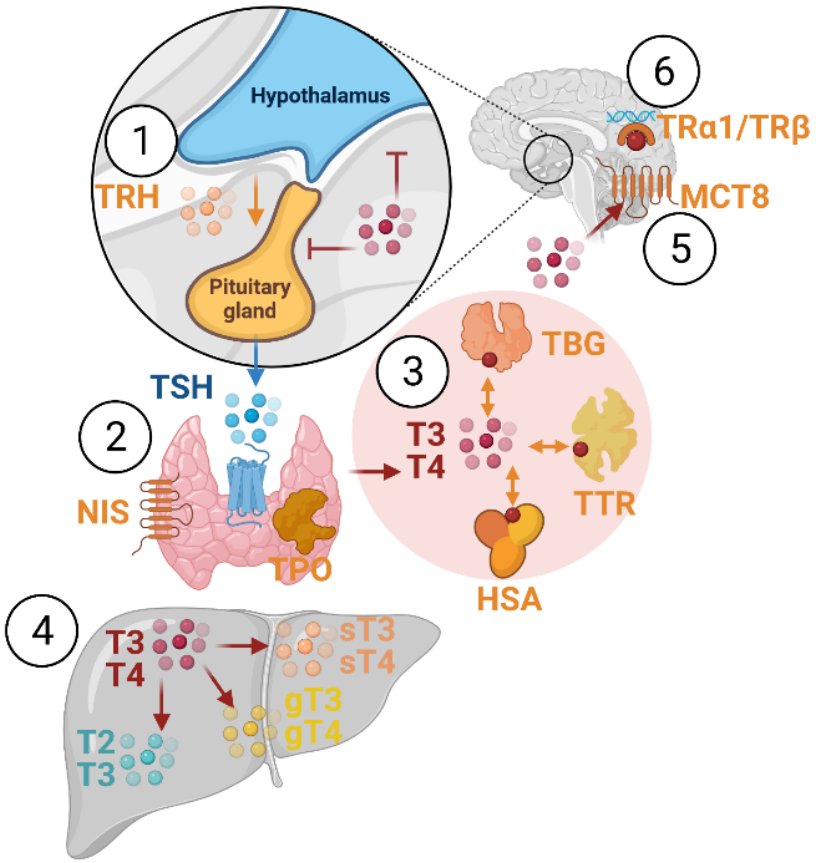
STUDY REPORT

for the fluorescent FITC-T4 Transthyretin (TTR) competitive binding assay, – Part 1

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



© BioRender.com

2022

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 in Part 1 of the validation study. The method was developed by VU Amsterdam, the Netherlands and implemented by EU-NETVAL laboratory Wageningen Food Safety Research in the Netherlands, for optimisation and experimental assessment.

Contact information

Toine FH Bovee, PhD
WFSR, Wageningen Food Safety Research
Akkermaalsbos 2
6708 WB, Wageningen
The Netherlands
Email: toine.bovee@wur.nl

https://www.wur.nl/en/research-results/research-institutes/food-safety-research.htm

Study report

Study Title:	Validation study of a fluorescent FITC-T4 transthyretin competitive binding assay (part 1)
Study ID:	1207366201-Validation study EU-NETVAL
Study Director:	Yoran Weide
Experimental starting date	05-04-2021
Experimental completion date	05-10-2021

	Study Director (Approval)	Supervisor (optional)
Signed		
Date	17-11-2022	17-11-2022

TABLE OF CONTENTS

1 STUDY SCOPE

This study is performed for PART 1 of the EURL ECVAM coordinated Thyroid Validation Study. After the full description of method “Fluorescent FITC-T4 transthyretin competitive binding assay” in a standard operating procedure, in this study the robustness and reliability of the method will be assessed by performing five valid runs with the reference (T4) and test items (PFOS, Triclosan, TBBPA, BPA and D-mannitol).

This study is supplemented with an extra requirement for the autofluorescence or quenching of the test items after performing PART 2.

2 STANDARD OPERATING PROCEDURE USED

SOP01- TTRfitc_v3.docx

This SOP describes in detail the used materials, equipment, chemicals, reagents, software, reference items, compounds of the test system and the working procedure of the assay. In Sections 3, 4, 5, and 6 a short description of the SOP is given.

3 MATERIALS, EQUIPMENT, CHEMICALS, REAGENTS AND SOFTWARE

3.1 Materials

3.1.1. Burret: Supelco LC column with Frit and Stopcock 300 mm x 10.5 mm ID x 13 mm OD

3.1.2. 96 wells black chimney plates polystyrene non-binding (Greiner Bio-One 655900)

3.2 Equipment

3.2.1. Fluorescence plate reader: ClarioStar BMG Labtech; settings λ 483 ± 14 nm excitation and λ 530 ± 30 nm

3.2.2. UV/VIS Spectrophotometer: Thermo Spectronic Helios ε; 490 nm

3.2.3. Centrifuge: Sorvall RC 3BP+

3.2.4. Plateshaker: Heidolph titramax 101

3.3 Chemicals

3.3.1. Pyridine (anhydrous) 99.8%, CAS 110-86-1

3.3.2. Triethylamine >99%, CAS 121-44-8

3.3.3. Lipophilic Sephadex, CAS 9041-37-6

3.3.4. Ammonium acetate > 98%, CAS 631-61-8

3.3.5. Ammonium bicarbonate >99.5%, CAS 1066-33-7

3.3.6. Sodium bicarbonate, CAS 144-55-8

3.3.7. Tris(hydroxymethyl)aminomethane, CAS 77-86-1

3.3.8. Sodium chloride, CAS 7647-14-5

3.3.9. EDTA >99%, CAS 6381-92-6

3.3.10. Ultrapure water

3.3.11. DMSO >99.5%, CAS 67-68-5

3.3.12. Acetic acid ≥99.7%, CAS 64-19-7

3.3.13. Hydrochloric acid 37%, CAS 7647-01-0

3.3.14. Sodium hydroxide ≥98%, CAS 1310-73-2

3.4 Reagents preparation

3.4.1. Preparation of PWT (pyridine water triethylamine) mixture

- Mixed 9 mL pyridine (3.3.1) with 1.5 mL ultrapure water (3.3.10) and 0.1 mL triethylamine (3.3.2)

3.4.2. Preparation of NH4-acetate (0.2 M)

- Weighed out 7.709 g ammonium acetate (3.3.4).
- Dissolved in approximately 400 mL ultrapure water (3.3.10) in a 500 mL volumetric flask.

- Adjusted the pH to 4.0 with acetic acid (3.3.12).
- Brought to a total volume of 500 mL with ultrapure water.

3.4.3. Preparation of NH_4HCO_3 (0.05 M)

- Dissolved 1.9765 g ammonium bicarbonate (3.3.5) in ultrapure water (3.3.10) in a 500 mL volumetric flask.

3.4.4. Preparation of $NaHCO_3$ (0.05 M)

- Dissolved 2.1005 g sodium bicarbonate (3.3.6) in approximately 400 mL ultrapure water (3.3.10) in a 500 mL volumetric flask.
- Adjusted the pH to 8.5 with 1 M NaOH (3.3.14).
- Brought to a total volume of 500 mL with ultrapure water.

3.4.5. Preparation of Tris-HCl buffer (0.1 M Tris, 0.1 M NaCl, 1 mM EDTA)

- Weighed out 12.11 g Tris (3.3.7).
- Weighed out 5.84 g NaCl (3.3.8).
- Weighed out 0.372 g EDTA (3.3.9).
- Dissolved the above substances in approximately 800 mL ultrapure water in a 1000 mL volumetric flask.
- Adjusted the pH to 8.0 with 1 M HCl (3.4.11).
- Brought to a total volume of 1000 mL with ultrapure water.
- Storage life at room temperature: 2 months.

3.4.6. Preparation column packing Sephadex

- Saturated Sephadex (3.3.3) overnight in ultrapure water (3.3.10) (1:10), i.e. 10 g Sephadex + 100 mL ultrapure water. Stored at 4°C (storage life: 3 years).

3.4.7. TTR stock solution (3.64 µM)

- Dissolved 1 mg TTR (5.1) in 5 mL cold (4°C) Tris-HCl buffer (3.4.5) by homogenizing head over head manually.
- Aliquoted the stock solution in portions of 100 µL, stored at -20°C. Storage life: 1 year.

3.4.8. TTR working solution (120 nM)

- Pipetted x volume of 3.64 µM TTR stock solution (3.4.7) into Tris-HCl buffer (3.4.5) to get 120 nM. Mixed carefully by homogenising the solution.

3.4.9. T4 stock solution (1000 µM)

- Dissolved 3.88 mg reference item T4 (4.1) in 5 mL DMSO (3.3.11). Vortexed briefly.

