United States Environmental Protection Agency

Advancing the Acceptance and Use of the Human Thyroid Microtissue Assay

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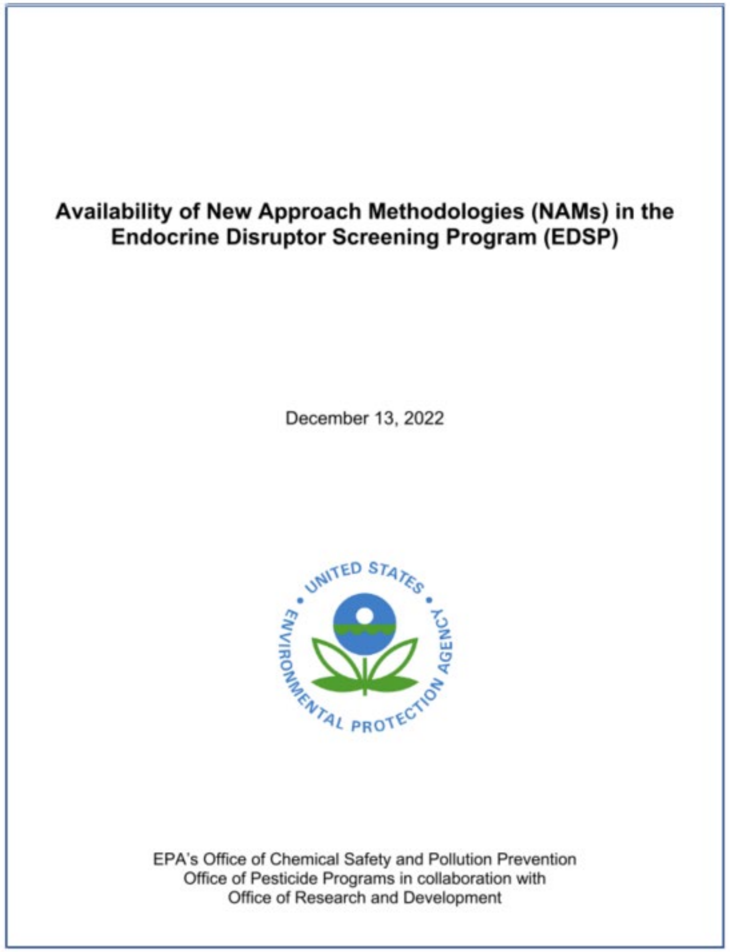
EPA at 50 — Progress for a Stronger Future

Availability of NAMs in the Endocrine Disruptor Screening Program


Availability of New Approach Methodologies (NAMs) in the Endocrine Disruptor Screening Program (EDSP)

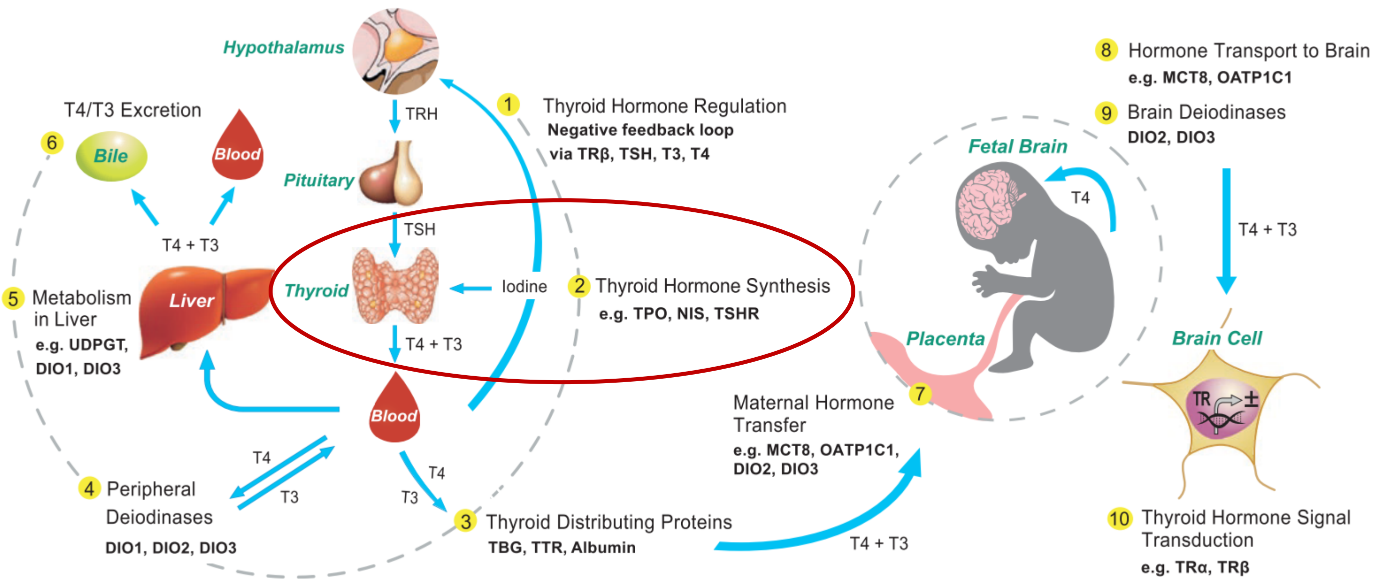
December 13, 2022

EPA's Office of Chemical Safety and Pollution Prevention
Office of Pesticide Programs in collaboration with
Office of Research and Development

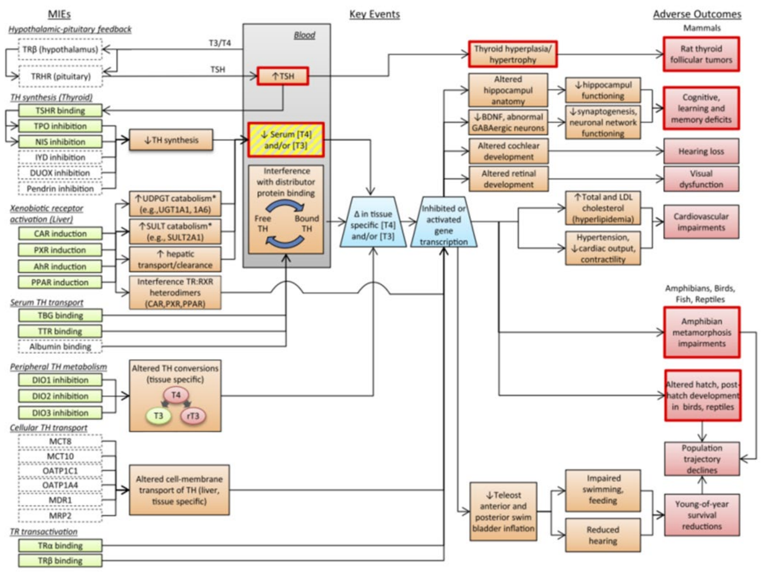
- The EDSP evaluates chemical effects on estrogen, androgen, and thyroid endocrine pathways.
- The validated Estrogen Receptor (ER) and Androgen Receptor (AR) pathway models may be used as an alternative to the Tier 1 screening assays.
- Additional NAMs including Integration of Bioactivity Exposure Ratios (IBER), QSAR models for ER and AR activity, and SeqAPASS for cross-species extrapolation may be used as Other Scientifically Relevant Information (OSRI) to prioritize chemicals for screening and hazard assessment.
- **Continue development of a Thyroid Pathway Framework that includes *in vitro* assays for thyroid-relevant targets to produce an integrated prediction model that may be used as OSRI for thyroid system perturbations.**

Thyroid ‘MIE’ Assays Do Not Directly Measure the ‘Key Event’ for Thyroid Hormone Synthesis

Sites of Interference for Thyroid Disrupting Chemicals



Thyroid AOP Network



Thyroid MIE	Assay	Environmental Chemicals Screened	Active Chemicals	% Active	Reference
TSHR	Engineered Cell Line	7871	825	10	TCPL: TOX21_TSHR_Agonist, TOX21_TSHR_Antagonist
TPO	Microsomal Enzyme	1074	150	14	K. Paul Friedman et al, ToxSci, 151(1), 2016, 160-180
NIS	Engineered Cell Line	293	137	47	J. Wang et al, EnvironSciTechn, 52, 2018, 5417-5426
NIS	Engineered Cell Line	768	167	22	J. Wang et al, Environment International, 126, 2019, 377-386
DIO 1	Recombinant Enzyme	292	18	6	M. Hornung et al, ToxSci, 162(2), 2018, 570–581
DIO 1	Recombinant Enzyme	1819	139	8	J. Olker et al, ToxSci, 168(2), 2019, 430-442
IYD	Recombinant Enzyme	1825	148	8	J. Olker et al, Toxicol In Vitro. 2021 Mar;71:105073.

DOI: 10.1210/endocr/bqaa106; DOI: 10.1289/EHP5297

Human Thyroid Microtissue Assay

Innovation

- Develop proof-of-concept assay
- Evaluate primary and immortalized thyrocytes

Validation

- Technical evaluation and standardization
- Validation for method transferability and reproducibility
- 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 (e.g., PFAS)

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

Filling Technology Gaps for *In Vitro* Thyroid Testing

OXFORD SOT Society of Toxicology
academic.oup.com/toxsci

TOXICOLOGICAL SCIENCES, 174(1), 2020, 63–78
doi: 10.1093/toxsci/kfz238
Advance Access Publication Date: December 6, 2019
Research Article

LifeNet Health®
Saving Lives. Restoring Health. Giving Hope.

