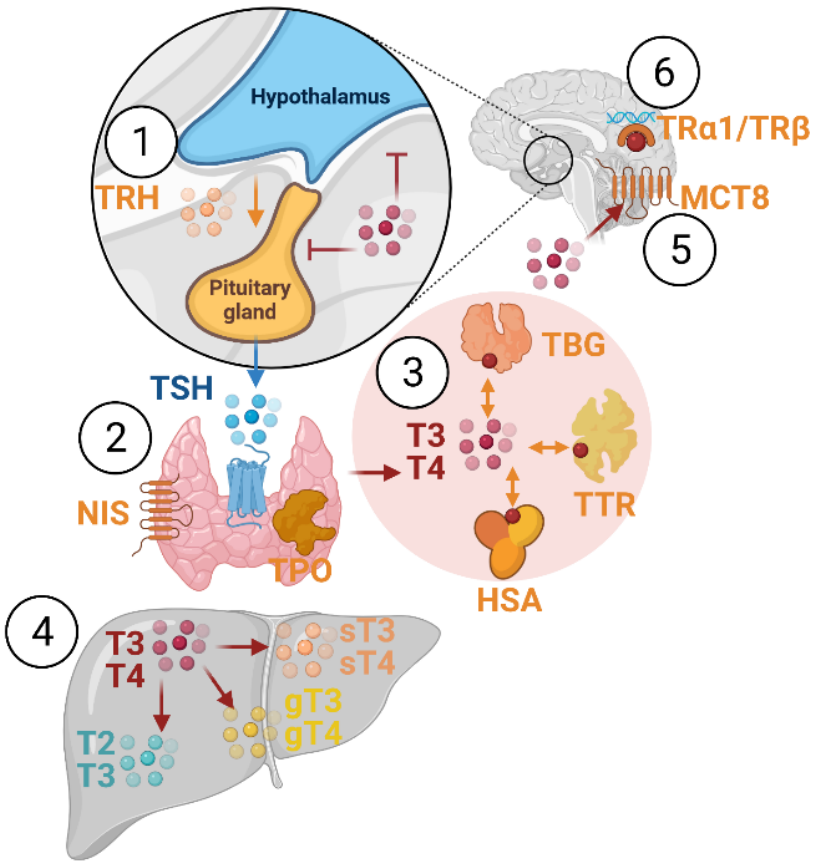
STANDARD OPERATING PROCEDURE

for the fluorescent FITC-T4 Transthyretin (TTR) competitive binding assay, version 1.0

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



2021

This Standard Operating Procedure (SOP) 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.

The SOP provided was used during Part 1 of the validation study. Later versions exist.

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.

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SOP-ID	: SOP01-TTRfitc
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Method number	: 3b
EU-NETVAL	: WFSR: Yoran Weide, Liza Portier and Toine Bovee
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Contents

1 OBJECTIVE AND SCOPE

This SOP describes the implementation of an *in vitro* TTR binding assay, in which items are tested for their potency to competitively inhibit the binding of fluorescently labelled thyroid hormone (FITC-T4) to its transporter protein transthyretin (TTR). In this SOP, test items are tested in default solvent DMSO. Using other solvents to enhance solubility of the test item should only be considered when interferences of the solvent can be excluded. As based on with a solvent control check.

2 DEFINITION

Thyroxine (T4) is a thyroid hormone, which is produced and released by the thyroid gland. Thyroxine-based hormones like T4 are primarily responsible for regulation of metabolism and play a major role in neurodevelopment. TTR is a transport protein in serum that delivers T4 at its target (tissue). Thyroid hormone (TH) disrupting compounds are potentially important environmental contaminants due to their possible adverse neurological and developmental effects on both humans and wildlife. Affecting the binding of TH to TTR is regarded as one of the possible mechanisms of thyroid system disruption by chemicals.

3 PRINCIPLE

Fluorescein isothiocyanate (FITC) is a fluorescent probe, which can be 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 TH disrupting compounds can be determined by calculating the IC50 and or Ki (inhibitory constant) values.