3.4.10. FITC-T4 working solution (220 nM)

- Pipetted x volume of 39.62 µM FITC-T4 (5.2) into Tris-HCl buffer (3.4.5) to get 220 nM.

3.4.11. Hydrochloric acid (1M)

- Diluted 83 mL 37% hydrochloric acid (3.3.13) in 1000 mL ultrapure water (3.3.10).

3.4.12. Sodium hydroxide (1M)

- Dissolved 40 g sodium hydroxide (3.3.14) in 1000 mL ultrapure water (3.3.10).

3.5 Software

- Excel Microsoft Office 365
- MARS Data Analysis, BMG Labtech
- Prism 5 for Windows, Version 5.02, registered trademark of GraphPad Software, Inc.

4 REFERENCE, TEST AND CONTROL ITEMS

4.1 Reference item: T4

L-Thyroxine (T4) ≥98% HPLC, powder, CAS 51-48-9

Sigma-Aldrich T2376, batch number BCCB2400

Expiration date: 28/02/2022

4.2 Test item: PFOS

Perfluoro octane sulfonic acid (PFOS) ≥98% HPLC, potassium salt, CAS 2795-39-3

Sigma-Aldrich 33829, batch number BCCB2805

Expiration date: 31-10-2023

4.3 Test item: Triclosan

Triclosan (Irgasan) ≥97%, powder, CAS 3380-34-5

Sigma-Aldrich 72779, batch number 058M4789V

Expiration date: n.a.

Quality release date: 25-06-2018

4.4 Test item: TBBPA

3,3’,5,5’-Tetrabromobisphenol A (TBBPA) 97%, powder, CAS 79-94-7

Sigma-Aldrich 330396, batch number MKCB9769

Expiration date: n.a.

Quality release date: 29-11-2016

4.5 Test item: BPA

Bisphenol A (BPA) ≥99%, powder, CAS 80-05-7

Sigma-Aldrich 239658, batch number MKCD7508

Expiration date: n.a.

Quality release date: 07-08-2017

4.6 Test item: D-mannitol

D-Mannitol ≥98%, powder, CAS 69-65-8

Sigma-Aldrich M4125, batch number WXBC2720V

Expiration date: 30-04-2022

5 THE TEST SYSTEM

5.1 Prealbumin (transthyretin, TTR)

Prealbumin (transthyretin, TTR) ≥95%, from human plasma, CAS 87090-18-4, Sigma-Aldrich P1742, batch numbers:

5.1.1. 23.03.21YW batch: SLB2163; Expiration date: n.a.; Quality release date: 19-12-2018

5.1.2. 10.06.21YW batch: SLCF2972; Expiration date: n.a.; Quality release date: 16-1-2020

5.2 Fluorescein isothiocyanate isomer (FITC)

Fluorescein isothiocyanate isomer I suitable for protein labelling ≥ 90% (FITC) powder, CAS 3326-32-7; Sigma-Aldrich F7250, batch number SLCB2729

6 PROCEDURE

Fluorescein isothiocyanate (FITC) is a fluorescent probe, which is linked to T4. Upon binding of the FITC-T4 probe to human transthyretin (TTR) as a test system, an increase in fluorescence is observed most likely due to the elimination of intramolecular fluorescence quenching of the FITC group by the iodine groups of the bound T4. This increase in fluorescence, however, is abolished by adding competitors for TTR-binding like endogenous ligand thyroxine (T4) as a reference item or xenobiotic TTR-binding compounds as test items. When test items induce a concentration-dependent decrease in fluorescence in a FITC-T4/TTR competitive binding assay, differences in potencies of these thyroid hormone (TH) disrupting compounds can be determined by calculating the IC50 and Ki20 (inhibitory constant) values.

6.1 Production and characterization of FITC-T4 batch #002

6.1.1 FITC-T4 production

1. Dissolved 10 mg FITC (5.2) in 0.5 mL PWT (3.4.1): 51.4 mM FITC.
2. Dissolved 10 mg T4 (4.1) in 1 mL PWT (3.4.1): 12.9 mM T4.
3. Mixed both solutions (0.5 mL 51.4 mM FITC and 1 mL 12.9 mM T4) and incubated for 1 hour at 37°C.
4. Pipetted the mixture (FITC + T4) into a new 50 mL polypropylene tube.
5. Precipitated the formed FITC-T4 by adding 20 mL of 0.2 M NH_4-acetate (pH 4.0) (3.4.2).
6. Centrifuged for 10 min at 1000 g.
7. Discarded the supernatant.
8. Added 20 mL ultrapure water and mixed vigorously.
9. Centrifuged for 10 min at 1000 g.
10. Discarded the supernatant.
11. Dissolved the pellet in 5 mL of 0.05 M NH_4HCO_3 (3.4.3).
12. Mixed thoroughly until FITC-T4 was dissolved

6.1.2 FITC-T4 purification

Prepared a column of Sephadex in a burette:

1. Equilibrated the swollen Sephadex (3.4.6) to room temperature.
2. Resuspended and then poured the Sephadex down with a glass rod into the burette.
3. The Sephadex was left to settle and pouring was continued until a 4.5 cm packing was achieved.
4. Equilibrated the Sephadex by passing 3 column volumes of 0.05 M $NaHCO_3$ (3.4.4).

Purified FITC-T4 over the Sephadex-column:

5. Added 0.5 mL of the FITC-T4 to the Sephadex-column.
6. Rinsed with 10 column volumes 0.05 M $NaHCO_3$ (3.4.4) (± 4.5 mL per column volume).
7. Eluted pellet of interest with 10 mL ultrapure water and collected the eluate temporarily in a 50 mL polypropylene tube.

6.1.3 FITC-T4 concentration

A small amount of the FITC-T4 eluate was diluted by a factor of 10 with ultrapure water. The absorbance of the diluted eluate was measured with the UV-VIS cuvette-based spectrophotometer. Next the concentration was calculated with:

$$Concentration\ FITC\text{-}T4\ \left[\frac{mol}{L}\right] = \frac{\mathrm{Ax} - \mathrm{A0}}{\varepsilon * l} * dilutionfactor$$

Ax = absorbance of FITC-T4

A0 = absorbance of blank (ultrapure water)

l = pathlength of cuvette in cm (1 cm)

ε = molar extinction coefficient of FITC = 7.8×10^4 $M^{-1}cm^{-1}$

The FITC-T4 eluate was aliquoted in volumes of 200 µl and stored at -80°C.

6.1.4 FITC-T4 characterization

A saturation curve was performed to determine the dissociation constant (Kd) of the FITC-T4/TTR complex for characterisation of FITC-T4 according to SOP01-TTRfitc_v3; 7.1.4. In short:

1. Prepared a dilution series of FITC-T4 with 15 different concentrations of FITC-T4 and a blank in Tris-HCl buffer (3.4.5).
2. Pipetted the different concentrations into a 96 well plate.
3. Measured fluorescence of the plate with the plate reader (3.2.1) with a warmed up bulb after the instrument setup was performed (6.2). The fluorescence was measured in relative fluorescence units (RFU).
4. Determined the fluorescence intensity for each FITC-T4 concentration with:

$$Y1 = [RFU\ FITC - T4\ with\ TTR] - [mean\ RFU\ FITC - T4\ without\ TTR]$$

5. Calculated the dissociation constant (Kd) by performing a non-linear regression on Y1 as a function of L (FITC-T4 concentrations) with:

$$Y1 = constant * \frac{(K_d + 30 + L) - \sqrt{(K_d + 30 + L)^2 - 120 * L}}{2}$$

30 is the final TTR protein concentration in nM

120 is the final TTR protein concentration in nM multiplied by 4 (quadratic equation)

6.2 Instrument setup

1. Used the plate of the FITC-T4 characterization (6.1.4) to determine the gain of the fluorescence plate reader (3.2.1) for FITC-T4 batch #002.
2. Set the wavelengths at λ 485 ± 20 nm excitation and λ 528 ± 20 nm emission.
3. Set the gain setting to distinguish the lowest concentration of FITC-T4 (1 nM) from the background (0 nM FITC-T4; blank; Tris-HCl buffer) based on the following criteria:

   [mean RFU of 1 nM FITC-T4] − [mean RFU blank] > +3SD of blank

6.3 Solubility of reference and test items

1. For stock solutions of the test items, a nominal amount (e.g. 0.5-600 mg/mL) of the test item was weighed into a clear glass vial, DMSO was added and a vortex was used to dissolve it. Every coming procedural step for solving the test items was noted (see TTRfitc-002).
   Solubility was checked visually. If it was not possible to solubilize, lower concentrations were prepared by adding extra DMSO.
2. Final concentrations of the stock solutions were registered in TTRftic-002 Preparation and pre-screening (range-finding) of test items.docx.