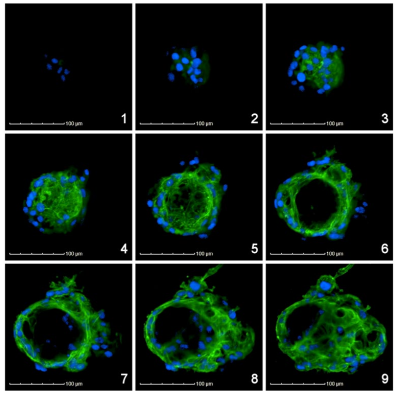
APPLIED IN VITRO TOXICOLOGY
Volume XX, Number XX, 2021

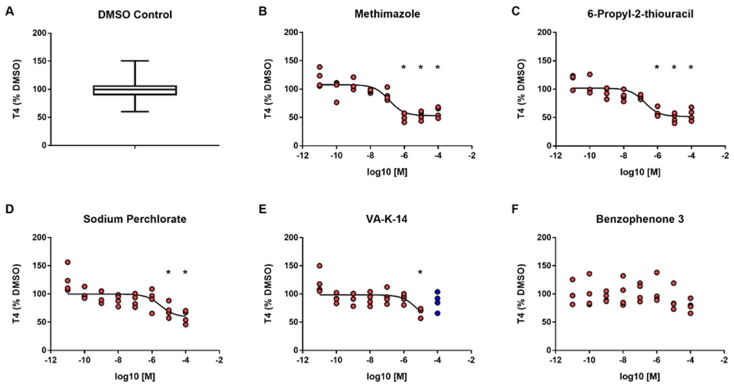
DOI: 10.1089/aivt.2020.0027

InSCREENeX

Development of an *In Vitro* Human Thyroid Microtissue Model for Chemical Screening

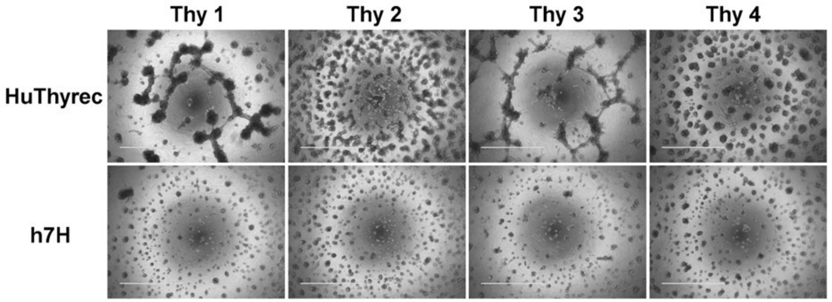
Chad Deisenroth,*,[1] Valerie Y. Soldatow,[†] Jermaine Ford,[‡] Wendy Stewart,* Cassandra Brinkman,* Edward L. LeCluyse,[†] Denise K. MacMillan,[‡] and Russell S. Thomas *

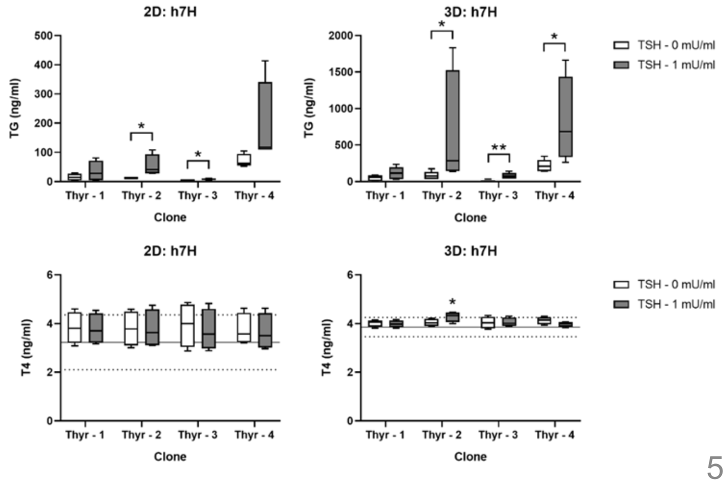




Characterization of Novel Human Immortalized Thyroid Follicular Epithelial Cell Lines

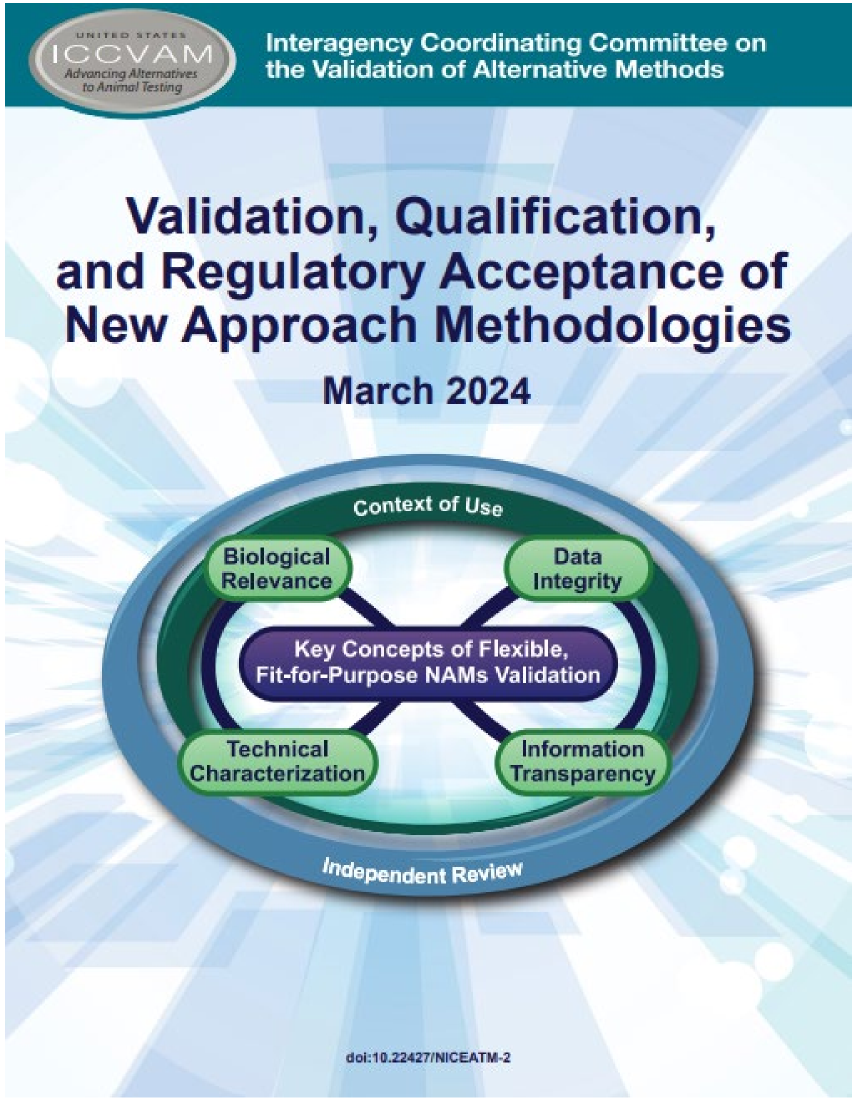
Kristen Hopperstad,[1,*] Theresa Truschel,[2,*] Tom Wahlicht,[2] Wendy Stewart,[1] Andrew Eicher,[1] Tobias May,[2] and Chad Deisenroth[1,†]





- A thyroid AOP 'key event' assay designed to evaluate disruption of thyroid hormone synthesis as a mode-of-action for endocrine-related hazard screening.
- Established commercial sources of primary human thyrocytes and immortalized cell lines which enhances method accessibility.

