Call for partner laboratories-deadline: 31st July

Method: *Fluorescent FITC-T4 Transthyretin Competitive Binding Assay for endocrine disruptors*

Transthyretin (TTR) is a thyroid hormone distributor protein (THDP) that is considered to be the most important THDP for delivery of TH to target tissues. TTR is one of the three plasma carrying proteins distributing TH from the thyroid gland to the peripheral target tissues.

In addition, TTR plays a major role in the transport of TH across the placenta and the blood-cerebrospinal-fluid-barrier (BCSFB). Mammalian TTR has high affinity for thyroxin (T4). Many xenobiotic compounds (including PCB- and PBDE-metabolites, PFAS, bisphenols, parabens, benzophenones) have been shown to be capable of competing with TH for binding to TTR. Consequently, T4 transport across the placenta and BCSFB may be decreased, and trans-barrier transport of xenobiotics may be increased.

FITC is a fluorescent probe; upon binding of T4-FITC to 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 IC_{50} and/or Ki (inhibitory constant) values.

This method is used in order to assess endocrine disruption and possible adverse neurological and developmental neurotoxicity.

The method had already been assessed under the EU NETVAL network for the pre-validation of thyroid assays. The OECD thyroid disruption methods expert group has positively evaluated this FITC-T4 TTR-binding assay as being sensitive, specific, and reproducible. The experts recognised TTR-binding as a relevant molecular initiating event for the identification and characterisation of thyroid hormone system disruptors and recommended further validation.

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Method description

This method evaluates the effect of test items for their potency to **competitively** inhibit the binding of fluorescently labelled thyroid hormone (FITC-T4) to its transporter protein transthyretin (TTR).

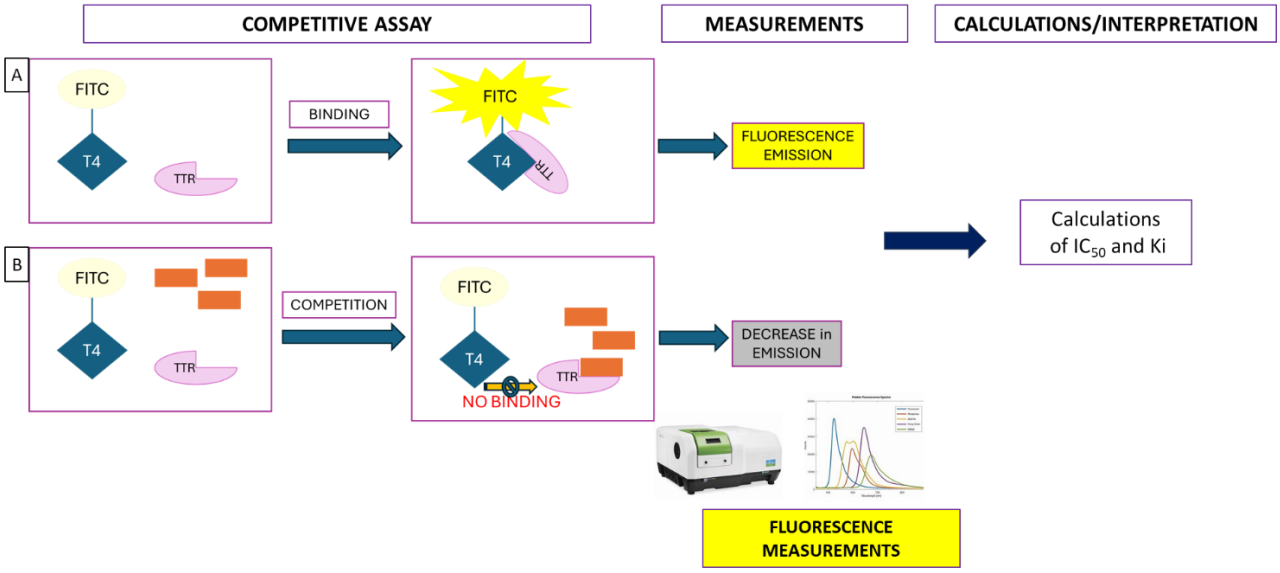

*Figure 1: FITC is a fluorescent probe; upon binding of T4-FITC to TTR as a test system, an **increase** in fluorescence is observed (A) most likely due to the elimination of intramolecular fluorescence quenching of the FITC group by the iodine groups of the bound T4. This increase in fluorescence, however, is **abolished** by adding **competitors** for TTR-binding (B).*

After 15 minutes of exposure at room temperature, with test chemicals with or without TTR, fluorescence measurements are performed and if a decrease is observed, IC_{50} and Ki are calculated to determine if test items are competitors of T4.

Readouts:

- Fluorescence Measurements

Prediction model

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 **IC_{50} and/or Ki** (inhibitory constant) values.

- If the concentration-response curve results in the calculation of an IC_{50} value, the test item interferes with T4 binding and is a competitor.

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- When the concentration-response curve does not result in the calculation of an IC_{50} value and the datapoints show a straight or increasing line, the test item does not interfere with T4 binding to TTR and is classified as a no responder (n.r.).