6.4 Preparation of the stock solution and dilutions of the test items

6.4.1 Reference item: T4

- 10 mM T4: weighed 48.86 mg T4 (4.1) and dissolved in 6.289 mL DMSO
- 1 mM T4: diluted 300 µL of 10 mM T4 into 2700 µL DMSO (stock solution)
- 100 µM T4: pipetted 100 µL of 1 mM T4 into 900 µL DMSO
- 30 µM T4: pipetted 30 µL of 1 mM T4 into 970 µL DMSO
- 10 µM T4: pipetted 100 µL of 100 µM T4 into 900 µL DMSO
- 3 µM T4: pipetted 100 µL of 30 µM T4 into 900 µL DMSO
- 1 µM T4: pipetted 100 µL of 10 µM T4 into 900 µL DMSO
- 0,3 µM T4: pipetted 100 µL of 3 µM T4 into 900 µL DMSO
- 0,1 µM T4: pipetted 100 µL of 1 µM T4 into 900 µL DMSO
- 0 µM T4: (blank DMSO as a SC, solvent control, 3.3.11)

6.4.2 Test item: PFOS

- 30 mM PFOS: weighed 18.86 mg PFOS (4.2) and dissolved in 1.168 mL DMSO

- 1 mM PFOS: pipetted 50 µL of 30 mM PFOS into 1450 µL DMSO
- 300 µM PFOS: pipetted 10 µL of 30 mM PFOS into 990 µL DMSO
- 100 µM PFOS: pipetted 100 µL of 1 mM PFOS into 900 µL DMSO
- 30 µM PFOS: pipetted 100 µL of 300 µM PFOS into 900 µL DMSO
- 10 µM PFOS: pipetted 100 µL of 100 µM PFOS into 900 µL DMSO
- 3 µM PFOS: pipetted 100 µL of 30 µM PFOS into 900 µL DMSO
- 1 µM PFOS: pipetted 100 µL of 10 µM PFOS into 900 µL DMSO
- 0 µM PFOS: (blank DMSO as a SC, solvent control, 3.3.11)

6.4.3 Test item: Triclosan

- 3 mM Triclosan: weighed 6.77 mg Triclosan (4.3) and dissolved in 7.794 mL DMSO
- 1 mM Triclosan: pipetted 500 µL of 3 mM Triclosan into 1000 µL DMSO
- 300 µM Triclosan: pipetted 100 µL of 3 mM Triclosan into 900 µL DMSO
- 100 µM Triclosan: pipetted 100 µL of 1 mM Triclosan into 900 µL DMSO
- 30 µM Triclosan: pipetted 100 µL of 300 µM Triclosan into 900 µL DMSO
- 10 µM Triclosan: pipetted 100 µL of 100 µM Triclosan into 900 µL DMSO
- 3 µM Triclosan: pipetted 100 µL of 30 µM Triclosan into 900 µL DMSO
- 1 µM Triclosan: pipetted 100 µL of 10 µM Triclosan into 900 µL DMSO
- 0 µM Triclosan: (blank DMSO as a SC, solvent control, 3.3.11)

6.4.4 Test item: TBBPA

- 1 mM TBBPA: weighed 1.90 mg TBBPA (4.4) and dissolved in 3.493 mL DMSO
- 10 µM TBBPA: pipetted 10 µL of 1 mM TBBPA into 990 µL DMSO
- 3 µM TBBPA: pipetted 300 µL of 10 µM TBBPA into 700 µL DMSO
- 1 µM TBBPA: pipetted 100 µL of 10 µM TBBPA into 900 µL DMSO
- 300 nM TBBPA: pipetted 100 µL of 3 µM TBBPA into 900 µL DMSO
- 100 nM TBBPA: pipetted 100 µL of 1 µM TBBPA into 900 µL DMSO
- 30 nM TBBPA: pipetted 100 µL of 300 nM TBBPA into 900 µL DMSO
- 10 nM TBBPA: pipetted 100 µL of 100 nM TBBPA into 900 µL DMSO
- 0 µM TBBPA: (blank DMSO as a SC, solvent control, 3.3.11)

6.4.5 Test item: BPA

- 2.5 M BPA: weighed 607.80 mg BPA (4.5) and dissolved in 1.065 mL DMSO
- 1 M BPA: pipetted 400 µL of 2.5 M BPA into 600 µL DMSO
- 300 mM BPA: pipetted 120 µL of 2.5 M BPA into 880 µL DMSO

- 100 mM BPA: pipetted 40 µL of 2.5 M BPA into 960 µL DMSO
- 30 mM BPA: pipetted 12 µL of 2.5 M BPA into 988 µL DMSO
- 10 mM BPA: pipetted 100 µL of 100 mM BPA into 900 µL DMSO
- 3 mM BPA: pipetted 100 µL of 30 mM BPA into 900 µL DMSO
- 1 mM BPA: pipetted 100 µL of 10 mM BPA into 900 µL DMSO
- 300 µM BPA: pipetted 100 µL of 3 mM BPA into 900 µL DMSO
- 100 µM BPA: pipetted 100 µL of 1 mM BPA into 900 µL DMSO
- 0 µM BPA: (blank DMSO as a SC, solvent control, 3.3.11)

6.4.6 Test item: D-mannitol

- 1 M D-mannitol: weighed 440.2 mg D-mannitol (4.6) and dissolved in 0.967 mL DMSO
- 300 mM D-mannitol: pipetted 300 µL of 1M D-mannitol into 700 µL DMSO
- 100 mM D-mannitol: pipetted 100 µL of 1 M D-mannitol into 900 µL DMSO
- 30 mM D-mannitol: pipetted 100 µL of 300 mM D-mannitol into 900 µL DMSO
- 10 mM D-mannitol: pipetted 100 µL of 100 mM D-mannitol into 900 µL DMSO
- 3 mM D-mannitol: pipetted 100 µL of 30 mM D-mannitol into 900 µL DMSO
- 100 µM D-mannitol: pipetted 100 µL of 10 mM D-mannitol into 900 µL DMSO
- 300 µM D-mannitol: pipetted 100 µL of 3 mM D-mannitol into 900 µL DMSO
- 0 µM D-mannitol: (blank DMSO as a SC, solvent control, 3.3.11)

6.6 Main assay

After characterization of FITC-T4 label multiple separate runs were performed per batch.

1. Prepared serial dilutions of test items in DMSO (according to 6.4), depending on potency and solubility as determined in TTRfitc-002. Aspired to have a dose response going from 0% to 100% relative fluorescence intensity (Y2; 6.9).
2. Pipetted the following according to Figure 1:

   With addition of TTR per well (columns 1-3 and 7-9):

   - 48 µL Tris-HCl buffer (3.4.5)
   - 2 µL x nM reference or test item

   Without addition of TTR per well (columns 4-6 and 10-12):

   - 98 µl Tris-HCl buffer (3.4.5)
   - 2 µL x nM reference or test item
3. Prepared a fresh 120 nM TTR working solution (3.4.8) (final concentration in assay: 30 nM) and pipetted directly to the plate according to Figure 1:

   With addition of TTR per well (columns 1-3 and 7-9):

   - 50 µl 120 nM of TTR working solution
4. Prepared a fresh 220 nM FITC-T4 working solution (3.4.10; 30% extra volume) (final concentration in assay: 110 nM) and pipetted directly to the plate according to Figure 1:

   With and without addition of TTR per well (all wells):

   - 100 µl working solution 220 nM FITC-T4
5. After pipetting, the plate was shaken on a plate shaker at 600 rpm for 5 minutes on room temperature in the dark.
6. Incubated the plate for 15 minutes on room temperature in the dark.
7. Measured fluorescence of the plate with the plate reader (3.2.1) with a warmed up bulb.

