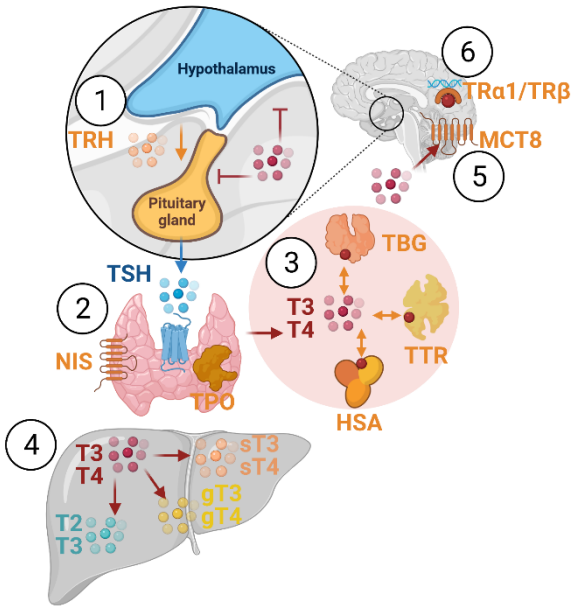
European Commission

STUDY REPORT

for the assessment of the human thyroid hormone receptor alpha (TRα) and beta (TRβ) reporter genes transactivation assay measuring agonist activity – Part 2

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

Caviola, E.



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.

This study report describes the experimental design and includes data generated in Part 2 of the validation study. The method was developed by INDIGO Biosciences, Inc. and subsequently implemented by the EU-NETVAL test facility Vitroscreen S.r.l. (Italy) within the validation study.

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Method 6a - Human Thyroid Hormone Receptor Alpha and Beta Reporter Assays Part II

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The following persons were responsible for key elements of the study within VitroScreen Laboratories:

Name - Surname Function	**Signature**	**Date**
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Euridice Santirocco Quality Assurance		06.02.2023
Marisa Meloni Test Facility Manager		

TIMING OF THE STUDY

TEST ITEMS ARRIVAL	03.11.2021
START OF EXPERIMENTAL PHASE: Part II	24.05.2022
END OF EXPERIMENTAL PHASE: Part II	11.11.2022
RAW DATA ANALYSIS: QA CONTROL	02.02.2023
STUDY REPORT DRAFT	06.02.2023

1. INTRODUCTION AND AIM OF THE STUDY

This study was performed for PART 2 of the EURL ECVAM coordinated Thyroid Validation Study. The proposed method is based on engineered cells expressing high levels of Thyroid Hormone Receptor alpha (NR1A1) or beta (NR1A2) and used to assess potential agonists in the activation of Thyroid Nuclear receptors, considered as potential endocrine disruptors, after 24h exposure.
In this study the predictivity of the method was evaluated using the following:

- 3,3',5-triiodo-L-Tyronine (T3) as Reference
- Sobetirome as Positive Control
- 17β-Estradiol (E2) as Negative Control
- Staurosporine as Positive Control for Viability
- DMSO 0.2% as Solvent Control

to test 30 coded chemicals (test items), assessing their capability to activate TR receptor as agonist ligands.

2. STUDY DESIGN

2.1 Study Scheduling

The PART II study presented three experimental steps:

- **Solubility test**
  Solubility test had the aim to conduct a preliminary determination of the highest soluble concentration and relative solvent for each test item.

- **Dose-range Finding**
  Dose-range Finding assay had the aim to establish the respective cytotoxicity thresholds of TIs and preliminar TR activation for subsequent TR activity assessment, starting from 1:500 highest soluble concentration and preparing a serial dilution of concentrations.
  For each TI, the highest non-cytotoxic concentration for each TI that shows higher activity (response threshold ≥10% from the reference item) was advanced to the TR Activity Assessment for further analysis.
  In case there were maximum two cytotoxic concentrations and there was no response (TRa activation <10% from reference item) at all the non cytotoxic concentrations, the TI was considered negative and no further test was performed.

  Required valid Dose-range Finding for each test item: 1

  In the following table I a summary of preformed Dose-Range Finding assay run for both TRα and TRβ is reported.

I. DOSE-RANGE FINDING ASSAY PERFORMED RUN							
Experimental Session	**RUN n.**	**Cell batch**	**Test Item arrival**	**Experiment Name**	**Date**	**n. Tested chemicals**	**Validity**
Part II	1	Trα: 240712c TRβ: 240802C	03.11.2021	**DRF_bis_01_R1**	11.10.2022	6 chemicals	valid
				DRF_bis_02_R1	13.10.2022	6 chemicals	valid
				DRF_bis_03_R1	17.10.2022	6 chemicals	valid
				DRF_bis_04_R1	20.10.2022	6 chemicals	valid
				DRF_bis_05_R1	24.10.2022	5 chemicals (1 chemical: not testable)	valid

- **TR Activity Assessment**
  The specific aim of TR activity assessment was to conduct a more finely tuned assessment of a "positive" TI activity metrics and to repeat the cytotoxicity assessment. Additional Positive and Negative Control items were included in the procedure for TR activity assessment.
  Required valid TR Activity Assessment Runs for each test item: 3

In the following table II a summary of preformed TR activity assay runs for both TRα and TRβ is reported.

II. TR activity ASSAY PERFORMED RUN							
Experimental Session	**RUN n.**	**Cell batch**	**Test Item arrival**	**Experiment Name**	**Date**	**n. Tested chemicals**	**Validity**
Part II	1	Trα: 240712c TRβ: 240802C	03.11.2021	**PART II_RUN 1**	03.11.2022	2 positive chemicals	valid
	2			**PART II_RUN 2**	07.11.2022	2 positive chemicals	valid
	3			**PART II_RUN 3**	10.11.2022	2 positive chemicals	valid

2.2 Experimental Design

The assays (both for dose-range findings and for TR activity) are performed in 2 days as reported in the following fig.1:

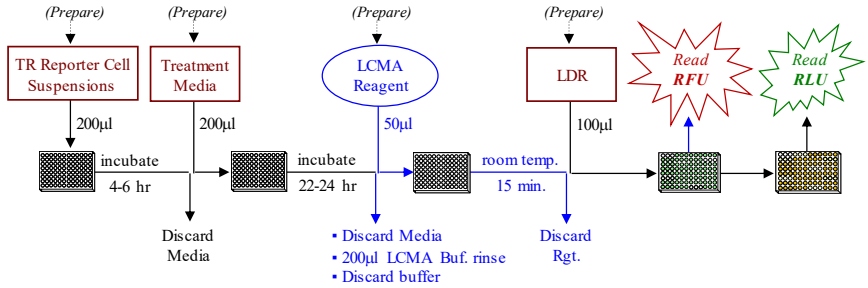


Figure 1. Overview of the workflow for dose range finding and TR assays**.** Text and arrows in blue font denote the LCM Assay for Cytotoxicity which is performed in multiplex with LDR Assay for TR activation assessment on the same plates.

In brief:

The preliminary solubility test by Molarity Method in elective solvent (DMSO, EtOH or DPBS) was performed, according to SOP, to assess the maximum concentration at which each test items was still soluble to establish the starting point for subsequent dose-range finding. Not soluble test items were excluded from testing.

Dose-range finding and TR activity assays were on 2-day experiment.
On Day 1. TRα and TRβ cells were seeded in distinct plates and incubated at 37°C, 5% CO2 and 90% RH for 4.5±0.5 h. After this time culture medium was discarded and substituted with media containing the treatments (controls, reference and test items) and the cells were incubated for 24±1 h in incubator.
In Tab. III and Tab IV the treatments applied for dose-range finding and TR assay respectively are reported.

III. Treatments for Dose-range Finding Assay				
Dose-Range Findings	**Controls**	Reference (REF)	3',3',5'-triiodo-L-Tyronine	0.1 µM
		Positive control Cytotoxicity (LCMA-PC)	Staurosporine	8 µM
		Solvent Control	DMSO	0.2%
		Background Cytotoxicity (LCMA-BKG)	DMSO (no cells)	0.2%
	Test Items	Soluble coded test chemicals		5 concentrations; 1:8 dilution factor; from 1:500 maximum solubility

IV. Treatments for TR Activity Assay				
TR Assay	**Controls**	Negative Control (NC)	17β-Estradiol	1.0 µM
		Positive control (PC)	Sobetirome	1.0 µM
		Reference (REF)	3',3',5'-triiodo-L-Tyronine	0.1 µM
		Reference Curve	3',3',5'-triiodo-L-Tyronine	8 concentration 1:3 dilution factor from 0.1µM
		Positive control Cytotoxicity (LCMA-PC)	Staurosporine	8 µM
		Solvent Control	DMSO	0.2%
		Background Cytotoxicity (LCMA-BKG)	DMSO (no cells)	0.2%
	Positive Test Items	T1	Chemical 791	7 concentrations; 1:4 or 1:3 dilution factor; from the highest not cytotoxic concentration with highest activatin of TR
		T2	Chemical 613	

On Day 2. At the end of exposure period, media with treatments were discarded and cytotoxicity was assessed by fluorescence-based LCMA methods and subsequentely the activation of TR receptors was measured by luminescence-based assay.

3. MATERIALS
3.1. TEST SYSTEM

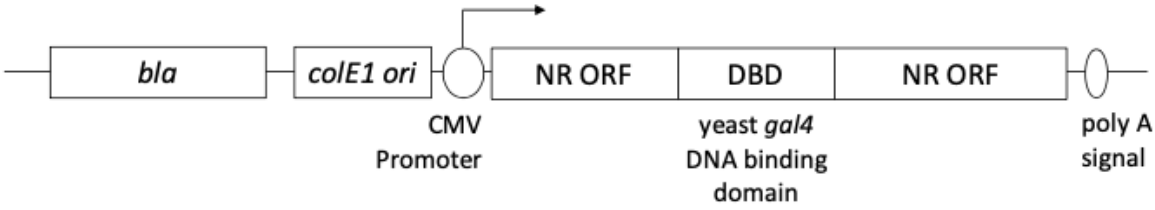
The assays for the assessment of the activation of Thyroid Hormone Receptor alpha (NR1A1) and beta (NR1A2) utilize Human Embryonic Kidney cells engineered to provide constitutive, high-level expression of the corresponding Thyroid Hormone Receptor. These reporter cells express hybrid thyroid hormone receptors in which their respective native N-terminal DNA Binding Domain (DBD) sequence have been replaced with that of the yeast GAL4 DBD sequence. Accordingly, the resident luciferase reporter gene is functionally linked to a tandem array of GAL4 upstream activation sequences (UAS). Thus, quantifying changes in luciferase expression in the treated *vs.* untreated reporter cells, following 24 hr exposure to a test item, provides a specific and sensitive measure of changes in TR activity without collateral induction/suppression of the target genes that are otherwise regulated by the activated TR transcription factors.

TRα and TRβ Reporter Cells are constructed through transient transfection of HEK293 cells. HEK293 is an immortalized cell line that has been identity-validated by, and sourced from, the American Type Culture Collection (ATCC; product #CRL-1573).