Validation of the method

Validation overview

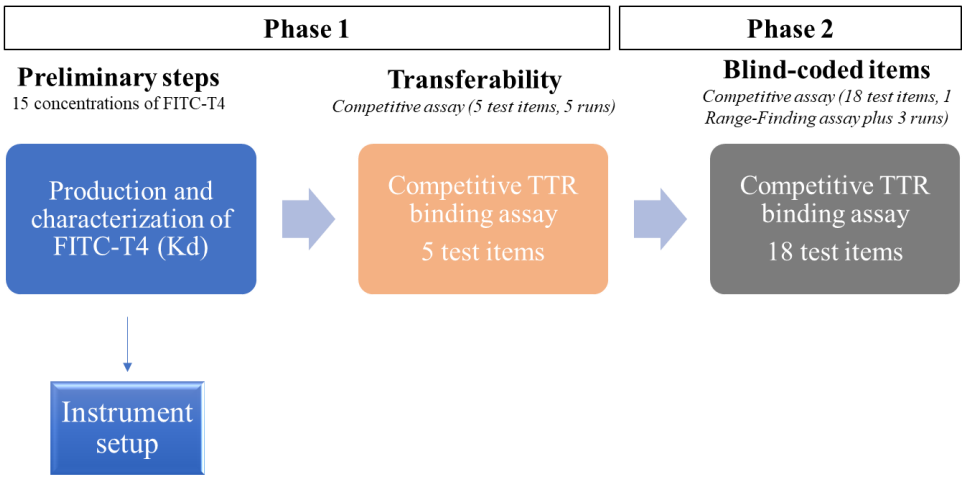

Figure 2: Validation process overview

Phase 1-Preliminary steps

Before performing the assay, the FITC-T4 batch needs to be produced and characterized => A saturation curve with 15 concentrations of FITC-T4 must be performed to determine the dissociation constant Kd (one 96 wells plate). This step needs to be performed for each batch of FITC-T4 produced and every 5 years. One batch can be used for all validation experiments. The required Kd value should be between 50 and 300nM.

	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 3: Plate layout of the saturation experiment of FITC-T4/TTR complex with final concentrations of FITC-T4 in nM

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Phase 1 (Transferability)

- Test 5 test items, 5 valid independent runs.
- Each run includes one reference plate with reference item (T4) and one test item, all other plates tested on the same time will contain 2 test items per plate. These plates must be run at the same time as the reference plate.

Steps:
-Solubility determination of the test items.
-Prepare a calibration curve of reference item (T4).
- Test 5 test items, 5 valid runs.

REFERENCE PLATE (to be performed for each run)

	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)	3000 nM TBBPA	3000 nM TBBPA	3000 nM TBBPA	3000 nM TBBPA	3000 nM TBBPA	3000 nM TBBPA
B	0.9766 nM	0.9766 nM	0.9766 nM	0.9766 nM	0.9766 nM	0.9766 nM	x M	x M	x M	x M	x M	x M
C	3.906 nM	3.906 nM	3.906 nM	3.906 nM	3.906 nM	3.906 nM	x M	x M	x M	x M	x M	x M
D	15.63 nM	15.63 nM	15.63 nM	15.63 nM	15.63 nM	15.63 nM	x M	x M	x M	x M	x M	x M
E	62.5 nM	62.5 nM	62.5 nM	62.5 nM	62.5 nM	62.5 nM	x M	x M	x M	x M	x M	x M
F	250 nM	250 nM	250 nM	250 nM	250 nM	250 nM	x M	x M	x M	x M	x M	x M
G	1000 nM	1000 nM	1000 nM	1000 nM	1000 nM	1000 nM	x M	x M	x M	x M	x M	x M
H	4000 nM	4000 nM	4000 nM	4000 nM	4000 nM	4000 nM	x M	x M	x M	x M	x M	x M
	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 4: Plate layout of the competitive binding experiment with T4 reference item and solvent control (SC) on the left, 3000 nM TBBPA as experiment control and one test item on the right (7 concentrations in triplicate).

TEST PLATE

	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)	62.5 nM T4	62.5 nM T4	62.5 nM T4	62.5 nM T4	62.5 nM T4	62.5 nM T4
B	x M	x M	x M	x M	x M	x M	x M	x M	x M	x M	x M	x M
C	x M	x M	x M	x M	x M	x M	x M	x M	x M	x M	x M	x M
D	x M	x M	x M	x M	x M	x M	x M	x M	x M	x M	x M	x M
E	x M	x M	x M	x M	x M	x M	x M	x M	x M	x M	x M	x M
F	x M	x M	x M	x M	x M	x M	x M	x M	x M	x M	x M	x M
G	x M	x M	x M	x M	x M	x M	x M	x M	x M	x M	x M	x M
H	x M	x M	x M	x M	x M	x M	x M	x M	x M	x M	x M	x M
	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 5: Plate layout of the competitive binding experiment with 2 test items (7 concentrations in triplicate), solvent control (SC)on the left, 62.5 nM T4 as experiment control on the right.

Phase 2 (Blind ring trial)

Test 18 test items: The test items will be sent to the labs by Pepper, blind-coded.

