United States Environmental Protection Agency

Advancing Translational Applications of Human Organotypic Thyroid Assays

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Communities of Practice Seminar Series
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Progress for a Stronger Future

Endocrine Disruptor Screening Program

Endocrine Pathway	Tier 1 Screening											Tier 2 Testing			
	In vitro					In vivo						In vivo			
	ER Binding	AR Binding	ER Transcriptional Activation*	Aromatase Inhibition	Steroidogenesis*	Uterotrophic*	Hershberger*	Pubertal Male	Pubertal Female	Amphibian Metamorphosis*	Fish Short Term Reproduction*	Rat 2-gen/ EOGRT*	MEOGRT*	LAGDA*	JQTT
E+	■		■		■	■			■		■	■	■	■	■
E-	■			■	■				■		■	■	■	■	■
A+		■			■		■	■			■	■	■	■	■
A-		■			■		■	■			■	■	■	■	■
HPT Axis								■	■	■		■		■	■

The current EDSP assay battery evaluates effects of chemical exposures on estrogen, androgen, and thyroid endocrine pathways

- No *in vitro* tests for thyroid endpoints
- No human representation for thyroid
- Too reliant on animal tests

***In vivo* endpoints for thyroid-related endocrine testing in guideline studies**

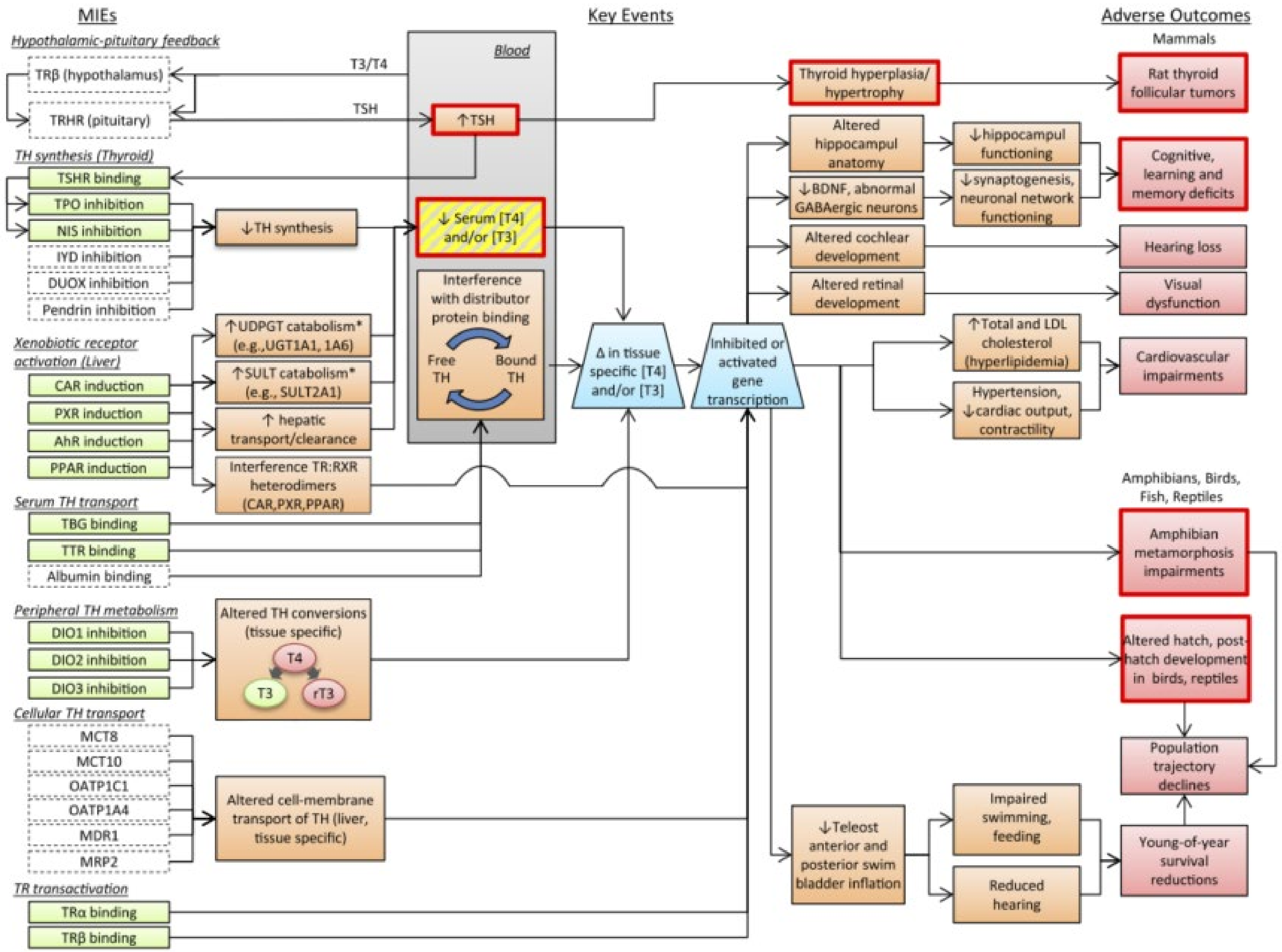
- Serum T3, T4 and TSH
- Thyroid and pituitary weights
- Thyroid histopathology

Screening Assay	Thyroid weight	Pituitary weight	Thyroid Histopathology	Serum TH levels
OECD TG 407	+	+	+	+ (optional)
OECD TG 408	-	-	+	-
OECD TG 416	+	+	-	-
OECD TG 422	-	-	+	-
OECD TG 441	-	-	-	+ (T3 and T4, optional)
OECD TG 443	+	+	+ (optional)	+ (T4 and TSH)
OECD TG 451			+	
OECD TG 452	+		+	
OECD TG 453	+		+	
EPA 15-day intact adult male rat assay	+	-	+	+
EPA Pubertal male	+	+	+	+ (T4 and TSH)
EPA Pubertal female	+	+	+	+ (T4 and TSH)

Jomaa, B. (2015).

DOI: 10.1289/EHP1304

Thyroid AOP Network: Broad Coverage of Mechanistic MIE-based Thyroid Assays



2013 Murk, A. J. *et al.* Mechanism-based testing strategy using in vitro approaches for identification of thyroid hormone disrupting chemicals. *Toxicology in vitro*.

2014 OECD. New Scoping Document on in vitro and ex vivo Assays for the Identification of Modulators of Thyroid Hormone Signalling. *OECD Series on Testing and Assessment, No. 207*

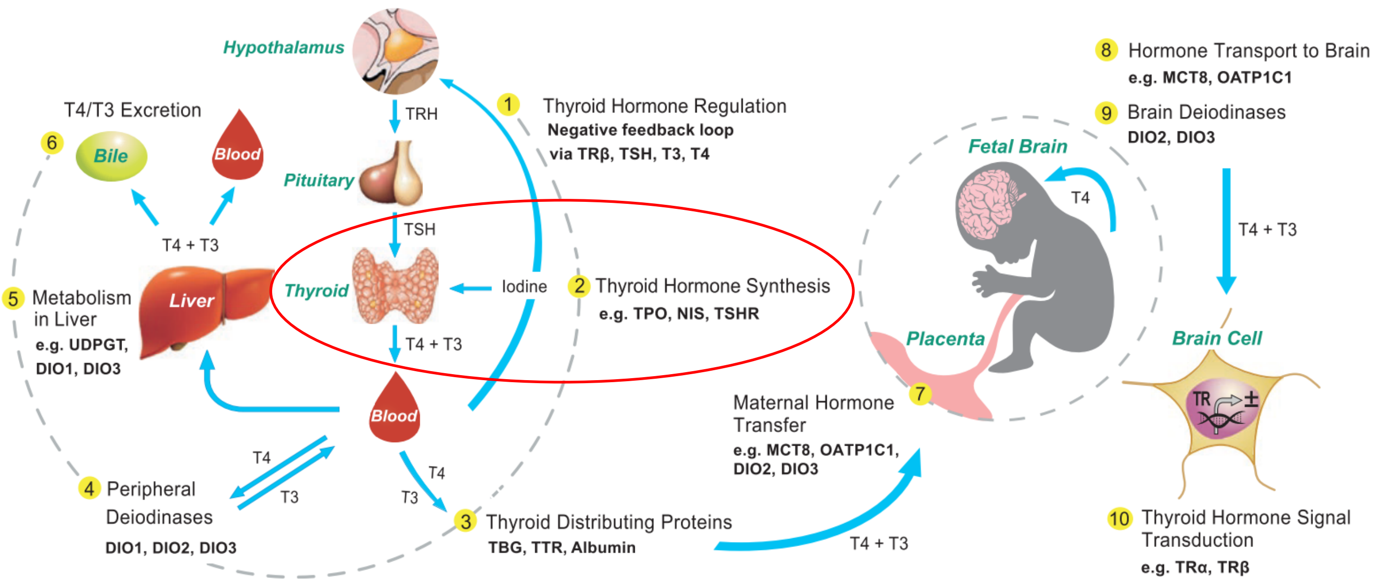
2019 Noyes, P.D. *et al.* Evaluating Chemicals for Thyroid Disruption: Opportunities and Challenges with in Vitro Testing and Adverse Outcome Pathway Approaches. *Environ Health Perspect.*

How can the human thyroid gland be represented *in vitro* to provide ‘key event’ coverage?

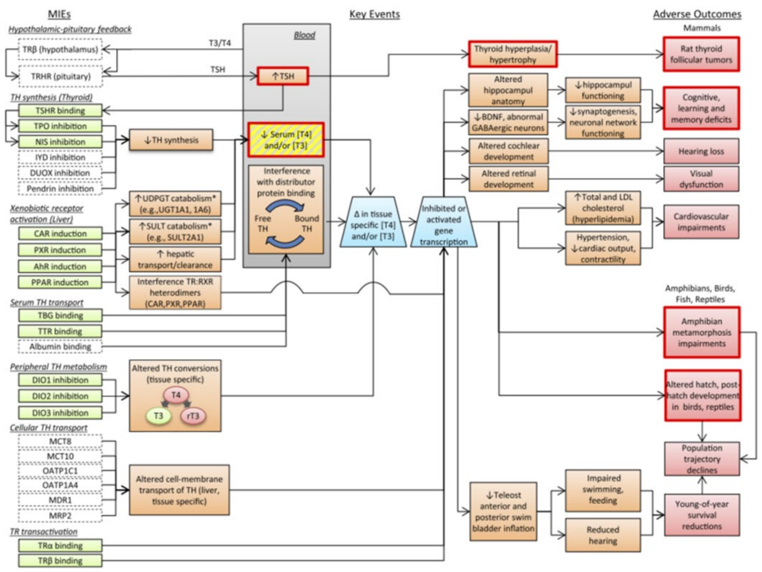
DOI: 10.1289/EHP5297

Challenges with *In Vitro* Thyroid Testing: Thyroid HTS Assays Do Not Directly Measure Thyroid Hormone Disruption

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

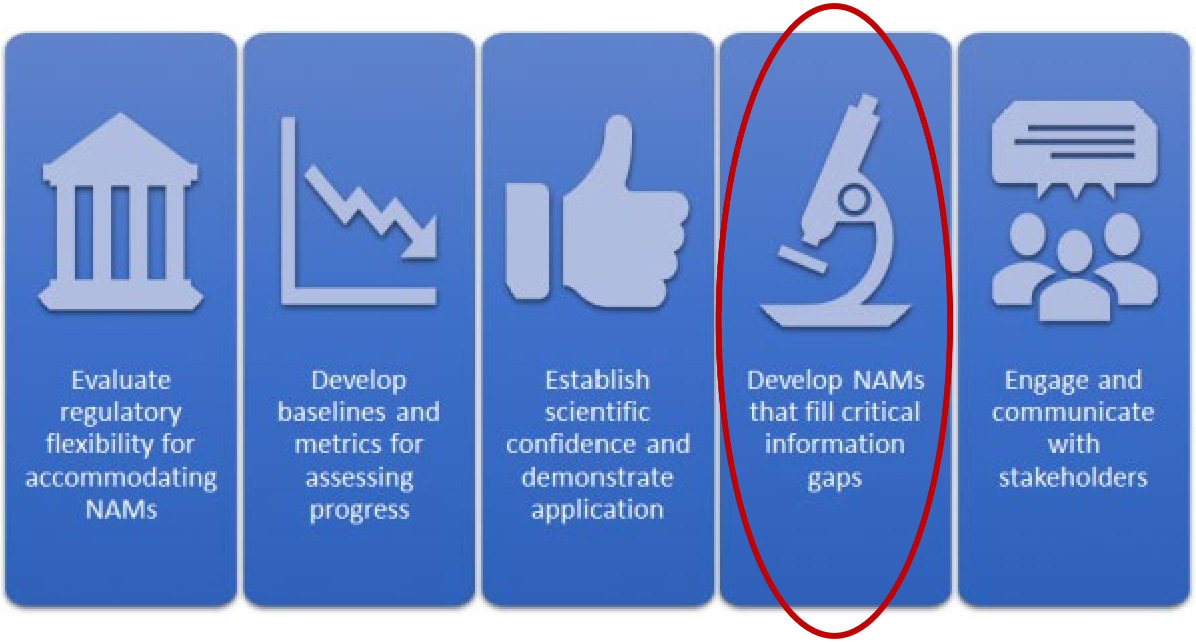
United States Environmental Protection Agency

Outline

- Development of a human thyroid organotypic culture model to address data gaps in screening and prioritization of thyroid disrupting chemicals
- Establishing confidence with an inter-laboratory prevalidation study of the human thyroid microtissue assay
- Orthogonal screening of prioritized chemicals in human thyroid microtissues for functional and mechanistic relevance

EPA New Approach Methods Work Plan: Reducing Use of Animals in Chemical Testing

New Approach Methods – any technology, methodology, approach, or combination that can provide information on chemical hazard and risk assessment to avoid the use of animal testing.



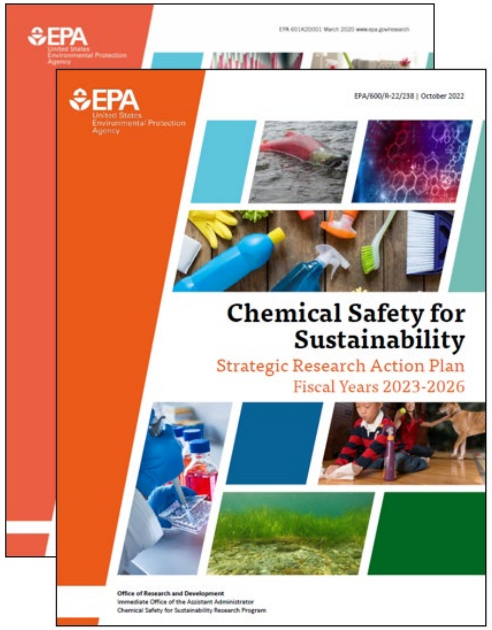
Five work plan objectives

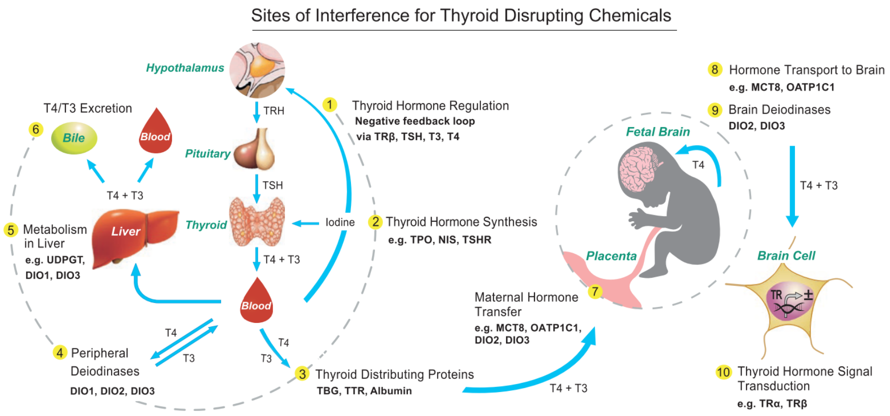
Examples of information gaps

- Inadequate coverage of biological targets.
- Minimal capacity for addressing xenobiotic metabolism in *in vitro* test systems.
- Limited capability to address tissue- and organ-level effects.
- Lack of robust integrated approaches to testing and assessment (IATAs) for complex biology.

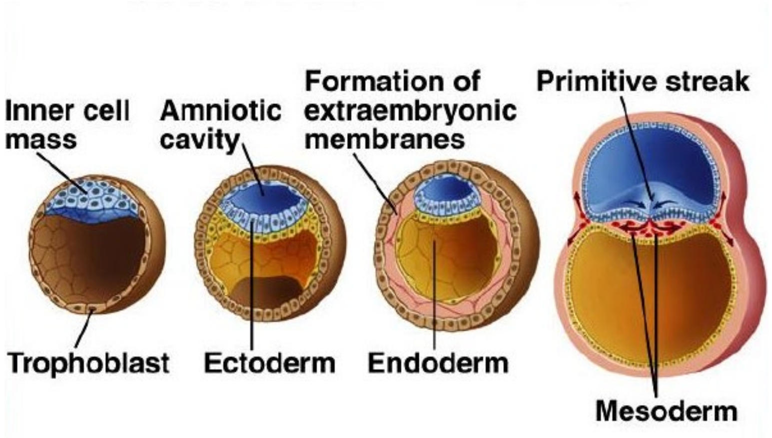
https://www.epa.gov/sites/production/files/2020-06/documents/epa_nam_work_plan.pdf

Chemical Safety for Sustainability – Virtual and Complex Tissue Models





Human Thyroid Disruption



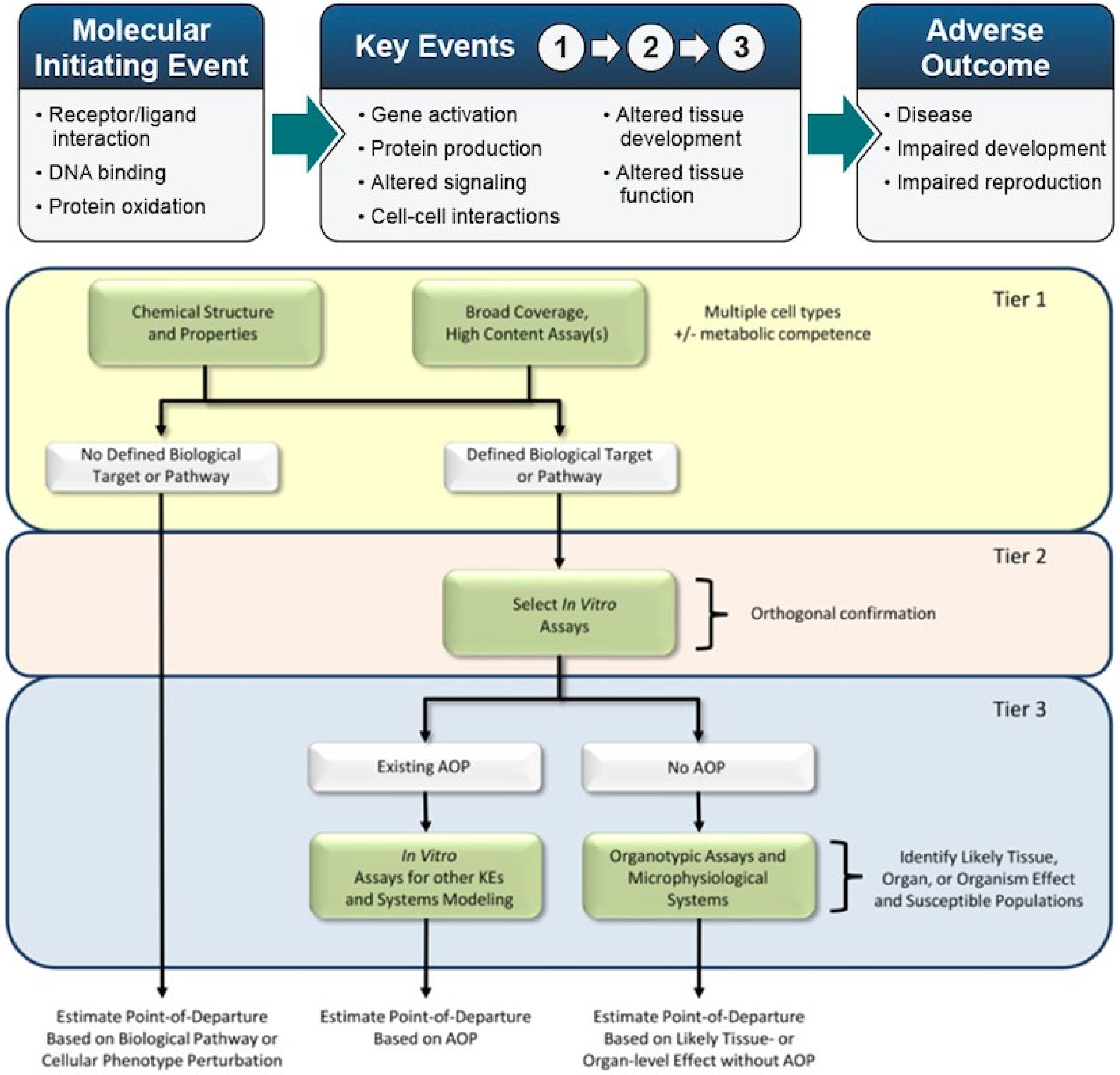
Human Developmental Toxicity

Research Area Objective: Develop, characterize, and apply organotypic and complex tissue models that bridge between *in vitro* and organismal assays for decision-relevant endpoints.