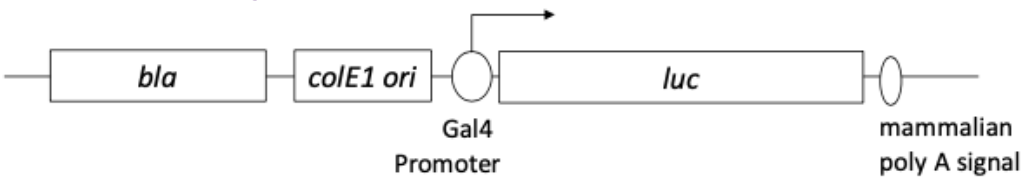
INDIGO Biosciences' Nuclear Receptor (NR) Reporter cells are constructed using a combination of proprietary vectors that express:

- a chimeric cDNA open reading frame (ORF) encoding a nuclear receptor ligand binding domain preceded by the yeast Gal4 DNA binding domain (DBD), and
- a luciferase (LUC) ORF functionally linked to the Gal4 promoter.

i. INDIGO Expression Construct for Hybrid Nuclear Receptors



ii. Luciferase Reporter Vector



Engineered TRα and TRβ Reporter Cells are produced by Indigo Biosciences and tested in sets of four replicate by treatment with 0 and 100 nM T3 for 24 hours to quantify TR

activity. Qualified Reporter Cells and kit components met or exceeded the following minimum performance criteria: Z' ≥ 0.50 S/B ≥ 1,000
TRα and TRβ Reporter cells are confirmed negative for mycoplasma. TRα and TRβ Reporter cells are confirmed negative for contaminating microbial agents.

Quality Control
The procedure is based on Engineered Proprietary Cells ready to use which cannot be cultured and amplified after experiment. Aliquot of **TRα** and **TRβ** cells were transferred to JRC (ISPRA) for cell type characterization.

TEST SYSTEM		
PART II DFR and TR Assay Runs		
NAME	**TRα**	**TRβ**
SUPPLIER	Indigo Biosciences	Indigo Biosciences
CAT. NUMBER	IB01001_EUC	IB01101_EUC
DATE OF ARRIVAL	27.07.2022	05.09.2022
BATCH N°	240712c	240802c
QUANTITY	200 µl/well cell suspension	200 µl/well cell suspension
EXPIRATION DATE	31/01/2023	28/02/2023

3.2. CULTURE CONDITION AND MEDIA

TRα and TRβ Reporter cell are ready to be used for TR activation assay. For the use, the cells are subjected to a rapid-thaw procedure in CRM (Cell Recovery Medium) to yield a cell suspension that is ready for immediate dispensing of 200µk with approx. 25.000 cells into the wells of a 96-well plate. For treatments CSM (Compound Screening Medium) is used to dilute chemicals Stocks (typically 500x concentrate) in order to achieve final testing concentration.

CULTURE MEDIA		
PART II DFR and TR Assay Runs		
NAME	CRM Medium	CSM Medium
SUPPLIER	Indigo Biosciences	Indigo Biosciences
CAT. NUMBER	IB01001_EUC and IB01101_EUC	IB01001_EUC and IB01101_EUC
DATE OF ARRIVAL	16.06.2022	16.06.2022
BATCH N.	240606Cpr	240606-7C
EXPIRATION DATE	31.12.2022	31.12.2022
STORAGE	-20°C	-20°C

3.3 TEST ITEMS: IDENTIFICATION AND CHARACTERIZATION

Coded chemicals (test items)					
N°	**Chemical code**	**State / Storage**	**MW approx.**	**Sample Weight (mg)**	**H hazard**
1	722	Solid RT	250	336	H302
2	457	Liquid RT	Aqueous solution (1 M)	1mL	H302+H332, H314, H351, H360, H362, H372, H411
3	908	Solid RT	350	615	H317, H319, H400
4	791	Solid -20°C	350	3 x 25mg original vial	none
5	084	Solid RT	175	325	H302, H351
6	480	Solid 4°C	500	3 x 10mg original vials	H302
7	521	Solid RT	125	327	H271, H302, H319, H373
8	304	Solid RT	250	312	H302, H317, H319
9	489	Solid RT	550	336	H410
10	184	Liquid RT	300	1mL	H360FD, H410
11	584	Solid RT	200	313	H302
12	139	Solid RT	275	338	H301+H311, H315, H319, H330, H335, H351, H410
13	676	Solid RT	300	340	H315, H319, H410
14	739	Solid 4°C	350	318	H315, H317, H319, H334
15	082	Solid 4°C	150	317	H302
16	183	Solid RT	125	312	H302, H351, H360D, H372
17	306	Solid RT	200	315	H302+H332, H318, H335, H341, H361d, H372, H411
18	814	Solid RT	325	329	H301, H361d, H372, H411

19	558	Solid 4°C	325	327	H302, H315, H317, H319, H334, H335
20	262	Solid 4°C	700	321	H315, H319, H361fd, H362, H373
21	797	Solid 4°C	275	325	none
22	535	Solid RT	275	336	H315, H319
23	613	Solid -20°C	750	258	H300 Fatal if swallowed
24	527	Solid 4°C	550	320	H301, H360F, H373, H400, H410
25	269	Solid RT inert gas	300	321	H301+H311+H331, H315, H319
26	717	Solid -20°C	475	316	none
27	832	Solid RT inert gas	200	325	H301, H330, H340, H350, H360fd, H372, H410
28	351	Solid RT	175	320	H317, H410
29	637	Solid RT	125	320	H302, H315, H318, H400
30	100	Solid RT inert gas	375	311	none

3.4 POSITIVE AND NEGATIVE CONTROLS: CHARACTERIZATION AND JUSTIFICATION OF USE

NAME	Staurosporine	DMSO	DMSO
CAS NUMBER	6299-74-1	67-68-5	67-68-5
INTENDED USE	CYTOTOXICITY POSITIVE CONTROL	SOLVENT CONTROL	Background no cell + solvent CONTROL
UNIVOCAL CODE	**LCMA-PC**	**SC**	**LCMA-BKG**
SUPPLIER	Indigo Biosciences	Sigma Aldrich Merck	Sigma Aldrich Merck
CAT. NUMBER	IB01001_EUC and IB01101_EUC	D8418	D8418
BATCH	220316 (kit) Staurosporine 230527	BCCH3300	BCCH3300
PHYSICAL FORM	Liquid	Liquid	Liquid
SOLVENT	DMSO	CSM Medium	CSM Medium
TREATMENT DOSE / CONCENTRATION	200 µl/well 8 µM	200 µl/well 0.2%	200 µl/well 0.2%
EXPIRATION	31/12/2022	Closed: 12/05/2025 Opened: 11/01/2023	Closed: 12/05/2025 Opened: 11/01/2023
CERTIFICATE OF ANALYSIS	n.a.	yes	yes
SAFETY INFORMATION	n.a	yes	yes
STORAGE	-80°C	RT	RT

NAME	Sobetirome	17β- Estradiol (E2)
CAS NUMBER	211110-63-3	50-28-2
INTENDED USE	POSITIVE CONTROL	NEGATIVE CONTROL
UNIVOCAL CODE	**PC**	**NC**
SUPPLIER	Indigo Biosciences	Indigo Biosciences
CAT. NUMBER	IB01001_EUC and IB01101_EUC	IB01001_EUC and IB01101_EUC
BATCH	240527	240531
PHYSICAL FORM	Liquid	Liquid
SOLVENT	DMSO	DMSO
DOSE CONCENTRATION	1 µM	1 µM
EXPIRATION	31/12/2022	31/12/2022
CERTIFICATE OF ANALYSIS	n.a.	n.a.
SAFETY INFORMATION	n.a	n.a
STORAGE	-80°C	-80°C

3.5 REFERENCES

NAME	3,3',5-triiodo-L-Tyronine, Sodium Salt	3,3',5-triiodo-L-Tyronine, Sodium Salt
CAS NUMBER	55-06-1	55-06-1
INTENDED USE	REFERENCE	REFERENCE
UNIVOCAL CODE	**REF EC100**	**RI (8 Concentrations)**
SUPPLIER	Indigo Biosciences	Indigo Biosciences
CAT. NUMBER	IB01001_EUC and IB01101_EUC	IB01001_EUC and IB01101_EUC
BATCH	240527	240527
PHYSICAL FORM	Liquid	Liquid
500x STOCK CONCENTRATION	50 µM	50 µM
SOLVENT	DMSO	DMSO
DOSE CONCENTRATION	0.10 µM	8 concentration 3 fold dilution (ref. Tab. Below)
EXPIRATION	31/12/2022	31/12/2022
CERTIFICATE OF ANALYSIS	n.a.	n.a.
SAFETY INFORMATION	n.a	n.a
STORAGE	-80°C	-80°C

REFERENCE TESTING CONCENTRATIONS		nM
RI T3	C1	100
	C2	33
	C3	11
	C4	3.7
	C5	1.2
	C6	0.41
	C7	0.14
	C8	0.046

3.6 SOLVENTS FOR TEST ITEMS

NAME	DMSO	ETHANOL	DPBS W/O Ca2+/Mg2+	CSM Medium
CAS NUMBER	67-68-5	64-17-5	n.a.	n.a.
INTENDED USE	SOLVENT I	SOLVENT II	SOLVENT III	FINAL SOLVENT
SUPPLIER	Sigma Aldrich Merck	Sigma Aldrich Merck	Sigma Aldrich Merck	Indigo
CAT. NUMBER	D8418	02851	D8537	IB01001_EUC and IB01101_EUC
BATCH	BCCH3300	BCCG2266	RNBL0128	231028-29J
PHYSICAL FORM	Liquid	Liquid	Liquid	Liquid
DOSE CONCENTRATION	Neat	Neat	Neat	Neat
EXPIRATION	Closed: 12.05.2025 Opened: 11.01.2023	30.09.2023	03.2024	31.05.2022
CERTIFICATE OF ANALYSIS	yes	yes	yes	n.a.
SAFETY INFORMATION	yes	yes	yes	n.a.
STORAGE	RT	RT	RT	-80°C

3.7 EQUIPMENT

The following equipment was used:

- Calibrated Incubator (37°C, 5% CO_2 and ≥ 70% humidified atmosphere) for mammalian cell culture (HERACELL 150 I or PANASONIC MCO-170AICUVH-PE)
- Cell culture-rated laminar flow hood.
- Sonication 37°C water bath
- Electronic calibrated 8-channel pipette, either an electronic repeat-dispensing or manual pipette and tips suitable for dispensing 50 µl, 100 µl, and 200 µl volumes (Integra Voyager II)
- Calibrated manual pipettes: 2.0, 10, 20, 100, 300 (8 channel) and 1000 µl maximum dispensing volume
- Vortex mixer

- Tecan Infinite M200 Plate reader capable of luminescence and fluorescence measurements, with the following paramaters:

Fluorescence Measure	Fluorescence filters Ex:485nm | Em:535nm
Luminescence Measure	Integration time: 500 ms

- Software: Excel version 16.47.1 and Prism 9

4. METHODS

The method is described in SOP version 2.0 on 13th May 2022, and briefly reported in the study plan.

Data elaboration are described in the SOP.

