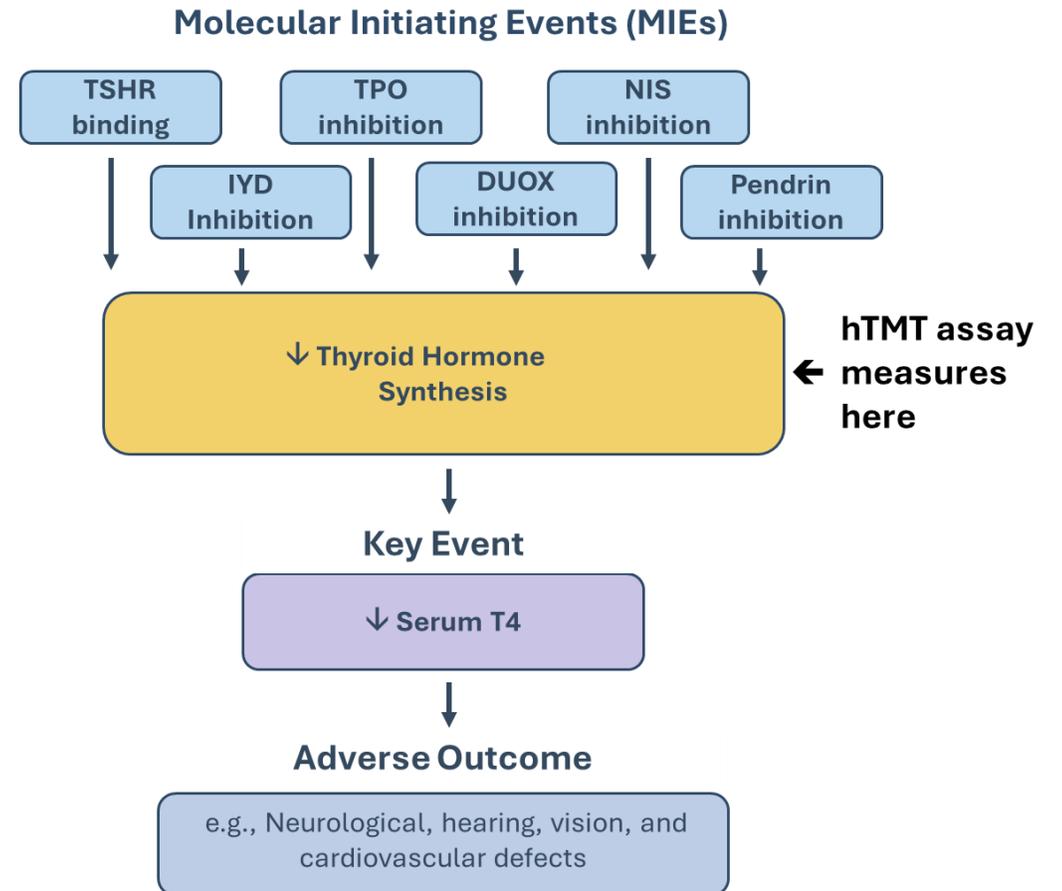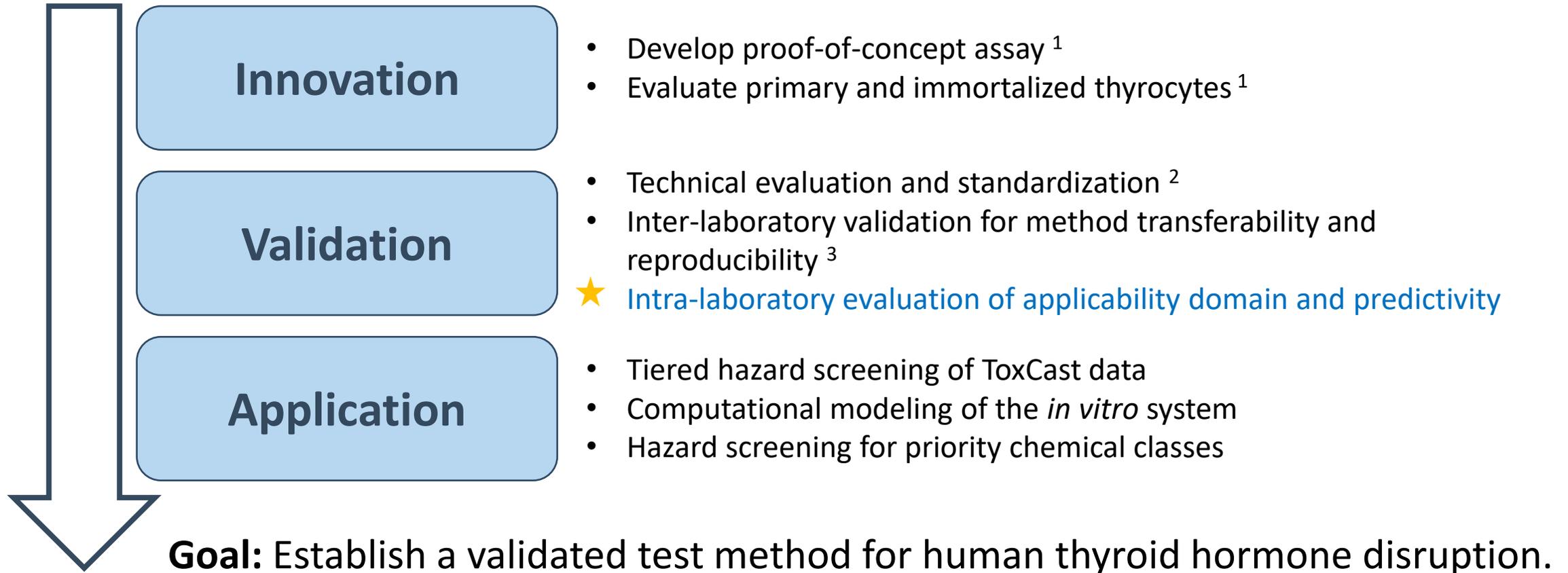# Gaps in Current Thyroid NAMs

- The Endocrine Disruptor Screening Program evaluates chemical effects on thyroid pathways.

- Existing thyroid NAMs focus on chemical – molecular initiating event (MIE) interactions.

- These assays do not indicate whether MIE interactions affect thyroid hormone synthesis.

- The **Human Thyroid Microtissue (hTMT) assay** provides an orthogonal, hormone-level assessment linked to downstream biological effects.

