



Thyretain[®]

TSI Reporter BioAssay



INTENDED USE

Thyretain TSI Reporter BioAssay is intended for the qualitative detection in serum of thyroid stimulating autoantibodies to the thyroid stimulating hormone (TSH) receptors (TSHRs) on the thyroid. The detection of these stimulating autoantibodies, in conjunction with other clinical and laboratory findings, may be useful as an aid in the differential diagnosis of patients with Graves' disease (GD).

SUMMARY AND EXPLANATION OF THE TEST

The synthesis and secretion of thyroid hormone by the thyroid gland is controlled by thyroid stimulating hormone (TSH), also called thyrotropin. TSH, secreted by the anterior pituitary, binds to the thyroid stimulating hormone receptors (TSHR), or thyrotropin receptors (TR), on the cells of the thyroid gland stimulating the synthesis and secretion of thyroid hormones.

Hyperthyroidism is characterized by an excessive synthesis and secretion of thyroid hormones. In the majority of cases this overproduction of hormones is caused by a class of autoantibodies to TSHR which mimic the action of TSH. These stimulating autoantibodies are referred to as thyroid stimulating immunoglobulins (TSI) and, when present, are indicative of GD.

In a normal functioning system, homeostasis is maintained by the hypothalamic-pituitary-thyroid axis. The hypothalamus senses low circulating levels of thyroid hormone and responds by releasing thyrotropin releasing hormone (TRH). The TRH stimulates the pituitary to produce and secrete TSH. The TSH, in turn, stimulates the thyroid to produce and release thyroid hormone until levels in the blood return to normal. Thyroid hormone exerts negative feed-back control over the hypothalamus as well as the anterior pituitary thus controlling the release of both TRH from hypothalamus and TSH from anterior pituitary gland. The two major hormones produced by the thyroid are thyroxine (T_4) and triiodothyronine (T_3). T_3 is formed through deiodination of T_4 and is the most active of the thyroid hormones, regulating most bodily processes. The TSI present in patients with GD mimic TSH causing an over-production of both hormones, leading to hyperthyroidism.

GD, one of the most common forms of hyperthyroidism, has an incidence of approximately 5 in 10,000 people per year, affecting 13 million, and targets women seven times as often as men.¹ Although there is currently no cure for GD, it is treatable by anti-thyroid drug therapies, radioactive iodine ablation or surgical removal of the thyroid gland, as cited by American Association of Clinical Endocrinologist guidelines.² Though the presence of TSI in serum of patients known to have GD is significant to the disease, the direct screening for this autoantibody has not been used as a primary tool in its diagnosis. The diagnosis of GD is typically derived from a panel of diagnostic tests, which includes the measurement of serum levels of TSH, T_3 , T_4 , and thyroid receptor antibodies (TRAb). There are two types of TRAb, however, TSI and Thyroid Blocking Immunoglobulins (TBI). The TBI binds to the TSHR and prevents or inhibits the stimulation and secretion of thyroid hormones by TSH, leading to hypofunctioning of the thyroid or hypothyroidism. The measurement of serum TRAb is flawed by its inability to distinguish TSI from TBI.

Thyretain TSI Reporter BioAssay (TSI Reporter) is a cell-based assay (or “bioassay”) which utilizes a genetically engineered cell line capable of specifically detecting serum TSI.

PRINCIPLE OF THE PROCEDURE

The Thyretain TSI Reporter BioAssay (TSI Reporter) utilizes a patented bioassay technology to detect TSI in human serum. Genetically engineered Chinese hamster ovary (CHO) cells, expressing a chimeric form³ of the human TSHR and a cyclic adenosine monophosphate (cAMP) induced luciferase reporter gene, are cryogenically preserved and provided in measured aliquots. The cells are seeded and grown for 15 to 18 hours to a confluent monolayer in a 96-well plate.

Patient sera, reference control, positive and normal controls are diluted with a proprietary Reaction Buffer, added to the cell monolayers and allowed to react with the cells for 3 hours. During this induction period, TSI, if present in the patient serum, bind to the chimeric human TSHR on the cell surface. This binding event induces a signaling cascade resulting in increased production of intra-cellular cAMP. This increased production of cAMP is evidenced by increased production of luciferase. At the conclusion of the 3-hour induction period the cells are lysed. Luciferase levels are then measured using a luminometer. A significant increase in luminescence over the Reference Control indicates the presence of TSI antibodies in the sample.

