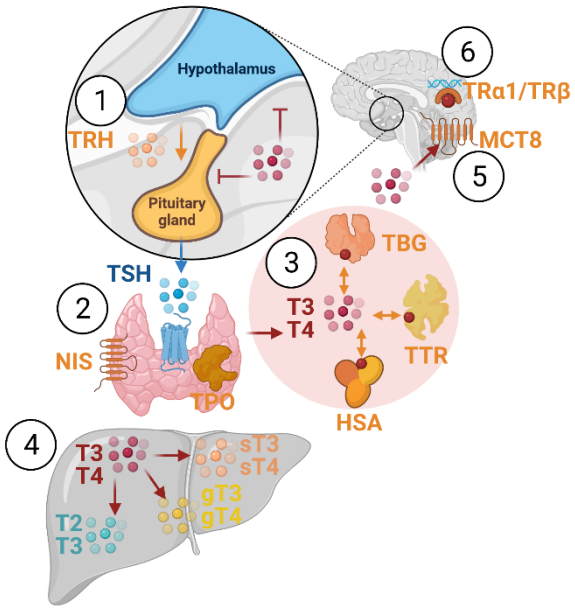
European Commission

STUDY REPORT

for the assessment of the human thyroid hormone receptor alpha (TRα) and beta (TRβ) reporter genes transactivation assay measuring agonist activity – Part 1

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 1 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 1

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TIMING OF THE STUDY

TEST ITEMS ARRIVAL	14.09.2020 and 27.10.2021
START OF EXPERIMENTAL PHASE: Part I	19.04.2021
END OF EXPERIMENTAL PHASE: Part I	21.05.2021
START OF EXPERIMENTAL PHASE: Part I bis	18.01.2022
END OF EXPERIMENTAL PHASE: Part I bis	03.02.2022
RAW DATA ANALYSIS: QA CONTROL	16.02.2022
STUDY REPORT	11.03.2022

1. INTRODUCTION AND AIM OF THE STUDY

This study was performed for PART 1 of the EURL ECVAM coordinated Thyroid Validation Study for Method 6a.
The proposed method 6a was performed using a kit developed by INDIGO Biosciences Inc. based on engineered human cells which express high levels of Thyroid Hormone Receptor alpha TRα (NR1A1) and beta TRβ (NR1A2).These cells were used in a 24h protocol exposure to assess the potential agonist activity on Thyroid Nuclear Receptors of 3 known chemicals versus a reference.

In this study the robustness and reliability of the method were assessed by performing 5 valid runs (of 7) testing the following test items:

- Sobetirome, known TR agonist
- 17β-Estradiol (E2) known not active compound
- 3',3',5'-triiodo-L-Tyronine (T3), natural TR ligand

Compared with the reference

- 3',3',5'-triiodo-L-Tyronine (T3), natural ligand supplied in the kit as reference

And the following controls:

- Sobetirome as Positive Control
- 17β-Estradiol (E2) as Negative Control
- Staurosporine as Positive Control for Cytotoxicity
- DMSO 0.2% as Solvent Control

2. STUDY DESIGN
2.1 Study Scheduling

The PART I study presented two experimental steps:

- **Solubility test and Dose-range Finding**.
  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 assay had the aim, starting from 1:500 highest soluble concentration and preparing a serial dilution of concentrations, to establish the respective cytotoxicity thresholds of TIs and preliminar TR activation for subsequent TR activity assessment.
  For each TI, The highest not cytotoxic concentration that showed no activity or activity against one, or both, of the TRs was advanced to the TR Activity Assessment for further analysis.
  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.**	**Run Name**	**Validity**	**Date**	**Cell batch**	**Test item**	**Test Item arrival**
Trα	Part I	1	**Dose Range Finding_RUN 1**	VALID	19.04.2021	220925	**T1**: Sobetirome CAS 211110-63-3. **T2**: 17β-Estradiol CAS 50-28-2 **T3**: 3'3'5'-Triiodo-L-Tyronine Sodium Salt CAS 55-06-1	14.09.2020
TRβ	Part I	1	**Dose Range Finding_RUN 1**	VALID	19.04.2021	220807		

- **TR Activity Assessment**: The specific aim of TR activity assessment was to conduct a more finely tuned assessment of a "positive" TI activity metrics, to confirm the absence of TR activation of "negative" TI 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: 5

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 RUNS								
	Experimental Session	**RUN n.**	**Run Name**	**Validity**	**Date**	**Cell batch**	**Test item**	**Test Item arrival**
Trα	**Part I**	1	**Part I_ RUN 1**	VALID	27.04.2021	220925	**T1**: Sobetirome CAS 211110-63-3 **T2**: 17β-Estradiol CAS 50-28-2 **T3**: 3'3'5'-Triiodo-L-Tyronine Sodium Salt CAS 55-06-1	14.09.2020
		2	**Part I_ RUN 2**	VALID	18.05. 2021			
		3	**Part I_ RUN 3**	NOT VALID	20.05.2021			
		4	**Part I_ RUN 4**	NOT VALID	20.05.2021			
	Part I Bis (perfromed after supplementary training)	5	**Part I bis_ RUN 1**	VALID	18.01.2022	230723	**T1** and **T2** same as Part I **T3**: 3'3'5'-Triiodo-L-Tyronine CAS 6893-02-3	Same as Part I 27.10.2021
		6	**Part I bis_ RUN 2**	VALID	24.01.2022			
		7	**Part I bis_ RUN 3**	VALID	31.01.2022			
TRβ	**Part I**	1	**Part I_ RUN 1**	NOT VALID	27.04.2021	220807	**T1**: Sobetirome CAS 211110-63-3 **T2**: 17β-Estradiol CAS 50-28-2 **T3**: 3'3'5'-Triiodo-L-Tyronine Sodium Salt CAS 55-06-1	14.09.2020
		2	**Part I_ RUN 2**	NOT VALID	18.05.2021			
	Part I Bis (perfromed after supplementary training)	3	**Part I bis_ RUN 1**	VALID	18.01.2022	230514	**T1** and **T2** same as Part I **T3:** 3'3'5'-Triiodo-L-Tyronine CAS 6893-02-3	Same as Part I 27.10.2021
		4	**Part I bis_ RUN 2**	VALID	24.01.2022			
		5	**Part I bis_ RUN 3**	VALID	31.01.2022			
		6	**Part I bis_ RUN 4**	VALID	03.02-2022			
		7	**Part I bis_ RUN 5**	VALID	03.02.2022			

As reported in Tab. II, during the execution of TR activity assays of Part I, 2 not valid runs for Trα and 2 not valid runs for TRβ occurred mostly due to too high technical variability. After troubleshooting and supplementary training, the experimental phase restarted and relative runs were recorded as Part I bis. Compared with PART I session, in PART I bis new batches of reagent an a different form of 3'3'5'-Triiodo-L-Tyronine as test item (CAS 6893-02-3 versus CAS 55-06-1 conjugated salt) were used.

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:

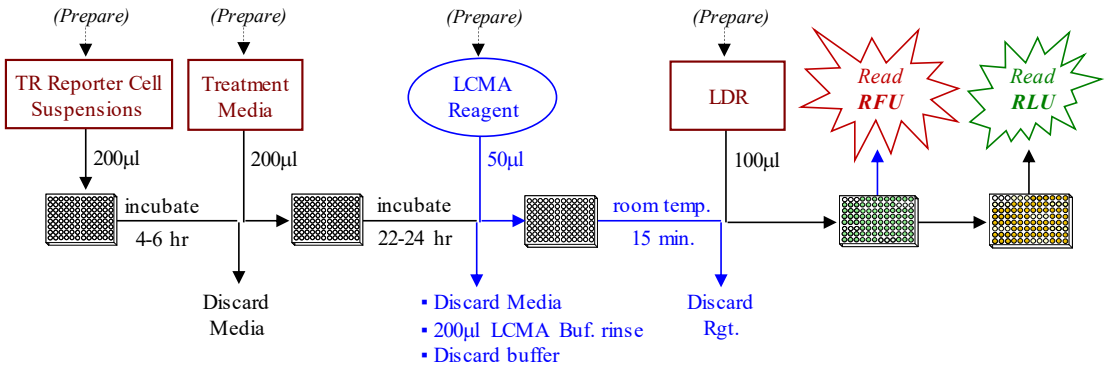


Figure 1. Overview of the workflow for dose range finding and TR assays. Text and arrows in blue font denote the LCM Assay for Cytotoxicity which is performed in multiplex with LDR Assay for TR activation assessment on the same plates.

In brief:

A preliminary solubility test by Molarity Method in elective solvent DMSO was performed to assess the maximum concentration at which each test items were still soluble to establish the starting point for subsequent dose-range finding.