mw 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	0 nM	0 nM	0 nM	0 nM	0 nM	0 nM	0 nM	0 nM	0 nM	0 nM	0 nM	0 nM
B	1 nM	1 nM	1 nM	1 nM	1 nM	1 nM	10 nM	10 nM	10 nM	10 nM	10 nM	10 nM
C	3 nM	3 nM	3 nM	3 nM	3 nM	3 nM	30 nM	30 nM	30 nM	30 nM	30 nM	30 nM
D	10 nM	10 nM	10 nM	10 nM	10 nM	10 nM	100 nM	100 nM	100 nM	100 nM	100 nM	100 nM
E	30 nM	30 nM	30 nM	30 nM	30 nM	30 nM	300 nM	300 nM	300 nM	300 nM	300 nM	300 nM
F	100 nM	100 nM	100 nM	100 nM	100 nM	100 nM	1000 nM	1000 nM	1000 nM	1000 nM	1000 nM	1000 nM
G	300 nM	300 nM	300 nM	300 nM	300 nM	300 nM	3000 nM	3000 nM	3000 nM	3000 nM	3000 nM	3000 nM
H	1000 nM	1000 nM	1000 nM	1000 nM	1000 nM	1000 nM	10 µM	10 µM	10 µM	10 µM	10 µM	10 µM
	With TTR			Without TTR			With TTR			Without TTR		
	110 nM FITC T4			110 nM FITC T4			110 nM FITC T4			110 nM FITC T4		
	T4						PFOS					

mw 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	0 nM	0 nM	0 nM	0 nM	0 nM	0 nM	100 nM T4	100 nM T4	100 nM T4	100 nM T4	100 nM T4	100 nM T4
B	10 nM	10 nM	10 nM	10 nM	10 nM	10 nM	100 pM	100 pM	100 pM	100 pM	100 pM	100 pM
C	30 nM	30 nM	30 nM	30 nM	30 nM	30 nM	300 pM	300 pM	300 pM	300 pM	300 pM	300 pM
D	100 nM	100 nM	100 nM	100 nM	100 nM	100 nM	1 nM	1 nM	1 nM	1 nM	1 nM	1 nM
E	300 nM	300 nM	300 nM	300 nM	300 nM	300 nM	3 nM	3 nM	3 nM	3 nM	3 nM	3 nM
F	1000 nM	1000 nM	1000 nM	1000 nM	1000 nM	1000 nM	10 nM	10 nM	10 nM	10 nM	10 nM	10 nM
G	3000 nM	3000 nM	3000 nM	3000 nM	3000 nM	3000 nM	30 nM	30 nM	30 nM	30 nM	30 nM	30 nM
H	10 µM	10 µM	10 µM	10 µM	10 µM	10 µM	100 nM	100 nM	100 nM	100 nM	100 nM	100 nM
	With TTR			Without TTR			With TTR			Without TTR		
	110 nM FITC T4			110 nM FITC T4			110 nM FITC T4			110 nM FITC T4		
	triclosan						TBBPA					

mw 3

	1	2	3	4	5	6	7	8	9	10	11	12
A	0 nM	0 nM	0 nM	0 nM	0 nM	0 nM	100 nM T4	100 nM T4	100 nM T4	100 nM T4	100 nM T4	100 nM T4
B	1000 nM	1000 nM	1000 nM	1000 nM	1000 nM	1000 nM	10 uM	10 uM	10 uM	10 uM	10 uM	10 uM
C	3000 nM	3000 nM	3000 nM	3000 nM	3000 nM	3000 nM	30 uM	30 uM	30 uM	30 uM	30 uM	30 uM
D	10 uM	10 uM	10 uM	10 uM	10 uM	10 uM	100 uM	100 uM	100 uM	100 uM	100 uM	100 uM
E	30 uM	30 uM	30 uM	30 uM	30 uM	30 uM	300 uM	300 uM	300 uM	300 uM	300 uM	300 uM
F	100 uM	100 uM	100 uM	100 uM	100 uM	100 uM	1 mM	1 mM	1 mM	1 mM	1 mM	1 mM
G	300 uM	300 uM	300 uM	300 uM	300 uM	300 uM	3 mM	3 mM	3 mM	3 mM	3 mM	3 mM
H	1 mM	1 mM	1 mM	1 mM	1 mM	1 mM	10 mM	10 mM	10 mM	10 mM	10 mM	10 mM
	With TTR			Without TTR			With TTR			Without TTR		
	110 nM FITC T4			110 nM FITC T4			110 nM FITC T4			110 nM FITC T4		
	BPA						D-Mannitol					

Figure 1: Plate layouts of the competitive binding experiment with: T4 (reference item) and PFOS, Triclosan, TBBPA, BPA and D-mannitol (test items). As plate controls, DMSO (0 nM) and 100 nM T4 were used. Note: concentrations were given in final concentrations in 96 well.

6.7 Check plate controls

Before data could be analyzed, the plate controls were determined and verified on their respective criteria.

6.7.1 Solvent control

Determined the relative fluorescence intensity (RFI) of the solvent control (SC; 0 nM) on plates 2 and 3 (*x*) (wells A1-A6, Figure 1) to the SC on plate 1 (wells A1-A6, Figure 1) as:

$$RFI\ solvent\ control = \frac{[mean\ RFU\ SC\ with\ TTR]plate\ x - [mean\ RFU\ SC\ without\ TTR]plate\ x}{[mean\ RFU\ SC\ with\ TTR]reference\ plate - [mean\ RFU\ SC\ without\ TTR]reference\ plate} * 100\%$$

This should be between 65 and 135%.

6.7.2 Positive control

Determined the relative fluorescence intensity (RFI) of the positive control 100 nM T4 (IC50) on plates 2 and 3 (wells A7-A12, Figure 1) to plate 1 (wells E1-E6, Figure 1) as:

$$RFI\ 100\ nM\ T4 = \frac{[mean\ RFU\ T4\ with\ TTR]plate\ x - [mean\ RFU\ T4\ without\ TTR]plate\ x}{[mean\ RFU\ T4\ with\ TTR]reference\ plate - [mean\ RFU\ T4\ without\ TTR]reference\ plate} * 100\%$$

This should be between 65 and 135%.

6.7.3 Positive plate control

Determined the relative fluorescence intensity (RFI) of the positive control 100 nM T4 compared to the solvent control (SC) per plate (wells E1-E6 compared to wells A1-A6, Figure 1) as:

$$RFI\ 100\ nM\ T4\ per\ plate = \frac{[mean\ RFU\ T4\ with\ TTR]plate\ x - [mean\ RFU\ T4\ without\ TTR]plate\ x}{[mean\ RFU\ \mathrm{SC}\ with\ TTR]plate\ x - [mean\ RFU\ \mathrm{SC}\ without\ TTR]plate\ x} * 100\%$$

6.8 Check for autofluorescence or quenching by the test item

Next, data of the test items were analyzed without the presence of TTR to check for autofluorescence or quenching by the test item on the assay.

1. Determined the Pearson correlation coefficient (r) for the values of "RFU test item without TTR" and the corresponding ^{10}log-transformed concentrations of the test item. Included the RFU values for the solvent control in this correlation. A test item concentration of 0 for the

solvent control was not used, because ^{10}log(0) is undefined. Instead, a concentration 1000x smaller than the lowest test concentration of the test item was used.

2. Tested if r significantly deviated from zero by performing a Student's t-test, with t=r/s_r and with $s_r = \sqrt{\frac{1-r^2}{n-2}}$, with n is the number of observations (n=24, according to Figure 1). If $|t| \geq t_{0.05(2),n-2}$, the test item significantly interferes with the readout of the experiment, making the experiment invalid. In case of n=24, the critical value of $t_{0.05(2),n-2}$ = 2.0739.
3. Determined the slope factor of "RFU test item without TTR" and the corresponding ^{10}log-transformed concentrations of the test item via linear regression (slope of background fluorescence). If the slope was not between -41.6 and 18.8 the test item significantly interfered with the readout of the experiment, making the experiment invalid.
4. First, the *t*-value was checked, if it fitted the requirement that $|t| < 2.0739$, then the test item did not affect the readout of the experiment, and IC50 and Ki20 values were determined. Second, if the data did or did not fit the requirement for the *t*-value, the slope of the background fluorescence was checked to be between -41.6 and 18.8. If this requirement fitted, then the test item did not affect the readout of the experiment, and IC50 and Ki20 values were determined.