Product: CSS.405.1.1 - Advancing Translational Applications and Acceptance of Human Organotypic Thyroid Assays.

DOI: 10.1210/endocr/bqaa106

EPA Computational Toxicology Blueprint: Tiered Hazard Screening and Prioritization

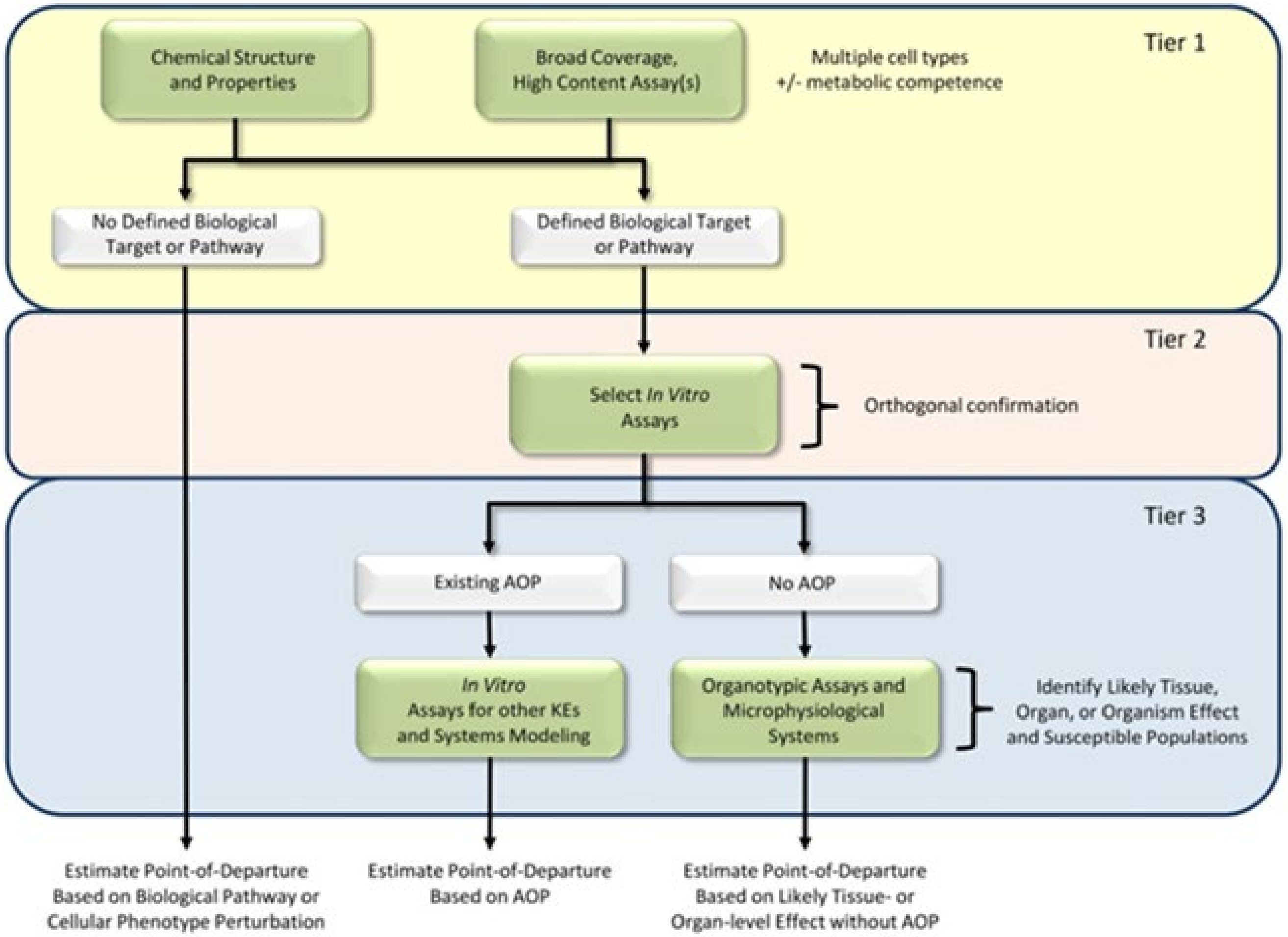


https://ntp.niehs.nih.gov/go/niceatm-aop; DOI: 10.1093/toxsci/kfz058

Tier 3 Experimental Approaches

- **Tier 1/2 Prioritized Chemicals**: Reduce HTS data uncertainty and provide more physiologically relevant insight into spatial and temporal toxicodynamics.
- **Organotypic Culture Models (OCMs)**: Primary cells or tissues in complex culture systems that more closely mimic organ structure and function.
- **Microphysiological Systems**: Microfluidic device containing OCMs in a controlled microenvironment.

Applying a Tiered Testing Paradigm to Identify Potential Human Thyroid Disruptors



CSS.405.1.1 - Advancing Translational Applications and Acceptance of Human Organotypic Thyroid Assays

- Inter-laboratory prevalidation study of the human thyroid microtissue assay.
- Orthogonal screening of prioritized thyroid HTS chemicals.
- Quantitative modeling of thyroid hormone synthesis perturbations in human thyroid microtissues.

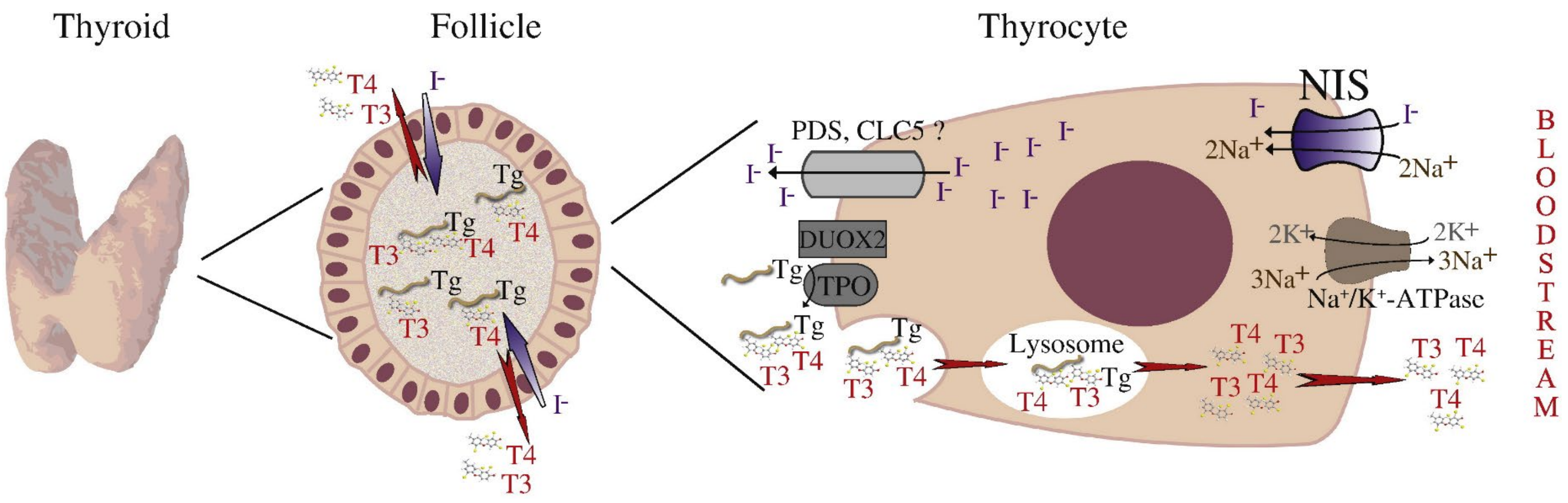
CSS.401.6.4 - Tiered Testing of Potential PFAS Inhibitors of NIS

- Orthogonal testing in the rat FRTL5 thyroid and human 3D thyroid microtissue assays for mechanistic confirmation and species comparison.

Addressing Partner Needs: Tiered testing strategies, Building confidence in new approach methods (NAMs), Vulnerable and sensitive lifestages and subpopulations

DOI: 10.1093/toxsci/kfz058

Challenges with *In Vitro* Thyroid Testing: Cell Type and Architecture are Critical Determinants for Hormone Synthesis



Cell Type

- No primary or thyroid cell lines, of any species, demonstrate appreciable capacity for thyroid hormone synthesis in 2D models
- Primary thyrocytes lose essential functions when cultured in conventional monolayer systems

Cell Architecture

- Follicular morphology is a critical feature for retaining hormone synthesis dynamics

DOI: 10.1016/j.bbamem.2013.08.013

Filling Technology Gaps for *In Vitro* Thyroid Testing

EPA United States Environmental Protection Agency

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

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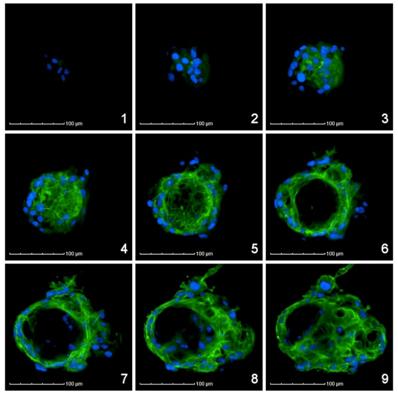
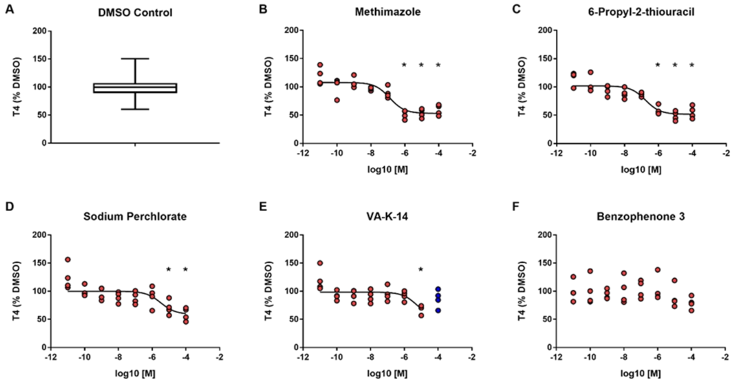
DOI: 10.1089/aivt.2020.0027

InSCREENex

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

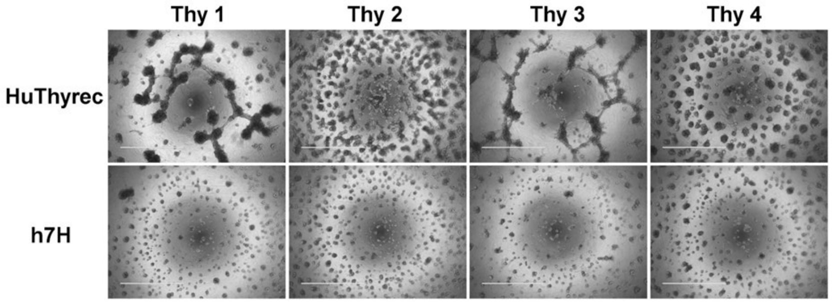
Chad Deisenroth ,*,[1] Valerie Y. Soldatow,[†] Jermaine Ford,[‡] Wendy Stewart,* Cassandra Brinkman,* Edward L. LeCluyse,[†] Denise K. MacMillan,[‡] and Russell S. Thomas *

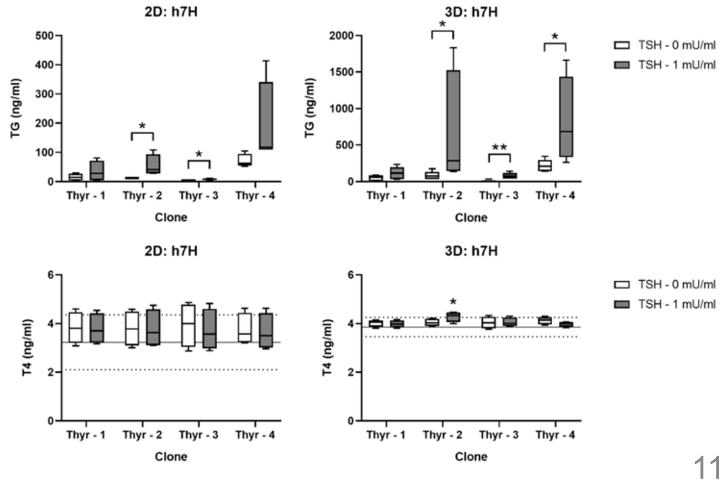




Characterization of Novel Human Immortalized Thyroid Follicular Epithelial Cell Lines

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





- A “Tier 3” assay designed to evaluate thyroid hormone synthesis perturbations as a mode-of-action for endocrine disruption in regulatory screening.
- Established commercial sources of primary human thyrocytes and immortalized cell lines.

Outline

- Development of a human thyroid organotypic culture model to address data gaps in screening and prioritization of thyroid disrupting chemicals
- Establishing confidence with an inter-laboratory prevalidation study of the human thyroid microtissue assay
- Orthogonal screening of prioritized chemicals in human thyroid microtissues for functional and mechanistic relevance

Test Method Validation Principles

- Validation provides confidence to regulatory stakeholders that a test method is **reliable**, **relevant**, and can be used for decision-making in a defined regulatory application.

  - **Reliability (Reproducibility)**: “Measures of the extent that a test method can be performed reproducibly within and between laboratories over time, when performed using the same protocol. It is assessed by calculating intra- and inter-laboratory reproducibility and intra-laboratory repeatability.”

  - **Relevance**: “Description of relationship of the test to the effect of interest and whether it is meaningful and useful for a particular purpose. It is the extent to which the test correctly measures or predicts the biological effect of interest. Relevance incorporates consideration of the accuracy (concordance) of a test method.”

OECD GD 34 (ENV/JM/MONO(2005)14)

EU-NETVAL Validation of In Vitro Thyroid Test Methods

- The European Union reference laboratory for alternatives to animal testing (EURL ECVAM) has compiled a number of *in vitro* thyroid methods with validation potential based on OECD scoping document (OECD, No. 207, 2017)

- The European Union Network of Laboratories for the Validation of Alternative Methods (EU-NETVAL) has 15 member labs participating in validation of 18 human-relevant mechanistic methods.