- First perform one range-finding experiment per test item.
- Perform main assays (3 valid runs for each test item) using concentrations determined during the range-finding experiment.

As described for phase 1, each run includes one reference plate with one test item, all other plates tested will contain 2 test items per plate tested at the same time as the reference plate.

Steps:
-Solubility determination of the test items.
-Prepare a calibration curve of reference item (T4).
- Test 18 test items, range-finding experiment, then 3 valid runs .

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Annex 1 : Necessary equipment and materials

Test system, label and reference items
Test system: Transthyretin (prealbumin, TTR) >95% from human plasma, CAS 87090-18-4
Label: Fluorescein 5-isothiocyanate isomer I (FITC) > 90%, CAS 3326-32-7
L-thyroxine (T4) >98%, CAS: 51-48-9

Test items:

- Bisphenol-A (BPA) (≥ 99%)
- TBBPA 97%, powder, CAS 79-94-7
- Triclosan (certified reference material, TraceCERT ®), CAS 3380-34-5
- Perfluorooctanesulfonic acid (PFOS) (95%)
- D-mannitol (≥ 98%), CAS 69-65-8

Chemicals

- Pyridine (anhydrous) 99.8%, CAS 110-86-1
- Triethylamine >99%, CAS 121-44-8
- Lipophilic Sephadex, CAS 9041-37-6
- Ammonium acetate > 98%, CAS 631-61-8
- Ammonium bicarbonate >99.5%, CAS 1066-33-7
- Sodium bicarbonate, CAS 144-55-8
- Tris(hydroxymethyl)aminomethane, CAS 77-86-1
- Sodium chloride, CAS 7647-14-5
- EDTA >99%, CAS 6381-92-6
- Ultrapure water
- DMSO >99.5%, CAS 67-68-5
- Acetic acid ≥99.7%, CAS 64-19-7
- Hydrochloric acid 37%, CAS 7647-01-0
- Sodium hydroxide ≥98%, CAS 1310-73-2

Materials

- 96 well black chimney polystyrene non-binding plates
- Burette Column with Frit and Stopcock 300 mm x 10.5 mm ID x 13 mm OD
- Semi-micro cuvette (10 mm, suitable for and based on UV-VIS spectrophotometer)
- Polypropylene tube, 50 mL
- Centrifuge (for 50 mL polypropylene tube)
- Polypropylene tube, 1.5 mL
- Amber glass vials
- Volumetric flask 500 mL
- Volumetric flask 1000 mL
- Clear glass vial

Equipment

- Microplate reader (fluorescence); with filter λ 485 ± 20 nm excitation and λ 528 ± 20 nm emission
- UV-VIS cuvette-based spectrophotometer with shaker ε; 490 nm
- Plate shaker

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Validation steps summary

- **Phase 1**
- **Preliminary steps:** Production and characterization of FITC-T4 batch, this step must be done before the assays, only once per batch.
- **Transferability:** 5 test items to be tested with 5 independent valid runs of each test item.

- **Phase 2 (Blind ring test):** 18 test items to be tested, with 3 independent valid runs of each test item, after a range-finding experiment that will determine concentrations to use in the main assays.
- In addition to the experimental operations, the partner lab will have to participate to a training organised in the Dev Lab facilities (2 days at Wageningen Food Safety Research (WFSR), Akkermaalsbos 2, 6708 WB Wageningen, the Netherlands; this training is foreseen end of September at earliest or October 2024).

- Partner lab will have to participate to regular online meetings with other labs and Pepper.
- For each phase, a study plan, raw and processed data and a report will be sent to Pepper.
- All runs (both invalid and valid runs) must be sent to Pepper.

ANNEX 2 : Required information for the Offer

In this offer, please provide the following information: financial conditions linked to the validation of the assay (consumables, workforce). In addition, specify in this offer the following logistic information: contingency plan and description of the quality system set up in your lab.

Method of interest	*"Fluorescent FITC-T4 Transthyretin Competitive Binding Assay for endocrine disruptors"*
Date of the proposal	*dd-mm-yyyy*
Laboratory Name and address	
Contact person information [name(s), email(s) and phone number(s)]	
Description of the work	*The way in which the laboratory intends to carry out the work must be described in brief, but in sufficient detail to demonstrate its understanding of the project.* *The availability of key equipment should be mentioned..*
Starting date and planning	*Expected planning for phase I and phase II, including experimental phases as well as reporting.*
Laboratory quality assurance	*QA guaranties of the laboratory must be described (GLP or other processes) .*
Contingency plan (to ensure continuity of the work)	*Indicate how the Continuity of work is ensured (e.g. illness, holidays of chief investigator and key persons ; response to materials delivery delays…)*
Apply for funding.	*Indicate the financial support you need to participate in the validation as a "test laboratory".* *Provide a separate estimate for each phase (transferability, ring trial).* *Note that Pepper's support is fungible between work and consumables.).*

To apply

Please sent your offer to : Philippe.hubert@ed-pepper.eu and to Torben.österlund@ed-pepper.eu before July 31st 2024.

If you have questions, please do not hesitate to contact us.

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Annex 3: References

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