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 substitute 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	T1	Sobetirome	5 concentrations; 1:8 dilution factor; from 1:500 maximum solubility
		T2	17β-Estradiol	
		T3	3',3',5'-triiodo-L-Tyronine	

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%
	Test Items	T1	Sobetirome	7 concentrations; 1:3 or 1:4 dilution factor; from the highest not cytotoxic concentration with highest activatin of TR
		T2	17β-Estradiol	
		T3	3',3',5'-triiodo-L-Tyronine	

On Day 2. At the end of exposure period, media with treatments were discarded and citotoxicity 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

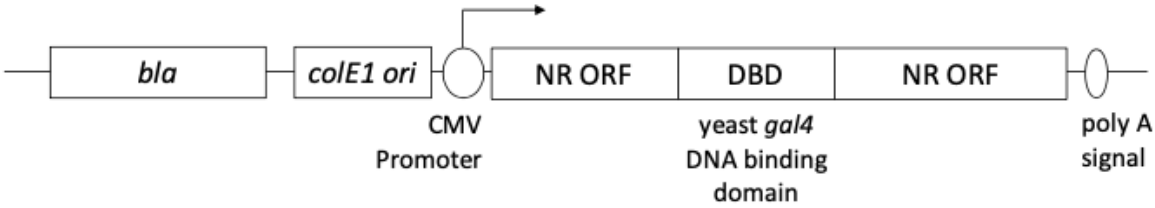
The assays for the assessment of the activation of Thyroid Hormone Receptor alpha (NR1A1) and beta (NR1A2) utilize Human Embryonic Kidney cells engineered to provide constitutive, high-level expression of the corresponding Thyroid Hormone Receptor. These reporter cells express hybrid thyroid hormone 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).

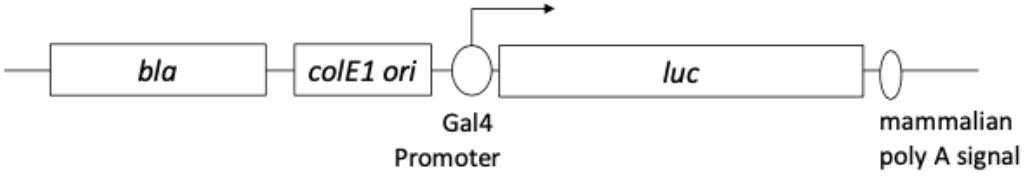
INDIGO Biosciences' Nuclear Receptor (NR) Reporter cells are constructed using a combination of proprietary vectors that express:

- 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, thus it's not possible to transfer an aliquot of the used batches for supplementary analysis and QC.

TEST SYSTEM		
PART I		
NAME	**TRα**	**TRβ**
SUPPLIER	Indigo Biosciences	Indigo Biosciences
CAT. NUMBER	IB01001_EUC	IB01101_EUC
DATE OF ARRIVAL	08/04/2021	08/04/2021
BATCH N°	220925	220807
QUANTITY	200 µl/well cell suspension	200 µl/well cell suspension
EXPIRATION DATE	31/10/2021	31/10/2021
PART I bis		
NAME	**TRα**	**TRβ**
SUPPLIER	Indigo Biosciences	Indigo Biosciences
CAT. NUMBER	IB01001_EUC	IB01101_EUC
DATE OF ARRIVAL	18/11/2022	18/11/2022
BATCH N°	230723	230514
QUANTITY	200 µl/well cell suspension	200 µl/well cell suspension
EXPIRATION DATE	31/05/2022	31/05/2022

3.1. 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 in culture plate. For treatments CSM (Compound Screening Medium) is used to dilute compounds Stocks (typically 500x concentrate) in order to achieve final testing concentration.

CULTURE MEDIA		
PART I		
NAME	CRM Medium	CSM Medium
SUPPLIER	Indigo Biosciences	Indigo Biosciences
CAT. NUMBER	IB01001_EUC	IB01101_EUC
DATE OF ARRIVAL	08/04/2021	08/04/2021
BATCH N.	230401	230318-19
EXPIRATION DATE	31/10/2021	31/10/2021
STORAGE	-20°C	-20°C
PART I bis		
NAME	CRM Medium	CSM Medium
SUPPLIER	Indigo Biosciences	Indigo Biosciences
CAT. NUMBER	IB01001_EUC	IB01101_EUC
DATE OF ARRIVAL	18/11/2022	18/11/2022
BATCH N.	231028	231028-29J
EXPIRATION DATE	31/05/2022	31/05/2022
STORAGE	-20°C	-20°C

3.2 TEST ITEMS: IDENTIFICATION AND CHARACTERIZATION

NAME	**Sobetirome**	**17β-Estradiol (E2)**
UNIVOCAL CODE	**T1**	**T2**
CAS NUMBER	211110-63-3	50-28-2
SUPPLIER	Sigma Aldrich Merck	Sigma Aldrich Merck
CAT. NUMBER	SML1900	E8875
BATCH	0000090784	SLCC8875
PHYSICAL FORM	Solid, white	Solid, white
MW	328.40	272.38
SOLVENT	DMSO	DMSO
TREATMENT DOSE / CONCENTRATION	200 µl/well Produced as 500x stocks (Ref. Appendix II for tested concentrations)	200 µl/well Produced as 500x stocks (Ref. Appendix II for tested concentrations)
EXPIRATION	n.a.	31/07/2022
COA	yes	yes
MSDS	yes	yes
STORAGE	-20°C	RT

NAME	3,3',5-triiodo-L-Tyronine Sodium Salt (T3)	3,3',5-triiodo-L-Tyronine (T3)
UNIVOCAL CODE	T3 (for PART I)	T3 (for PART I bis)
CAS NUMBER	55-06-1	6893-02-3
SUPPLIER	Sigma Aldrich Merck	ERM
CAT. NUMBER	T6397	ERM-AC469
BATCH	BCCB5600	sample n.1160
PHYSICAL FORM	Solid, white	Solid, white
MW	672.96	650.97
SOLVENT	DMSO	DMSO
TREATMENT DOSE / CONCENTRATION	200 µl/well Produced as 500x stocks (Ref. Appendix II for tested concentrations)	200 µl/well Produced as 500x stocks (Ref. Appendix II for tested concentrations)
EXPIRATION	28/04/2023	28/04/2023
COA	yes	yes
MSDS	yes	yes
STORAGE	-20°C	-20°C

3.3 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	CITOTOXICITY 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	276855-100ml	D5879 (part I) 276855-100ml (part I bis)
BATCH	211015 (Part I) 230527 (Part I bis)	STBK2718	SHBL1941 (Part I) STBK2718 (Part I bis)
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	31/10/2021 (Part I) 05/2022 (Part I bis)	Closed: 10/2024 Opened: 29/02/2022	21/01/2022 (part I) 29/02/2022 (part I bis)
CERTIFICATE OF ANALYSIS	n.a.	n.a.	n.a.
SAFETY INFORMATION	n.a	n.a	n.a
STORAGE	-80°C	-80°C	-80°C

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	230402 (Part I) 231101 (Part I bis)	230402 (Part I) 231101 (Part I bis)
PHYSICAL FORM	Liquid	Liquid
SOLVENT	DMSO	DMSO
DOSE CONCENTRATION	1 µM	1 µM
EXPIRATION	31/10/2021 (Part I) 05/2022 (Part I bis)	31/10/2021 (Part I) 05/2022 (Part I bis)
CERTIFICATE OF ANALYSIS	n.a.	n.a.
SAFETY INFORMATION	n.a	n.a
STORAGE	-80°C	-80°C

3.3 REFERENCES

NAME	3,3',5-triiodo-L-Tyronine, Sodum 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	230402 (Part I) 231101 (Part I bis)	230402 (Part I) 231101 (Part I bis)
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	31/10/2021 (Part I) 05/2022 (Part I bis)	31/10/2021 (Part I) 05/2022 (Part I bis)
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

4. METHODS

The method is described in SOP and briefly reported in the study plan.

Three deviations are reported.

Execution of TR assay: testing concentrations.
In SOP and study plan, ref. par 6.3.2 Experimental Protocol: TR Activity Assessment, DAY 1 – Cell seeding and Treatment, point n. 3, the indicated dilution factor for the preparation of test item testing concentrations is 1:3.
In case of TRα, considering the known activity range of 3,3',5-triiodo-L-Tyronine and sobetirome, as verified in PART I bis_RUN 1 experiment, the application of dilution factor 1:3 resulted in uncomplete dose-response sigmoidal curve, lacking of absence of signal (i.e. RA% >10% = no activity). In order to obtain a full response data set, the dilution factor was modified in 1:4 for all the test items on TRα.

Data elaboration are described in the SOP.
In 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 and should be reported in the SOP.

Acceptance Criteria are described in the SOP.
Regarding the criteria for run validation, different FA were adopted on the suggestion of method developer Indigo Biosciences Inc. as "at least to TRα MAC FA ≥ 300, and TRβ MAC to FA ≥ 500" instead of "at least to TRα MAC FA ≥ 600, and TRβ MAC to FA ≥ 1000" to include possible inter laboratory variability. This modification has to be evaluated by ECVAM on the basis of obtained results and eventually reported in SOP review.

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-ECVAL for evaluation and statistical analysis (ref. Appendix I for file list).

5.1. Dose-range finding

The performed solubility test assessed 50 mM in DMSO as the highest concentration at which all the test items resulted soluble and this concentration was considered the 500x concentrated stock as starting concentration for dose-range finding.

The results of Dose-range finding run are reported in Tab. V and VI for TRα and TRβ. The relative activation % (RA%) of each receptor, the viability % (%LC) and the selected concentration for subsequent TR activity assay are reported. The criterion for selection was the highest not cytotoxic concentration with maximum activation.

V. Dose-range finding Results for TRα

	nM	% LC	%RA	
T1	100000.00	70.8	13.33	
Sobetirome	**12500.00**	**92.7**	**62.17**	**Selected**
	1562.50	92.5	60.47	
	195.31	103.5	49.94	
	24.41	98.3	7.85	
T2	100000.00	30.8	0.00	
17β-Estradiol	**12500.00**	**88.1**	**0.17**	**Selected**
	1562.50	96.6	0.02	
	195.31	104.8	0.05	
	24.41	95.5	0.00	
T3	100000.00	73.1	27.90	
3'3'5'- Triiodo-L-thyronine	12500.00	82.1	47.45	
	1562.50	82.2	51.48	
	195.31	**92.2**	**75.47**	**Selected**
	24.41	86.0	66.47	

In bold the selected concentrations for TRα activity assay.