  - Block 1: Central Regulation
  - Block 2: Thyroid Hormone Synthesis
  - Block 3: Secretion and Transport
  - Block 4: Metabolism and Excretion
  - Block 5: Local Cellular Concentrations
  - Block 6: Cellular Responses
  - Block 7: Relevant Short Term Alternative Methods Integrating Multiple MOAs
  - Block 8: Integrative Cellular In Vitro Methods

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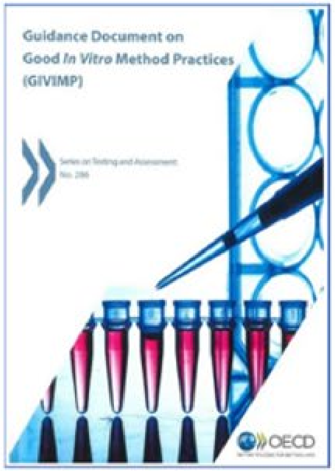
Update on the EU-NETVAL Validation Effort

Experimental work & deliverables

PART 1: Definition of the *in vitro* method

- ✓ Produce the related set of SOPs and related spreadsheets
- ✓ Further development of the method if so needed
- ✓ Aim: assessing robustness and reliability
- ✓ Few chemicals: Minimum the reference and control items
- ✓ Experimental study of 5 valid runs

Deliverables: SOP(s), Study plan, Study report, Assessment Report



PART 2: Relevance

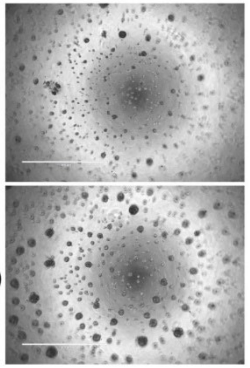
- ✓ Started when the 'definition' had proven to be successful
- ✓ Aim: assessing overall relevance, based on the underlying mechanisms of the selected *in vitro* methods
- ✓ A set of 30 blind-coded chemicals is tested in all methods
- ✓ Experimental study of 3 valid runs per test item

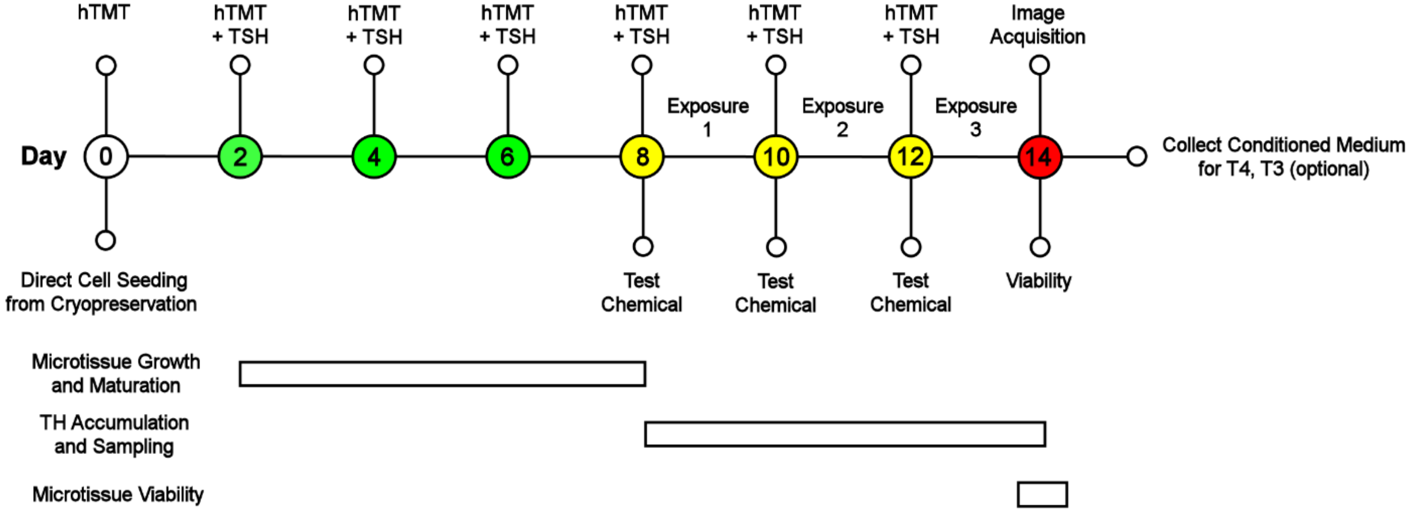
Deliverables: SOP(s), Study plan, Study report

FINAL OVERALL data collection
October 2022 – April 2023

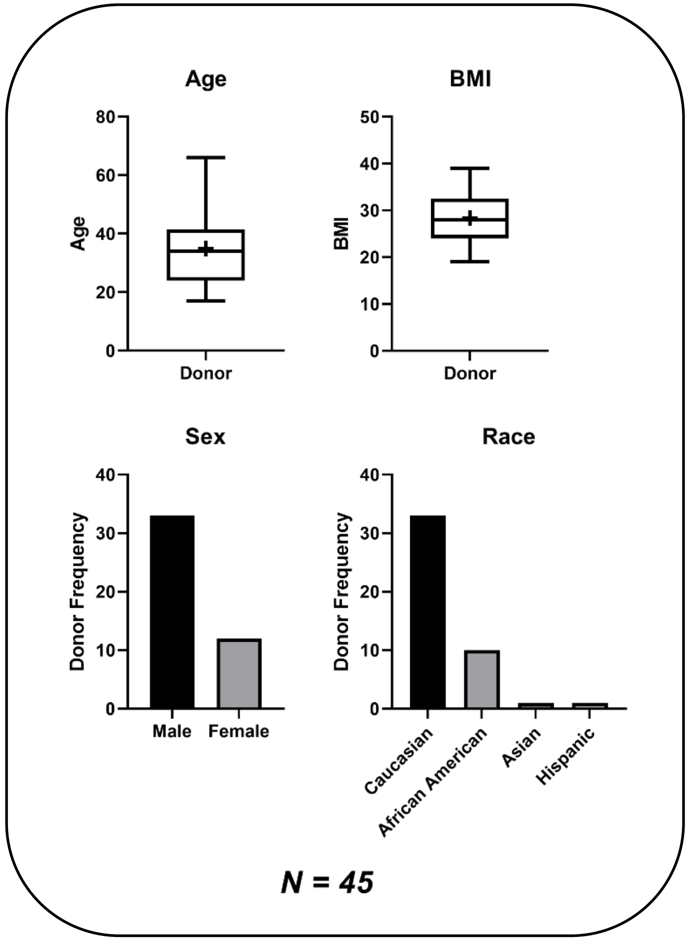
European Commission

Improving the Platform: Human Thyroid Microtissue Assay v2.0





Method modifications for improved performance, accessibility, and implementation.



Expansion of the EPA Thyroid Donor Biobank

Human Thyroid Microtissue Assay Workflow – Designed for Reproducibility

Matrigel coating.

Eppendorf ThermoStat C

- Microplates maintained at consistent temperature.

Medium changes and treatments.

Integra ViaFlo 96/384

- Semi-automated medium exchange for minimal pipetting error and microtissue damage.

Chemical dosing.

Labcyte Echo 555

- Precise chemical dosing of assay plates using acoustic dispensing.

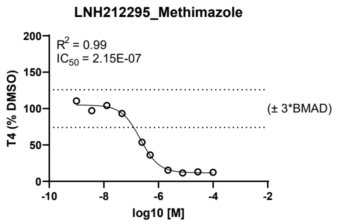
Data collection.

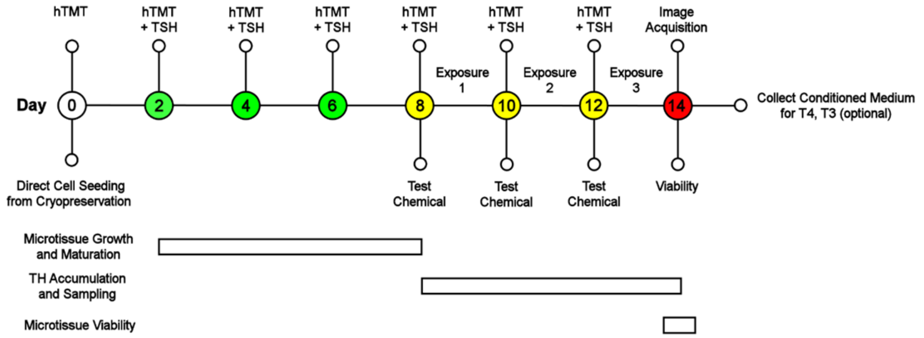
BMG CLARIOstar Plus

- Multi-mode plate reader.

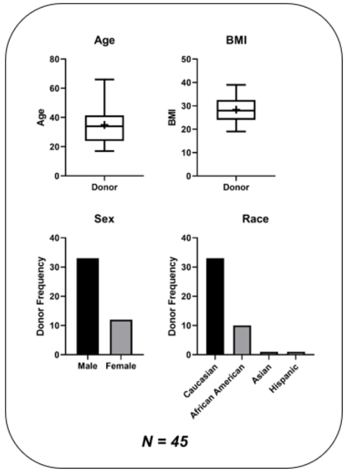
Data modeling and analysis.

- Routine: GraphPad Prism
- Screening: ToxCast Pipeline

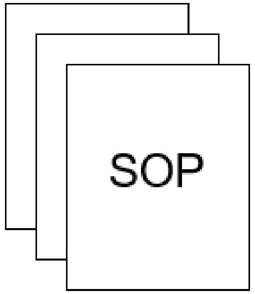




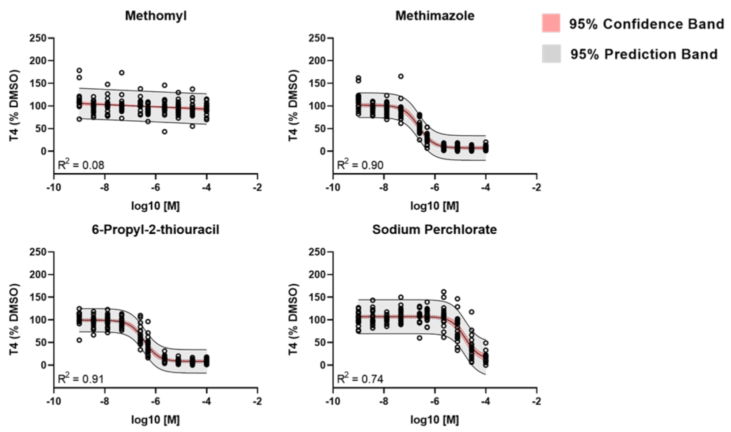
Increasing Confidence in a Human Thyroid Microtissue New Approach Method (NAM)



Characterizing the EPA Donor Biobank

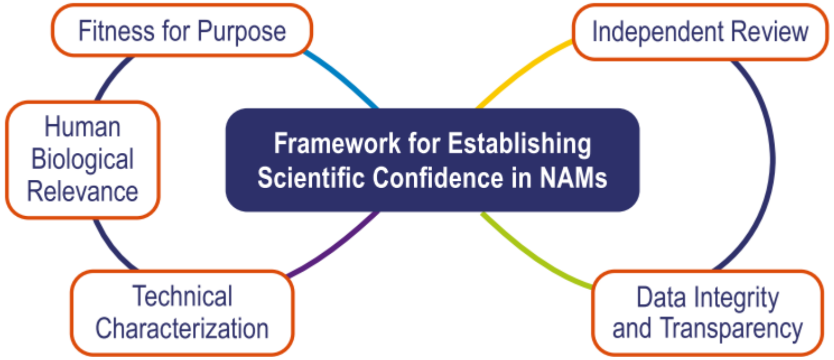


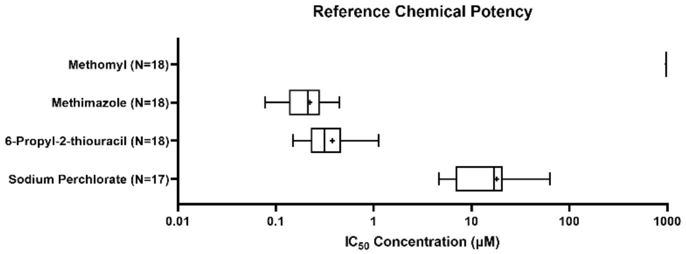
Independent Peer Review by Validation Management Team



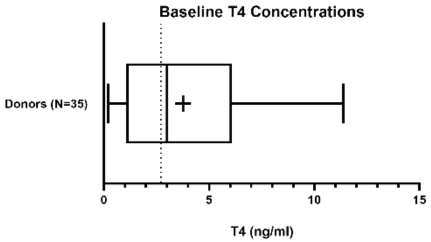
Independent Donor Performance Summary (*N* = 18)

Evaluating Human Donor Variability





Selecting Reference Chemicals



95% CI of median	
Actual confidence level	95.64%
Lower confidence limit	1.500
Upper confidence limit	4.410

Establishing Donor Acceptance Criteria

Long Range Goal: Establish a validated OECD Test Guideline for human thyroid hormone disruption

doi.org/10.1007/s00204-022-03365-4

Inter-laboratory Prevalidation of the Human Thyroid Microtissue Assay

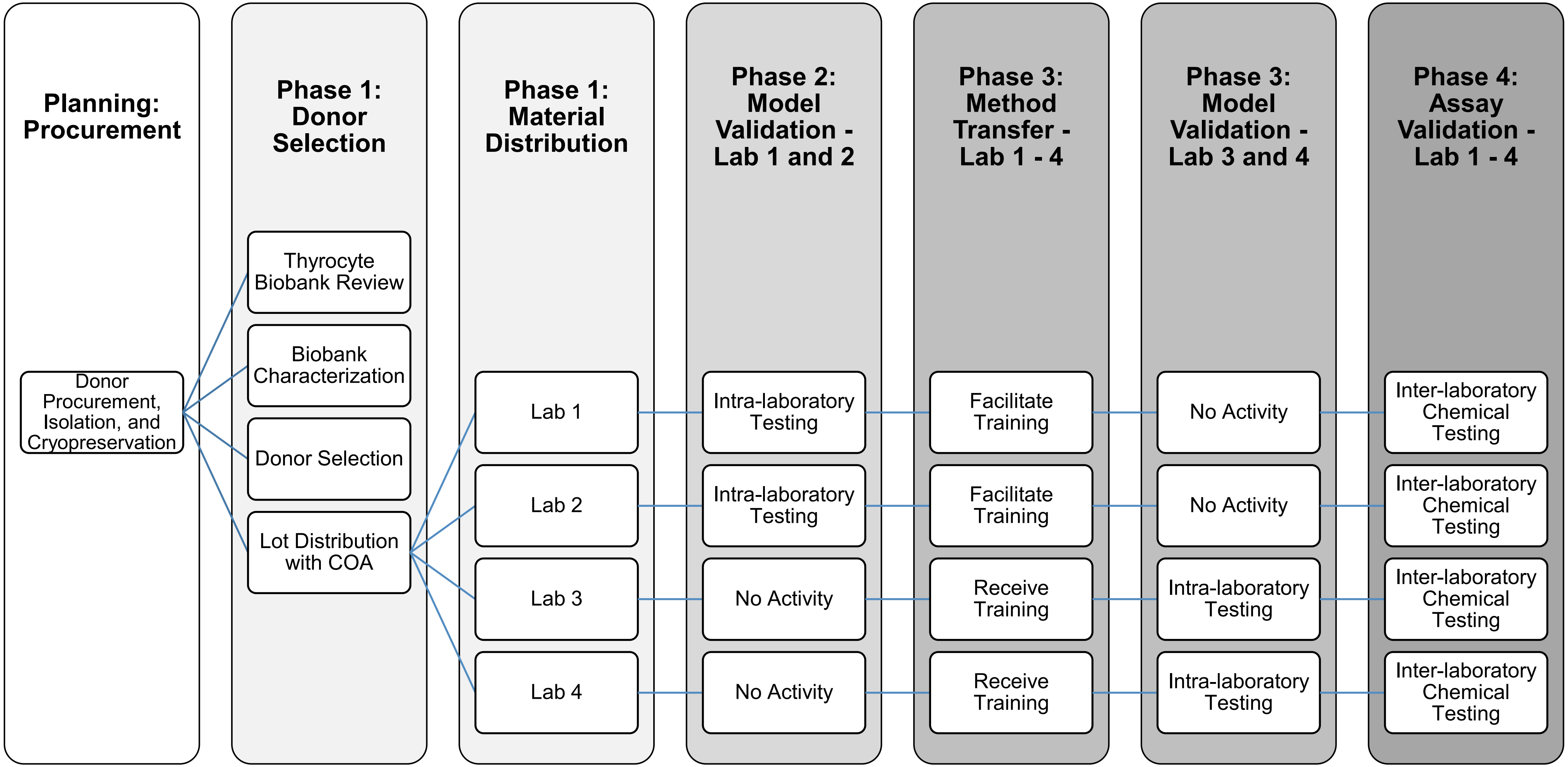
Goal: To structure and support a preliminary assessment of the test method reliability and relevance.

NICEATM

Objectives

1. Collaborative effort on the study design, analytical approaches, chemical selection, and data interpretation.
2. Test method standardization.
3. Test method transfer, training and intra-laboratory model performance evaluation.
4. Limited inter-laboratory reference chemical testing and assay performance evaluation.

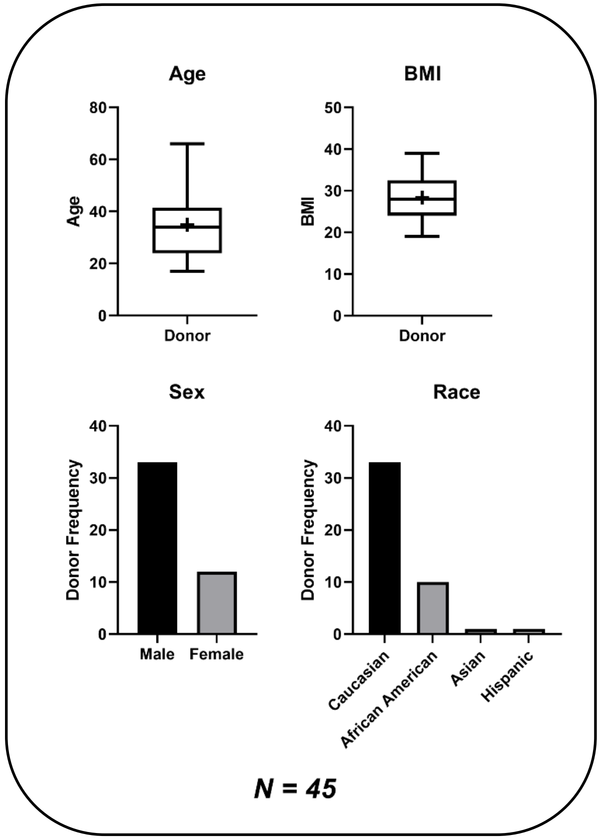
Experimental Plan



EPA to provide standardized cells and reagents to all partner labs for the study:

1. Cryopreserved Primary Human Thyroid Follicular Epithelial Cells – *Lot validation in progress.*
2. Thyroid Stimulating Hormone – *Lot validated.*
3. Human anti-TSHR Recombinant Antibody (clone K1-70) – *Lot validated.*

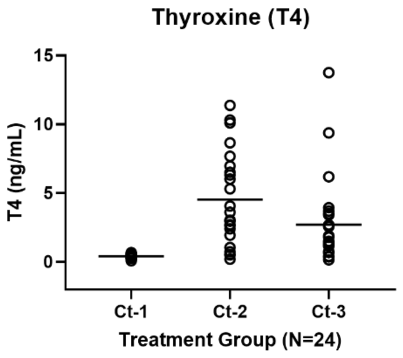
EPA Thyroid Donor Biobank – Donor Characterization and Qualification

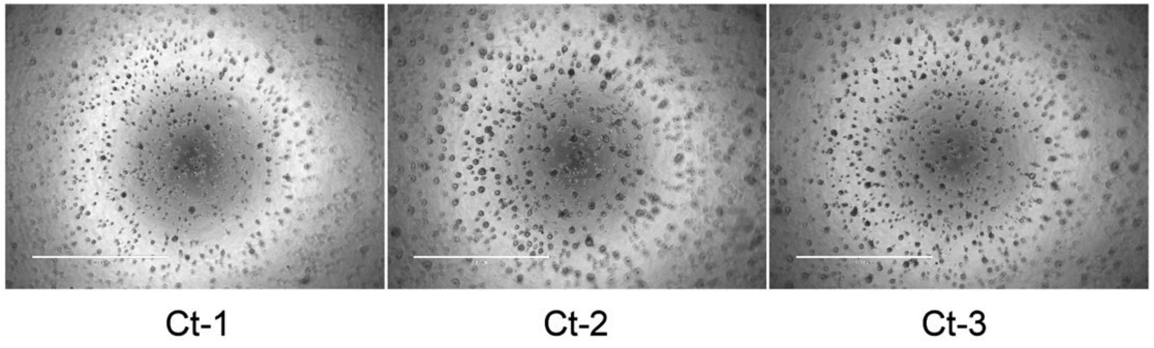


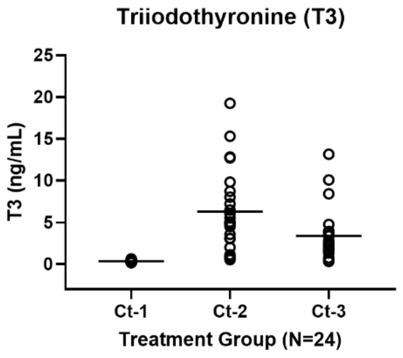
Biobank Summary (August 2017 – Present)	
Donors	45
Median Age	34 (17-66)
Sex	Male (33); Female (12)
Race	Caucasian (33), African American (10), Hispanic (1), Asian (1)
Median BMI	28 (19-39)
Serologies	CMV, EBV
Euthyroid	44/45
Median Lot Vial Count	10 (0-70)

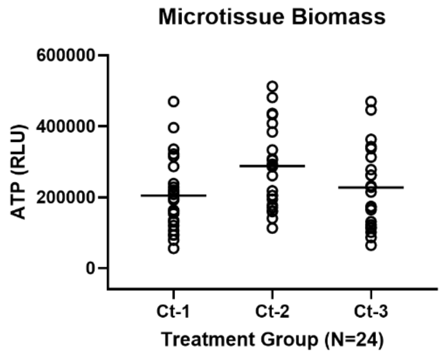
What donors should be tested for the model and performance validation phases?

