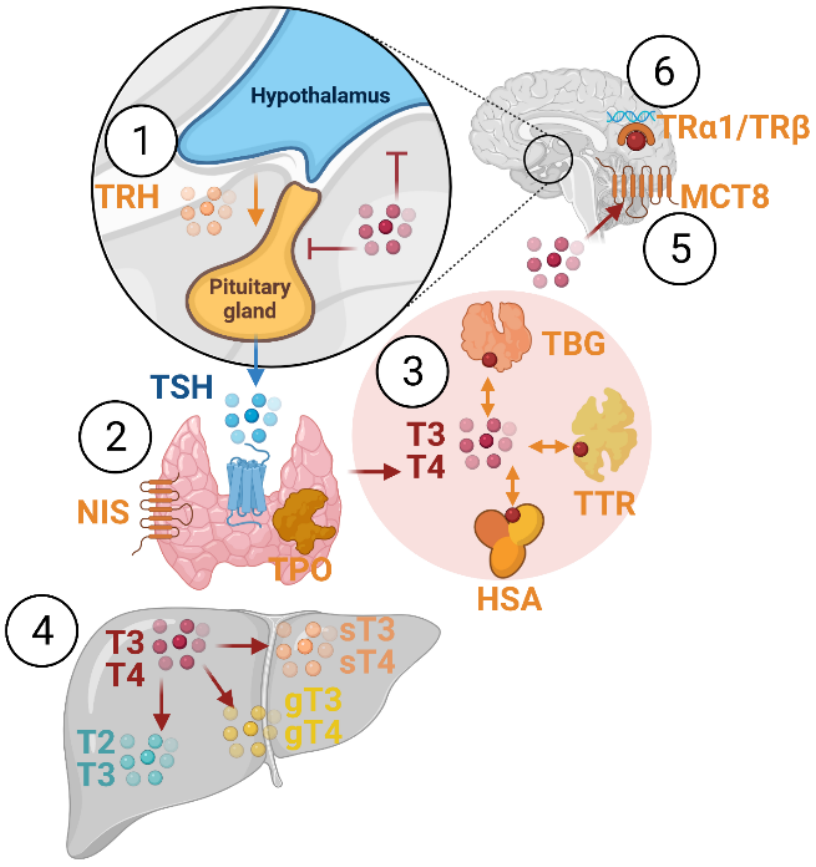
STUDY REPORT

for the thyroid hormone receptor transactivation (TRβ-CALUX®) assay, measuring agonist and antagonist activities – Part 1 & 2 - BDS

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



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This study report has been prepared within the context of a collaboration agreement signed in 2019 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. For information on the methodology and quality underlying the data presented in this report, users should contact the referenced source.

This study report describes the experimental design and includes data generated for the TRβ-CALUX® assay in Part 1 and Part 2 of the validation study, by the method developer BioDetection Systems BV, the Netherlands.

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Validation of the transcriptional (anti-)TRβ CALUX bioassay for the detection of thyroidogenic and anti-thyroidogenic chemicals

final report

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Content

1 Acronyms

$\%VC_R$	Reproducibility variation coefficient
%CV	Coefficient of variance
BDS	BioDetection Systems BV (the Netherlands)
DMSO	Dimethylsulfoxide
EC_{10}	The molar concentration of a chemical which produces 10% of the maximum possible response for that chemical
EC_{50}	The molar concentration of a chemical which produces 50% of the maximum possible response for that chemical
EURL ECVAM	European Centre for the Validation of Alternative Methods
EDCs	Endocrine Disrupting Chemicals
TRβ	Thyroid Receptor β
IC_{20}	The molar concentration of a chemical which produces 80% of the maximum possible response for that chemical (20% inhibition)
IC_{50}	The molar concentration of a chemical which produces 50% of the maximum possible response for that chemical (50% inhibition)
NC	Negative control
OECD	Organisation for Economic Co-operation and Development
PC	Positive control
PC_{10}	The concentration of a chemical at which its response equals 10% of the maximum response of the reference standard
PC_{50}	The concentration of a chemical at which its response equals 50% of the maximum response of the reference standard.
PC_{80}	The concentration of a chemical at which its response equals 80% of the maximum response of the reference standard.
SC	Solvent control
SD	Standard Deviation
SOP	Standard Operating Procedure
TA	Transcriptional Activation
TC_{max}	Maximum relative induction of the test chemical (relative to the maximum induction of reference standard).
TC_{min}	Minimum relative induction of the test chemical (relative to the maximum induction of reference standard).
TCx	Test chemical x

2 Introduction

Chemicals having an impact on human development and reproduction are of major concern. Testing of such endocrine disruptors requires the use of large numbers of animal experiments. Therefore, the development and validation of alternative approaches for the assessment of developmental and reproductive hazards is of high priority. In recent years, transactivation assays for (anti)estrogenicity and (anti)androgenicity have been developed and validated according to OECD principles. OECD performance-based Test Guidelines (TGs) for both Estrogen Receptor Transactivation Assays (ERTA) such as the ERa CALUX bioassay and Androgen Receptor Transactivation Assays (ARTA) such as AR CALUX have been developed. The basis for these OECD TG 455 (ERa CALUX) and TG458 (AR CALUX) are results obtained from inter-laboratory validation studies.

In addition to the development of methods to study endocrine disruption, incorporation of test systems to evaluate disruptors of thyroid hormone signalling pathways has been limited, mainly because of the complexity of the thyroid system. As a starting point for the selection and validation of *in vitro* methods addressing various aspects of thyroid disruption, EURL ECVAM has compiled a number of Thyroid Hormone Disruption (THD) *in vitro* methods with validation potential, based on the OECD scoping paper (OECD, 2014) for identification of modulators of thyroid hormone signalling.

To evaluate promising methods addressing the various phases of the thyroid hormone signalling, EURL ECVAM organised a Thyroid Validation Study. One of the methods to be evaluated is the transcriptional (anti-)TRβ CALUX bioassay for the detection of thyroidogenic and anti-thyroidogenic chemicals. In the present report, the described (anti-)TRβ CALUX validation experiments and outcomes have been performed for PART 1 and PART 2 of the EURL ECVAM coordinated Thyroid Validation Study. After the full description of method “TR-CALUX” in a set of standard operating procedures (SOPs), three valid runs were generated for the reference and control items and 14 coded test items allowing the assessment of within and between run reproducibility and overall reliability and relevance of the method to identify the (anti-)thyroidogenic potential of chemicals.

3 Validation process

The TRβ CALUX bioassay was validated for agonistic and antagonistic properties. Initially, Standard Operating Procedures (SOPs) and calculation documents were developed and drafted. These SOPS were used and evaluated during the validation of the bioassay. The results of the validation study may lead to adaptations and refinements in the constructed SOPs/calculation documents.

The validation study was divided into two parts. During the first part, agonist and antagonist reference and control samples were evaluated in the (anti-)TRβ CALUX bioassay. In the second part, the response of 14 blind coded test items to induce agonistic or antagonistic activity in the TRβ CALUX bioassay was evaluated. Based on the results of these analyses, the test items were classified for agonism or antagonism. During both parts, a pre-screen test was performed after which the test items were tested comprehensively. The validation study started the first of August 2022 and was completed the 25th of November 2022.

Ad 1 *Pre-screen testing*
The objective of pre-screen testing is to:
- determine the solubility of test items
- determine cytotoxicity at concentrations of test items evaluated for (anti)thyroidogenic activity during pre-screen testing
- classify test items for agonism or antagonism (qualitative; positive or negative response)
- derive a refined concentration-range of test items for comprehensive testing

During pre-screen testing, the concentrations and dilution series of test items for comprehensive testing are selected. In first instance, the solubility of test items is evaluated. Concentrations showing insolubility during the preparation of stock or work solutions for pre-screen experiments, as determined by visual inspection, shall not be selected for comprehensive testing. Furthermore, concentrations showing cytotoxicity as determined using the cytotox CALUX bioassay and observed visually after exposure are not selected for further testing.

Ad 2 *Comprehensive testing*
The objective of the comprehensive runs is to:
- determine cytotoxicity at concentrations of test items evaluated for (anti-)thyroidogenic activity during comprehensive testing
- classify test items for agonism or antagonism (qualitative: positive or negative response)
- classify test items for agonism or antagonism (quantitative: agonism: EC_{50}, PC_{10}; antagonism: IC_{50}, PC_{80})
- assess the performance of the test (determination of the thyroidogenic and anti-thyroidogenic potency of test items)

The concentrations and dilution series to be used during comprehensive testing are based on the results of the pre-screen testing. The refined concentration series derived following pre-screen testing will only contain non-cytotoxic concentrations and concentrations that are completely soluble in the solvent applied.

Part 1 of the validation (reference and control compounds) starts with pre-screen testing followed by 2 comprehensive runs for all compounds. Part 2 of the validation (14 blind coded compounds) also starts with pre-screen testing. Test items classified as positive during pre-screen testing were analysed comprehensively in 2 independent runs (agonism and antagonism). Test items classified as negative during pre-screen testing were evaluated comprehensively in 2 independent runs (agonism) or 1 run (antagonism).

4 Material and methods

For this validation study, BDS constructed all required documentations (standard operating procedures (SOPs), data calculation documents and forms; see Annex A). To optimise the constructed SOPs, data calculation documents or forms, results and findings of the present study may lead to adjustments or updates of the indicated documents.

The TRβ CALUX cells were not received from EURL ECVAM but taken from the same batch of frozen TRβ CALUX cells as supplied to EURL ECVAM by BDS at the start of the study. Reference, positive control and negative control standards (see table 2) evaluated for their (anti-)thyroidogenic potential in part 1 of the validation study were purchased by BDS. Test items to be evaluated for their (anti-)thyroidogenic potential in part 2 of the validation study were obtained coded from EURL ECVAM. In table 1, the received blind coded test items are given including their physical state, molecular weight (approximate) and volume received.

In Annex B, most essential equipment to perform (anti-)TRβ CALUX bioassays is indicated. The developed SOPs for working with cultured or seeding cells, evaluation of stock and medium stability and agonistic/ antagonistic testing include a full list of materials and equipment required.

Table 1 List of test items (blind coded) received from EURL ECVAM to be used for the validation of the TRβ CALUX bioassay to detect thyroidogenic agonists and antagonists

BDS code (-)	Chemical code (-)	State / storage (-)	Molecular weight (appr.) (M)	Sample weight (mg)
43395	496	Solid; RT	250	319
43396	425	Liquid; ; RT	1 M (aquaous solution)	1 ml
43397	493	Solid; RT	250	337
43398	751	Liquid; RT	300	1 mL
43399	177	Solid; RT	275	535
43400	265	Solid; RT	300	330
43401	420	Solid; 4°C	350	336
43402	255	Solid; 4°C	150	307
43403	282	Solid; RT	325	377
43404	657	Solid; 4°C	700	326
43405	212	Solid; 4°C	275	310
43406	585	Solid; -20°C	750	250
43407	870	Solid; RT (inert gas)	200	309
43408	9	Solid; -20°C	350	2 x 25 mg original vials

The validation of the TRβ CALUX bioassay was performed as indicated by EURL ECVAM and described in SOP03-TRb-01_v8 (agonistic mode) and SOP04-TRb01_v12 (antagonistic mode) with minor changes:

1 The reference compound stock solutions were not prepared fresh prior to each pre-screen or comprehensive study. Instead, at the start of the validation study, stock solutions for both agonist and antagonist reference compounds were prepared in DMSO after which the required dilution series in DMSO were prepared. These dilutions series were aliquoted in smaller portions and stored frozen at -20^{0}C. Prior to performing a series of pre-screen or comprehensive measurements, one stored reference dilution series was recovered from the freezer and allowed to adjust to room temperature before being used. Once used, this freshly thawed reference dilution series was not frozen again at -20^{0}C but stored at 4^{0}C. This reference dilution series was used for a maximum

of 4 weeks after which it was discarded, and a new aliquoted reference dilution series was taken from the freezer and used for further measurements.

2 Instead of using cultured cells, a batch of seeding cells was used in the present study.

3 Cytotoxicity was evaluated by using the cytotox CALUX as well as by visual inspection of the TRβ cells. The cytotoxicity control (Triton X-100) was not analysed on the TRβ cells. Instead, the cytotox CALUX reference standard TBT was used. During the validation study, evaluation of cytotoxicity using both visual observations and cytotox CALUX analyses was only performed during pre-screen testing and the first comprehensive runs. During the second run of comprehensive testing, cytotoxicity was only evaluated visually.

4 During part 2 antagonistic measurements, the light emitted by the DMSO solvent controls showed to be relatively low. Since the analysed light units of the solvent controls is used as the maximum response in the antagonistic mode of the TRβ CALUX bioassay, the calculated relative induction of the reference compound and test items was overestimated (relatively high and more than 100%). For further evaluation of antagonistic properties during comprehensive testing of blind coded test items, the relative induction of the reference compound and test items were normalised based on the response observed in the highest dilutions of the reference series and test items. In case of normalisation of test items, this was only performed in case at the highest 2-3 dilutions of test item, a plateau could be observed

5 Agonistic analyses were performed according to the plate-setup indicated in the SOP for part 1 pre-screen analyses only. For all other agonist measurement (part 1 and 2), the solvent control in wells E2-G2 was replace by the highest concentration C8 of the reference compound T3.

In table 2, TRβ CALUX bioanalysis information is summarised.

Table 2 TRβ CALUX bioanalysis information

	TRβ CALUX (agonism)	anti-TRβ CALUX (antagonism)
Cell line	TRβ CALUX (human U2OS cells)	TRβ CALUX (human U2OS cells)
Cell medium (seeding cells)	D-MEM/F12 medium without phenol red (DMEM/F12) containing 5% dextran-coated charcoal-treated fetal calf serum (DCC-FCS)	D-MEM/F12 medium without phenol red (DMEM/F12) containing 5% dextran-coated charcoal-treated fetal calf serum (DCC-FCS)
Cell medium (exposing cells)	D-MEM/F12 medium without phenol red (DMEM/F12)	D-MEM/F12 medium without phenol red (DMEM/F12)
Vehicle	DMSO	DMSO
Vehicle control	0.1% DMSO final concentration	0.1% DMSO final concentration
Final conc. vehicle	0.1%	0.1%
Reference standard	3,3',5- Triiodo-L-Thyronine (T3) CAS: 6893-02-3	Diclazuril CAS: 101831-37-2
Positive control	L-Thyroxine (T4) CAS: 51-48-9	3,3',5,5'-Tetrabromobisphenol A (TBBPA) CAS: 79-94-7
Negative control	Bisphenol A (BPA) CAS: 80-05-7	3,3',5- Triiodo-L-thyronine (T3) CAS: 6893-02-3
No. of test items part 1	5	5
No. of test items part 2	14	14
Minimum no. of runs per test item	Positive test item: 1 pre-screen run 2 comprehensive runs Negative test item: 1 pre-screen run 2 comprehensive runs	Positive test item: 1 pre-screen run 2 comprehensive runs Negative test item: 1 pre-screen run 1 comprehensive run
Concentrations tested	Pre-screen testing: see Annex C (part 1) see Annex D (part 2) Comprehensive testing: see Annex C (part 1) see Annex D (part 2)	Pre-screen testing: see Annex C (part 1) see Annex D (part 2) Comprehensive testing: see Annex C (part 1) see Annex D (part 2)
Cell density	10.000/well	10.000/well
Incubation time	22-24 hr.	22-24 hr.

Note. all concentration of reference standards, positive control, negative control are analysed in triplicate.

5 Results

5.1 Part 1 – reference standards and positive/negative control standards

5.1.1 Pre-screen testing

5.1.1.1 Solubility in DMSO and solubility and stability in medium

Reference standards and positive/negative control standards were initially dissolved in DMSO at a concentration of 0.1 M as described in the SOP05-TRb01. In case the test item proved not to dissolve at a concentration of 0.1 M in DMSO, the test item was weighed again and dissolved in DMSO at a concentration that is three times lower than the previous. In table 3, the final soluble stock concentrations of reference and control standards in DMSO are given.

Once the solubility in DMSO was determined, the solubility and stability of the reference and control standards in medium was determined. In table 3, the final soluble and stable concentration of the compounds in medium is presented (1000-times diluted from stock solution in DMSO; 0.1% exposure concentration). These concentrations of reference and control standards were used as the highest test concentrations for pre-screen testing.

Table 3 Solubility/stability of reference and positive/negative control standards in DMSO and cell culture medium

Reference/control standards	Solubility in DMSO[a]	Solubility / stability in medium (T=0 and T=24 hrs)	
	Conc. stock (M)	Conc. in well (M)	Conc. stock (M)
T3	1.0E-01	3.0E-06	3.0E-03*
T4	1.0E-01	1.0E-05	1.0E-02
BPA	1.0E-01	1.0E-04	1.0E-01
Diclazuril	1.0E-01	1.0E-04	1.0E-01
TBBPA	1.0E-01	1.0E-04	1.0E-01

a completely soluble after 1 minute of vortexing

* T3 is not stable in DMSO after freezing and thawing. After thawing, T3 is not dissolved in DMSO anymore at 0.1 M. Therefore, it was decided to prepare a new T3 stock in DMSO of 3E-03 M to be used in prescreen testing.

