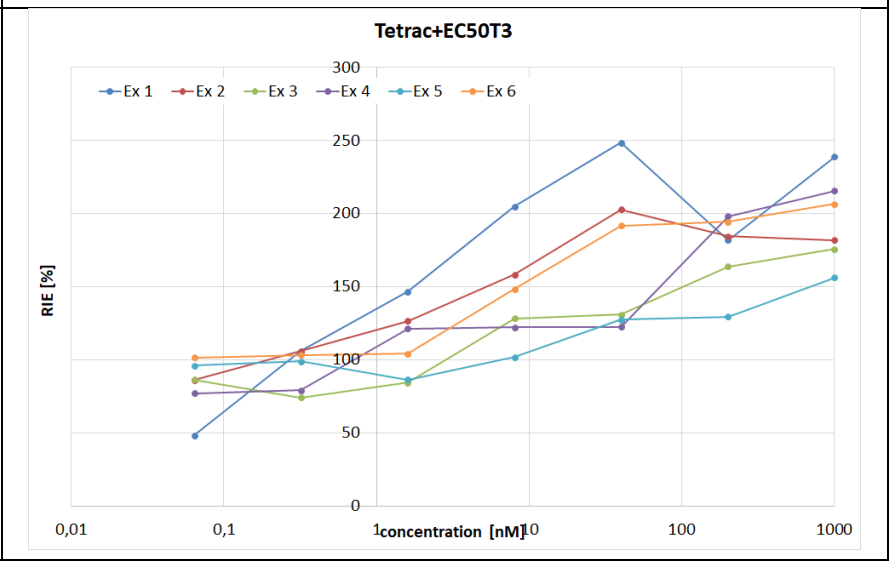

T3+EC50T3
Ex 1
Ex 2
Ex 3
Ex 4
Ex 5
Ex 6
RIE [%]
concentration [nM]

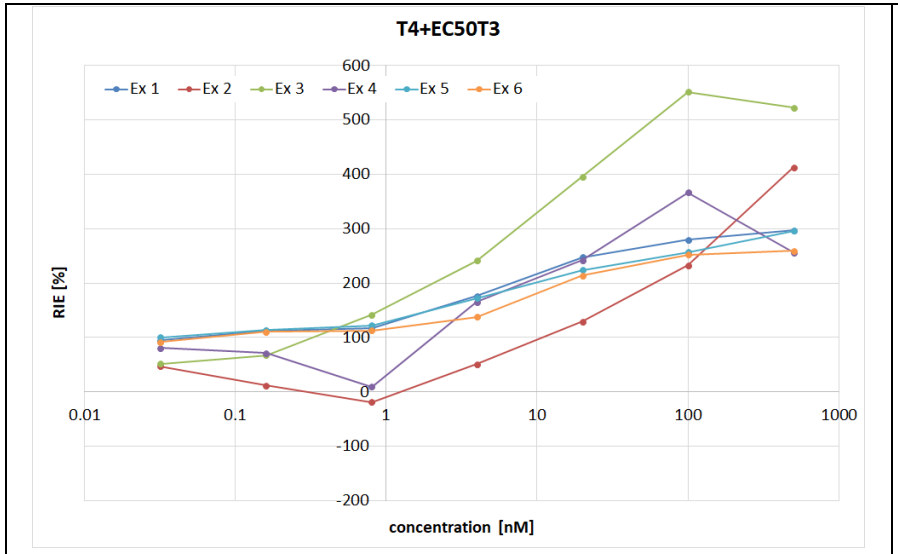

T3+EC50T3
Ex 1
Ex 2
Ex 3
Ex 4
Ex 5
Ex 6
RIE [%]
concentration [nM]

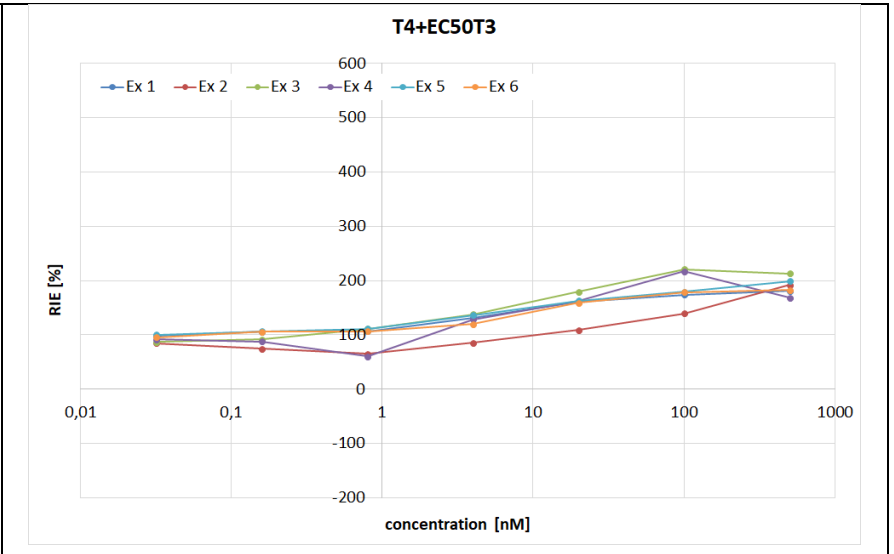

DPH+EC50T3
Ex 1
Ex 2
Ex 3
Ex 4
Ex 5
Ex 6
RIE [%]
concentration [nM]