4 Test system, reference item, chemicals and reagents

4.1 Test system and reference item

4.1.1 Test system: Transthyretin (prealbumin, TTR) >95% from human plasma, CAS 87090-18-4

4.1.2 Reference item: L-thyroxine (T4) > 98%, CAS: 51-48-9

4.2 Chemicals

4.2.1 Pyridine (anhydrous) 99.8%, CAS 110-86-1
4.2.2 Triethylamine >99%, CAS 121-44-8
4.2.3 Fluorescein 5-isothiocyanate isomer I (FITC) > 90%, CAS 3326-32-7
4.2.4 Lipophilic Sephadex, CAS 9041-37-6
4.2.5 Ammonium acetate > 98%, CAS 631-61-8
4.2.6 Ammonium bicarbonate >99.5%, CAS 1066-33-7
4.2.7 Sodium bicarbonate, CAS 144-55-8
4.2.8 Tris(hydroxymethyl)aminomethane, CAS 77-86-1
4.2.9 Sodium chloride, CAS 7647-14-5
4.2.10 EDTA >99%, CAS 6381-92-6
4.2.11 Ultrapure water
4.2.12 DMSO >99.5%, CAS 67-68-5
4.2.13 Acetic acid ≥99.7%, CAS 64-19-7
4.2.14 Hydrochloric acid 37%, CAS 7647-01-0
4.2.15 Sodium hydroxide ≥98%, CAS 1310-73-2

4.3 Reagents preparation

4.3.1 Preparation of PWT (pyridine water triethylamine) mixture:
- 9 mL pyridine (4.2.1)
- 1.5 mL ultrapure water (4.2.11)
- 0.1 mL triethylamine (4.2.2)

4.3.2 Preparation of NH_4-acetate (0.2M)

1. Weigh out 7.709 g of ammonium acetate (4.2.5) and record the weight.
2. Dissolve approximately in 400 mL ultrapure water (4.2.11) in a volumetric flask 500 mL.
3. Adjust the pH to 4.0 with acetic acid (4.2.13).
4. Bring the volume to a total volume of 500 mL with ultrapure water.

4.3.3 Preparation of NH_4HCO_3 (4.2.6)

1. Dissolve 1.9765 g in 500 mL volumetric flask in ultrapure water (4.2.11) (0.05 M)

4.3.4 Preparation of $NaHCO_3$ (4.2.7)

1. Dissolve 2.1005 g in approximately 400 mL volumetric flask (5.11) in ultrapure water (4.2.11) (0.05 M).
2. Adjust pH to 8.5 with 1 M NaOH (4.2.15).
3. Bring to a total volume of 500 mL with ultrapure water.

4.3.5 Preparation of Tris-HCl buffer (0.1 M Tris, 0.1 M NaCl, 1 mM EDTA):

1. Weight out 12.11 g Tris (4.2.8) and record weight.
2. Weight out 5.84 g NaCl (4.2.9) and record weight.
3. Weight out 0.372 g EDTA (4.2.10) and record weight.
4. Dissolve the above substances in approximately 800 mL ultrapure water in a volumetric flask 1000 mL.
5. Adjust the pH to 8.0 with 1 M HCl (4.3.10).
6. Bring the volume to a total volume of 1000 mL with ultrapure water.
7. Storage life at room temperature: 2 months.

4.3.6 Preparation column packing Sephadex

1. Saturate Sephadex (4.2.4) overnight in ultrapure water (1:10), i.e. 10 g Sephadex + 100 mL ultrapure water
2. Store at 4°C (Storage life 3 years).

4.3.7 TTR stock solution (3.64 µM) in TRIS-buffer:
Careful: TTR is a tetramer protein and thereby a labile substance. Careful handling is required to prevent dimerization or monomerization of the protein. DO NOT VORTEX!
- dissolve 1 mg TTR (4.1.1) in 5 mL cold (4°C) Tris-HCl buffer (4.3.5)
- aliquot the stock solution in portions of 100 µL, store at -20°C. Storage life: 1 year

4.3.7.1 TTR working solution (120 nM) in TRIS-buffer:
- 90 µL TTR stock solution (4.3.7) + 2660 µL Tris-HCl buffer (4.3.5)
- mix careful by homogenising the solution

4.3.8 Reference item T4 stock solution (1000µM):
- dissolve 3.88 mg of reference item T4 (4.1.2) in 5 mL DMSO (4.2.12)
- vortex briefly

4.3.9 FITC-T4 working solution (220 nM):
- pipette x volume of FITC-T4 produced in 7.1 and concentration determined in 7.1.3 into Tris-HCl buffer (4.3.5) to get 220 nM