5.1.1.2 Cytotoxicity

Cytotoxicity was determined during all pre-screen runs and during the first agonist and antagonist comprehensive runs by applying the cytotox CALUX bioassay and by visual inspection of the cells after exposure. During the second comprehensive measurements, cytotoxicity was only evaluated visually after exposure. The cytotox CALUX bioassay was performed parallel to the TRβ CALUX and anti-TRβ CALUX measurements using plate-setups similar to the TRβ CALUX analyses, described in SOP03-TRb01 and SOP04-TRb01. Prior to lysing exposed TRβ CALUX cells, the viability of the cells was evaluated visually using a phase-contrast reverse microscope. The results of both cytotoxicity tests performed during pre-screen and comprehensive analyses, are summarised in table 4 and table 5.

Visual evaluation of cytotoxicity showed similar results to cytotoxicity determined using the cytotox CALUX bioassay. Graphical representations of cytotox CALUX analysis results are given in Annex E. Blind coded reference and positive/negative control samples showing no cytotoxicity during pre-screen testing also did not show cytotoxicity during agonistic or antagonistic comprehensive testing. Only Diclazuril and TBBPA showed cytotoxicity during pre-screen testing. Based on cytotoxicity pre-screen testing, the highest concentration of samples to be evaluated for agonism and antagonism were selected. During comprehensive agonistic testing, TBBPA showed cytotoxicity at the highest test concentration although at the same concentration during pre-screen testing, cytotoxicity was not observed. During comprehensive antagonistic testing, the 2 highest concentrations of TBBPA tested showed cytotoxicity. Again,

the lowest concentration did not show cytotoxicity during pre-screen testing whereas the highest concentration was not tested for cytotoxicity during pre-screen testing.

Note: The cytotox CALUX bioassay constitutively expresses luciferase, emitting light constantly. A dose-depend reduction of emitted light is indicative for cytotoxic effects of the samples under investigation. Since both the TRβ CALUX and cytotox CALUX comprise the same U2OS basic cell, cytotoxicity observed in the cytotox CALUX bioassay upon exposure to a test item is representative to cytotoxicity caused by the same test item in the TRβ CALUX bioassay.

5.1.1.3 Pre-screen analyses

Following solubility testing, the agonistic and antagonistic properties of the reference and positive/negative control standards were evaluated by exposing TRβ CALUX cells (agonistic and antagonistic mode) to test item serial dilutions.

In Annex C.1, the final pre-screen testing exposure concentrations of the reference and positive/negative control standards are given. After 24-hours of exposure, cells were inspected visually for signs of cytotoxicity. Next, cells were lysed, luminescence was measured following addition of the substrate luciferase and the analysis data were evaluated for compliance of the acceptance criteria (see SOP03-TRb01 (agonism) and SOP04-TRb01 (antagonism)). All microtiterplates were evaluated for determination of refined concentration series of reference and positive/negative control standards to be used for comprehensive testing although some of the microtiterplates analysed for antagonism did not meet the acceptance criteria (|CV| of estimated log(IC50) outside acceptance range; see table 15).

To further evaluate the analysis results, the average of each accepted triplicate analysis was calculated after which the relevant average solvent blank was subtracted. Next, all results were expressed as percentage of response, relative to the analysis results of the reference standard in each respective run (agonism: maximum induction of reference standard set at 100% response; antagonism: maximum induction of the solvent control set at 100% response). The statistical software package Graphpad Prism 5.0 was used to fit raw data (non-linear regression, variable slope, 4 parameters, robust fit, Hill equation) and to quantify the thyroidogenic potential (RPC_{max}, EC_{50}, EC_{10}, PC_{50}, PC_{10}) or anti-thyroidogenic (RPC_{min}, IC_{50}, IC_{20}, PC_{50}, PC_{80}) potential of the tested reference and control samples items. In Annex F (figures F.1.A and F.1.C-F.1.G (agonism); figures F.2.A and F.2.C-F.2.G (antagonism)), graphical representations of all pre-screen runs performed are given (relative induction (%) as function of exposure concentrations).

The procedure for selection of start concentrations and dilution factors for comprehensive testing is given in the standard operating procedures. With respect to the start concentrations of control samples for comprehensive evaluations, the highest concentration showing no cytotoxicity is used for comprehensive testing in case none or only one concentration of control sample showed a response and met the criteria stated above. If a sample showed 2 or more concentrations meeting the criteria above, 3 times the lowest concentration showing the maximum relative induction (agonism) or minimum relative induction (antagonism) is used for comprehensive testing. In table 4 (agonism) and 5 (antagonism), the selected maximum stock concentrations of reference and positive/negative control standards to be used for comprehensive testing are presented.

With respect to the dilution factors to be used during comprehensive testing, the dilution steps to be applied are depending on the number of test concentrations showing a response above 10% (agonism) or below 80% (antagonism). In short, if test items show no concentrations where the relative induction is equal or above 10% (agonism) or equal or below 80% (antagonism), a dilution series with steps of 5 will be used in comprehensive testing. If only 1 concentration is found that meets these criteria, a dilution series with steps of 2 will be used. If 2 or more concentrations are found that meet these criteria, a dilution series with steps of 3 and 3.3 are used in comprehensive testing. In table 4 (agonism) and table 5 (antagonism) the selected dilution steps are given.

Table 4 Evaluation of cytotoxicity detected during pre-screen testing and selection of the maximum concentrations of reference and positive/negative control standards to be used for agonistic comprehensive testing

Test item	Prescreen cytotoxicity[a]	Highest stock conc. without cytotox (M)	Response TRβ CALUX Yc < 80%	Dilution steps for compr. testing	Stock conc. selected for compr. testing	Comprehensive cytotoxicity[a,b]
T3	No	3.00E-03	>2 dilutions	3 – 3.3	1.00E-06	No
T4	No	1.00E-02	>2 dilutions	3 – 3.3	1.00E-04	No
BPA	No	1.00E-01	no	5	1.00E-01	No
Diclazuril	Yes	1.00E-02	no	5	1.00E-02	No
TBBPA	Yes	1.00E-03	no	5	1.00E-03	Yes (1.0e-3)

a Evaluation of cytotoxicity by visual inspection or by applying the cytotox CALUX bioassay showed similar results.

b Between brackets, the stock concentration showing cytotoxicity during comprehensive testing, is indicated

Table 5 Evaluation of cytotoxicity detected during pre-screen testing and selection of the maximum concentrations of reference and positive/negative control standards to be used for antagonistic comprehensive testing

Test item	Prescreen cytotoxicity[a]	Highest stock conc. without cytotox (M)	Response anti-TRβ CALUX Yc < 80%	Dilution steps for compr. testing	Stock conc. selected for compr. testing	Comprehensive cytotoxicity[a,b]
T3	No	3.00E-03	no	5	3.00E-03	No
T4	No	1.00E-02	no	5	1.00E-02	No
BPA	No	1.00E-01	>2 dilutions	3 - 3.3	1.00E-01	No
Diclazuril	Yes	1.00E-02	>2 dilutions	3 - 3.3	1.00E-02	No
TBBPA	Yes	1.00E-03	>2 dilutions	3 - 3.3	3.00E-03	Yes (1.0e-3; 3.0E-3)

a Evaluation of cytotoxicity by visual inspection or by applying the cytotox CALUX bioassay showed similar results.

b Between brackets, the stock concentration showing cytotoxicity during comprehensive testing, is indicated

5.1.2 Comprehensive testing

The objective of the comprehensive analyses is to:

- classify test chemicals as agonist or antagonist (positive or negative)
- determine the agonistic and antagonistic analysis characteristics (agonism: IF, RPC_{max}, EC_{50}, EC_{10}, PC_{50}, PC_{10}; antagonism: IF, RPC_{min}, IC_{50}, IC_{20}, PC_{50}, PC_{80})
- assess the performance of the (anti)TRβ CALUX bioassay (determination of coefficient of variance and reproducibility)

5.1.2.1 Agonism

During the agonist pre-screen of the reference standards and positive/negative control samples, the dilution steps as well as the start concentrations for all test items were determined. The selected start concentrations as well as the dilution steps are given in table 4. In Annex C.2 the refined concentration series of the evaluated compounds used for each of the independent comprehensive test runs are presented.

Following exposure and analysis of luminescence, data from each independent comprehensive experiment was evaluated for compliance to the acceptance criteria (see table 14). Microtiter plates or individual test items that were rejected because they did not meet the acceptance criteria, were re-analysed (SOP03-TRb01). Only acceptable data was evaluated. All test items were analysed two times in two independent comprehensive runs.

To further evaluate the analysis results, the average of each accepted triplicate analysis was calculated after which the relevant average solvent blank was subtracted. Next, all results were expressed as percentage relative to the analysis response of the reference standard in each respective run (maximum induction of reference standard set at 100% response). The statistical software package Graphpad Prism 5.0 was used to fit raw data (non-linear regression,

variable slope, 4 parameters, robust fit, Hill equation) and to quantify the thyroidogenic potential (RPC_{max}, EC_{50}, EC_{10}, PC_{50}, PC_{10}) of the tested reference and control samples items. In Annex F (figures F.1.B and F.1.C-F.1.G (agonism)), graphical representations of all comprehensive runs performed are given (relative induction (%) as function of exposure concentrations). Fit statistics and reference standards and positive/negative control samples characteristics are summarised in Annex G.1.

Two of the tested reference and positive/negative control standards elicited an agonistic response (T3 and T4). Furthermore, all test items showed reproducible results. For each of the two independent consecutive comprehensive test runs, test items were classified according to the agonistic classifier given in table 6. The final classification of the reference and positive/negative control standards based on the agonist classifiers, is presented in table 8.

Table 6 Agonistic classifiers for classification of test items

Agonism For each run, a test item is considered		
A	Positive	when the relative induction (Yc) of the test item is equal or exceeds 10% (REF RPC_{10}) for two or more consecutive concentrations
B	Negative	In all other cases

5.1.2.2 Antagonism

During the antagonist pre-screen of the reference standards and positive/negative control samples, the dilution steps as well as the start concentration for all test items were determined. The selected start concentrations as well as the dilution steps are given in table 5. In Annex C.3 the refined concentration series of the evaluated compounds used for each of the independent comprehensive test runs are presented.

Following each independent comprehensive run, data were evaluated for compliance to the acceptance criteria (see table 15). All microtiterplates were used for further evaluation of agonistic properties of the test items although some of the microtiterplates did not meet the acceptance criteria (|CV| of estimated log(IC50) outside acceptance range) (SOP04-TRb01). All compounds were analysed two times in two independent comprehensive runs.

To identify whether an observed anti-thyroidogenic activity is a specific response, all reference standards and positive/negative control samples were tested for anti-thyroidogenicity twice on the same microtiter plate. During the first exposure (top of the microtiter plate), TRβ CALUX cells were exposed to the refined concentration series of compounds in the presence of the approximate EC_{50} concentration of the TRβ agonist T3. In the second exposure (bottom of the microtiter plate), TRβ CALUX cells were exposed to the same test item in the presence of an excess TRβ agonist T3 (100-times approximate EC_{50} concentration).

Reference and test item analysis results, including specificity analysis results, were evaluated by calculating the average response of each accepted triplicate analysis. Next, the average response observed after exposure to the maximum concentration of the antagonist reference compound (100% inhibition) was subtracted. All results were expressed as percentage of response, relative to the maximum induction of the reference standard in each respective run (0% inhibition; solvent control). The statistical software package Graphpad Prism 5.0 was used to fit raw data (non-linear regression, variable slope, 4 parameters, robust fit, Hill equation). Using the same software package, quantitative assessment of anti-androgenic activity (RPC_{min}, IC_{50}, IC_{20}, PC_{50}, PC_{80}) of the evaluated reference standards and positive/negative control samples was performed. In addition, specificity was determined plotting the relative induction of the standard antagonistic analysis (Y_c) against the relative induction of the specificity analysis (S_c) and evaluating the correlation coefficient R^2. For all tested compounds, graphical representations of valid comprehensive test runs and their specificity

evaluation are given in Annex F (figures F.2.B and F.2.C-F.2.G (antagonism); F.3 (specificity)). Fit statistics and reference standards and positive/negative control samples characteristics are summarised in Annex G.2.

All analysis results of reference and positive/negative control samples were reproducible. Three of the tested reference and positive/negative control samples showed a clear dose-dependent inhibition of response (BPA, Diclazuril and TBBPA). To evaluate whether the observed antagonistic effect caused by the 3 compounds is specific, the specificity of the response was tested by addition of an excess of agonist reference compound T3. This specificity test showed that all 3 compounds are specific antagonists. Final classification of the tested compounds for each of the two independent consecutive comprehensive test runs, was based on the antagonistic classifier given in table 7. Final classification of all reference and positive/negative control samples based on the antagonistic classifiers is presented in table 8.

Table 7 Antagonistic classifiers for classification of test items

Antagonism For each run, a test item is considered		
A	Positive	when the following two conditions are met: • the relative induction (Yc) of the test item is less or equal to 80% (REF RPC_{80}) for two or more consecutive concentrations and • the coefficient of determination (R^2) is less or equal to 0.9 for the linear regression of relative induction of the test item's specificity control (Sc) versus the relative induction (Yc)
B	Negative	In all other cases

5.1.3 Final classification

Based on the results of all pre-screen and comprehensive test runs, the test items were classified using the classifiers given in table 6 (agonism) and table 7 (antagonism). In table 8, the final classification of all test items are presented.

Table 8 Final classification agonists

TRβ CALUX (agonism)		anti-TRβ CALUX (antagonism)	
Test item	**Classification**	**Test item**	**Classification**
T3	positive	T3	negative
T4	positive	T4	negative
BPA	negative	BPA	positive
Diclazuril	negative	Diclazuril	positive
TBBPA	negative	TBBPA	positive

5.2 Part 2 – blind coded test items

5.2.1 Pre-screen

5.2.1.1 Solubility

Blind coded test items were initially dissolved in DMSO at a concentration of 0.1 M as described in the SOP05-TRb01. In case the test item proved not to dissolve at a concentration of 0.1 M in DMSO, the test item was weighed again and dissolved in DMSO at a concentration that is three times lower than the previous. In table 9, the final soluble stock concentrations of test item in DMSO are given.

Once the solubility in DMSO was determined, the solubility and stability of the test item in medium was determined. In table 9, the final soluble and stable concentration of the test items in medium are presented (1000-times diluted from stock solution in DMSO; 0.1% exposure concentration). These concentrations of test items were used as the highest test concentrations for pre-screen testing. The solubility and stability in medium of sample 43408 was not determined as directed by ECVAM.

Table 9 Solubility of test items in DMSO and cell culture medium

BDS code	Chemical code	Solubility in DMSO[a]	Solubility / stability in medium (T=0 and T=24 hrs)	
(-)	(-)	Conc. stock (M)	Conc. in well (M)	Conc. stock (M)
43395	496	1.0E-01	1.0E-04	1.0E-01
43396	425	1.0E-01	1.0E-04	1.0E-01
43397	493	1.0E-01	1.0E-04	1.0E-01
43398	751	1.0E-01	1.0E-04	1.0E-01
43399	177	1.0E-01	1.0E-04	1.0E-01
43400	265	1.0E-01	1.0E-04	1.0E-01
43401	420	1.0E-01[b]	1.0E-04	1.0E-01
43402	255	1.0E-01	1.0E-04	1.0E-01
43403	282	1.0E-01	1.0E-04	1.0E-01
43404	657	3.0E-02	3.0E-05	3.0E-02
43405	212	1.0E-01[c]	1.0E-04	1.0E-01
43406	585	1.0E-01	1.0E-04	1.0E-01
43407	870	1.0E-01	1.0E-04	1.0E-01
43408	9	1.0E-01	-	1.0E-01

a completely soluble after 1 minute of vortexing
b completely soluble after soluble after ultrasonic treatment
c completely soluble after standing for 24 hours

5.2.1.2 Cytotoxicity

Cytotoxicity was determined by visual inspection of exposed cells and cytotox CALUX analysis during all pre-screen runs and during the first agonist and antagonist comprehensive runs. During the second comprehensive runs, cytotoxicity was only evaluated visually. The cytotox CALUX bioassay was performed parallel to the TRβ CALUX and anti-TRβ CALUX measurements using plate-setups similar to the TRβ CALUX analyses, described in SOP03-TRb01 and SOP04-TRb01. Prior to lysing exposed TRβ CALUX cells, the viability of the cells was evaluated visually using a phase-contrast reverse microscope. The results of both cytotoxicity tests performed during pre-screen and comprehensive analyses, are summarised in table 10 and table 11.