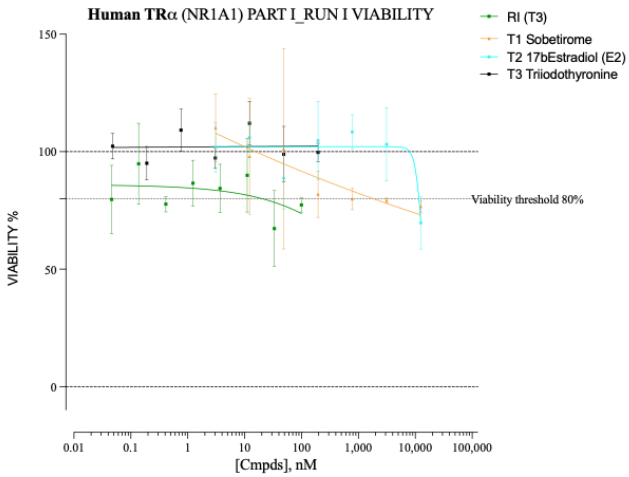
VI. Dose-range finding Results for TRβ				
	nM	**% LC**	**% RA**	
T1	100000,00	83,2	14,80	
Sobetirome	12500,00	102,7	35,90	
	1562,50	**90,8**	**46,28**	**Selected**
	195,31	98,3	37,31	
	24,41	88,9	14,82	
T2	100000,00	29,6	0,03	
17β-Estradiol	**12500,00**	**92,9**	**0,07**	**Selected**
	1562,50	114,0	0,04	
	195,31	111,0	0,10	
	24,41	110,6	0,02	
T3	100000,00	86,0	30,39	
3'3'5'- Triiodo-L-thyronine	12500,00	90,3	38,61	
T3	1562,50	95,7	49,73	
	195,31	**81,5**	**44,37**	**Selected (2nd)**
	24,4	**98,5**	**52,10**	**Selected (1st) ***

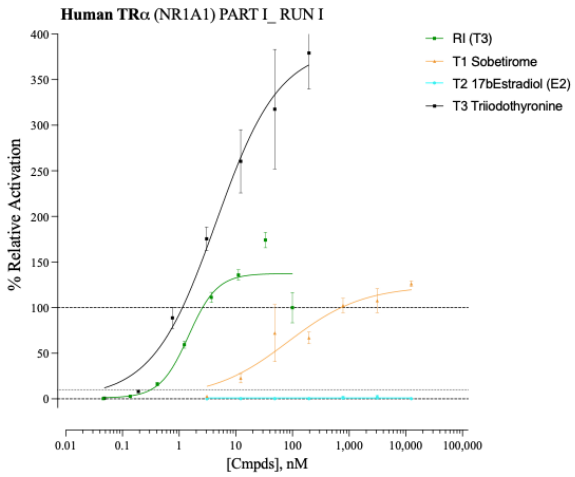
In bold the selected concentrations for TRβ activity assay. *Tested only in PART I_ RUN 1 then substituted with the second choice.

5.2. TR Activity assay: TRα

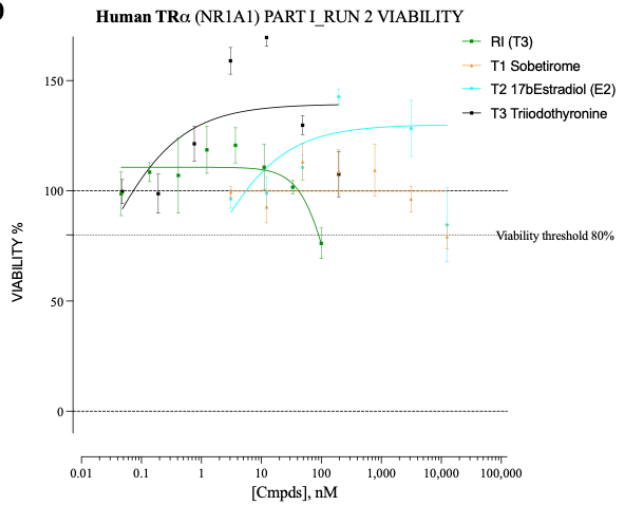
In fig. 2a-2e the results of cytotoxicity LCMA assay (expressed as viability %) and Activation of TRα (Relative Activation %) plotted against tested concentrations are reported for the test compounds (reference or test items) for each valid run.