Increasing Confidence in the Human Thyroid Microtissue Assay as a New Approach Method



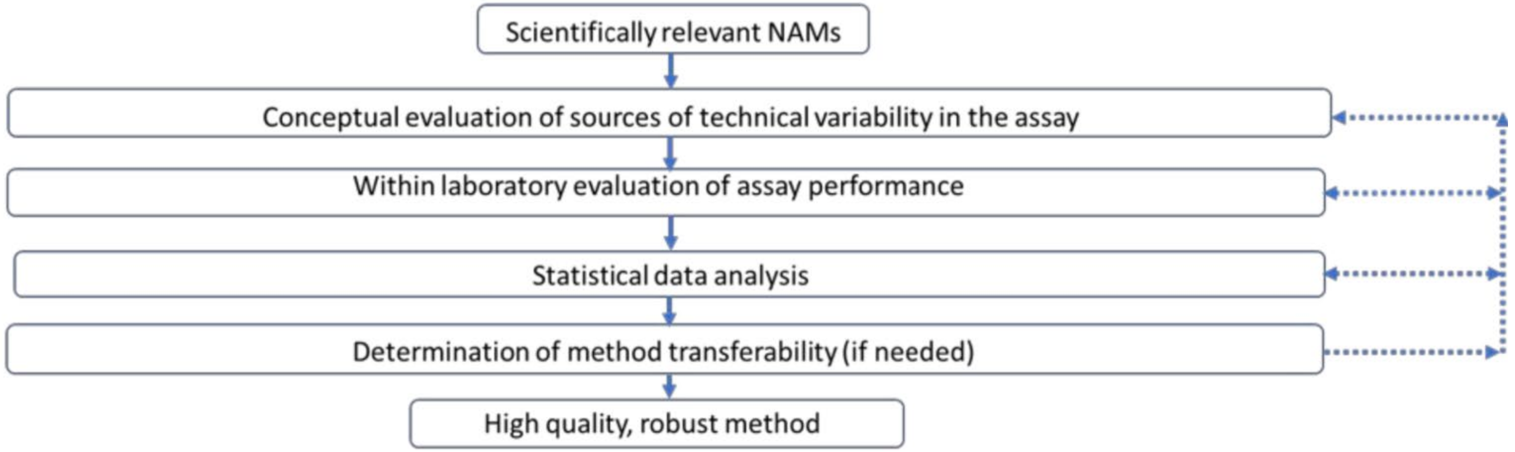
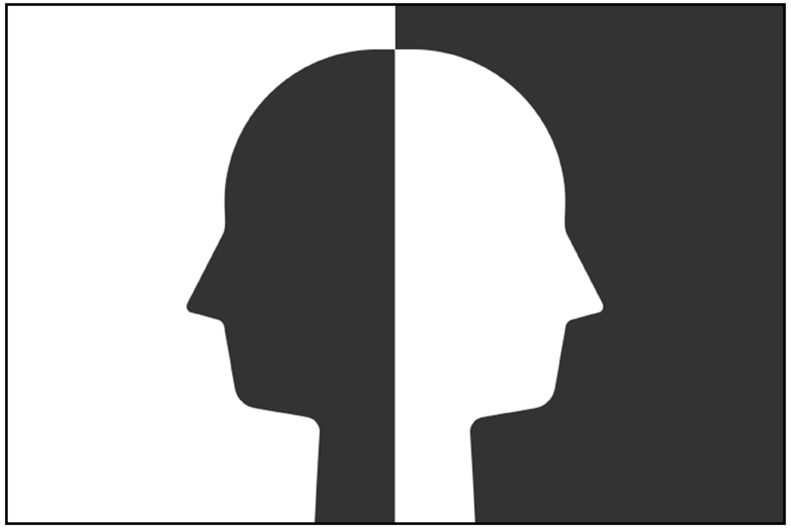


Fig 2: Framework for developing robust NAMs.

Technical Characterization: Standardizing Organotypic Assays is Challenging

“I want an assay that is reproducible”



“I want an assay that predicts a range of human responses”

Goal: Minimize technical variability to increase confidence in the ‘true’ biological performance.

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Standardization of the Human Thyroid Microtissue Assay

OXFORD
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academic.oup.com/toxsci

Toxicological Sciences, 2024, 1–19
https://doi.org/10.1093/toxsci/kfae014
Advance Access Publication Date: February 4, 2024
Research article

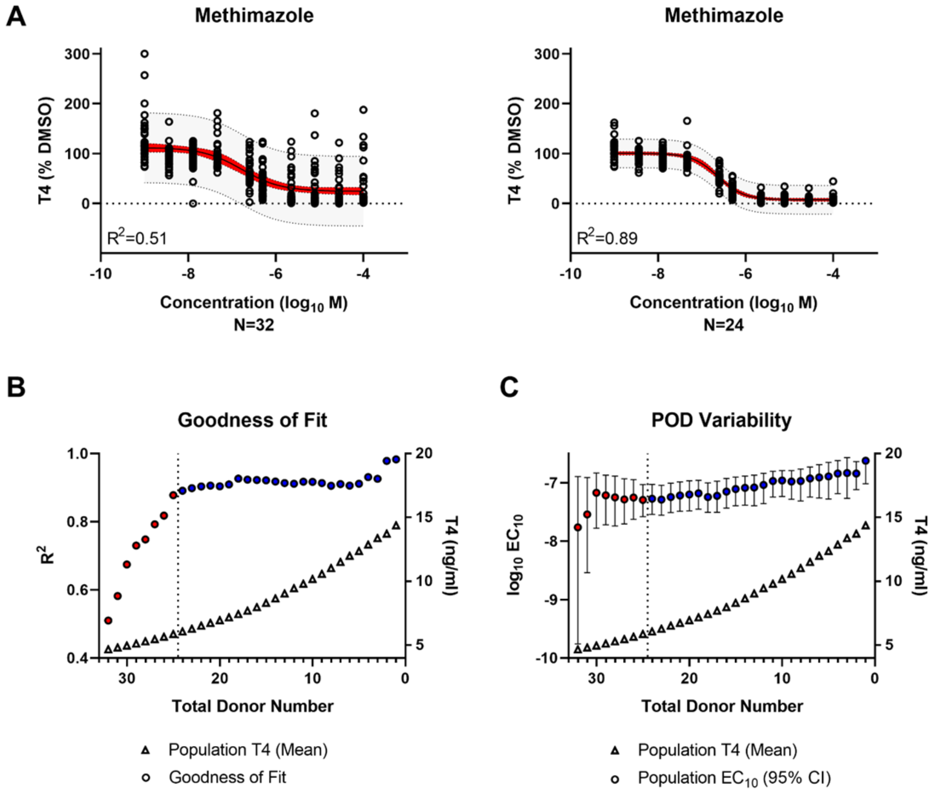
Technical evaluation and standardization of the human thyroid microtissue assay

Briana Foley,[1] Kristen Hopperstad,[1] John Gamble,[1,2] Scott G. Lynn,[3] Russell S. Thomas,[1] Chad Deisenroth[1,*]

- Defined optimal donor procurement specifications.
- Established qualification criteria for primary human thyrocytes.
- Established minimum assay performance guidelines.
- Set benchmark ranges for reference chemical responses.

Donor Cohort Demographic Summary

Donors	32
Age	34 (17-61)
Sex	Male (24), Female (8)
Race	Caucasian (25), African American (7)
BMI	28 (18-37)



Inter-laboratory Validation of the Human Thyroid Microtissue Assay

Goal: To assess the test method reliability and reproducibility.

NICEATM

Independent Validation Management Team

Objectives

1. SOP refinement.
2. Test method training, transfer and intra-laboratory model performance evaluation.
3. Limited inter-laboratory reference chemical testing and assay performance evaluation.

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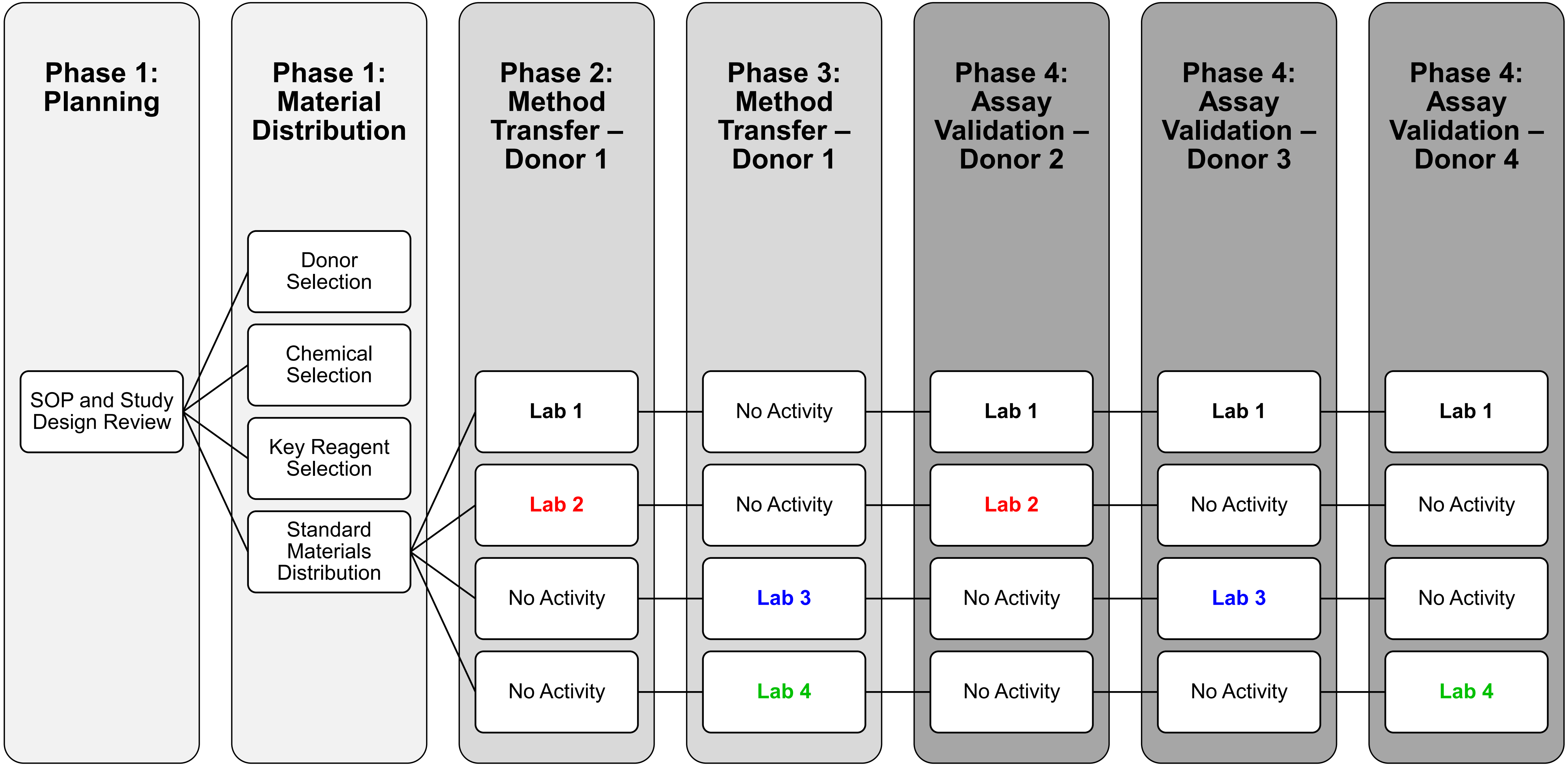
Experimental Plan