4.3.10 Hydrochloric acid (1M):
- dilute 83 mL 37% hydrochloric acid (4.2.14) in 1000 mL ultrapure water

4.3.11 Sodium hydroxide (1M):
- dissolve 40g sodium hydroxide (4.2.15) in 1000 mL ultrapure water

5 EQUIPMENT

5.1 Microplate reader (fluorescence); with filter λ 485 ± 20 nm excitation and λ 528 ± 20 nm emission
5.2 UV-VIS cuvette-based spectrophotometer with shaker ε; 490 nm
5.3 96 Wells black chimney plates polystyrene non-binding
5.4 Burette Column with Frit and Stopcock 300 mm x 10.5 mm ID x 13 mm OD
5.5 Plate shaker
5.6 Semi-micro cuvette (10 mm, suitable for and based on UV-VIS spectrophotometer)
5.7 Polypropylene tube, 50 mL
5.8 Centrifuge (for 50 mL polypropylene tube)
5.9 Polypropylene tube, 1.5 mL
5.10 Amber glass vials
5.11 Volumetric flask 500 mL
5.12 Volumetric flask 1000 mL
5.13 Clear glass vial

6 SAFETY PRECAUTIONS

Handling chemicals, test and reference items

Weighing and dissolving all chemicals, test and reference items should be done with care. Wear disposable gloves. Work as much as possible in a safety cabinet or fume hood. Materials that are contaminated with chemicals, test or reference items should be disposed in toxic waste. Store test and reference items in a fridge (cooled and dark).

7 PROCEDURE

General

Prior to testing of test items for TTR binding potency which can be performed in multiple separate runs (7.3 and 7.4) the production and characterisation of FITC-T4 label should be performed. After production of a batch FITC-T4 label characterisation is performed once (7.1 and 7.2). After characterisation the FITC-T4 should be aliquoted, stored and can be used for multiple separate runs (7.3 and 7.4).

7.1 Production and characterisation of FITC-T4 label batch for multiple separate runs

7.1.1 Preparing FITC-T4

1. Dissolve 10 mg FITC (4.2.3) in 0.5 mL PWT (4.3.1) in an amber glass vial (5.10), 51.4 mM FITC.
2. Dissolve 10 mg T4 (4.1.2) in 1 mL PWT (4.3.1) in an amber glass vial (5.10), 12.9 mM T4.
3. Mix both solutions (FITC + T4) in a new amber glass vial (5.10) and incubate for 1 hour at 37°C.
4. Pipette the mixture (FITC-T4) into a new 50 mL polypropylene tube (5.7).
5. Precipitate the formed FITC-T4 by adding 20 mL of 0.2 M NH_4-acetate (pH 4.0) (4.3.2).
6. Centrifuge for 10 min at 1000 g (5.8).
7. Discard supernatant.
8. Add 20 mL ultrapure water and mix vigorously.
9. Centrifuge for 10 min at 1000 g (5.8).
10. Discard supernatant.
11. Dissolve the pellet in 5 mL of 0.05 M NH_4HCO_3 (4.3.3).
12. Mix thoroughly until FITC-T4 is dissolved (bright clear dark orange colour), see Figure 1.
13. Optional: when mixture does not dissolve easily, incubate at 37°C for a few minutes.

Figure 1: FITC-T4 before purification

7.1.2 FITC-T4 purification

Prepare a column of Sephadex in a burette:

1. Equilibrate the swollen Sephadex (4.3.6) to room temperature.
2. Resuspend/mix and then pour the Sephadex down with a glass rod into the burette (5.4).
3. Let the Sephadex settle and continue pouring until a 4.5 cm packing is achieved.
4. Equilibrate the Sephadex by passing 3 column volumes of 0.05 M $NaHCO_3$ (4.3.4).
5. Add 0.5 mL of the FITC-T4 to the Sephadex-column.
6. Rinse with 10 column volumes 0.05 M $NaHCO_3$ (4.3.4) (± 4.5 mL per column volume), Figure 3.
7. Elute pellet of interest (Figure 4) with 10 mL ultrapure water and collect the eluate temporarily in a 50 mL polypropylene tube (5.9).

Figure 3:

After rinsing 10 column volumes

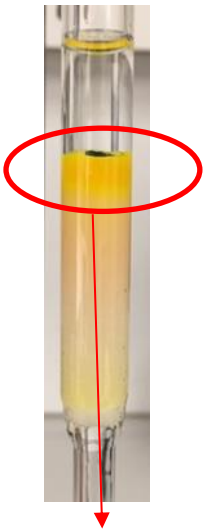

Figure 4:

Pellet of interest

Figure 2:

After rinsing 2 column volumes

7.1.3 **Determination of the FITC-T4 concentration**

When measuring the absorbance of FITC-T4 eluate, a small amount should be diluted by a factor of 10 with ultrapure-water.