REAGENTS AND MATERIALS PROVIDED

Thyretain TSI Reporter BioAssay kit contains the following:

Cat. #40-25000.v2/40-50000.v2

- **CHO Mc4 FreshFrozenCells®**, 1.0 mL: Cryovials containing CHO Mc4 cells cryogenically preserved in cryoprotective medium containing DMSO. Reagent is stored at –70°C or lower.
- **Cell Attachment Solution (CAS)**, 200 mL: A proprietary reagent that promotes rapid cell attachment is used to treat the wells of a 96-well plate prior to planting the cells. Reagent is stored at 2°C to 30°C .
- **Growth Medium (GM)**, 200 mL: Hamm’s F-12 cell culture medium containing 10% FBS. Reagent is stored at 2°C to 8°C .
- **Reaction Buffer (RB)**, 500 mL: A proprietary buffer that augments the reaction of TSI with the TSHR. Reagent is stored at 2°C to 8°C.
- **Control Set:**
 - ▶ **Positive Control**, 0.5 mL: TSI-containing human serum which yields a value that is ≥140% of the Reference Control. Reagent is stored at –70°C or lower.
 - ▶ **Reference Control**, 0.5 mL: A bTSH-containing solution against which controls and test specimens are compared. Reagent is stored at –70°C or lower.
 - ▶ **Normal Control**, 0.5 mL: Human serum that is negative for the presence of TSI which yields a value that is <140% of the Reference Control. Reagent is stored at –70°C or lower.
- **Luciferase Assay Reagent Set:**
 - ▶ **Luciferase Substrate**, 1 vial: A lyophilized beetle luciferin substrate which is converted by luciferase to oxyluciferin and light. Reagent is stored at –20°C or lower.
 - ▶ **Luciferase Assay Buffer Solution**, 1 vial, 10 mL: A cell culture lysis buffer.

MATERIALS REQUIRED BUT NOT PROVIDED

- –70°C or lower freezer or liquid nitrogen Dewar
 - NOTE: A chest freezer is preferred over an upright freezer. Assay failure is a potential issue due to repeated exposure of the cells to temperature changes over time when an upright freezer is used.**
- Humidified, 5% CO₂, 35°C to 37°C Incubator
- Bio-safety Cabinet Class II
- Luminometer capable of reading a 96 multi-well plate (MWP)
- Luminometer calibrator plate
- Microscope, inverted
- Calibrated Pipettes
 - ▶ Multi-Channel 20 to 200 µL
 - ▶ Single 20 to 200 µL
 - ▶ Single 100 to 1000 µL
 - ▶ Sterile Pipette Tips
 - ▶ 5, 10, 25mL Sterile Serological Pipettes
- Sterile Transfer Pipette
- Pipetman® variable volume pipetters
- 96 MWP, Black, Clear Bottom (Costar #3603)
- Sterile Reagent Reservoirs
- Sterile Absorbent Pad
- Water bath, 35°C to 37°C
- Sterile screw-cap tubes (15- or 50-mL)
- 13-mm Test Tubes
- Vortex Mixer
- Timer
- Household Bleach

REAGENT STORAGE INSTRUCTIONS

CHO Mc4 FreshFrozenCells	Store at –70°C or lower
Control Set (Positive, Reference, and Normal)	
Cell Attachment Solution NOTE: The solution may show precipitation if stored at refrigerated temperatures. The material may be dissolved when the solution is warmed in a 35°C to 37°C water bath. It is recommended that this solution be stored at room temperature to avoid precipitation	Store at 2°C to 30°C.
Growth Medium	Store at 2°C to 8°C
Reaction Buffer	
Luciferase Assay Reagent Set	Store at –20°C or lower

STABILITY

Reagents and components will retain their full potency through the expiration date shown on the label of each bottle when stored at recommended temperatures.

WARNINGS AND PRECAUTIONS

- For *in vitro* diagnostic use
- This Kit contains materials of human (e.g., human serum) and bovine (e.g., bTSH) origin. All bovine materials have been certified to be of United States origin. All human serum controls have been tested for

HBsAg, HIV-1, -2 and HCV antibodies and found to be negative. Despite this screening, all human serum controls and patient samples should be considered potentially hazardous and handled with extreme care.