In brief, RLU (Relative Luminescence Units) mean, standard deviation and coefficient of variation (CV%) were calculated for each treatment (test items, reference, controls). Fold activation (FA) of T3 at EC100 (0.10 µM) was calculated dividing average RFU $EC100_{T3}$ with average RFU of solvent control (SC). Signal background (from wells without cells) was subtracted from every treatment then the Relative-Activation percent (%RA) was calculated dividing background-subtracted RFU of treatments with the one of T3 at EC100 (set as 100%). Z' value was calculated using the RI aRLU and its corresponding SD, and the SC aRLU and its corresponding SD in the following formula: Z' = 1 - [3*(SDT3 0.10 µM + SDSC) / (aRLUT3 0.10 µM - aRLUSC)].
The %RA values of test items and reference were plot against corresponding concentrations using a non-linear regression curve-fitting model (variable slope, 4 parameters, least squares fit) using PRIMS version 9.4.1

The calculation of Assay Metrics, %CV log (EC50) for Reference Item (RI) was calculated on Log(EC50) mean and standard error expressed in M rather than nM. This calculation was necessary to meet the acceptance criteria.

For viability, RFU (Relative Fluorescence Units) mean, standard deviation and coefficient of variation (CV%) were calculated for each treatment (test items, reference, controls) then back-ground was substracted. Viability was calculated for every treatment (test items, reference, controls) dividing average background-subtracted RFU of each treatment with background-subtracted RFU of Solvent Control.

5. RESULTS

All raw and elaborated data are stored in files maintained in VitroScreen server/archive. Elaborated data (excel and Prism files) are transferred to EURL-ECVAM for evaluation and statistical analysis (ref. Appendix I for file list).

5.1. Solubility Test

The results of the Solubility Test are reported in following Tab.V.

Tab V. Solubility Test Results

Chemical code	Solvent	START CONC 500X C1 (mM)	Chemical code	Solvent	START CONC 500X C1 (mM)
722	DMSO	50	**183**	DMSO	50
457	DMSO	50	**306**	Not soluble	Not soluble
908	DMSO	50	**814**	DMSO	50
791	DMSO	50	**558**	DMSO	50
084	DMSO	50	**262**	DMSO	25
480	DMSO	50	**797**	DMSO	50
521	DMSO	50	**535**	DMSO	50
304	DMSO	50	**613**	DMSO	50
489	DMSO	50	**527**	DMSO	25
184	DMSO	50	**269**	DMSO	50
584	DMSO	50	**717**	DMSO	25
139	DMSO	50	**832**	DMSO	50
676	DMSO	50	**351**	DMSO	50
739	DMSO	12,5	**637**	DMSO	50
82	DMSO	50	**100**	DMSO	50

The solubility test was performed following molarity method described in SOP. In the case of chemicals 306, the test was performed by performing solubilization at 3 decreasing concentrations (50, 25 and 12,5 mM) starting in DMSO, as elective solvent, followed by Ethanol and, in the end, in DPBS. The chemicals resulted completely insoluble at any conditions so it was excluded from test method.

5.2. Dose-range finding (DRF)

For each soluble chemical, the concentration obtained from solubility test was considered the 500x concentrated stock as starting concentration for Dose-Range Finding.

Initially, two experiments (corresponding to the test of 12 chemicals) were performed but the results did not match acceptance criteria due to too low cell viability (not conform cell system, due to logistic issues). The produced data were excluded from validation and are not reported. The part II of validation was re-started using conform cell TRα and TRβ batches. The repeated Dose Range Finding experiments were identified as "Part II_DRF bis.

5.2.1 TRα DRF Results

The results of the single run of Dose-Range Finding on TRα cells are reported in Tab. VI. Data elaboration were performed as reported in the SOP and summarized in Par. 4.Methods.

The relative activation % (%RA), the viability % (%LC) and the selected concentration for subsequent TR activity assay are reported. The criterion for selection was the highest not cytotoxic concentration (viability threshold for not cytotoxic compound %LC≥80%) with maximum TR activation. No invalid runs occurred.

VI. Dose-Range Finding Results for TRα

Chemicals	nM	% LC	%RA	
722	100000.00	64.7	0.03	Negative chemical
	12500.00	102.4	0.01	
	1562.50	101.7	0.02	
	195.31	109.5	0.00	
	24.41	103.8	-0.01	
457	100000.00	31.0	0.01	Negative chemical
	12500.00	128.4	0.02	
	1562.50	133.7	0.03	
	195.31	136.4	0.04	
	24.41	134.9	0.02	
908	100000.00	11.7	0.02	Negative chemical
	12500.00	115.6	0.03	
	1562.50	117.4	0.05	
	195.31	111.1	0.05	
	24.41	102.6	0.05	
791	100000.00	26.2	6.15	**Positive chemical** **Selected starting concentration**
	12500.00	92.3	126.68	
	1562.50	**96.4**	**154.44**	
	195.31	99.7	125.73	
	24.41	96.2	38.91	

084	100000.00 12500.00 1562.50 195.31 24.41	114.9 127.4 121.7 120.4 117.3	0.08 0.00 0.13 0.29 0.22	Negative chemical
480	100000.00 12500.00 1562.50 195.31 24.41	126.9 118.3 115.9 109.2 108.0	0.04 0.09 0.20 0.24 0.14	Negative chemical
521	100000.00 12500.00 1562.50 195.31 24.41	102.2 108.4 115.2 112.8 108.9	-0.03 0.02 -0.01 -0.02 0.05	Negative chemical
304	100000.00 12500.00 1562.50 195.31 24.41	35.9 126.6 137.7 138.3 135.6	-0.01 0.01 0.01 0.02 -0.04	Negative chemical
489	100000.00 12500.00 1562.50 195.31 24.41	0.5 113.8 126.6 126.7 115.5	-0.04 0.00 0.04 0.00 0.02	Negative chemical
184	100000.00 12500.00 1562.50 195.31 24.41	6.6 117.9 127.4 138.4 135.8	-0.04 0.01 0.03 0.01 0.00	Negative chemical
584	100000.00 12500.00 1562.50 195.31 24.41	116.6 154.7 167.2 165.4 160.4	0.01 0.03 0.03 0.02 0.03	Negative chemical
139	100000.00 12500.00 1562.50 195.31 24.41	0.3 146.6 150.2 162.8 146.4	0.01 0.06 0.05 0.06 0.01	Negative chemical

676	100000.00	0.5	-0.05	Negative chemical
	12500.00	15.9	-0.07	
	1562.50	96.1	-0.04	
	195.31	102.5	-0.04	
	24.41	92.7	0.01	
739	25000.00	90.3	-0.06	Negative chemical
	3125.00	153.5	-0.02	
	390.63	172.8	-0.02	
	48.83	179.9	0.01	
	6.10	164.0	-0.04	
082	100000.00	122.6	-0.03	Negative chemical
	12500.00	126.3	-0.01	
	1562.50	134.9	0.03	
	195.31	150.0	-0.04	
	24.41	143.2	-0.03	
183	100000.00	78.6	-0.01	Negative chemical
	12500.00	84.5	0.02	
	1562.50	104.3	-0.01	
	195.31	114.7	0.03	
	24.41	105.3	0.00	
814	100000.00	67.8	0.01	Negative chemical
	12500.00	170.58	0.00	
	1562.50	172.99	0.00	
	195.31	169.68	0.03	
	24.41	146.40	0.03	
558	100000.00	-0.2	0.03	Negative chemical
	12500.00	123.4	-0.01	
	1562.50	149.8	0.01	
	195.31	144.6	0.04	
	24.41	134.5	0.04	
262	50000.00	-0.4	0.00	Negative chemical
	6250.00	109.1	0.01	
	781.25	98.2	-0.02	
	97.66	94.3	0.00	
	12.21	99.7	-0.02	
797	100000.00	52.5	0.01	Negative chemical
	12500.00	134.9	0.02	
	1562.50	176.0	0.00	
	195.31	174.0	0.02	
	24.41	177.0	-0.01	

535	100000.00 12500.00 1562.50 195.31 24.41	133.5 157.5 177.6 182.6 172.7	0.04 0.05 0.03 0.06 0.05	Negative chemical
613	100000.00 12500.00 **1562.50** 195.31 24.41	0.5 79.9 **80.6** 91.0 93.0	-0.02 38.07 **50.17** 27.12 -0.01	**Positive chemical** **Selected starting concentration**
527	50000.00 6250.00 781.25 97.66 12.21	2.5 121.19 144.91 151.34 143.01	-0.04 0.05 0.10 0.10 0.02	Negative chemical
269	100000.00 12500.00 1562.50 195.31 24.41	26.8 117.7 122.1 123.4 121.7	-0.02 0.01 0.03 0.04 0.02	Negative chemical
717	50000.00 6250.00 781.25 97.66 12.21	0.4 4.2 106.7 110.9 113.2	-0.02 -0.04 -0.01 -0.01 -0.03	Negative chemical
832	100000.00 12500.00 1562.50 195.31 24.41	-0.6 37.4 166.1 176.8 155.6	-0.02 -0.03 0.01 -0.01 -0.03	Negative chemical
351	100000.00 12500.00 1562.50 195.31 24.41	99.2 135.0 147.5 145.3 129.9	0.02 0.04 0.06 0.04 0.04	Negative chemical
637	100000.00 12500.00 1562.50 195.31 24.41	65.3 81.3 88.4 92.7 87.1	0.03 0.04 0.00 0.02 0.02	Negative chemical

100	100000.00	91.1	0.04	Negative chemical
	12500.00	142.58	0.03	
	1562.50	145.01	0.02	
	195.31	141.20	0.03	
	24.41	144.05	0.00	
306	Not soluble - not tested			

In green the selected concentrations for TRα activity assay.
Among the 29 tested chemicals, only two, code 791 and 613, resulted able to activate TRα at not-cytotoxic concentrations.

5.2.2 TRβ DRF Results

The results of the single run of Dose-Range Finding on TRβ cells are reported in Tab. VII. Data elaboration were performed as reported in the SOP and summarized in Par. 4.Methods. The relative activation % (%RA), the viability % (%LC) and the selected concentration for subsequent TR activity assay are reported. The criterion for selection was the highest not cytotoxic concentration (viability threshold for not cytotoxic compound %LC≥80%) with maximum TR activation. No invalid runs occurred.