1. Measure the absorbance of diluted eluate (FITC-T4) in a cuvette (5.6) with the UV-VIS cuvette-based spectrophotometer (5.2) and use ultrapure-water as a blank.
2. Calculate the concentration with the molar extinction coefficient 7.8x10^4 M/cm (8.1.2):

$$Concentration\ FITC\text{-}T4\ \left[\frac{mol}{L}\right] = \frac{\mathrm{Ax} - \mathrm{A0}}{7.8\mathrm{x}10^4\ * l}\ * dilutionfactor$$

$$Ax = absorbance\ of\ FITC - T4$$

$$A0 = absorbance\ of\ blank\ (ultrapure\ water)$$

$$l = pathlength\ of\ cuvette\ in\ cm$$

3. Aliquot eluate (FITC-T4) preferably in volumes of 200 µL in in a 1.5 mL polypropylene tube (5.9)

4. Store batch of FITC-T4 label at -80°C, concentration (7.1.3) and dissociation constant (7.1.4) should be retested after 5 years. Expiration time is not known yet.

7.1.4 **Characterisation of the FITC-T4 label**

A saturation curve should be performed to determine the dissociation constant (Kd) of the FITC-T4/TTR complex for characterisation of FITC-T4. This characterisation should be performed once after each newly produced batch of FITC-T4 label or retested after 5 years.

- Prepare different FITC-T4 working solutions in 15 concentrations (1 to 15) and a blank (16) according to Table 1 (column A and B).

	A. Working solution FITC-T4 concentration		**B. Working solution FITC-T4 concentration**
1	2000 nM	**9**	200 nM
2	1800 nM	**10**	100 nM
3	1200 nM	**11**	60 nM
4	1000 nM	**12**	20 nM
5	600 nM	**13**	10 nM
6	500 nM	**14**	6 nM
7	400 nM	**15**	2 nM
8	300 nM	**16**	blank (Tris-HCl buffer (4.3.5)

Table 1: Prepare FITC-T4 working solutions (7.1.3) in 15 concentrations and a blank (table 1 A and B) by diluting it with Tris-HCl buffer (4.3.5).

- Pipette the following into a 96 wells plate (5.3) according to Figure 5 to get final concentrations of FITC-T4 (Table 2).

With the addition of TTR per well:

1. 50 µL Tris-HCl buffer (4.3.5)
2. 100 µL working solution x nM FITC-T4 (table 1 A and B) according to Figure 5.
3. 50 µL 120 nM TTR working solution (4.3.7.1) (final concentration 30 nM)

Without addition of TTR per well:

1. 100 µL Tris-HCl buffer (4.3.5)
2. 100 µL working solution x nM FITC-T4 (table 1 A and B) according to Figure 5.

	A. Working solution FITC-T4 concentration	**B. Final concentration in well FITC-T4 (Fig 5.)**		**C. Working solution FITC-T4 concentration**	**D. Final concentration in well FITC-T4 (Fig 5.)**
1	2000 nM	1000 nM	**9**	200 nM	100 nM
2	1800 nM	900 nM	**10**	100 nM	50 nM
3	1200 nM	600 nM	**11**	60 nM	30 nM
4	1000 nM	500 nM	**12**	20 nM	10 nM
5	600 nM	300 nM	**13**	10 nM	5 nM

6	500 nM	250 nM	**14**	6 nM	3 nM
7	400 nM	200 nM	**15**	2 nM	1 nM
8	300 nM	150 nM	**16**	blank (Tris-HCl buffer, 4.3.5)	0 nM (blank, 4.3.5)