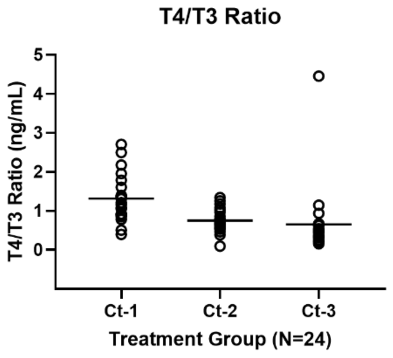
Ensuring High Quality Cells - Donor Characterization and Qualification Parameters

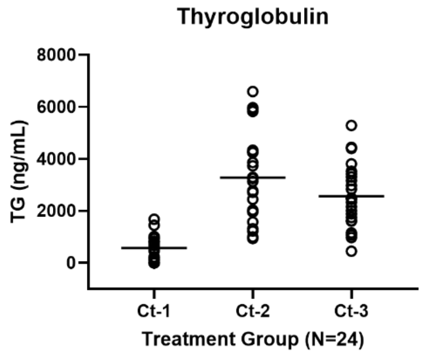


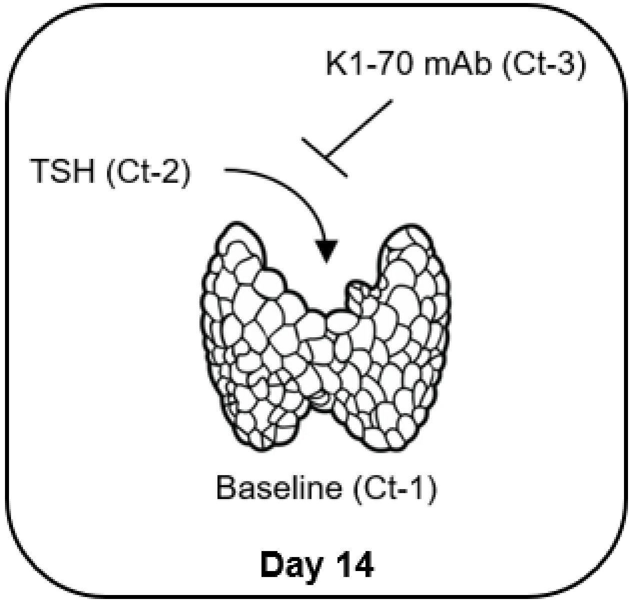












LifeNet Health LifeSciences Division

CERTIFICATE OF ANALYSIS DRAFT

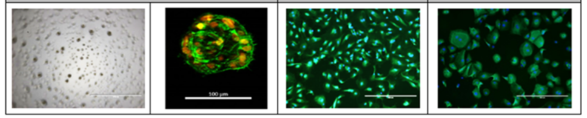
CELL TYPE	Primary Human Thyrocytes		
Product Code		Lot Number	THYxxxxxxx
Thyroid Disorder	Not Reported	Passage Number	P1
Cryopreservation Media	Serum-Free Cryopreservation Medium	Cryopreservation Method	Controlled Rate Freezer
Storage Condition:	Cryopreserved in Vapor Phase Liquid Nitrogen (< -150°C)		

DONOR DEMOGRAPHICS								
Age	Sex	Race	BMI	Tobacco Use	Alcohol Use	Drug Use	Medication Use	Cause of Death
43	M	C	23.4	None	None	None	Azelastine HCL	CNS Tumor

QUALITY TEST	SPECIFICATION	RESULT
Morphology	Microtissues; Follicle-like morphology (≥50µm)	(Attached image)
Viable Cell Count	≥ 3.0 x 10^6 viable cells per vial (Trypan Blue)	xxx
Viability	≥70 %post thaw (Trypan Blue)	xxx
QC Culture Period	14 days	Pass
Purity (ICC)	Positive: ≥ 90% KRT7; ≥53% TG; Negative marker (Calcitonin, VE-Cadherin or VEGF-R2)	Pass
Virus	Negative: HIV-1, Hepatitis-B, and Hepatitis-C	Pass
Sterility	Negative: bacteria, yeast, and fungi	(ND)

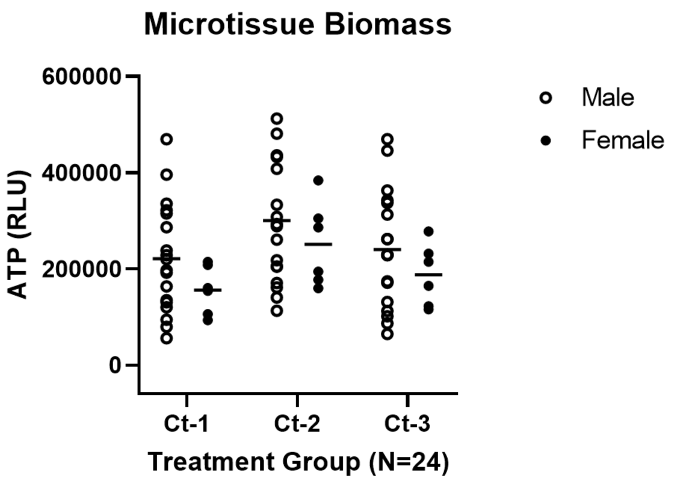
IN VITRO THYROID MICROTISSUE FUNCTION DATA (ELISA TEST RESULTS)			
Thyroglobulin (TG) on Day 7 Min Spec ≥1000ng/mL TG		Thyroxine (T_4) on Day 14 Min Spec ≥3ng/ml T_4	
0 IU/mL TSH	1 mIU/mL TSH	0 IU/mL TSH	1 mIU/mL TSH
332ng/ mL	1615 ng/ mL	<LOQ	6 ng/ mL

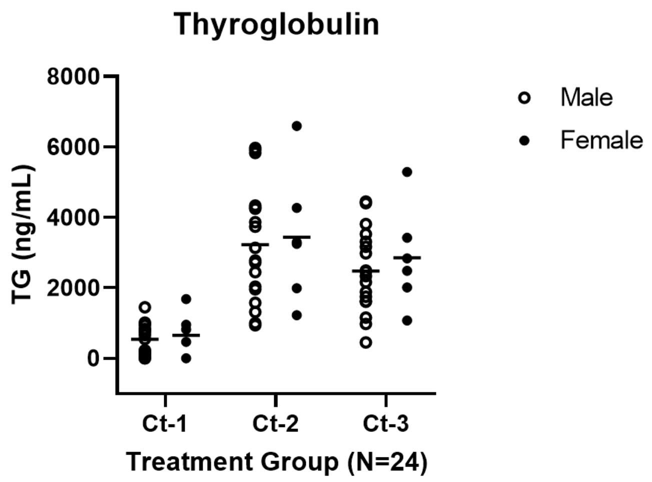
MORPHOLOGY		ICC (TG)	ICC (CK 7)

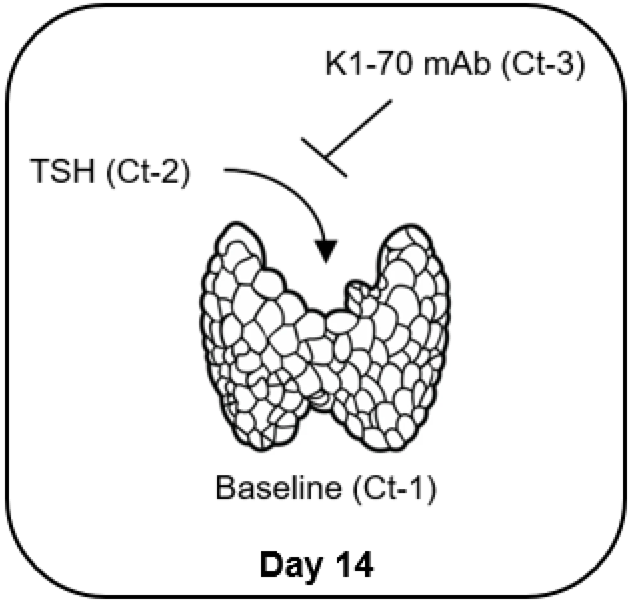


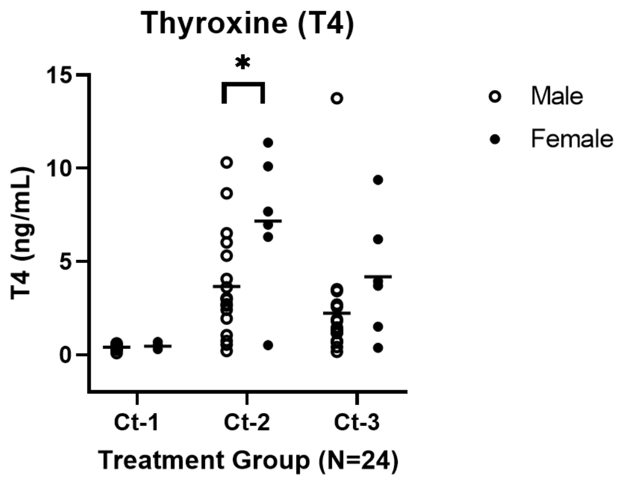
- Microtissue Morphology
- Microtissue Biomass
- TSH Receptor Sensitivity
- Thyroglobulin Synthesis
- Hormone Synthesis
- Reference Chemical Response

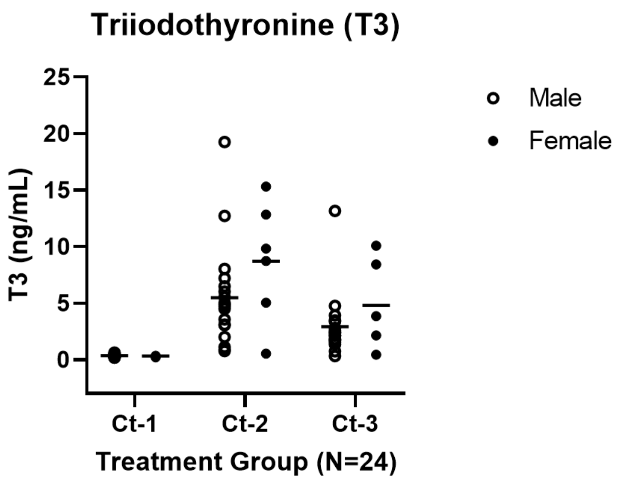
What Impact Does Donor Sex Have on Microtissue Performance?

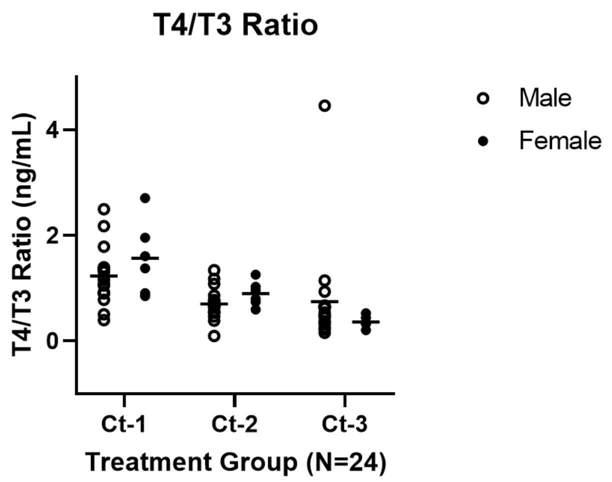




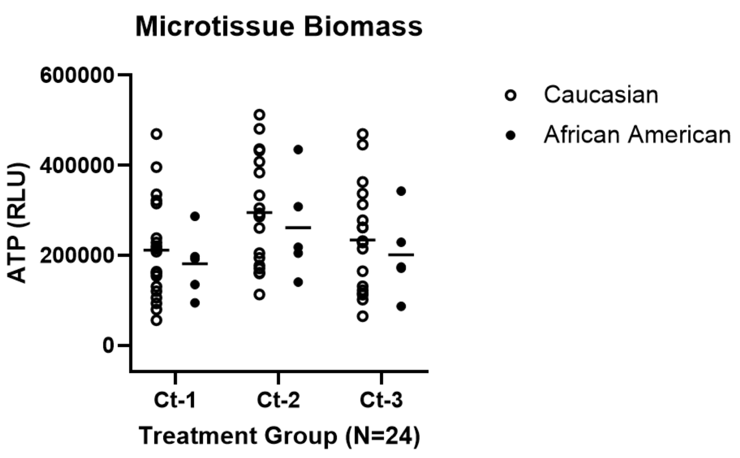


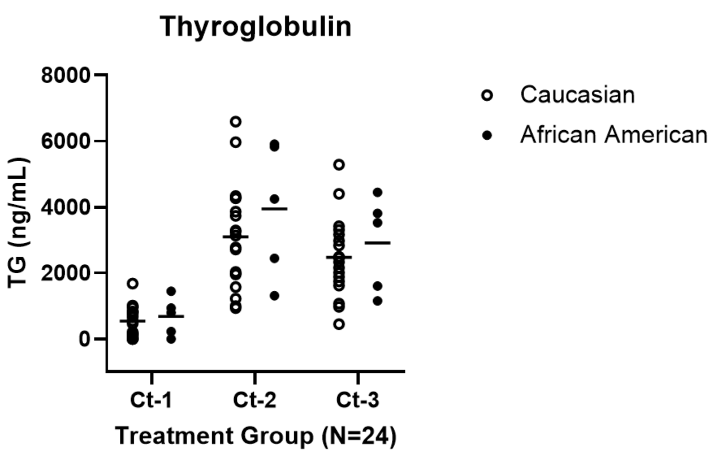


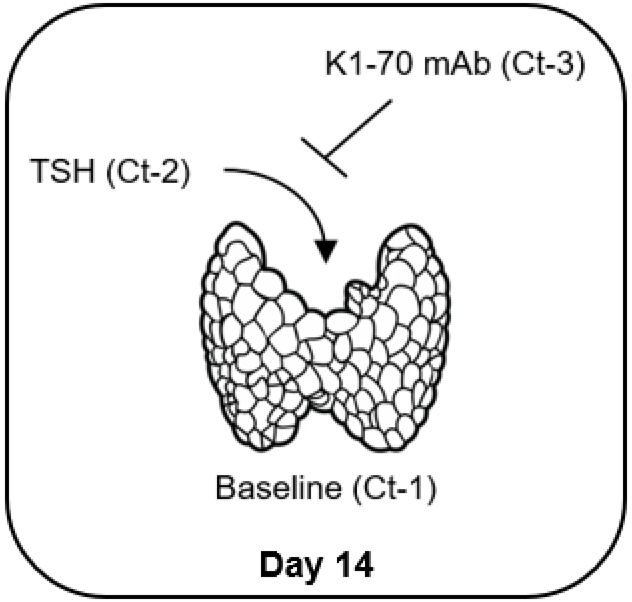


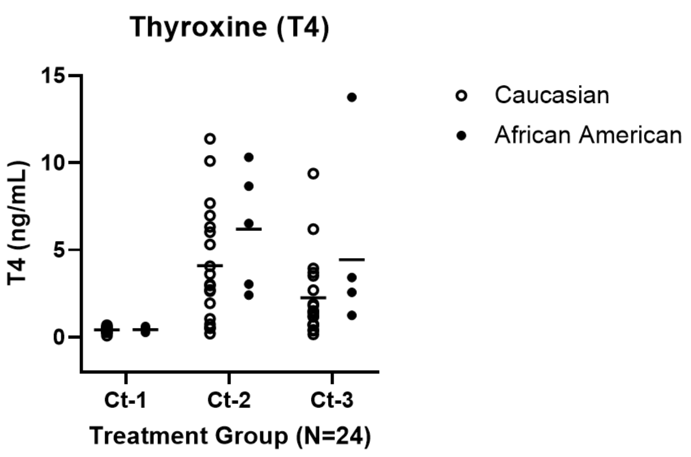


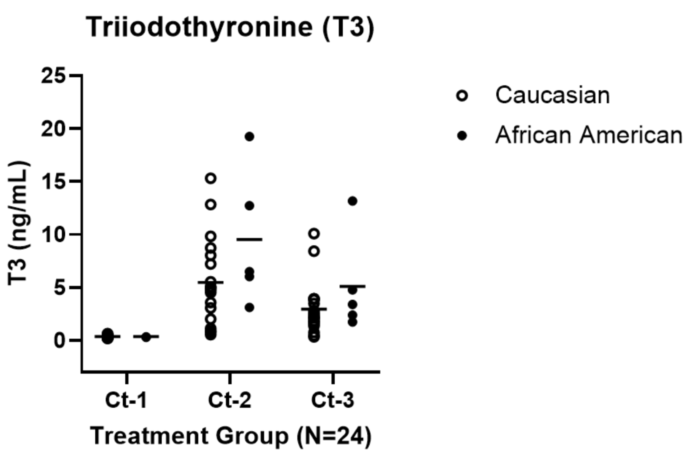
What Impact Does Donor Race Have on Microtissue Performance?