Molecular Initiating Events (MIEs)

TSHR binding | TPO inhibition | NIS inhibition

IYD Inhibition | DUOX inhibition | Pendrin inhibition

↓ Thyroid Hormone Synthesis

hTMT assay measures here

Key Event

↓ Serum T4

Adverse Outcome

e.g., Neurological, hearing, vision, and cardiovascular defects

Human Thyroid Microtissue Assay

Innovation
- Develop proof-of-concept assay [1]
- Evaluate primary and immortalized thyrocytes [1]

Validation
- Technical evaluation and standardization [2]
- Inter-laboratory validation for method transferability and reproducibility [3]
- ⭐ Intra-laboratory evaluation of applicability domain and predictivity

Application
- Tiered hazard screening of ToxCast data
- Computational modeling of the *in vitro* system
- Hazard screening for priority chemical classes

**Goal:** Establish a validated test method for human thyroid hormone disruption.

[1]Deisenroth, Chad, et al. "Development of an in vitro human thyroid microtissue model for chemical screening." *Toxicological Sciences* 174.1 (2020): 63-78.

[2]Foley, B., et al. "Technical Evaluation and Standardization of the Human Thyroid." *Toxicological Sciences*.
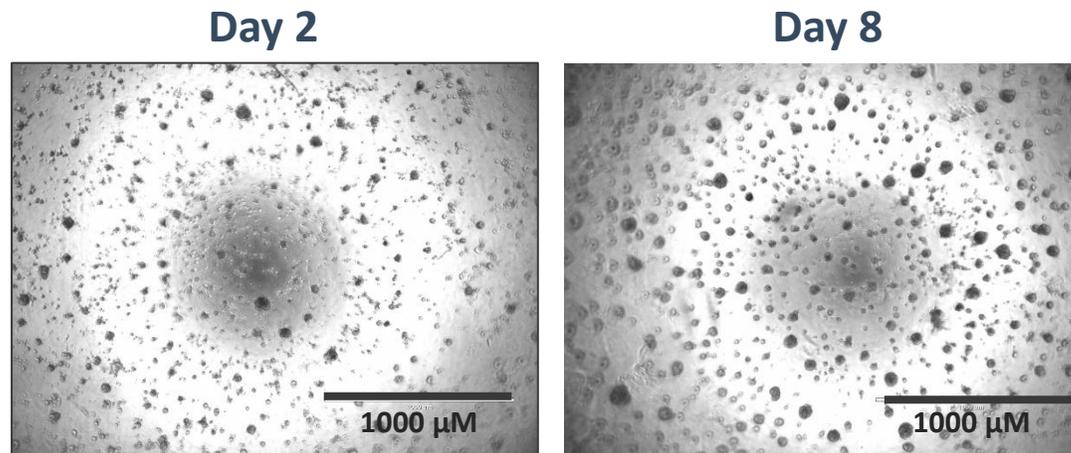
[3]Deisenroth, Chad, et al. "Interlaboratory validation of the human thyroid microtissue assay." *Toxicological Sciences* (2025): kfaf166.

Human Thyroid Microtissue Assay

14-day hTMT Assay Workflow
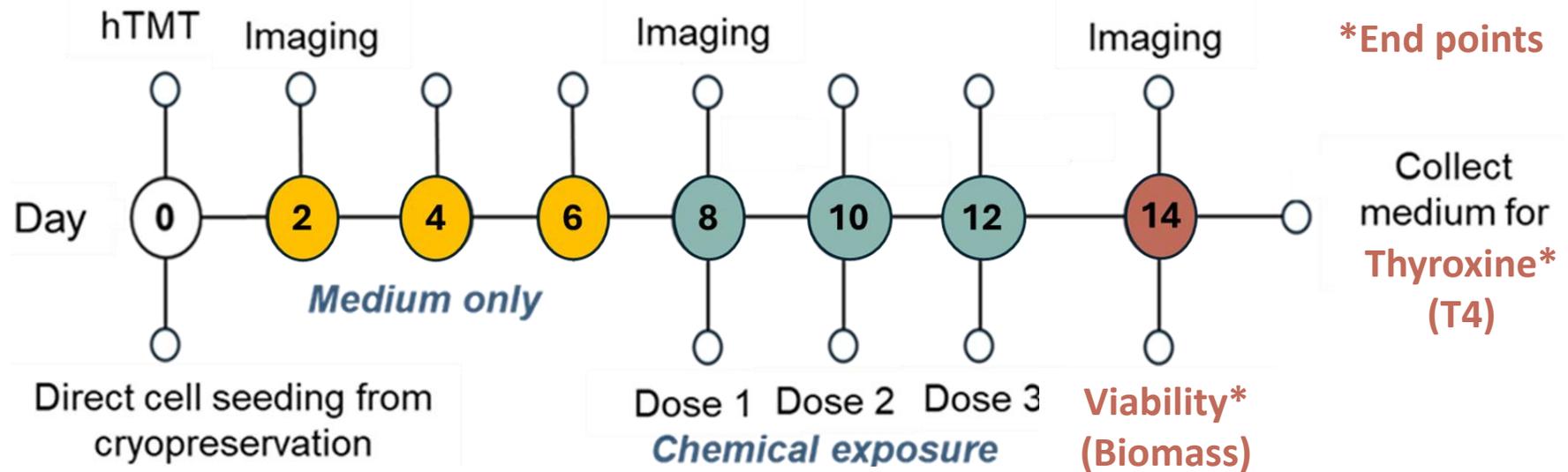
Imaging of Cell Morphology

Reference Chemicals for Proficiency Testing[1]

Chemical	Mechanism	IC$_{50}$ (μM)
Methimazole	TPO inhibitor	0.29 – 0.94
6-Propyl-2-thiouracil	TPO inhibitor	0.46 – 1.15
Sodium perchlorate	NIS inhibitor	2 - 6
Methomyl (reference negative)	NA	NA

[1]Foley, B., et al. "Technical Evaluation and Standardization of the Human Thyroid." *Toxicological Sciences*.

Intra-laboratory Validation of the hTMT Assay

Objectives:

1. Assess the predictive accuracy of the hTMT assay across an expanded chemical test set.

2. Evaluate assay performance across experimental replicates.

3. Quantify predictive performance metrics (sensitivity, specificity, and balanced accuracy).

Chemical Test Set

Chemical Selection

- Subject Matter experts used systematic, weighted-evidence literature review to identify chemicals with thyroid pathway activity or inactivity, spanning thyroid hormone synthesis and related mechanisms.

- Independent *in vitro* studies contributed additional chemicals with evidence of T4 activity or inactivity.