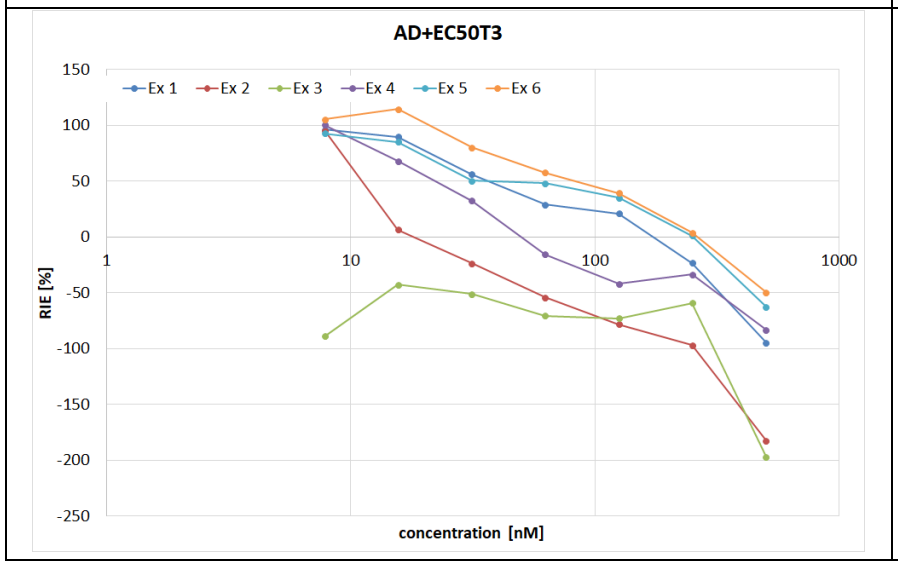

DPH+EC50T3
Ex 1
Ex 2
Ex 3
Ex 4
Ex 5
Ex 6
RIE [%]
concentration [nM]

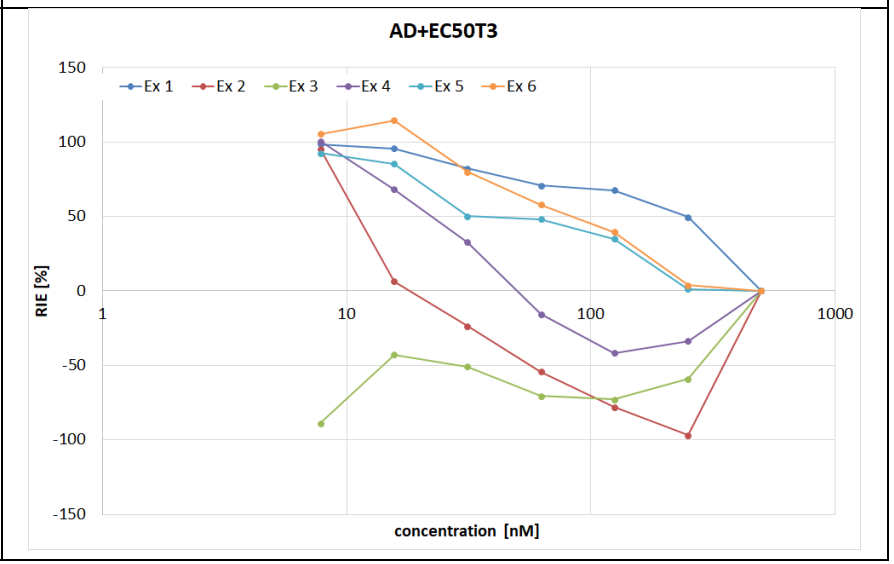

Tetrac+EC50T3
Ex 1
Ex 2
Ex 3
Ex 4
Ex 5
Ex 6
RIE [%]
concentration [nM]