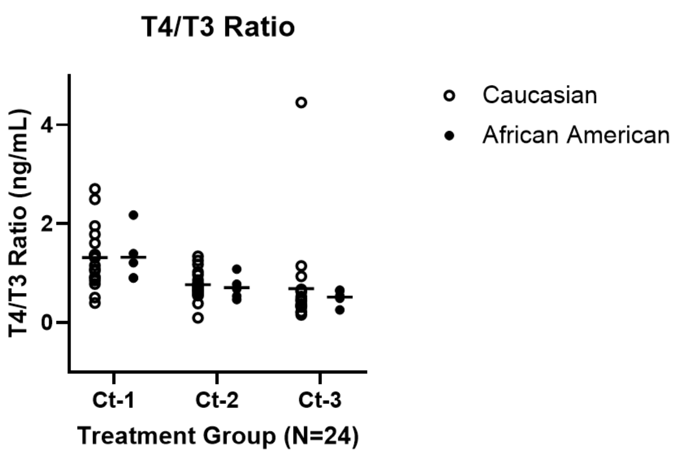












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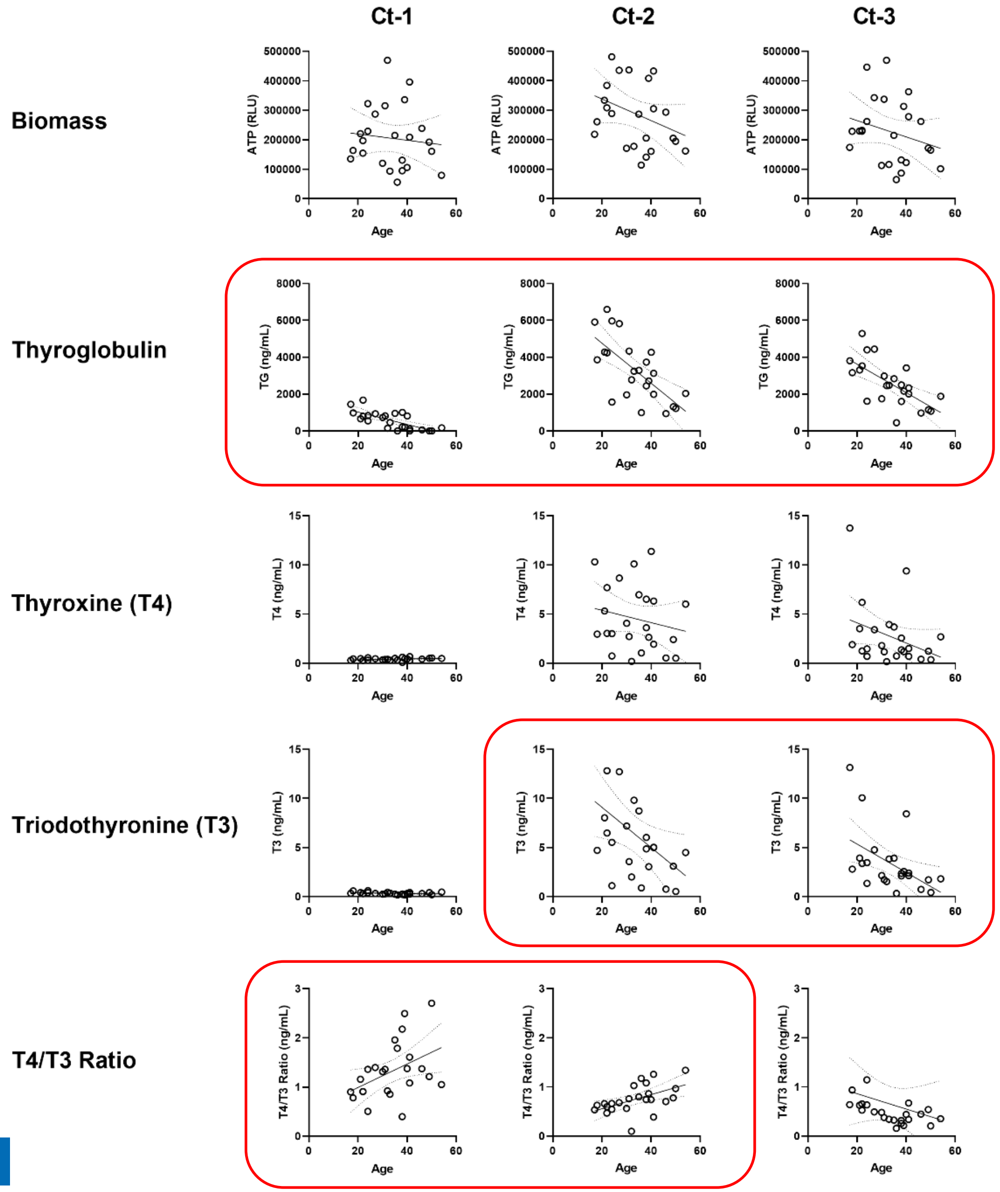
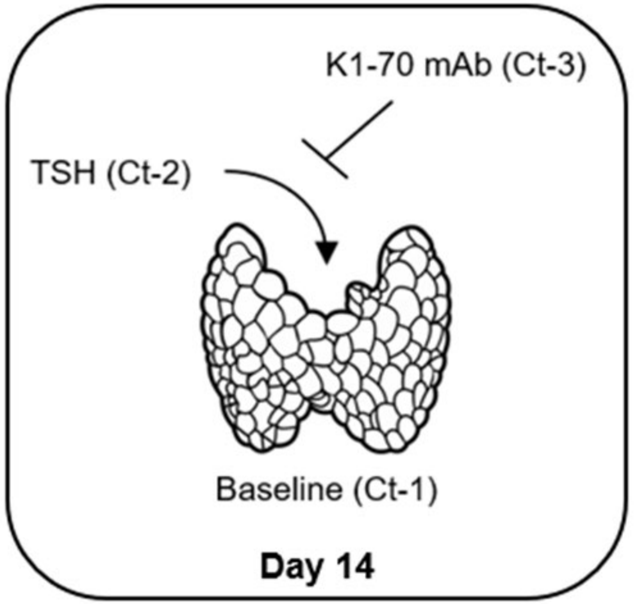
What Impact Does Donor Age Have on Microtissue Performance?

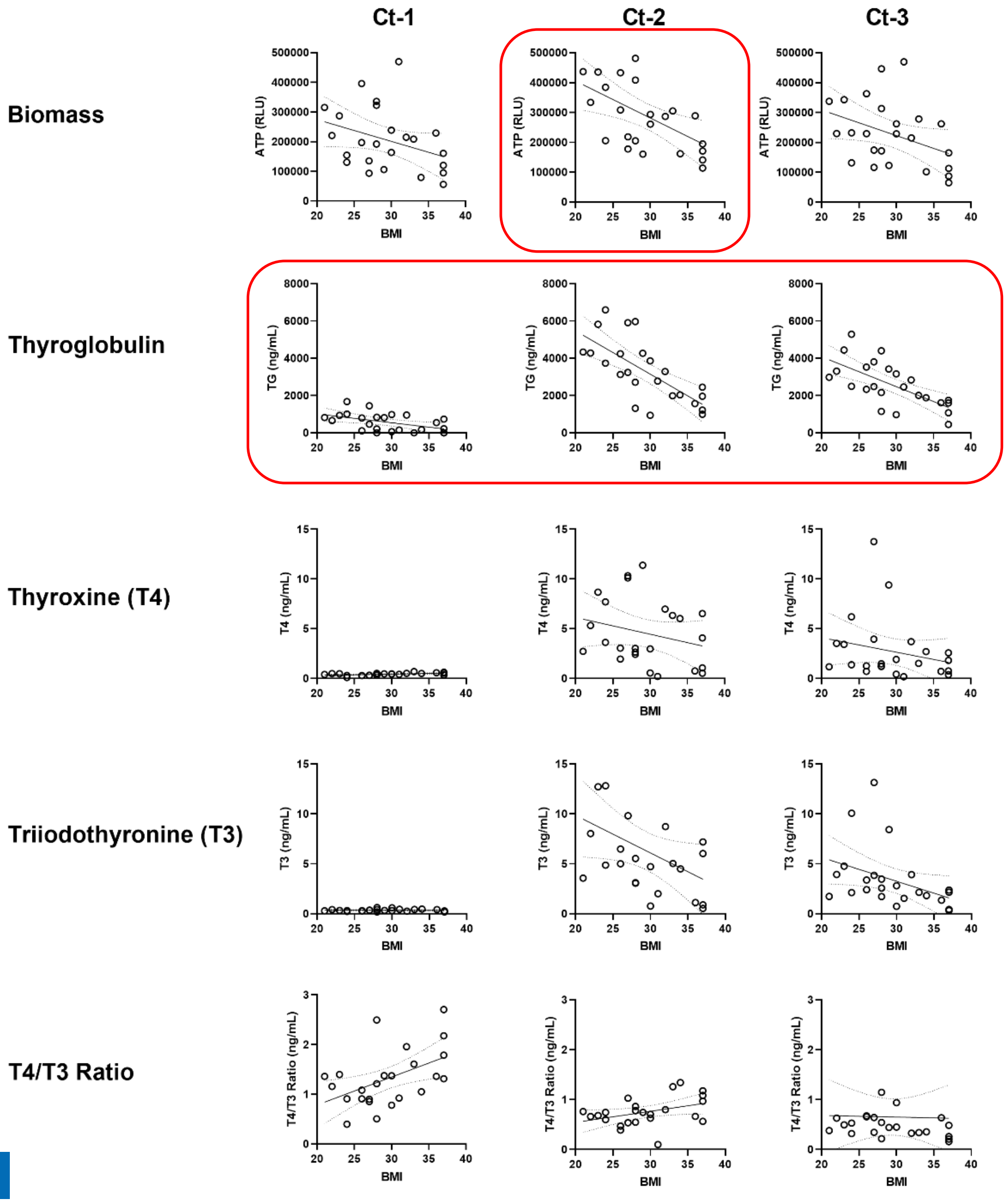


Generally, there is an inverse relationship between *in vitro* performance and age.

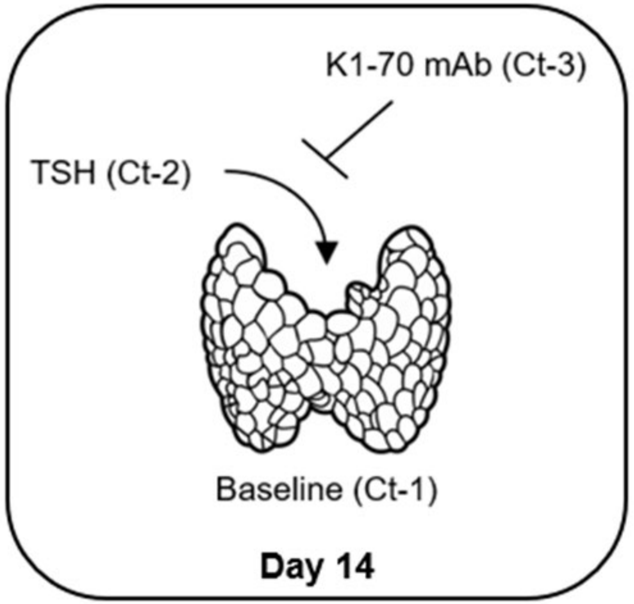


- Evaluation of 24 donors (and counting) at Day 14.
- Thyroglobulin expression is inversely related to age.
- T3 synthesis is inversely related to age when stimulated with TSH.
- T4/T3 ratio is positively related with age when stimulated with TSH.

What Impact Does Donor Body Mass Index (BMI) Have on Microtissue Performance?

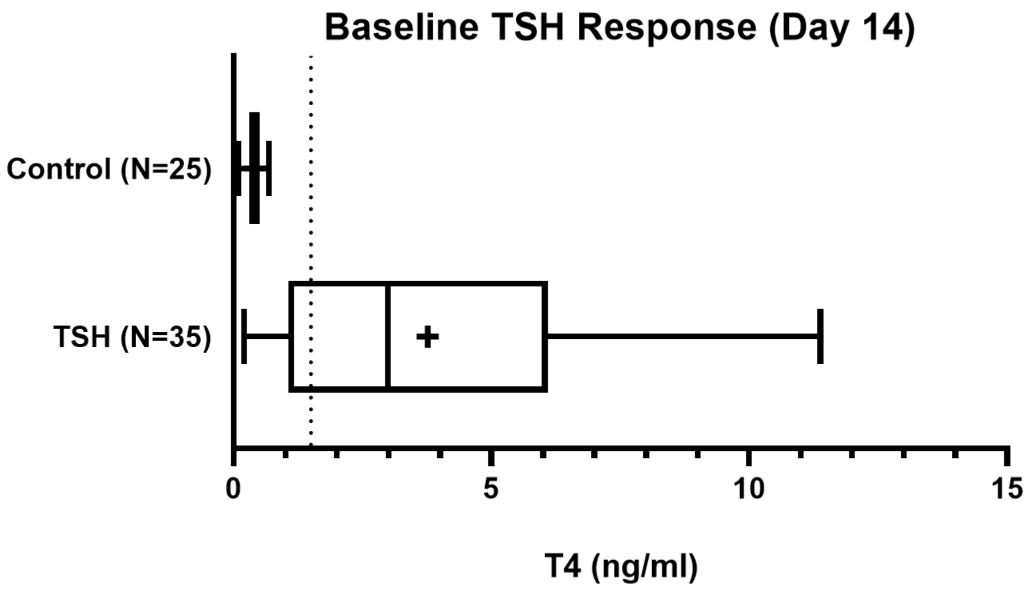


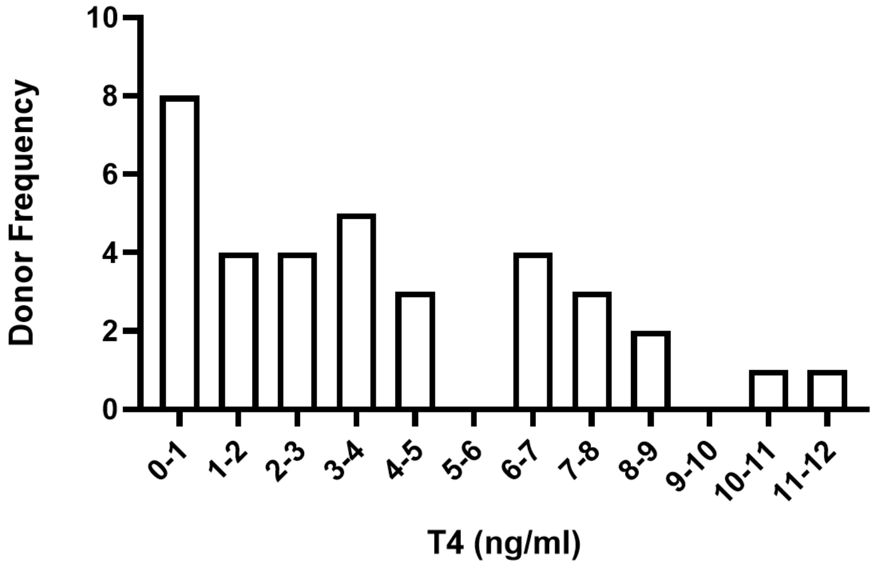
Generally, there is an inverse relationship between *in vitro* performance and BMI.



- Evaluation of 24 donors (and counting) at Day 14.
- Biomass is inversely related to BMI when stimulated with TSH.
- Thyroglobulin expression is inversely related to BMI.

Donor Qualification – Setting Minimum Criteria for Hormonogenic Competence





T4 (ng/ml)	Control	TSH
Minimum	0.09	0.20
25% Percentile	0.30	1.07
Median	0.45	3.00
75% Percentile	0.51	6.10
Maximum	0.69	11.38
Range	0.59	11.18

CI (95%)		
Lower confidence limit	0.32	**1.50**
Upper confidence limit	0.50	4.41

- Independent donors evaluated.
- Human serum total T4 reference range: 54 – 115 ng/ml.
- Minimum T4 synthesis threshold set at 1.5 ng/ml.
- 29% of donors do not qualify.

Reference Compound List

							Bioactivity		Cytotoxicity		Exposure	
Chemical_Name	DTXSID	CASRN	Classification	Target Gene	T4 phenotype	T3 phenotype	TOXCAST_NUMBER_OF_ASSAYS/TOTAL	TOXCAST_PERCENT_ACTIVE	TOXCAST_Cytotoxicity_Lower Bound_uM	TOXCAST_Cytotoxicity_Median_uM	EXPOCAST	EXPOCAST_MEDIAN_EXPOSURE_PREDICTION_MG/KG-BW/DAY
Dimethyl Sulfoxide	DTXSID2021735	67-68-5	Control_Solvent	NA	Baseline	Baseline	8/440	1.8	1000	1000	Y	2.67E-06
TSH	NA	NA	Control_Agonist	TSHR	Increase	Increase	0	0	NA	NA	NA	NA
K1-70 mAb	NA	NA	Control_Antagonist	TSHR	Decrease	Decrease	0	0	NA	NA	NA	NA
Methomyl	DTXSID1022267	16752-77-5	Reference_Negative	NA	No Change	No Change	17/964	1.8	1000	1000	Y	3.86E-07
Methimazole	DTXSID4020820	60-56-0	Reference_Antagonist	TPO	Decrease	Decrease	21/978	2.2	1000	1000	Y	2.59E-06
6-Propyl-2-thiouracil	DTXSID5021209	51-52-5	Reference_Antagonist	TPO	Decrease	Decrease	37/1054	3.5	1000	1000	Y	5.05E-07
Sodium Perchlorate	DTXSID1034185	7601-89-0	Reference_Antagonist	NIS	Decrease	Decrease	2/41	4.9	NA	NA	NA	NA

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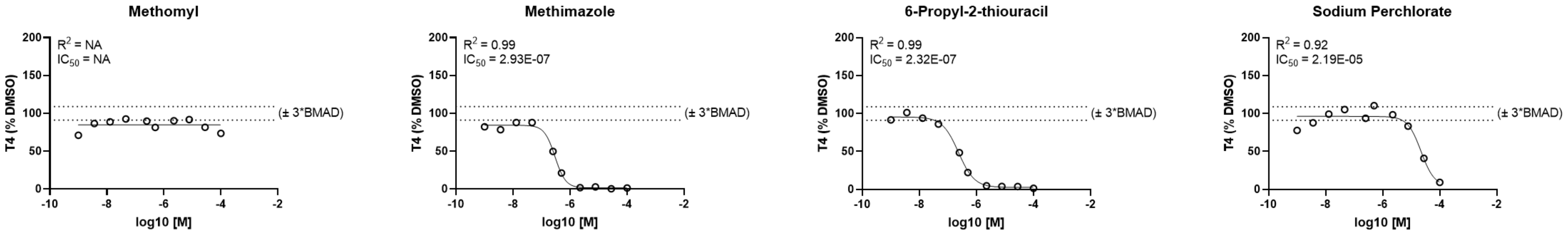
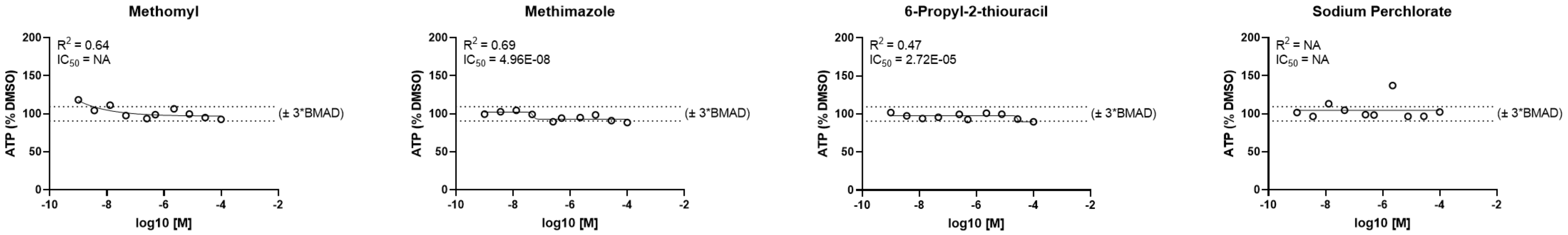
Donor Characterization – Reference Chemical Performance

Thyroxine (T4)

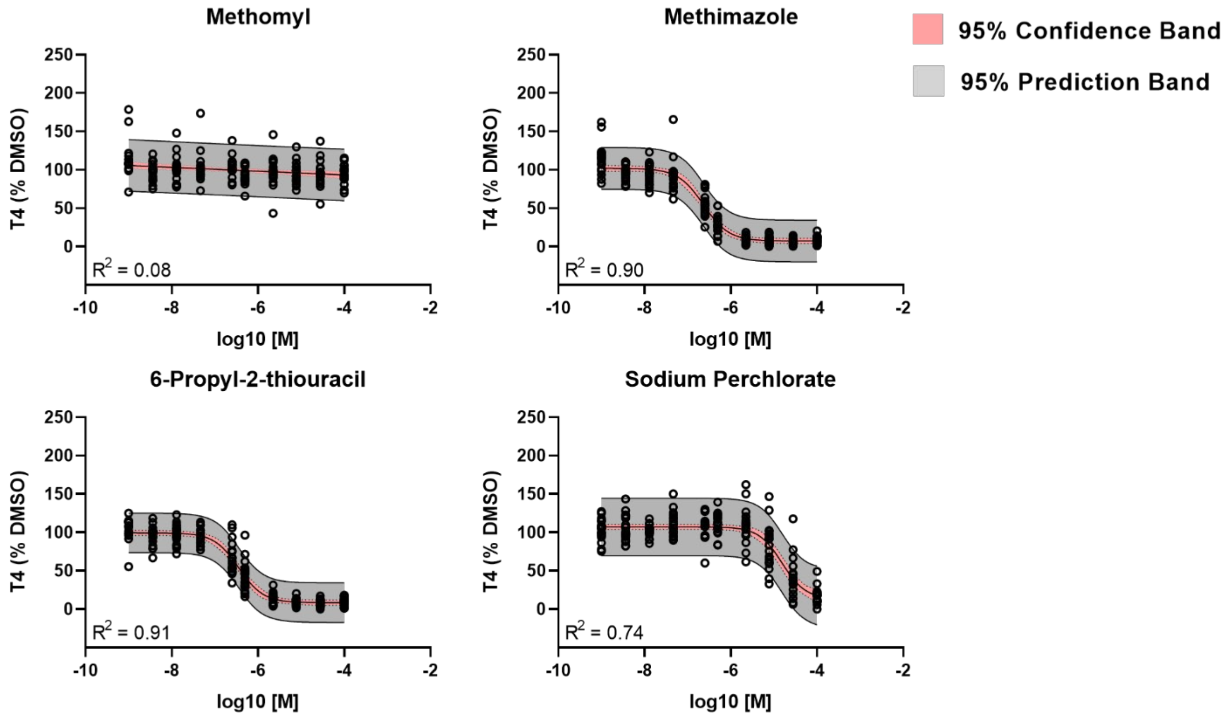


Cytotoxicity



- Evaluation of chemical effects on thyroxine (T4) synthesis and cytotoxicity at day 14.
- All donors evaluated independently.
- Donor-dependent data normalization is critical.