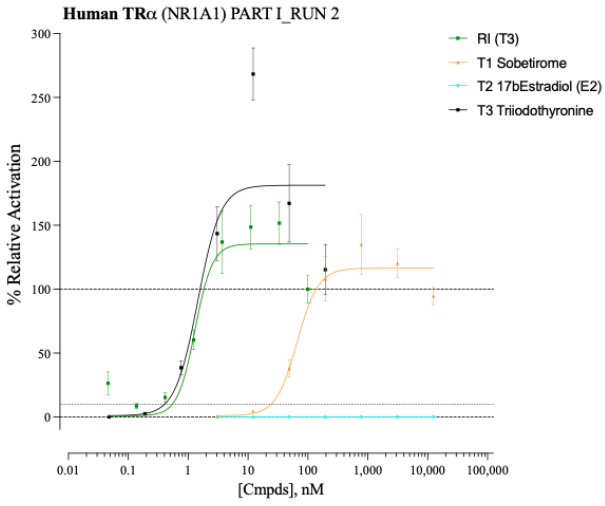
2a



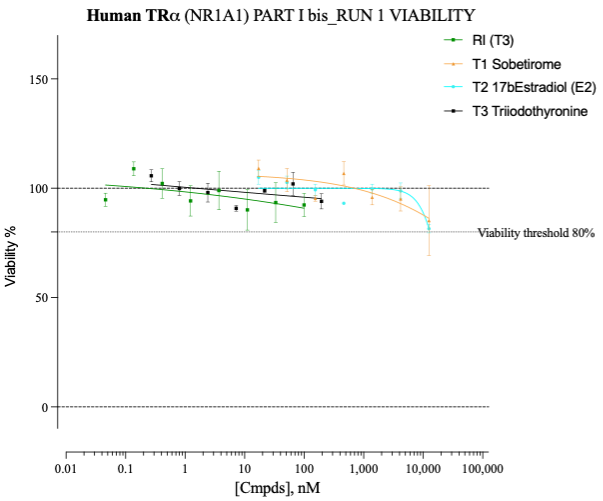


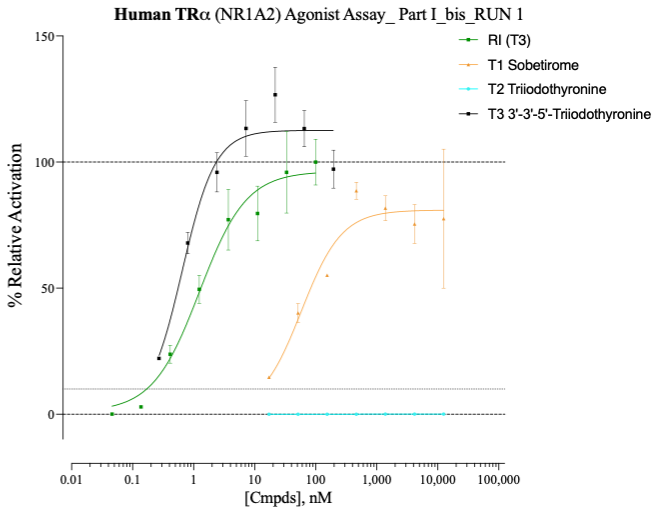
2b



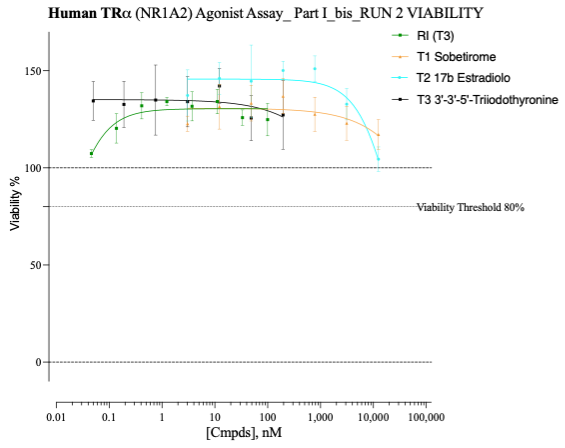


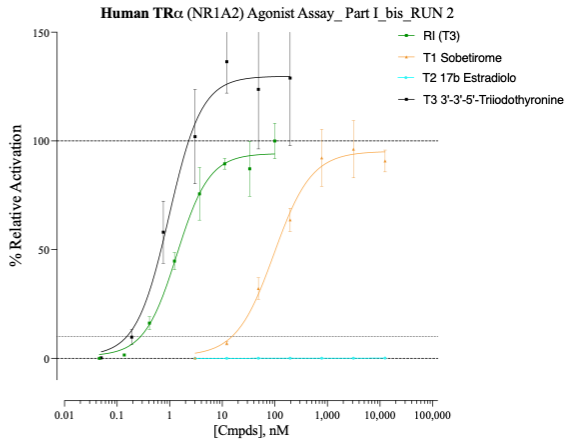
2c



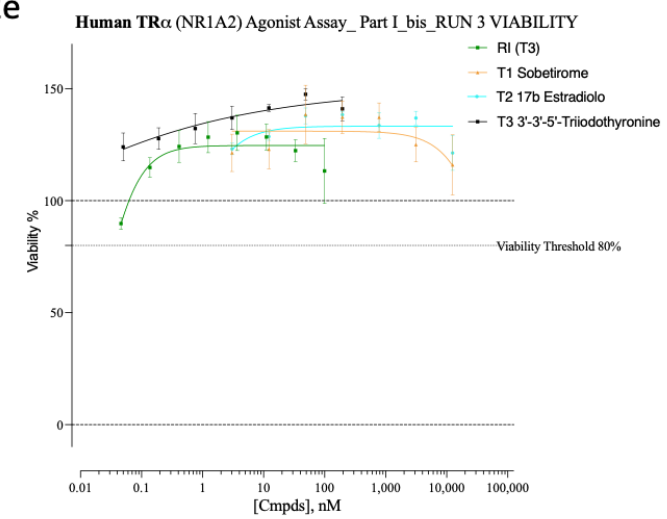


2d





2e



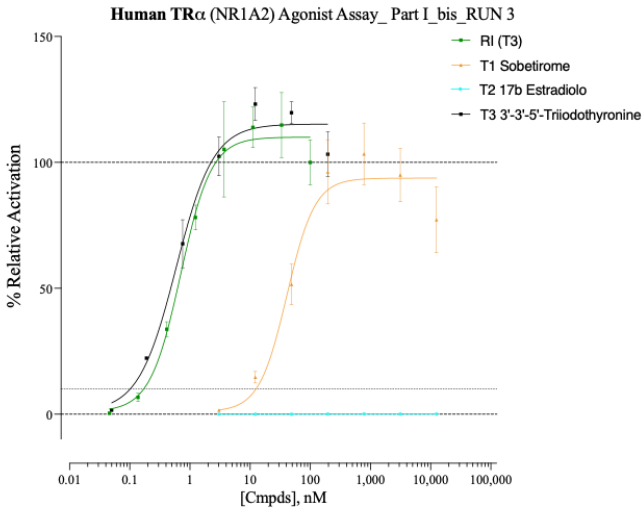


Fig 2a-e. Results of 5 valid run on TRα cells. Viability (on left) expressed as viability % (LC%) and activation of TRα (on right) expressed as % Relative Activation

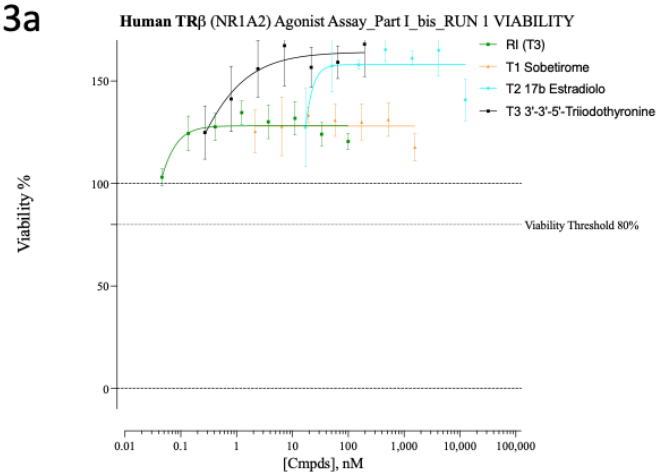
In tab. VII, the sigmoidal curve indicating the activation of receptor TRα, the EC50 and the relative 95% CI are reported for the test compounds (reference or test items) for each valid run.

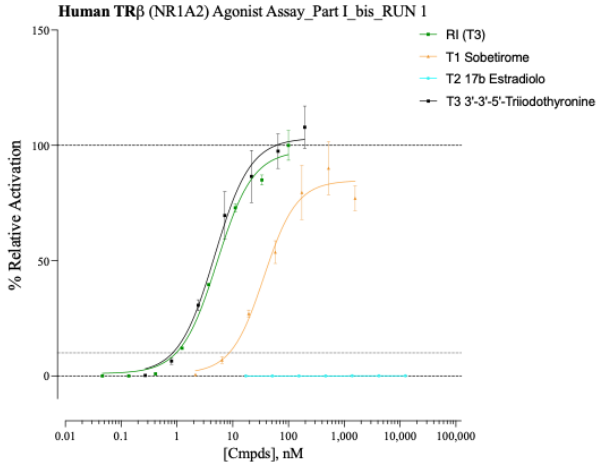
VII. Calculated EC50 for TRα						
	Test Compound		**RUN**	**Sigmoidal dose-resp.**	**Calculated EC50 (nM)**	**95% CI (nM) (profile likelihood)**
TRα	RI	3'3'5' Triiodo-L-thyronine (Reference)	PART I_RUN 1	YES	1.47	0.9677 - 2.248
			PART I_RUN 2	YES	1.31	0.8988 - 1.781
			PART I bis_RUN 1	YES	1.25	0.8633 - 1.970
			PART I bis_RUN 2	YES	1.37	1.078 - 1.769
			PART I bis_RUN 3	YES	0.70	0.5564 - 0.8867
	T1	Sobetirome	PART I_RUN 1	YES	83.02	30.50 - 684.9
			PART I_RUN 2	YES	66.27	49.27 - 97.48
			PART I bis_RUN 1	YES (not complete)	58.49	35.88 - 94.01
			PART I bis_RUN 2	YES	96.68	70.92 - 132.4
			PART I bis_RUN 3	YES	41.04	27.23 - 57.69
	T2	17β-Estradiol	PART I_RUN 1	NO	//	(Very wide)
			PART I_RUN 2	NO	2.47 E-81	(Very wide)
			PART I bis_RUN 1	NO	1.01E+09	//
			PART I bis_RUN 2	NO	~ 4.77e-127	(Very wide)
			PART I bis_RUN 3	NO	~ 4.482e-139	(Very wide)
	T3	3'3'5' Triiodo-L-thyronine	PART I_RUN 1	YES	4.47	2.465 - 11.30
			PART I_RUN 2	YES	1.46	0.7172 - ???
			PART I bis_RUN 1	YES (not complete)	0.65	0.4904 - 0.8620
			PART I bis_RUN 2	YES	0.97	0.61 - 1.62
			PART I bis_RUN 3	YES	0.59	0.45 - 0.76

5.2. TR Activity assay: TRβ

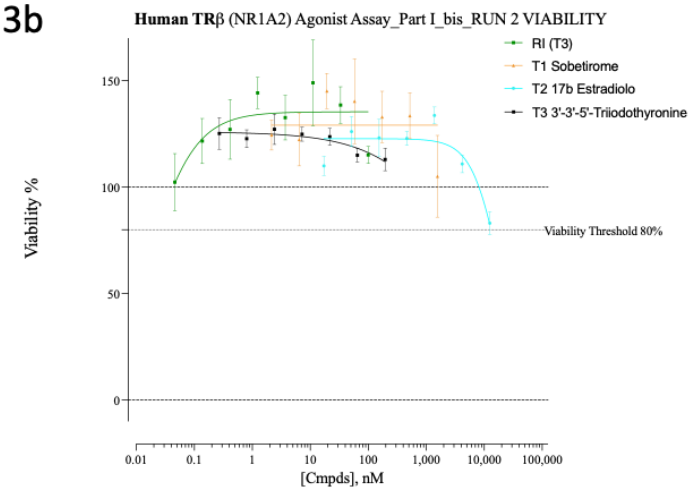
In fig. 3a-3e the results of cytotoxicity LCMA assay (expressed as viability %) and Activation of TRβ (Relative Activation %) plotted against tested concentrations are reported for the test compounds (reference or test items) for each valid run