6.9 Process data to determine IC50 and calculate Ki20 value

1. Determined the relative fluorescence intensity (RFI, Y2) [%] as:

$$Y2 = relative\ fluorescence\ intensity = \frac{[RFU\ FITC\text{-}T4\ with\ TTR]treatment - [mean\ RFU\ FITC\text{-}T4\ without\ TTR]treatment}{[mean\ RFU\ FITC\text{-}T4\ with\ TTR]\ solvent\ control - [mean\ RFU\ FITC\text{-}T4\ without\ TTR]\ solvent\ control} * 100\%$$

2. Made a two-column table with final concentrations (A) of the test item and their corresponding relative fluorescence intensity values (Y2).
3. Calculated the median inhibition concentration (IC50) by performing a non-linear regression on Y2 as a function of A according to Hill equation:

$$Y2 = Y_{min} + \frac{(Y_{max} - Y_{min})}{1 + \left(\frac{IC50}{A}\right)^{HillSlope}}$$

4. Based on the values for IC50 and HillSlope, IC20 value was calculated for 20% of inhibited FITC-T4 binding, according to

$$IC20 = IC50 * \left(\frac{100 - 20}{20}\right)^{\frac{1}{HillSlope}}$$

5. Based on the IC20 value, the dissociation constant of the inhibitor-TTR complex (Ki20) was calculated according to:

$$Ki20 = \frac{Kd * PL20 * IC20}{L20 * PI20} - P20$$

PL20 is the concentration of the protein-ligand complex (i.e. FITC-T4 bound to TTR) at 20% inhibition, which was calculated under the bioassay conditions as:

$$PL20 = \left(\frac{100 - 20}{100}\right) * \frac{(Kd + 140) - \sqrt{(Kd + 140)^2 - 13200}}{2}$$

- 140 is the sum of the total concentrations in the test system of protein and ligand (TTR + FITC-T4) in nM

- 13200 is the product of the total concentrations in the test system of protein ligand (TTR x FITC-T4) in nM^2 multiplied by 4 (quadratic equation)

L20 is the concentration of free ligand FITC-T4 at 20% inhibition, which can be calculated under the bioassay conditions as:

$$L20 = 110 - PL20$$

- 110 being the total concentration in the test system of the ligand (FITC-T4) in nM

PI20 is the concentration of the protein-inhibitor complex (i.e. test item bound to TTR) at 20% inhibition, which can be calculated under the bioassay conditions as:

$$PI20 = 30 - PL20 * \left(\frac{Kd}{L20} + 1\right)$$

- 30 is the total concentration in the test system of protein (TTR) in nM

P20 is the concentration of free protein TTR at 20% inhibition, which can be calculated under the bioassay conditions as:

$$P20 = \frac{Kd * PL20}{L20}$$

6.10 Acceptance criteria

6.10.1 Instrument setup

RFU of 1 nM FITC-T4 subtracted by RFU of blank (Tris-HCl buffer) should be > +3SD of blank

6.10.2 Kd value of FITC-T4

The Kd value of FITC-T4 should be between 50 and 300 nM.

6.10.3 Negative experiment control (IC0)

Test plate (plate x) to reference plate (plate 1) solvent control should be between 65 and 135%.

6.10.4 Positive experiment control (IC50)

Test plate (plate x) to reference plate (plate 1) control of 100 nM T4 should be between 65 and 135%.

6.10.5 Autofluorescence and quenching by test item

The Pearson correlation coefficient should be determined with Student's t-test and the t-value should be lower than 2.0739. The slope of the background fluorescence of the test item should be between -41.6 and 18.8. If both requirements are not met, the experiment is invalid.

6.10.6 IC50 value of reference item

The IC50 of T4 should be between 40 and 160 nM.

7 RESULTS

7.1 Test system preparation

7.1.1 Instrument setup for FITC-T4 batch #002

Before any experiments could be performed, the setup of the fluorescence plate reader was determined. The wavelengths were set at λ 485 ± 20 nm excitation and λ 528 ± 20 nm emission. The gain of the instrument was tested at 500 and 750, see Table 1 for the results.

Table 1.Fluorescence intensities (FI) of the lowest FITC-T4 concentration (1 nM) and the blank (0 nM FITC-T4) in the absence of TTR at different gains of the fluorescence plate reader with the calculated values required for the criterium.

	Gain 500		Gain 750	
	Average FI	3SD	Average FI	3SD
1 nM FITC-T4	29.0 ± 1.0	-	33.3 ± 1.2	-
0 nM FITC-T4	28.0 ± 0.0	0.0	27.7 ± 0.6	1.7
Subtracted	1.0		5.7	

For the gain of 500 the criteria was met, because the subtracted value (= 1.0) is larger than 3 times the SD of the blank (= 0.0) (6.10.1), but nevertheless there is no significant difference between the fluorescence intensity of the lowest FITC-T4 concentration (1 nM) and that of the blank. For the gain of 750 the criteria was also met, because the subtracted value (= 5.7) is larger than 3 times the SD of the blank (= 1.7) (6.10.1), with a significant difference between the measured fluorescence intensities. Therefore, a gain of 750 was used for the entire experiment.

7.1.2 Characterization of FITC-T4 batch #002

FITC-T4 batch #002 was produced and characterized according to 6.1. The concentration of FITC-T4 batch #002 (further referred to as FITC-T4) was determined with the UV/VIS spectrophotometer and is 39.62 µM. The dissociation constant of FITC-T4 was determined with a saturation curve of the FITC-T4/TTR complex, which is shown in Figure 2., based on the model mentioned in 6.1.4.5. The calculated dissociation constant of FITC-T4 is 164.2 nM, which fits the requirements (6.10.2).

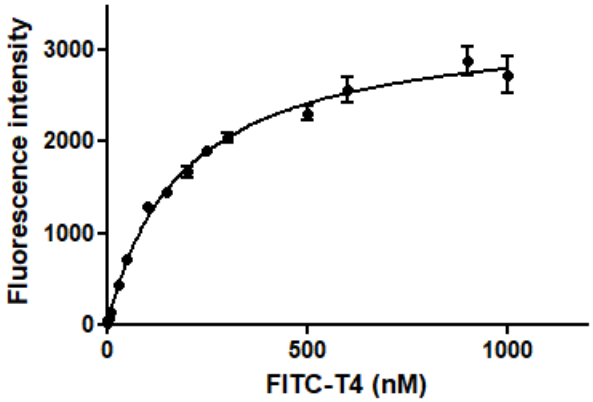


Figure 2. Saturation curve of the FITC-T4/TTR complex.

7.2 Solubility of test items

Solubility of all reference and test items was tested and noted in TTRfitc-002 Preparation and pre-screening (range-finding) of test items.docx.

7.2.1 Reference item: T4

L-thyroxine was soluble in DMSO at a concentration of 10 mM and 2 µl of 10 mM T4 was soluble in 200 µl Tris-HCl buffer.

7.2.2 Test item: PFOS

PFOS was soluble in DMSO at a concentration of 30 mM and 2 µl of 30 mM PFOS was soluble in 200 µl Tris-HCl buffer.

7.2.3 Test item: Triclosan

Triclosan was soluble in DMSO at a concentration of 3 mM and 2 µl of 3 mM Triclosan was soluble in 200 µl Tris-HCl buffer.

7.2.4 Test item: TBBPA

TBBPA was soluble in DMSO at a concentration of 1 mM and 2 µl of 1 mM TBBPA was soluble in 200 µl Tris-HCl buffer.

7.2.5 Test item: BPA

BPA was soluble in DMSO at a concentration of 2.5 M and 2 µl of 2.5 M BPA was precipitating in 200µl Tris-HCl buffer. However, 2 µl of 100 mM BPA was soluble in 200 µl Tris-HCl buffer.

7.2.6 Test item: D-mannitol

D-Mannitol was insoluble in DMSO at a concentration of 2.5 M, but soluble at 1 M. 2 µl of 1 M D-Mannitol was soluble in 200 µl Tris-HCl buffer.

7.3 Concentration ranges for the test items

Range-finding experiments of PFOS, Triclosan and TBBPA were not required, because historical data and literature of Weiss et al. (2015) utilized the following concentration ranges:

- PFOS: 10 nM – 10 µM
- Triclosan: 10 nM – 10 µM
- TBBPA: 100 pM – 100 nM

Range-finding experiments of BPA and D-mannitol were performed and noted in TTRfitc-002 Preparation and pre-screening (range-finding) of test items.docx. The following concentration ranges were found suitable:

- BPA:1 µM – 1 mM
- D-mannitol: 10 µM – 10 mM

7.4 Results assessment

The range finding and subsequent TTR binding screening assay of all 6 test items were executed in several independent runs per test item. All experiments with test items, included negative (DMSO) and positive (100 nM T4) experiment controls, which was given an experiment number.

7.4.1 Negative and positive experiment controls

All experiments included negative (IC0) and positive experiment (IC50) controls. In Table 2 the negative control (SC, DMSO) and positive experiment control (100 nM T4) are given. The negative control is given by the solvent control at which no competitive binding occurs, this means that all TTR is saturated with FITC-T4, resulting in 100% binding and thus a maximum fluorescence of FITC-T4. The fluorescence intensity (FI) of the SC on the reference plate (MW1) is set to 100% to which all FIs of SC on the other plates are compared, resulting in a relative fluorescence intensity expressed in percentages, which should be between 65 and 135% to be accepted (6.10.3). All percentages for all experiment numbers fall between those values, meaning that all experiments are accepted based on the negative control.

Next to that, all plates per experiment contained 100 nM T4 as a positive control (Figure 1). On plate 1 this concentration is part of the calibration series of T4 (Figure 1) and the 100 nM is close to the IC50 value of T4 (Table 4). The response curve of T4 is very steep around the IC50, which makes this positive control very sensitive, and as a result, the criterion of 65 to 135% rather strict (6.10.4). The fluorescence intensity (FI) of 100 nM T4 on the reference plate (MW1) is set to 100% to which all FIs of 100 nM T4 on the other plates are compared, resulting in a relative fluorescence intensity expressed in percentages, which should be between 65 and 135% to be accepted (6.10.4). Similar to the negative control, all experiments are accepted based on the positive control.