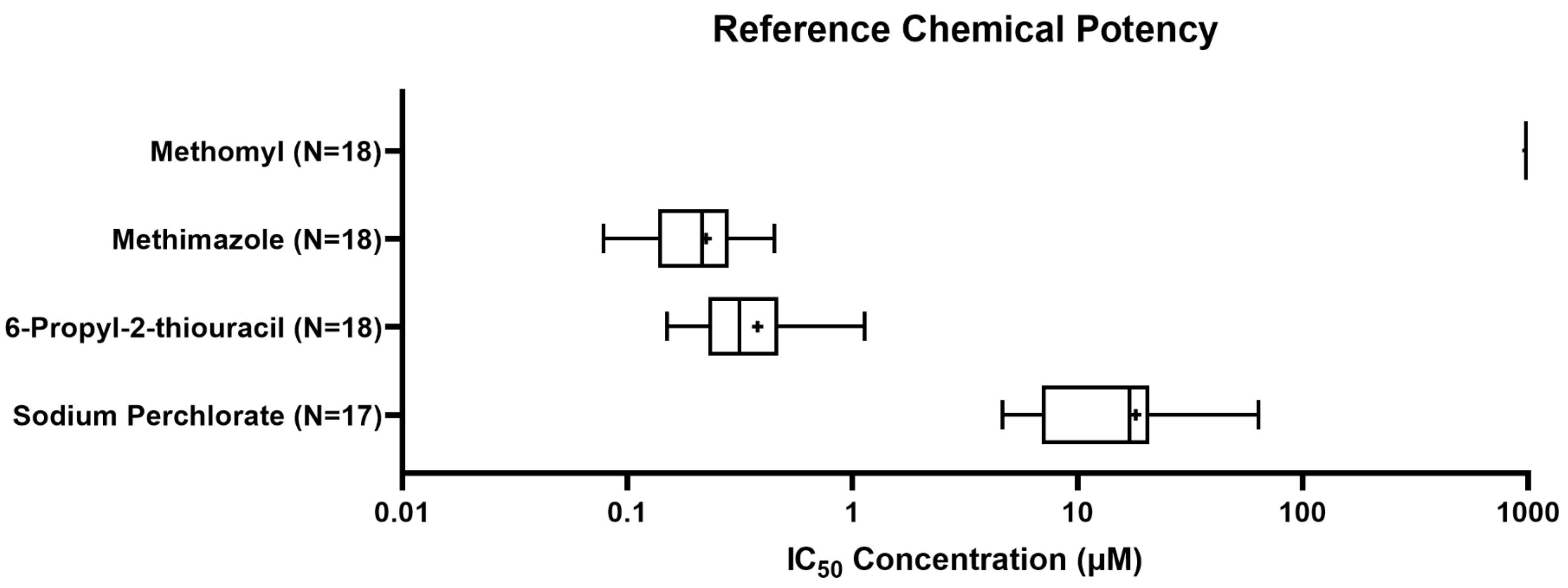
Reproducibility is Supported in a Variable-Donor Assay Platform with Qualified Donors



Independent Donor Performance Summary (*N* = 18)

	Methomyl		Methimazole		6-Propyl-2-thiouracil		Sodium Perchlorate	
	Best-fit values	CI (95%)	Best-fit values	CI (95%)	Best-fit values	CI (95%)	Best-fit values	CI (95%)
Bottom	97.9	65.1 to ???	7.3	3.6 to 10.8	8.4	4.7 to 11.9	13.3	-5.2 to 25.4
Top	Unstable	(Very wide)	101.8	97.8 to 106.5	99.3	95.9 to 102.9	107.1	103.6 to 110.6
Log IC50 (M)	-9.605	???	-6.6	-6.7 to -6.6	-6.4	-6.5 to -6.4	-4.8	-5.0 to -4.6
IC50 (µM)	**Unstable**	**???**	**0.23**	**0.18 to 0.28**	**0.36**	**0.30 to 0.42**	**15.3**	**10.9 to 23.6**

Evaluating Human Biological Variability to Benchmark Expectations in a Variable-Donor Assay Platform



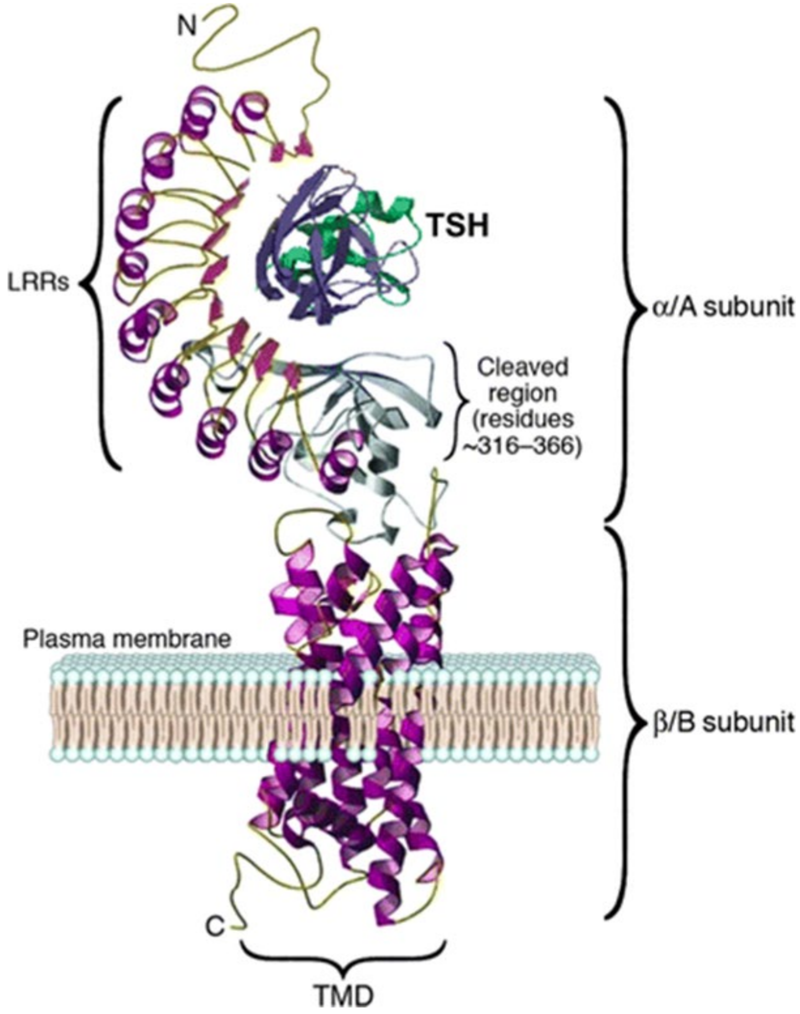
IC50 (µM)	Methomyl	Methimazole	6-Propyl-2-thiouracil	Sodium Perchlorate
Minimum	1000	0.08	0.15	4.6
25% Percentile	1000	0.14	0.23	6.9
Median	1000	0.22	0.32	17.0
75% Percentile	1000	0.28	0.47	20.7
Maximum	1000	0.45	1.13	63.7
Range	0	0.37	0.98	59.1

CI (95%)				
Lower confidence limit	**1000**	**0.14**	**0.23**	**7.1**
Upper confidence limit	**1000**	**0.28**	**0.46**	**19.5**

Outline

- Development of a human thyroid organotypic culture model to address data gaps in screening and prioritization of thyroid disrupting chemicals
- Establishing confidence with an inter-laboratory prevalidation study of the human thyroid microtissue assay
- Orthogonal screening of prioritized chemicals in human thyroid microtissues for functional and mechanistic relevance

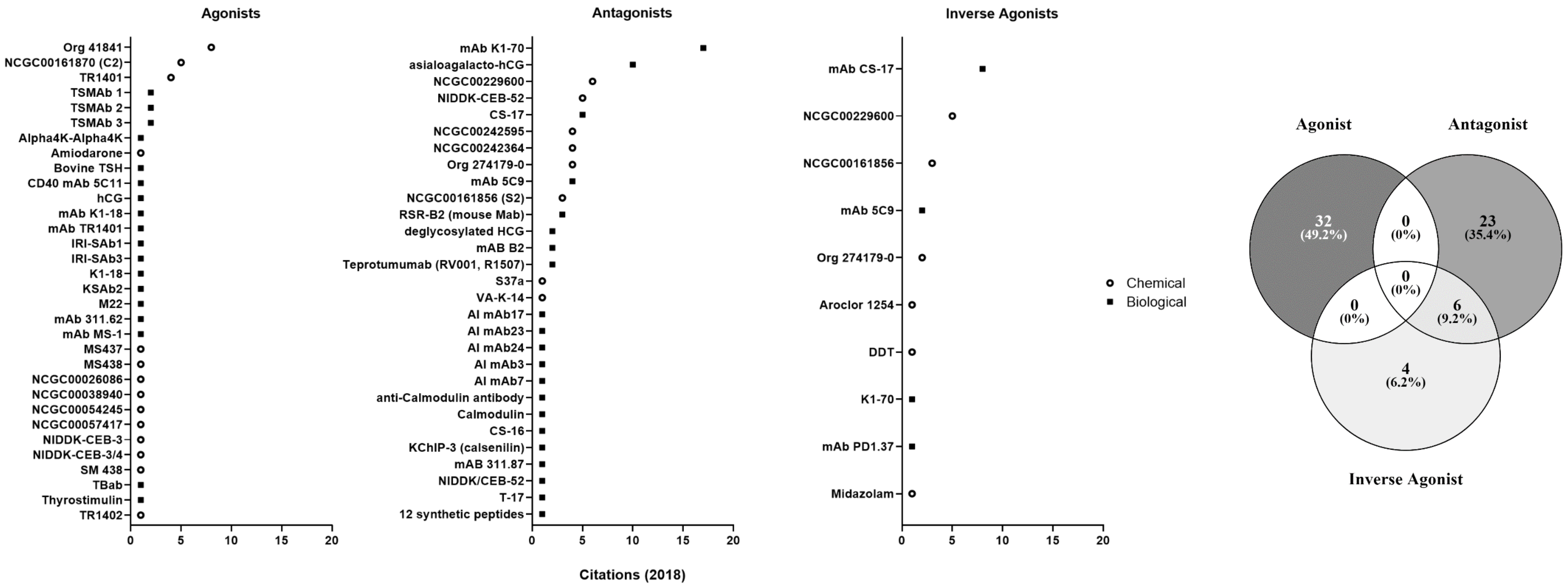
Is the Thyroid Stimulating Hormone Receptor (TSHR) a Target for Environmental Chemicals?



- TSHR is a G-protein-coupled receptor expressed primarily in thyrocytes.
- The primary ligand is Thyroid Stimulating Hormone (TSH).
- Biological and chemical modulators
  - TSH and TSHR autoimmune antibodies bind to the ectodomain (α subunit)
  - Small molecule ligands bind to the transmembrane domain (β subunit)
- Modulator classifications
  - Agonist – Activation from basal state
  - Antagonist – Inhibition of activated state
  - Inverse Agonist – Inhibition of basal state (constitutive activity)
- Toxicological outcomes
  - May contribute to hyperthyroidism (TSHR agonism) or hypothyroidism (TSHR antagonism) and associated adverse effects.

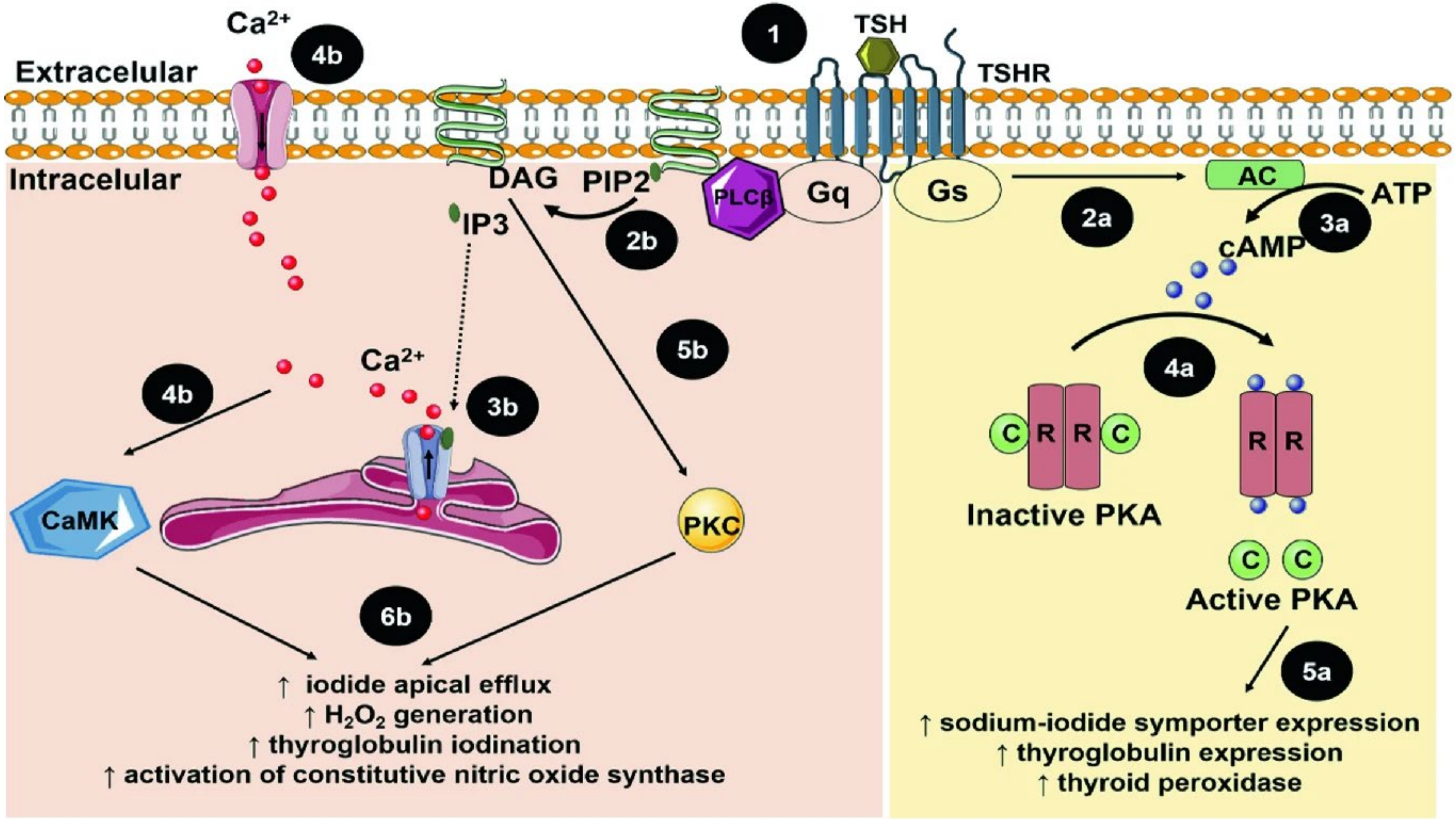
DOI: 10.1007/s12026-012-8312-8.