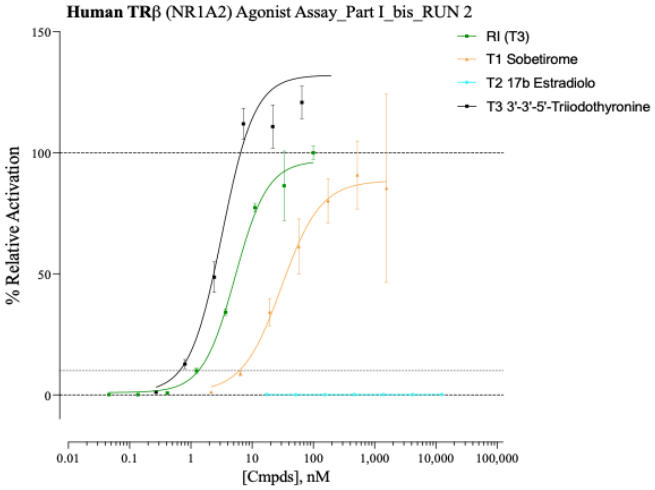
3a



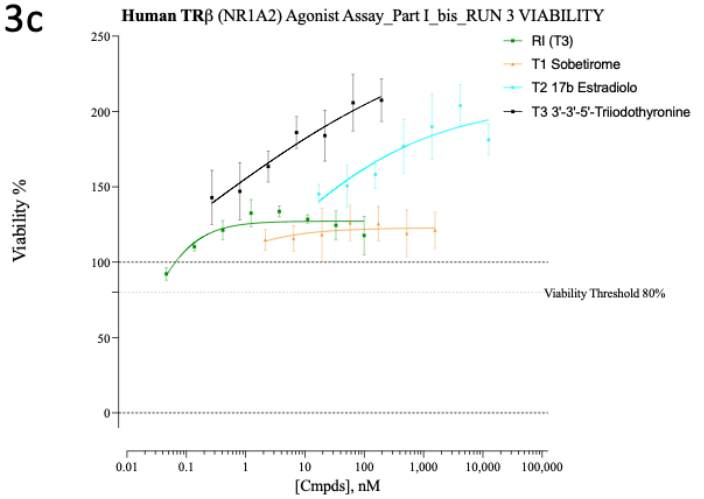


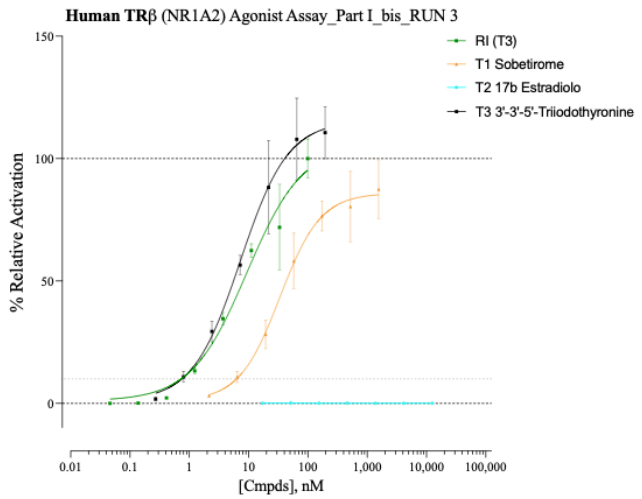
3b



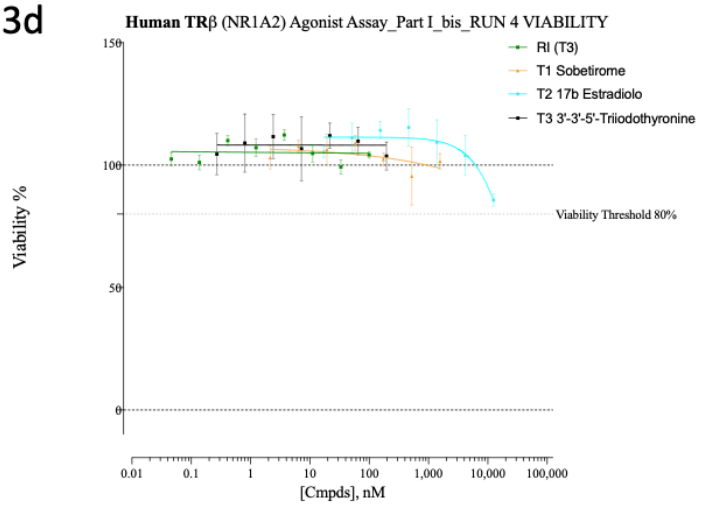


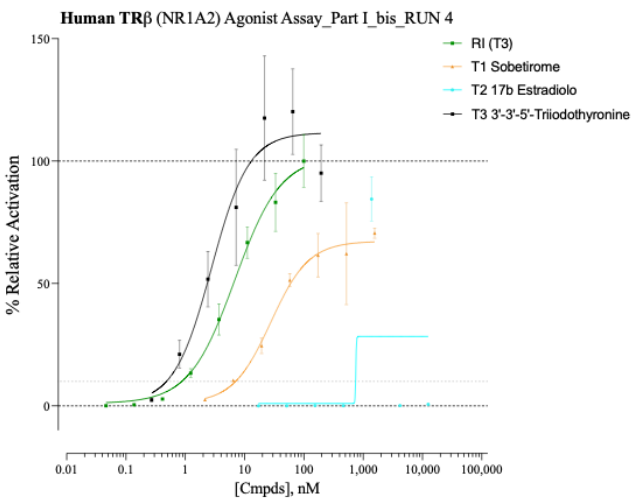
3c



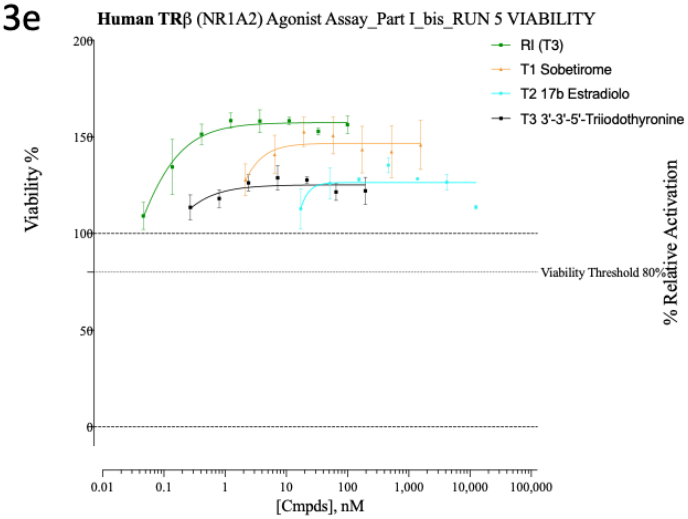


3d





3e



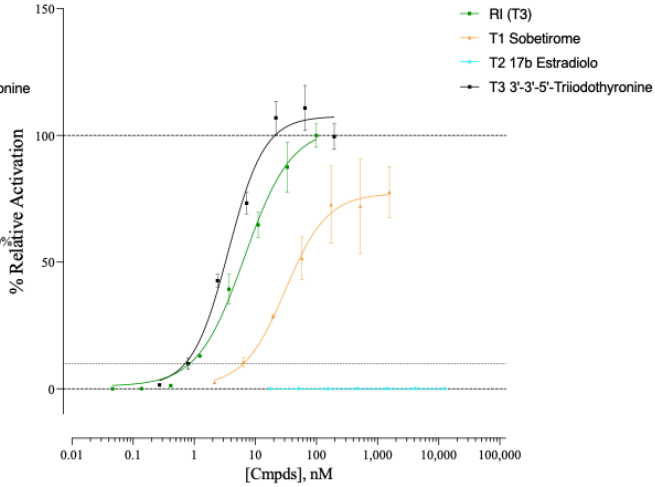


Fig 3a-e. Results of 5 valid run on TRβ cells. Viability (on left) expressed as viability % (LC%) and activation of TRβ (on right) expressed as % Relative Activation

In tab. VIII, the sigmoidal curve indicating the activation of receptor TRβ, the EC50 and the relative 95% CI are reported for the test compounds (reference or test items) for each valid run.

VIII. Calculated EC50 for TRβ						
	Test Compound		**RUN**	**Sigmoidal dose-resp.**	**Calculated EC50 (nM)**	**95% CI nM (profile likelihood)**
TRβ	RI	3'3'5' Triiodo-L-thyronine (Reference)	PART I bis_RUN 1	YES	5.25	4.51 - 6.21
			PART I bis_RUN 2	YES	5.29	4.43 to 6.45
			PART I bis_RUN 3	YES	9.38	5.34 - 27.29
			PART I bis_RUN 4	YES	6.88	5.07 - 10.42
			PART I bis_RUN 5	YES	6.66	5.25 - 8.87
	T1	Sobetirome	PART I bis_RUN 1	YES	36.17	27.23 - 48.21
			PART I bis_RUN 2	YES	30.30	17.15 to 60.68
			PART I bis_RUN 3	YES	33.84	24.25 - 50.03
			PART I bis_RUN 4	YES	27.66	18.60 - 44.76
			PART I bis_RUN 5	YES	31.14	20.20 - 51.59
	T2	17β-Estradiol	PART I bis_RUN 1	NO	1.08E+08	242034 - ???
			PART I bis_RUN 2	NO	457143	//
			PART I bis_RUN 3	NO	4.02E-10	//
			PART I bis_RUN 4	NO	~ 752.4	(Very wide)
			PART I bis_RUN 5	NO	9.05E-45	(Very wide)
	T3	3'3'5' Triiodo-L-thyronine	PART I bis_RUN 1	YES	4.74	3.68 - 6.46
			PART I bis_RUN 2	YES	3.25	2.36 - 5.09
			PART I bis_RUN 3	YES	7.27	5.03 - 11.59
			PART I bis_RUN 4	YES	2.77	1.67 - 4.60
			PART I bis_RUN 5	YES	3.62	2.94 - 4.46

6. CONCLUSIONS

This study was performed for PART 1 of the EURL ECVAM coordinated Thyroid Validation Study for Method 6a.
The proposed method 6a was performed using a kit developed by INDIGO Biosciences Inc. based on engineered human cells which express high levels of Thyroid Hormone Receptor alpha TRα (NR1A1) and beta TRβ (NR1A2).These cells were used in a 24h protocol exposure to assess the potential agonist activity on Thyroid Nuclear Recepotos of 3 known chemicals versus a reference.

In this study the robustness and reliability of the method were assessed by performing 5 valid runs (of 7) testing Viability and TR activation capability of:

- Sobetirome, known TR agonist
- 17β-Estradiol (E2) known Not active compound
- 3',3',5'-triiodo-L-Tyronine (T3), natural TR ligand

Compared with:

- 3',3',5'-triiodo-L-Tyronine (T3), natural ligand supplied in the kit as reference

On the basis of the obtained results:

- Sobertirome, as expected, activated both TRα and TRβ resulting as agonist compound
- 3',3',5'-triiodo-L-Tyronine, as expected, activated both TRα and TRβ resulting as agonist compound. Data will be compared to 3',3',5'-triiodo-L-Tyronine tested as Reference to evaluate the robustness of the method.
- 17β-Estradiol (E2) as expected, did not induced any cytotoxicity at the tested concentration but did not activate TRα or TRβ, resulting as not active compound on TR.

The complete dataset will be evaluated by EURL-ECVAM to assess reproducibility of the method and SOP consistency before PART II (screening of 30 coded compounds).