Visual evaluation of cytotoxicity showed similar results to cytotoxicity determination using the cytotox CALUX bioassay. Graphical representations of cytotox CALUX analysis results are given in Annex E. Test items showing no cytotoxicity during pre-screen testing also did not show cytotoxicity during agonistic or antagonistic comprehensive testing. During comprehensive agonistic testing, 2 compounds (43399 and 43400) showed cytotoxicity at the highest concentration tested. These concentrations were not tested during pre-screen testing but were chosen based on the selection rules for concentration to be tested during comprehensive analyses. During comprehensive antagonistic testing, 3 compounds (43399, 43400 and 43406) showed cytotoxicity at the highest concentration tested. Also, these concentrations were not tested during pre-screen testing but were chosen based on the selection rules for concentration to be tested during comprehensive analyses.

Table 10 Evaluation of cytotoxicity detected during pre-screen testing and selection of the maximum concentrations of test items to be used for agonistic comprehensive testing

Test item	Prescreen cytotoxicity[a]	Highest stock conc. without cytotox (M)	Response TRβ CALUX Yc < 80%	Dilution steps for compr. testing	Stock conc. selected for compr. testing	Comprehensive cytotoxicity[a,b]
43395	No	1.00E-01	No	5	1.0E-01	No
43396	No	1.00E-01	No	5	1.0E-01	No
43397	No	1.00E-01	No	2	1.0E-01	No
43398	No	1.00E-01	No	5	1.0E-01	No
43399	Yes	1.00E-03	No	5	3.0E-03	Yes (3.0E-3)
43400	Yes	1.00E-03	No	5	3.0E-03	Yes (3.0E-3)
43401	No	1.00E-01	No	5	1.0E-01	No
43402	No	1.00E-01	No	5	1.0E-01	No
43403	Yes	1.00E-02	>2 dilutions	3 – 3.3	3.0E-02	No
43404	No	3.00E-02	No	5	3.0E-02	No
43405	No	1.00E-01	No	3 – 3.3	1.0E-01	No
43406	Yes	1.00E-02	>2 dilutions	3 – 3.3	3.0E-04	No
43407	Yes	1.00E-03	No	5	3.0E-03	No
43408	Yes	1.00E-02	>2 dilutions	3 – 3.3	3.0E-05	No

a Evaluation of cytotoxicity by visual inspection or by applying the cytotox CALUX bioassay showed similar results.

b Between brackets, the stock concentration showing cytotoxicity during comprehensive testing, is indicated

Table 11 Evaluation of cytotoxicity detected during pre-screen testing and selection of the maximum concentrations of test items to be used for antagonistic comprehensive testing

Test item	Prescreen cytotoxicity[a]	Highest stock conc. without cytotox (M)	Response anti-TRβ CALUX Yc < 80%	Dilution steps for compr. testing	Stock conc. selected for compr. testing	Comprehensive cytotoxicity[a,b]
43395	No	1.00E-01	1	2	1.0E-01	No
43396	No	1.00E-01	>2 dilutions	3 - 3.3	1.0E-01	No
43397	No	1.00E-01	No	5	1.0E-01	No
43398	No	1.00E-01	No	5	1.0E-01	No
43399	Yes	1.00E-03	No	5	3.0E-03	Yes (3.0E-3)
43400	Yes	1.00E-03	1	2	3.0E-03	Yes (3.0E-3)
43401	No	1.00E-01	No	5	1.0E-01	No
43402	No	1.00E-01	No	5	1.0E-01	No
43403	Yes	1.00E-02	No	5	3.0E-02	No
43404	No	3.00E-02	>2 dilutions	3 - 3.3	3.0E-02	No
43405	No	1.00E-01	No	3 - 3.3	1.0E-01	No
43406	Yes	1.00E-02	No	5	3.0E-02	Yes (6.0e-3; 3.0E-2)
43407	Yes	1.00E-03	No	5	3.0E-03	No
43408	Yes	1.00E-02	No	5	3.0E-02	No

a Evaluation of cytotoxicity by visual inspection or by applying the cytotox CALUX bioassay showed similar results.

b Between brackets, the stock concentration showing cytotoxicity during comprehensive testing, is indicated

5.2.1.3 Pre-screen analyses

Following solubility testing, the agonistic and antagonistic properties of the test items were evaluated by exposing TRβ CALUX cells (agonistic and antagonistic mode) to dilution series of the test items.

In Annex D.1, the final pre-screen testing exposure concentrations of the test items are given. After 24-hours of exposure, cells were inspected visually for signs of cytotoxicity. Next, cells were lysed, luminescence was measured following addition of the substrate luciferase and the analysis data was evaluated for compliance of the acceptance criteria (see SOP03-TRb01 and SOP04-TRb01). All microtiterplates were used for determination of test item refined concentration series to be used for comprehensive testing although some of the microtiterplates did not meet the acceptance criteria (EC50 of T3 reference compound, IC50 of Diclazuril reference compound or |CV| of log(IC50) outside acceptance range; see table 14 and 15).

To further evaluate the analysis results, the average of each accepted triplicate analysis was calculated after which the relevant average solvent blank was subtracted. Next, all results were expressed as percentage relative to the analysis response of the reference standard in each respective run (maximum induction of reference standard or solvent control set at 100% response for agonistic and antagonistic studies respectively). The statistical software package Graphpad Prism 5.0 was used to fit raw data (non-linear regression, variable slope, 4 parameters, robust fit, Hill equation) and to quantify the thyroidogenic potential (RPC_{max}, EC_{50}, EC_{10}, PC_{50}, PC_{10}) or anti-thyroidogenic (RPC_{min}, IC_{50}, IC_{20}, PC_{50}, PC_{80}) potential of the test items. In Annex H, (figures H.1 (agonism) H.2 (antagonism)), graphical representations of all pre-screen runs performed are given (relative induction (%) as function of exposure concentrations).

The procedure for selection of start concentrations and dilution factors for comprehensive testing is given in the standard operating procedures. With respect to the start concentrations of test items for comprehensive evaluations, the highest concentration showing no cytotoxicity is used for comprehensive testing in case none or only one concentration of test item showed a response (agonism: >10%; antagonism: <80%) and met the criteria stated above. If a test item showed 2 or more agonistic (>10%) or antagonistic (<80%) concentrations meeting the criteria indicated above, 3 times the lowest concentration showing the maximum relative induction (agonism) or minimum relative induction (antagonism) was used for comprehensive testing. In table 10 (agonism) and 11 (antagonism), the selected maximum stock concentrations of reference and positive/negative control standards to be used for comprehensive testing are presented.

With respect to the dilution factors to be used during comprehensive testing, the dilution steps to be applied are depending on the number of test concentrations showing a response above 10% (agonism) or below 80% (antagonism). In short, if test items show no concentrations where the relative induction is equal or above 10% (agonism) or equal or below 80% (antagonism), a dilution series with steps of 5 will be used in comprehensive testing. If only 1 concentration is found that meets these criteria, a dilution series with steps of 2 will be used. If 2 or more concentrations are found that meet these criteria, a dilution series with steps of 3 and 3.3 are used during comprehensive testing. In table 10 (agonism) and table 11 (antagonism) the selected dilution steps are given.

5.2.2 Comprehensive testing

The objective of the comprehensive runs is to:

- classify test chemicals as agonist or antagonist (positive or negative)
- determine the agonistic and antagonistic analysis characteristics (agonism: IF, RPC_{max}, EC_{50}, EC_{10}, PC_{50}, PC_{10}; antagonism: IF, RPC_{max}, IC_{50}, IC_{20}, PC_{50}, PC_{80})
- assess the performance of the (anti)TRβ CALUX bioassay (determination of coefficient of variance and reproducibility)

5.2.2.1 Agonism

During the agonist pre-screen of the test items, the dilution steps as well as the start concentration for all test items were determined (see table 10). In Annex D.2 the refined concentration series of the selected test items used for each of the independent comprehensive test runs are presented. Following exposure and analysis of luminescence, data from each independent comprehensive experiment was evaluated for compliance to the acceptance criteria. All microtiterplates were used for further evaluation of agonistic properties of the test items although some of the microtiterplates did not meet the acceptance criteria (EC50 of reference T3 outside acceptance range; see table 14). All test items were analysed two times in two independent comprehensive runs.

For further evaluation of analysis results, the average of each accepted triplicate analysis was calculated after which the relevant average solvent blank was subtracted from each individual measurement. Next, all results were expressed as percentage relative to the analysis response of the reference standard in each respective run (maximum induction of reference standard set at 100% response). The statistical software package Graphpad Prism 5.0 was used to fit raw data (non-linear regression, variable slope, 4 parameters, robust fit, Hill equation). Using the same software package, quantitative assessment of androgenic activity (RPC_{max}, EC_{50}, EC_{10}, PC_{50}, PC_{10}) was performed. In Annex H (figures H.1 (agonism)), graphical representations of all comprehensive runs performed are given (relative induction (%) as function of exposure concentrations). Fit statistics and blind coded test item characteristics are summarised in Annex I.1.

For all test items, the analysis results obtained during 2 comprehensive test runs showed to be reproducible. Three of the blind coded test items elicited an agonistic response (43397, 43406 and 43408). For each of the two independent consecutive comprehensive test runs, test items were classified according to the agonistic classifier given in table 6. The final classification of blind coded test items based on the agonist classifiers, is presented in table 12.

5.2.2.2 Antagonism

During the antagonist pre-screen of the test items the dilution steps as well as the start concentration for all test items were determined (see table 11). In Annex D.3 the refined concentration series of the test items used for each of the independent comprehensive test runs are presented. Following each independent comprehensive run, data was evaluated for compliance to the acceptance criteria. All microtiterplates were used for further evaluation of antagonistic properties of the test items although some of the microtiterplates did not meet the acceptance criteria (|CV| of estimated log(IC50), IF of Diclazuril or R2 specificity outside acceptance range). Test items that were classified following pre-screen testing as positives, were analysed two times in 2 independent comprehensive runs. Test items that were classified as negatives were only analysed comprehensively once.

To identify whether any anti-thyroidogenic activity observed is a specific response, all test items showing anti-thyroidogenic activity were evaluated for specificity by testing these test items twice on the same microtiter plate. During the first exposure (top of the microtiter plate),

TRβ CALUX cells were exposed to the refined concentration series of test items in the presence of the approximate EC_{50} concentration of the TRβ agonist T3. In the second exposure (bottom of the microtiter plate), TRβ CALUX cells were exposed to the same test item in the presence of an excess TRβ agonist T3 (100-times approximate EC_{50} concentration).

Reference and test item analysis results, including specificity analysis results, were evaluated by calculating the average of each accepted triplicate analysis. Next, the average response observed after exposure TRβ CALUX cells to the maximum concentration of the antagonist reference compound (100% inhibition) was subtracted from all individual measurements. During antagonistic analyses of blind coded test items, it was observed that in many cases the luminescence measured in outer rows of the 96-microtiterplate differed from luminescence observed in inner-wells of 96-microtiterplates (luminescence lower in outer wells). Because the solvent control triplicates are located at outer wells, setting the maximum induction at 100% based on the solvent control results in an overestimation of the relative response of reference compound and test items. Therefore, for further evaluation of antagonistic properties, it was decided that the relative induction of the reference compound and test items were calculated based on the response observed in the two highest dilutions of the reference compound and test items respectively. In case of normalisation of test items, this was only performed if at the highest 2 dilutions of test item, a plateau could be observed.

The statistical software package Graphpad Prism 5.0 was used to fit raw data (non-linear regression, variable slope, 4 parameters, robust fit, Hill equation). Using the same software package, quantitative assessment of anti-androgenic activity (RPC_{min}, IC_{50}, IC_{20}, PC_{50}, PC_{80}) of the evaluated reference standards and positive/negative control samples was performed. In addition, specificity was determined plotting the relative induction of the standard antagonistic analysis (Y_c) against the relative induction of the specificity analysis (S_c) and evaluating the correlation coefficient R^2. For all tested items, graphical representations of valid comprehensive test runs and their specificity evaluation are given in Annex H (figures H.2 (antagonism); H.3 (specificity)). Fit statistics and blind coded test item characteristics are summarised in Annex I.2.

All analysis results of the 14 blind coded test items were reproducible. Five of the blind coded test items showed a dose-dependent antagonistic response in the anti-TRβ CALUX bioassay (43395; 43396; 43400; 43404 and 43405). To evaluate whether the observed antagonistic effect caused by the 5 test items is specific, the specificity of the response was tested by addition of an excess of agonist reference compound T3. This specificity test indicated that 2 test items showed non-specific antagonism whereas 3 test items showed specific antagonists. Final classification of the blind coded test items for each of the two independent consecutive comprehensive test runs, was based on the antagonistic classifier given in table 7. Final classification of all reference and positive/negative control samples based on the antagonistic classifiers is presented in (table 12).

5.3 Final classification

Based on the results of all pre-screen and comprehensive test runs, the test items were classified using the classifiers given in table 6 (agonism) and table 7 (antagonism). In table 12, the final classification of all test items is presented.

Table 12 Final classification agonism and antagonism

TRβ CALUX (agonism)		anti-TRβ CALUX (antagonism)	
Test item	**Classification**	**Test item**	**Classification**
43395	negative	43395	positive
43396	negative	43396	inconclusive[a]
43397	positive	43397	negative
43398	negative	43398	negative
43399	negative	43399	negative
43400	negative	43400	negative[b]
43401	negative	43401	negative
43402	negative	43402	negative
43403	negative	43403	negative
43404	negative	43404	negative[b]
43405	negative	43405	positive
43406	positive	43406	negative
43407	negative	43407	negative
43408	positive	43408	negative

a – first comprehensive analysis indicated only 1 concentration showing a result below 80% whereas the second comprehensive analysis indicated >2 concentrations causing a relative induction below 80%. Therefore, the compound is classified as inconclusive. Normally, a third analysis has to be performed for confirmation.

b – test item showed an antagonistic response in the anti-TRb CALUX bioassay. Specificity testing showed that the observed antagonistic response of the test item was non-specific and hence, the test item showed non-specific antagonism. Based on specificity test, test item is classified as “negative” based on the antagonistic classifiers.

5.4 Intra-lab coefficient of variance and reproducibility

For agonistic and antagonistic analyses, the intra-laboratory coefficient of variance (%CV) was calculated based on the log(EC_{50}) and log(PC_{10}) (agonism) and the log(IC_{50}) and log(PC_{80}) (antagonism) for the reference standard T3 and Diclazuril respectively. For agonistic runs, a total of 9 analysed plates containing the agonist reference T3 were evaluated and for antagonistic runs, also a total of 9 analysed plates containing the reference standard Diclazuril were evaluated. In table 13, the log(EC_{50}), log(PC_{10}), log(IC_{50}) a log(PC_{80}), values for the reference standards are given. From the obtained data, the %CV for the agonistic and antagonistic runs were calculated. In all cases, the %CVs for the agonistic or antagonistic reference compounds were below 5%.

The acceptance range for the EC50 and IC50 concentrations of the agonist and antagonist reference item T3 and Diclazuril as indicated in the acceptance criteria, are between 2.0E-11 – 2.0E-10 and 1.9E-07 – 1.9E-06 M respectively. Evaluation of the EC50 values measured during the present agonistic studies showed that 2 of the T3 reference series showed EC50 values slightly above the indicated criteria (2.9E-10 and 3.1E-10 M). For the antagonistic analyses, only 1 Diclazuril reference series showed and IC50 below the indicated acceptance range.

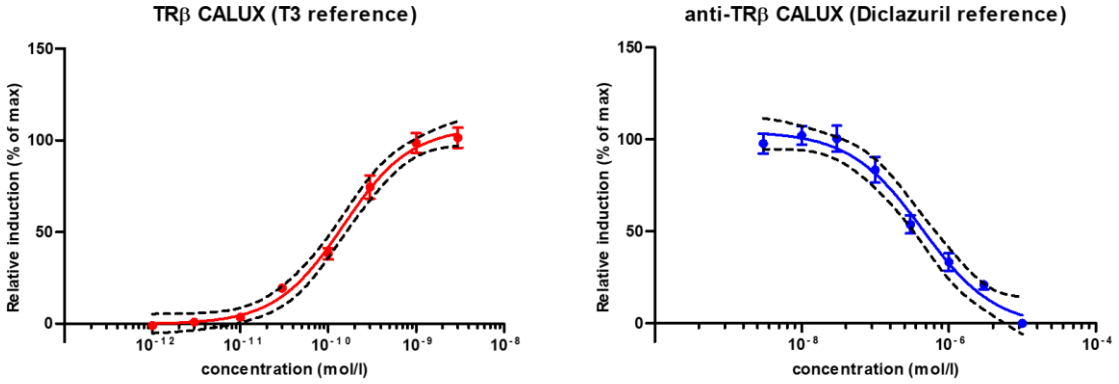
Not for all reference standards the acceptance criteria as indicated in the standard operation procedures (SOP03-TRb01_v8 agonism; SOP04-TRb01_v12 antagonism) were met. For antagonistic measurements, the |CV| of estimated log(IC_{50}) (antagonism) for the reference item Diclazuril regularly exceeded the indicated limit of 3% (see tables 15). Closer evaluation of results showed that relatively high standard deviations between triplicate measurements were observed and as a result, the calculated |CV| log(IC50) did not meet the acceptance criteria in 4 out of 9 antagonistic analysis runs. Furthermore, during 3 of the 4 antagonistic comprehensive test-runs, analysis results for the PC, NC and VC deviated from results obtained during the other antagonistic measurements. With respect to the observed high standard deviation between triplicate measurements on a plate, they were at least partially the result of low relative light units often measured in the outer rows of the 96-microtiterplates. Relatively low luminescence in the outer-exposed rows of 96-microtiterplates is observed more often, also for test items resulting in relatively high standard deviation in triplicate measurements of the same test concentration on a plate.