Table 2: Working solutions FITC-T4 column A and C to get final concentrations FITC-T4 column B and D.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1000 nM	1000 nM	1000 nM	1000 nM	1000 nM	1000 nM	100 nM	100 nM	100 nM	100 nM	100 nM	100 nM
B	900 nM	900 nM	900 nM	900 nM	900 nM	900 nM	50 nM	50 nM	50 nM	50 nM	50 nM	50 nM
C	600 nM	600 nM	600 nM	600 nM	600 nM	600 nM	30 nM	30 nM	30 nM	30 nM	30 nM	30 nM
D	500 nM	500 nM	500 nM	500 nM	500 nM	500 nM	10 nM	10 nM	10 nM	10 nM	10 nM	10 nM
E	300 nM	300 nM	300 nM	300 nM	300 nM	300 nM	5 nM	5 nM	5 nM	5 nM	5 nM	5 nM
F	250 nM	250 nM	250 nM	250 nM	250 nM	250 nM	3 nM	3 nM	3 nM	3 nM	3 nM	3 nM
G	200 nM	200 nM	200 nM	200 nM	200 nM	200 nM	1 nM	1 nM	1 nM	1 nM	1 nM	1 nM
H	150 nM	150 nM	150 nM	150 nM	150 nM	150 nM	blank (0nM)	blank (0nM)	blank (0nM)	blank (0nM)	blank (0nM)	blank (0nM)
	With TTR			Without TTR			With TTR			Without TTR		
	FITC T4 saturation			FITC T4 saturation			FITC T4 saturation			FITC T4 saturation		
	saturation experiment 30 nM TTR						saturation experiment 30 nM TTR					

Figure 5: Plate layout saturation experiment FITC-T4/TTR. Final concentration in nM FITC-T4 (table 2 B and D)

After pipetting into the 96 wells plate (5.3) subsequently:

1. Mix the plate by using the plate shaker (5.5) at 600 rpm for 5 minutes kept in the dark.
2. Incubate for 15 minutes at room temperature kept in the dark.
3. Measure the plate with the microplate reader (5.1), load the protocol for instrument setup (7.2) and make sure the light bulb is warmed-up.
4. Process the data to determine the dissociation constant (Kd)

Processing data to determine the dissociation constant (Kd)

- For each FITC-T4 concentration, subtract the average fluorescence in the absence of TTR from the triplicate fluorescence values in the presence of TTR to obtain the fluorescence enhancement (Y1) (8.1.1):

$$Y1 = \ fluorescent\ enhancement = [RFU\ \ FITC\text{-}T4\ with\ TTR] - [mean\ RFU\ FITC\text{-}T4\ without\ TTR]$$

- make a two-column table with all tested ligand concentrations of FITC-T4 (L) and their corresponding fluorescent enhancement values (Y1)
- calculate the dissociation constant (Kd value) by performing a non-linear regression on Y as a function of L according to the following quadratic equation (based on the assay conditions) (8.1.3):

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

used variables:

- 30 is the final concentration of protein (TTR) in nM (7.1.4 and 7.4)
- 120 is the concentration of protein (TTR) in nM multiplied by 4 (quadratic equation)

Result assessment of the Kd value required should be between 50 and 300 nM (8.2.5).

7.2 Instrument setup for fluorescent FITC-T4

1. Perform experiment in 96 wells black chimney plates polystyrene non-binding (5.3) and measure from the top.
2. Set the wavelengths at λ 485 ± 20 nm excitation and λ 528 ± 20 nm emission and use the appropriate filter(s) (cubes) or a linear variable filter monochromator for both excitation and emission.
3. The fluorescence range to be measured must fall within the dynamic range of the instrument (consult the instrument manual).

Following settings are only put per batch of FITC-T4 label during characterisation (7.1.4) and should remain for multiple runs of 7.3 and 7.4:

4. The gain setting should be set to distinguish the lowest concentration 1 nM of FITC-T4 separated from background (blank Tris-HCl buffer) (4.3.5) with the following criteria (8.2.7):
   RFU of 1 nM FITC-T4 subtracted by RFU of blank should be > +3SD of blank.

7.3 Preparation and pre-screening of reference and test items

After characterisation of FITC-T4 label multiple separate runs can be performed per batch.
All reference and test items are prepared in the same concentration of DMSO (4.2.12).

7.3.1 Prepare Test Item stock solutions

1. Prepare a stock solution of test item by weighing a nominal amount (e.g. 25-50 mg/mL) in DMSO into a clear glass vial (5.13) and use a vortex. And note every coming procedural step for solving test items.

- using other solvents than DMSO (4.2.12) to dissolve test items is not preferred and should however be assessed for interference of this assay by adding a solvent control check (Figure 6 and Figure 7)

2. Visually check if dissolved. If not dissolved heat and/or ultrasound may be applied to aid solubility.

- If not possible to solubilise, prepare a lower (e.g., 1:2) stock solution, (or dilute existing stock) and again check solubility.

