Inter-laboratory validation of the Xenopus Embryonic Thyroid Signalling Assay

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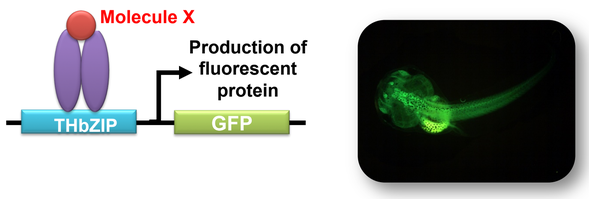
With support from France and financial participation from Watchfrog and the French ministry responsible for ecology, a validation plan has being developed for the Xenopus Embryonic Thyroid signalling Assay (XETA). Laboratories from OECD member states participate in the validation study, including laboratories from Europe, North America, and Asia. The objectives of the validation are : 1) to establish the relevance of the assay by assessing its sensitivity to detect disruption by compounds active at different points within the thyroid system, and 2) to assess the reproducibility of the assay across participating laboratories.

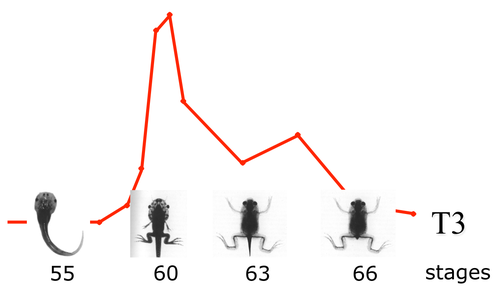
XETA is an aqueous assay based on the genetic detection of a chemical's impact on transgenic *Xenopus laevis* at embryonic stages. This transgenic line can detect the activity of Thyroid Hormone (TH) agonists that activate TH receptors, as well as antagonists of thyroid axis that work through various mechanisms. The XETA provides a rapid response (<72 h), allowing an efficient method for screening thyroid disruptors. The basis of the assay is the measurement of GFP fluorescence in the THbZIP-GFP tadpoles. Each translucent tadpole expresses a basal fluorescence. In contact with a thyroid disruptor, the GFP transcription is down or up regulated, revealing the action of chemicals on the thyroid system.

In addition to serving as a quick screen for thyroid active chemicals, XETA, could serve as a potential alternative method to the *in vivo* Amphibian Metamorphosis Assay (OECD 231). This test is based on the study of the metamorphosis of *X. laevis* tadpoles after three weeks of exposure to a given chemical, and includes histology of the thyroid gland. XETA could provide an alternative test that can be performed quickly, providing information that would be useful for screening. It is intended that this assay will be applied to testing in the context of REACH and other international testing programs.

XETA : an *in vivo* transcriptional assay

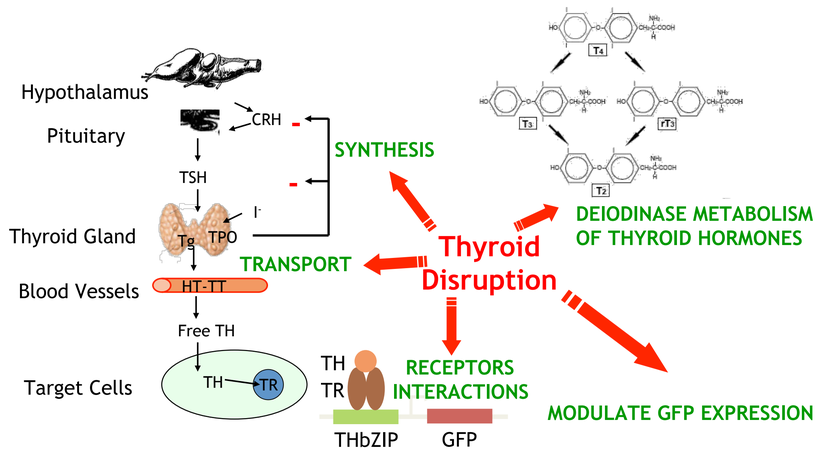


A tadpole from the THbZIP transgenic line carries a genetic construct with GFP expression driven by the THbZIP promoter. The THbZIP gene is directly regulated by thyroid hormones during xenopus metamorphosis.



Xenopus metamorphosis is the criteria chosen by the OECD to identify thyroid disruptors in the test guideline 231. This developmental process is dependant on a functional thyroid hormone axis and undisturbed action of the hormone in the tissues.