As indicated previously (section 5.2.2.2), the maximum fold-induction determined for each plate was low and often did not meet acceptance criteria during comprehensive antagonistic measurements in part 2 of the validation study (see Annex I, table I.2). In case the measured inducibility is low, any lower activity observed in outer rows may contribute significantly to higher relative standard deviations in triplicate measurements. Although not all analysis results from the reference compounds met the acceptance criteria, the data were used for further evaluation of agonistic and antagonistic potency in the TRβ CALUX.

In figure 1, the average percentage of induction of the reference agonist T3 and reference antagonist Diclazuril obtained from all runs (pre-screen and comprehensive) are given. In the table below the figure, the 95% confidence intervals for the top / bottom, log[EC50] / log[IC50] and Hill-slope are given. Clearly, a strong intra-laboratory consistency is observed.

Table 13 Intra-laboratory coefficient of variance (%CV) based on the reference standards T3 log(EC_{50}) and log(PC_{10}) values for agonistic studies and the reference standards Diclazuril log(IC_{50}) and log(PC_{80}) values for antagonistic studies, determined during all pre-screen and comprehensive runs.

	Agonism		Antagonism	
	log(PC10)	log(EC50)	log(PC80)	log(IC50)
Part 1 - prescreen 1.1	-10.77	-10.02	-7.04	-6.23
Part 1 - comprehensive 1.1	-10.79	-9.90	-7.19	-6.13
Part 1 - comprehensive 1.2	-10.87	-10.11	-7.10	-6.39
Part 2 -prescreen 2.1	-10.10	-9.51	-7.10	-6.22
Part 2 - prescreen 2.2	-10.19	-9.57	-7.62	-6.82
Part 2 - comprehensive 2.1	-10.73	-9.89	-6.64	-6.00
Part 2 - comprehensive 2.2	-10.77	-9.89	-6.77	-6.30
Part 2 - comprehensive 2.3	-10.83	-9.85	-6.75	-6.40
Part 2 - comprehensive 2.4	-10.80	-9.77	-7.27	-6.30
avg	-10.65	-9.84	-7.05	-6.31
sd	0.29	0.19	0.30	0.23
%CV	2.73	1.97	4.28	3.59



	Agonism	Antagonism
Top / bottom	97 to 117	-22 to 18
Log[EC_{50}] / Log [IC_{50}]	-10 to -9.7	-6.6 to -6.1
Hill Slope	0.77 to 1.48	-1.3 to -0.42
R^2	0.9796	0.9508

Figure 1 Average T3 (agonism; left) and Diclazuril (antagonism; right) dose-response curves from all valid pre-screen and comprehensive runs. Solid lines indicate average fit. Dotted lines indicate 95% confidence. In the table, the 95% confidence intervals of fitted T3 and Diclazuril dose-response data are given.

Table 14 Reference item T3 analysis characteristics indicated as acceptance criteria for agonism pre-screen / comprehensive testing

	%CV (LOG[EC50])	EC50 (M)	PC RI%	NC RI%	IF	Z-factor
Part 1 prescreen	0.56	9.5E-11	92	0.5	8.7	0.56
Part 1 compr. 1	0.77	1.3E-10	48	1.3	8.7	0.65
Part 1 compr. 2	0.75	7.7E-11	87	2.6	7.1	0.68
Part 2 prescreen 1	0.46	3.1E-10	48	2.6	9.9	0.65
Part 2 prescreen 2	0.35	2.7E-10	52	3.2	10.6	0.76
Part 2 compr. 1	0.60	1.3E-10	58	3.9	9.7	0.68
Part 2 compr. 2	0.58	1.3E-10	68	4.0	9	0.68
Part 2 compr. 3	1.18	1.4E-10	49	3.4	12.2	0.48
Part 2 compr. 4	0.87	1.7E-10	62	5.3	9.9	0.63

Table 15 Reference item Diclazuril analysis characteristics indicated as acceptance criteria for antagonism pre-screen / comprehensive testing.

	%CV (LOG[IC50])	IC50 (M)	PC RI%	NC RI%	VC RI%	IF	Z-factor	R2 specificity
Part 1 prescreen	3.38	5.8E-07	-16	199	6.1	5.1	0.36	
Part 1 compr. 1	3.96	7.4E-07	-13	200	15	4.3	0.90	0.4549
Part 1 compr. 2	2.37	4.0E-07	-22	243	8.1	8.5	0.63	0.3654
Part 2 prescreen 1	2.49	6.0E-07	-16	244	7.4	6.4	0.56	
Part 2 prescreen 2	3.35	1.5E-07	-17	240	9.3	5.9	0.65	
Part 2 compr. 1	2.47	9.9E-07	-68	1039	45	2.2	0.74	0.8489
Part 2 compr. 2	1.68	4.9E-07	-68	1050	45	2.2	0.76	0.8256
Part 2 compr. 3	1.35	3.9E-07	-76	1152	42	2.1	0.76	0.8294
Part 2 compr. 4	4.67	5.0E-07	-16	325	25	5.5	0.65	0.5100

6 Discussion and recommendations

The TRβ CALUX is a stably transfected *in vitro* transactivation assay to detect thyroid receptor agonists and antagonists. TRβ CALUX cells express a functioning human thyroid receptor and contain a reporter gene (luciferase) under the control of an thyroid responsive element. An increase or decrease of thyroidogenic signalling, results in corresponding changes in the expression of luciferase activity in TRβ CALUX cells and is measured with a luminometer. Accordingly, TRβ CALUX cells can be used to detect chemicals with (anti)-thyroidogenic properties.

Organised and coordinated by EURL ECVAM, the TRβ CALUX bioassay was subject to validation according to OECD guidelines. The goal of the study was to determine whether the *in vitro* transactivation TRβ CALUX can be used for identification of modulators of thyroid hormone signalling and has validation potential. Prior to performing the validation study, BDS constructed standardised operating procedures and calculation documents. Furthermore, acceptance criteria were drafted. Both the SOPs, calculation documents and acceptance criteria are evaluated during the present validation study and results from the study may lead to adjustments, changes or refinements of procedures and acceptance criteria.

The validation study consisted of an intra-laboratory study performed in 2 parts. During the first part of the validation study, the thyroidogenic activities of the reference standards and positive/negative control standards for both the agonistic and antagonistic mode of the TRβ CALUX bioassay, were evaluated. During the second part of the validation study, several blind coded test items were obtained from EURL ECVAM and tested for their agonistic and antagonistic properties using the TRβ CALUX. For both parts, the validation consists of pre-screen testing and comprehensive testing. For antagonistic studies, additional specificity tests were included. Following comprehensive studies, test items were classified and positives, negatives or inclusive according to the classifiers indicated in the SOP.

In the present report, the results of the validation study performed by BDS are presented. The analyses were performed according to the SOP instructions except for the following procedures.

1 The reference compound stock solutions were not prepared fresh prior to each pre-screen or comprehensive study. Instead, at the start of the validation study, stock solutions for both agonist and antagonist reference compounds were prepared in DMSO after which the required dilution series in DMSO were prepared. These dilutions series were aliquoted in smaller portions and stored frozen at -20^{0}C. Prior to performing a series of pre-screen or comprehensive measurements, one stored reference dilution series was recovered from the freezer and allowed to adjust to room temperature before being used. Once used, this freshly thawed reference dilution series was not frozen again at -20^{0}C but stored at 4^{0}C. This reference dilution series was used for a maximum of 4 weeks after which it was discarded, and a new aliquoted reference dilution series was taken from the freezer and used for further measurements.

2 The present validation study was not performed using cultured cells, but with seeding cells. Seeding cells are CALUX cells cultured in large quantity after which they are frozen in liquid nitrogen in small aliquots sufficient to fill 4-6 96-well microtiterplates and expose the plates 1 day after seeding. The cells are not different from cells that are cultured; only the procedures for freezing and thawing are different. The major advantage of using seeding cells is that all cells have the same passage. Furthermore, when running multiple CALUX bioassays, no cell cultures of different CALUX cells have to be maintained.

3 Cell toxicity (cytotoxicity) was evaluated by using the cytotox CALUX cells as well as by visual inspection of the TRβ CALUX cells. The cytotoxicity control (Triton X-100) was not analysed on the TRβ CALUX cells. Instead, the cytotox CALUX reference standard TBT was used as positive control for cytotoxicity and tested in the cytotox CALUX bioassay.

During agonistic and antagonistic testing in part 1 and part 2 of the present validation study, cytotoxicity of test items was evaluated using the cytotox CALUX bioassay and visual inspection of the cells after exposure during pre-screen runs and the first comprehensive run. During the second comprehensive testing for agonism and antagonism, cytotoxicity was only evaluated using visual inspection of the cells.

4 During antagonistic studies of test items (part 2), it was observed that luminescence in outer rows of the 96-microtiterplates differed from luminescence emitted from inner-wells. Since all triplicate analyses of the DMSO (or solvent control) are located at outer rows, the average emitted light of the solvent control is relatively low. In contrast, not all triplicate measurements of reference standards and test items are located at the outer rows and hence, the average emitted light from reference standards and test items is different from the average emitted light from the solvent control. Since the measured light from the triplicate solvent control samples is used to set the maximum relative induction at 100%, the relative inductions of Diclazuril and test items are often overestimated. Because antagonism is scored in case the response of a test item decreases with 20% (80% relative induction), it was decided to normalise and calculate the relative induction of the reference compound and test items based on the response observed in the two highest dilutions of the reference series and test items respectively. In case of normalisation of test items, this was only performed if at the highest 2 dilutions of test item, a plateau could be observed.

5 Agonistic analyses were performed according to the plate-setup indicated in the SOP for part 1 pre-screen analyses only. For all other agonist measurement (part 1 and 2), the solvent control in wells E2-G2 was replace by the highest concentration C8 of the reference compound T3. As a consequence, the background induction of the SC used to correct for background activity of compounds on plate 1 was not based on the average of the results of the 6 wells E2-J2, but on the average of the results of the 3 wells E2-G2.

The intra-laboratory coefficient of variation (%CV) was assessed, based on the log(EC_{50}) and log(PC_{10}) (agonism) and the log(IC_{50}) and log(PC_{80}) (antagonism) for the reference standard T3 and Diclazuril respectively. For the TRβ CALUX bioassay, the %CV was found to be below 2.7% whereas for the anti- TRβ CALUX bioassay, the %CV was below 4.3%. These results indicate robustness and reproducibility of the (anti-) TRβ CALUX bioassay.

In the presented validation study, the agonist and antagonist reference compounds and positive and negative control items behaved as expected. For both the agonistic and antagonistic analyses, the reference compounds and positive control items showed to be active compounds and were classified as positives. The negative control standards did not show activity and were classified as negatives. With respect to the antagonist positive control compound TBBPA, the present validation study showed that the concentration of TBBPA indicated in SOP for antagonistic testing to be used as positive control concentration, is cytotoxic and therefore not suitable. The TBBPA concentration to be used as positive control should show an anti-thyroidogenic response at non-cytotoxic concentrations. Therefore, the TBBPA concentration indicated in the SOP for antagonistic testing has to be changed. The concentration TBBPA as positive control is suggested to be changed to a stock concentration of 1.0E-4 M (well concentration 1.0E-7M).

During part 2 of the validation study, agonistic pre-screening of the 14 blind coded test showed 3 compounds with agonistic properties. Following comprehensive testing of all test items, these three compounds were the only compounds that could be classified as positive samples. Following antagonistic pre-screening of the 14 blind coded test items, 5 test items showed antagonistic properties and were selected for further comprehensive testing. Following specificity testing of the 5 test items initially indicated as potential antagonists during pre-screen testing, 2 test items were shown to be non-specific antagonist. Furthermore, for one potential antagonist only 1 dilution showed a response below 80% during one of the comprehensive

studies. Based on these results, from 5 test items initially indicated as potential antagonist during pre-screen testing, 2 test items were classified as positive antagonists (specific antagonists), 1 test items was classified as inconclusive for which a third comprehensive test should be performed, and 2 test items were classified as negative antagonists (non-specific antagonists).

The calculation rules and procedures to evaluate analyses results in the present validation study deviated slightly from the SOPs. First of all, during the validation study it was observed that the amount of emitted light from outer wells was in many cases significantly lower than the measured response in inner wells. The reason for this is not entirely known. It has been hypothesized that disturbance in air flow during incubation in CO2 incubators could affect the behaviour of the cells. Therefore, a first measure that can be taken is to minimise the opening and closing of the CO2 incubator (to be indicated relevant the SOPs). Because of the observed lower response in outer wells, the standard deviation in triplicate analyses in the present study is relatively high. Especially during antagonistic measurements, relatively high standard deviations in triplicate measurement were observed and hence, the calculated |CV| Log(IC50) was also high. To solve this, the standard deviation in triplicate analyses should be reduced. As indicated above, strict rules with respect to opening CO_2 incubators containing exposed 96-well microtiterplates may be required. Another option is, in addition to keeping strict rules with respect to opening of the incubators, to alter the acceptance criteria and allow a |CV| for agonistic measurements of 2.5% and for antagonistic measurements of 5% (The suggested acceptance criterium for agonistic measurements is half the acceptance criterium of antagonistic measurements because the maximum induction during agonistic measurements (presence T3 EC100 concentration) is twice the maximum induction during antagonistic measurements (presence T3 EC50 concentration)).

Furthermore, the impact of different responses in outer wells is of significance for the anti-TRβ CALUX bioassay. In this assay, the solvent control (DMSO) is located at outer wells. This solvent control is used to set the relative induction of the reference compound at 100%. But if the measured light in the solvent control is lower than the measured light in the highest dilutions of the reference item and test items, the initial activity in cells exposed to the lowest concentrations of reference or test item is not calculated to be 100% but much higher. Evaluation of antagonism which is determined when a response is below 80% relative induction, can be impaired by the incorrect calculation of relative induction (100%). To overcome this problem, the maximum response of reference compounds and test items was set at 100% using the response of the highest 2 dilutions of the reference compound or test items respectively (lowest concentrations tested). To anticipate any problems arising from lower activities observed in solvent controls due to the “outer-well-effects”, using the response of the lowest two concentrations (highest 2 dilutions) of the reference compound Diclazuril to calculate relative inductions of the reference compound is suggested. For test items a similar approach is suggested: the response of the lowest two concentrations (highest 2 dilutions) of a test item is used to calculate the relative inductions of this test item rather than using the solvent control response measured on this plate.

Another observation in the present validation study is the relatively low induction rate (fold induction) observed in the antagonistic modus of the TRβ CALUX bioassay. Especially during the first comprehensive analyses of part 2, the induction factor (IF) was low and did not meet acceptance criteria (antagonistic comprehensive runs 1, 2 and 3). These antagonistic measurement series were all performed at the same time. In addition to the low IF, the PC, NC and VC analysis results in these series of analyses also deviated from other antagonistic comprehensive measurements performed on other days. Many factors could contribute to deviating analysis results, such as impaired cell performance or any other external factor influencing the performance of the cells. Based on the evaluation of the acceptance criteria, a third comprehensive antagonistic analysis run should have been performed.

The R2 specificity acceptance criterion for the reference compound Diclazuril (R2 ≤ 0.7) was also not met during the 3 antagonistic comprehensive runs mentioned above. In all 3 cases, the R2 was below 0.9. R2 specificity is also used as classifier for positive competitive antagonists. In this case, compounds are considered as competitive antagonist in case the R2 ≤ 0.9. The more stringent rule for the antagonist reference compound was based on the acceptance criterion previously determined for the anti-AR CALUX reference compound flutamide for which ample of data was available. For Diclazuril however, not much data was available on specificity. Therefore, the indicated R2 specificity limit of 0.7 might be to strict. Additional measurements and information is required to set a value for the R2 specificity of the anti-TRβ CALUX reference compound Diclazuril. Until more data is available, it is recommended to set the R2 specificity at 0.9 as for classification of test items.

To improve the IF in antagonistic analyses, it was investigated if using a higher concentration of the T3 agonist as fixed concentration in all antagonistic measurements instead of the EC50 now described in the SOP would be feasible. In Annex J the results of this study are summarised. Using a fixed concentration of T3 resulting in 60% induction in the agonistic modus (EC60) improved the fold induction significantly without a major change in the IC50 of Diclazuril. Therefore, it is proposed to change the concentration of T3 added to all wells in the antagonistic modus of the TRβ CALUX bioassay from the EC50 concentration into the EC60 concentration. For specificity testing this also implies to change T3 concentration from the 100xEC50 concentration into the 100xEC60 concentration. It should be noted that during the second antagonistic comprehensive run of part 2, the IF was found to be significantly higher than during the first comprehensive runs. This increase of IF was not because the EC60 concentration of agonist reference compound T3 was used for these analyses. In the present validation study, only addition of the EC50 concentration of the agonist reference compound T3 was used during measurement of antagonism. Finding higher IF-values during the second antagonistic comprehensive run also indicates that the first runs (all performed at the same time) deviated from normal analyses as indicated above.

