ARTICLE IN REVIEW:

US EPA Evaluation and Standardization of the Human Thyroid Microtissue Assay

SUMMARY: The US Environmental Protection Agency's (EPA's) mandate to reduce reliance on vertebrate animal testing has prompted it to identify, evaluate, and set performance standards for new approach methods (NAMs) capable of modeling human-relevant exposures and health outcomes *in vitro*. One such NAM, the human thyroid microtissue assay, as described previously,[2] utilizes primary human thyrocytes to replicate both structural and functional features of the human thyroid gland (uniquely including hormone production) to enable testing for potential thyroid-disrupting chemicals. As a variable-donor assay platform, it becomes important to balance any potential influence from technical variability against the assay's ability to predict a range of human responses. Thus, the objectives of this study were to establish minimum acceptance criteria for donor qualification, set benchmark ranges for reference chemical responses, and to evaluate the influence of donor-demographic variability on assay performance. Thyrocytes from 32 demographically-diverse research-consented euthyroid donors were cultured for 14 days in thyroid stimulating hormone (TSH)-free human thyroid microtissue (hTMT) medium within 3 treatment groups (without TSH [Tx-1], with TSH [Tx-2], and with TSH + anti-TSHR K1-70 antibody [Tx-3]). Of these, Tx-2 most closely simulated the biological state of the thyroid gland. Performance endpoints were biomass (measured as relative light units [RLUs] of adenosine triphosphate [ATP]); thyroglobulin, T4, and T3 (each measured using ELISA); and the calculated T4/T3 ratio. Finally, the influence of variability in donor demographic parameters (age, sex, race, and body mass index [BMI]) on each performance endpoint was modeled using multiple linear regressions. The results of this study suggested T3 and T4 levels as the most suitable basis for donor thyrocyte qualification and related selection criteria were established based on performance lower confidence limits (Table 1). Next, reference chemical responses successfully modeled hormone synthesis inhibition at significant human-relevant targets along multiple pathways (Figure 1). Finally, only donor age and BMI exhibited a significant inverse relationship with hormone synthesis and ideal cutoffs were modeled using the newly established qualification criteria.

T3 and T4 hormone levels were deemed the most suitable criteria for thyrocyte donor qualification

Its capacity to produce thyroid hormone is a unique and critical feature of the human thyroid microtissue assay, thus qualified donor thyrocytes should yield reliable levels. Related selection criteria were established from lower confidence limits in the Tx-2 group (Table 1), and a strong correlation between T3 and T4 levels (r = 0.88) suggested coordinated and proportional synthesis of these hormones across independent donors (data not shown). Ultimately, the T3 and T4 selection criteria provided the most ideal separation of qualified (n=24) vs non-qualified (n=8) donors.

Reference chemical responses indicated mechanistic inhibition of hormone synthesis at several "significant targets" along multiple pathways[1]
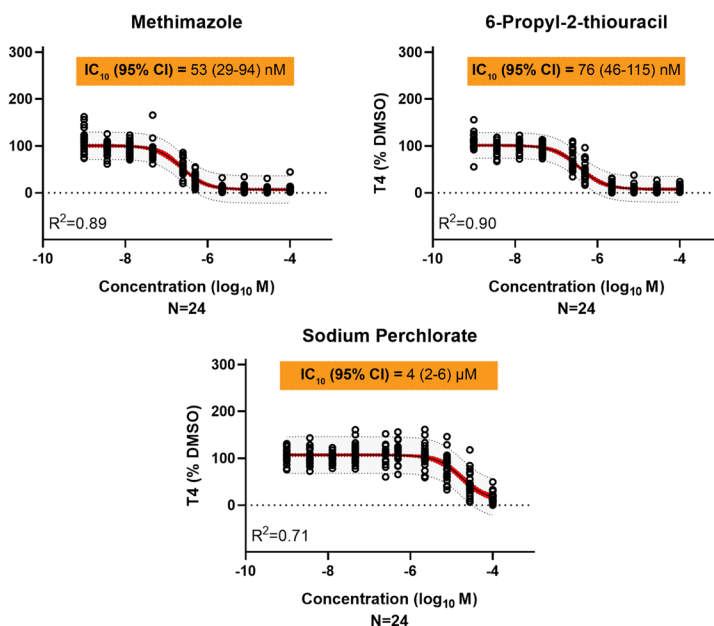
The human thyroid microtissue assay integrates known mechanisms of thyroid hormone synthesis into a single model (Figure 1), and thus "fills an important key event gap [in the] thyroid adverse outcome pathway network".[1] Notably, application of the donor selection criteria (Table 1) improved data modeling and decreased uncertainty in the chemical potency benchmark determinations (Figure 1).

Only donor age and BMI exhibited a significant inverse relationship with hormone synthesis

Thyrocytes derived from older donors, or those with a higher body mass, were less responsive to thyroid stimulating hormone (TSH) in terms of thyroglobulin synthesis and its downstream products, T3 and T4. Influences from donor sex or race were nonsignificant across all models. Modeling these data against the newly-established donor selection criteria (Table 1) suggested that donors aged ≤56 years and with BMI ≤37 kg/m² are more likely to exhibit reliable performance in the human thyroid microtissue assay.

References

1. 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; (E-pub ahead of print). https://doi.org/10.1093/toxsci/kfae014

2. Deisenroth C, Soldatow VY, Ford J, et al. Development of an In Vitro Human Thyroid Microtissue Model for Chemical Screening. Toxicol Sci. 2020; 174(1):63-78. https://doi.org/10.1093/toxsci/kfz238

Methimazole — IC_{10} (95% CI) = 53 (29-94) nM, R^2=0.89, N=24

6-Propyl-2-thiouracil — IC_{10} (95% CI) = 76 (46-115) nM, R^2=0.90, N=24

Sodium Perchlorate — IC_{10} (95% CI) = 4 (2-6) µM, R^2=0.71, N=24

Table 1. Donor Thyrocyte Qualification Parameters

	Biomass (RLU)	Thyroglobulin (ng/mL)	T4 (ng/mL)	T3 (ng/mL)	T4/T3 Ratio (ng/mL)
Median	230197	3405	3.03	4.94	0.57
Lower Confidence Limit (99%)	189321	1961	1.07	2.02	0.45
Qualification Criteria	≥180000	≥1900	≥1.0	≥2.0	≥0.4
Priority	Optional	Optional	Required	Recommended	Optional

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Figure 1 (left). Population-level reference chemical concentration-response modeling for the qualified (N=24) donor cohort. Values were normalized by donor and plotted in aggregate for modeling. The red band represents the 95% confidence interval of the model fit. The gray band indicates the 95% prediction interval where 95% of future donors would be expected to exhibit bioactivity. Figure reproduced from portions of Figures 6, 7, and 8 and data from Table 5 with permission under an open access license.[1]

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