3. Continue until the solution is soluble.
4. Record the concentration of soluble stock solution.

7.3.2 Prepare Reference Item (T4) working solutions

1. Using the reference item (T4) stock solution (4.3.8), prepare a calibration curve in the following work concentrations in amber glass vials (5.10) using DMSO (4.2.12).

- 100 µM T4: pipette 100 µL of 1000 µM T4 stock solution (4.3.8) into 900 µL DMSO
- 30 µM T4: pipette 30 µL of 1000 µM T4 stock solution (4.3.8) into 970 µL DMSO
- 10 µM T4: pipette 100 µL of 100 µM T4 into 900 µL DMSO
- 3 µM T4: pipette 100 µL of 30 µM T4 solution into 900 µL DMSO
- 1 µM T4: pipette 100 µL of 10 µM T4 solution into 900 µL DMSO
- 0,3 µM T4: pipette 100 µL of 3 µM T4 solution into 900 µL DMSO
- 0,1 µM T4: pipette 100 µL of 1 µM T4 solution into 900 µL DMSO
- 0 µM T4: (blank DMSO as a SC, solvent control, 4.2.12)

2. Prepare a plate according to (7.4) for each of the 8 concentrations of the reference item (7.3.2) using the plate layout (Figure 6).

7.3.3 Range Finding (pre-screen) of test items

1. Prepare test item (pre-screening) serial dilutions in the following work concentrations in amber glass vials (5.10) using DMSO (4.2.12).

- highest in DMSO soluble concentration of test item stock (7.3.1) in molar or mg/ml when molecular weight is not known
- 10x dilution test item: pipette 10 µL of test item stock into 90 µL DMSO, vortex briefly
- 10^2x dilution test item: pipette 10 µL of 10x dilution test item into 90 µL DMSO, vortex briefly

- 10^3x dilution test item: pipette 10 µL of 10^2x dilution test item into 90 µL DMSO, vortex briefly
- 10^4x dilution test item: pipette 10 µL of 10^3x dilution test item into 90 µL DMSO, vortex briefly
- 10^5x dilution test item: pipette 10 µL of 10^4x dilution test item into 90 µL DMSO, vortex briefly
- 10^6x dilution test item: pipette 10 µL of 10^5x dilution test item into 90 µL DMSO, vortex briefly
- Solvent control (SC, blank DMSO 4.2.12 or other solvent)

2. Prepare 96 well plates according to 7.4 , one 96 well plate with reference and a test item as displayed in Figure 6 and (multiple) plates for test items as displayed in Figure 7.

A plate with only test items like Figure 7 should include a plate control with final concentration of 100 nM T4

3. Visually check if test items are soluble in the Tris-HCl buffer(4.3.5) (microscopic observations may also be used for confirmation). Wells should be excluded in which insolubility is observed.
4. After pipetting, shake the plate on a plate shaker (5.5) at 600 rpm for 5 minutes on room temperature in the dark.
5. Incubate the treated plate for 15 minutes on room temperature
6. Perform the endpoint measurement as described with settings as determined in 7.2.
7. Check if any binding competition is present and select a range of which possible concentrations would provide a dose response curve from 0 to 100% binding competition (preferably in 3 and 10-fold like reference item, T4 series, 7.3.2) to be used for final testing (7.4) by determining the relative fluorescence enhancement (Y2) [%] as (8.1.5):

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

Binding competition is present when *Y2* (relative fluorescence enhancement) is lower than 100% and decreases with increasing concentrations of test items to a maximum of 0%.

7.4 The competitive TTR binding screening assay

After characterisation of FITC-T4 label multiple separate runs can be performed per batch.

1. Prepare serial dilutions of test items in DMSO, depending on potency and solubility as determined in (7.3) range finder. It should be pursued to have a dose response going from 0% to 100% relative fluorescence enhancement (Y2).
2. Pipette the following according to Figure 6.

With addition of TTR per well:

- 48 µL Tris-HCl buffer (4.3.5)
- 2 µL [T4] reference item or test item [x] (take final concentrations into account)

Without addition of TTR per well:

- 98 µl TRIS buffer (4.3.5)
- 2 µL [T4] standard or test item [x] (take final concentrations into account)

FITC-T4 working solution should be prepared in excess with **30% extra volume** that is minimally needed and should be added within 2 minutes and FITC-T4 working solution as “sinking” of FITC-T4 might appear:

3. Prepare a fresh 220 nM FITC-T4 working solution (30% extra volume) in Tris-HCl buffer (4.3.5) (final concentration 110 nM).
4. Prepare a fresh 120 nM TTR working solution (4.3.7.1) (final concentration 30 nM) and keep on ice.