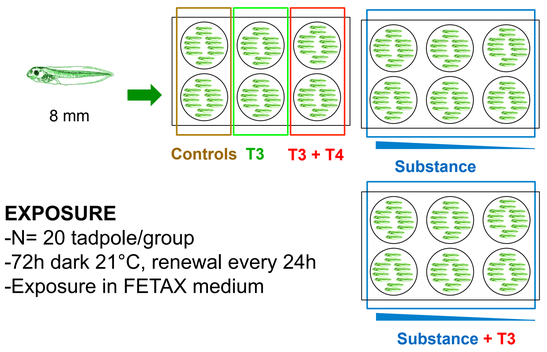
Disruption of human and amphibian thyroid system



Thyroid disruptors act at different levels of the thyroid axis and will lead to the modification of target genes expression. By revealing this terminal downstream event, any disruption of the thyroid system will be highlighted by changing levels of GFP expression.

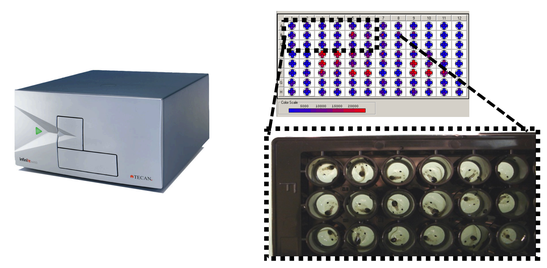
Protocol outline

EXPOSURE in 6 well plates



EXPOSURE
-N= 20 tadpole/group
-72h dark 21°C, renewal every 24h
-Exposure in FETAX medium

AUTOMATED READING in 96 well-plate fluorescent reader



Groups of 20 tadpoles are exposed to the chemicals in 6 well plate, 10 tadpoles per well. Five concentrations of the chemical are tested with or without spiking with 3.25 µg/l of T3. Inhibitors of the deiodinases or receptor antagonists are detected in the T3 spiked mode. After 72h of exposure, the tadpoles are anesthetised and placed in a 96 well plate. The fluorescence is quantified using an automated fluorescence plate reader.

OECD inter laboratory validation phase one

Goals of phase one

-Provide a detailed protocol for the test method
-Demonstration of the inter-laboratory transferability
-Provide data on intra-laboratory and inter-laboratory reproducibility
-Demonstration of the test method's performance using a first set of reference chemicals
-Provide a set of data for expert review

-Three labs involved :
Japan (T. Iguchi), USA (D. Buscholtz), France (Watchfrog)

-Chemicals :
-3 active molecules**:**
T4 (natural thyroid receptor agonist) ,
TRIAC (synthetic thyroid receptor agonist),
PTU (TPO and deiodinase inhibitor)
-1 inactive molecule :
Cefuroxime (cephalosporin antibiotic)
-5 concentrations of each substance +/- T3
-Chemical analysis to verify that the substances were tested at the correct concentrations.

Statistical analysis of the phase one results

Country	Statistical Test	T4 (mg/l)									
		-T3					+T3				
		5	2	1	0,1	0,01	5	2	1	0,1	0,01
USA	Dunnett										
	JONCKHEERE										
	WILLIAMS										
Japan	Dunnett										
	JONCKHEERE										
	WILLIAMS										
France	Dunnett										
	JONCKHEERE										
	WILLIAMS										

Country	Statistical Test	PTU (mg/l)									
		-T3					+T3				
		100	30	10	3	1	100	30	10	3	1
USA	Dunnett										
	JONCKHEERE										
	WILLIAMS										
Japan	Dunnett										
	JONCKHEERE										
	WILLIAMS										
France	Dunnett										
	JONCKHEERE										
	WILLIAMS										

Country	Statistical Test	TRIAC (mg/l)									
		-T3					+T3				
		5	2	1	0,1	0,01	5	2	1	0,1	0,01
USA	Dunnett										
	JONCKHEERE										
	WILLIAMS										
Japan	Dunnett										
	JONCKHEERE										
	WILLIAMS										
France	Dunnett										
	JONCKHEERE										
	WILLIAMS										

Country	Statistical Test	CEFUROXIME (mg/l)									
		-T3					+T3				
		100	30	10	3	1	100	30	10	3	1
USA	Dunnett										
	JONCKHEERE										
	WILLIAMS										
Japan	Dunnett										
	JONCKHEERE										
	WILLIAMS										
France	Dunnett										
	JONCKHEERE										
	WILLIAMS										