Table 2. Per experiment number the fluorescent intensity (FI) of the negative (SC, DMSO) and positive control (100 nM T4) on plate 1 (MW1), the relative fluorescence intensities given in percentages of the negative and positive controls compared to plate 1 are given. For both controls the values should be between 65 and 135% to be accepted. [a]TTR batch A was used (5.1.1). [b]TTR batch B was used (5.1.2).

	Negative control (DMSO, IC0)			Positive control (100 nM T4, IC50)		
Experiment number	FI of SC on MW1	MW2 to 1 (%)	MW3 to 1 (%)	FI of T4 on MW1	MW2 to 1 (%)	MW3 to 1 (%)
TTRfitc-003[a]	1681	94.2	95.7	922	100.3	97.3
TTRfitc-004[a]	1669	102.5	95.6	939	108.3	94.2
TTRfitc-005[a]	1645	101.2	97.7	950	96.2	102.1
TTRfitc-006[b]	658	107.3	97.1	287	95.5	107.4
TTRfitc-007[b]	672	90.4	96.9	266	96.4	86.2

7.4.2 Positive plate control

In Table 3 the positive plate control (RFI of 100 nM T4) and the IC50 values of T4 per experiment are given. The RFI of 100 nM T4 per plate is calculated according to 6.7.3, in which the FI of 100 nM T4 (IC50) is compared to the FI of the solvent control (IC100). These values are also given in the figures with the response curve per test item as horizontal lines per experiment number.

Furthermore, the IC50 value of the calibration curve of T4 on plate 1 is determined per experiment. This value should be between 40 and 160 nM (6.10.6) for an experiment to be accepted. All IC50 values fall between those values, meaning that all experiments are accepted based on this criterion.

Table 3. Plate control of 100 nM T4 per plate (MWx) per experiment number and IC50 of T4 on plate 1. [a]TTR batch A was used (5.1.1). [b]TTR batch B was used (5.1.2).

	Plate control (RFI of 100 nM T4 (%))			
Experiment number	MW1	MW2	MW3	IC50 of T4 (nM)
TTRfitc-003[a]	54.84	58.43	55.75	113.1
TTRfitc-004[a]	56.24	59.41	55.40	112.5
TTRfitc-005[a]	57.76	54.89	60.36	134.6
TTRfitc-006[b]	43.54	38.73	48.18	106.1
TTRfitc-007[b]	39.56	42.16	35.21	90.2
Test items per plate	T4	Triclosan	BPA	
	PFOS	TBBPA	D-mannitol	

7.5 Test items results

In this section results of all test items are shown. For each test item one figure and one table show the results, in some cases supplemented with an explanation.

For all five runs per test item applies that the first three runs (TTRfitc-003, -004, and -005) were performed with TTR batch A (5.1.1) and the last two runs (TTRfitc-006 and -007) were performed with TTR batch B (5.1.2).

Results of the five performed test runs are shown in the figure: background fluorescence and response curve. The analysis per test item started with the check for a background effect of the test item on the assay (6.8). The t-values of the Student's T-test (6.8.2) and the slope of the background fluorescence (6.8.3) are shown in the table. With the criteria for the *t*-value and a range for the slope, three scenarios are possible:

i. When the *t*-value is smaller than $|2.074|$ (6.10.5), independent of the value of the slope, the result is considered valid.
ii. When the *t*-value does not meet the criterium, i.e. is greater than $|2.074|$, but the value of the slope is between the set criterion, i.e. between -41.6 and 18.8 (6.10.5), the result is still considered valid.
iii. When the *t*-value and the value of the slope both do not meet the criteria, the test is rejected. Therefore, the result is considered not valid.

Independent of the *t*-values and the slope, a concentration-response curve fit was performed to calculate an IC50 value for the test item (6.9) and in addition an IC20 value and dissociation constant (Ki20). The results of the response curve show two outcomes:

i. The concentration-response curve resulted in the calculation of an IC50 value, the IC50 value is given.
ii. The concentration-response curve did not result in the calculation of an IC50 value and the datapoints show a straight line, the test item does not interfere with T4 binding to TTR and is stated as no responder (n.r.).

7.5.1 Reference item: T4 (L-thyroxine)

In every experiment T4 was tested for its potency.

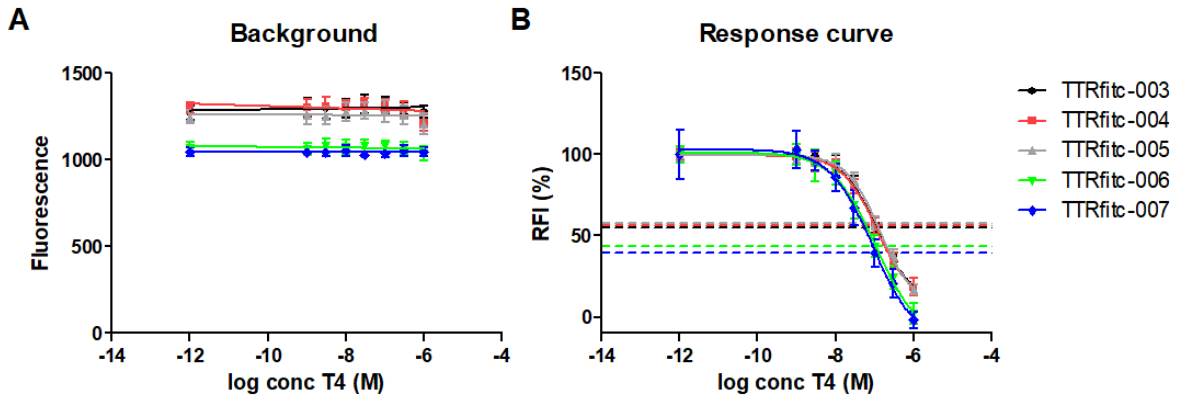


Figure 3.(a) Background fluorescence and (b) response curve in the competitive binding experiment for reference item T4.

Table 4. t-value based on the correlation of the background fluorescence, slope of the background fluorescence, and calculated binding values of reference item T4.

T4	Background		Binding values			
	t-value	Slope	Log IC50 (M)	IC50 (nM)	IC20 (nM)	Ki20 (nM)
TTRfitc-003	0.693	3.6	-6.946	113.1	27.0	47.9
TTRfitc-004	-1.254	-7.0	-6.949	112.5	27.3	48.5
TTRfitc-005	-0.190	-1.1	-6.871	134.6	35.0	66.4
TTRfitc-006	-0.585	-2.3	-6.974	106.1	18.5	28.2
TTRfitc-007	-0.035	-0.1	-7.045	90.2	15.7	21.7
Mean			-6.957 ± 0.062	111.3 ± 16.0	24.7 ± 7.7	42.5 ± 17.8
CV of LogIC50			0.90%			

Based on the criteria for autofluorescence, these results are valid. As was stated before, all IC50 values fall between the set criterium.

7.5.2 Test item: PFOS

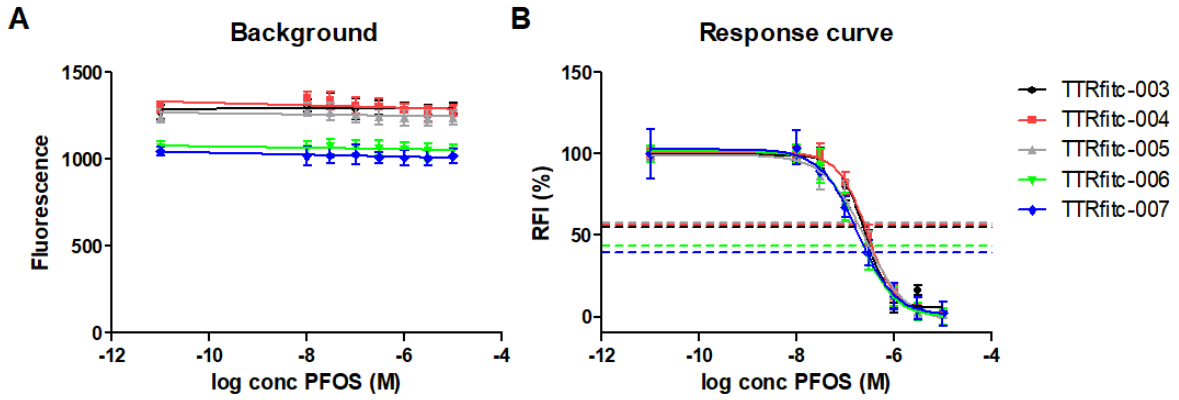


Figure 4.(a) Background fluorescence and (b) response curve in the competitive binding experiment for test item PFOS.