With and without addition of TTR per well:

- 100 µl working solution 220 nM FITC-T4 (4.3.9)

With addition of TTR per well:

- 50 µl 120 nM of TTR working solution (4.3.7.1)

	mw 1											
	1	2	3	4	5	6	7	8	9	10	11	12
A	SC (0 nM)	SC (0 nM)	SC (0 nM)	SC (0 nM)	SC (0 nM)	SC (0 nM)	SC (0 nM)	SC (0 nM)	SC (0 nM)	SC (0 nM)	SC (0 nM)	SC (0 nM)
B	1 nM	1 nM	1 nM	1 nM	1 nM	1 nM	x nM	x nM	x nM	x nM	x nM	x nM
C	3 nM	3 nM	3 nM	3 nM	3 nM	3 nM	x nM	x nM	x nM	x nM	x nM	x nM
D	10 nM	10 nM	10 nM	10 nM	10 nM	10 nM	x nM	x nM	x nM	x nM	x nM	x nM
E	30 nM	30 nM	30 nM	30 nM	30 nM	30 nM	x nM	x nM	x nM	x nM	x nM	x nM
F	100 nM	100 nM	100 nM	100 nM	100 nM	100 nM	x nM	x nM	x nM	x nM	x nM	x nM
G	300 nM	300 nM	300 nM	300 nM	300 nM	300 nM	x nM	x nM	x nM	x nM	x nM	x nM
H	1000 nM	1000 nM	1000 nM	1000 nM	1000 nM	1000 nM	x nM	x nM	x nM	x nM	x nM	x 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		
	reference item [T4] calibration curve						test item [A]					

Figure 6: Plate layout competitive binding experiment with T4 reference item and test item. Note: concentrations are given in final concentrations in 96 well.

	mw x											
	1	2	3	4	5	6	7	8	9	10	11	12
A	SC (0 nM)	SC (0 nM)	SC (0 nM)	SC (0 nM)	SC (0 nM)	SC (0 nM)	100 nM T4	100 nM T4	100 nM T4	100 nM T4	100 nM T4	100 nM T4
B	x nM	x nM	x nM	x nM	x nM	x nM	x nM	x nM	x nM	x nM	x nM	x nM
C	x nM	x nM	x nM	x nM	x nM	x nM	x nM	x nM	x nM	x nM	x nM	x nM
D	x nM	x nM	x nM	x nM	x nM	x nM	x nM	x nM	x nM	x nM	x nM	x nM
E	x nM	x nM	x nM	x nM	x nM	x nM	x nM	x nM	x nM	x nM	x nM	x nM
F	x nM	x nM	x nM	x nM	x nM	x nM	x nM	x nM	x nM	x nM	x nM	x nM
G	x nM	x nM	x nM	x nM	x nM	x nM	x nM	x nM	x nM	x nM	x nM	x nM
H	x nM	x nM	x nM	x nM	x nM	x nM	x nM	x nM	x nM	x nM	x nM	x 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		
	test item [B]						test item [C]					

Figure 7: Plate layout competitive binding experiment with test items, solvent control (SC) and 100 nM T4 as plate controls. Note: concentrations are given in final concentrations in 96 well.

5. After pipetting, shake the plate on a plate shaker (5.5) at 600 rpm for 5 minutes on room temperature in the dark.
6. Incubate for 15 minutes on room temperature in the dark.

7. Measure fluorescence with the plate reader (5.1) with the protocol according to 7.2 and make sure the light bulb is warmed-up.

7.5 Check for autofluorescence or quenching by the test item

1. Determine 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. Include the RFU values for the solvent control in this correlation. Do not use a test item concentration of 0 for the solvent control, because ^{10}log(0) is undefined. Instead, fill out a concentration 1000x smaller than the lowest test concentration of the test item.

2. Test if r significantly deviates 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* 6 and *Figure* 7). 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. (8.2.9)

3. If fluorescence does not change with increasing concentrations of test item (i.e. $|t| < t_{0.05(2),n-2}$) the test item does not affect the readout of the experiment, and IC50 and Ki values may be determined.