- The final test set consisted of **49 putative thyroid-active and thyroid-inactive chemicals.**

Evidence Sources and Thyroid Pathway Activity for the Test Set

Source	Number of Active Chemicals	Number of Inactive Chemicals
European Center for the Validation of Alternative Methods (ECVAM)[1]	28	32
European Food Safety Authority (EFSA)[2]	12	-
U.S. EPA hTMT Reference Chemicals[3]	3	1
U.S. EPA hTMT T4 Inhibitors[4]	3	-

A Priori Classification Workflow

Published literature & independent *in vitro* studies
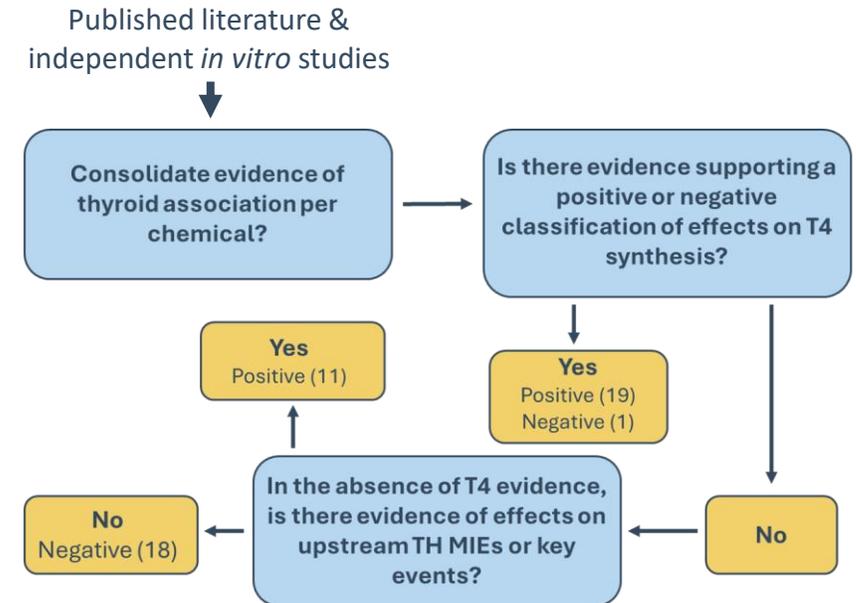
A Priori Classification Summary

A priori classification	Predicted Bioactivity	Number of chemicals
Positive	T4 disruptive	30
Negative	Non-disruptive	19

[1]Bernasconi, Camilla, et al. "Chemical selection for the Thyroid Validation Study coordinated by EURL ECVAM and involving EU-NETVAL laboratories." ALTEX (2025).
[2]European Food Safety Authority (EFSA), et al. "Establishment of cumulative assessment groups of pesticides for their effects on the thyroid." EFSA Journal 17.9 (2019): e05801.EPA.
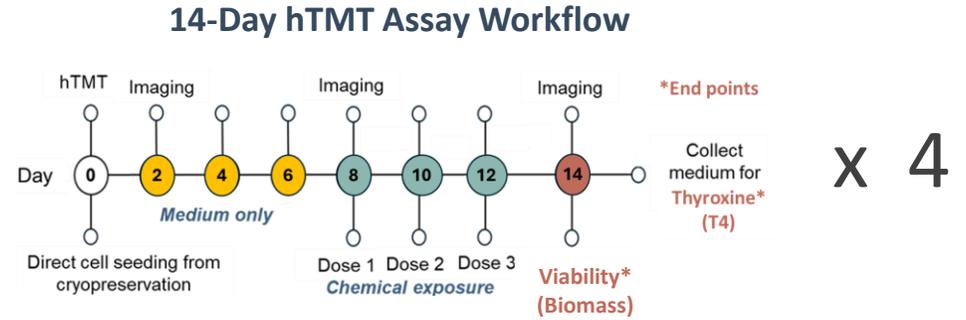[3]Foley, B., et al. "Technical Evaluation and Standardization of the Human Thyroid." Toxicological Sciences.
[4]Deisenroth, Chad, et al. "Interlaboratory validation of the human thyroid microtissue assay." Toxicological Sciences (2025): kfaf166.
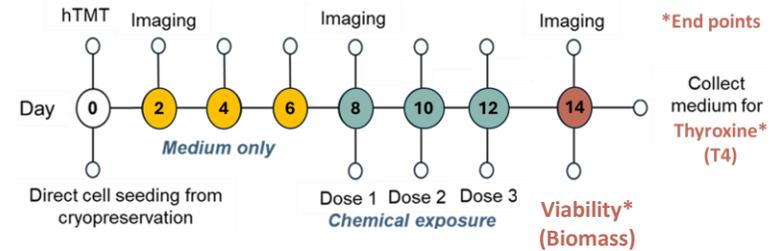
Experimental Design

Experimental replicates consisted performing the standard hTMT assay on **four separate timelines**.

Each replicate consisted of **six 96-well plates** to accommodate the full chemical test set.

x 4

Experimental Design

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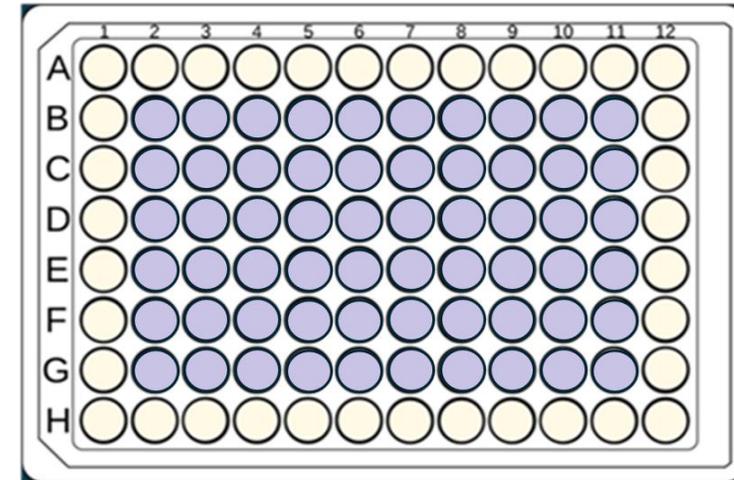**Each replicate** consisted of **six 96-well plates** to accommodate the full chemical test set.

Each plate consisted of:
- Pooled donor cells suspended in medium **without thyroid stimulating hormone (TSH)** supplementation and seeded in the **inner 60 wells** previously coated in Matrigel® Matrix.