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 raw data and documents produced during the study are archived for a period of one year in VitroScreen's Archive. At the end of this period, VitroScreen will dispose all the material, if not previously agreed with the Sponsor.

Study material	
Raw data and documents	*Archiving period 1year*
Specimens (paraffins)	
Test Item and Controls Specimens (if different from paraffins)	*Disposal after 30 days from the end of the study, according to VitroScreen's SOPs*

APPENDIX

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

APPENDIX II:

- Tested concentrations of test items in Dose-range finding run.
- Tested concentrations of test items in TR assay runs

APPENDIX III:

- Sponsor information

APPENDIX I

In Tab. IX the list of supplied data in excel and Prism files for both valid and not valid runs are reported.

IX. LIST OF SUPPILED DATA FILES

RUN	FILE	Type	Valid data	
			Trα	TRβ
PART I_RUN 1	RIC 04-19_6a_part I_TR ASSAY_RUN 1_TRa_TRbnv	excel	yes	no
	RIC 04-19_TR_Alpha_ agonist_RUN 1_M	PRISM	yes	-
	RIC 04-19_TR_Alpha_ agonist_RUN 1_nM	PRISM	yes	-
	RIC 04-19_TR_Beta_ agonist_RUN 1_nM_nv	PRISM	-	no
PART I_RUN 2	RIC 04-19_6a_part I_TR ASSAY_RUN 2_TRa_TRbnv	excel	yes	no
	RIC 04-19_TR_Alpha_ agonist_RUN 2_M	PRISM	yes	-
	RIC 04-19_TR_Alpha_ agonist_RUN 2_nM	PRISM	yes	-
	RIC 04-19_TR_Beta_ agonist_RUN 2_nM_nv	PRISM	-	no
PART I_RUN 3	RIC 04-19_6a_part I_TR ASSAY_RUN 3_TRanv	excel	no	-
	RIC 04-19_TR_Alpha_ agonist_RUN 3_M_nv	PRISM	no	-
	RIC 04-19_TR_Alpha_ agonist_RUN 3_nM_nv	PRISM	no	-
PART I_RUN 4	RIC 04-19_6a_part I_TR ASSAY_RUN 4_TRanv	excel	no	-
	RIC 04-19_TR_Alpha_ agonist_RUN 4_nMnv	PRISM	no	-
PART I bis_RUN 1	RIC 04-19_PART_I_bis_TRassay_RUN 1_TRa_TRb	excel	yes	yes
	RIC 04-19_PART_I_bis_TRassay_RUN 1_TRa_M	PRISM	yes	-
	RIC 04-19_PART_I_bis_TRassay_RUN 1_TRa_nM	PRISM	yes	-
	RIC 04-19_PART_I_bis_TRassay_RUN 1_TRb_M	PRISM	-	yes
	RIC 04-19_PART_I_bis_TRassay_RUN 1_TRb_nM	PRISM	-	yes
PART I bis_RUN 2	RIC 04-19_PART_I_bis_TRassay_RUN 2_TRa_TRb	excel	yes	yes
	RIC 04-19_PART_I_bis_TRassay_RUN 2_TRa_M	PRISM	yes	-
	RIC 04-19_PART_I_bis_TRassay_RUN 2_TRa_nM	PRISM	yes	-
	RIC 04-19_PART_I_bis_TRassay_RUN 2_TRb_M	PRISM	-	yes
	RIC 04-19_PART_I_bis_TRassay_RUN 2_TRb_nM	PRISM	-	yes
PART I bis_RUN 3	RIC 04-19_PART_I_bis_TRassay_RUN 3_TRa_TRb	excel	yes	yes
	RIC 04-19_PART_I_bis_TRassay_RUN 3_TRa_M	PRISM	yes	-
	RIC 04-19_PART_I_bis_TRassay_RUN 3_TRa_nM	PRISM	yes	-
	RIC 04-19_PART_I_bis_TRassay_RUN 3_TRb_M	PRISM	-	yes
	RIC 04-19_PART_I_bis_TRassay_RUN 3_TRb_nM	PRISM	-	yes
PART I bis_RUN 4	RIC 04-19_PART_I_bis_TRassay_RUN 4_TRb	excel	-	yes
	RIC 04-19_PART_I_bis_TRassay_RUN 4_TRb_M	PRISM	-	yes
	RIC 04-19_PART_I_bis_TRassay_RUN 4_TRb_nM	PRISM	-	yes
PART I bis_RUN 5	RIC 04-19_PART_I_bis_TRassay_RUN 5_TRb	excel	-	yes
	RIC 04-19_PART_I_bis_TRassay_RUN 5_TRb_M	PRISM	-	yes
	RIC 04-19_PART_I_bis_TRassay_RUN 5_TRb_nM	PRISM	-	yes

APPENDIX II

In tab. X the tested concentrations of test items in dose-range finding run are reported

X. Dose-range Findings: tested concentrations			
Test Item	Stock 500X (mM)	Concentration	Final 1X (nM) Dilution 1:8
T1: Sobetorime T2: 17β-Estradiol T3: 3',3',5'-triiodo-L-Tyronine	50,0	C1	100000.0
		C2	12500.0
		C3	1562.5
		C4	195.3
		C5	24.4

In tab. XI and XII the tested concentrations of test items in TR assay runs are reported

	XI. TRα assay: tested concentration			
	Test Item	Concentration	Final 1X (nM) Dilution 1:4	Final 1X (nM) Dilution 1:3*
TRα	T1: Sobetorime	C1	12500.0	12500.0
		C2	3125.0	4166.7
		C3	781.3	1388.9
		C4	195.3	463.0
		C5	48.83	154.32
		C6	12.21	51.44
		C7	3.052	17.147
	T2: 17β-Estradiol	C1	12500.0	12500.0
		C2	3125.0	4166.7
		C3	781.3	1388.9
		C4	195.3	463.0
		C5	48.83	154.32
		C6	12.21	51.44
		C7	3.052	17.147
	T3: 3',3',5'-triiodo-L-Tyronine	C1	195.3	195.3
		C2	48.83	65.10
		C3	12.21	21.70
		C4	3.052	7.234
		C5	0.763	2.411
		C6	0.191	0.804
		C7	0.048	0.268

*for PART I bis_RUN 1 TRα: dilution factor 1/3 (dilution factor too low, uncomplete sigmoidal curve)

	XII. TRβ Assay: tested concentration			
	Test Item	Concentration	Final 1X (nM)	
			Dilution 1:3	Dilution 1:3
TRβ	T1: Sobetorime	C1	1562.5	--
		C2	520.8	--
		C3	173.6	--
		C4	57.9	--
		C5	19.29	--
		C6	6.43	--
		C7	2.143	--
	T2: 17β-Estradiol	C1	12500.0	--
		C2	4166.7	--
		C3	1388.9	--
		C4	463.0	--
		C5	154.32	--
		C6	51.44	--
		C7	17.147	--
	T3: 3',3',5'-triiodo-L-Tyronine	C1**	195.3	24.4
		C2	65.10	8.14
		C3	21.70	2.71
		C4	7.234	0.904
		C5	2.411	0.301
		C6	0.804	0.100
		C7	0.268	0.033

**tested only in PART I _RUN 1 TRβ: from 24.4 nM (statarting concentration too low: uncomplete sigmoidal curve)

APPENDIX III

SPONSOR INFORMATION

JRC - EURL ECVAM
Via E. Fermi 2749
21027 Ispra VA
ITALY

DATE: 27th April 2022

JUSTIFICATION
For each test item, the graphs representing the TRα activation in all valid runs are added. The paragraph numbering is modified

MODIFIED POINT
Pag. 20

Par. 5.2.1 TR activity Assay: TRα
In fig. 2f the results of TRα activation in all valid runs for each test item compared with RI curves are reported.