VII. Dose-range finding Results for TRβ				
	nM	**% LC**	**% RA**	
722	100000.00	59.1	0.02	Negative chemical
	12500.00	99.9	0.00	
	1562.50	101.2	0.00	
	195.31	100.3	0.00	
	24.41	100.0	0.03	
457	100000.00	35.9	0.04	Negative chemical
	12500.00	131.0	0.02	
	1562.50	137.3	0.00	
	195.31	134.3	0.03	
	24.41	124.4	0.03	
908	100000.00	6.4	0.03	Negative chemical
	12500.00	101.5	0.05	
	1562.50	112.9	0.04	
	195.31	103.8	0.04	
	24.41	104.3	0.01	
791	100000.00	27.6	4.95	**Positive chemical** **Selected starting concentration**
	12500.00	122.2	97.37	
	1562.50	**120.0**	**105.48**	
	195.31	117.4	93.64	
	24.41	105.9	56.47	

084	100000.00 12500.00 1562.50 195.31 24.41	119.7 145.0 159.6 163.4 153.8	0.13 0.23 0.71 1.14 0.92	Negative chemical
480	100000.00 12500.00 1562.50 195.31 24.41	149.8 151.9 158.4 147.2 148.4	-0.02 0.09 0.05 0.04 0.02	Negative chemical
521	100000.00 12500.00 1562.50 195.31 24.41	70.6 81.5 94.6 101.4 100.7	0.03 0.00 0.00 -0.01 -0.01	Negative chemical
304	100000.00 12500.00 1562.50 195.31 24.41	35.8 109.0 121.0 124.9 128.9	-0.01 0.05 0.02 0.00 0.00	Negative chemical
489	100000.00 12500.00 1562.50 195.31 24.41	-0.2 84.9 100.8 104.5 97.3	-0.03 0.07 0.04 0.02 0.05	Negative chemical
184	100000.00 12500.00 1562.50 195.31 24.41	3.5 95.8 116.5 118.8 100.2	-0.03 -0.06 0.07 -0.06 -0.07	Negative chemical
584	100000.00 12500.00 1562.50 195.31 24.41	113.5 147.0 163.1 161.2 144.0	-0.07 -0.01 -0.01 0.03 0.01	Negative chemical
139	100000.00 12500.00 1562.50 195.31 24.41	-0.1 130.9 134.8 123.1 119.5	-0.03 -0.02 0.04 0.07 0.04	Negative chemical

676	100000.00 12500.00 1562.50 195.31 24.41	0.1 37.6 123.2 120.2 115.8	0.00 0.00 0.00 0.00 0.00	Negative chemical
739	25000.00 3125.00 390.63 48.83 6.10	124.3 146.5 150.1 157.8 139.2	0.01 0.00 0.02 0.00 0.02	Negative chemical
082	100000.00 12500.00 1562.50 195.31 24.41	112.3 123.3 135.5 125.6 123.4	0.06 0.04 0.06 0.06 0.06	Negative chemical
183	100000.00 12500.00 1562.50 195.31 24.41	101.7 92.3 93.3 94.7 117.4	0.02 -0.01 -0.01 -0.02 -0.02	Negative chemical
814	100000.00 12500.00 1562.50 195.31 24.41	73.1 137.1 142.7 140.8 134.7	0.01 0.09 0.00 0.00 -0.02	Negative chemical
558	100000.00 12500.00 1562.50 195.31 24.41	0.0 111.4 143.2 145.3 130.6	0.03 0.04 0.05 0.02 0.03	Negative chemical
262	50000.00 6250.00 781.25 97.66 12.21	0.0 82.3 75.4 83.4 91.6	0.01 0.00 0.00 0.02 0.00	Negative chemical
797	100000.00 12500.00 1562.50 195.31 24.41	38.8 95.1 117.7 120.7 115.4	0.01 0.01 0.03 0.02 0.00	Negative chemical

535	100000.00 12500.00 1562.50 195.31 24.41	67.5 70.1 81.0 90.1 76.1	0.05 0.05 0.06 0.02 0.06	Negative chemical
613	100000.00 12500.00 **1562.50** 195.31 24.41	-0.4 107.9 **99.3** 118.2 121.6	0.07 50.65 **56.24** 33.60 0.04	**Positive chemical** **Selected starting concentration**
527	50000.00 6250.00 781.25 97.66 12.21	1.1 148.6 200.4 196.7 181.9	0.03 0.05 0.10 0.05 0.08	Negative chemical
269	100000.00 12500.00 1562.50 195.31 24.41	72.3 177.0 185.1 179.8 164.2	0.03 0.06 0.13 0.07 0.05	Negative chemical
717	50000.00 6250.00 781.25 97.66 12.21	12.5 6.9 110.7 116.6 103.0	0.00 -0.01 -0.01 0.01 0.01	Negative chemical
832	100000.00 12500.00 1562.50 195.31 24.41	-0.8 45.4 172.5 169.9 150.3	0.00 0.01 0.03 0.01 0.04	Negative chemical
351	100000.00 12500.00 1562.50 195.31 24.41	110.9 141.0 132.9 125.6 119.7	0.04 0.05 0.05 0.02 0.07	Negative chemical
637	100000.00 12500.00 1562.50 195.31 24.41	89.4 111.2 120.3 125.4 125.9	-0.03 -0.02 -0.02 0.00 0.03	Negative chemical

100	100000.00	113.2	-0.01	Negative chemical
	12500.00	168.1	-0.02	
	1562.50	181.2	0.01	
	195.31	179.4	0.01	
	24.41	172.2	0.02	
306		Not soluble - not tested		

In green the selected concentrations for TRβ activity assay.

Among the 29 tested chemicals, only two, code 791 and 613, resulted able to activate TRβ at not-cytotoxic concentrations.

5.2.3 DRF Metrics for RI

Concerning the activation of TRα and TRβ by natural ligand 3'3'5'-Triiodo-L-thyronine as Reference Item at EC100, the metrics related to acceptance criteria of all the performed runs are reported in Tab. VIII.

Tab. VIII Metrics for RI (T3 EC100)

			TRα		TRβ	
			FA	Z'	FA	Z'
DFR EXP.	N.	Chemical code	≥300	≥0.5	≥500	≥0.5
DFR 01	1	**722**	4059	0.69	2570	0.62
	2	**457**				
	3	**908**				
	4	**791**	1583	0.63	882	0.55
	5	**84**				
	6	**480**				
DFR 02	7	**521**	1471	0.57	2755	0.55
	8	**304**				
	9	**489**				
	10	**184**	3558	0.60	968	0.62
	11	**584**				
	12	**139**				
DFR 03	13	**676**	2251	0.90	6280	0.80
	14	**739**				
	15	**82**				
	16	**183**	2944	0.65	3130	0.72
	17	**814**				

	18	**558**				
DFR 04	19	**262**	2906	0.73	5035	0.65
	20	**797**				
	21	**535**				
	22	**613**	1130	0.78	2461	0.76
	23	**527**				
	24	**269**				
DFR 05	25	**717**	1804	0.80	4661	0.80
	26	**832**				
	27	**351**				
	28	**637**	2498	0.75	2545	0.80
	29	**100**				
		306	Not soluble - not tested			

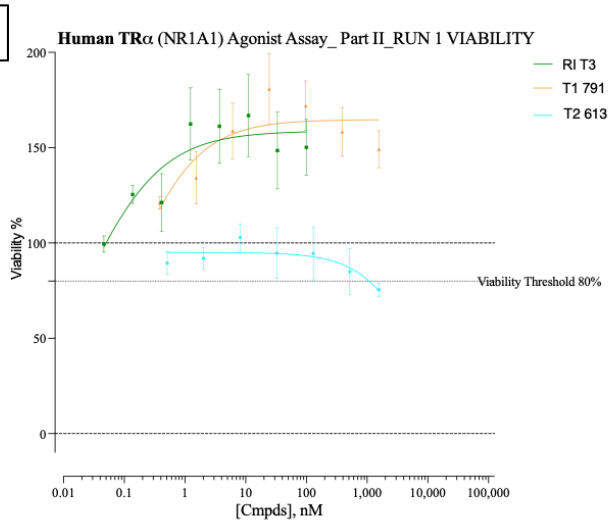
5.3. TR Activity assay

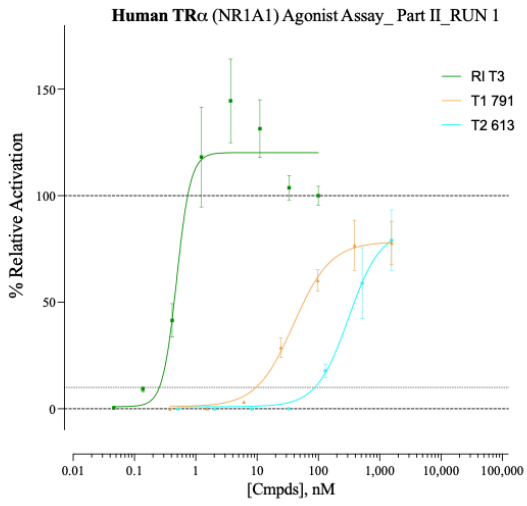
TR Activation assay was performed only on the positive chemicals 791 and 613.
On the basis of the results of Dose Range Finding, for the production of the TR testing concentrations, dilution factor of 4-fold and 3-fols will be applied on 791 and 613 respectively with the aim to obtain a complete dose-response curve.
Total 3 valid runs were performed. No invalid runs occurred.

5.3.1 TRα

In fig. 2a-2c the results of cytotoxicity LCMA assay (expressed as viability %) and Activation of TRα (Relative Activation %) plotted against tested concentrations (nM) are reported for the reference and positive chemicals, for each valid run.

2a





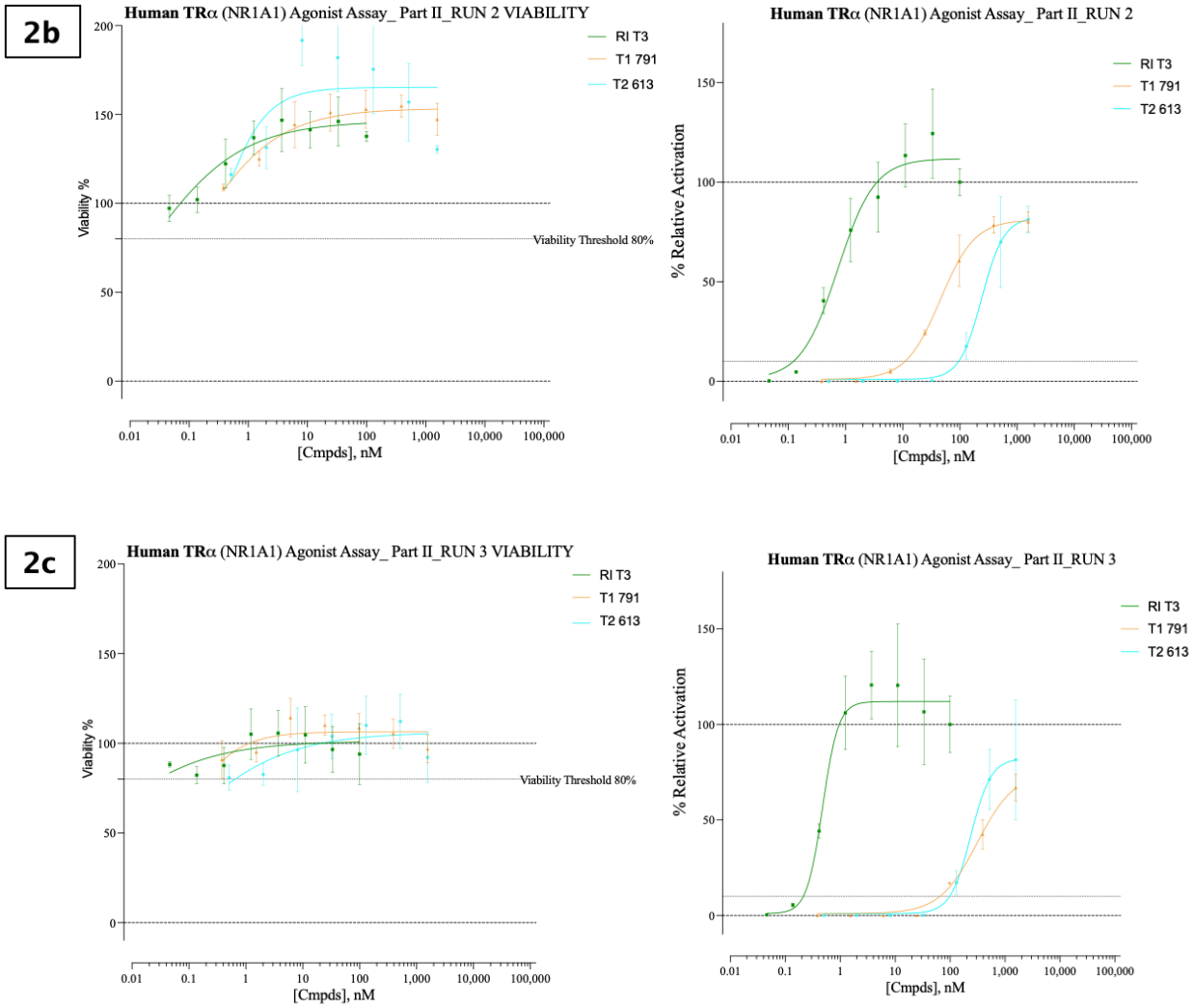


Fig 2a-c. Results of 3 valid runs on TRα cells for positive chemicals 791 and 613. Viability (on left) expressed as viability % (LC%) and activation of TRα (on right) expressed as % Relative Activation

In Fig. 2d, for positive chemicals, the graphs representing the TRα activation in all valid runs are added compared with Reference (RI).