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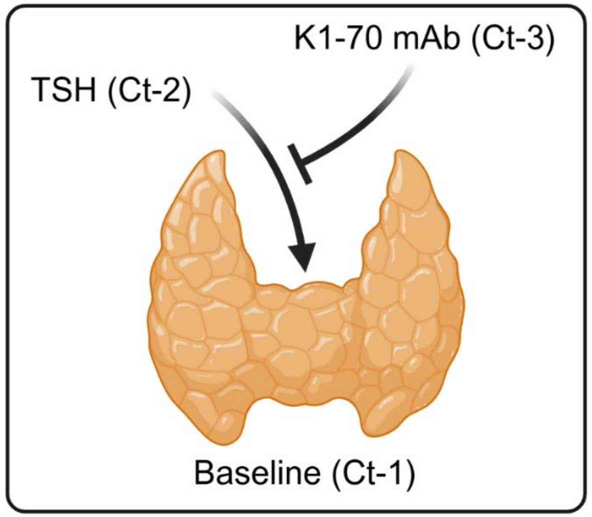
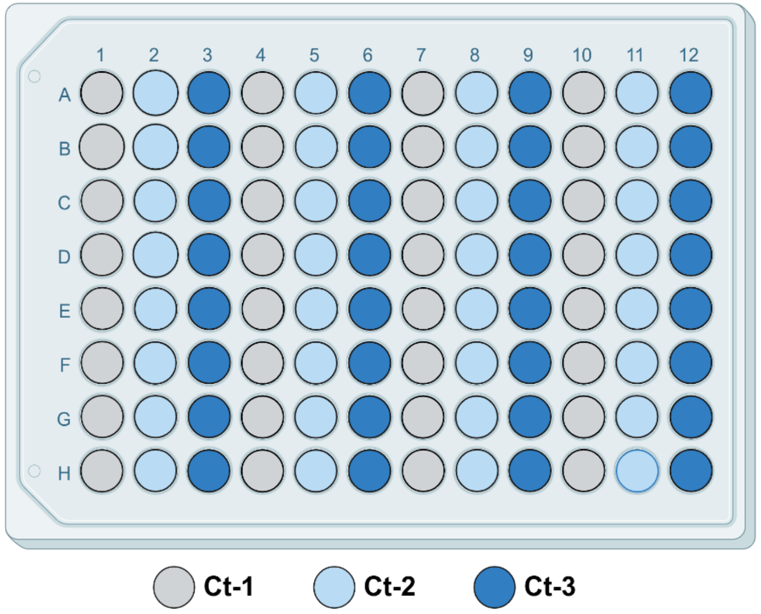
Donor Selection

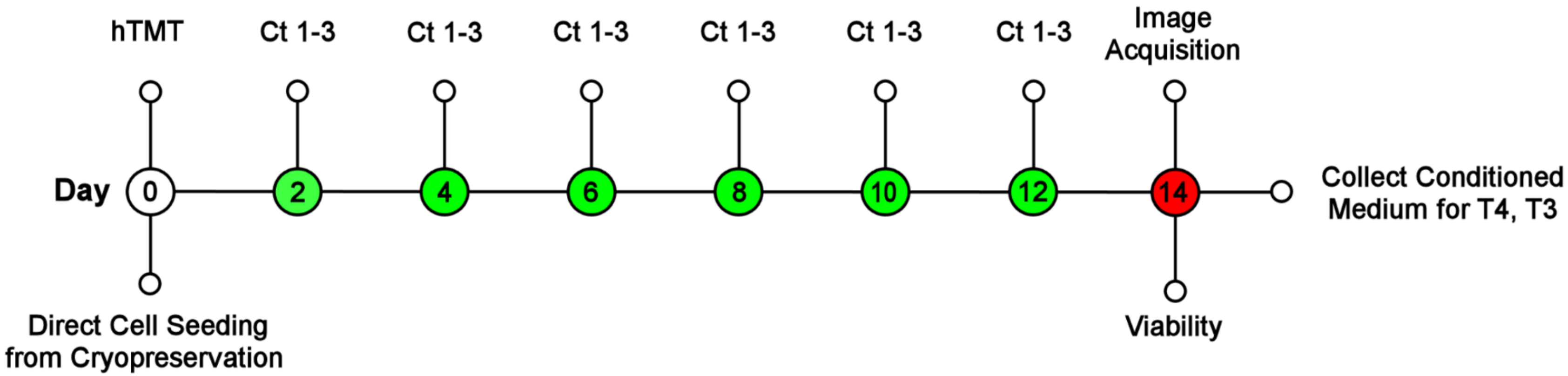
Lab	Study Phase	Donor ID	Age	Sex	Race	BMI	Vials per Lab	Experimental Replicates per Lab
1,2,3,4	2,3	EPATHY0035	22	F	Caucasian	24	8	3-5
1,2	4	EPATHY0015	40	F	Caucasian	29	6	4
1,3	4	EPATHY0025	44	F	Caucasian	20	6	4
1,4	4	EPATHY0058	37	M	African American	34	6	4

- Phase 2-3: One qualified donor evaluated across all laboratories.
- Phase 4: Three qualified donors run in the method developer lab (Lab 1) to benchmark results. One matched donor per partner lab for comparison.
- All selected donors met previously reported qualification criteria (https://doi.org/10.1093/toxsci/kfae014).