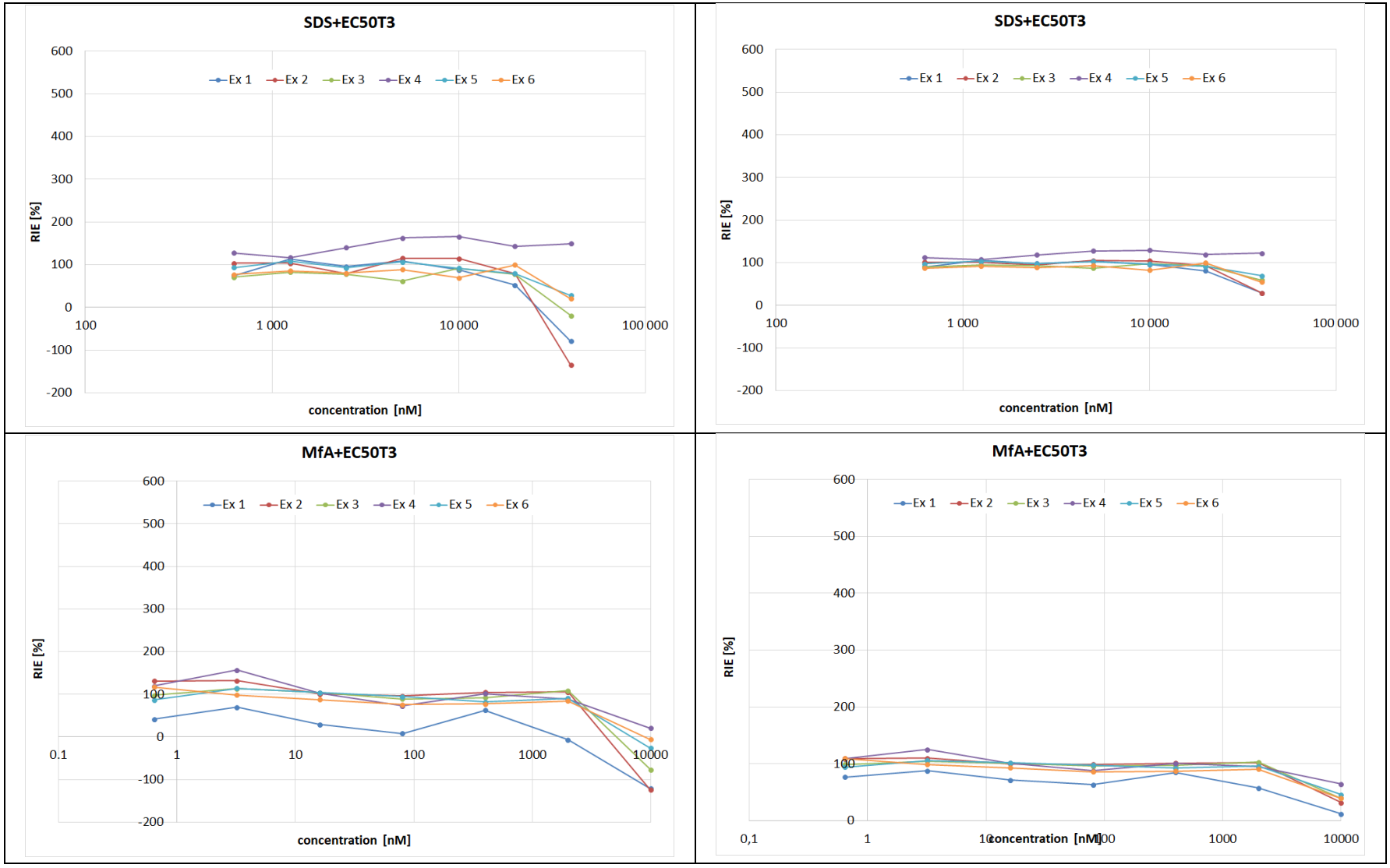

Tetrac+EC50T3
Ex 1
Ex 2
Ex 3
Ex 4
Ex 5
Ex 6
RIE [%]
concentration [nM]

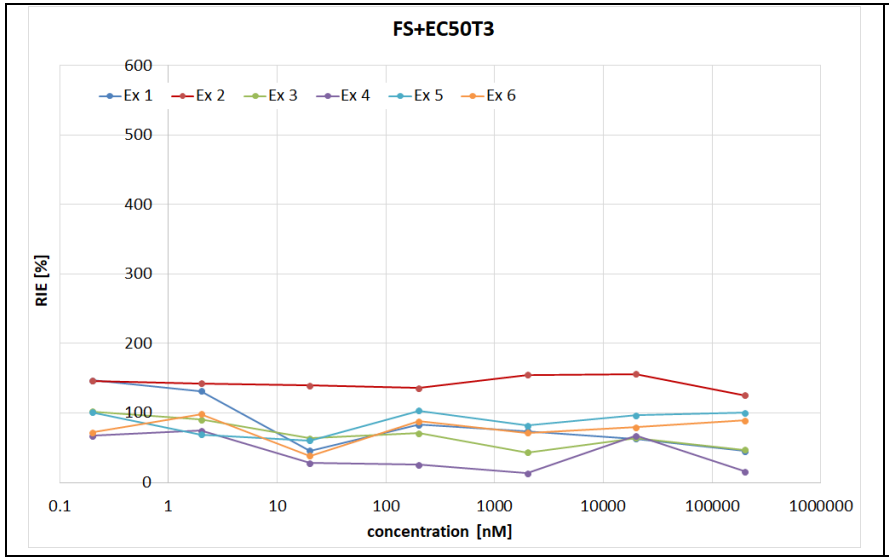

T4+EC50T3
Ex 1
Ex 2
Ex 3
Ex 4
Ex 5
Ex 6
RIE [%]
600
500
400
300
200
100
0
-100
-200
0.01
0.1
1
10
100
1000
concentration [nM]