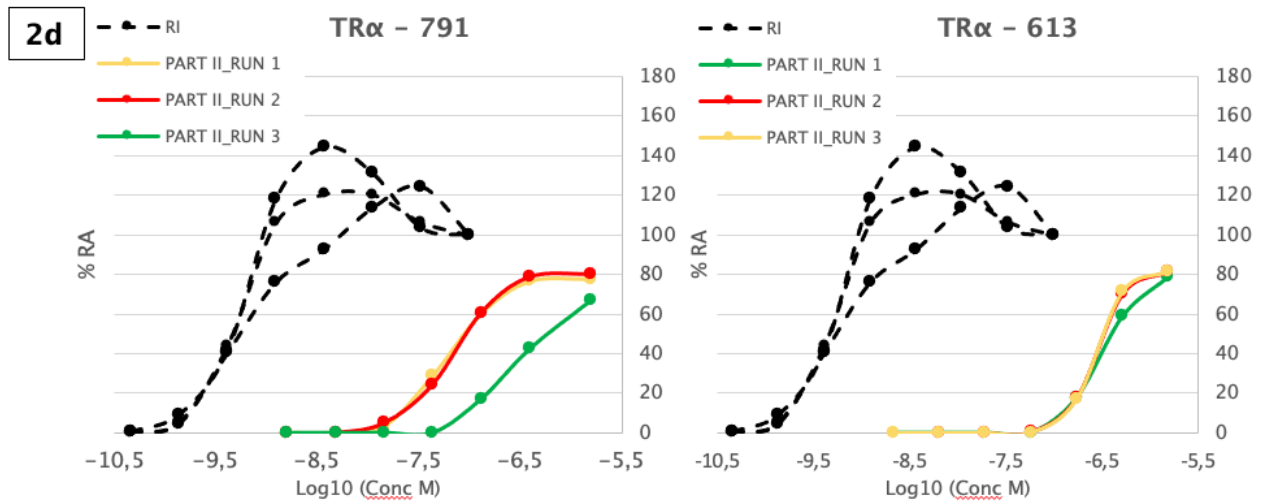


Fig 2d. Results of 3 valid runs on TRα cells for positive chemicals 791 and 613. % Relative Activation (%RA) for positive compound compared with RI (dot line)

In tab. IX. the presence of sigmoidal curve, indicating the activation of receptor TRα, the EC50 and the relative 95% CI are reported for the tested reference or positive chemicals for each valid run. To give an extended dataset, the results obtained in Dose-Range Finding test are inserted (in grey) for the positive chemicals, although the experimental conditions (i.e. number of concentrations and dilution factor) are different from TR assay.

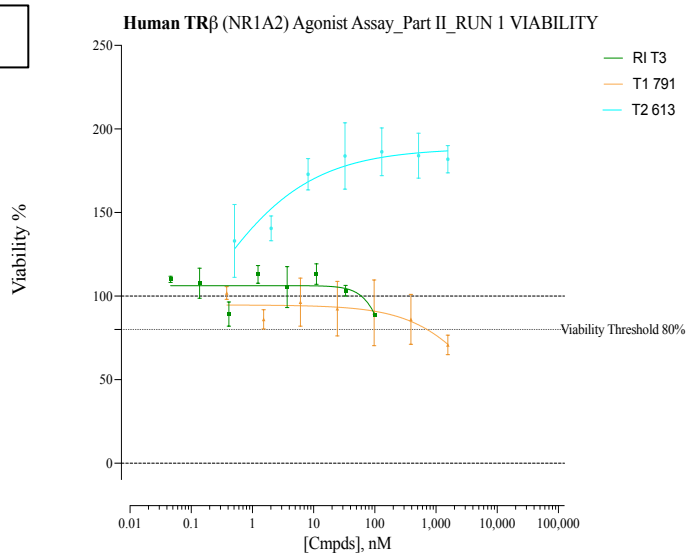
IX. Calculated EC50 for TRα						
	Test Chemicals		RUN	Sigmoidal dose-resp.	Calculated EC50 (nM)	95% CI (nM) (profile likelihood)
TRα	RI	3'3'5' Triiodo-L-thyronine (Reference)	PART II_RUN 1	YES	0.49	??? to 0.6628
			PART II_RUN 2	YES	0.74	0.489 to 1.148
			PART II_RUN 3	YES	0.48	0.361 to 0.662
	T1	**791**	DRF_bis_01_R1	YES (incomplete)	46.12*	11.28 to 153.0*
			PART II_RUN 1	YES	39.43	29.62 to 53.56
			PART II_RUN 2	YES	45.62	36.41 to 57.69
			PART II_RUN 3	YES	310.50	221.9 to 542.7
	T2	**613**	DRF_bis_04_R1	YES	183.2*	Very wide*
			PART II_RUN 1	YES	310.60	203.5 to 595.8
			PART II_RUN 2	YES	241.1	??? to 355.5
			PART II_RUN 3	YES	236.0	??? to 427.6

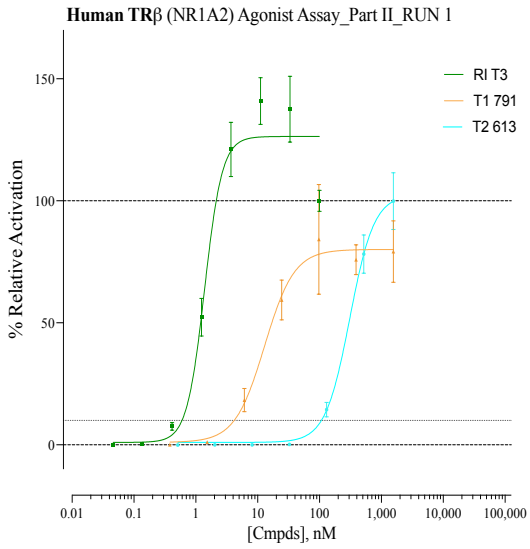
* Dose-Range Findings EC50 and 95% CI were calculated excluding %RA of cytotoxic concentrations

5.3.2 TRβ

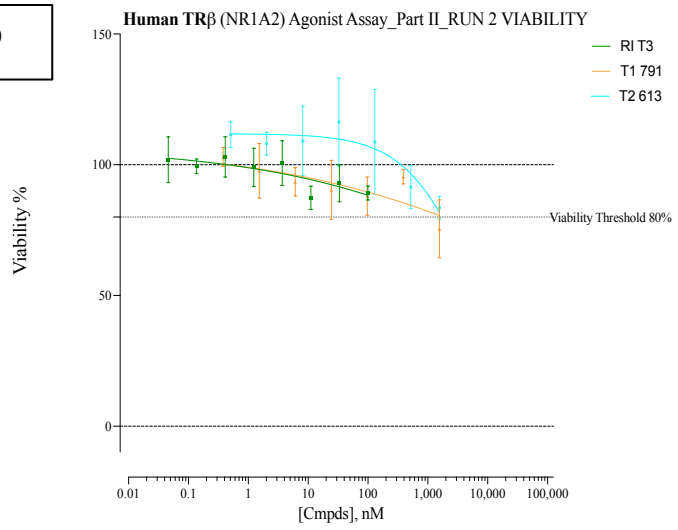
In fig. 3a-3c the results of cytotoxicity LCMA assay (expressed as viability %) and Activation of TRβ (Relative Activation %) plotted against tested concentrations are reported for the test chemicals (reference or test items) for each valid run.

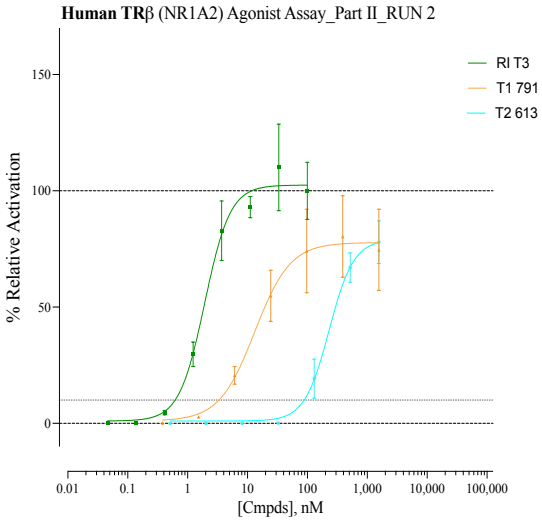
3a





3b





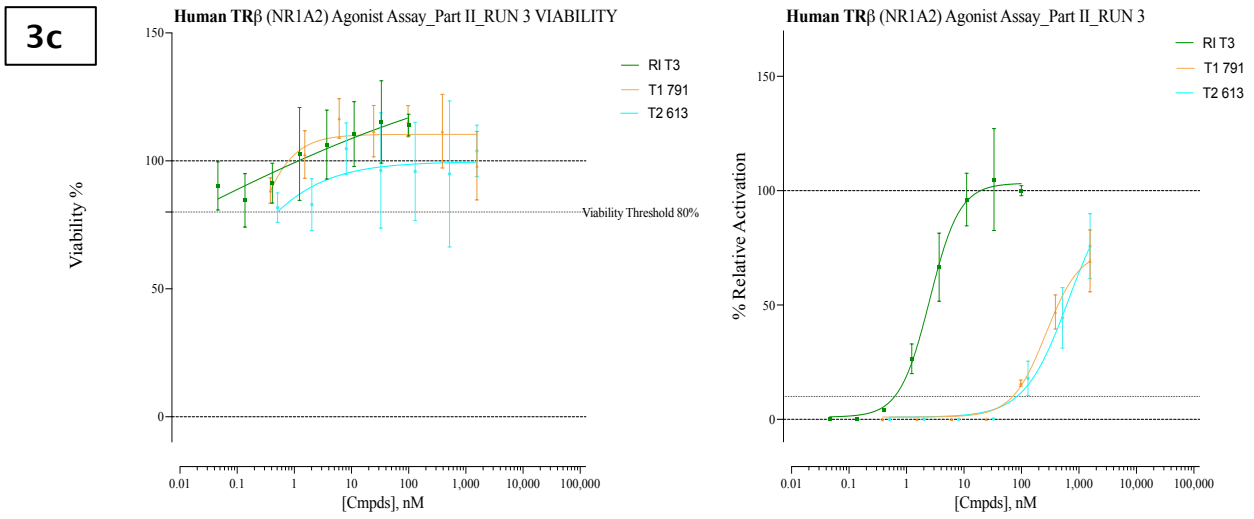


Fig 3a-c. Results of 3 valid runs on TRβ cells. Viability (on left) expressed as viability % (LC%) and activation of TRβ (on right) expressed as % Relative Activation

In Fig. 2d, for positive chemicals, the graphs representing the TRβ activation in all valid runs are added compared with Reference (RI).