Phase 2-3: Human Thyroid Microtissue Assay – Method Transfer

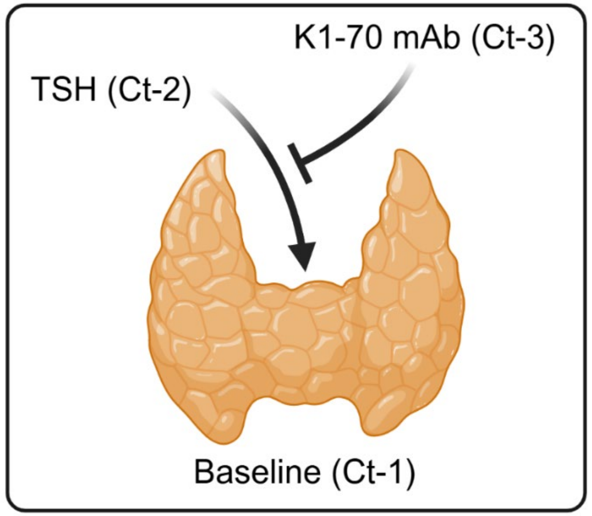


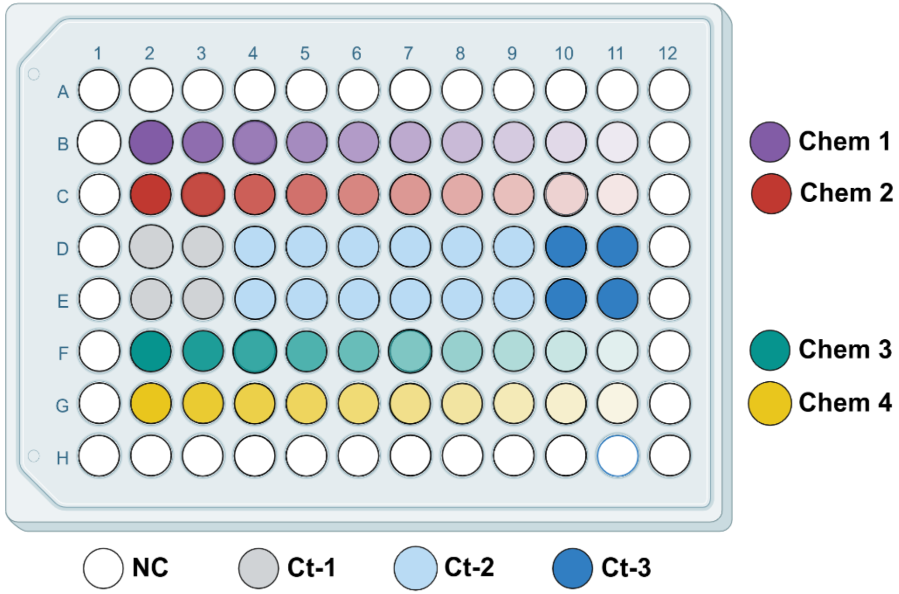


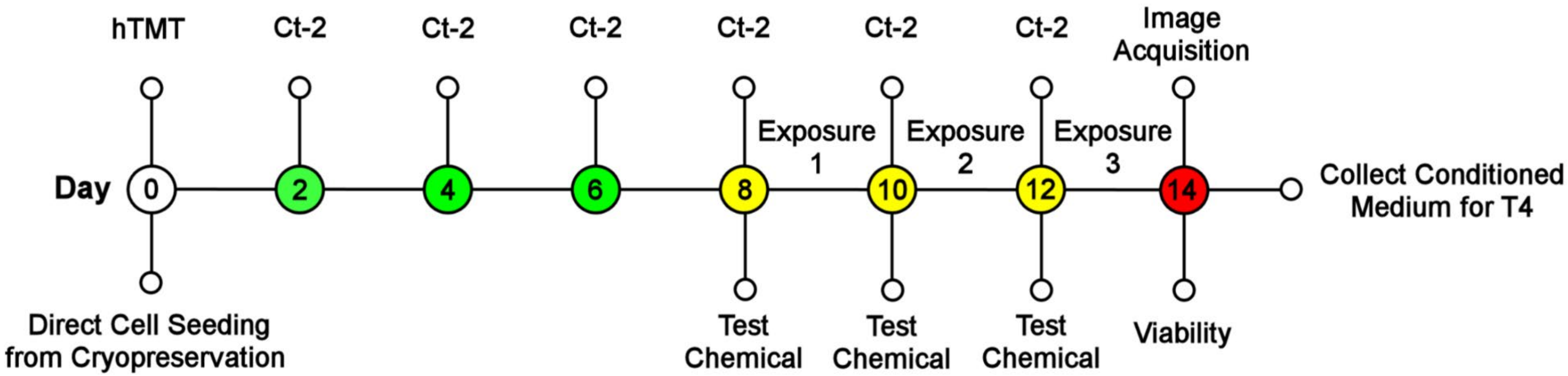


Control (Ct) groups include controls for: baseline activity (Ct-1), TSHR agonism (Ct-2), and TSHR antagonism (Ct-3).

Phase 4: Human Thyroid Microtissue Assay – Method Validation



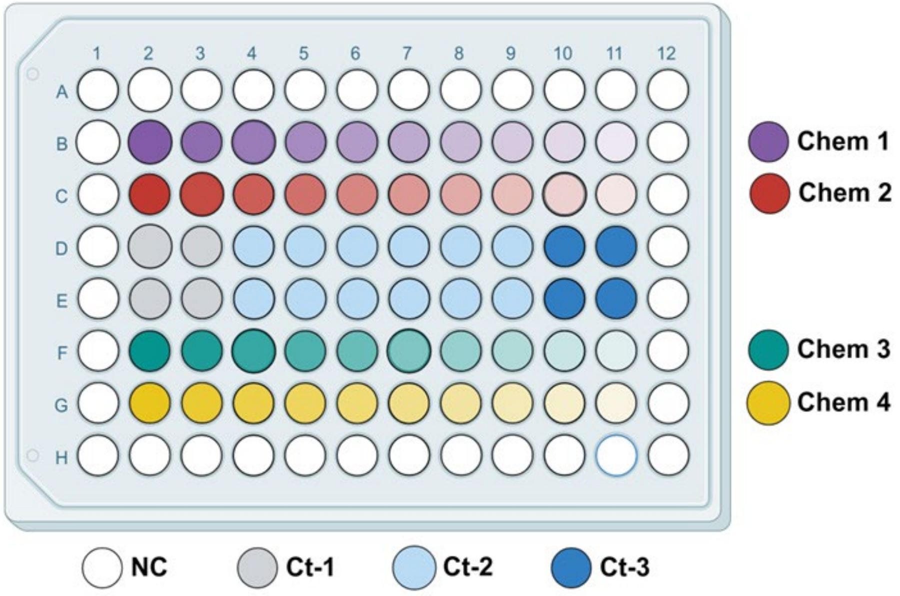




Control (Ct) groups include controls for: baseline activity (Ct-1), TSHR agonism (Ct-2), and TSHR antagonism (Ct-3).

Reference Chemicals

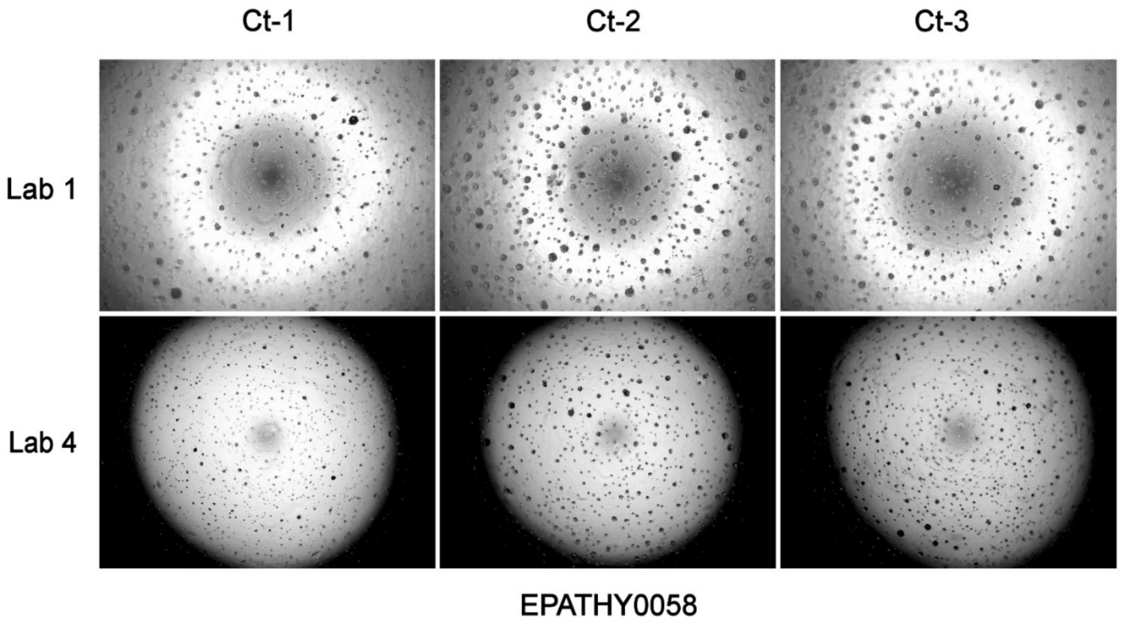
CASRN	Chemical Name	CoA Purity	Concentration (mM)	Assay Classification
16752-77-5	Methomyl	99.5	20.70	Negative
60-56-0	Methimazole	100	20.21	Positive (Antagonist)
51-52-5	6-Propyl-2-thiouracil	99.2	20.40	Positive (Antagonist)
7601-89-0	Sodium perchlorate	99.5	20.40	Positive (Antagonist)
67-68-5	Dimethyl sulfoxide	100	na	Solvent Control



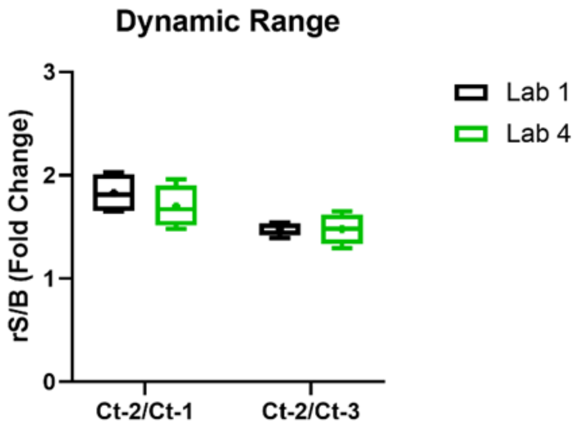
- Chemicals were independently procured by NICEATM via MRIGlobal (Kansas City, MO) and shipped to all laboratories with unique chemical codes for each chemical x laboratory pair.
- Blinding of coded chemicals was maintained for the duration of phase 4 until data collection and analysis had been completed for all laboratories.