14-Day hTMT Assay Workflow

x 4

Cells in TSH (-), 3×10^4 p/well
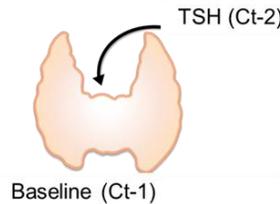
TSH (-) only

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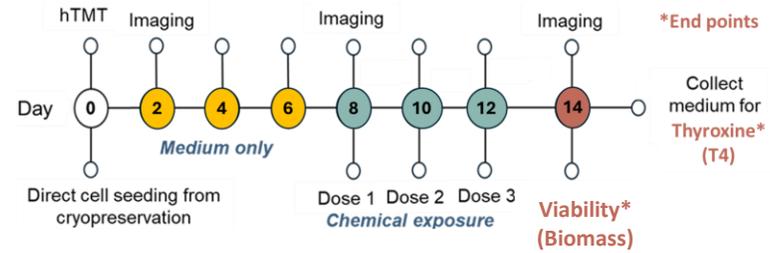
Each replicate consisted of **six 96-well plates** to accommodate the full chemical test set.
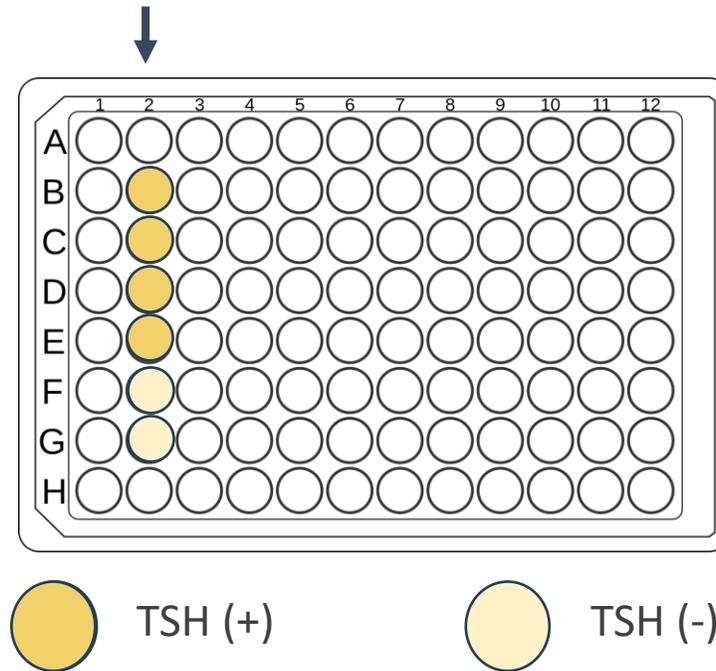
Each plate consisted of:
- Pooled donor cells suspended in medium **without thyroid stimulating hormone (TSH)** supplementation and seeded in the **inner 60 wells** previously coated in Matrigel® Matrix.

- **TSH controls:**
  - **Ct-2** (medium with TSH supplementation)
  - **Ct-1** (medium without TSH supplementation)

14-Day hTMT Assay Workflow

x 4

TSH (Ct-2)

Baseline (Ct-1)

⬤ TSH (+)   ◯ TSH (-)
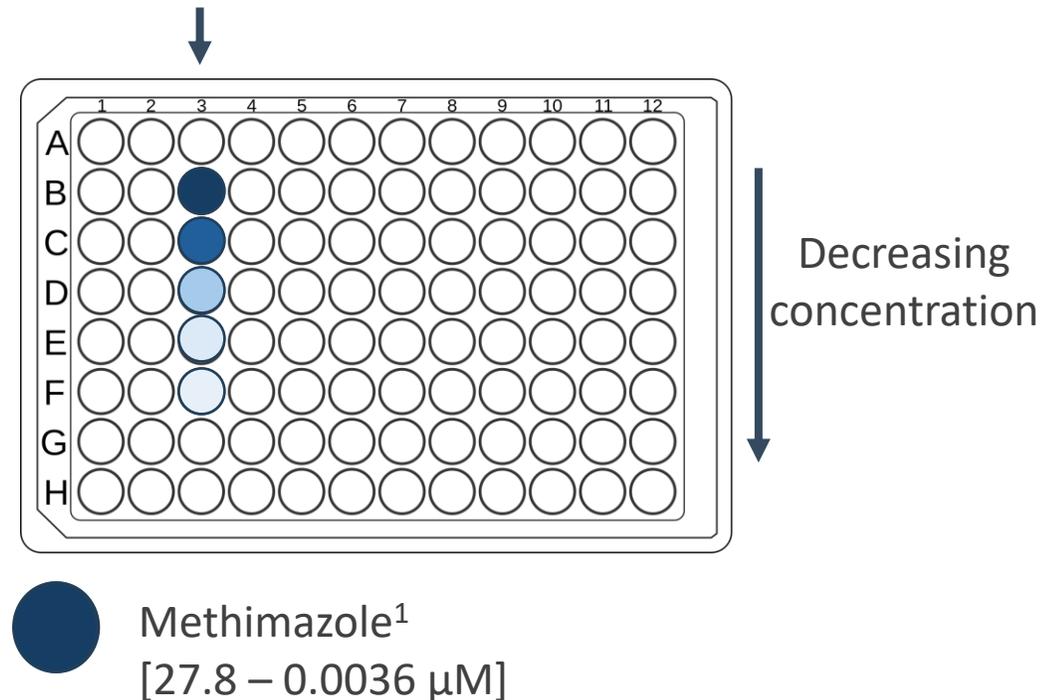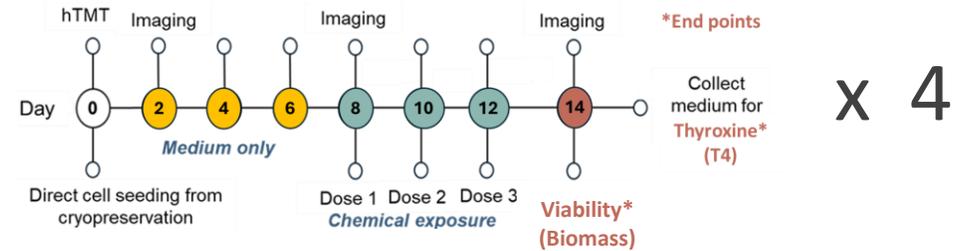
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  - **Ct-1** (medium without TSH supplementation)

- **Reference chemical control :**
  - **Methimazole** (anti-thyroid drug and TPO inhibitor)