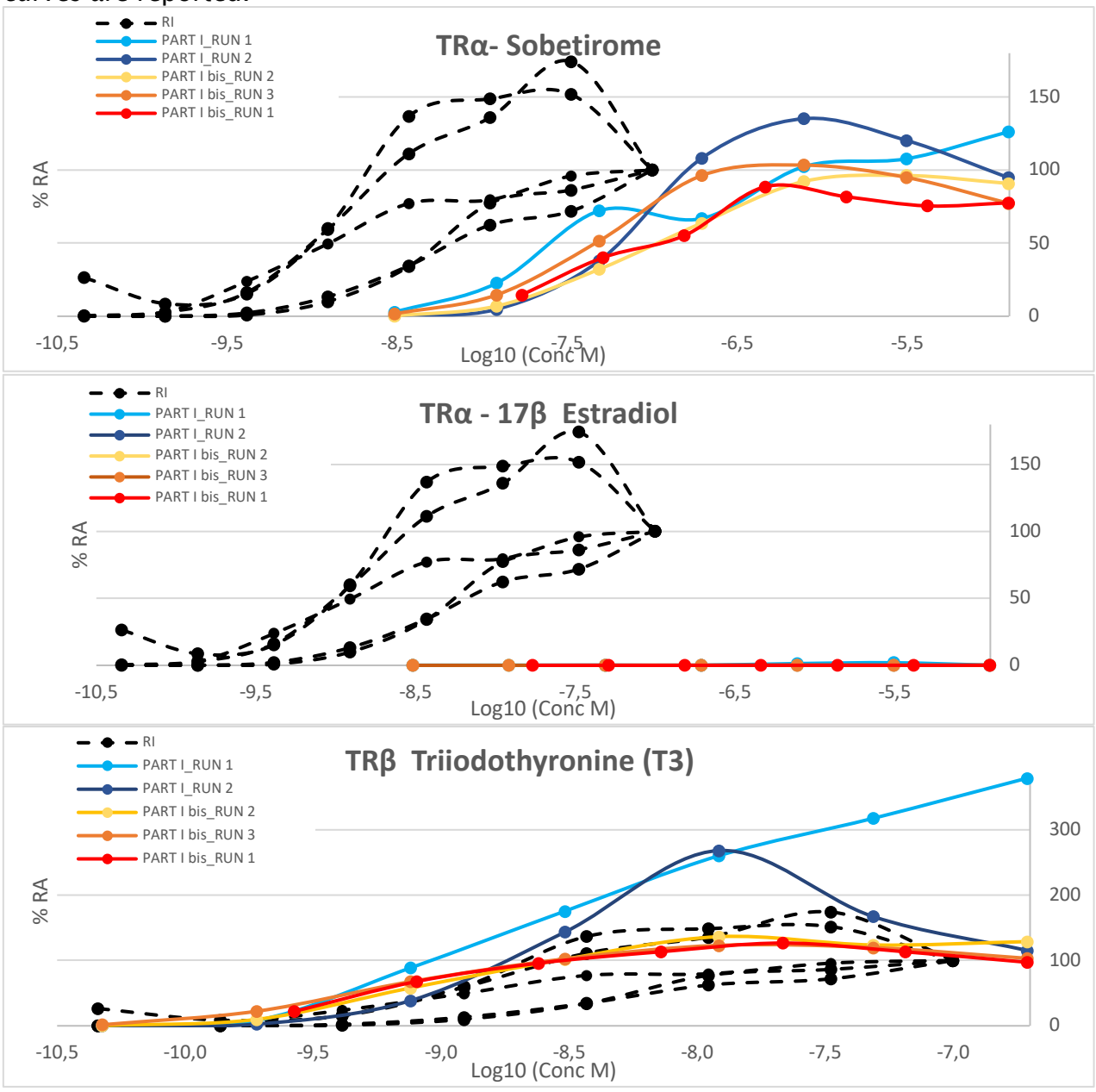


Fig 2f. Results of 5 valid run on TRα cells. % Relative Activation (%RA) for each test item compared with RI (dot line)

JUSTIFICATION

Since for negative compounds no dose response is present, EC50 value cannot be calculated. The data relative to EC50 and 95% CI of 17β-estradiol are removed from table of TRα.

MODIFIED POINT

Pag. 22, Tab. VII

VII. Calculated EC50 for TRα						
	Test Compound		RUN	Sigmoidal dose-resp.	Calculated EC50 (nM)	95% CI (nM) (profile likelihood)
TRα	RI	3'3'5' Triiodo-L-thyronine (Reference)	PART I_RUN 1	YES	1.47	0.9677 - 2.248
			PART I_RUN 2	YES	1.31	0.8988 - 1.781
			PART I bis_RUN 1	YES	1.25	0.8633 - 1.970
			PART I bis_RUN 2	YES	1.37	1.078 - 1.769
			PART I bis_RUN 3	YES	0.70	0.5564 - 0.8867
	T1	Sobetirome	PART I_RUN 1	YES	83.02	30.50 - 684.9
			PART I_RUN 2	YES	66.27	49.27 - 97.48
			PART I bis_RUN 1	YES (not complete)	58.49	35.88 - 94.01
			PART I bis_RUN 2	YES	96.68	70.92 - 132.4
			PART I bis_RUN 3	YES	41.04	27.23 - 57.69
	T2	17β-Estradiol	PART I_RUN 1	NO	Not Calculable	
			PART I_RUN 2	NO	Not Calculable	
			PART I bis_RUN 1	NO	Not Calculable	
			PART I bis_RUN 2	NO	Not Calculable	
			PART I bis_RUN 3	NO	Not Calculable	
	T3	3'3'5' Triiodo-L-thyronine	PART I_RUN 1	YES	4.47	2.465 - 11.30
			PART I_RUN 2	YES	1.46	0.7172 - ???
			PART I bis_RUN 1	YES (not complete)	0.65	0.4904 - 0.8620
			PART I bis_RUN 2	YES	0.97	0.61 - 1.62
			PART I bis_RUN 3	YES	0.59	0.45 - 0.76

JUSTIFICATION
For each test item, the graphs representing the TRβ activation in all valid runs are added. The paragraph numbering is modified

MODIFIED POINT
Pag. 23

Par. 5.2.2 TR activity Assay TRβ
In fig. 3f the results of TRβ activation in all valid runs for each test item compared with RI curves are reported.

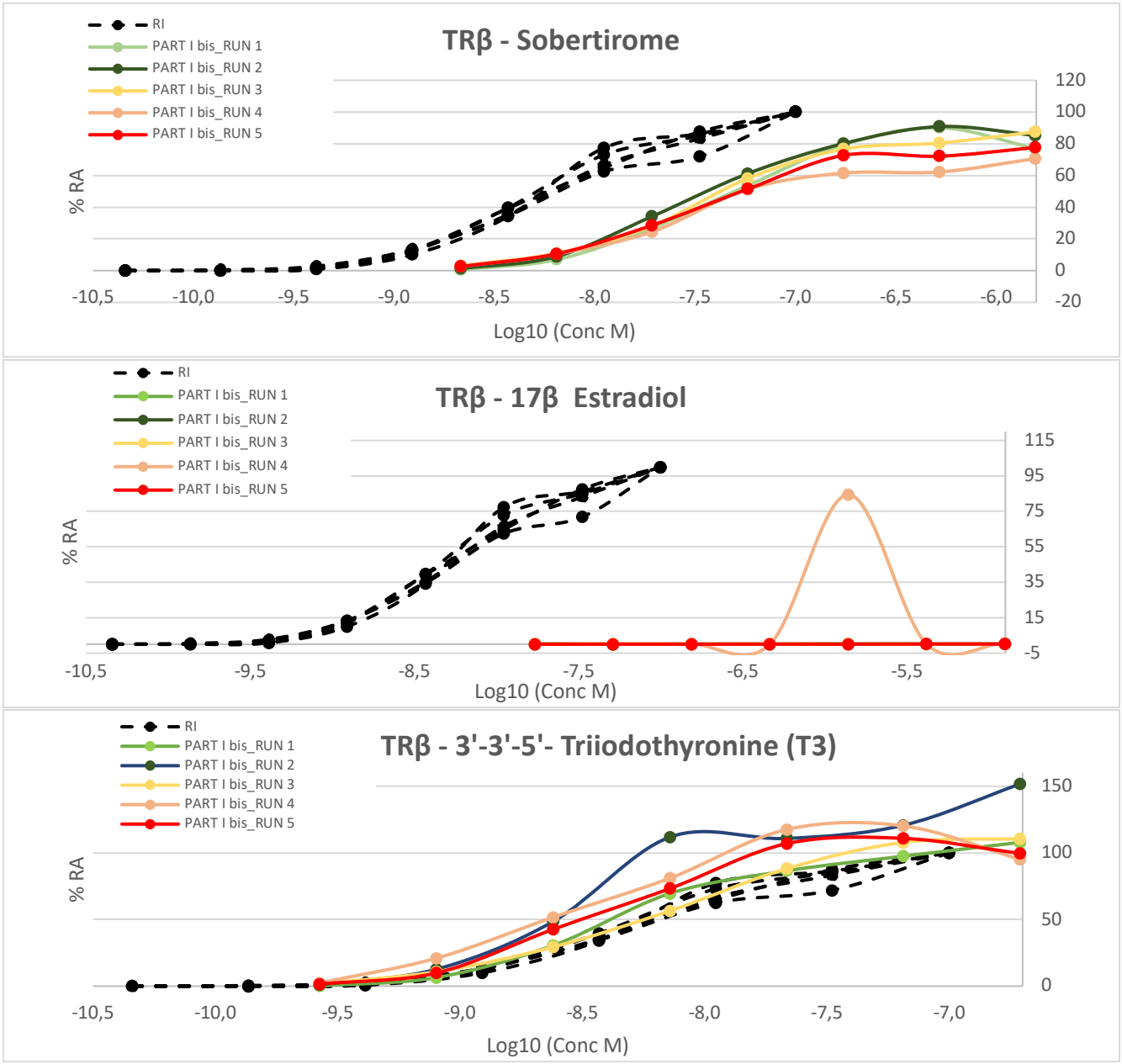


Fig 3f. Results of 5 valid run on TRβ cells. % Relative Activation (%RA) for each test item compared with RI (dot line)

In case of 17β estradiol, the activation of TR receptor in single concertation in RUN 4 probably derives from TI cross contamination and has not to be considered.