- No known test method can offer complete assurance that infectious agents are absent; therefore, all human blood derivatives, reagents and human specimens should be handled as if capable of transmitting infectious disease. It is recommended that reagents and human specimens should be handled in accordance with the OSHA Standard on Bloodborne Pathogens.⁴
 - ▶ Cell cultures may have some potential to be hazardous. Personnel working with these cultures must be properly trained in safe handling techniques⁵ and have experience with tissue culture before attempting this procedure.
 - ▶ All procedures must be conducted in accordance with the CDC 5th edition Biosafety in Microbiological and Biomedical Laboratories, 2007, and CLSI Approved Guideline M29-A, Protection of Laboratory Workers from Instrument Biohazards and Infectious Disease Transmitted by Blood, Body Fluids, and Tissue.
- All specimens and materials used to process them should be considered potentially infectious and handled in a manner which prevents infection of laboratory personnel.
 - ▶ Biosafety Level 2 or other appropriate biosafety practices should be used when handling these materials.
 - ▶ Decontamination of specimens and cultures is most effectively accomplished using a 1:10 dilution of household bleach.
 - ▶ Although Control reagents have been shown to be non-infectious (Positive and Normal Controls) and of United States origin (Reference Control), the same precautions taken in handling and disposing of other infectious materials should be employed in their use.
- Never pipette reagents or clinical samples by mouth; avoid all contact of clinical samples with broken skin.
- Avoid splashing and the generation of aerosols with clinical samples.
- Use aseptic technique and sterile equipment and materials for all cell culture procedures.
- The **CHO Mc4 FreshFrozenCells** must be properly stored (–70° C or below) at all times to maintain optimum performance. The swift transfer of freezer vials to and from the freezer or liquid nitrogen storage is mandatory. **Repeated exposure to temperature fluctuations may affect cell viability and/or assay performance.**
- The **CHO Mc4 FreshFrozenCells** are single-use only and cannot be re-frozen once thawed.
- Extreme care should be taken to ensure that the level of CO₂ in the incubator is accurately calibrated to 5%. Prolonged exposure to excessively high (>5.5%) or low (<4.5%) CO₂ conditions could affect assay performance.
- The handling and preparation of the **CHO Mc4 FreshFrozenCells** and cell culture reagents must be performed using aseptic technique, unless otherwise noted.
- All reagents should be pre-warmed to ambient temperature before use. This includes **Growth Medium, Reaction Buffer, and Luciferase Assay Reagent Set**.
- The TSI Reporter Controls are supplied at working strength. Any dilution of these reagents will decrease sensitivity.
- Reagents should be used prior to their expiration date.
- Each multi-well plate should be used only once. Do not re-use previously assayed plate.
- Microbial contamination of the **CHO Mc4 FreshFrozenCells** and cell culture reagents may cause a decrease in sensitivity.
- Use of other reagents than those specified with the components of this Kit, especially those that contain sodium azide, may lead to erroneous results.
- The Growth Medium contains the pH indicator phenol red. Repeated exposure of the medium to the air may cause an increase in pH, evidenced by an increasingly deep red color. Limit the exposure of the medium to air as pH levels above 7.9 may affect the assay performance.
- Testing should be performed in an area with adequate ventilation.

- Dispose of containers and unused contents in accordance with Federal, State and Local regulatory requirements.
- Wear suitable protective clothing, gloves, and eye/face protection when handling the contents of this kit.
- Wash hands thoroughly after handling.
- For additional information on hazard symbols, safety, handling and disposal of the components within this kit, please refer to the Safety Data Sheet (SDS) located at quidel.com

SPECIMEN COLLECTION AND PREPARATION

Proper collection and handling of the patient specimen are the most important factors in successful TSI detection. Specimen collection and processing should be attempted only by personnel trained in such procedures. Care should be taken during all specimen collection and handling to avoid generation of aerosols.

Serum is required for the TSI Reporter. For additional specimen collection and processing recommendations please refer to CLSI Document H3-A6.⁶

Specimen Transport and Storage

Serum specimens should be transported to the laboratory at 2°C to 8°C using cold packs, wet ice, foam refrigerant, or other coolants. The specimen should be processed and tested as soon as possible or stored for up to 72 hours at 2°C to 8°C before testing. If testing does not occur before 72 hours the specimen may be aliquoted and frozen at –20° C for up to 2 months. Extended storage, beyond 2 months, should occur at temperatures that are –70°C or lower or in a liquid nitrogen Dewar.