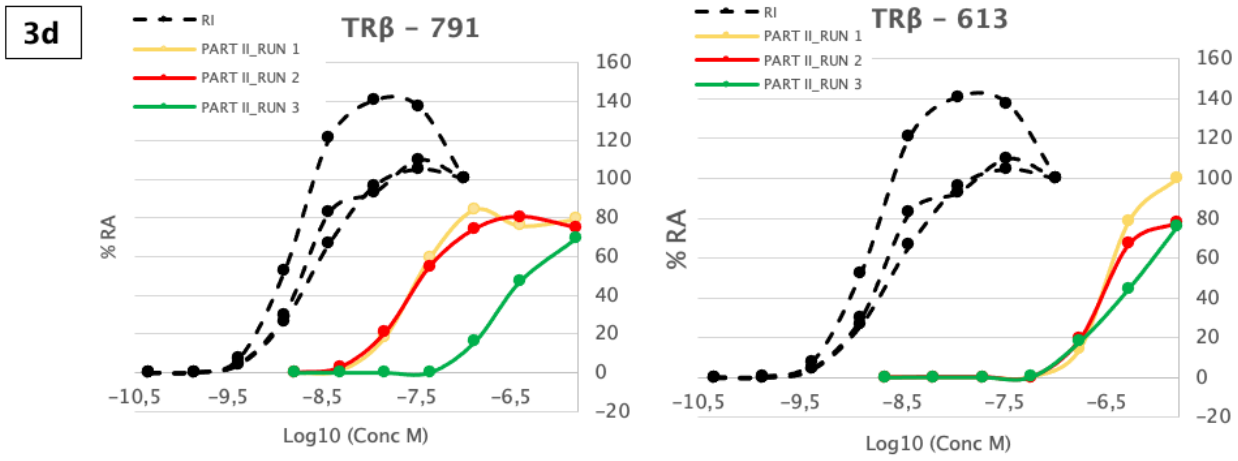


Fig 3d. Results of 3 valid runs on TRβ cells for positive chemicals 791 and 613. % Relative Activation (%RA) for each test item compared with RI (dot line)

In tab. X. the presence of sigmoidal curve, indicating the activation of receptor TRβ, the EC50 and the relative 95% CI are reported for the tested reference or positive chemicals for each valid run. To give an extended dataset, the results obtained in Dose-Range Finding test are inserted (in grey) for the positive chemicals, although the experimental conditions (i.e. number of concentrations and dilution factor) are different from TR assay.

X. Calculated EC50 for TRβ						
	Test Chemicals		RUN	Sigmoidal dose-resp.	Calculated EC50 (nM)	95% CI (nM) (profile likelihood)
TRβ	RI	3'3'5' Triiodo-L-thyronine (Reference)	PART II_RUN 1	YES	1.385	1.143 to 1.733
			PART II_RUN 2	YES	1.93	1.540 to 2.444
			PART II_RUN 3	YES	2.528	1.939 to 3.316
	T1	**791**	DRF_bis_01_R1	YES (incomplete)	20.41*	???*
			PART II_RUN 1	YES	12.94	8.776 to 18.69
			PART II_RUN 2	YES	13.21	8.066 to 21.99
			PART II_RUN 3	YES	268.1	188.1 to 460.6
	T2	**613**	DRF_bis_04_R1	YES	184.4*	Very wide*
			PART II_RUN 1	YES	308.5	258.4 to 363.6
			PART II_RUN 2	YES	230.6	179.5 to 293.9
			PART II_RUN 3	YES	656.9	312.3 to ???

* Dose-Range Findings EC50 ans 95% CI were calculated excluding %RA of cytotoxic concentrations

5.3.4 TR Activity assay for Reference Item (RI): TRα

Concerning the activation of TRα by natural ligand 3'3'5'-Triiodo-L-thyronine as Reference Item, the metrics related to acceptance criteria of all the performed runs are reported in Tab. XI.

				PART II TR ASSAY		
Tab. XI RI Metrics for Trα assay			Run n.	**1**	**2**	**3**
2	FA of REF-EC100 (T3; 0.10 µM)	≥ 300 FA	On P1	1756	1022	1817
			On P2	765	1246	1141
3	RI-EC50	≤ 10 nM (≤ 1.0E-08 M)	-	0.49	0.74	0.48
4	%CV log (EC50) for RI	< 3%	-	-0.80	0,92	0.68
5	PC %RA (Sobetirome at EC100; 1 µM)	≥ 50% RA	-	63.61	77.21	70.74
6	NC %RA (17-b-Estradiol; 1 µM)	< 10% RA	-	0.12	0.03	0.11
7	Z' for REF-EC100 (T3; 0.10µM)	≥ 0.5	On P1	0.87	0.79	0.56
			On P2	0.77	0.73	0.73

In fig. 4 the results of TRα activation in valid runs for RI are reported.

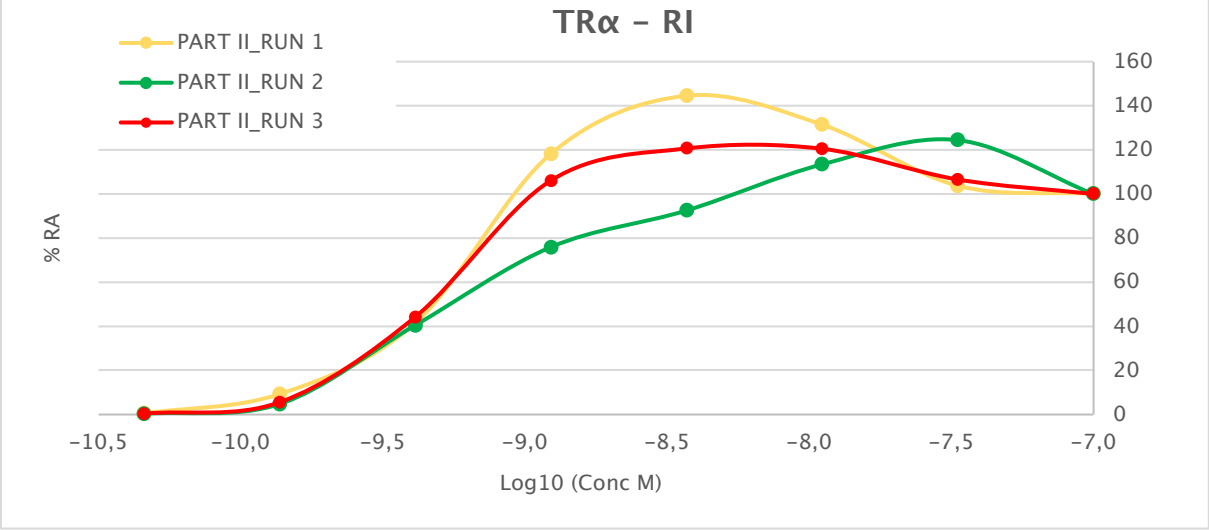


Fig 4. Results of 3 valid run on TRα cells. % Relative Activation (%RA) for RI

5.3.5 TR Activity assay for Reference Item (RI): TRα

Concerning the activation of TRβ by RI, the metrics related to acceptance criteria of all the performed runs are reported in Tab. XII.

				PART II TR ASSAY		
Tab. XII RI Metrics for Trβ assay			Run n.	1	2	3
2	FA of REF-EC100 (T3; 0.10 µM)	≥ 300 FA	On P1	922	1000	4381
			On P2	564	736	2123
3	RI-EC50	≤ 10 nM (≤ 1.0E-08 M)	-	1.39	1.93	2.53
4	%CV log (EC50) for RI	< 3%	-	0.50	0.55	0.64
5	PC %RA (Sobetirome at EC100; 1 µM)	≥ 50% RA	-	61.79	68.30	70.52
6	NC %RA (17-b-Estradiol; 1 µM)	< 10% RA	-	0.08	0.00	0.12
7	Z' for REF-EC100 (T3; 0.10µM)	≥ 0.5	On P1	0.87	0.63	0.93
			On P2	0.74	0.60	0.76

In fig. 5 the results of TRβ activation in valid runs for RI are reported

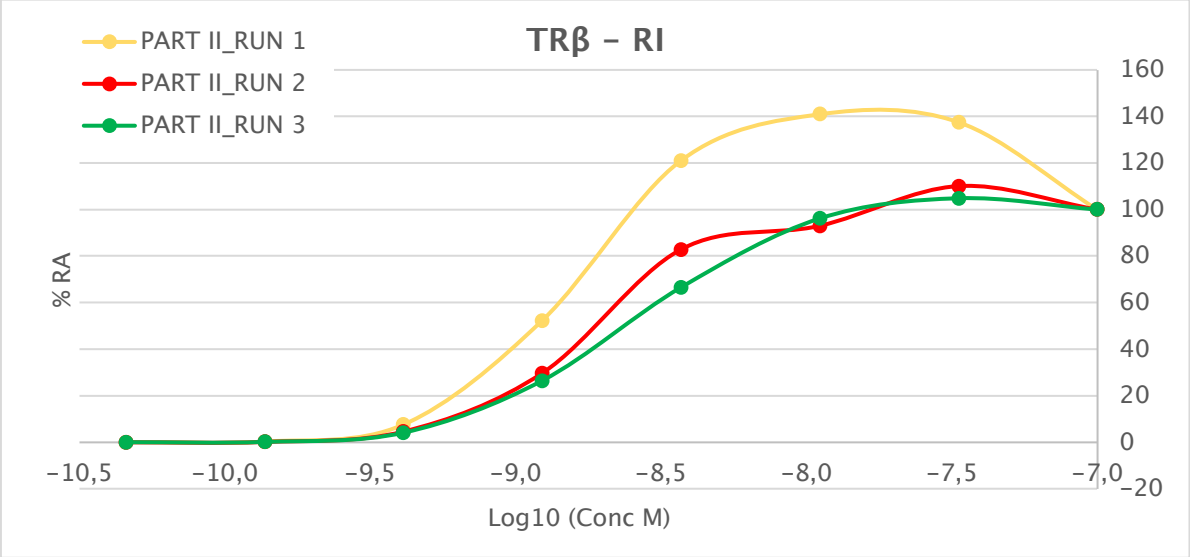


Fig 5. Results of 3 valid run on TRβ cells. % Relative Activation (%RA) for RI

6. CONCLUSIONS

This study was performed for PART 2 of the EURL ECVAM coordinated Thyroid Validation Study. The proposed method is based on engineered cells expressing high levels of Thyroid Hormone Receptor alpha (NR1A1) and beta (NR1A2) and used to assess potential agonists in the activation of Thyroid Nuclear receptors, considered as potential endocrine disruptors, after 24h exposure.