JUSTIFICATION
Since for negative compounds no dose response is present, EC50 value cannot be calculated. The data relative to EC50 and 95% CI of 17β-estradiol are removed from table of TRβ

MODIFIED POINT
Pag. 25, Tab. VIII

VIII. Calculated EC50 for TRβ

	Test Compound		RUN	Sigmoidal dose-resp.	Calculated EC50 (nM)	95% CI nM (profile likelihood)
TRβ	RI	3'3'5' Triiodo-L-thyronine (Reference)	PART I bis_RUN 1	YES	5.25	4.51 - 6.21
			PART I bis_RUN 2	YES	5.29	4.43 to 6.45
			PART I bis_RUN 3	YES	9.38	5.34 - 27.29
			PART I bis_RUN 4	YES	6.88	5.07 - 10.42
			PART I bis_RUN 5	YES	6.66	5.25 - 8.87
	T1	Sobetirome	PART I bis_RUN 1	YES	36.17	27.23 - 48.21
			PART I bis_RUN 2	YES	30.30	17.15 to 60.68
			PART I bis_RUN 3	YES	33.84	24.25 - 50.03
			PART I bis_RUN 4	YES	27.66	18.60 - 44.76
			PART I bis_RUN 5	YES	31.14	20.20 - 51.59
	T2	17β-Estradiol	PART I bis_RUN 1	NO	Not Calculable	
			PART I bis_RUN 2	NO	Not Calculable	
			PART I bis_RUN 3	NO	Not Calculable	
			PART I bis_RUN 4	NO	Not Calculable	
			PART I bis_RUN 5	NO	Not Calculable	
	T3	3'3'5' Triiodo-L-thyronine	PART I bis_RUN 1	YES	4.74	3.68 - 6.46
			PART I bis_RUN 2	YES	3.25	2.36 - 5.09
			PART I bis_RUN 3	YES	7.27	5.03 - 11.59
			PART I bis_RUN 4	YES	2.77	1.67 - 4.60
			PART I bis_RUN 5	YES	3.62	2.94 - 4.46

JUSTIFICATION
Additional paragraphs are added to show the performances of Reference Item on both TRα and TRβ related to acceptance criteria in all performed runs (valid and not valid)

MODIFIED POINT
Pag. 25

5.3.1 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 both valid and not valid (in grey) are reported in Tab. XIII. After invalid runs, a supplementary training on method was performed and FA criteria (n.2) was reduced.

				PART I RUN 1	PART I RUN 2	PART I RUN 1	PART I RUN 4		PART I bis RUN 1	PART I bis RUN 2	PART I bis RUN 3
Tab. XIII RI Metrics for Trα assay			**Run n.**	1	2	3	4		5	6	7
2	FA of REF-EC100 (T3; 0.10 µM)	**≥ 600 FA**	On P1	1267.9	1539.6	1478.5	981.4	**≥ 300 FA**	1539.1	2397.7	1482.5
			On P2	776.4	1986.8	533.5	793.0		1095.5	1895.2	2472.1
3	RI-EC50	≤ 10 nM (≤ 1.0E-08 M)	-	1.5	1.31	1.60	2.57	as PART I	1.25	1.37	0.70
4	%CV log (EC50) for RI	< 3%	-	0.99	0.68	0.70	2.19		0.86	0.56	0.53
5	PC %RA (Sobetirome at EC100; 1 µM)	≥ 60% RA	-	180.9	96.21	60.15	24.94		82.70	67.80	62.69
6	NC %RA (17-b-Estradiol; 1 µM)	< 10% RA	-	0.3	0.14	0.10	0.08		0.12	0.14	0.16
7	Z' for REF-EC100 (T3; 0.10µM)	≥ 0.5	On P1	0.50	0.68	0.75	0.41		0.73	0.76	0.73
			On P2	0.72	0.52	0.55	0.50		0.74	0.58	0.63

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

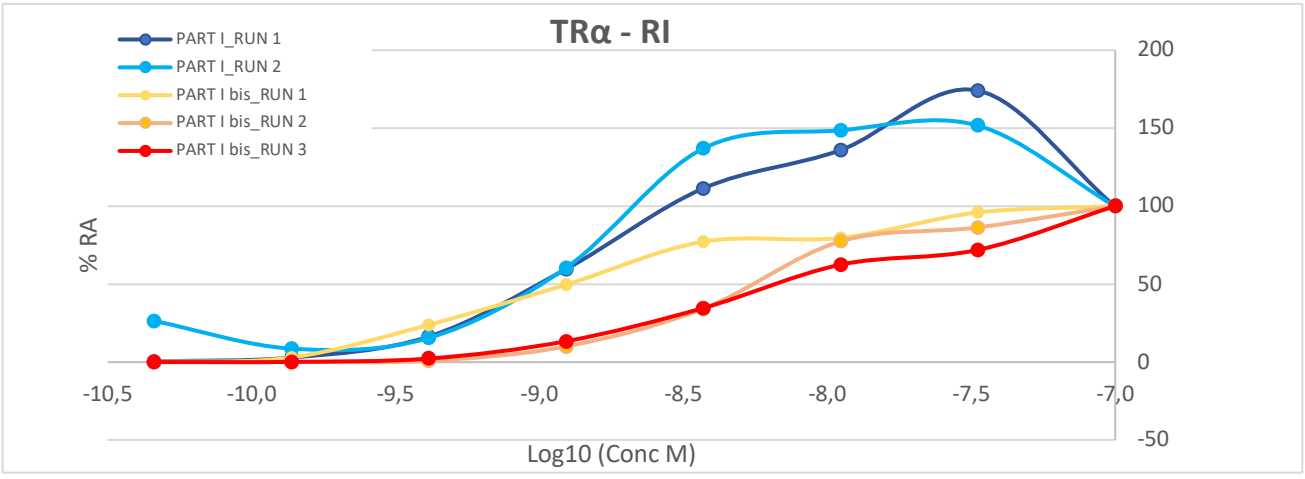


Fig 4. Results of 5 valid run on TRα cells. % Relative Activation (%RA) for RI

5.3.2 TR Activity assay for Reference Item (RI): TRβ

Concerning the activation of TRβ by RI, the metrics related to acceptance criteria of all the performed runs both valid and not valid (in grey) are reported in Tab. XIV. After invalid runs, a supplementary training on method was performed and FA criteria (n.2) was reduced.

				PART I RUN 1	PART I RUN 2		PART I bis RUN 1	PART I bis RUN 2	PART I bis RUN 3	PART I bis RUN 4	PART I bis RUN 5
XIV. RI Metrics for Trβ assay			Run n.	1	2		3	4	5	6	7
2	FA of REF-EC100 (T3; 0.10 µM)	**≥ 1000 FA**	On P1	291.1	192.6	**≥ 500 FA**	1667.8	840.43	1482.5	5373.86	4212.29
			On P2	229.4	274.8		2364.8	592.79	2472.1	2200.90	2483.44
3	RI-EC50	≤ 40 nM (≤ 4.0E-08 M)	-	3.19	6.32	as PART I	5.25	5.29	9.38	6.88	9.38
4	%CV log (EC50) for RI	< 3%	-	0.56	0.83		0.56	0.46	1.61	0.83	1.61
5	PC %RA (Sobetirome at EC100; 1 µM)	≥ 60% RA	-	227.18	83.05		59.89	61.87	71.97	67.99	71.97
6	NC %RA (17-b-Estradiol; 1 µM)	< 10% RA	-	0.31	-0.05		0.13	0.41	0.16	0.11	0.16
7	Z' for REF-EC100 (T3; 0.10µM)	≥ 0.5	On P1	0.92	0.64		0.81	0.91	0.73	0.67	0.86
			On P2	0.53	0.61		0.50	0.86	0.63	0.67	0.86

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

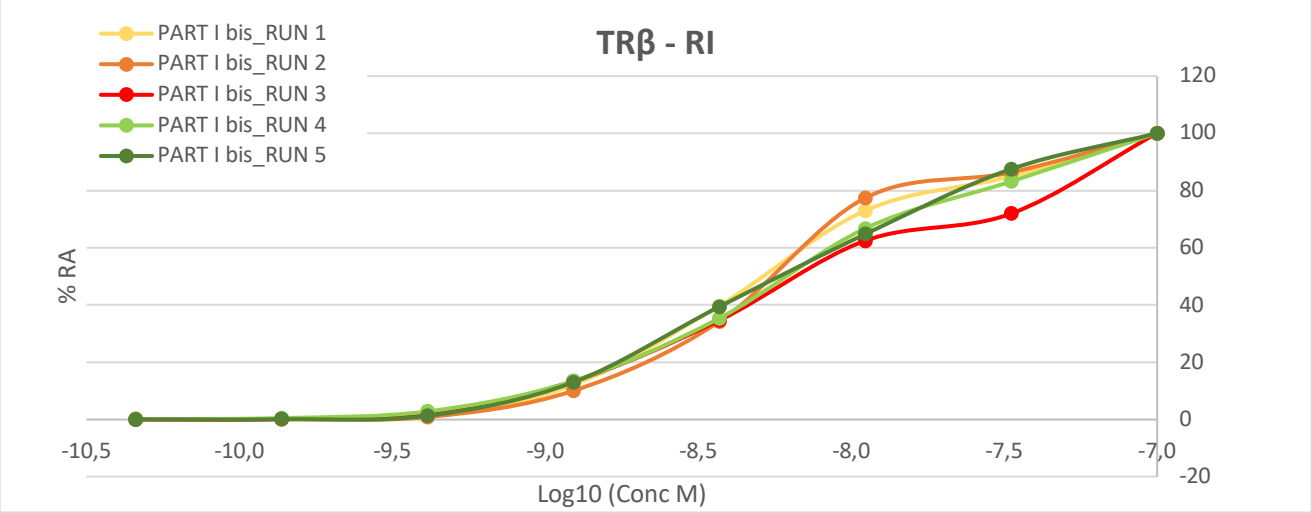


Fig 5. Results of 5 valid run on TRβ cells. % Relative Activation (%RA) for RI

QUALIFICA *QUALIFICATION*	**FIRMA** *SIGNATURE*	**DATA** *DATE*
Direttore di Studio *Study Director*		**27.04.2022**
Assicurazione Qualità *Quality Assurance*		**27.04.2022**
Direttore del Centro di Saggio Testing Facility Manager		**27.04.2022**

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