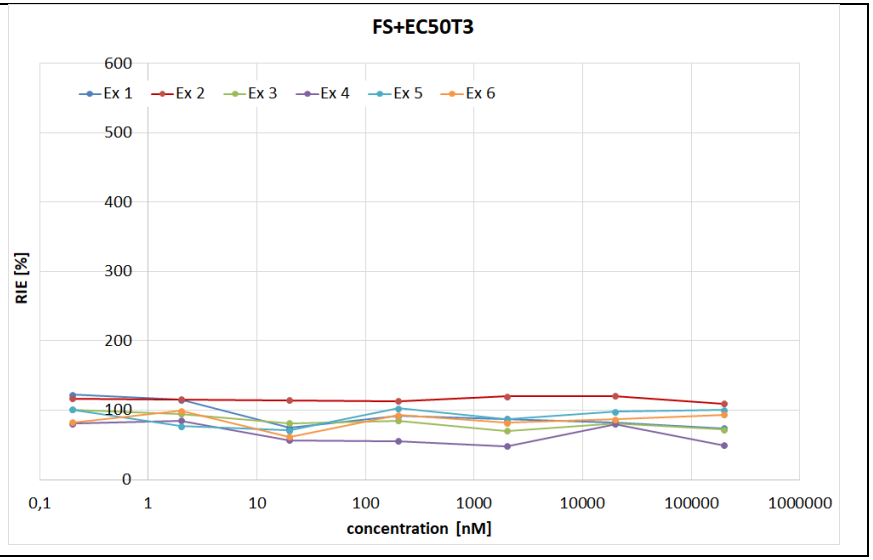

T4+EC50T3
Ex 1
Ex 2
Ex 3
Ex 4
Ex 5
Ex 6
RIE [%]
600
500
400
300
200
100
0
-100
-200
0,01
0,1
1
10
100
1000
concentration [nM]


AD+EC50T3
Ex 1
Ex 2
Ex 3
Ex 4
Ex 5
Ex 6
RIE [%]
150
100
50
0
-50
-100
-150
-200
-250
1
10
100
1000
concentration [nM]


AD+EC50T3
Ex 1
Ex 2
Ex 3
Ex 4
Ex 5
Ex 6
RIE [%]
150
100
50
0
-50
-100
-150
1
10
100
1000
concentration [nM]


SDS+EC50T3
Ex 1
Ex 2
Ex 3
Ex 4
Ex 5
Ex 6
RIE [%]
600
500
400
300
200
100
0
-100
-200
100
1 000
10 000
100 000
concentration [nM]
SDS+EC50T3
Ex 1
Ex 2
Ex 3
Ex 4
Ex 5
Ex 6
RIE [%]
600
500
400
300
200
100
0
-100
-200
100
1 000
10 000
100 000
concentration [nM]
MfA+EC50T3
Ex 1
Ex 2
Ex 3
Ex 4
Ex 5
Ex 6
RIE [%]
600
500
400
300
200
100
0
-100
-200
0.1
1
10
100
1000
10000
concentration [nM]
MfA+EC50T3
Ex 1
Ex 2
Ex 3
Ex 4
Ex 5
Ex 6
RIE [%]
600
500
400
300
200
100
0
0,1
1
concentration [nM]
1000
10000


FS+EC50T3
Ex 1
Ex 2
Ex 3
Ex 4
Ex 5
Ex 6
RIE [%]
600
500
400
300
200
100
0
0.1
1
10
100
1000
10000
100000
1000000
concentration [nM]


FS+EC50T3
Ex 1
Ex 2
Ex 3
Ex 4
Ex 5
Ex 6
RIE [%]
600
500
400
300
200
100
0
0,1
1
10
100
1000
10000
100000
1000000
concentration [nM]

7. ACCEPTANCE CRITERIA

7.1. Verification of the EC50 value of T3

Six verifications were performed – three in the presence of antibiotics and three without antibiotics.
All of them met acceptance criterion for the EC50 T3 value "The mean EC50 value should be -10±0.4 log10(Molar) units (in the range from -10.4 to -9.6 log10(Molar) units)" (Table 2)**.**
The EC50 values of T3 obtained in all these tests was in the range: from -10.3705 to -10,0702 log10(Molar) units.