Finally, the EC50 concentration of the agonist reference item T3 slightly exceed the acceptance criteria for T3 EC50 as indicated in SOP03-TRb01 twice. In the present validation study, the T3 reference stock solution was not prepared fresh prior to each pre-screen or comprehensive run. Instead, at the start of the validation study, a stock solutions agonist reference compounds T3 was prepared in DMSO after which the required dilution series in DMSO were prepared, aliquoted and stored frozen at -20^{0}C. Prior to performing a series of pre-screen or comprehensive measurements, one stored reference dilution series was recovered from the freezer and used. Once used, this freshly thawed reference dilution series was not frozen again at -20^{0}C but stored at 4^{0}C and used for a maximum of 4 weeks after which it was discarded, and a new aliquoted reference dilution series was taken from the freezer. To avoid possible deiodination of T3 and as a result a decrease in bioactivity and a shift of measured EC50 values towards higher concentration, it strongly advised to use freshly thawed T3 reference dilution series once and discard after use.

Suggestion for SOP adaptations and refinements:

1 Opening of CO2 incubators should be minimised as much as possible in order to not disturb the airflow

2 Change the concentration of the positive control TBBPA indicated in the SOP for antagonistic testing (SOP04-TRb01) into 1.0E-4 M (stock concentration)

3 In the antagonistic modus of the TRβ CALUX bioassay, calculation of the relative induction of Diclazuril reference compound and test items is based on the response of the highest 2 dilutions (lowest 2 concentrations) of the Diclazuril reference compound and test items respectively. With respect to calculation of the relative induction of test

items, using the 2 highest dilutions (lowest 2 concentrations) of test item can only be used in case at these dilutions/concentrations, a maximum plateau can be observed

4 Change the fixed concentration of T3 used during antagonistic measurements from the EC50 concentration into the EC60 concentration of T3. Also change the concentration T3 during specificity measurements from 100xEC50 to 100xEC60 concentration

5 Change the acceptance criterion for the calculated |CV| of estimated reference log(EC50) agonism) to 2.5%

6 Change the acceptance criterion for the calculated |CV| of estimated reference log(IC50) (antagonism) to 5%

7 Change the acceptance criterion for the specificity R^2 between S_c^n and Y_c for Diclazuril to 0.9

7 Conclusion

The present validation study of the human TRβ mediated stably transfected transactivation *in vitro* assay to detect thyroid receptor agonists and antagonists (TRβ CALUX) showed good reproducibility and robustness. Out of the 14 test items evaluated for agonistic and antagonistic androgen activity, 3 were classified as agonists and 2 were classified as antagonist. One test item was classified as inconclusive for antagonism. The results of the present validation study also give reason to adjust or refine some points in the SOPs (acceptance criteria) and calculation documents.

Annex A - List of Standard Operating Procedures (SOPs), calculation documents and forms drafted by BDS for the transcriptional (anti-)TRβ CALUX bioassay validation study

Standard Operating Procedures (SOPs)	Document type
SOP supplement - Definitions an abbreviation	Word
SOP01-TRb01_v3 - Working with U2OS CALUX seeding cells	Word
SOP02-TRb01_v3 - Working with U2OS CALUX cultured cells	Word
SOP03-TRb01_v8 - Transactivation assay thyroidogenic potential using TRβ-CALUX cells	Word
SOP04-TRb01_v12 - Transactivation assay anti-thyroidogenic potential using TRβ-CALUX cells	Word
SOP05-TRb01_v3 - Test item stock solution solubility and medium stability	Word
SOP06-TRb01_v2 - List of definitions, abbreviations and calculation parameters	Word

Calculation documents	Document type
DAT02-TRb06_v7 - mg per ml	Excel
DAT02-TRb06_v8 - mol per l	Excel
DAT04-TRb06_v9 - mg per ml	Excel
DAT04-TRb06_v10 - mol per l	Excel
DAT05-TRb06_v10 - mg per ml	Excel
DAT05-TRb06_v10 - mol per l	Excel
DAT06-TRb06_v3 - mg per ml	Excel
DAT06-TRb06_v3 - mol per l	Excel
DAT07-TRb06_v5	Excel
DAT08-TRb06_v2	Excel
DAT09-TRb06_v4	Excel

Forms	Document type
FRM01-TRb06_v1 - medium	Word
FRM02-TRb06_v1 - CELLS	Word
FRM02-TRb15-v1 - medium stability	Word
FRM04-TRb06_v1 - QC-CELLS	Word
FRM07-TRb06_v2 - TI-for-PS	Word
FRM08-TRb06_v2 - TI-for-CT mg per ml	Word
FRM08-TRb06_v2 - TI-for-CT mol per l	Word
FRM09-TRb01_v2 - Reference and control items stock solutions	Word

Annex B - List of most essential equipment required to perform (anti-)TRβ CALUX bioanalyses

Material - consumables	Supplier *	Cat. Number
Caps with Teflon inlay for flat bottom vials	e.g. Fisher Scientific	11792428
Cell culture flasks 75 cm^2	e.g. Corning	3275 or 430641
Cryovials (1.5 to 2 ml)	e.g. Greiner	121261
Filter sterilization unit	e.g. Nalgene	
Flat bottom transparent culture plates, 24 wells	e.g. Greiner	Cell star® 662160
Flat bottom transparent culture plates, 96 wells	e.g. Greiner	Cell star® 655180
Plastic tubes (12-15 ml) with cap, sterile	e.g. Greiner/Falcon	
Plastic tubes (50 ml) with cap, sterile	e.g. Greiner/Falcon	
Reagent-reservoirs (sterile)	e.g. Costar	4870
Sterile pipette filtertips, for different volumes (e.g. 1-10µl, 100µl, 200 µl, 1000 µl).		
Sterile pipettes, 1, 2, 5, 10 and 25 ml		
Sterile pipette tips, 10, 100, 200, 1000 and 5000 µl		
Vials, flat bottom, 1,5 ml	e.g. Fisher Scientific	11565874

Materials - equipment
Analytical balance suitable to accurately weigh 10 mg.
Centrifuge with swing-out rotor for 50 ml centrifuge tubes
CO_2 incubator
Haemocytometer e.g. Bürker-Türk counting chamber
Inverted microscope (culture microscope) with 4´, 10´ and preferably 20´ objective
Low temperature storage such as liquid nitrogen container or -150 °C freezer, for storage of cryovials.
Luminometer for 96-well plates, preferably with 2 injectors
Multichannel pipette for 30 ml, 100 ml and 200 ml
pH meter
Pipettes for accurate pipetting volumes between 1 and 5000 ml
Pipettes, different types e.g. 0.5-10 µl, 100-200 µl and 1000 µl.
Plate shaker for horizontal shaking at 300 rpm.
Safety-cabinet or glove box for the safe handling of chemicals.
Vortex
Water bath, set at 37°C

* When there is no supplier or catalogue number suggested, there are no specific requirements

Luminometer setting:
1) Inject 100 µl illuminate mix
2) Measure the light response for 4 seconds
3) Inject 100 µl NaOH

A full list of materials and equipment required for performing CALUX bioanalyses are given in the various standard operating procedures (SOPs; see Annex A)

Annex C - Reference and positive/negative control standards concentration series used for agonistic and antagonistic testing (part 1)

Table C.1 Concentration series of reference and positive/negative control samples used for pre-screen testing

Test item	Dilution							
	1	10	100	1000	10000	100000	1000000	10000000
	Concentration in the well (M)							
T3	3.00E-06	3.00E-07	3.00E-08	3.00E-09	3.00E-10	3.00E-11	3.00E-12	3.00E-13
T4	1.00E-05	1.00E-06	1.00E-07	1.00E-08	1.00E-09	1.00E-10	1.00E-11	1.00E-12
BPA	1.00E-04	1.00E-05	1.00E-06	1.00E-07	1.00E-08	1.00E-09	1.00E-10	1.00E-11
Diclazuril	1.00E-04	1.00E-05	1.00E-06	1.00E-07	1.00E-08	1.00E-09	1.00E-10	1.00E-11
TBBPA	1.00E-04	1.00E-05	1.00E-06	1.00E-07	1.00E-08	1.00E-09	1.00E-10	1.00E-11

Table C.2 Concentration series of reference and positive/negative control samples used for agonistic comprehensive testing

Test item	Dilution							
	C1	C2	C3	C4	C5	C6	C7	C8
	Concentration in the well (M)							
T3	1.00E-09	3.03E-10	1.00E-10	3.03E-11	1.00E-11	3.03E-12	1.00E-12	3.03E-13
T4	1.00E-07	3.03E-08	1.00E-08	3.03E-09	1.00E-09	3.03E-10	1.00E-10	3.03E-11
BPA	1.00E-04	2.00E-05	4.00E-06	8.00E-07	1.60E-07	3.20E-08	6.40E-09	1.28E-09
Diclazuril	1.00E-05	2.00E-06	4.00E-07	8.00E-08	1.60E-08	3.20E-09	6.40E-10	1.28E-10
TBBPA	1.00E-06	2.00E-07	4.00E-08	8.00E-09	1.60E-09	3.20E-10	6.40E-11	1.28E-11

Table C.3 Concentration series of reference and positive/negative control samples used for antagonistic comprehensive testing

Test item	Dilution							
	C1	C2	C3	C4	C5	C6	C7	C8
	Concentration in the well (M)							
T3	3.00E-05	6.00E-06	1.20E-06	2.40E-07	4.80E-08	9.60E-09	1.92E-09	3.84E-10
T4	1.00E-05	2.00E-06	4.00E-07	8.00E-08	1.60E-08	3.20E-09	6.40E-10	1.28E-10
BPA	1.00E-04	5.00E-05	2.50E-05	1.25E-05	6.25E-06	3.13E-06	1.56E-06	7.81E-07
Diclazuril	3.00E-05	1.00E-05	3.00E-06	1.00E-06	3.00E-07	1.00E-07	3.00E-08	1.00E-08
TBBPA	3.00E-06	1.00E-06	3.00E-07	1.00E-07	3.00E-08	1.00E-08	3.00E-09	1.00E-09

Annex D - Test items concentration series used for agonistic and antagonistic testing (part 2)

Table D.1 Concentration series of test items used for pre-screen testing

Test item	Dilution							
	1	10	100	1000	10000	100000	1000000	10000000
	Concentration in the well (M)							
43395	1.00E-04	1.00E-05	1.00E-06	1.00E-07	1.00E-08	1.00E-09	1.00E-10	1.00E-11
43396	1.00E-04	1.00E-05	1.00E-06	1.00E-07	1.00E-08	1.00E-09	1.00E-10	1.00E-11
43397	1.00E-04	1.00E-05	1.00E-06	1.00E-07	1.00E-08	1.00E-09	1.00E-10	1.00E-11
43398	1.00E-04	1.00E-05	1.00E-06	1.00E-07	1.00E-08	1.00E-09	1.00E-10	1.00E-11
43399	1.00E-04	1.00E-05	1.00E-06	1.00E-07	1.00E-08	1.00E-09	1.00E-10	1.00E-11
43400	1.00E-04	1.00E-05	1.00E-06	1.00E-07	1.00E-08	1.00E-09	1.00E-10	1.00E-11
43401	1.00E-04	1.00E-05	1.00E-06	1.00E-07	1.00E-08	1.00E-09	1.00E-10	1.00E-11
43402	1.00E-04	1.00E-05	1.00E-06	1.00E-07	1.00E-08	1.00E-09	1.00E-10	1.00E-11
43403	1.00E-04	1.00E-05	1.00E-06	1.00E-07	1.00E-08	1.00E-09	1.00E-10	1.00E-11
43404	3.00E-05	3.00E-06	3.00E-07	3.00E-08	3.00E-09	3.00E-10	3.00E-11	3.00E-12
43405	1.00E-04	1.00E-05	1.00E-06	1.00E-07	1.00E-08	1.00E-09	1.00E-10	1.00E-11
43406	1.00E-04	1.00E-05	1.00E-06	1.00E-07	1.00E-08	1.00E-09	1.00E-10	1.00E-11
43407	1.00E-04	1.00E-05	1.00E-06	1.00E-07	1.00E-08	1.00E-09	1.00E-10	1.00E-11
43408	1.00E-04	1.00E-05	1.00E-06	1.00E-07	1.00E-08	1.00E-09	1.00E-10	1.00E-11

Table D.2 Concentration series of test items used for agonistic comprehensive testing

Test item	Dilution							
	C1	C2	C3	C4	C5	C6	C7	C8
	Concentration in the well (M)							
43395	1.00E-04	2.00E-05	4.00E-06	8.00E-07	1.60E-07	3.20E-08	6.40E-09	1.28E-09
43396	1.00E-04	2.00E-05	4.00E-06	8.00E-07	1.60E-07	3.20E-08	6.40E-09	1.28E-09
43397	1.00E-04	5.00E-05	2.50E-05	1.25E-05	6.25E-06	3.13E-06	1.56E-06	7.81E-07
43398	1.00E-04	2.00E-05	4.00E-06	8.00E-07	1.60E-07	3.20E-08	6.40E-09	1.28E-09
43399	3.00E-06	6.00E-07	1.20E-07	2.40E-08	4.80E-09	9.60E-10	1.92E-10	3.84E-11
43400	3.00E-06	6.00E-07	1.20E-07	2.40E-08	4.80E-09	9.60E-10	1.92E-10	3.84E-11
43401	1.00E-04	2.00E-05	4.00E-06	8.00E-07	1.60E-07	3.20E-08	6.40E-09	1.28E-09
43402	1.00E-04	2.00E-05	4.00E-06	8.00E-07	1.60E-07	3.20E-08	6.40E-09	1.28E-09
43403	3.00E-05	1.00E-05	3.00E-06	1.00E-06	3.00E-07	1.00E-07	3.00E-08	1.00E-08
43404	3.00E-05	6.00E-06	1.20E-06	2.40E-07	4.80E-08	9.60E-09	1.92E-09	3.84E-10
43405	1.00E-04	3.33E-05	1.00E-05	3.33E-06	1.00E-06	3.33E-07	1.00E-07	3.33E-08
43406	3.00E-07	1.00E-07	3.00E-08	1.00E-08	3.00E-09	1.00E-09	3.00E-10	1.00E-10
43407	3.00E-06	6.00E-07	1.20E-07	2.40E-08	4.80E-09	9.60E-10	1.92E-10	3.84E-11
43408	3.00E-08	1.00E-08	1.00E-09	1.00E-10	1.00E-11	1.00E-12	1.00E-13	1.00E-14

Table D.3 Concentration series of test items used for antagonistic comprehensive testing

Test item	Dilution							
	C1	C2	C3	C4	C5	C6	C7	C8
	Concentration in the well (M)							
43395	1.00E-04	5.00E-05	2.50E-05	1.25E-05	6.25E-06	3.13E-06	1.56E-06	7.81E-07
43396	1.00E-04	3.33E-05	1.00E-05	3.33E-06	1.00E-06	3.33E-07	1.00E-07	3.33E-08
43397	1.00E-04	2.00E-05	4.00E-06	8.00E-07	1.60E-07	3.20E-08	6.40E-09	1.28E-09
43398	1.00E-04	2.00E-05	4.00E-06	8.00E-07	1.60E-07	3.20E-08	6.40E-09	1.28E-09
43399	3.00E-06	6.00E-07	1.20E-07	2.40E-08	4.80E-09	9.60E-10	1.92E-10	3.84E-11
43400	3.00E-06	1.50E-06	7.50E-07	3.75E-07	1.88E-07	9.38E-08	4.69E-08	2.34E-08
43401	1.00E-04	2.00E-05	4.00E-06	8.00E-07	1.60E-07	3.20E-08	6.40E-09	1.28E-09
43402	1.00E-04	2.00E-05	4.00E-06	8.00E-07	1.60E-07	3.20E-08	6.40E-09	1.28E-09
43403	3.00E-05	6.00E-06	1.20E-06	2.40E-07	4.80E-08	9.60E-09	1.92E-09	3.84E-10
43404	3.00E-05	1.00E-05	3.00E-06	1.00E-06	3.00E-07	1.00E-07	3.00E-08	1.00E-08
43405	1.00E-04	3.33E-05	1.00E-05	3.33E-06	1.00E-06	3.33E-07	1.00E-07	3.33E-08
43406	3.00E-05	6.00E-06	1.20E-06	2.40E-07	4.80E-08	9.60E-09	1.92E-09	3.84E-10
43407	3.00E-06	6.00E-07	1.20E-07	2.40E-08	4.80E-09	9.60E-10	1.92E-10	3.84E-11
43408	3.00E-05	6.00E-06	1.20E-06	2.40E-07	4.80E-08	9.60E-09	1.92E-09	3.84E-10

Annex E - Graphical representations of cytotoxicity evaluation of reference and positive/negative control samples and blind coded test items using the cytotox CALUX bioassay