Literature Review – TSHR Modulator Landscape



	TSH Receptor Modulators			
	Agonist	Antagonist	Inverse Agonist	Total
Chemical	14	8	6	28
Biological	18	21	4	43

TSHR Signaling Pathways in Thyroid Follicular Epithelial Cells

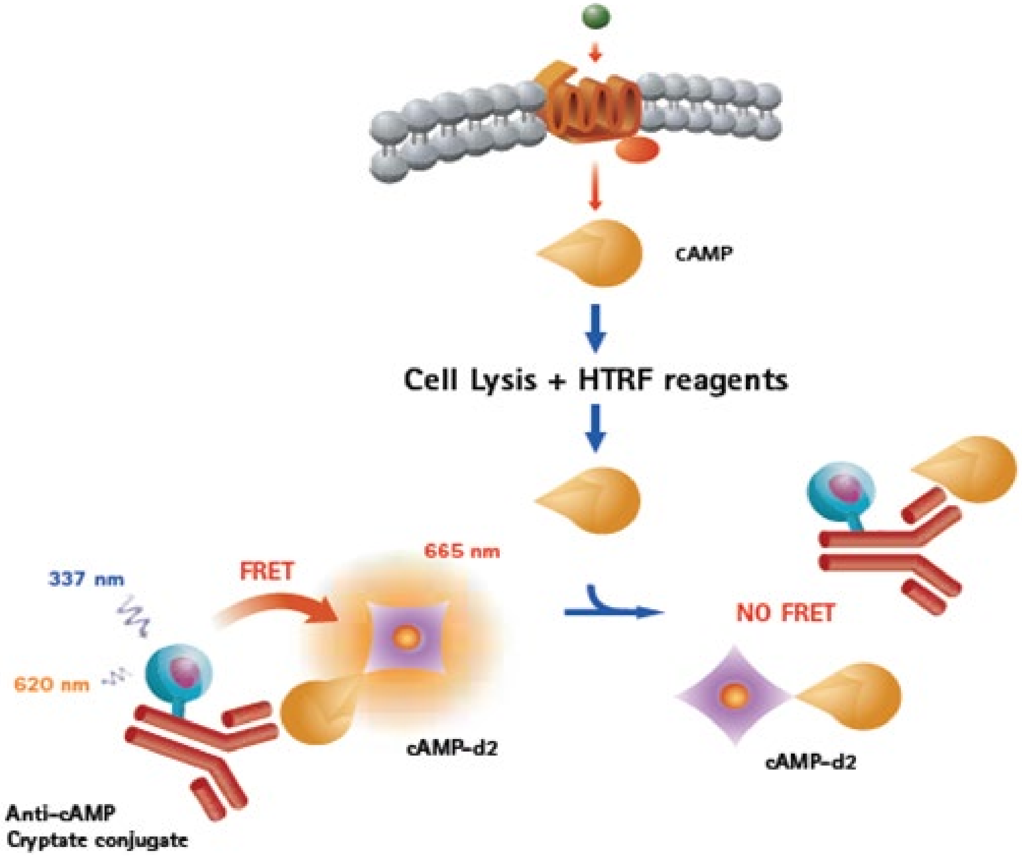


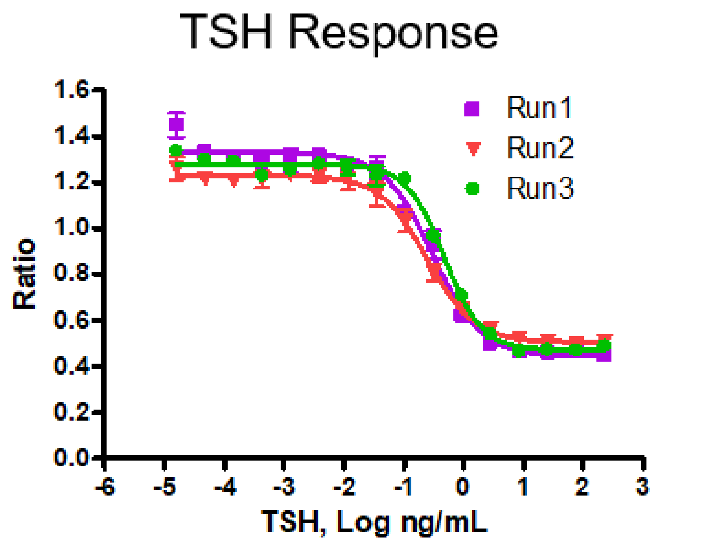
Non-Canonical

Canonical

DOI: 10.1007/978-3-030-44436-5_3.

Tox21 TSHR Assay – Screening the Tox21 Chemical Library





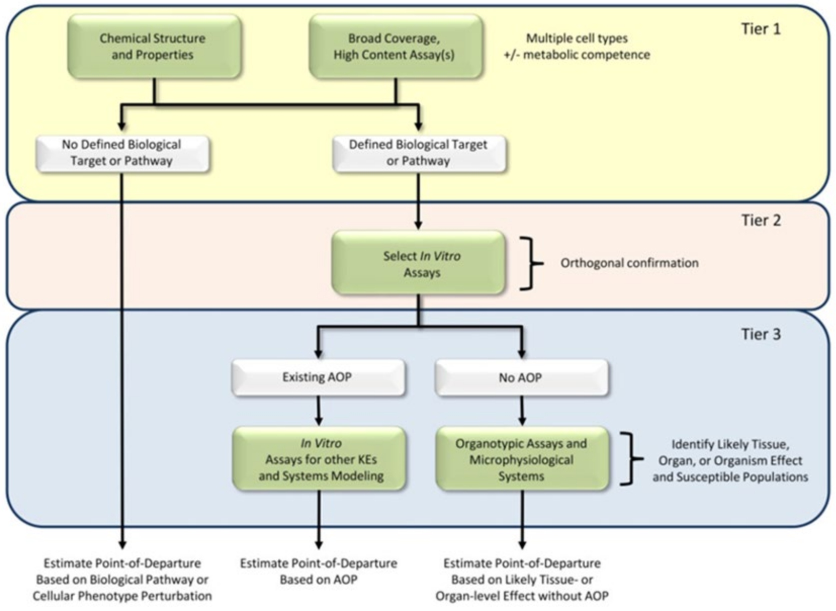
	Run1	Run2	Run3
HILLSLOPE	-1.069	-1.086	-1.368
EC50	0.3024	0.2461	0.4732

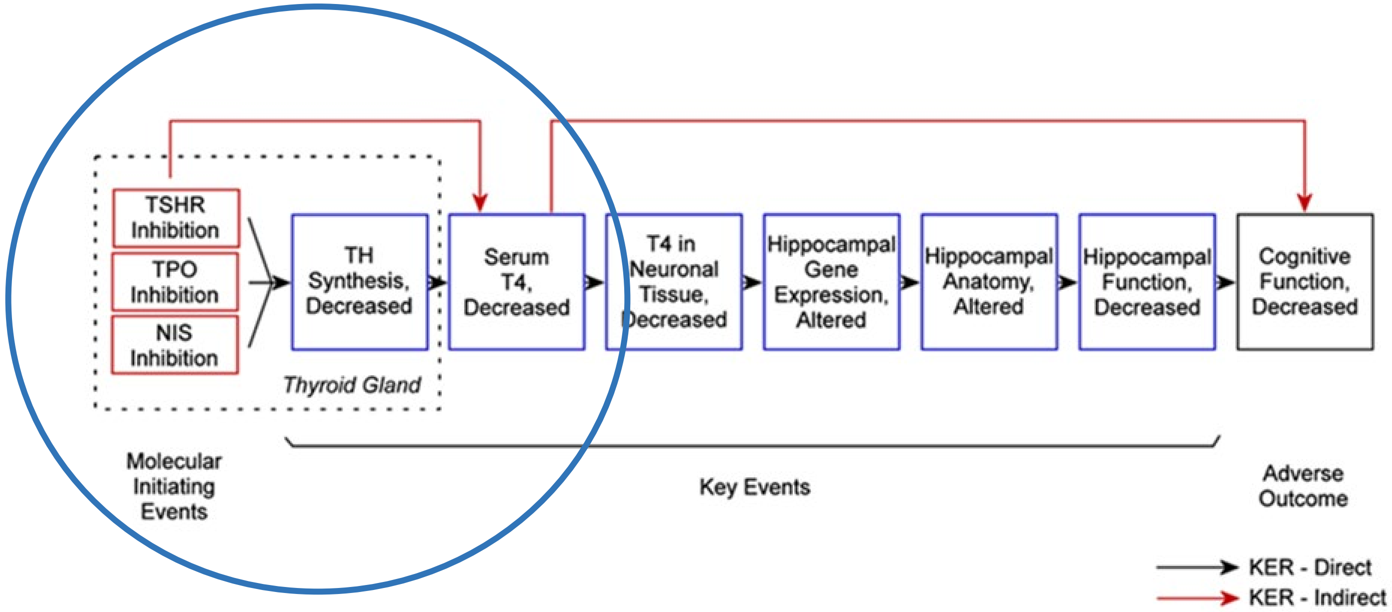
Bioactivity hit rate: 825 of 7871 chemicals (10%)

Assay	Cell Type	TSHR Expression	Test Chemical Exposure	Endpoint	Detection Technology
ACTOne-Gs TSHR GPCR HEK293	Human Embryonic Kidney Cell Line	Recombinant	30 min	cAMP	HTRF

https://www.cisbio.eu/

Tier 3 Screening of TSHR-Prioritized Chemicals in Human Thyrocyte Assays





TSHR Hit Prioritization Workflow	• Chemical selection based on bioactivity, structural diversity, HTS assay specificity and promiscuity
Orthogonal Screening for MIE Effects	• Confirmation of TSHR bioactivity in normal human thyrocytes using a native protein biomarker
Secondary Screening for Key Event Effects	• Extension of MIE perturbation (TSHR) to apical key event (T4 synthesis)

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Tox21 TSHR Assay – Active Chemicals Prioritization Workflow

Tox21_TSHR Assay

- TOX21_TSHR_Agonist_ratio - 489
- TOX21_TSHR_Antagonist_ratio - 336
- Dual Agonist/Antagonist - 49

Tox21 Screened Inventory: 7871 Chemicals

Quality Control

- DMSO solution availability
- Purity and degradation

Filters

- Remove mixtures, unavailable chemicals, flagged inventory, QC fails, dyes, and detergents
- Remove ToxPrints associated with fluorescence artifacts
- Remove promiscuous compounds

Selection Parameters

- High Bioactivity Signal
- Non-cytotoxic
- Structural Diversity

Prioritized Chemicals

	Chemicals	Agonist Mode	Antagonist Mode
Agonists	54	54	-
Agonist-Antagonist	8	8	8
Antagonist	46	-	46
Inactive	10	10	10
Total	**118**	**72**	**64**

Prioritized chemicals: 108 of 825 (~13%)

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TSHR Screening Workflow

Phase 1

Orthogonal Screening for MIE Effects

2D Primary Thyrocytes – Thyroglobulin Assay

Agonist Mode – 72 chems	Antagonist Mode – 64 chems

Phase 2

Secondary Screening for Key Event Effects

3D Thyroid Microtissues – T4 Assay

Agonist Mode – active chems	Antagonist Mode – active chems

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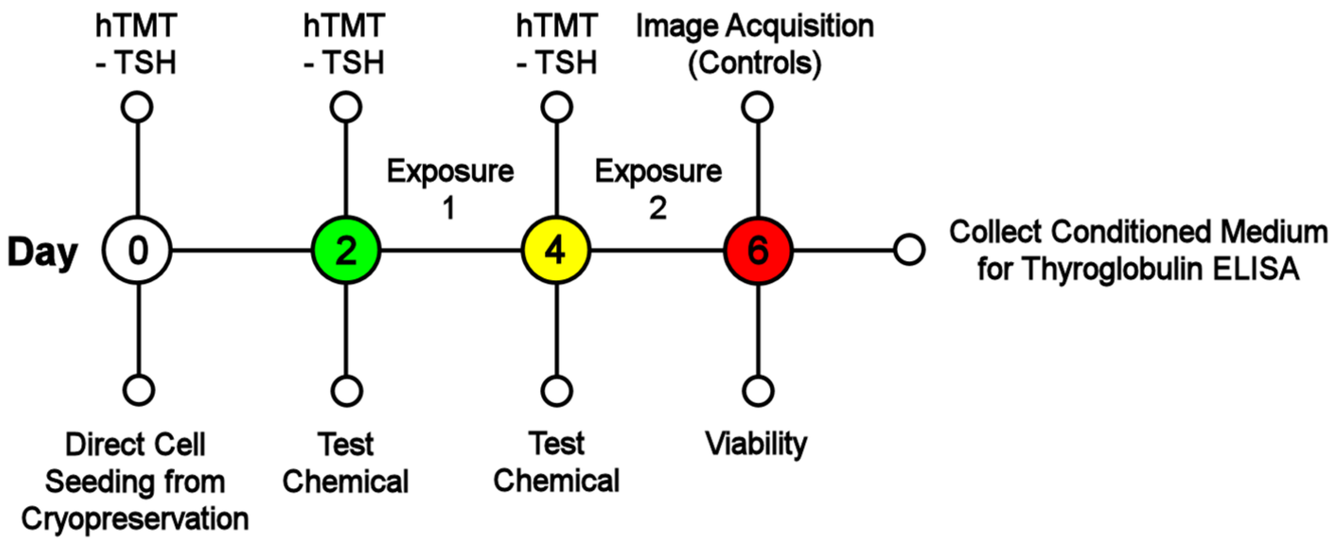
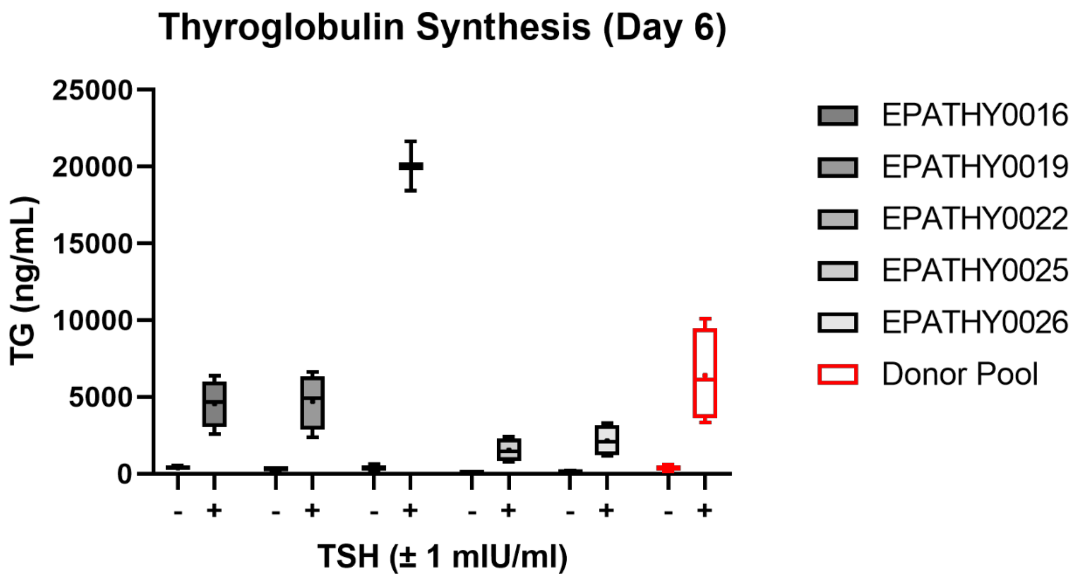
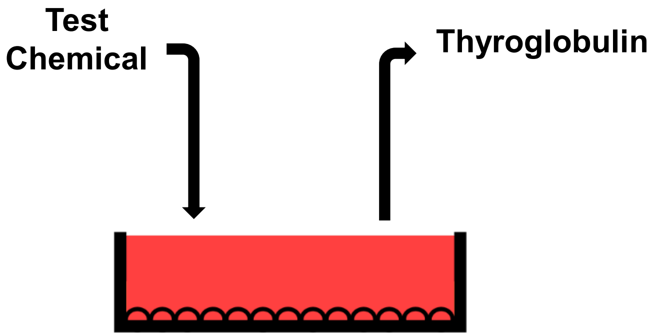
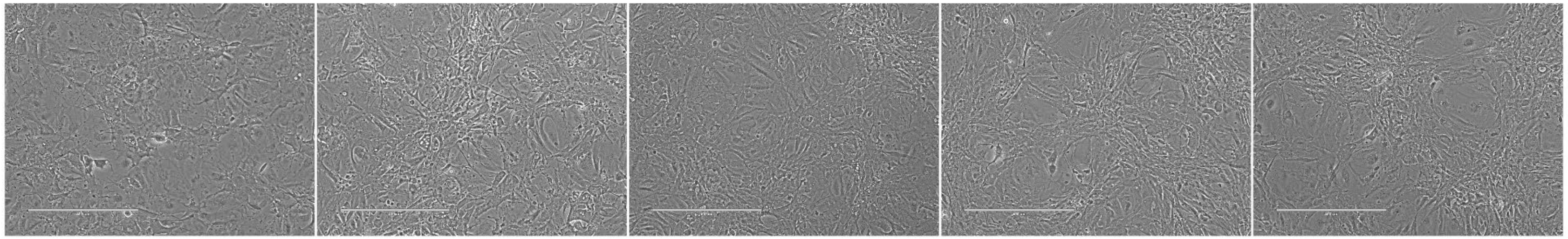
2D Thyroglobulin Assay (TSHR Agonist Variant) - Workflow



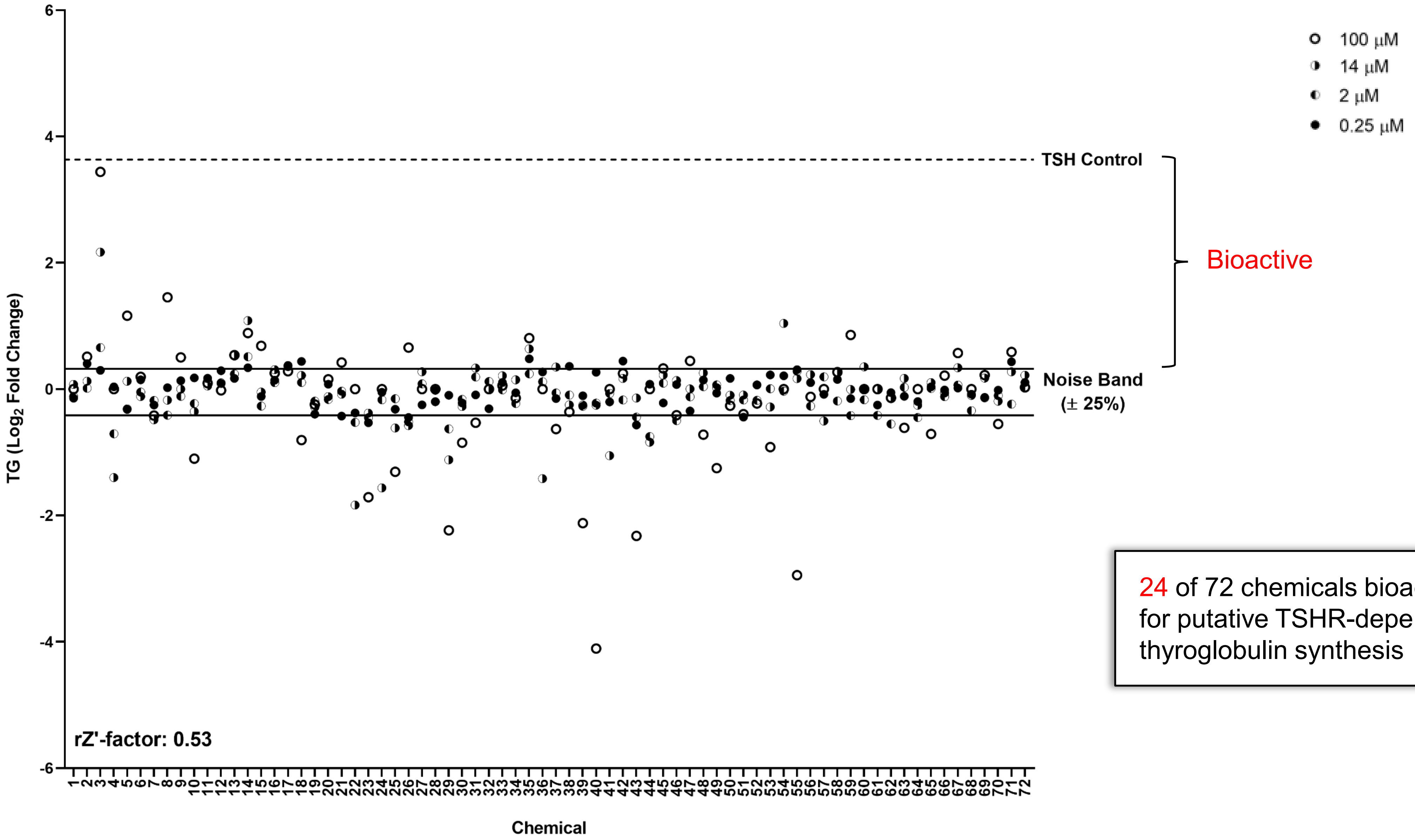




Donor ID	Sample	Age	Sex	Race	BMI	Euthyroid (Y/N)	Passage
EPATHY0016	Human Thyroid	23	M	Asian	36	Y	P0
EPATHY0019	Human Thyroid	20	M	Caucasian	28	Y	P0
EPATHY0022	Human Thyroid	34	F	African American	29	Y	P0
EPATHY0025	Human Thyroid	44	F	Caucasian	20	Y	P0
EPATHY0026	Human Thyroid	24	M	Hispanic	26	Y	P0


