The Endocrine Disruptor Assay: Thyroid peroxidase (TPO)

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The endocrine system is a vital messenger system of the body, that utilizes a plethora of hormones. These signalling molecules travel through the bloodstream, as a communication tool to exert effects on distant cells, tissues, and organs. This signalling is essential for controlling many processes within the body, from early ones like embryonic development and organ formation to the functional and metabolic control of tissue and organ functions in thyroid-controlled activities and sexual function in the androgenic axis of the human body. The chemical substances of natural or synthetic origin, which can alter the function of endocrine signaling systems could result in adverse effects on the health of humans and animals are termed endocrine disruptors (ED) (New Scoping Document on in *Vitro and Ex Vivo Assays* for the Identification of Modulators of Thyroid Hormone Signalling, 2017).

The thyrogenic adverse effects lead to metabolic disorders, while the adverse effects on androgenic axis leads to developmental disorders. It is significant that each chemical exposed to life/environment and ecosystem must be assessed for lack of adverse effects on both these systems. In long run, established adverse effects of these significant controllers may obviate any need for Developmental Toxicity studies in animals, while they may be coupled with a few other *in vitro/in silico tests.*

EDs remain a matter of global concern and a challenge to the global community, not only towards life, but also towards ecology & environment. Hence, various regulatory agencies throughout the world are actively employing various screening programs like the Endocrine Disruptor Screening Program (EDSP) to limit exposure and raise the flag regarding the nature of the chemical substance. Bisphenol A usage ban in baby bottles and toys is a result of such efforts *(Bisphenol A (BPA): Use in Food Contact Application | FDA, n.d.).*

*I*n the EDSP screening several chemicals (pharmaceutical, crop-care, Specialty / performance chemicals, their metabolites and degradants are subjected to screening for their potential to impact the three important hormones' androgen, estrogen, and thyroid. Several strategies have been laid down to study reproductive steroids Estrogen and Androgen. However, due to the complexity of the thyroid system, a limited number of assays are available to screen thyroid hormones.

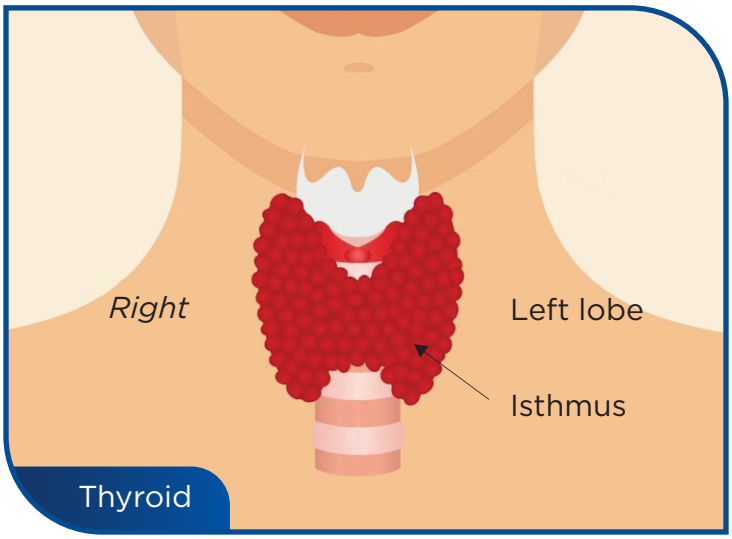


Fig 1 Thyroid organ

Etymology for thyroid

Thyroid borrowed from New Latin *thyroīdēs,* shortened from *thyreoīdēs,* borrowed from Greek *thyreoeidḗs* "shield-shaped (of the cartilage in the larynx)," from *thyreós* "stone put against a door to keep it shut, oblong shield" (probably originally noun derivative of an adjective meaning "shaped like a door," from *thýra* "door" + -eos, suffix of appurtenance) + *-oeidēs* (Source: merriam-webster.com/dictionary/thyroid)(*Thyroid | Definition of Thyroid by Merriam-Webster,* n.d.)

In a true sense, they do function as a door for all metabolic processes.

This key haem containing enzyme involved in thyroid hormone synthesis, thyroid peroxidase (TPO) is one of the major target for enzymes in the series of key events related to the thyroid functioning and thus significant for establishing thyrogenic ED potential. This enzyme catalyzes mono- and diiodination of L-tyrosine (L-Tyr) to generate 3-Iodo-L-tyrosine (MIT) and 3,5-Diiodo-L-tyrosine (DIT), respectively, followed by the coupling of iodinated tyrosine rings to generate thyroid hormones, 3,3′5-Triiodo-L-thyronine (T3) and Levothyroxine (T4) as shown in fig 2 (Tater et al., 2021)

With the development of the field of alternatives and increased importance on 3Rs (Replace, Reduce and Refine) principles, demand for developing in vitro assays that closely represent in vivo situations, is also soaring high.

Thus, we at JRF, have developed a robust, sensitive, and rapid *in vitro* system to evaluate the effect of chemicals on the multiple catalytic activities of thyroid peroxidase.