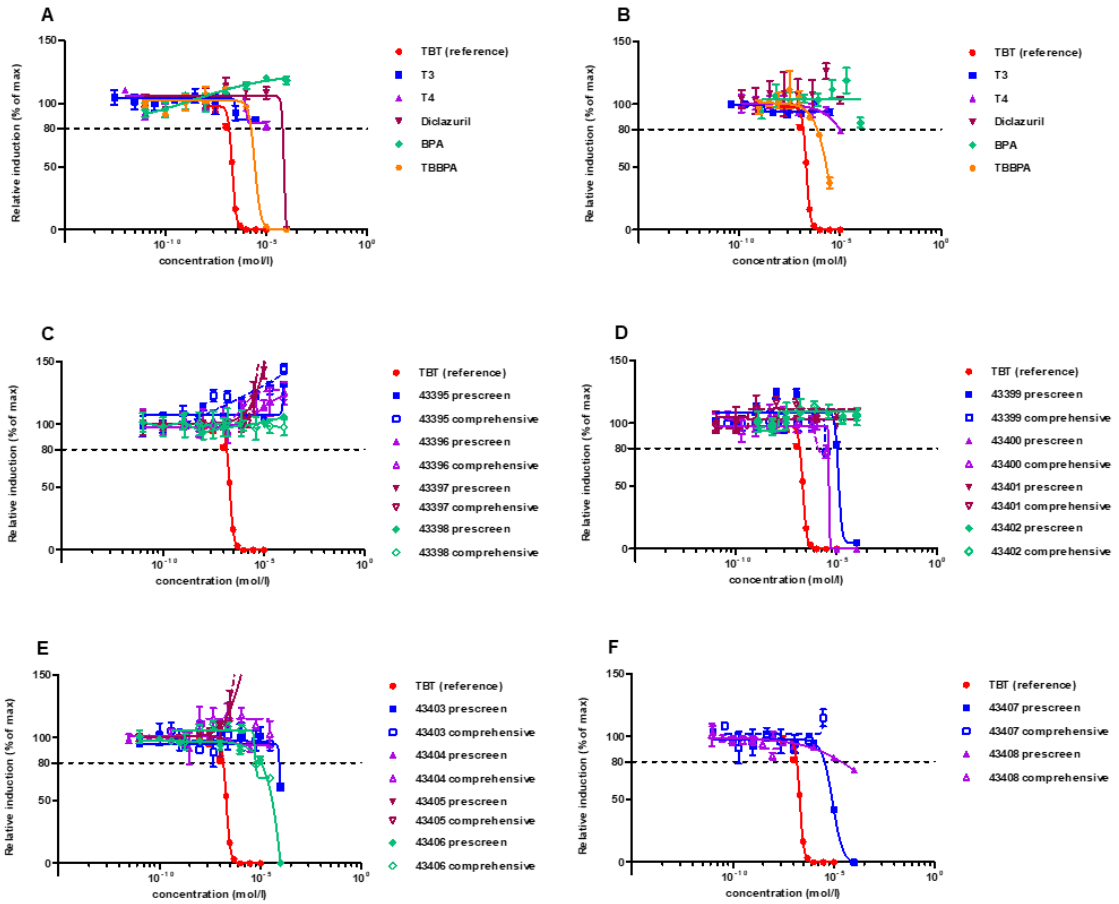


Figure E.1 Graphical representation of cytotox CALUX analysis results.
Figures A - B: part 1 cytotoxicity evaluation of reference and positive/negative control samples during pre-screen and comprehensive testing (figure A and B respectively)
Figures C - D: part 2 cytotoxicity evaluation of blind coded test items

To evaluate cytotoxic potency of reference and positive/negative control samples and blind coded test items in the present validation study, the cytotox CALUX bioassay is used. The cytotox CALUX bioassay comprises human bone cell lines (U2-OS), incorporating the firefly luciferase gene that is constitutively expresses and hence, light is constantly emitted. Exposure of the cytotox CALUX cells to compounds causing cytotoxicity, result in a reduction of luminescence. Compound concentrations causing >20% reduction of luminescence are considered cytotoxic.

Annex F - Graphical representations of all agonistic and antagonistic pre-screen and comprehensive analysis results of reference and positive/negative control standards (part 1)

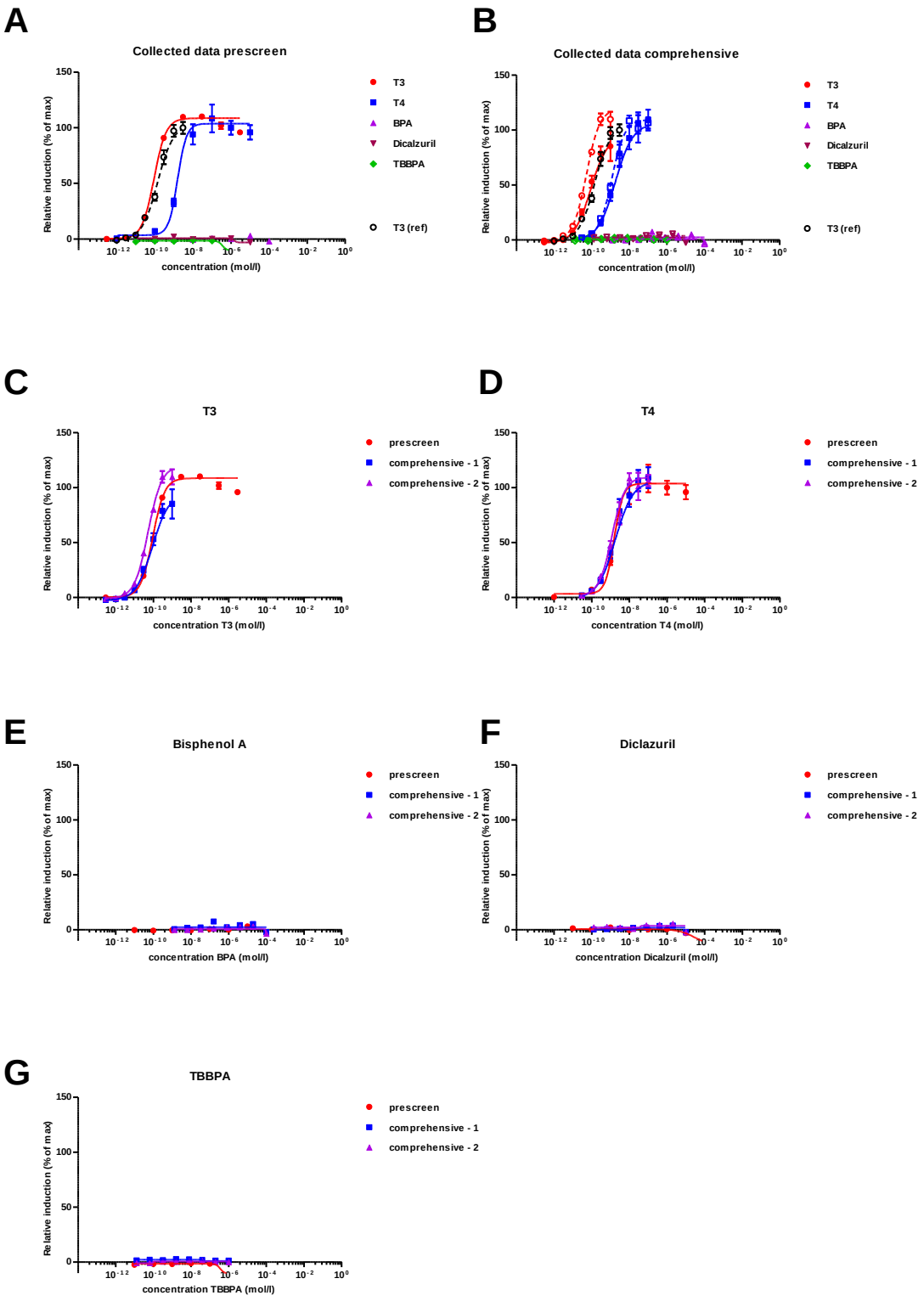


Figure F.1 Graphical representation of agonistic analyses results (part 1, reference standards and positive/negative control samples)

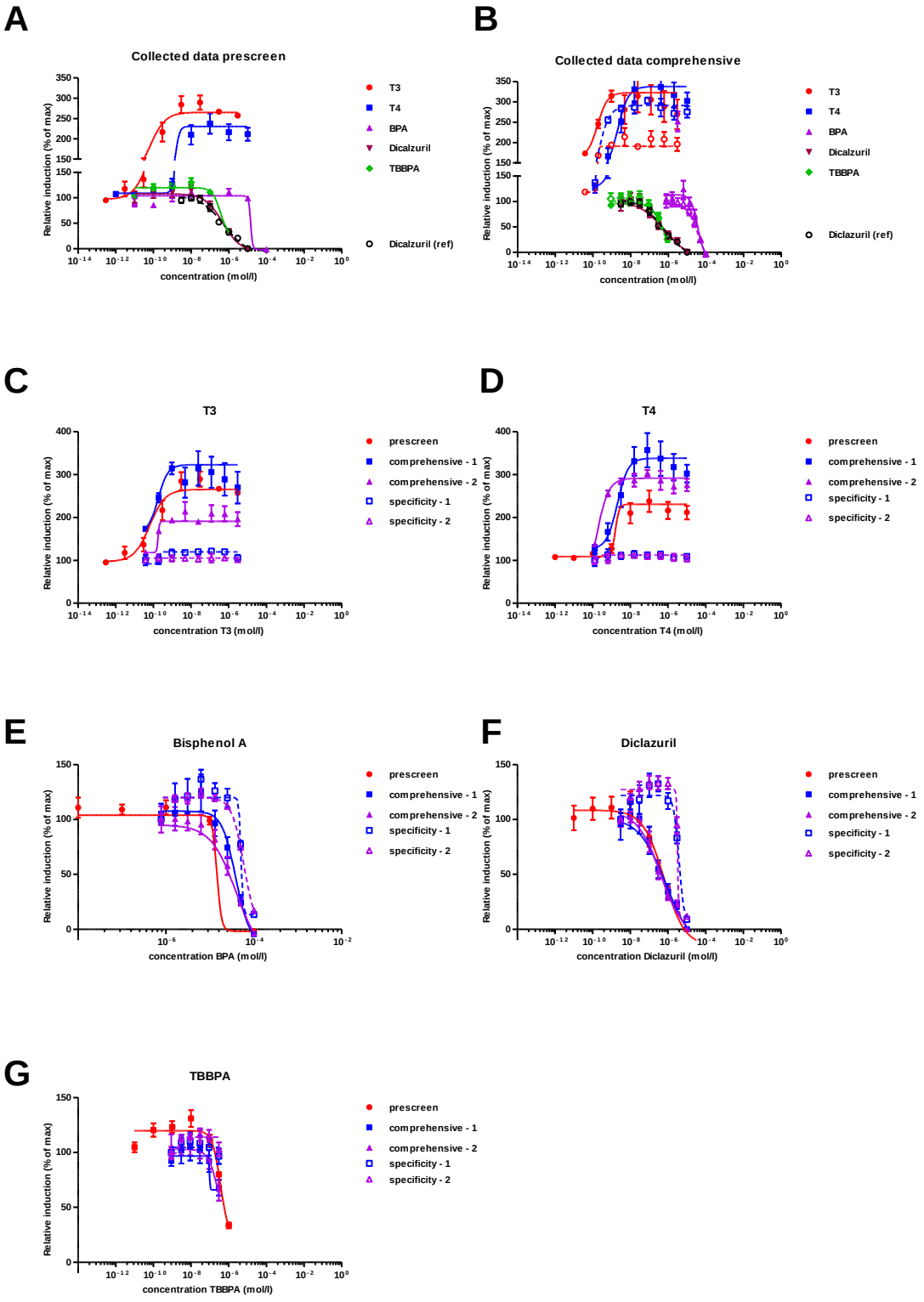


Figure F.2 Graphical representation of antagonistic analyses results (part 1, reference standards and positive/negative control samples)

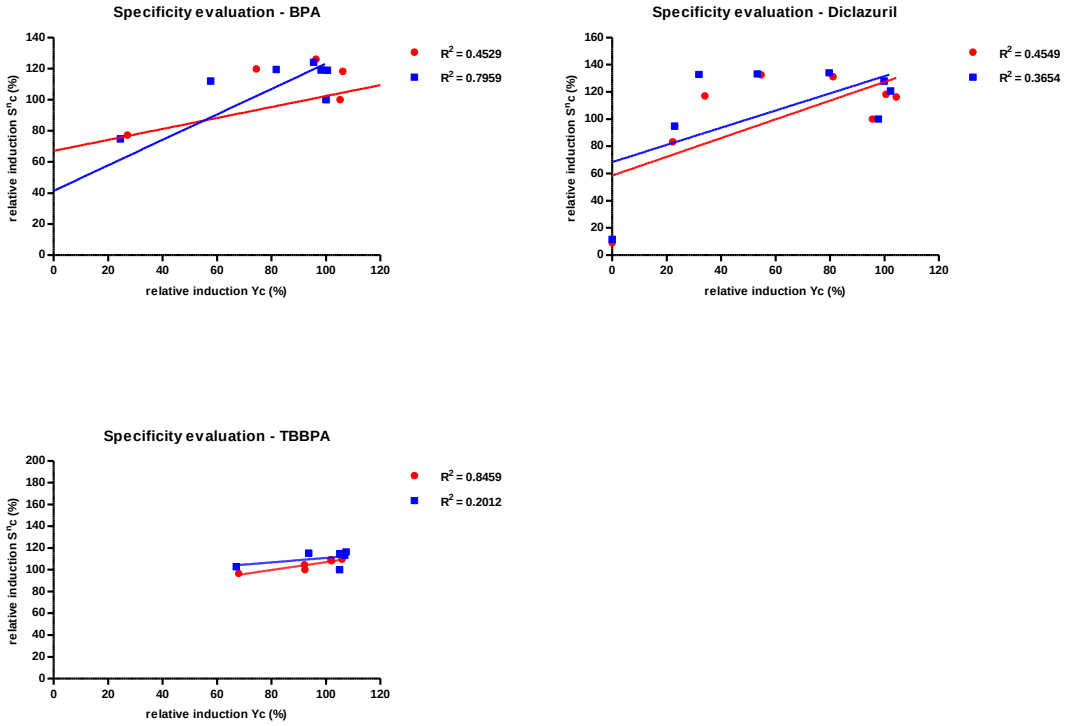


Figure F.3 Graphical representation of antagonistic specificity analysis results part 1

Annex G - Analysis characteristics agonistic and antagonistic comprehensive testing of reference and positive/negative control standards (part 1)

Annex G.1 Analysis characteristics agonistic comprehensive testing of reference standard and positive/negative control items.

	Calc. document	Plate	plate IF	z-factor	RPCmax (%)	Respons Yc > 10%
Comprehensive 1						
T3	TRb compr. 1	1	8.7	0.65	85.2	>2
T4	TRb compr. 1	2	8.2	0.69	109.1	>2
BPA	TRb compr. 1	2	8.2	0.69	<10	No
Diclazuril	TRb compr. 1	3	8.1	0.61	<10	No
TBBPA	TRb compr. 1	3	8.1	0.61	<10	No
Comprehensive 2						
T3	TRb compr 2	1	7.1	0.68	109.8	>2
T4	TRb compr 2	2	8.6	0.53	108.4	>2
BPA	TRb compr 2	2	8.6	0.53	<10	No
Diclazuril	TRb compr 2	3	8.5	0.50	<10	No
TBBPA	TRb compr 2	3	8.5	0.50	<10	No

	Calc. document	Plate	EC10 (M)	EC50 (M)	PC10 (M)	PC50 (M)	|CV| (log[EC50]) (%)
Comprehensive 1							
T3	TRb compr. 1	1	9.92E-12	8.82E-11	1.29E-11	1.04E-10	1.0
T4	TRb compr. 1	2	2.17E-10	1.79E-09	1.74E-10	1.61E-09	1.2
BPA	TRb compr. 1	2	(-)	(-)	(-)	(-)	(-)
Diclazuril	TRb compr. 1	3	(-)	(-)	(-)	(-)	(-)
TBBPA	TRb compr. 1	3	(-)	(-)	(-)	(-)	(-)
Comprehensive 2							
T3	TRb compr 2	1	9.01E-12	5.25E-11	7.81E-12	4.04E-11	0.42
T4	TRb compr 2	2	2.02E-10	1.18E-09	1.71E-10	1.00E-09	0.99
BPA	TRb compr 2	2	(-)	(-)	(-)	(-)	(-)
Diclazuril	TRb compr 2	3	(-)	(-)	(-)	(-)	(-)
TBBPA	TRb compr 2	3	(-)	(-)	(-)	(-)	(-)

Annex G.2 Analysis characteristics antagonistic comprehensive testing of reference standard and positive/negative control items.