In this study the predictivity of the method was evaluated using the following:

- 3,3',5-triiodo-L-Tyronine (T3) as Reference
- Sobetirome as Positive Control
- 17β-Estradiol (E2) as Negative Control
- Staurosporine as Positive Control for Viability
- DMSO 0.2% as Solvent Control

to test 30 coded chemicals, assessing their capability to activate TR receptor as agonist ligands.

The results can be summarised as follow:

- Of the 30 coded chemicals tested for solubility, 29 were determined as soluble in suitable solvent (DMSO) and the starting concentration for subsequent Dose-Range Finding was determined for each one. Only 1 chemical (code: 306) resulted completely not soluble at all the experimental conditions and was excluded from testing.

- Starting from maximum soluble concentration, each soluble chemical was tested in Dose-Range Finding (1 valid run) to individuate the cytotoxicity threshold and the preliminary positive response for TRα and TRβ activation, to determine the starting concertation for subsequent TR assay. Of the 29 soluble chemicals, only 2 (code: 791 and 613) were able to activate TRs (both TRα and TRβ) resulting as potential agonist. For each positive chemical, the highest not cytotoxic concentration with the highest effect on TR activation was selected as starting concertation for further analysis. The remaining 27 chemicals were considered as negative and no further TR assay was performed.

- The 2 positive chemicals (code: 791 and 613) were tested in TR assay (3 valid runs) to conduct a more finely tuned assessment of positive activity metrics and to verify cytotoxicity. Both chemicals, 791 and 613, resulted as potential agonist for both TRα and TRβ in all the 3 performed runs. The calculated EC50 resulted quite reproducible in 2 of 3 runs (run 1 and 2). In order to increase the dataset, the EC50 from Dose Range finding was added and, although the experimental conditions are quite different in term of number of tested concentrations and dilution factor, the obtained value is comparable to those derived for TR assay.

- In case of chemical 791, for both TRα and TRβ, in 1 of 3 runs a shifting of dose response curve and a corresponding different EC50 were obtained. Considering that the same test item solution and deriving serial dilutions were tested on both TRα and TRβ cells, it is plausible that the shifting was due to a pipetting error during the preparation of the highest testing concentration in the specific run (RUN 3).

7. REFERENCES

- Zhang JH. Chung TD. Oldenburg KR. (1999) A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. J Biomol Screen: 4(2). 67-73.
- Human Thyroid Hormone Receptor TRα (NR1A1) TRβ (NR1A2) - Technical Manual
- Technical Manual LCMA kit (TM_LCMA)

8. ARCHIVING

The study material will be archived as follow:

Study material	
Raw data and documents	*Maintained in Archive, at disposal for evaluation, until authorization for elimination will be released by the Sponsor*
Test Item and Controls	*Disposal after 30 days from the end of the experimental phase.*

APPENDIX

APPENDIX I:
- list of supplied excel and GraphPad PRISM files for evaluation

APPENDIX II:

- Tested concentrations of test chemicals in Dose-range finding run.
- Tested concentrations of test chemicals in TR assay runs
- Plate layout

APPENDIX III:

- Sponsor information

APPENDIX I

In Tab. XIII the list of supplied data in excel and Prism files is reported.

XIII. LIST OF SUPPILED DATA FILES				
RUN	FILE	Type	Valid data	
			Trα	TRβ
Dose-Range Finding (bis)				
PART II_DRF_RUN 1 Experiment 1	RIC 04-19_6a_PART II_DRF_BIS_01_R1_T1-T3	excel	yes	yes
	RIC 04-19_6a_PART II_DRF_BIS_01_R1_T4-T6	excel	yes	yes
	RIC 04-19_PART_II_DRF_BIS_01_R1_TRA_agonist_T1-T6_M	PRISM	yes	yes
	RIC 04-19_PART_II_DRF_BIS_01_R1_TRA_agonist_T1-T6_nM	PRISM	yes	yes
	RIC 04-19_PART_II_DRF_BIS_01_R1_TRA_agonist_T1-T6_nM CITOTOX	PRISM	yes	yes
	RIC 04-19_PART_II_DRF_BIS_01_R1_TRB_agonist_T1-T6_M	PRISM	yes	yes
	RIC 04-19_PART_II_DRF_BIS_01_R1_TRB_agonist_T1-T6_nM	PRISM	yes	yes
	RIC 04-19_PART_II_DRF_BIS_01_R1_TRB_agonist_T1-T6_nM CITOTOX	PRISM	yes	yes
PART II_DRF_RUN 1 Experiment 2	RIC 04-19_6a_PART II_DRF_BIS_02_R1_T1-T3	excel	yes	yes
	RIC 04-19_6a_PART II_DRF_BIS_02_R1_T4-T6	excel	yes	yes
	RIC 04-19_PART_II_DRF_BIS_02_R1_TRA_agonist_T1-T6_M	PRISM	yes	yes
	RIC 04-19_PART_II_DRF_BIS_02_R1_TRA_agonist_T1-T6_nM	PRISM	yes	yes
	RIC 04-19_PART_II_DRF_BIS_02_R1_TRA_agonist_T1-T6_nM CITOTOX	PRISM	yes	yes
	RIC 04-19_PART_II_DRF_BIS_02_R1_TRB_agonist_T1-T6_M	PRISM	yes	yes
	RIC 04-19_PART_II_DRF_BIS_02_R1_TRB_agonist_T1-T6_nM	PRISM	yes	yes
	RIC 04-19_PART_II_DRF_BIS_02_R1_TRB_agonist_T1-T6_nM CITOTOX	PRISM	yes	yes
PART II_DRF_RUN 1 Experiment 3	RIC 04-19_6a_PART II_DRF_BIS_03_R1_T1-T3	excel	yes	yes
	RIC 04-19_6a_PART II_DRF_BIS_03_R1_T4-T6	excel	yes	yes
	RIC 04-19_PART_II_DRF_BIS_03_R1_TRA_agonist_T1-T6_M	PRISM	yes	yes
	RIC 04-19_PART_II_DRF_BIS_03_R1_TRA_agonist_T1-T6_nM	PRISM	yes	yes
	RIC 04-19_PART_II_DRF_BIS_03_R1_TRA_agonist_T1-T6_nM CITOTOX	PRISM	yes	yes
	RIC 04-19_PART_II_DRF_BIS_03_R1_TRB_agonist_T1-T6_M	PRISM	yes	yes
	RIC 04-19_PART_II_DRF_BIS_03_R1_TRB_agonist_T1-T6_nM	PRISM	yes	yes
	RIC 04-19_PART_II_DRF_BIS_03_R1_TRB_agonist_T1-T6_nM CITOTOX	PRISM	yes	yes
PART II_DRF_RUN 1 Experiment 4	RIC 04-19_6a_PART II_DRF_BIS_04_R1_T1-T3	excel	yes	yes
	RIC 04-19_6a_PART II_DRF_BIS_04_R1_T4-T6	excel	yes	yes
	RIC 04-19_PART_II_DRF_BIS_04_R1_TRA_agonist_T1-T6_M	PRISM	yes	yes
	RIC 04-19_PART_II_DRF_BIS_04_R1_TRA_agonist_T1-T6_nM	PRISM	yes	yes
	RIC 04-19_PART_II_DRF_BIS_04_R1_TRA_agonist_T1-T6_nM CITOTOX	PRISM	yes	yes
	RIC 04-19_PART_II_DRF_BIS_04_R1_TRB_agonist_T1-T6_M	PRISM	yes	yes
	RIC 04-19_PART_II_DRF_BIS_04_R1_TRB_agonist_T1-T6_nM	PRISM	yes	yes
	RIC 04-19_PART_II_DRF_BIS_04_R1_TRB_agonist_T1-T6_nM CITOTOX	PRISM	yes	yes

PART II_DRF_RUN 1 Experiment 5	RIC 04-19_6a_PART II_DRF_BIS_05_R1_T1-T3	excel	yes	yes
	RIC 04-19_6a_PART II_DRF_BIS_05_R1_T4-T6	excel	yes	yes
	RIC 04-19_PART_II_DRF_BIS_05_R1_TRA_agonist_T1-T6_M	PRISM	yes	yes
	RIC 04-19_PART_II_DRF_BIS_05_R1_TRA_agonist_T1-T6_nM	PRISM	yes	yes
	RIC 04-19_PART_II_DRF_BIS_05_R1_TRA_agonist_T1-T6_nM CITOTOX	PRISM	yes	yes
	RIC 04-19_PART_II_DRF_BIS_05_R1_TRB_agonist_T1-T6_M	PRISM	yes	yes
	RIC 04-19_PART_II_DRF_BIS_05_R1_TRB_agonist_T1-T6_nM	PRISM	yes	yes
	RIC 04-19_PART_II_DRF_BIS_05_R1_TRB_agonist_T1-T6_nM CITOTOX	PRISM	yes	yes
TR ASSAYS				
PART II_RUN 1	RIC 04-19_PART_II_TRassay_RUN 1	excel	yes	yes
	RIC 04-19_PART_II_TRassay_RUN 1_TRa	PRISM	yes	yes
	RIC 04-19_PART_II_TRassay_RUN 1_TRa_nM	PRISM	yes	yes
	RIC 04-19_PART_II_TRassay_RUN 1_TRa_nM CITOTOX	PRISM	yes	yes
	RIC 04-19_PART_II_TRassay_RUN 1_TRb	PRISM	yes	yes
	RIC 04-19_PART_II_TRassay_RUN 1_TRb_nM	PRISM	yes	yes
	RIC 04-19_PART_II_TRassay_RUN 1_TRb_nM CITOTOX	PRISM	yes	yes
PART II_RUN 2	RIC 04-19_PART_II_TRassay_RUN 2	excel	yes	yes
	RIC 04-19_PART_II_TRassay_RUN 2_TRa	excel	yes	yes
	RIC 04-19_PART_II_TRassay_RUN 2_TRa_nM	PRISM	yes	yes
	RIC 04-19_PART_II_TRassay_RUN 2_TRa_nM CITOTOX	PRISM	yes	yes
	RIC 04-19_PART_II_TRassay_RUN 2_TRb	PRISM	yes	yes
	RIC 04-19_PART_II_TRassay_RUN 2_TRb_nM	PRISM	yes	yes
	RIC 04-19_PART_II_TRassay_RUN 2_TRb_nM CITOTOX	PRISM	yes	yes
PART II_RUN 3	RIC 04-19_PART_II_TRassay_RUN 3	excel	yes	yes
	RIC 04-19_PART_II_TRassay_RUN 3_TRa	excel	yes	yes
	RIC 04-19_PART_II_TRassay_RUN 3_TRa_nM	PRISM	yes	yes
	RIC 04-19_PART_II_TRassay_RUN 3_TRa_nM CITOTOX	PRISM	yes	yes
	RIC 04-19_PART_II_TRassay_RUN 3_TRb	PRISM	yes	yes
	RIC 04-19_PART_II_TRassay_RUN 3_TRb_nM	PRISM	yes	yes
	RIC 04-19_PART_II_TRassay_RUN 3_TRb_nM CITOTOX	PRISM	yes	yes