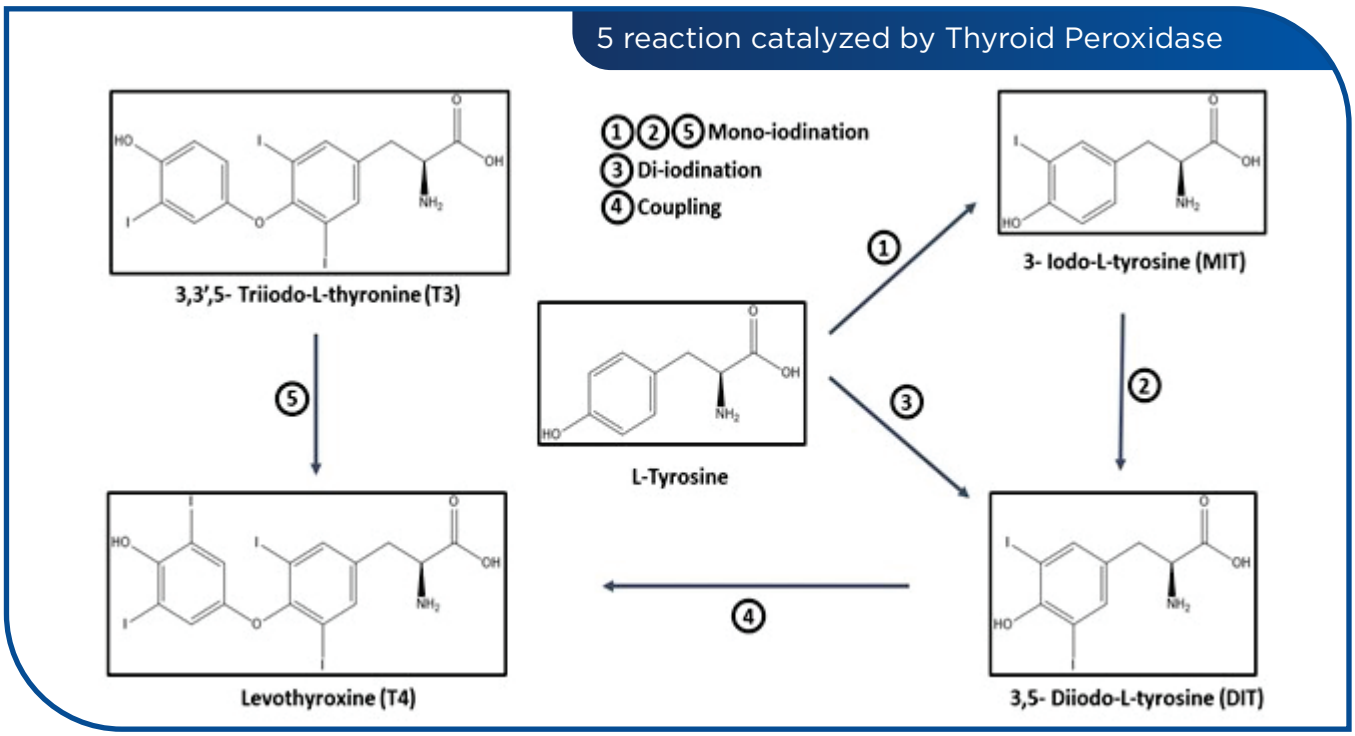


Fig 2. Graphical representation of 5 reactions catalysed by TPO enzyme.

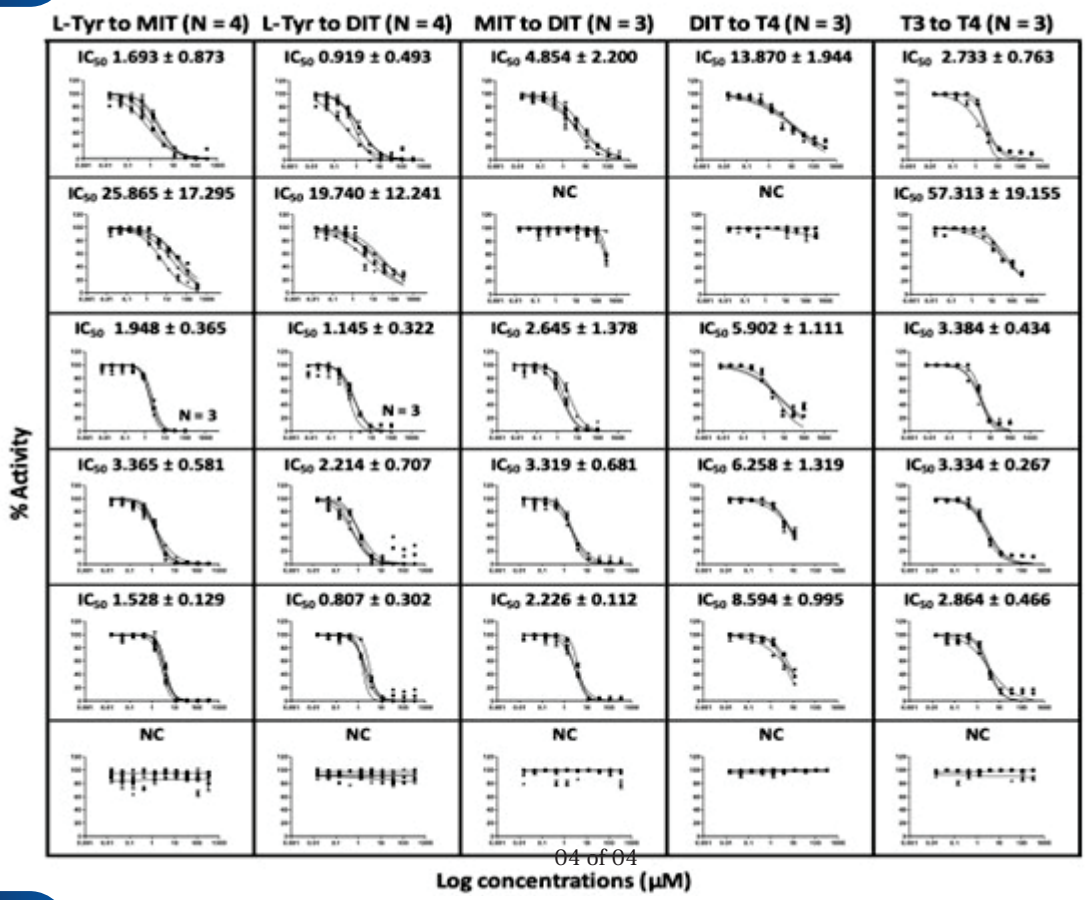
(Tater et al., 2021)