7.2. Pre-screen experiments

Fourteen Pre-screen tests were performed. For all of them, except two, the first acceptance criterion "%DR or %AR for UC(PCM) should not be more than 15% different from TI SC" was met. One of exceptions was the test of Tetrac (with the mistake made during the plating) which gave unacceptable criterion for both %DR and %AR calculations. The second one was the test of AD, where the criterion was met only for %AR calculations, not %DR calculations.
For all these tests, REF for EC50 T3 calculated based on both %AR and %DR got exactly the same results and all of them met the acceptance criterion: "RPE for EC50 T3 should be in the range of 30-70%".
For all tests that met both acceptance criteria, RPE value for EC50 T3 was in the range 42-69% (Table 7).

Table 7. Validity of Pre-screen tests based on results calculated using %AR and %DR as well as RPE value for EC50 T3

Item	**%DR/%AR for UC(PCM) < 15% of %DR/%AR for TI SC**	**RPE for EC50 T3**
T3	accepted/accepted	66
DPH	accepted/accepted	42
	accepted/accepted	52
Tetrac	**not accepted/not accepted**	49
	accepted/accepted	56
T4	accepted/accepted	58
AD	**not accepted**/accepted	33
	accepted/accepted	47
	accepted/accepted	56
SDS	accepted/accepted	61
	accepted/accepted	46
MfA	accepted/accepted	69
	accepted/accepted	57
FS	accepted/accepted	49

7.3. T-Screen tests

AGONISM
Because some experiments were not valid, each item was tested six times using five plates in every round - half a plate per one item and reference item tested twice: plate 1: Ref(T3) together with T3 (as TI); plate 1a; Ref(T3) together with FS (as TI)).
Six rounds multiply by five plates and two items per plate gives 60 determinations. Eight out of 60 performed determinations were invalid or not finished due to accident (Table 8).

Table 8. Validity of AGONISM experiments (Ex- experiment) for all item tested.

Experiments in which RPE met acceptance criterion (YES accepted; NO not accepted)						
Item tested	**Ex 1**	**Ex 2**	**Ex 3**	**Ex 4**	**Ex 5**	**Ex 6**
Ref(T3)	NO	YES	YES	YES	YES	YES
Ref(T3) plate 1a	YES	YES	YES	YES	NO	YES
T3	NO	YES	YES	YES	YES	YES
DPH	YES	YES	YES	YES	YES	YES
Tetrac	YES	YES	YES	NO	YES	YES
T4	YES	YES	YES	accident	YES	YES
AD	YES	YES	YES	accident	YES	YES
SDS	YES	YES	YES	YES	YES	YES
MfA	YES	YES	YES	YES	YES	NO
FS	YES	YES	YES	YES	NO	YES

RPE calculated based on results for negative control (NC) were comparable to RPE for untreated control (UC(PCM) in every experiment except of one, i.e. in experiment 5 on plate 1a (RPE for NC was -2 and for UC(PCM) -12) (Table 9).
RPE values calculated for positive control (PC/A) showed correct direction of cell response, i.e. stimulation but in half of experiments response was higher than response for the highest concentration of Ref(T3).
RPE values calculated for cytotoxic control (SDS) also showed correct direction of cell response, i.e.inhibition and it was in the range from -60 to -36.
Criteria EC50 for Ref(T3) calculated based on results obtained for two plates per every round met acceptance criterion being always in the range from -10.4 to -9.6 log10(Molar) units. Also, Z-factor that was always ca. 0.9 met acceptance criterion (accepted above 0.5).

Table 9. RPE values for all controls used in AGONISM experiments designed on Plate 1 and Plate 1a (Ex – experiment)

Control (RPE)	**Plate 1**						**Plate 1a**						**Range from/to (only accepted round)**
	Ex 1	**Ex 2**	**Ex 3**	**Ex 4**	**Ex 5**	**Ex 6**	**Ex 1**	**Ex 2**	**Ex 3**	**Ex 4**	**Ex 5**	**Ex 6**	
NC	-11	-1	-1	-3	-3	-1	0	0	-1	-3	-2	1	-3 / 1
UC (PCM)	-16	-3	-1	-4	-6	-6	4	1	-3	0	-12	-5	12/4
SC(right)/SC(left)	-13/13	-1/1	2/-2	3/-3	0/0	4/-4	1/-1	-1/1	-3/3	3/-3	1/-1	-2/2	
SDS	-58	-40	-39	-50	-59	-53	-44 (%AR) -43 (%DR)	-36	-40	-55	-56	-60	-60/-36
PC/A	104	117	80	84	126	86	103	90	96	106	126 (%AR) 123 (%DR)	99	80/126
EC50 for Ref(T3)	-10,072	-9,984	-10,115	-10,121	-10,211	-10,127	-10,260	-10,036	-9,926 (%AR) -9.942 (%DR)	-9,905	-10,161	-10,205	-10.026 /-9.686
EC50 for T3	-10,046 (%AR) -10.048 (%DR)	-9,922	-10,026	-10,142	-9,686	-10,108							
Z-factor	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	-
ACCEPTANCE	NO	YES	YES	YES	YES	YES	YES	YES	YES	YES	NO	YES	-