APPENDIX II

In tab. XIV the tested concentrations of all soluble chemicals in dose-range finding run are reported

XIV. TRα and TRβ Dose-range Findings: tested concentrations			
Test chemicals	Stock 500X (mM)	Concentration	Final 1X (nM) Dilution 1:8
722, 457, 908, 791, 084, 480, 521, 304, 489, 184, 584, 139, 676, 082, 183, 814, 558, 797, 535, 613, 269, 832, 351, 637, 100	50.0	C1	100000.0
		C2	12500.0
		C3	1562.5
		C4	195.3
		C5	24.4
262, 527, 717	25.0	C1	50000.00
		C2	6250.00
		C3	781.25
		C4	97.66
		C5	12.21
739	12.5	C1	25000.00
		C2	3125.00
		C3	390.63
		C4	48.83
		C5	6.10

In tab. XV the tested concentrations of positive chemicals in TR assay are reported

	XV. TRα and TRβ assay: tested concentrations		
	Test chemicals	**Concentration**	**Final 1X (nM)**
TRα TRβ	**T1: 791**	**Dilution factor: 1/4**	
		C1	1562.5
		C2	390.6
		C3	97.7
		C4	24.4
		C5	6.1
		C6	1.5
		C7	0.4
	T2: 613	**Dilution factor: 1/3**	
		C1	1562.5
		C2	520.8
		C3	173.6
		C4	57.9
		C5	19.3
		C6	6.4
		C7	2.1

Plate layouts for Dose Range Finding (DRF)

The layouts are valid for both TRα and TRβ

Plate 1

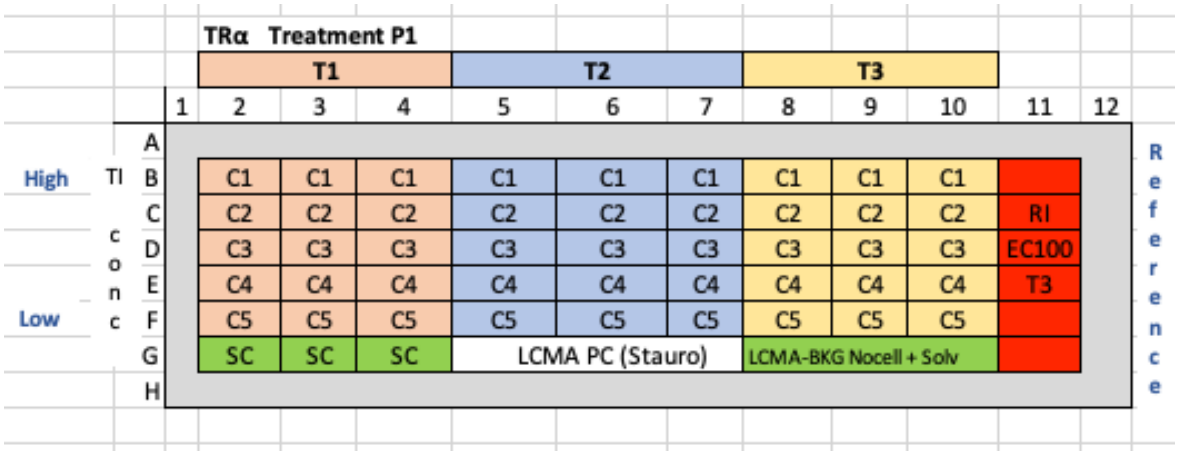
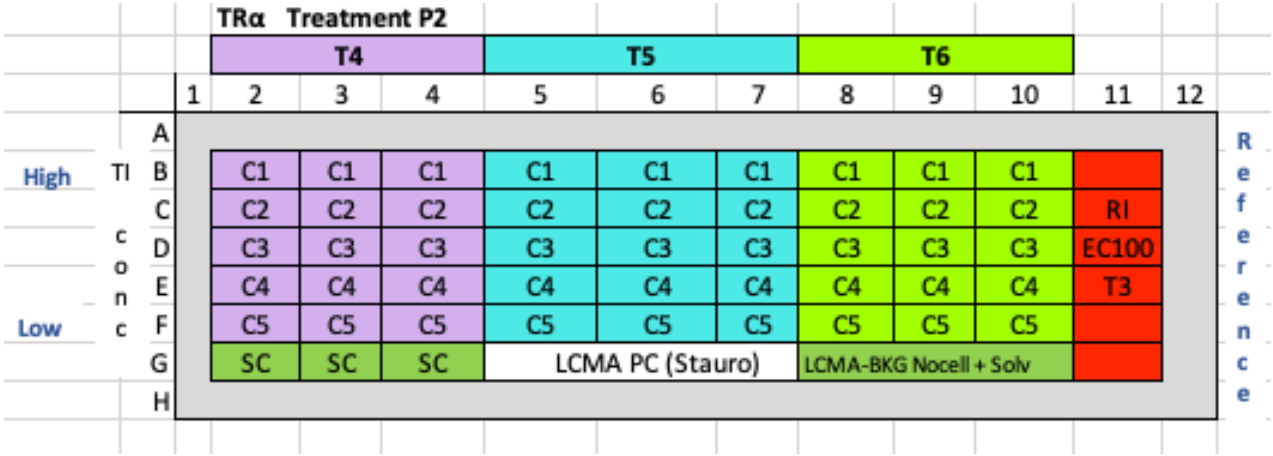


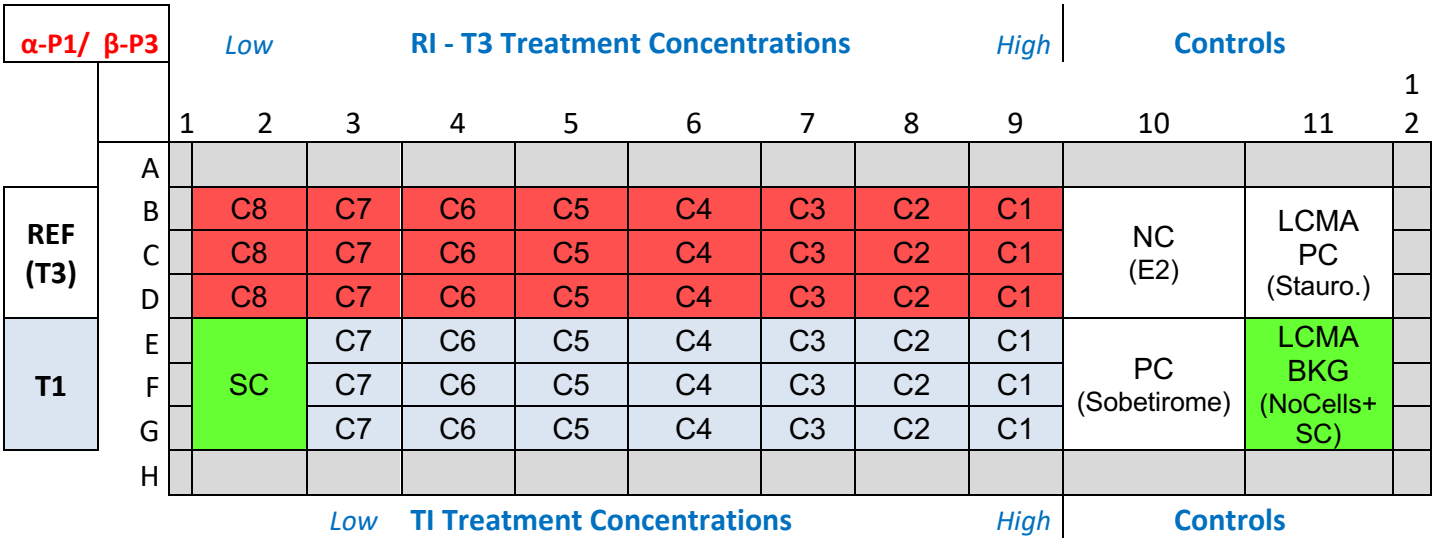
Plate 2

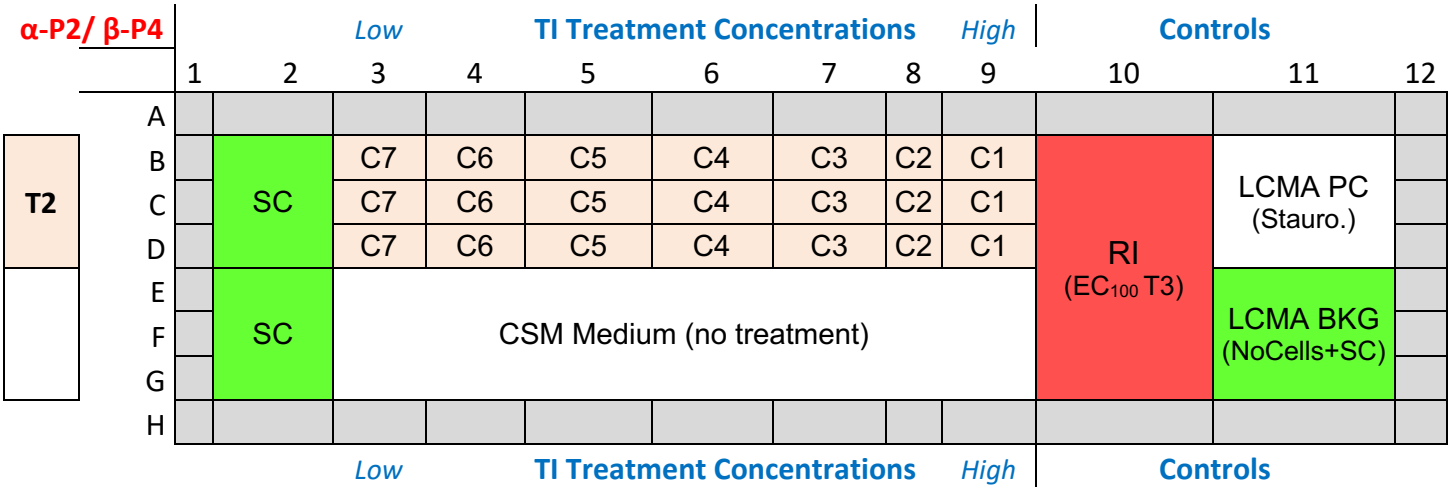


T= Test Item; C=concentration (C1: highest; C5: lowest); SC=Solvent Control;
LCMA PC= Citotoxicity Control (Staurosporine); LCMA-BKG: signal background, no cell;
RI EC100T3= T3 0.10 µM (EC100)

Plate layouts for TR Activity Assay

The layouts are valid for both TRα and TRβ





T= Test Item; C=concentration (C1: highest; C5: lowest); SC=Solvent Control; LCMA PC= Citotoxicity Control (Staurosporine); LCMA-BKG: signal background, no cell; RI EC100T3= T3 0.10 µM (EC100); REF: 8 concentration of T3; NC: Negative Control 17β-estradiol; PC: Positive Control

APPENDIX III

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