*Table 5. t-value based on the correlation of the background fluorescence, slope of the background fluorescence, and calculated binding values of test item PFOS. * value outside criteria.*

PFOS	Background		Binding values			
	t-value	Slope	Log IC50 (M)	IC50 (nM)	IC20 (nM)	Ki20 (nM)
TTRfitc-003	-0.227	1.4	-6.602	249.8	111.2	242.6
TTRfitc-004	-3.181*	-7.2	-6.538	289.8	112.4	245.2
TTRfitc-005	-1.626	-3.6	-6.586	259.3	68.6	144.1
TTRfitc-006	-0.934	-3.9	-6.727	187.7	58.7	121.1
TTRfitc-007	-1.329	-5.8	-6.741	181.7	53.8	109.8
Mean			-6.639 ± 0.090	233.7 ± 47.1	80.9 ± 28.7	172.6 ± 66.3
CV of LogIC50			1.36%			

Based on the criteria for autofluorescence, these results are valid.

Even though, the t-value of TTRfitc-004 for PFOS is outside the criteria (larger than $|2.074|$), the slope of the background fluorescence is between -41.6 and 18.8, so the result is still considered valid.

7.5.3 Test item: Triclosan

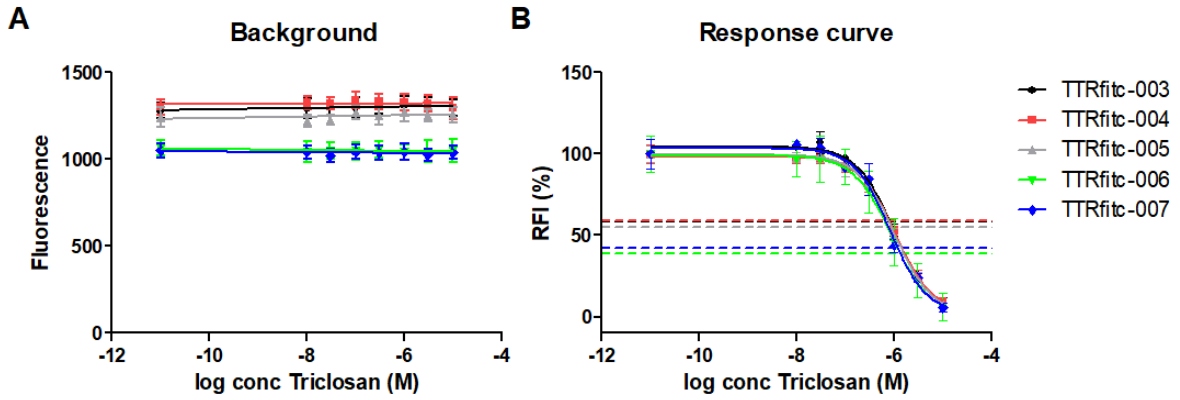


Figure 5. (a) Background fluorescence and (b) response curve in the competitive binding experiment for test item Triclosan.

Table 6. t-value based on the correlation of the background fluorescence, slope of the background fluorescence, and calculated binding values of test item Triclosan.

Triclosan	Background		Binding values			
	t-value	Slope	Log IC50 (M)	IC50 (nM)	IC20 (nM)	Ki20 (nM)
TTRfitc-003	0.855	4.4	-6.003	993.3	296.3	670.8
TTRfitc-004	0.240	1.2	-5.963	1089.0	326.9	741.6
TTRfitc-005	0.915	4.2	-5.981	1045.0	314.3	712.5
TTRfitc-006	-0.499	-2.7	-6.042	907.9	258.9	584.3
TTRfitc-007	-0.507	-2.1	-6.074	842.8	265.0	598.3
Mean			-6.013 ± 0.045	975.6 ± 100.3	292.3 ± 29.8	661.5 ± 69.0
CV of LogIC50			0.75%			

Based on the criteria for autofluorescence, these results are valid.

7.5.4 Test item: TBBPA

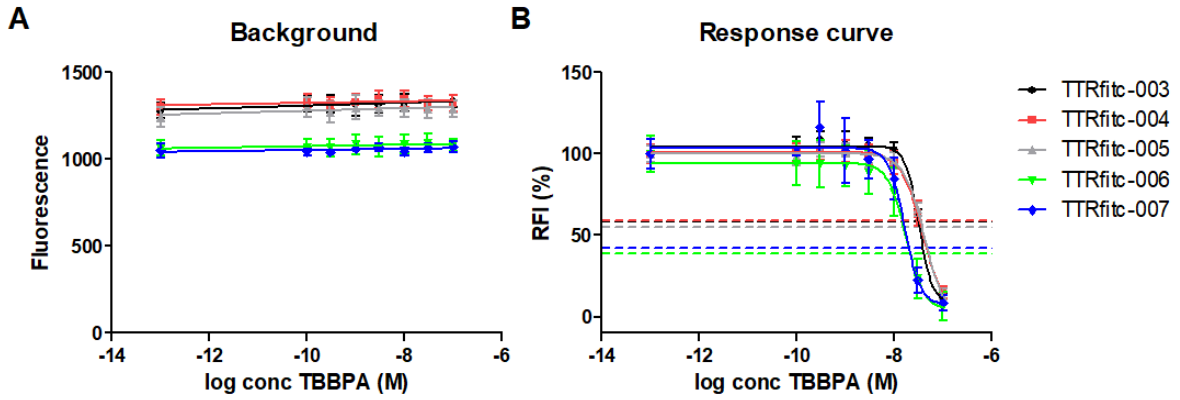


Figure 6. (a) Background fluorescence and (b) response curve in the competitive binding experiment for test item TBBPA.

Table 7. t-value based on the correlation of the background fluorescence, slope of the background fluorescence, and calculated binding values of test item TBBPA.

TBBPA	Background		Binding values			
	t-value	Slope	Log IC50 (M)	IC50 (nM)	IC20 (nM)	Ki20 (nM)
TTRfitc-003	1.760	8.5	-7.490	32.4	21.0	34.0
TTRfitc-004	0.967	4.4	-7.412	38.8	19.1	29.6
TTRfitc-005	1.353	7.6	-7.369	42.8	21.5	35.2
TTRfitc-006	0.851	4.3	-7.756	17.6	9.8	8.1
TTRfitc-007	1.229	3.5	-7.784	16.5	9.9	8.2
Mean			-7.562 ± 0.195	29.6 ± 12.1	16.3 ± 5.9	23.0 ± 13.7
CV of LogIC50			2.58%			

Based on the criteria for autofluorescence, these results are valid.

7.5.5 Test item: BPA

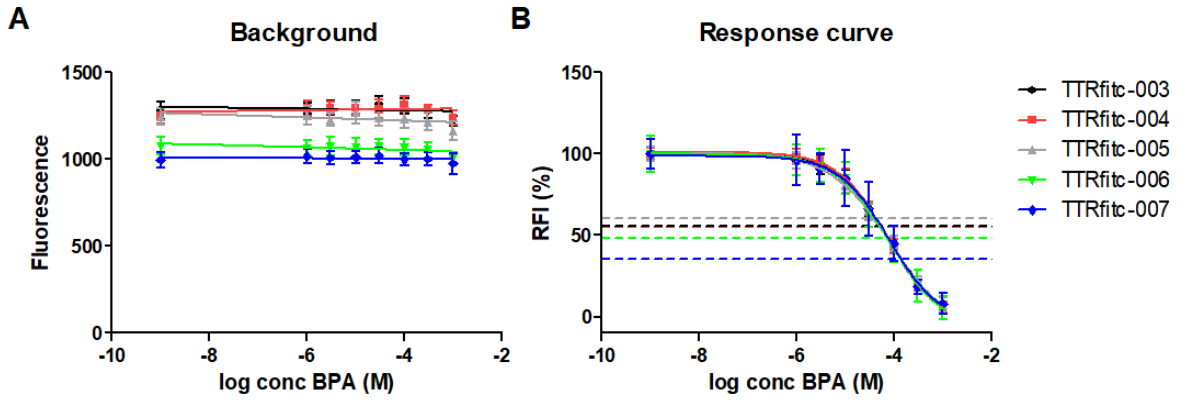


Figure 7. (a) Background fluorescence and (b) response curve in the competitive binding experiment for test item BPA.

Table 8. t-value based on the correlation of the background fluorescence, slope of the background fluorescence, and calculated binding values of test item BPA.