Unlike previously employed methods used to study TPO which analyze the conversion of single TPO substrate or pseudo substrates, JRF developed battery of TPO assay make use of physiologically more relevant substrates of TPO, defining an exact discrete key event (Tyrosine to MIT, MIT to DIT, DIT to T3 and T4). This in vitro assay also offers a unique perspective to study multiple reactions catalyzed by the TPO. This enables the researcher to study the actual quantification of iodine organification as well of synthesis at all the steps for the formation of the thyroid hormones.

This LC-MS/MS-based highly sensitive, selective, and rapid detection method combined with simple assay set up makes this approach highly attractive for accurate screening of xenobiotic.

Kinetic analysis of each TPO catalyzed reaction showed distinct Km and Vmax values, which were used to get turnover number (Kcat) and Kcat/Km (catalytic efficiency) for all these key events. Overall, using this strategy, JRF published a set of assays for the first time to characterise the TPO function. These assays can be analyzed uniquely to study five discrete events in thyroid hormone synthesis. The assay can be used to investigate as well as discriminate the inhibition potential of known and unknown TPO inhibitors.

A



B

Test Chemicals	L-Tyr to MIT (N=4)		L-Tyr to DIT (N=4)		MIT to DIT (N=3)		DIT to T4 (N=3)		T3 to T4 (N=3)	
	IC50	SD	IC50	SD	IC50	SD	IC50	SD	IC50	SD
AGN	1.693	0.873	0.919	0.493	4.854	2.200	13.870	1.944	2.733	0.763
DZN	25.865 *	17.295	19.740 *	12.241	NC	-	NC	-	57.313	19.155
ETU	1.948 *	0.365	1.145 *	0.322	2.645	1.378	5.902	1.111	3.384	0.434
PTU	3.365	0.581	2.214	0.707	3.319	0.681	6.258	1.319	3.334	0.267
GST	1.528	0.129	0.807	0.302	2.226	0.112	8.594	0.995	2.864	0.466
TST	NC	-	NC	-	NC	-	NC	-	NC	-

*N=3

L-Tyr: L-tyrosine disodium salt, MIT: 3-Iodo-L-tyrosine, DIT: 3,5 Diiodo-L-tyrosine, T3: 3,3',5- Triiodo-L-thyronine, T4: Levothyroxine, AGN: Apigenin, DZN: Daidzein, ETU: Ethylenethiourea, PTU: 6-Propyl -2-thiouracil, GST: Genistein, TST: Testosterone, IC50: Half maximum inhibitory concentration, NC: Not converged

Fig 3: Inhibition of multiple TPO catalyzed reactions by AGN, DDZ, ETU, GST, and PTU

Graphs illustrating inhibitor concentration v/s % activity were plotted as per four parametric fits using Graph-pad prism. The individual reaction catalyzed by TPO, with the corresponding substrate is indicated. TST was taken as a negative control. IC50 values (inhibitor concentration which produces 50 % enzyme inhibition) for each reaction with respective inhibitors are shown. (Tater et al., 2021)

In vitro toxicology is becoming popular as alternatives to animal methods models are being developed by scientists throughout the world and JRF is happy to contribute a small part in these mammoth tasks.

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About the Author:

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Dr. Rahul Date is a Biochemist with more than 20 years of experience, leading R&D team at JRF. His team is focused on developing various in vitro assays specifically in the field of skin sensitisation, ADME assays and endocrine disruptor. His team has recently published work on TPO assay CRTOX journal.

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