ANTAGONISM

Because some experiments were not valid, each item was tested six times using five plates in every round - half a plate per one item and reference item tested twice: plate 1: Ref(DPH)/EC50T3 together with T3/EC50T3 (as TI); plate 1a; Ref(DPH)/EC50T3 together with FS/EC50T3 (as TI)).
Six rounds multiply by five plates and two items per plate gives 60 determinations and all of them were valid, mainly due to only two acceptance criteria, i.e. 1. for Z-factor (Z-factor > 0.5); 2. for RPE for S/T3 (RPE for S/T3 should be in the range of 30-70%) (Table 10).

Table 10. Validity of ANTAGONISM experiments (Ex- experiment) for all item tested.

In which experiment RIE met acceptance criterion (YES accepted; NO not accepted)?						
Item tested	Ex 1	Ex 2	Ex 3	Ex 4	Ex 5	Ex 6
Ref(DPH)+EC50T3	YES	YES	YES	YES	YES	YES
Ref(DPH) +EC50T3 plate 1a	YES	YES	YES	YES	YES	YES
T3+EC50T3	YES	YES	YES	YES	YES	YES
DPH+EC50T3	YES	YES	YES	YES	YES	YES
Tetrac+EC50T3	YES	YES	YES	YES	YES	YES
T4+EC50T3	YES	YES	YES	YES	YES	YES
AD+EC50T3	YES	YES	YES	YES	YES	YES
SDS+EC50T3	YES	YES	YES	YES	YES	YES
MfA+EC50T3	YES	YES	YES	YES	YES	YES
FS+EC50T3	YES	YES	YES	YES	YES	YES

RIE calculated based on results for negative control (NC) were similar to RIE calculated for S/T3 in every experiment except of one, i.e. in experiment 3 on plate 1a (RIE for NC was -15 and for S/T3 110/90). Based on these results additional acceptance criterion should be set: RIE for NC not lower than 80%.
PC was not determined in the SOP but during the PART 1 Ref(DPH)C1=EC50 T3 was used. It means that RIE for PC should be equal RIE Ref(DPH)C1+EC50T3 ±15%, i.e. in the range -15/15. Four out of 10 experiment would not have met this criterion, i.e. Ex 1 and Ex 5 on the plate 1 and Ex 2 and Ex 5 on the plate 1a.
RIE values calculated for Ref(T3) showed correct direction of cell response, i.e. stimulation and it was in the range from 213-572.
RPE calculated for S/T3 calculated based on results obtained for two plates per every round was in the range from 37-58%. Also, Z-factor, that was always ca. 0.9, met acceptance criterion (accepted above 0.5) (Table 11A).

Table 11. RIE values for all controls used in ANTAGONISM experiments designed on Plate 1 and Plate 1a (Ex – experiment). A – Results calculated using the highest DPH concentration C1 (calculations according to the SOP "*T-screen assay using GH3 cell line*" Section 3.4.2-2 ; B – results calculated using the UC values instead of the highest DPH concentration C1 (the alternative calculations)

A

Control	**Plate 1**						**Plate 1a**						**Range from/to** (only accepted round)
	Ex 1	**Ex 2**	**Ex 3**	**Ex 4**	**Ex 5**	**Ex 6**	**Ex 1**	**Ex 2**	**Ex 3**	**Ex 4**	**Ex 5**	**Ex 6**	
NC	100	105	103	107	106	112	115	131	-15	81	91	100	-15/131
UC (PCM)	-65 (%AR) -66 (%DR)	-238	-116	-70	-76	-71	-111 (%AR) -112 (%DR)	-175	-91	-67 (%AR) -68 (%DR)	-35	-60	-238/-35
S/T3(right) / SCT3(left)	98/102	16/184	121/79	91/109 (%AR) 90/110 (%DR)	91/109	96/104	100/100	97/103	110/90	126-74 (%AR) 127-73 (%DR)	67/133	111/89	16/184
REF(T3) [RPE for S/T3]	220 [58]	489 [47]	298 [52]	283 [48]	225 [58]	273 [50]	304 (%AR) 305 (%DR) [51]	572 [37]	251 [56]	233 (%AR) 234 (%DR) [56]	213 [55]	242 [53]	213/572 [37/58]
PC	17	-9	-4	4	37	1	-2 (%AR) -3 (%DR)	-21	9	-8	63	7	-21/63
Z-factor	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	-
ACCEPTANCE	YES	YES	YES	YES	YES	YES	YES	YES	YES	YES	YES	YES	-