- **Repeated freezing and thawing of serum samples should be avoided since this may affect specimen biological activity, leading to erroneous results.**
- **Samples may go through a maximum of three freeze/thaw cycles.**
- **Samples collected for retrospective analysis should be aliquoted upon receipt and immediately frozen.**

All potentially infectious agents should be transported according to International Air Transport Association (IATA), International Civil Aviation Organization, (ICAO), Titles 42 and 49 of the US Code of Federal Regulations, or other regulatory requirements, as may be applicable.

PROCEDURE

Preliminary Comments and Precautions

- Adhere to the recommended volumes in the following procedure to ensure that accurate results are obtained.
- When medium is decanted from the cells, it is important that all medium is removed. Decant medium with enough force to completely remove it from each well. Visually examine the wells for medium removal before proceeding.
NOTE: It is imperative that all medium be removed from the wells before moving to the next step in the procedure.

REGARDING CELL CULTURE TESTING:

- To prevent aspiration of water from the water bath into the vial, do not allow the water bath level to reach the junction of the vial/cap.
- Aseptic technique must be used throughout the first 15 to 18 hours of the procedure. Microbial contamination increases the risk of assay failure.
- Do not allow the monolayers to dry between steps; this can be avoided by handling one plate at a time.

- Uniform heating of the cells is a requirement. The plates are to be placed side-by-side in the incubator rather than stacked. Stacking the plates will cause poor assay performance and greatly increase the risk for both inter- and intra-plate variation as well as assay failure.
- The plates must be carefully handled in order to avoid uneven distribution of the cells. Use of non-vibrating surfaces is a necessity to ensure uniform distribution of the cells in the wells.
- A confluent monolayer is one where cells are in contact with each other forming a continuous sheet of adherent cells on the bottom of the plate well. The confluency of the monolayer is assessed prior to use with a microscope at 100X magnification.

REGARDING CONTROL SET:

- It is good practice to examine the results of the Positive and Normal Controls before examining the test results of the specimens. However different lots might have a different positive control range. Please check the positive control reference range label for the test range before the test. If one or both of the controls fail to perform as expected, review the steps and conditions under which the test was performed to determine the cause(s). Do not report results until controls perform as expected.

TSI Reporter Procedure

Day 1: *Carry out aseptically*

1. Calculate the number of plates needed to perform the assay – each plate can support 21 specimens tested along with the Positive, Reference and Normal Controls. All testing is performed in duplicate.
2. Aliquot 5 mL per plate of Growth Medium (GM) into a suitable, sterile container (i.e., 50-mL centrifuge tube).
3. Place aliquoted GM into a 35°C to 37°C water bath 5 minutes prior to use.
4. Add 100 µL of Cell Attachment Solution (CAS) using a multi-channel pipette to each of the 48 inner wells of each plate, and treat for 1 to 10 minutes at 20°C to 25°C.
5. Decant the CAS from the wells onto a sterile absorbent pad inside the biological hood.
6. Thaw one vial of CHO Mc4 FreshFrozenCells per plate in a 35°C to 37°C water bath for 2 to 4 minutes.
7. Transfer the entire volume of thawed cells to the pre-warmed GM using a sterile transfer pipette. Rinse transfer pipette into the GM by aspirating and expelling 3 times.
8. Close the tube and mix the cell suspension by inverting several times.
9. Pour the suspension to a sterile reagent reservoir.
10. Add 100 µL of the cell suspension to each of the inner 48 wells of each plate using a 100-µL multi-channel pipette. It is necessary to mix the suspension in the reagent reservoir by pipetting up and down frequently to ensure the cells stay in suspension and are uniformly distributed among the wells.
11. Incubate all seeded plates for 15 to 18 hours in a humidified, 5% CO₂, 35°C to 37°C incubator. In order to avoid microbial contamination, handle the plates so that the lid is not opened outside of the Bio-safety Cabinet.

Day 2: *Steps 1-16 can be carried out on the benchtop*

1. Place Reaction Buffer (RB) in a 35°C to 37°C water bath 5 minutes prior to use.
2. Remove the plate(s) from the incubator.
3. Examine the monolayers in each well microscopically using a magnification of 100X to 200X.
 - a. Plates exhibiting signs of microbial contamination should be discarded.
 - b. Monolayers must be confluent to be used in the assay. Mark any wells for disqualification based on sub-confluence.

NOTE: *Plates can remain in the incubator for up to 18 hours or until confluency is reached (a minimum of 15 hours). Plates that fail to reach confluency should be disqualified from use.*

 - a. Individual wells containing piled or layered cells should be disqualified from use based on over-confluence.