14-Day hTMT Assay Workflow

x 4

Decreasing concentration

Methimazole[1]
[27.8 – 0.0036 μM]

[1]Foley, B., et al. "Technical Evaluation and Standardization of the Human Thyroid." *Toxicological Sciences*.
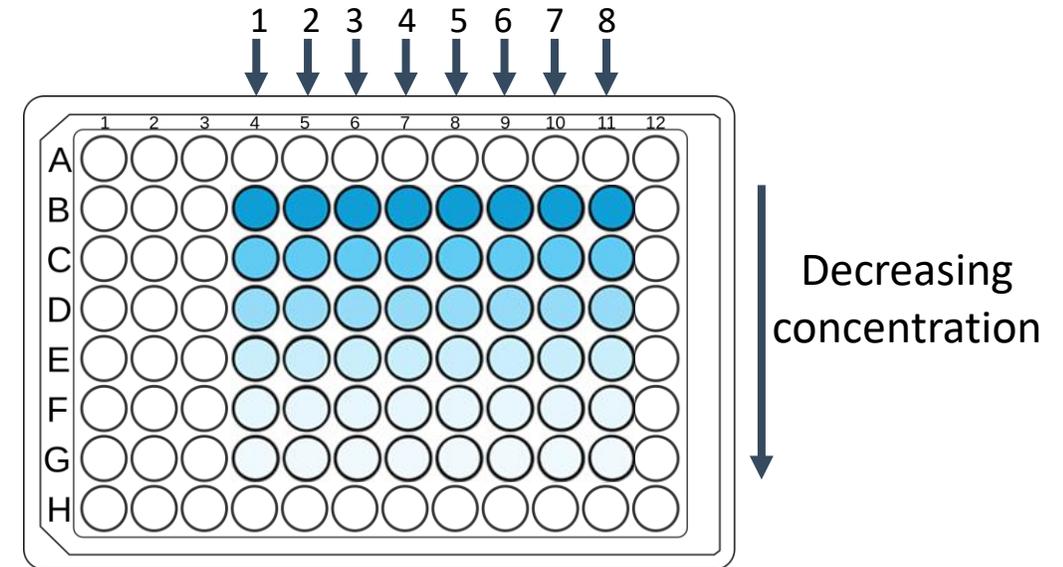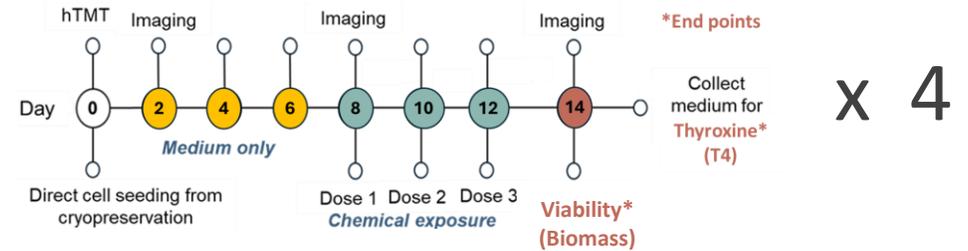
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  - **Ct-2** (medium with TSH supplementation)
  - **Ct-1** (medium without TSH supplementation)
- **Reference chemical control :**
  - **Methimazole** (anti-thyroid drug and TPO inhibitor)
- **8 unique test chemicals:**
  - Alphabetically randomized
  - Tested in a six-point, 10-fold serial dilution series.
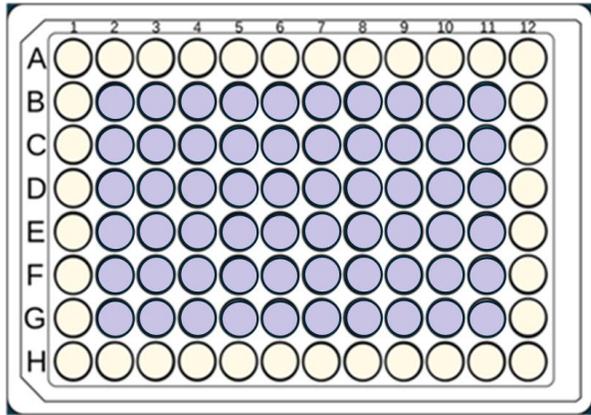

14-Day hTMT Assay Workflow

x 4

Decreasing concentration

Test chemical
Max concentration ranged from 25 – 100 μM, depending on chemical-specific solubility limits.

11

Experimental Design

Day 0

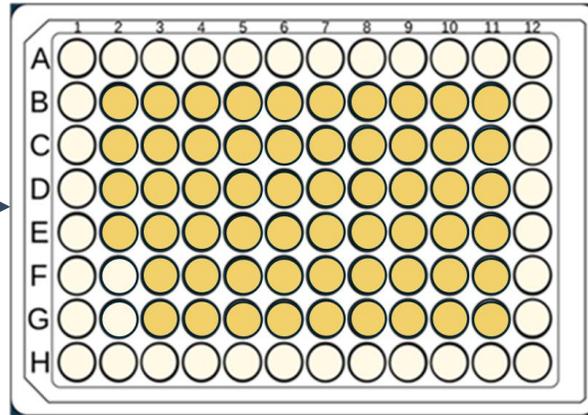Primary human thyrocytes from 5 qualified donors pooled & seeded in 96-well plates.

- Seeded
- Not seeded

Donor specifications

Age Range	20 - 54
Body Mass Index (BMI) Range	19 - 34
Sex (n)	Male (4), Female (1)
Race (n)	Caucasian (4), African American (1)

Days 2*, 4, 6

Wells treated with medium with (+) or without TSH (-) supplementation.