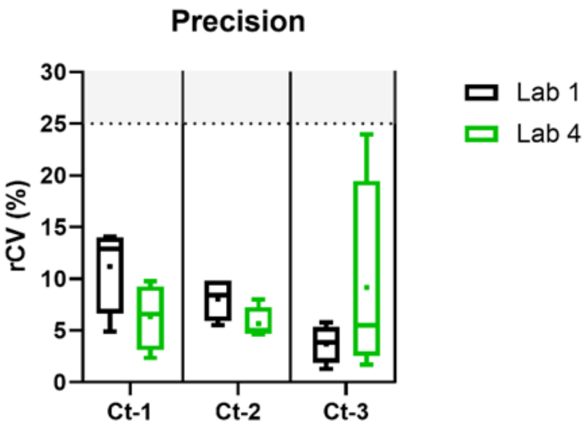
EPATHY0058 – Microtissue Biomass

Donor EPATHY0058
Race: African American
Sex: Male
Age: 37
BMI: 34
Thyroid Disease: None Reported
Qualified: Yes

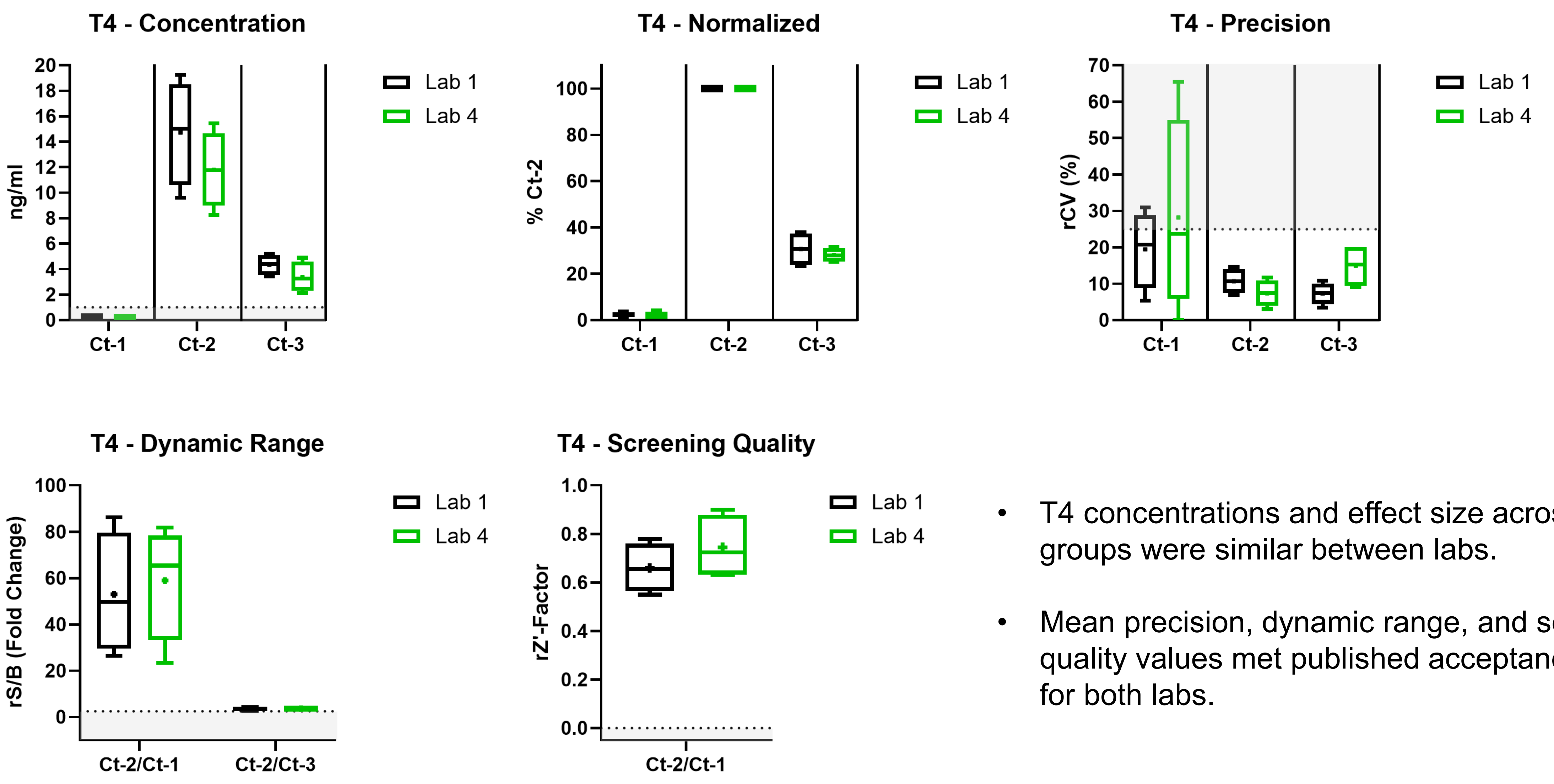


- Microtissue frequency, distribution, and size heterogeneity were similar between labs.
- Dynamic range indicated consistent relative responses to TSH stimulation.
- Precision of responses indicated consistent cell seeding.



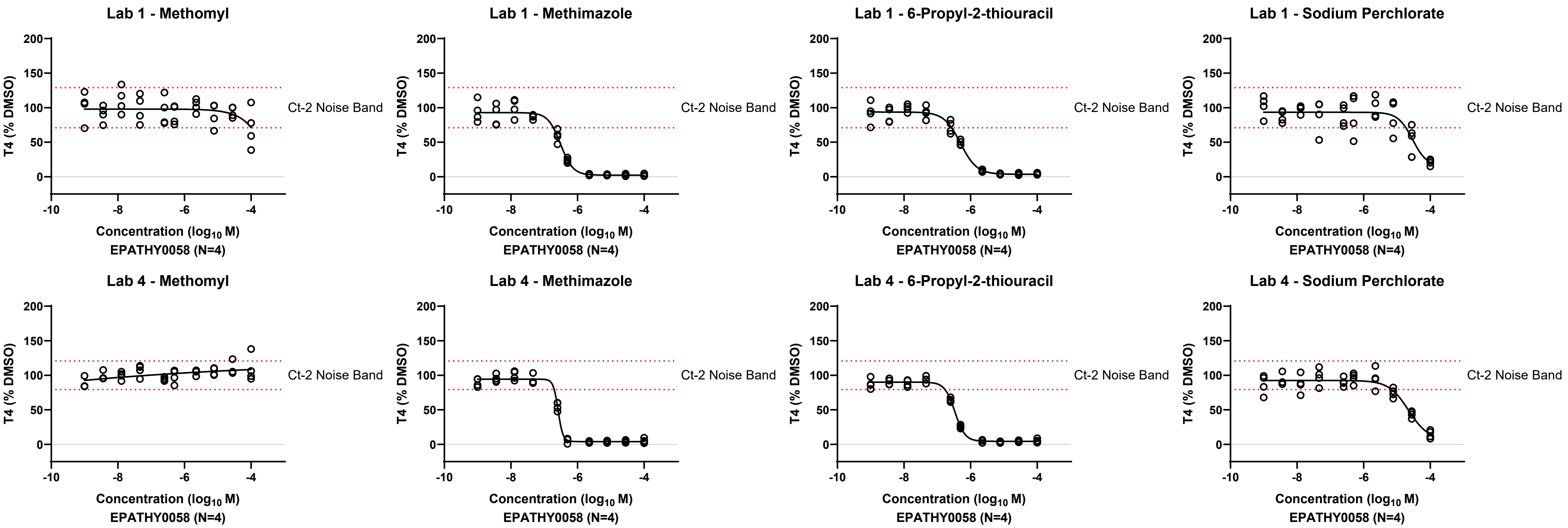


EPATHY0058 – Assay Performance Metrics



- T4 concentrations and effect size across control groups were similar between labs.
- Mean precision, dynamic range, and screening quality values met published acceptance criteria for both labs.

EPATHY0058 – Reference Chemicals (T4)



	EPATHY0058 - T4 AC50 (µM)	
Chemical	**Lab 1**	**Lab 4**
Methomyl	2.84	NA
Methimazole	0.306	0.260
6-Propyl-2-thiouracil	0.494	0.336
Sodium Perchlorate	29	22

- Effect size and potency were generally equivalent across labs.
- True Positive actives: 6/6. True Negative: 1/2.
- False positive was observed for Methomyl in Lab 1 at the highest tested concentration.

Data Interpretation Procedure

4.1.12: Reference Chemical Evaluation – Data Interpretation Procedure

- Purpose: To classify the T4 bioactivity and cytotoxicity of the test chemicals.

Hit Call		
T4	Cytotoxicity	Test Chemical Classification
0	0	Inactive
1	0	Active; non-cytotoxic
0	1	Inactive; cytotoxic
1	1	If T4 LOEC < ATP LOEC; flag as active, cytotoxic
		If T4 LOEC ≥ ATP LOEC; flag as equivocal, cytotoxic
		If T4 LOEC > ATP LOEC; flag as inactive, cytotoxic

- Evaluation was performed on the hit calls made from the thyroid hormone and cytotoxicity measurements, modeling, and analyses across data aggregated from all experiments (N=4).