EPATHY0016 EPATHY0019 EPATHY0022 EPATHY0025 EPATHY0026

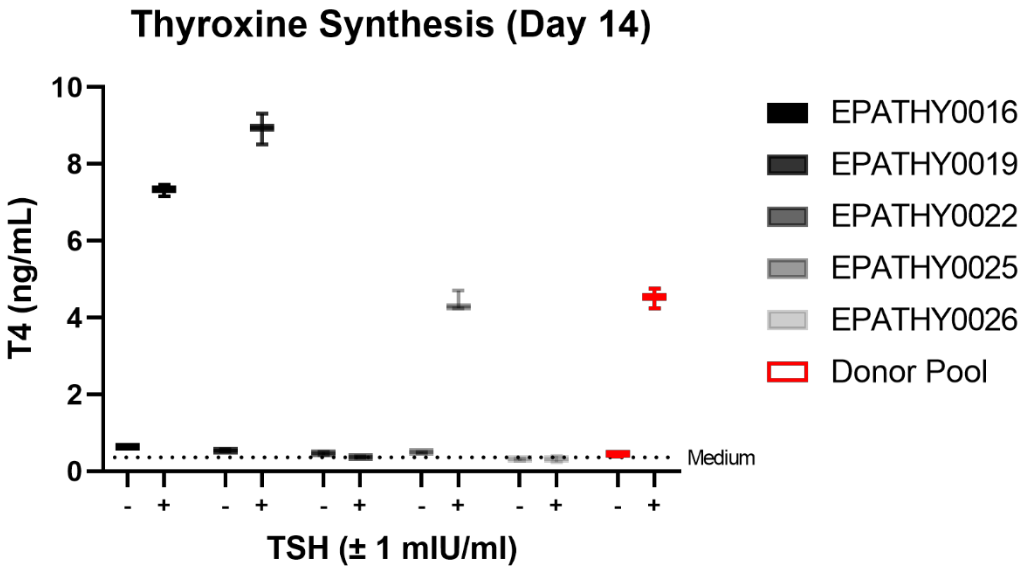
2D Thyroglobulin Assay - Screen Results

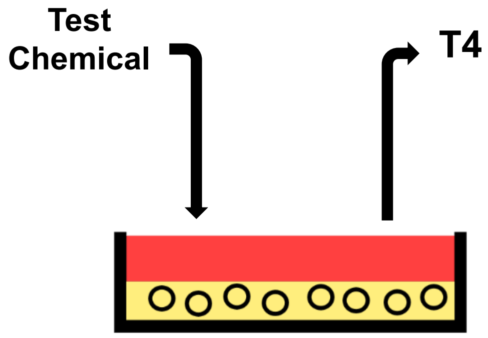


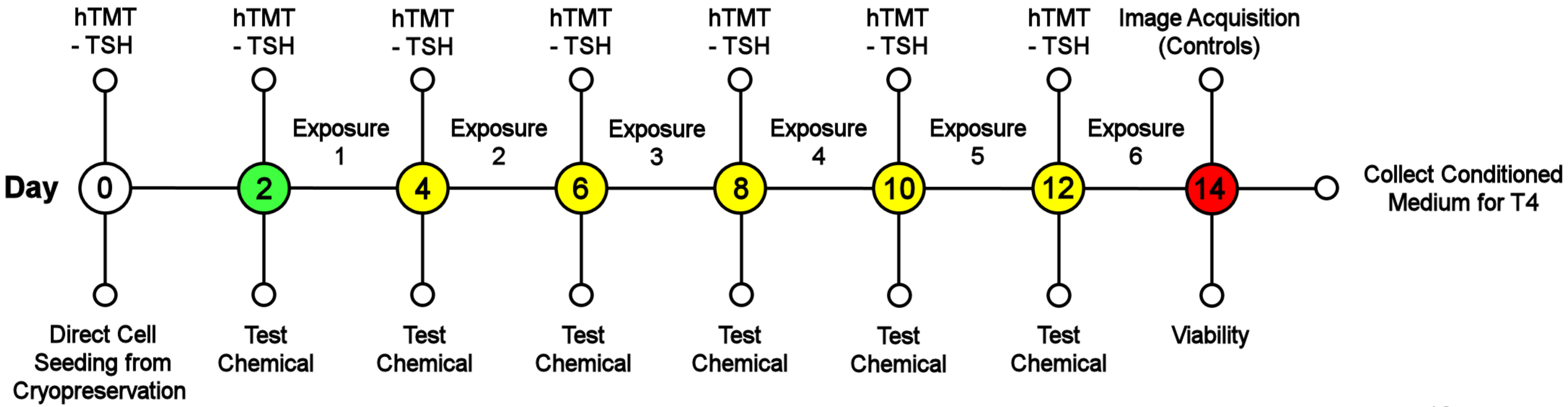
24 of 72 chemicals bioactive for putative TSHR-dependent thyroglobulin synthesis

3D Thyroid Microtissue Assay (TSHR Agonist Variant) - Workflow

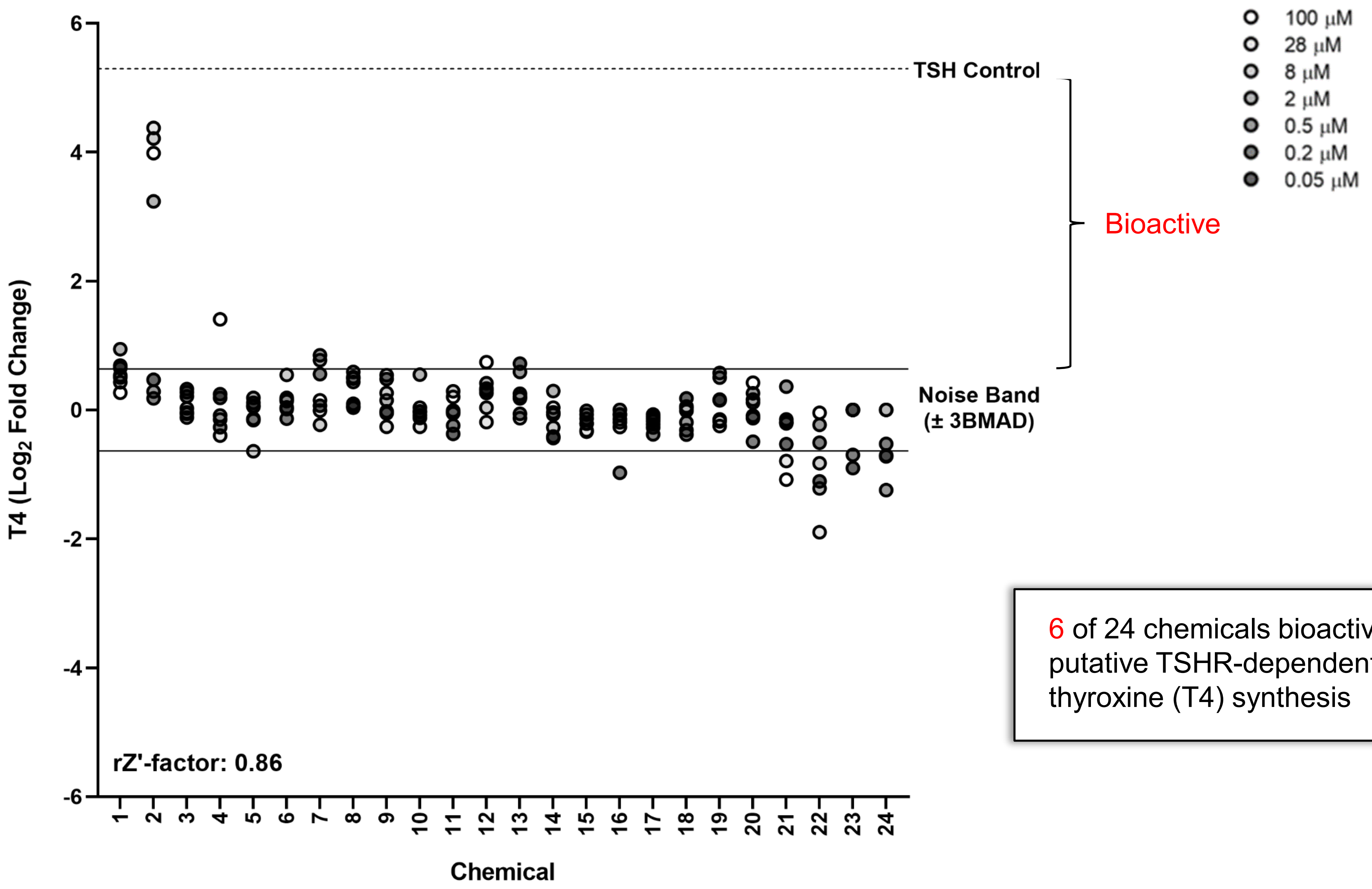
Donor ID	Sample	Age	Sex	Race	BMI	TG	T4
EPATHY0016	Human Thyroid	23	M	Asian	36	+	+
EPATHY0019	Human Thyroid	20	M	Caucasian	28	+	+
EPATHY0022	Human Thyroid	34	F	African American	29	+	-
EPATHY0025	Human Thyroid	44	F	Caucasian	20	+	+
EPATHY0026	Human Thyroid	24	M	Hispanic	26	+	-







3D Thyroid Microtissue Assay (TSHR Agonist Variant) – Screen Results



6 of 24 chemicals bioactive for putative TSHR-dependent thyroxine (T4) synthesis

3D Thyroid Microtissue Assay (TSHR Agonist Variant) – Representative Effects

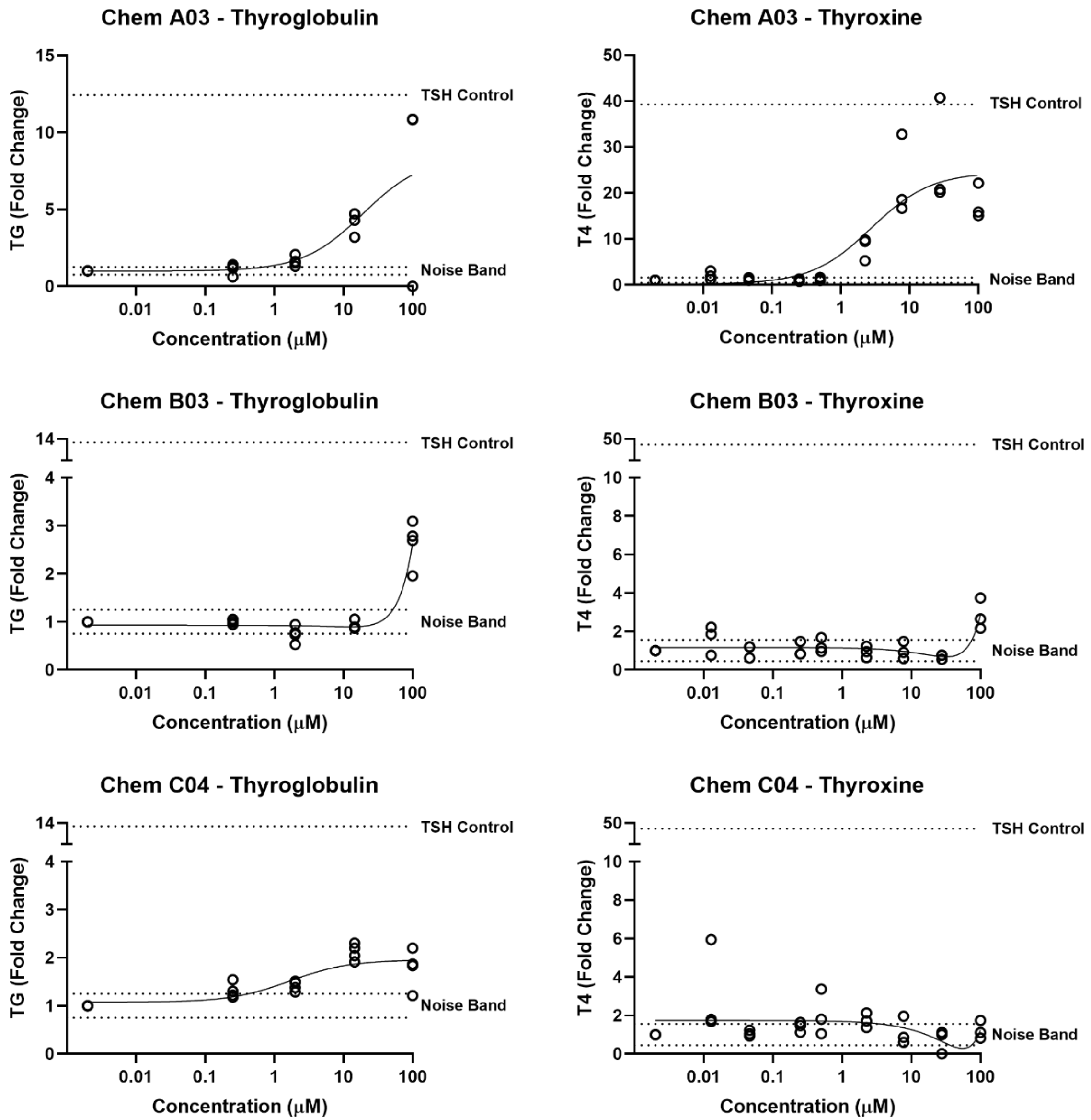
MIE

Key Event

Strong

Moderate

Weak

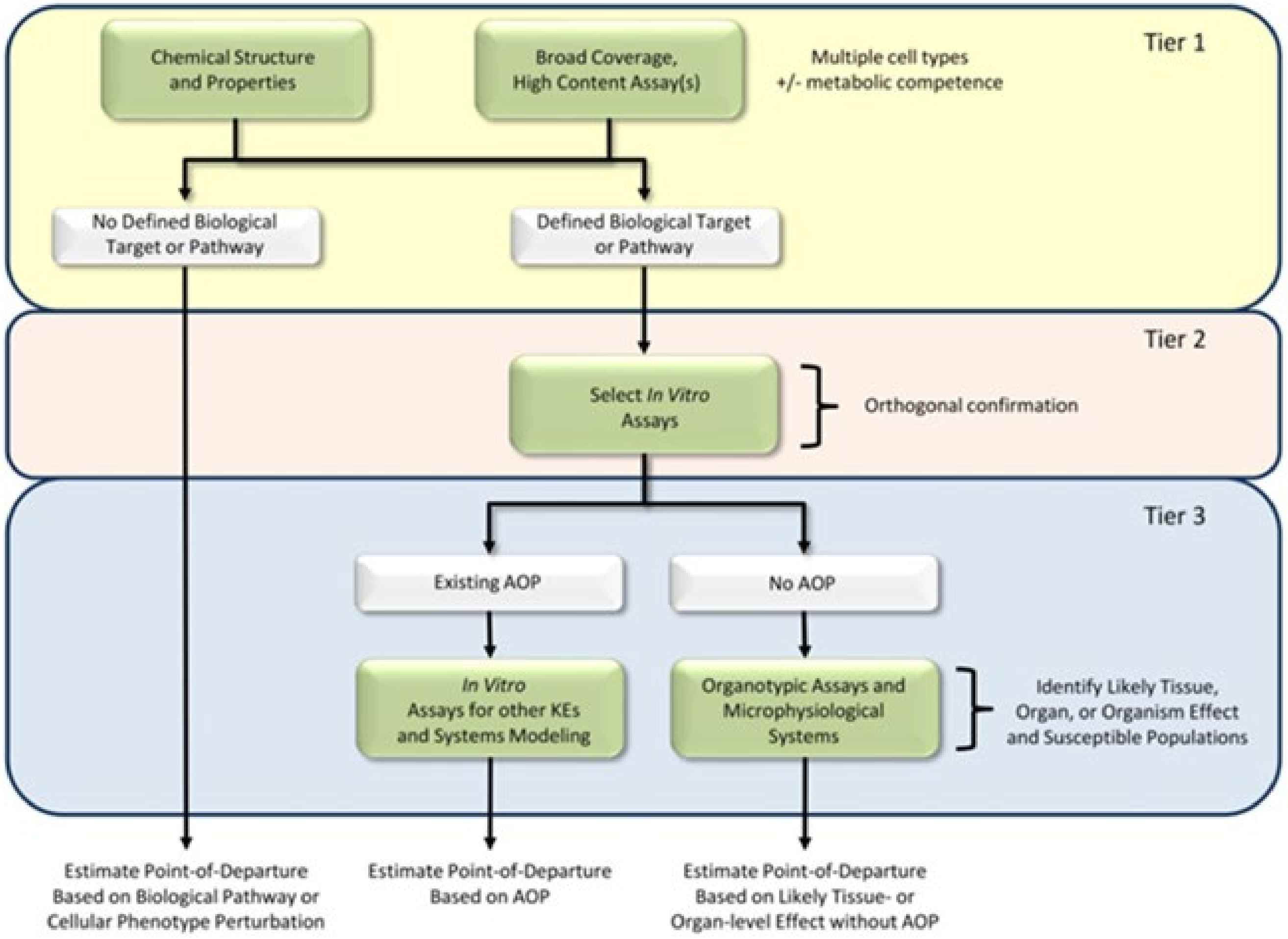


TSHR Hit Prioritization Workflow

Orthogonal Screening for MIE Effects

Secondary Screening for Key Event Effects

A Tiered Testing Paradigm to Identify Potential TSHR-dependent Human Thyroid Disruptors



Tox21 Screening Library	• 7871 Chemicals
Tier 2: TSHR Screening Assay Bioactivity	• 825 Chemicals
TSHR Hit Prioritization Workflow (Agonist)	• 72 Chemicals
Tier 3: Orthogonal Screening for MIE Effects	• 24 Chemicals
Tier 3: Secondary Screening for Key Event Effects	• 6 Chemicals

6 chemicals identified as potential human thyroid agonists

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Frederic Schorsch
Olivier Blanck

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