- TSH (+), Ct-2 medium
- TSH (-), Ct-1 medium

Image acquisition *

Days 8*, 10, 12

Wells treated with TSH (+) or TSH (-) medium, containing chemical concentrations or vehicle (DMSO)

Decreasing Concentration
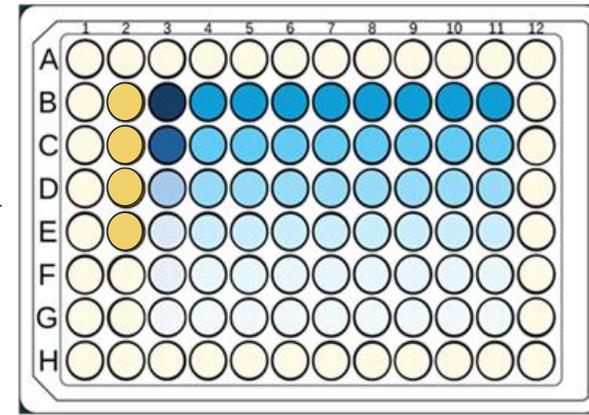
- Ct-2 medium + DMSO
- Ct-1 medium + DMSO
- Ct-2 medium + Methimazole
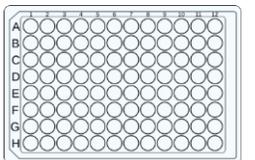- Ct-2 medium + Test chemicals

Days 14*

Medium collection

ELISA

T4 endpoint

Test Plate

CT-Glo ATP Assay

Viability (biomass) endpoint

x 6

12

Analytical Workflow

1. Performance Evaluation

Experimental replicates were evaluated against predefined acceptance criteria[1]:

- Dynamic range (rCV)
- Precision (rCV)
- Screening quality (rZ' factor)

Reference chemical control (Methimazole) was evaluated against proficiency benchmark[1]:
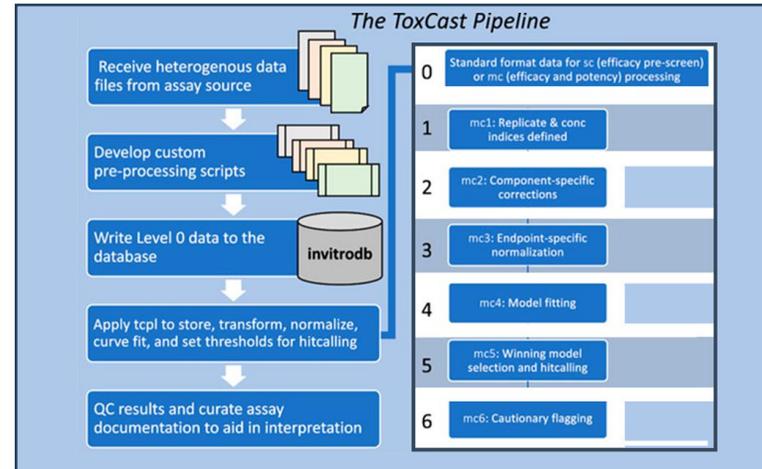
- IC50 = 0.19 – 0.28 µM

Biological performance was assessed by[1]:

- TSH-induced biomass increase
- Donor cohort meeting minimum qualification criterion for T4 production

2. Endpoint Analysis

T4 and Viability Endpoints were processed using the EPA Toxcast Pipeline (tcpl)

The ToxCast Pipeline

Receive heterogenous data files from assay source

Develop custom pre-processing scripts

Write Level 0 data to the database — invitrodb

Apply tcpl to store, transform, normalize, curve fit, and set thresholds for hitcalling

QC results and curate assay documentation to aid in interpretation

- 0 — Standard format data for sc (efficacy pre-screen) or mc (efficacy and potency) processing
- 1 — mc1: Replicate & conc indices defined
- 2 — mc2: Component-specific corrections
- 3 — mc3: Endpoint-specific normalization
- 4 — mc4: Model fitting
- 5 — mc5: Winning model selection and hitcalling
- 6 — mc6: Cautionary flagging

Hit call	T4	Viability
≥ 0.9	**Active** for T4 disruption	**Cytotoxic**
< 0.9	**Inactive** for T4 disruption	**Non-cytotoxic**

3. Predictivity Assessment

For non-cytotoxic chemicals, assay outcomes for T4 were compared to *a priori* classifications within a confusion matrix

	A Priori Classification	
Assay Outcome	**Positive**	**Negative**
Positive	True Positive (TP)	False Positive (FP)
Negative	False Negative (FN)	True Negative (TN)

Calculated Predictive Metrics:

- **Sensitivity** = % correctly identified positives (TP/ TP + FN)
- **Specificity** = % correctly identified negatives (TN/ TN + FP)
- **Balanced Accuracy:** Mean of sensitivity and specificity

[1]Foley, B., et al. "Technical Evaluation and Standardization of the Human Thyroid." *Toxicological Sciences*.

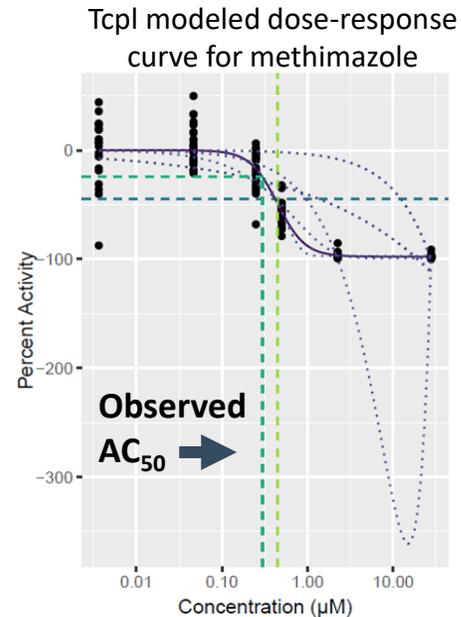# Assay performance criteria & proficiency benchmarks were met

❖ Experimental replicates met predefined assay acceptance criteria.

❖ The reference chemical control, methimazole closely aligned with the established proficiency benchmark range.

hTMT Assay Performance Acceptance Criteria[1]

Metric	Acceptance Criteria for T4[1]	T4 Median ± SD (n = 24)	T4 Range (n = 24)
Dynamic Range (rS/B)	≥ 2.5 ✅	32.3 ± 14.5	9.4 – 66.7
Precision (rCV)	≤ 25 ✅	5.9 ± 3.9	0.15 – 17.2
Screening Quality (rZ' Factor)	> 0 ✅	0.9 ± 0.04	0.8 - 1

Proficiency Benchmark for Methimazole[1]

Tcpl modeled dose-response curve for methimazole

Observed AC$_{50}$ ➡

Winning Model (hill)
Losing Models
Cutoff
BMD
AC50

Metric	AC$_{50}$ (µM)
Proficiency Testing Benchmark[1]	**0.19 – 0.28** ✅
Assay Result	0.44

[1]Foley, B., et al. "Technical Evaluation and Standardization of the Human Thyroid." *Toxicological Sciences*.
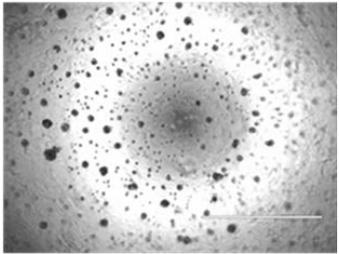
Thyroid stimulating hormone (TSH) reproducibly increased biomass & T4 production

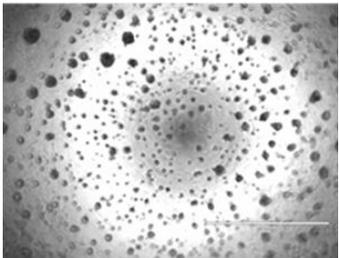❖ TSH significantly increased biomass in the Ct-2 control.

