

Development of a Novel Reporter Cell Line and a Standardized Protocol for the Detection of Thyroid Stimulating Immunoglobulin (TSI)

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Abstract

Introduction: Current methods for the detection of Thyroid Hormone Receptor Autoantibodies (TRAbs), such as the KRONUS TSH Receptor Autoantibody Coated-Tube RIA Kit, do not distinguish between TSI and other TRAbs that bind but do not stimulate. TSI are of particular importance because of their role in Grave's hyperthyroidism, where they mimic the function of thyroid stimulating hormone. The development of a cell line and standardized protocol that could be used to specifically detect and differentiate TSI from other TRAbs would be a powerful tool to assist physicians in the diagnosis of Grave's disease. **Methods:** This novel cell line was developed by stably transfecting Chinese hamster ovary cells with a human thyrotropin/rat luteinizing hormone (LH) chimeric receptor and a firefly luciferase reporter gene. Selection of the clone was based on luciferase expression in response to TSI and results from cell passage stability data. A standardized assay protocol was developed based on results from analytical performance studies which included precision and reproducibility studies. **Results:** Stability studies showed that the cell line retained a constant level of luciferase activity in response to TSI over twenty-six passages regardless of the presence or absence of selection media. Results from our cross-reactivity and interference studies showed that that follicle stimulating hormone, LH, and other hormones did not interfere or cross-react at clinically relevant levels. A reproducibility study was conducted at 3 sites between 4 technicians in order to ascertain the coefficient of variation (CV) for a panel of specimens containing various levels of TSI. The average CV was shown to be 20.8%. In a precision study involving 1 technician who performed the assay over 20 days, the average CV of a panel of specimens was 12%. The experimental CHO cell line was compared to the KRONUS TSH Receptor Autoantibody Coated-Tube RIA Kit using a series of clinical specimens. This set of clinical specimens showed an overall agreement between the two methods of 94.6%. **Conclusions:** Our results show that this cell based reporter system is stimulated by TSI, is stable, and performs significantly similar to an FDA cleared predicate device.

Introduction

Hyperthyroidism is a disorder characterized by the excessive synthesis and secretion of thyroid hormones. In the majority of cases this overproduction of hormones is caused by a class of autoantibodies to the thyroid stimulating hormone receptor (TSHR) which mimic the action of thyroid stimulating hormone (TSH). These stimulating autoantibodies are referred to as thyroid stimulating immunoglobulins (TSI) and, when present, are indicative of Graves' disease (GD). GD has an incidence of approximately 5 in 10,000 people per year, affecting 13 million in the United States, and targeting women seven times as often as men¹. Despite these statistics, there was no diagnostic test for the specific detection of TSI until the development of Diagnostic Hybrids product Thyretain™. Thyretain™ is intended for the qualitative detection and measurement in serum of thyroid stimulating autoantibodies to TSHRs on the thyroid.

In previous studies, we described the development of a novel cell line used for the detection of TSI². In the studies described here we highlight the development of the Thyretain™ standardized protocol which uses our novel cell line to specifically detect TSI. The measurement of these stimulating autoantibodies may be useful in the differential diagnosis of patients with GD.

Methods – Development of the Thyretain™ Standardized Protocol

On day one each 96 well plate is treated with Cell Attachment Solution followed by inoculation with CHO Mc4 FreshFrozenCells®. Plates are then incubated for 15 to 18 hours in a humidified, 5% CO₂, 35° to 37° C incubator. Plates are removed from the incubator and inspected to ensure that the monolayer is confluent and appears healthy. Medium is removed from the wells and the cells are rinsed with pre-warmed (37°C) Reaction Buffer followed by the addition of 100µL Reaction Buffer to each well. Specimens and controls are then prepared by adding 1 part specimen or control to 10 parts Reaction Buffer. 100µL of each specimen or control is added to the appropriate wells in triplicate and the plate is placed in a humidified, 5% CO₂, 35° to 37° C incubator for 3 hours. Following the incubation period, all liquid is decanted from the plate followed by blotting on an absorbent pad to remove excess liquid. 75µL of Luciferase Substrate (containing lysis reagents) is added to each well. After a 10 minute incubation at room temperature, the plate is read on a multi-well plate luminometer. The turn around time for the assay is less than 24 hours.

Thyretain™ Assay Principle

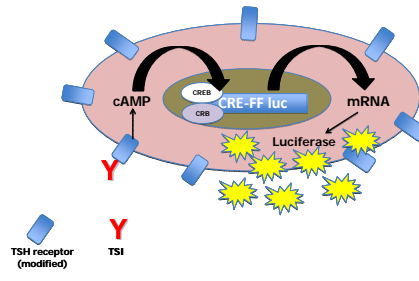


Fig 1: **Thyretain™ Assay Principle.** The binding of TSI to a chimeric TSH receptor on the surface of genetically modified Chinese hamster ovary cells induces a signaling cascade that begins with an increase in intracellular cAMP, and results in luciferase expression.