cd Table 11 - B

Control	Plate 1						Plate 1a						Range from/to (only accepted round)
	Ex 1	**Ex 2**	**Ex 3**	**Ex 4**	**Ex 5**	**Ex 6**	**Ex 1**	**Ex 2**	**Ex 3**	**Ex 4**	**Ex 5**	**Ex 6**	
NC	100	101	101	104	103	107	107	111	40	89	93	100	40/111
UC (PCM)	0	0	0	0	0	0	0	0	0	0	0	0	0
S/T3(right) / SCT3(left)	99/101	75/125	110/90	94/106	95/105	98/102	100/100	99/101	105/95	116/84	76/124	107/93	75/125
REF(T3) [RPE for S/T3]	172 [58]	215 [47]	192 [52]	207 (%AR) 208 (%DR) [48]	171 [58]	201 [50]	197 [51]	272 [37]	179 [56]	179 (%AR) 189 (%DR) [56]	183 [55]	189 [53]	171/215 [37/58]
PC	50	68	52	43	64	42	52 (%AR) 51 (%DR)	56	53	36 (%AR) 35 (%DR)	73	42	36/73 (%AR) 35/73 (%DR)
Z-factor	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	-
ACCEPTANCE	YES	YES	YES	YES	YES	YES	YES	YES	YES	YES	YES	YES	-

8. CONCLUSIONS

1. T-Screen test can be performed in medium both with and without antibiotics
   Results of the verifications of the EC50 value of T3 obtained in medium without antibiotics was comparable to these obtained with antibiotics. Also, no microbiological contaminations was observed during the culture without antibiotics.
   Only one difference was observed in the routine culture of GH3 cells in medium with and without antibiotics (but should be further confirmed): GH3 cells seem to proliferate a bit slower in medium without antibiotics as compared to culture with antibiotics.
2. Length of the period between a passage of GH3 cells and the start of the 48-h preincubation in PCM medium has no effect on results of the T-screen test.
   Results of the T-Screen tests performed in both schedule variants (1 – starting three days after passage and 2 – starting one day after passage) were comparable. Therefore the SOP can provide both options and this allows more flexibility for the user.
3. Passage number seems to have no effect on the results of the study.
   Because it was difficult to perform all elements of the study (Td determination, the verification of the EC50 value of T3 and six rounds/experiments) using cells up to 20. Passage, it could be advised to use the cells up to 25. passage to be able to perform maximum acceptable number of rounds (up to eight rounds) using one batch of restored cells.
4. Although the doubling time (Td) of GH3 cells met the acceptance criteria when determined in two schedule variants (1 – from Monday to Tuesday/next week; 2 – from Friday to Monday/next week), the results obtained for the first variant was on the border of acceptance. Thus, it is advised to perform the measurements only in the second schedule variant as it was recommended in the SOP "*Handling and Maintenance of GH3 cell line*" Section 2.4
5. Both % AlamarBlue reduction (%AR) and % Dye reduction (%DR) calculations can be used.
   Calculations of 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) gave different results/values. However, the %AR and %DR values, when used for calculations of the relative proliferative effect (RPE) and the relative inhibitory effect (RIE) gave exactly the same results. However, it is worth knowing that %DR value seems to be more stringent – some of acceptance criteria, e.g. for solvent control or Z-factor (SC) are based on %AR and %DR and some experiments did not meet acceptance criteria when calculated based on %DR but was accepted as calculated based on %AR. They were as follows:
   - One Pre-screen test for AD - acceptance criteria for UC or SC
   - Two T-screen tests – acceptance criteria for Z-factor (1. Round 6 for SDS/MfA; 2. Round 4 for DPH/Tetrac)

   It is therefore suggested to omit the calculation of %AR from the data analysis and to refine the acceptance criteria so that they do not lead to invalid results.
6. Calculation of the Cell proliferation (%CP) is unnecessary for the determination of the range of concentrations in the of pre-Screen test. Also, the %CP value differs depending on the method of calculation (%AR vs %DR). It is therefore suggested to omit the calculation of %CP from the data analysis.
7. Results of the T-Screen test for agonist chemicals seem to be reproducible making the test promising for determination of agonistic potential of chemicals. The reference item and

agonistic test items (which?) caused a strong concentration-dependent stimulation of GH3 cell growth.