❖ Donor qualification for T4 production was maintained after pooling multiple donor lots.
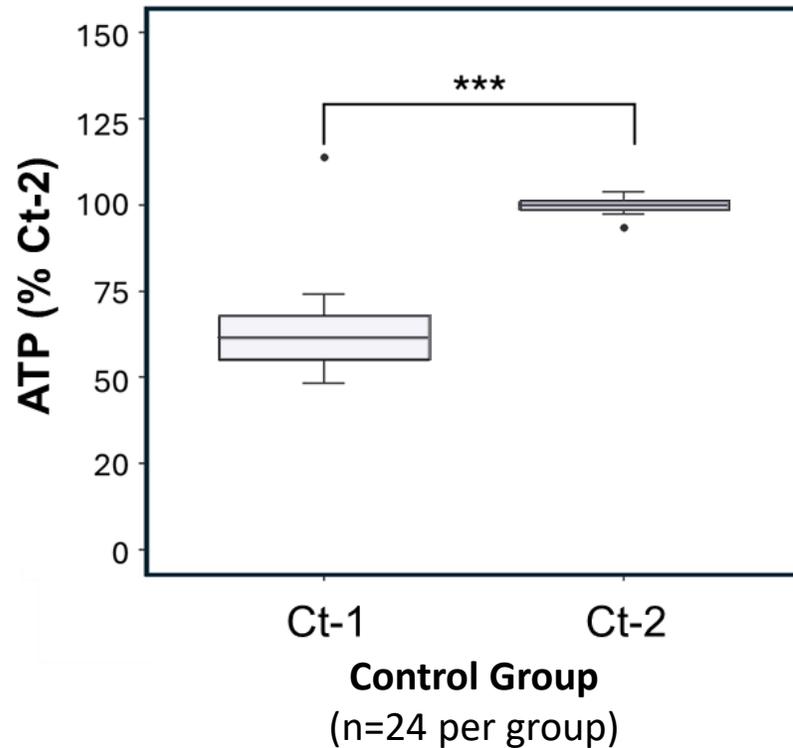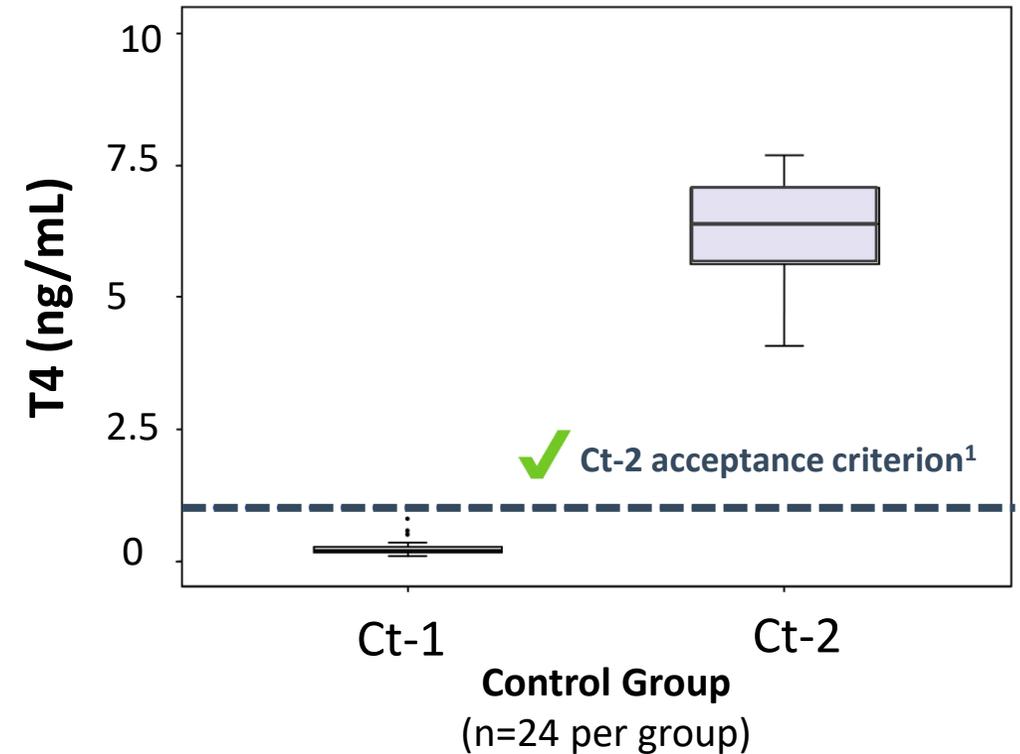
Representative Morphology

Ct-1 (TSH -)

Ct-2 (TSH +)

[1]Foley, B., et al. "Technical Evaluation and Standardization of the Human Thyroid." *Toxicological Sciences*.

Chemical activity aligns with *a priori classification*

❖ Most chemicals showed assay responses consistent with their *a priori* classification.

- Most *a priori* positives exhibited T4-disruptive activity.

- Most *a priori* negatives did not disrupt T4 production.

- Discordant responses of *a priori* negatives were primarily associated with cytotoxicity.