Interference and Cross-Reactivity

Hormone	WHO - NIBSC Code	Starting Concentration (mIU/mL)	Ending Concentration (mIU/mL)	Normal Physiological Range in Healthy Adults Concentration (mIU/mL)	Concentration at which Cross-reactivity Occurs (mIU/mL)
Luteinizing Hormone (LH)	80/552	10,000	2.4	5 to 20	625
Human Chorionic Gonadotropin (hCG)	75/589	650,000	158.7	0.1 to 8,000	40,625
Follicle Stimulating Hormone (FSH)	83/575	8,000	3.9	1.4 to 116.3	2,000
Thyroid Stimulating Hormone (TSH)	03/192	9,500	0.000039	0.0003 to 0.0030	≥ 0.35

Fig 2: **Interference and Cross-Reactivity.** To address cross-reactivity and interference, serial dilutions of the hormones were made by spiking each into normal and positive serum respectively followed by testing in the Thyretain™ assay. Results showed that there was no interference or cross-reactivity at clinically relevant levels for the hormones tested.

Precision

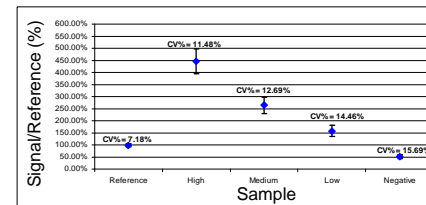


Fig 3: **Precision.** A manufactured panel of samples with varying amounts of TSI was analyzed by a single technician over 20 days. Results were reported as the ratio of the luminescence of the sample to that of the pooled normal. Results showed that the CV% values ranged from 7.18% for the reference control to 15.69% for the TSI negative specimen.

Reproducibility

	Tech 1 (Site 1)		Tech 2 (Site 2)		Tech 3 (Site 3)		Tech 4 (Site 4)	
	SRR%	CV%	SRR%	CV%	SRR%	CV%	SRR%	CV%
Sample A	270.1%	11.5%	439.9%	10.5%	280.1%	13.6%	289.5%	10.6%
Sample B	293.1%	14.3%	495.1%	14.6%	374.3%	14.7%	345.1%	11.8%
Sample C	48.2%	14.9%	67.9%	14.7%	44.3%	20.2%	48.6%	22.5%
Sample D	158.6%	10.6%	198.0%	11.0%	143.6%	14.4%	142.7%	12.1%

Fig 4: **Reproducibility.** A manufactured panel of samples with varying amounts of TSI were analyzed by four technicians at three different sites. Samples were analyzed multiple times per plate twice a day over an eight day period for a total n of 180 for the entire experiment. Results are reported as a sample to reference ratio (SRR%) luminescence. Results showed that between the sites and users Samples A, B, C, and D had average CV% values of 23.7, 23.7, 24.6, and 17.9% respectively.

Stability of Cell Clone

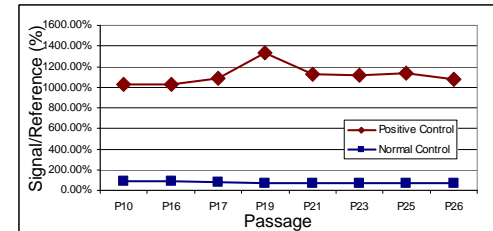


Fig 5: **Stability of Selected Clone.** Cells were maintained for 26 passages in growth media without selection. Aliquots of cells from each passage were frozen to make working stocks for the purpose of this study, and also to create stocks for the next passage. For the study shown here, a frozen stock was thawed, dispensed into 96 well plates, grown for 24 hours, starved for 24 hours, and induced with the positive, normal, and pooled normal serum prior to lysis and luminescent analysis. Results were reported as the ratio of the positive or normal luminescence to the pooled normal luminescence. Results showed that the selected clone performed consistently over 26 passages.

Comparison to Predicate Device

		KRONUS CT TRAb	
		+	-
Thyretain™ TSI Reporter BioAssay	+	44	2
	-	11	184
Positive Percent Agreement		80.0%	
Negative Percent Agreement		98.9%	

Fig 6: **Comparison to Predicate Device.** Clinical specimens (241) were analyzed in the Thyretain™ TSI Reporter Bioassay and the KRONUS CT RIA assay. Results showed an 80.0% Positive Percent Agreement (PPA), 98.9% Negative Percent Agreement (NPA), and an overall agreement of 94.6%.

Conclusions

Our results show that this cell based reporter system is stimulated by TSI, is stable, and performs in a substantially equivalent manner to a predicate device. Discrepancies between the KRONUS CT RIA and the Thyretain™ assay, reflected in the PPA, can, in part, be explained by the presence of "blocking" antibodies. Since the KRONUS CT RIA and other non-specific TRAb based tests bind all TRAbs, a positive result from these tests can be due to the presence of blocking antibodies.