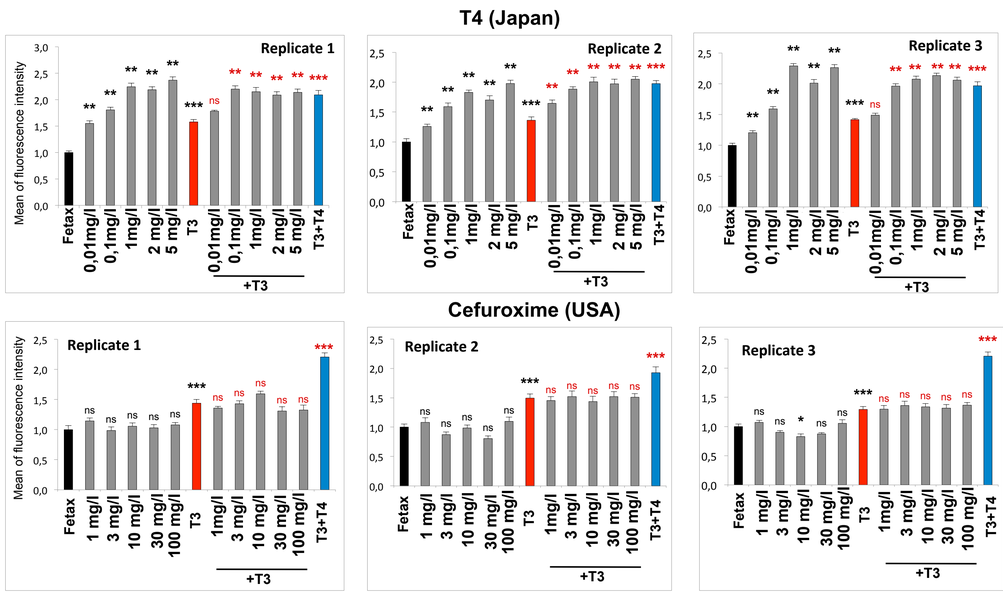
Significant effects were found at low concentrations levels by the three laboratories for the active chemicals.
The concentrations giving statistical difference compared to the controls are indicated in green for the active chemicals and in red for the inert chemical. Three statistical approaches were used to analyse the combined raw data of the three replicates for each laboratory. For the active chemicals the determined LOECs are consistent across labs and across the different statistical tests. The results for the inert chemical are more variable and influenced by the statistical test. The results of the phase 2 trial with a larger set of chemicals will allow the final selection of the statistical analysis.

6 concentrations, 3 Replicates, 10 Tadpoles				
Effect %	Williams	JT	Dunnett	Dunn
10	60%	41%	39%	15%
15	90%	57%	75%	28%
25	100%	71%	99%	38%
50	100%	96%	100%	49%

6 concentrations, 3 Replicates, 20 Tadpoles				
Effect %	Williams	JT	Dunnett	Dunn
10	61%	41%	42%	16%
15	90%	56%	77%	28%
25	100%	68%	99%	37%
50	100%	96%	100%	52%

Selected power of the three statistical tests.
Statistical analysis shows that the number of tadpoles per group could be reduced from 20 to 10 without affecting the statistical power.

Examples results of replicates



A high level of reproducibility is observed among replicates.
A dose response is observed for T4 with the identification of the lowest dose as an active concentration in every replicate. No effects are observed for cefuroxime, only one tested concentration in one of the three replicates was statistically different from the control. The histograms represent the mean fluorescence intensity of 20 tadpoles. The errors bars indicate the SEM. Statistical analysis : ANOVA followed by a Dunnet's test for parametric data, Dunn's test for non-parametric data. **:p<0,01 ***: p<0,001.

Conclusions :

-Significant effects of PTU, TRIAC and T4 were found at low concentrations in all three labs.
-A similar inter and intra laboratory reproducibility was observed.
-The test was successfully transferred to two new labs.
-Based on the statistical analysis, the number of tadpoles required in the assay is only 10 per concentration.
-A three replicate design gives the statistical power to detect a 15% increase in fluorescence.
-A larger set of data is required to allow the definitive statistical approach to be selected.

Goals for phase 2 :

Set the definitive XETA experimental design and statistical analysis by creating a dataset.

-Include new labs.
-Test chemicals with other modes of action.
-Test additional inert chemicals.