	Document name	Plate	plate IF	z-factor	RPCmin (%)	Respons Yc < 80%
Comprehensive 1						
T3	Anti-TRb compr. 1	2	4.9	0.77	>80	No
T4	Anti-TRb compr. 1	3	4.8	0.55	>80	No
BPA	Anti-TRb compr. 1	4	5.1	0.64	-4.4	>2
Diclazuril	Anti-TRb compr. 1	1	4.3	0.90	0.0	>2
TBBPA	Anti-TRb compr. 1	5	5.7	0.60	-7.2	>2
Comprehensive 2						
T3	Anti-TRb compr. 2	2	7.1	0.64	>80	No
T4	Anti-TRb compr. 2	3	6.1	0.63	>80	No
BPA	Anti-TRb compr. 2	4	6.0	0.57	0.0	>2
Diclazuril	Anti-TRb compr. 2	1	8.5	0.63	0.0	>2
TBBPA	Anti-TRb compr. 2	5	5.8	0.44	0.0	>2

	Document name	Plate	IC20 (M)	IC50 (M)	PC80 (M)	PC50 (M)	|CV| (log[IC50]) (%)	Specificity R^2
Comprehensive 1								
T3	Anti-TRb compr. 1	2	(-)	(-)	(-)	(-)		
T4	Anti-TRb compr. 1	3	(-)	(-)	(-)	(-)		
BPA	Anti-TRb compr. 1	4	2.08E-05	3.68E-05	2.39E-05	3.65E-05	2.3	0.4529
Diclazuril	Anti-TRb compr. 1	1	7.92E-08	7.49E-07	6.36E-08	4.71E-07	4.0	0.4549
TBBPA	Anti-TRb compr. 1	5	2.15E-07	5.79E-07	1.66E-07	4.63E-07	2.4	0.7913
Comprehensive 2								
T3	Anti-TRb compr. 2	2	(-)	(-)	(-)	(-)		
T4	Anti-TRb compr. 2	3	(-)	(-)	(-)	(-)		
BPA	Anti-TRb compr. 2	4	1.58E-05	3.97E-05	1.07E-05	2.76E-05	3.6	0.7959
Diclazuril	Anti-TRb compr. 2	1	5.76E-08	3.96E-07	7.93E-08	4.03E-07	2.3	0.3654
TBBPA	Anti-TRb compr. 2	5	1.45E-07	3.51E-07	1.59E-07	3.95E-07	3.1	0.7517

Annex H - Graphical representations of all agonistic and antagonistic pre-screen and comprehensive analysis results of test items (part 2)

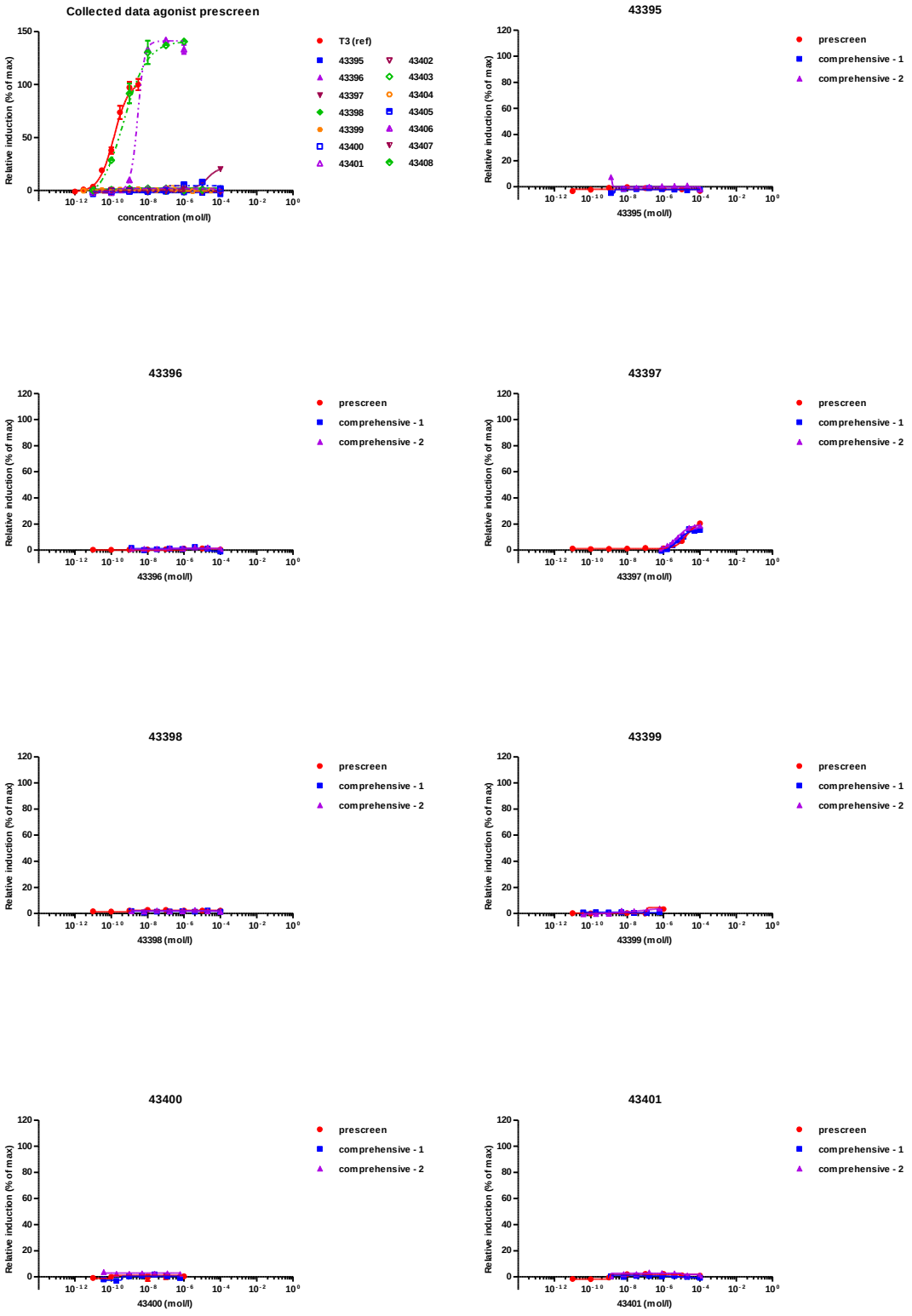


Figure H.1 Graphical representation of agonistic analyses results (part 2, test items)

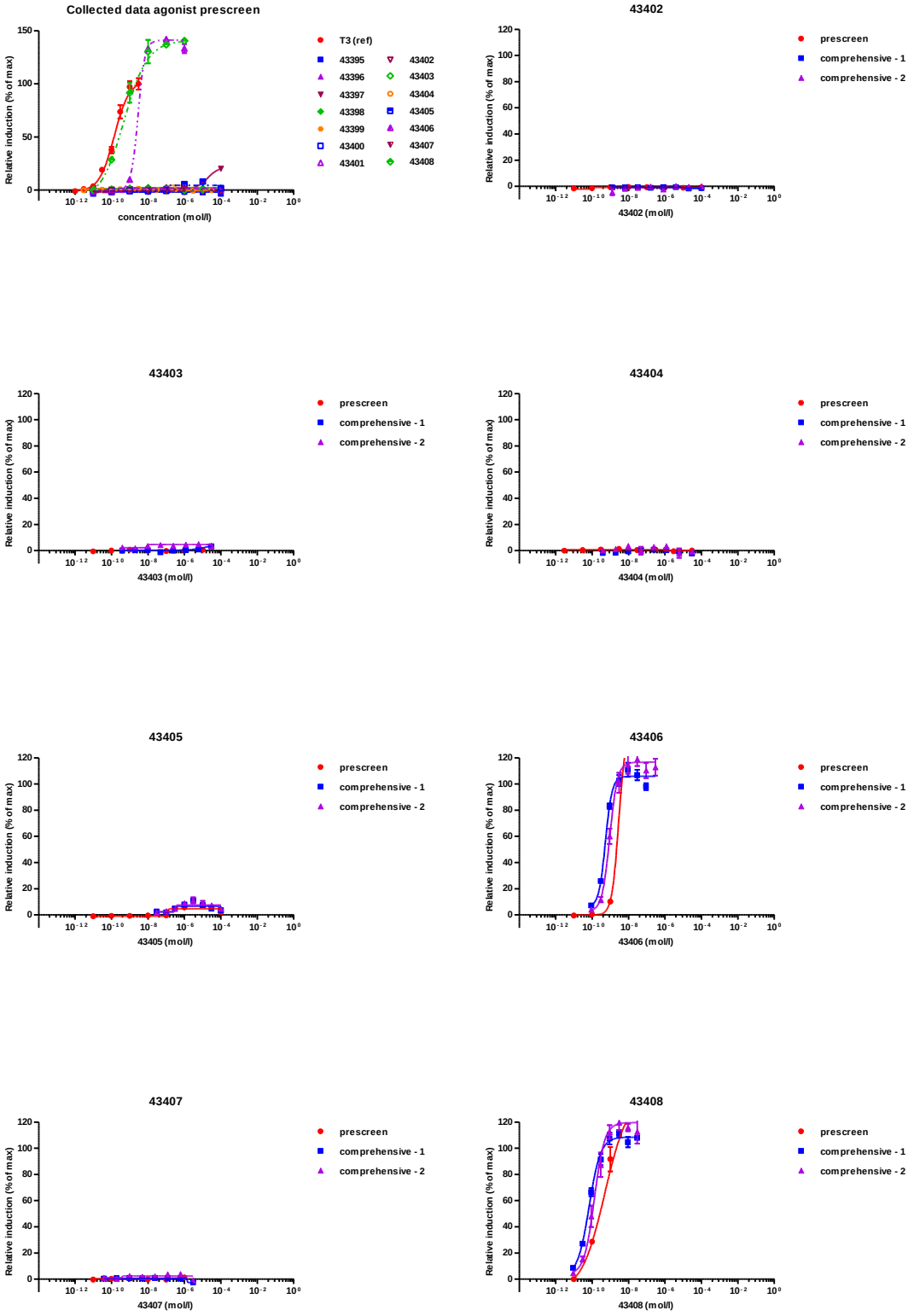


Figure H.1 Graphical representation of agonistic analyses results (part 2, test items)

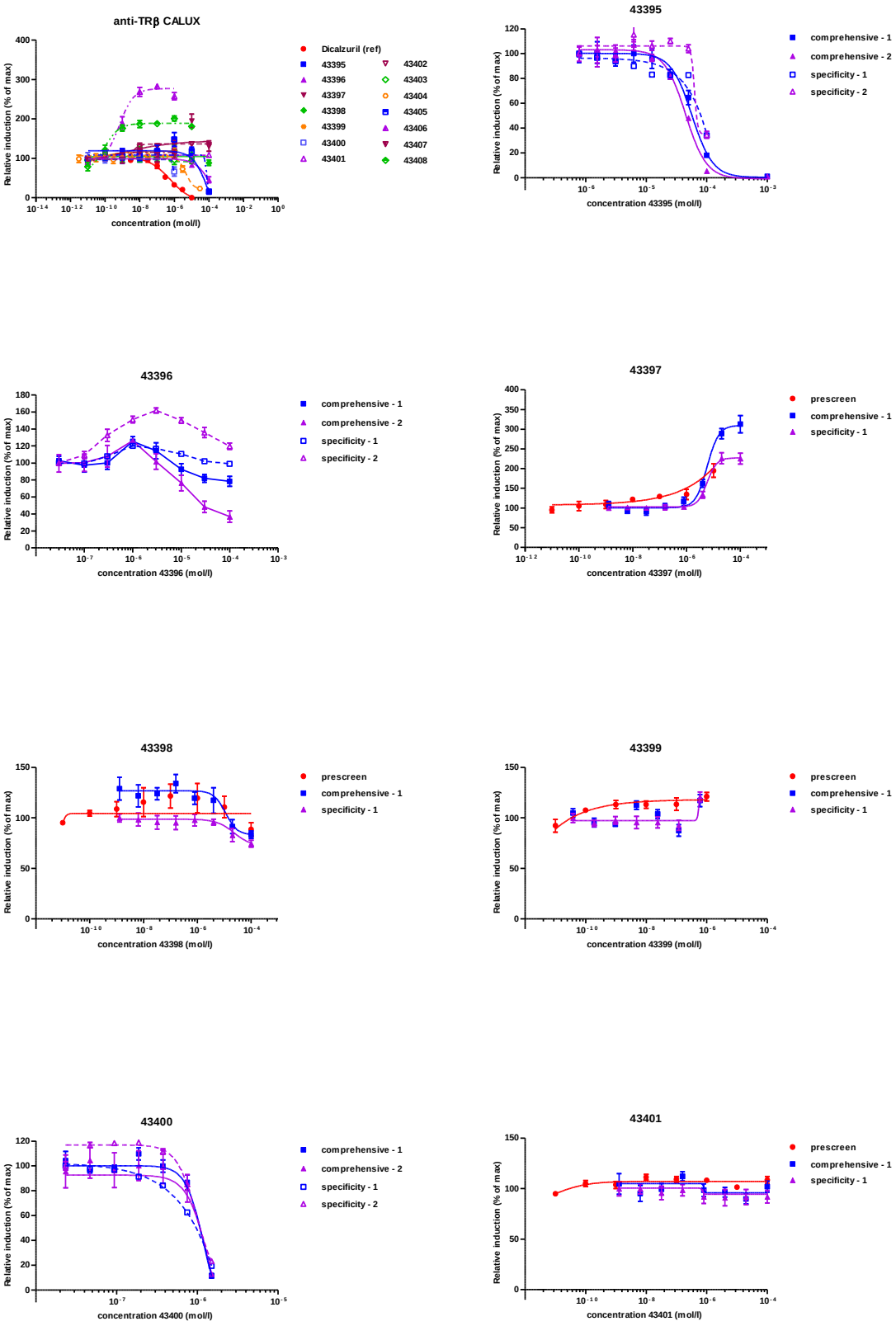


Figure H.2 Graphical representation of antagonistic analyses results (part 2, test items)

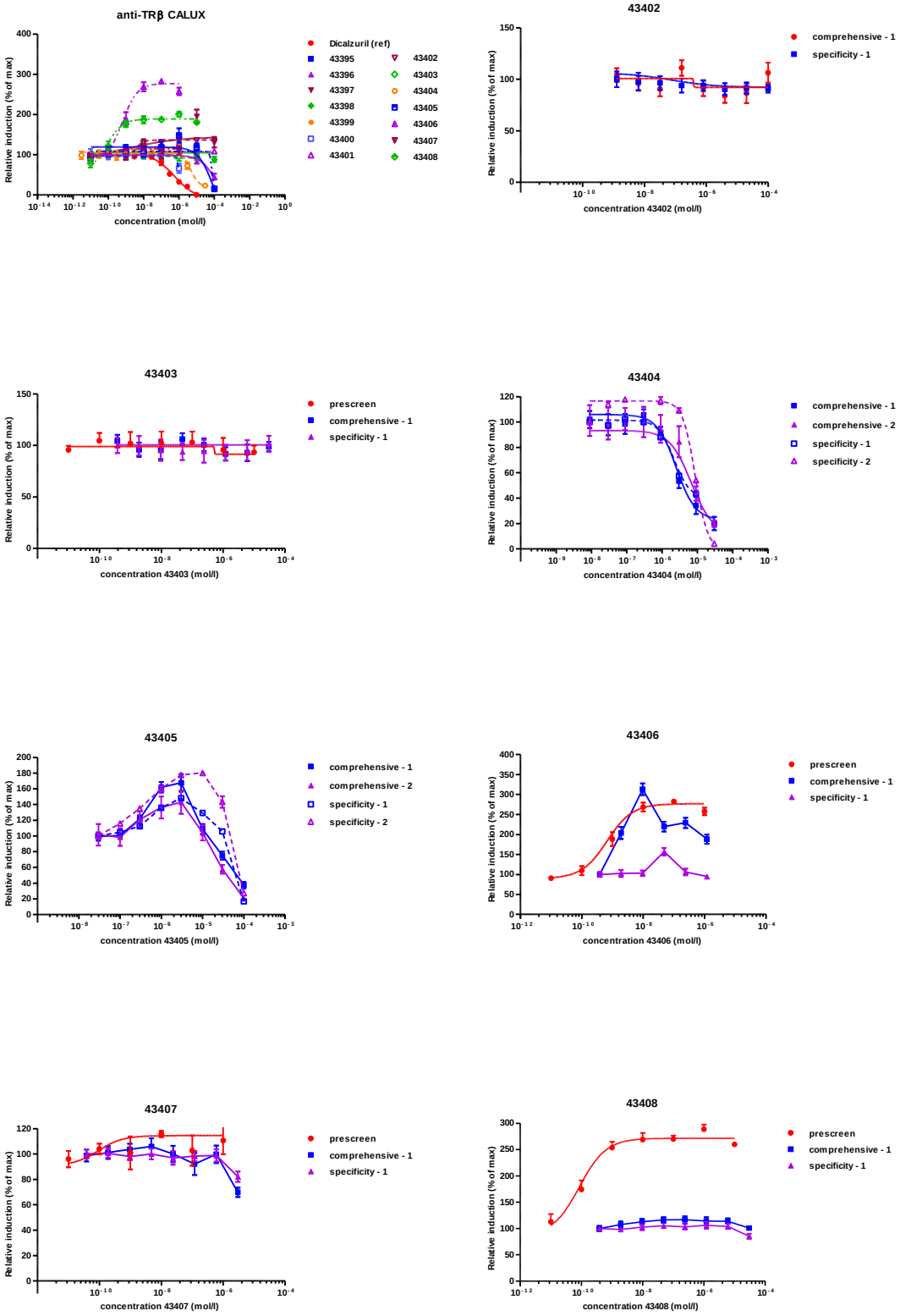


Figure H.2 Graphical representation of antagonistic analyses results (part 2, test items)

Note: for sample 43408 the maximum induction during pre-screen testing is much higher than during comprehensive testing. The reason for this is that for calculating the relative induction during comprehensive testing, the response of the lowest 2 concentrations tested was used as maximum induction. In contrast, during pre-screen testing, the lowest concentrations used for calculation the relative induction are lower and at these concentrations, the response of test item 43408 was significantly lower. Test item 43408 shows agonistic effects and at concentrations tested for antagonism, the agonistic response is at its maximum but it is used as maximum response for calculation relative induction of antagonistic effects.