8. Results of the T-Screen test for antagonist chemicals were ambiguous, probably due to very weak or no effect of reference item (DPH). Also, AD that was expected to be antagonist also showed weak antagonistic effect. Without a good reference chemical usefulness of the antagonistic part of the T-Screen test is questioned.
   After appling the altentative calculations, using the UC values instead of the highest DPH concentration C1, the results were less variable confirming that DPH is not good reference item.
9. FS at the concetration 100 nM would be probably better negative control than MfA.
   Although MfA showed no effect in the concentration chosen, at the highest concentration tested (10 µM) it showed antagonistic effect.
10. SDS, also used as the control of cytotoxicity, at the highest noncytotoxic concentration (40 µM) showed antagonistic effect.

9. ADITIONAL INFORMATION

9.1. The equipment used for the study

- Fridge-freezers ((1) IMP 95-62-32 and (2) IMP 95-62-40)
- Freezer below -135 °C (MDF-C2156VAN-PE; IMP 808-029-20)
- Freezer below -70 °C (Platinum 340V; IMP 486-007)
- Temperature sensor and control system for freezers below -135 °C and -70 °C (LB-707T; IMP 808-029-20A)
- CO_2 humidified incubators at 37°C +/- 2 °C, 5% CO_2 +/- 0.5% (the upper one/IMP 801-199-20 for "clean" culture and the lower one/IMP 801-199-20B for exposed cells)
- Temperature and humidity monitoring: IMP 801-199-20C
- Water bath (AJL Vibra (1); IMP 801-322-16)
- Pipette Aid (1) Easypet 3; IMP 801-011-84B; (2) SWIFTPET; IMP 31-543-195)
- Pipette Eppendorf 100-1000 µl (IMP-801-368-06B)
- Pipettes Eppendorf 10-100 µl (IMP IMP-801-386-06A)
- Pipettes Eppendorf 0.5-10 µl (IMP 31-543-251)
- 8-channel pipettes ((1) BRAND; IMP 31-543-43 and (2) Eppendorf IMP 801-368-06D)
- pH-metr (Lab860 IMP 31-83-41)
- Centrifuge (Megafuge; IMP 801-50-46)
- Vacuum pump (Witko; IMP 801-426-02)
- Laminar Flow Hood Sterile (ThermoSafe 18; IMP 801-307-10)
- Analytical balance (Sartorius R200D; IMP 31-514-2) and mass standards IMP 35-164-9 and IMP 35-514-2A
- Vortex (IKA Vibrax IMP 43-12-57)
- Autoclave (H+P 250T; IMP 801-42-4)
- Thermometer (J211/21)
- Thermometer min-max (ZTM-55-1; ZTM-55-2; ZTM-45-1; ZTM-45-2)
- Thermohygrometer
- Microscopes (Leica; AS 06-18 and Olympus IX70; IMP 801-11-77)
- Microplate reader (MultiscanGO; IMP 801-0-021-30) with the setting: continuous shaking 1 min; multiple wavelengths: 570 nm and 600 nm)
- Clear glass vials for the preparation of stock solutions (Agilent #5183-4448 (with caps # 13-425) and Sarstedt #86.1509 (without caps)

- 96-well plates (Nunc # 167 008)
- 24-well plates (Falcon#353504)
- Culture Flasks (T75; Nunc # 156499)
- Culture Flasks (T25; Nunc #156367)
- Serological pipettes with filter Falcon (2 mL#357507), 5 mL#357543), 10 mL# 357551), and 25 mL#357525)
- Serological pipettes without filter 2 mL (Greiner#710183)
- Sterile, pipette tips without filter 10 µL (Axygen# T-300-L), 200 µL (Starlab# S1111-0006), 1000 µL (CAPP#5130130)
- Conical tubes 15 mL (Greiner #188271), and 50 mL (Greiner#227261)
- Syringe filters (0.22 µm) (TPP #99722)
- Bottle filter 500 ml, 0.22 PES + a bottle (TPP#99500)
- Polypropylene Cluster Tubes, 1.1 mL, 8 strip (Axygen#MTS-11-8-C)

9.2. Definitions and abbreviations

DMSO – Dimethyl sulfoxide
EtOH - ethanol
DF – dilution factor
N/A – not analyzed
TI – test item
SC – solvent control
RPE – the relative proliferative effect
RIE – the relative inhibitory effect
EC50 – concentration at which 50% of maximum relative proliferation is observed
IC50 – concentration at which 50% of maximum relative inhibition is observed

10. ARCHIVING INFORMATION

The study plan, original of the documents, notes, raw data, copies of certificates, the report and the most important electronic files will be archived and stored in the GLP Archive at the Nofer Institute of Occupational Medicine, for a period of 10 years from the end of the study.
The Reference control and test items used for the study will be retained by the test facility until the expiry date is reached or until the end of the validation study (if the date is not determined).

17./11./2021r. Joanna Kord
Study Director
date and signature

18./11./2021 r.
Test Facility Manager
date and signature

17/11/2021r. Stanicka
Head of Quality Assurance Unit
date and signature

THE END OF REPORT

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