7.6 Processing data to determine IC50/ Ki value

1. Determine the relative fluorescence enhancement (Y2) [%] as (8.1.5):

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

2. Make a two-column table with final concentrations (A) of the test item and their corresponding fluorescent enhancement values (Y)

3. Calculate the median inhibition concentration (IC50) by performing a non-linear regression on *Y* as a function of *A* according to Hill equation (8.1.6):

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

4. Based on the values for IC50 and HillSlope, ICx values can be calculated for any percentage (x) of inhibited FITC-T4 binding, according to (8.1.7):

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

5. Based on the ICx value, the dissociation constant of the inhibitor-TTR complex (Ki) can be calculated according to (8.1.8):

$$Ki = \frac{Kd * PLx * ICx}{Lx * PIx} - Px$$

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

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

used variables:

- 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)

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

$$Lx = 110 - \left(\frac{100 - x}{x}\right) * \frac{(Kd + 140) - \sqrt{(Kd + 140)^2 - 13200}}{2}$$

used variables:

- 110 being the total concentration in the test system of the ligand (FITC-T4) in nM
- 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 and ligand (TTR x FITC-T4) in nM^2 multiplied by 4 (quadratic equation)

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

$$PIx = 30 - PLx * \left(\frac{Kd}{Lx} + 1\right)$$

used variables:

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

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

$$Px = \frac{Kd * PLx}{Lx}$$

8 RESULTS

8.1 Calculations

8.1.1 Fluorescent enhancement:

$$Y1 = \ fluorescent\ enhancement = [RFU\ \ FITC\text{-}T4\ with\ TTR] - [mean\ RFU\ FITC\text{-}T4\ without\ TTR]$$

8.1.2 FITC-T4 concentration determination with cuvette based UV-VIS spectrophotometer:

$$Concentration\ FITC\text{-}T4\ \left[\frac{mol}{L}\right] = \frac{\mathrm{Ax} - \mathrm{A0}}{7.8\mathrm{x}10^4\ * l}\ * dilutionfactor$$

$$Ax = absorbance\ of\ FITC - T4$$

$$A0 = absorbance\ of\ blank\ (ultrapure\ water)$$

$$l = pathlength\ of\ cuvette\ in\ cm$$

8.1.3 Estimation of dissociation constant (Kd) for FITC-T4-TTR complex:

Solve the quadratic equation describing Y1 as a function of L by non-linear regression:

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

used variables:

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

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

8.1.4 Relative fluorescence enhancement (%):

$$Y2 = relative\ fluorescence\ enhancement$$

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

8.1.5 Concentration-response curve fit with IC50 estimation:

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

8.1.6 ICx estimation:

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

with x is percentage inhibition

8.1.7 Estimation of dissociation constant (Ki) for inhibitor-TTR complex:

$$Ki = \frac{Kd * PLx * ICx}{Lx * PIx} - Px$$

8.1.8 Solvent control (SC) plate to plate control:

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

8.1.9 Plate to plate positive control 100 nM T4:

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

8.1.10 Check for autofluorescence or quenching by test item with Student's T-test:

$$s_r = \sqrt{\frac{1 - r^2}{n - 2}}$$

8.2 Results assessment

8.2.1 Solvent control (SC) test plate to reference plate control (7.4)
Test plate (plate x) to reference plate (plate 1) solvent control (8.1.9) should be between 65 and 135%.

8.2.2 Solvent control (SC) test plate to test plate control (7.4)
Test plate (plate x) to test plate (plate y) solvent control (see 8.1.9) should be between 65 and 135%.

8.2.3 Positive test plate to reference plate control (7.4)
Test plate (plate x) to reference plate (plate 1) control 100 nM T4 (8.1.10) should be between 65 and 135%.

8.2.4 Positive test plate to test plate control (7.4)
Test plate (plate x) to test plate (plate y) control 100 nM T4 (see 8.1.10) should be between 65 and 135%.

8.2.5 Kd value requirements (7.1.4)
The Kd value (7.1.4) should be between: 50 and 300 nM.

8.2.6 IC50 value requirements of reference item (7.4)
The IC50 of T4 should be between 40 and 160 nM.

8.2.7 Setting the gain of the instrument (7.2)
RFU of 1 nM FITC-T4 subtracted by RFU of blank (Tris-HCl buffer) should be > +3SD of blank (7.2)

8.2.8 Auto fluorescence and quenching by test item check with Pearson correlation coefficient and Student's t-test (7.5)
In case of n=24, the critical value of $t_{0.05(2),n-2}$ = 2.0739

9 LITERATURE

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