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Bioactivity Classifications

Lab	Donor	Chemical	T4	Cytotoxicity	Classification_Expected	Classification_Observed	Concordance	Reproducibility (%)
1	EPATHY0015	6-Propyl-2-thiouracil	1	0	Active; non-cytotoxic	Active; non-cytotoxic	1	70.8
2	EPATHY0015	6-Propyl-2-thiouracil	1	0	Active; non-cytotoxic	Active; non-cytotoxic	1	
1	EPATHY0025	6-Propyl-2-thiouracil	1	0	Active; non-cytotoxic	Active; non-cytotoxic	1	
3	EPATHY0025	6-Propyl-2-thiouracil	1	0	Active; non-cytotoxic	Active; non-cytotoxic	1	
1	EPATHY0058	6-Propyl-2-thiouracil	1	0	Active; non-cytotoxic	Active; non-cytotoxic	1	
4	EPATHY0058	6-Propyl-2-thiouracil	1	1	Active; non-cytotoxic	Active; cytotoxic**	0	
1	EPATHY0015	Methimazole	1	0	Active; non-cytotoxic	Active; non-cytotoxic	1	
2	EPATHY0015	Methimazole	1	0	Active; non-cytotoxic	Active; non-cytotoxic	1	
1	EPATHY0025	Methimazole	1	1	Active; non-cytotoxic	Active; cytotoxic**	0	
3	EPATHY0025	Methimazole	1	1	Active; non-cytotoxic	Active: cytotoxic**	0	
1	EPATHY0058	Methimazole	1	0	Active; non-cytotoxic	Active; non-cytotoxic	1	
4	EPATHY0058	Methimazole	1	0	Active; non-cytotoxic	Active; non-cytotoxic	1	
1	EPATHY0015	Methomyl	0	0	Inactive	Inactive	1	
2	EPATHY0015	Methomyl	1	0	Inactive	Active; non-cytotoxic**	0	
1	EPATHY0025	Methomyl	0	1	Inactive	Inactive; cytotoxic**	0	
3	EPATHY0025	Methomyl	0	0	Inactive	Inactive	1	
1	EPATHY0058	Methomyl	1	0	Inactive	Active; non-cytotoxic**	0	
4	EPATHY0058	Methomyl	0	0	Inactive	Inactive	1	
1	EPATHY0015	Sodium perchlorate	1*	0	Active; non-cytotoxic	Active; non-cytotoxic	1	
2	EPATHY0015	Sodium perchlorate	1	0	Active; non-cytotoxic	Active; non-cytotoxic	1	
1	EPATHY0025	Sodium perchlorate	1	1	Active; non-cytotoxic	Equivocal: cytotoxic**	0	
3	EPATHY0025	Sodium perchlorate	1	0	Active; non-cytotoxic	Active; non-cytotoxic	1	
1	EPATHY0058	Sodium perchlorate	1	0	Active; non-cytotoxic	Active; non-cytotoxic	1	
4	EPATHY0058	Sodium perchlorate	1	0	Active; non-cytotoxic	Active; non-cytotoxic	1	

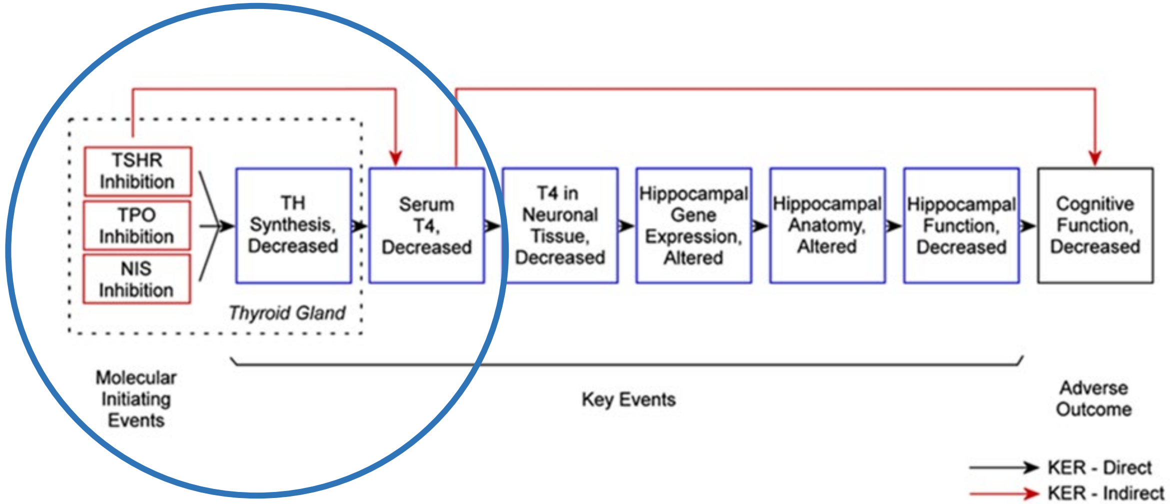
* Based on bioactivity that exceeds the upper bound cutoff.
** Incorrectly classified.
Hit calls: 0= inactive, 1= active
Concordance: 0= no, 1= yes

- Sensitivity for T4 bioactivity was 92%.
- The overall reproducibility of bioactivity classifications (% concordance) was 71%.

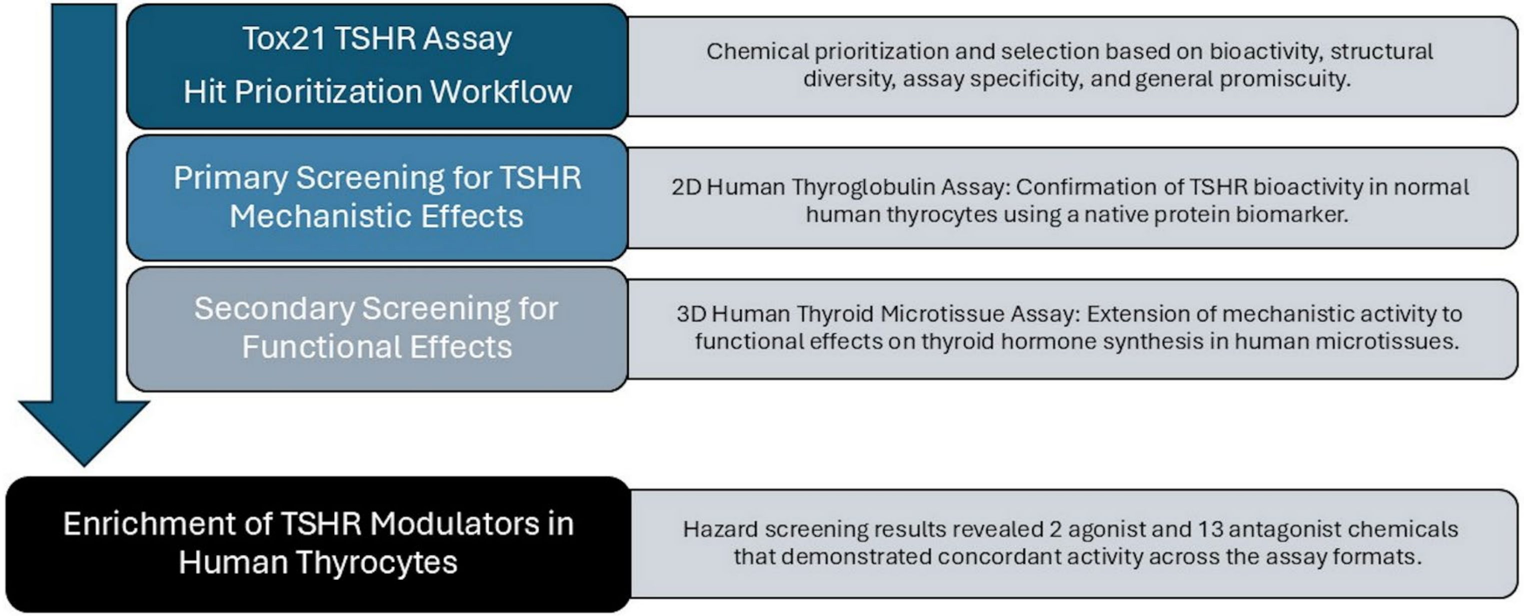
Application: Screening of TSHR-Prioritized Chemicals in Human Thyrocyte Assays

Orthogonal Screening for Thyroid Stimulating Hormone Receptor Modulators in Human Thyroid Assays

Briana Foley[1], Kristen Breaux[1], Mahmoud Shobair[1], Madison Feshuk[1], Ann M. Richard[1,2], Russell S. Thomas[1], Katie Paul Friedman[1], Chad Deisenroth[1§]



Integration of thyroid microtissues into an AOP-based tiered screening paradigm to support the context of use.



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Lab 4

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United States ICCVAM Advancing Alternatives to Animal Testing
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Thyroid Microtissue References