BPA	Background		Binding values			
	t-value	Slope	Log IC50 (M)	IC50 (nM)	IC20 (nM)	Ki20 (nM)
TTRfitc-003	-0.877	-4.5	-4.114	$7.7 \cdot 10^4$	$1.4 \cdot 10^4$	$3.2 \cdot 10^4$
TTRfitc-004	0.605	3.0	-4.121	$7.6 \cdot 10^4$	$1.5 \cdot 10^4$	$3.4 \cdot 10^4$
TTRfitc-005	-1.468	-8.7	-4.080	$8.3 \cdot 10^4$	$1.2 \cdot 10^4$	$2.7 \cdot 10^4$
TTRfitc-006	-1.448	-7.4	-4.093	$8.1 \cdot 10^4$	$1.4 \cdot 10^4$	$3.2 \cdot 10^4$
TTRfitc-007	-0.400	-1.8	-4.096	$8.0 \cdot 10^4$	$1.6 \cdot 10^4$	$3.6 \cdot 10^4$
Mean			-4.101 ± 0.017	$7.9 \cdot 10^4 \pm 0.3 \cdot 10^4$	$1.4 \cdot 10^4 \pm 0.1 \cdot 10^4$	$3.3 \cdot 10^4 \pm 0.3 \cdot 10^4$
CV of LogIC50			0.40%			

Based on the criteria for autofluorescence, these results are valid.

7.5.6 Test item: D-mannitol

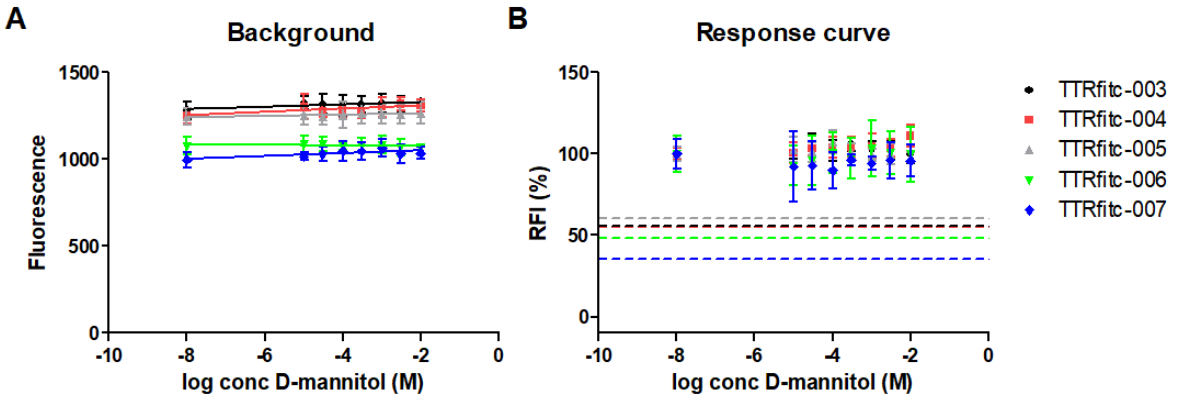


Figure 8. (a) Background fluorescence and (b) response curve in the competitive binding experiment for test item D-mannitol.

Table 9. t-value based on the correlation of the background fluorescence, slope of the background fluorescence, and calculated binding values of test item D-mannitol. n.r.: no response.

	Background		Binding values			
D-mannitol	t-value	Slope	Log IC50 (M)	IC50 (nM)	IC20 (nM)	Ki20 (nM)
TTRfitc-003	1.387	6.7	n.r.	n.r.	n.r.	n.r.
TTRfitc-004	1.928	9.4	n.r.	n.r.	n.r.	n.r.
TTRfitc-005	0.647	3.7	n.r.	n.r.	n.r.	n.r.
TTRfitc-006	-0.316	-1.3	n.r.	n.r.	n.r.	n.r.
TTRfitc-007	1.734	8.1	n.r.	n.r.	n.r.	n.r.
Mean			-	-	-	-
CV of LogIC50			-			

Based on the criteria for autofluorescence, these results are valid.

However, the response curve shows a straight line, meaning that D-mannitol did not show any binding potency. Therefore, an IC50 value could not be calculated and IC20 and Ki20 values are not given.

7.5.7 Overall results

*Table 10. Criteria on the background fluorescence, concentration of the tested ranges and calculated binding values of all test items. n.r.: no response. *out of the 5 experiments the amount of times that the value falls within the set criteria.*

	Criteria*		Range	IC50 and Ki20 values		
Test item	t-value	Slope	Log conc (M)	Log IC50 (M)	CV of log IC50	Ki20 (nM)
T4	5/5	5/5	-9.0 – -6.0	-6.957 ± 0.062	0.90%	42.5 ± 17.8
PFOS	4/5	5/5	-8.0 – -5.0	-6.639 ± 0.090	1.36%	172.6 ± 66.3
Triclosan	5/5	5/5	-8.0 – -5.0	-6.013 ± 0.045	0.75%	661.5 ± 69.0
TBBPA	5/5	5/5	-10.0 – -7.0	-7.562 ± 0.195	2.58%	23.0 ± 13.7
BPA	5/5	5/5	-6.0 – -3.0	-4.101 ± 0.017	0.40%	$3.3 \cdot 10^4 \pm 0.3 \cdot 10^4$
D-mannitol	5/5	5/5	-5.0 – -2.0	n.r.		

8 CONCLUSIONS

This study is performed for PART 1 of the EURL ECVAM coordinated Thyroid Validation Study. In this part a successful validation was carried out by performing five valid runs for the 6 test items. All test items could be tested with our Fluorescent FITC-T4 transthyretin competitive binding assay.

The SOP was followed as described, however, some adjustments were made to complement the SOP further and to improve the readability. But the biggest improvement was the addition of the second criterion for the autofluorescence or quenching of the test item, further explained in PART 2 of this validation study. This last improvement is also of influence to this study, otherwise one of the five runs of PFOS was stated invalid.

The characteristics of manually made FITC-T4 label does not seem to vary from batch to batch based on its Kd value (see batch #001 vs batch #002 supplementary data TTRfitc-suppl-01).

All IC50, IC20, and Ki20 values that were found in this validation study for T4, PFOS, Triclosan and TBBPA are very similar to the values reported by Hamers et al. (2020) (Table 11). The log10 coefficient of variation (CV) of reference and positive test items in this study were <5% for log IC50 in M (Table 10), which indicates a high reproducibility among the five valid runs. This also means that the use of two different batches of TTR does not have a significant effect on the log IC50 value.

Table 11. IC50, IC20, and Ki20 values from part 1 validation study in comparison to values reported by Hamers et al. (2020). REF: reference, TI: Test item, n.r.: no response.

Test item		Hamers et al. (2020)			EU-NETVAL WFSR part 1 validation (2022)			
Code	Name	IC50 (nM)	IC20 (nM)	Ki20 (nM)	IC50 (nM)	IC20 (nM)	Ki20 (nM)	
REF	T4	100	21	32	111	25	43	plate01
TI1	PFOS	250	82	160	234	81	173	plate01
TI2	Triclosan	1100	440	930	976	292	661	plate02
TI3	TBBPA	34	14	17	30	16	23	plate02
TI4	BPA		not tested		79322	14056	32502	plate03
TI5	D-mannitol		not tested		n.r.	n.r.	n.r.	plate03

9 RECOMMENDATIONS

The T4 concentration curve that was tested does not show a full dose response curve. The concentration curve of T4 should be adjusted to 4000, 1000, 250, 62.5, 15.63, 3.91 and 0.98 nM, see supplementary data TTRfitc-suppl-02.

Also an experiment control for 100% binding competition at 0% relative fluorescence intensity should be added, which can be obtained with 3000 nM of TBBPA.

10 RECORDS AND ITEMS TO BE RETAINED

All experiments got a unique number: TTRfitc-xxx, xxx will be an increasing number. All documents, raw and processed data; including solubility testing and range finding of test items, test system characterization and TTR binding screening assay were saved with this unique number.

Documents will be stored or archived and data files that will be submitted to EURL ECVAM. NB: all valid results were sent to EURL ECVAM, there were no invalid results in this study (part 1).

11 REFERENCES

Weiss JM, Andersson PL, Zhang J, Simon E, Leonards PE, Hamers T, Lamoree MH. Tracing thyroid hormone-disrupting compounds: database compilation and structure-activity evaluation for an effect-directed analysis of sediment. *Anal Bioanal Chem.* 2015 Jul;407(19):5625-34. doi: 10.1007/s00216-015-8736-9

Hamers T, Kortenkamp A, Scholze M, Molenaar D, Cenijn PH, Weiss JM. Transthyretin-Binding Activity of Complex Mixtures Representing the Composition of Thyroid-Hormone Disrupting Contaminants in House Dust and Human Serum. *Environ Health Perspect.* 2020 Jan;128(1):17015. doi: 10.1289/EHP5911