Chemicals grouped by predefined (*a priori*) T4 activity

Point color indicates assay response: **Blue** = T4, **Gray** = viability & non-cytotoxic, **Red** = viability & cytotoxic
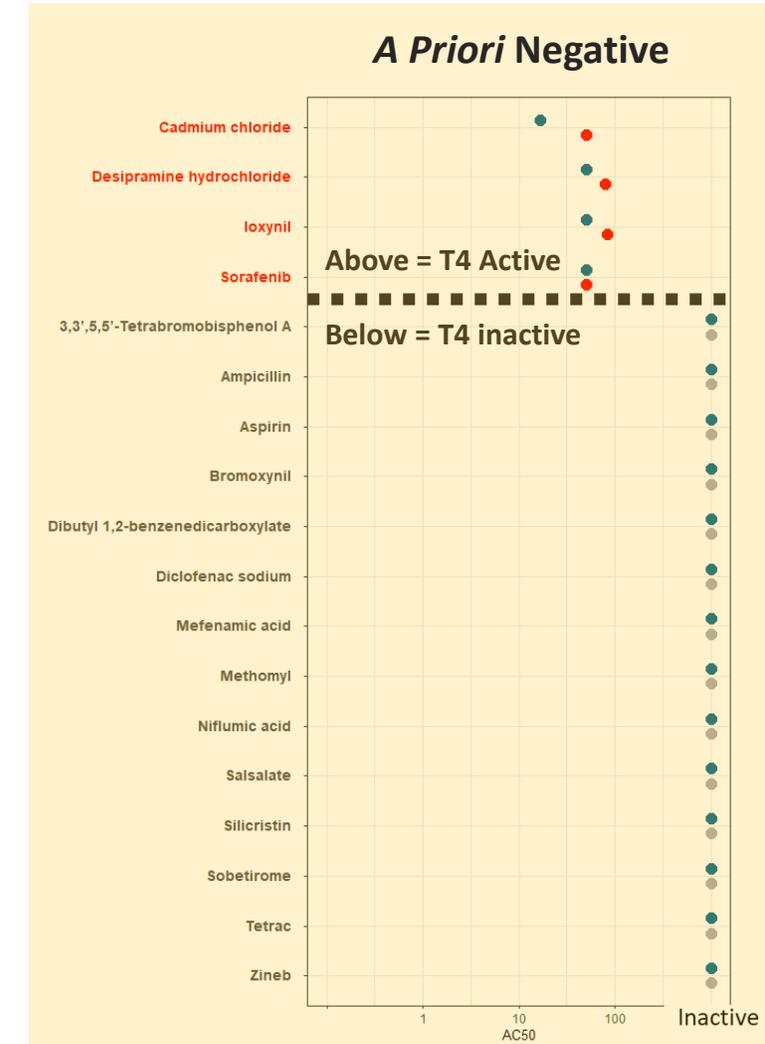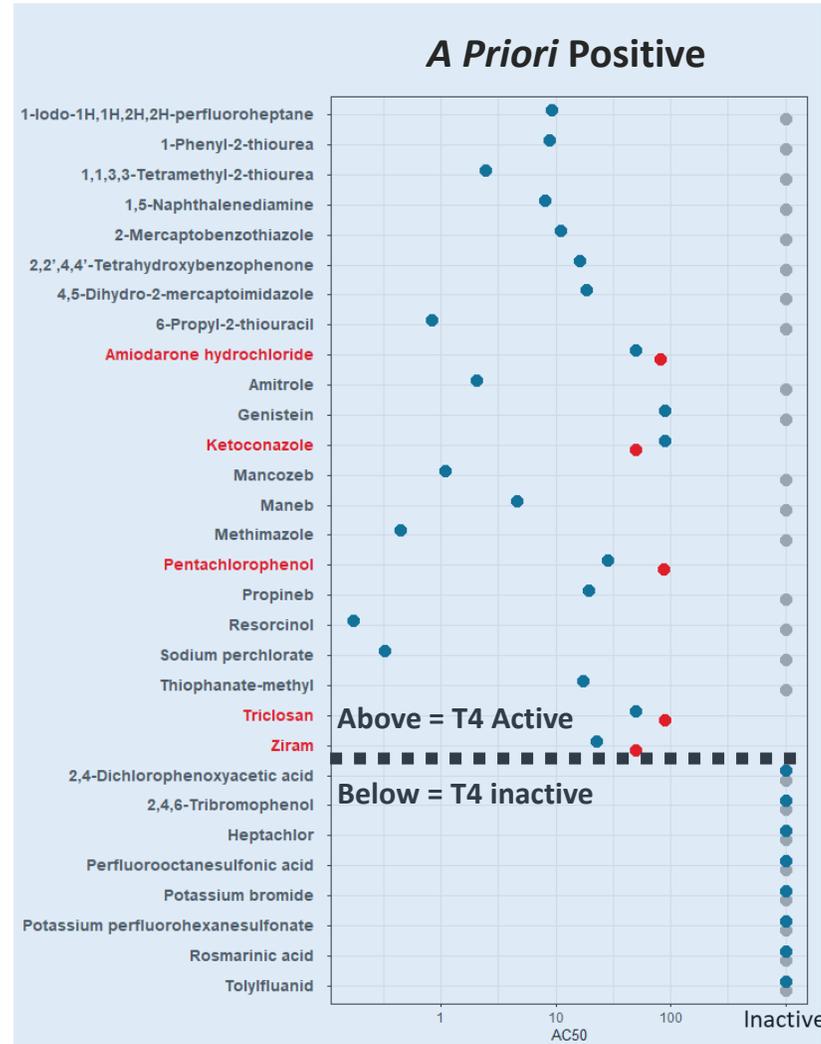
hTMT assay demonstrated predictive performance for T4 disruption

❖ The hTMT assay identified T4 disruptive chemicals with 68% sensitivity and 100% specificity.

- Nine cytotoxic chemicals were excluded from analysis.

- The assay correctly classified 17 of 25 *a priori* T4-positive chemicals.

- The assay correctly classified all 15 *a priori* T4-negative chemicals.

Assay Outcome vs. *A Priori* Classification
(n = 40 chemicals)

Assay Outcome	*A Priori* Classification	
	Positive	Negative
Positive	17	0
Negative	8	15

Predictive Performance Metrics
(non-cytotoxic subset)

Metric	Result
Sensitivity	68%
Specificity	100%
Balanced accuracy	84%

Conclusions

Intra-laboratory Validation and Predictivity Assessment of the hTMT assay:

- **Advances thyroid New Approach Methods (NAMs)** by addressing a key event gap in the thyroid adverse outcome pathway through functional assessment of hormone synthesis disruption.

- **Demonstrates reliable predictive performance** representing an important step towards assay validation for identification and evaluation of T4-disruptive chemicals.

- **Supports assay robustness and reproducibility**, as donor qualification was maintained across a pooled donor cohort and assay performance consistently met predefined acceptance criteria and proficiency benchmarks.

Acknowledgments

Mentorship & Guidance (EPA-OCSPP):

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Briana Foley

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Amanda Jurgelewicz

Kristen Breaux

Additional Support:

Parisa Sadrpour (EPA-OCSPP)

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American Society for Cellular and Computational Toxicology

OAK RIDGE INSTITUTE FOR SCIENCE AND EDUCATION
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Thank you for your time! Questions are welcome.

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OCSPP (Office of Chemical Safety and Pollution Prevention)

ORD (Office of Research and Development