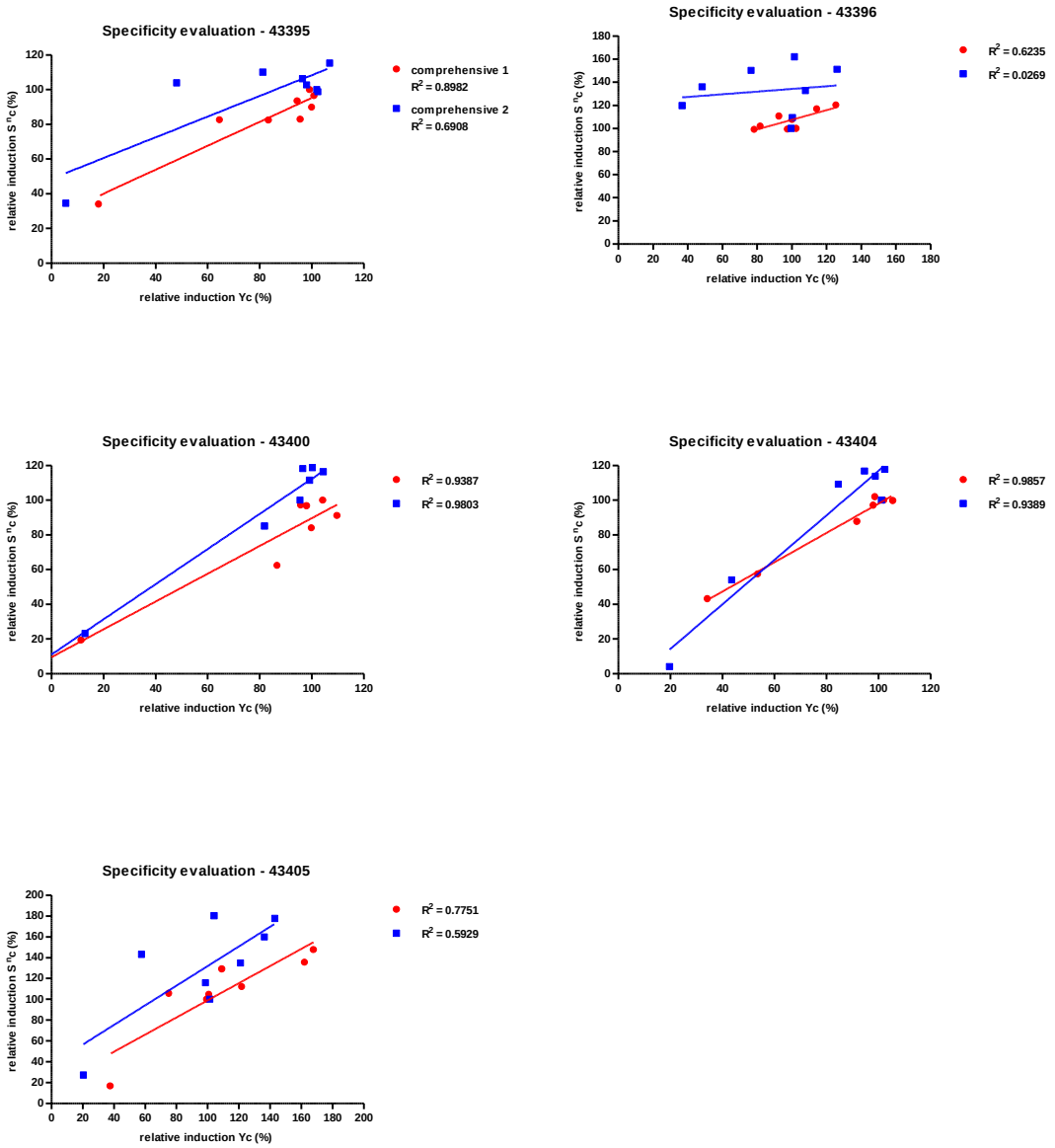


Figure H.3 Graphical representation of antagonistic specificity analyses results (part 2, test items)

Annex I - Analysis characteristics agonistic and antagonistic comprehensive testing of test items (part 2)

Annex I.1 Analysis characteristics agonistic comprehensive testing of blind coded test items.

	Document name	Plate	plate IF	z-factor	RPCmax (%)	Respons Yc > 10%	EC10 (M)	EC50 (M)	PC10 (M)	PC50 (M)	|CV| (log[EC50]) (%)
Comprehensive 1											
43395	TRb comp. 1-1	1	9.7	0.68	<10	No	(-)	(-)	(-)	(-)	(-)
43396	TRb comp. 1-1	2	9.2	0.70	<10	No	(-)	(-)	(-)	(-)	(-)
43397	TRb comp. 1-1	2	9.2	0.70	16.0	No	1.05E-06	5.56E-06	9.60E-06	(-)	1.3
43398	TRb comp. 1-1	3	10.7	0.81	<10	No	(-)	(-)	(-)	(-)	(-)
43399	TRb comp. 1-1	3	10.7	0.81	<10	No	(-)	(-)	(-)	(-)	(-)
43400	TRb comp. 1-1	4	9.7	0.63	<10	No	(-)	(-)	(-)	(-)	(-)
43401	TRb comp. 1-1	4	9.7	0.63	<10	No	(-)	(-)	(-)	(-)	(-)
43402	TRb comp. 1-2	1	9.0	0.68	<10	No	(-)	(-)	(-)	(-)	(-)
43403	TRb comp. 1-2	2	9.2	0.64	<10	No	(-)	(-)	(-)	(-)	(-)
43404	TRb comp. 1-2	2	9.2	0.64	<10	No	(-)	(-)	(-)	(-)	(-)
43405	TRb comp. 1-2	3	10.9	0.72	<10	No	(-)	(-)	(-)	(-)	(-)
43406	TRb comp. 1-2	3	10.9	0.72	105.9	>2	2.13E-10	5.38E-10	1.43E-10	4.87E-10	0.44
43407	TRb comp. 1-2	4	10.2	0.69	<10	No	(-)	(-)	(-)	(-)	(-)
43408	TRb comp. 1-2	4	10.2	0.69	108.5	>2	1.39E-11	6.83E-11	1.08E-11	5.89E-11	0.62
Comprehensive 2											
43395	TRb comp. 2-1	1	12.2	0.48	<10	No	(-)	(-)	(-)	(-)	(-)
43396	TRb comp. 2-1	2	9.6	0.62	<10	No	(-)	(-)	(-)	(-)	(-)
43397	TRb comp. 2-1	2	9.6	0.62	19.7	No	8.64E-07	5.39E-06	5.83E-06	(-)	2.6
43398	TRb comp. 2-1	3	9.2	0.77	<10	No	(-)	(-)	(-)	(-)	(-)
43399	TRb comp. 2-1	3	9.2	0.77	<10	No	(-)	(-)	(-)	(-)	(-)
43400	TRb comp. 2-1	4	9.4	0.72	<10	No	(-)	(-)	(-)	(-)	(-)
43401	TRb comp. 2-1	4	9.4	0.72	<10	No	(-)	(-)	(-)	(-)	(-)
43402	TRb comp. 2-2	1	9.9	0.63	<10	No	(-)	(-)	(-)	(-)	(-)
43403	TRb comp. 2-2	2	9.3	0.53	<10	No	(-)	(-)	(-)	(-)	(-)
43404	TRb comp. 2-2	2	9.3	0.53	<10	No	(-)	(-)	(-)	(-)	(-)
43405	TRb comp. 2-2	3	11.2	0.67	<10	No	(-)	(-)	(-)	(-)	(-)
43406	TRb comp. 2-2	3	11.2	0.67	116.7	>2	3.19E-10	9.29E-10	2.55E-10	7.88E-10	0.56
43407	TRb comp. 2-2	4	10.2	0.59	<10	No	(-)	(-)	(-)	(-)	(-)
43408	TRb comp. 2-2	4	10.2	0.59	119.6	>2	3.19E-11	1.33E-10	2.13E-11	1.02E-10	0.75

Annex I.2 Analysis characteristics antagonistic comprehensive testing of blind coded test items.

	Calc. document	plate IF	z-factor	RPCmin (%)	Respons Yc < 80%	IC20 (M)	IC50 (M)	PC80 (M)	PC50 (M)	|CV| (log[IC50]) (%)	Specificity R^2
Comprehensive 1											
43395	anti-TRb comp. 2-1	2.2	0.79	18.0	>2	3.30E-05	5.71E-05	3.32E-05	5.73E-05	7.0	0.8982
43396	anti-TRb comp. 2-1	2.3	0.59	78.3	1	1.14E-05	2.77E-05	3.34E-05	(-)	0.16	0.6235
43397	anti-TRb comp. 2-1	2.2	0.55	>80	No	(-)	(-)	(-)	(-)		(-)
43398	anti-TRb comp. 2-1	2.4	0.78	>80	No	5.57E-06	1.12E-05	(-)	(-)		(-)
43399	anti-TRb comp. 2-1	2.5	0.65	>80	No	(-)	(-)	(-)	(-)		(-)
43400	anti-TRb comp. 2-2	2.4	0.80	0.0	>2	8.42E-07	1.21E-06	7.83E-07	1.08E-06	3.9	0.9387
43401	anti-TRb comp. 2-2	2.4	0.53	>80	No	(-)	(-)	(-)	(-)		(-)
43402	anti-TRb comp. 2-2	2.5	0.71	>80	No	(-)	(-)	(-)	(-)		(-)
43403	anti-TRb comp. 2-2	2.3	0.61	>80	No	(-)	(-)	(-)	(-)		(-)
43404	anti-TRb comp. 2-2	2.2	0.54	20.0	>2	1.05E-06	2.51E-06	1.52E-06	3.91E-06	1.5	0.9857
43405	anti-TRb comp. 2-3	2.3	0.57	37.6	>2	1.67E-05	2.97E-05	2.71E-05	5.30E-05	4.1	0.7751
43406	anti-TRb comp. 2-3	2.3	0.69	>80	No	(-)	(-)	(-)	(-)		(-)
43407[a]	anti-TRb comp. 2-3	2.2	0.68	69.8	No	4.05E-06	5.30E-06	2.88E-06	(-)		(-)
43408	anti-TRb comp. 2-3	2.2	0.73	>80	No	(-)	(-)	(-)	(-)		(-)
Comprehensive 2											
43395	TRb comp. 2-4	7.8	0.52	5.5	>2	2.58E-05	4.48E-05	2.72E-05	4.56E-05	2.6	0.6908
43396	TRb comp. 2-4	5.5	0.56	36.9	>2	5.80E-06	1.24E-05	1.04E-05	2.67E-05	3.0	0.0269
43400	TRb comp. 2-4	5.8	0.65	0.0	>2	9.37E-07	1.45E-06	6.81E-07	1.08E-06	1.0	0.9803
43404	TRb comp. 2-4	7.6	0.54	19.7	>2	2.51E-06	6.67E-06	2.09E-06	7.19E-06	2.8	0.9389
43405	TRb comp. 2-4	8.3	0.56	20.5	>2	1.25E-05	2.45E-05	1.92E-05	3.42E-05	2.8	0.5929

a Only 1 (highest) test concentration response <80%

Annex J Evaluation alternative concentration T3-agonsist in exposure medium during antagonistic analyses

Because of the low induction factor found in antagonistic testing, higher concentrations of T3 to be used as fixed concentration agonist in supplemented medium for antagonistic tests were tested for their applicability. The EC50, EC60, EC70 and EC80 of agonist reference item T3 were evaluated in this experiment. The concentrations of T3 resulting in 50%, 60%, 70% or 80% response in the TRβ CALUX bioassay were derived from agonist TRβ CALUX analysis results obtained in the present study (part 1 agonist comprehensive testing). In table H-1, the calculated EC50, EC60, EC70 and EC80 concentration of T3 are given. Based on these calculated values, the concentrations of T3 indicated in table H-2 were prepared and used to evaluate the impact of higher fixed concentration of T3 on the induction factor during antagonistic measurements.

All indicated EC concentrations were prepared fresh. At first T3 was dissolved in DMSO at 0.003 M. This solution was diluted to a 3.0E-7 M solution by making a 100-times dilutions (990 µl DMSO + 10 µl stock) and repeating this with the new dilution made. From the 3.0E-7 M solution, 3 other dilutions were prepared (table H-2). Anti-TRβ CALUX measurements were performed at different concentrations of T3 present in the incubation mixture, using the antagonist Diclazuril reference compound.

Results

The results indicate that the fold induction is higher when using the EC60 concentration of T3 is used instead of the EC50 concentration of T3 as fixed concentration present in all exposure mixtures in the anti-TRβ CALUX bioassay. Higher concentrations of T3 present as fixed concentration during the antagonistic measurement (EC70 and EC80 concentrations) did not increase the fold induction significantly. The doses response curves of all Diclazuril dilution series incubated with the different T3 concentrations, were evaluated in GraphPad (see figure H-1). In table H-3, the calculated IC50 concentrations of Diclazuril in the presence of different fixed concentrations of T3 are presented. In addition, the observed induction actors are also given. Based on these results, increasing the fixed concentration of T3 agonist does increase the induction factor without significantly changing the IC50 value for Diclazuril.

Table J.1 Concentration of agonist reference compound T3 resulting in 50%, 60%, 70% or 80% activity in the TRβ CALUX bioassay.

EC value	Concentration in well (M)	Concentration in stock (M)
EC50	1.0E-10	1.0E-07
EC60	1.4E-10	1.4E-07
EC70	2.0E-10	2.0E-07
EC80	3.1E-10	3.1E-07

Table J.2 Concentration of agonist reference compound T3 prepared to evaluate the impact on the IC50 concentration and induction factor of Diclazuril in the ani-TRβ CALUX bioassay.

EC value	Concentration in well (M)	Concentration in stock (M)
EC50	1.0E-10	1.0E-07
EC60	1.5E-10	1.5E-07
EC70	2.0E-10	2.0E-07
EC80	3.0E-10	3.0E-07

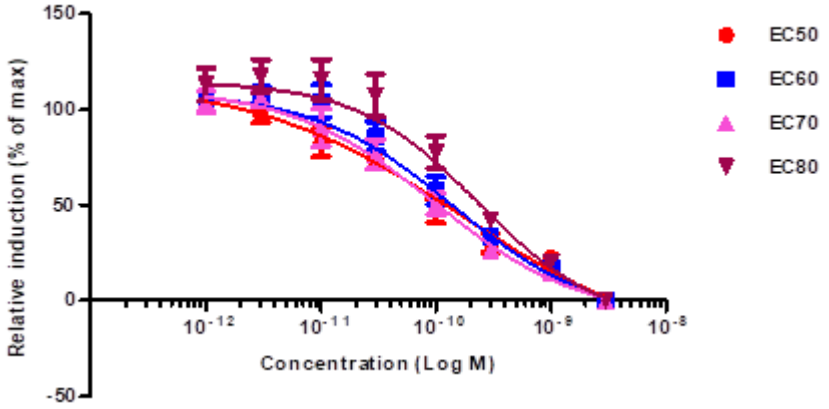


Figure J.1 Response curves of Diclazuril in the anti-TRβ CALUX in the presence of different fixed concentrations of the agonist reference compound T3

Table J.3 Effect on the induction factor and Diclazuril IC50 concentration when performing anti- TRβ CALUX bioanalyses at different fixed concentrations of T3 agonist (EC50, EC60, EC70 and EC80 concentrations)

EC value	Max FI (-)	IC50 (M)
EC50	6.9	1.7E-6
EC60	13.1	1.4E-6
EC70	15.7	9.8E-7
EC80	16.3	2.3E-6

In conclusion, it is recommended that the EC60 concentration of T3 is used in the future for antagonistic TRβ CALUX measurements instead of the EC50 concentration of T3. The concentration T3 during specificity testing will also change to 100xEC60.