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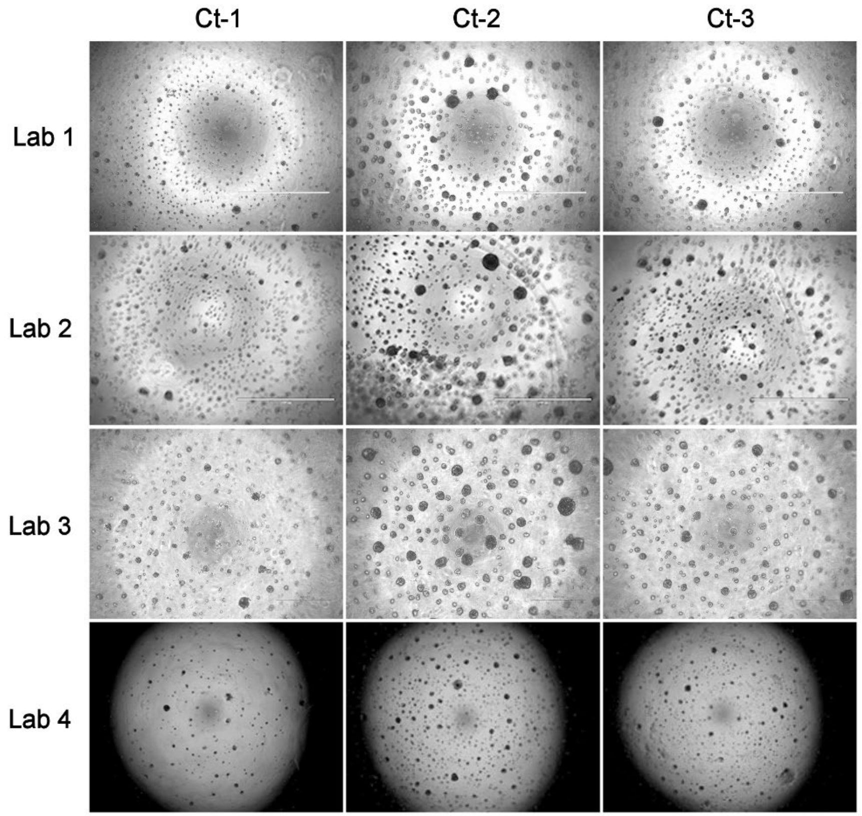
- Hopperstad K, Truschel T, Wahlicht T, Stewart W, Eicher A, May T, Deisenroth C. Characterization of Novel Human Immortalized Thyroid Follicular Epithelial Cell Lines. Appl In Vitro Toxicol. 2021 Jun 16;7(2):39-49. doi: 10.1089/aivt.2020.0027. PMID: 35663474; PMCID: PMC9157743.

- Foley B, Hopperstad K, Gamble J, Lynn SG, Thomas RS, Deisenroth C. Technical evaluation and standardization of the human thyroid microtissue assay. Toxicol Sci. 2024 Apr 29;199(1):89-107. doi: 10.1093/toxsci/kfae014. PMID: 38310358; PMCID: PMC11784494.

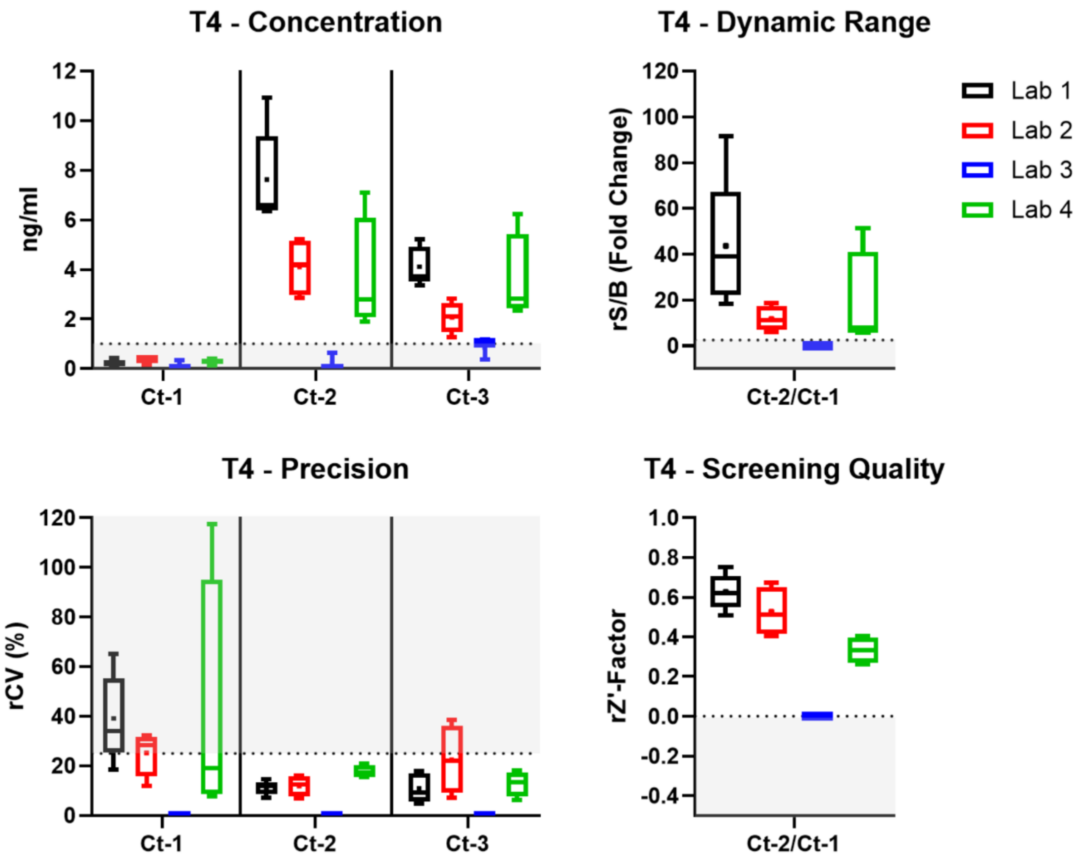
- Foley B, Breaux K, Shobair M, Feshuk M, Richard AM, Thomas RS, Paul Friedman K, Deisenroth C. Orthogonal Screening for Thyroid Stimulating Hormone Receptor Modulators in Human Thyroid Assays. Toxicol Sci. 2025. Accepted for publication.

Supplementary

Phase 2-3: Human Thyroid Microtissue Assay – Method Transfer



EPATHY0035



- All labs consistently reproduced microtissue formation with high precision.
- 3 of 4 labs reproduced thyroid hormone synthesis that met all acceptance criteria.

ToxCast Pipeline (TCPL) Data Analysis

- The EPA ToxCast program has historically modeled medium- and high-throughput screening data from a heterogenous source of chemicals, assay technologies, and experimental designs. This has yielded concentration-response data of essentially every permutation imaginable, presenting challenges for accurate data modeling and analysis.

- The ToxCast data pipeline (TCPL) was originally developed to format, normalize, model, and analyze these datasets. It is a standard tool for consistent and reproducible curve-fitting that generally works for all types of data.

- The most recent update of TCPL uses 10 different models to maximize the best fitting curves to the empirical data (doi.org/10.3389/ftox.2023.1275980). Continuous hit calls are made to determine if statistically significant effects are observed based on uncertainties in the data modeling and parameters for defining assay-dependent biological effects.

- Thyroid data will be accessible to the public on the EPA CompTox Chemicals Dashboard (https://comptox.epa.gov/dashboard/).

Bioactivity Classifications (TCPL Analysis)

Lab	Donor	Chemical	T4	Cytotoxicity	Classification_Expected	Classification_Observed	Concordance	Reproducibility (%)
1	EPATHY0015	6-Propyl-2-thiouracil	1	0	Active; non-cytotoxic	Active; non-cytotoxic	1	91.7
2	EPATHY0015	6-Propyl-2-thiouracil	1	0	Active; non-cytotoxic	Active; non-cytotoxic	1	
1	EPATHY0025	6-Propyl-2-thiouracil	1	0	Active; non-cytotoxic	Active; non-cytotoxic	1	
3	EPATHY0025	6-Propyl-2-thiouracil	1	0	Active; non-cytotoxic	Active; non-cytotoxic	1	
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1	EPATHY0015	Methimazole	1	0	Active; non-cytotoxic	Active; non-cytotoxic	1	
2	EPATHY0015	Methimazole	1	0	Active; non-cytotoxic	Active; non-cytotoxic	1	
1	EPATHY0025	Methimazole	1	0	Active; non-cytotoxic	Active; non-cytotoxic	1	
3	EPATHY0025	Methimazole	1	0	Active; non-cytotoxic	Active; non-cytotoxic	1	
1	EPATHY0058	Methimazole	1	0	Active; non-cytotoxic	Active; non-cytotoxic	1	
4	EPATHY0058	Methimazole	1	0	Active; non-cytotoxic	Active; non-cytotoxic	1	
1	EPATHY0015	Methomyl	0	0	Inactive	Inactive	1	
2	EPATHY0015	Methomyl	0	0	Inactive	Inactive	1	
1	EPATHY0025	Methomyl	0	1	Inactive	Inactive; cytotoxic**	0	
3	EPATHY0025	Methomyl	0	0	Inactive	Inactive	1	
1	EPATHY0058	Methomyl	0	0	Inactive	Inactive	1	
4	EPATHY0058	Methomyl	0	0	Inactive	Inactive	1	
1	EPATHY0015	Sodium perchlorate	1	0	Active; non-cytotoxic	Active; non-cytotoxic	1	
2	EPATHY0015	Sodium perchlorate	0	0	Active; non-cytotoxic	Inactive**	0	
1	EPATHY0025	Sodium perchlorate	1	0	Active; non-cytotoxic	Active; non-cytotoxic	1	
3	EPATHY0025	Sodium perchlorate	1	0	Active; non-cytotoxic	Active; non-cytotoxic	1	
1	EPATHY0058	Sodium perchlorate	1	0	Active; non-cytotoxic	Active; non-cytotoxic	1	
4	EPATHY0058	Sodium perchlorate	1	0	Active; non-cytotoxic	Active; non-cytotoxic	1	

* Based on bioactivity that exceeds the upper bound cutoff.
** Incorrectly classified.
Hit calls: 0= inactive, 1= active
Concordance: 1= yes, 0= no

- Sensitivity for T4 bioactivity was 96%.
- The overall reproducibility of bioactivity classifications (% concordance) was 92%.