4. Return plate(s) to the incubator following examination.
5. Calculate the amounts of serum specimen, control and Reaction Buffer (RB) needed to perform the assay as follows:
 - a. Prepare a 1:11 dilution of each specimen as follows:
 - i. Label a 13-mm test tube with patient identifier.
 - ii. Add 400 μ L of RB to each tube.
 - iii. Thaw frozen specimens in a 35°C to 37°C water bath for 7 to 10 minutes.
 - iv. Vortex vigorously for 15 seconds.
 - v. Add 40 μ L of each serum to the RB in the appropriately labeled tube.
 - vi. Vortex vigorously for 15 seconds.
 - b. Prepare a 1:11 dilution of each control and reference as follows (multiply the volumes listed in Steps 5.b.iii and 5.b.v (below) by the number of plates tested to prepare final volume needed for the run):
 - i. Thaw the appropriate volume of Controls and Reference in a 35°C to 37°C water bath for 7 to 10 minutes.
 - ii. Label a 13-mm test tube with control identifier.
 - iii. Add 400 μ L of RB to each tube.
 - iv. Vortex controls and reference vigorously for 15 seconds.
 - v. Add 40 μ L of each serum to the RB in the appropriately labeled tube.
 - vi. Vortex vigorously for 15 seconds.

The following Steps 6 through 16 are to be performed one plate at a time. They should also be performed sequentially in order to avoid drying of the cell monolayer.

6. Remove the plate(s) from the incubator.
7. Decant the GM from the plate into an appropriate waste container.
8. Rinse the cells by adding 100 μ L of pre-warmed RB to each well using a multi-channel pipette. The medium should be gently dispensed down the side of the well in order to avoid disruption of the monolayer.
9. Decant the RB into an appropriate waste container.
10. Add 100 μ L of pre-warmed RB to each well using a multi-channel pipette. The medium should be gently dispensed down the side of the well in order to avoid disruption of the monolayer.
11. Add 100 μ L of the diluted controls, reference and specimens (prepared above) to the appropriate RB containing wells (in duplicate) using a 20 μ L to 200 μ L pipette.
12. Incubate each plate for 3 hours in a humidified, 5% CO₂, 35°C to 37°C incubator.
13. Remove an appropriate number of Luciferase Assay Reagent Set kits from the freezer approximately 30 minutes prior to use. Two kits provide enough substrate for 5 plates.
14. Thaw the Luciferase Assay Buffer Solution (buffer) in a 35°C to 37°C water bath for 15 minutes. Store at 20°C to 25°C until used.
15. Store the Luciferase Substrate at 20°C to 25°C until used.
16. Pour the buffer (white bottle) into the lyophilized Luciferase Substrate (amber bottle). Replace the cap, and invert gently 6 times to mix.
17. Transfer the substrate solution to a reagent reservoir and cover to keep the substrate solution in the dark. Minimize exposure of the substrate solution to light as this may cause a decrease in activity.
18. Process each plate separately to completion as follows:

NOTE: The lysis process for the next plate should not be carried out until the previous plate is completed and enough time has elapsed to allow for the previous plate to be analyzed in the luminometer.

 - a. Decant the contents of the plate into an appropriate waste container.
 - b. Remove the remaining volume of medium from the wells by blotting the plate upside down on an absorbent pad.

- c. Add 75 µL of Luciferase Substrate to each well using a multi-channel pipette.
- d. Cover the MWP and allow to stand at 20°C to 25°C for 10 minutes.
NOTE: It is critical to maintain the cell lysis temperature above 20°C. The test result will be affected if the lysis temperature falls to 19°C or less.
- e. Read the plate in a luminometer that is programmed to read the inner 48 wells of the plate at an integration time of 1 second per well.

Quality Control

- Positive and Normal Controls should be run and calculated with each plate of specimens to confirm the assay performance.
- A reference range is provided with each Control Set which establishes the maximum and minimum acceptable values for the Positive Controls when it is compared to the Reference Control. The positive control range may change with each lot of the positive control. Please check the value for the positive control on the positive control reference range label prior to evaluating control validity.
- If assay controls fail to perform correctly (i.e., above or below established range), results for that plate are considered invalid. Contact Diagnostic Hybrids, Inc. Technical Support if an assay run is invalid.

RESULTS

Calculation of Results

The average Relative Light Unit (RLU) of each specimen is calculated using the values from the duplicate wells.

$$\text{Average RLU} = (\text{Well 1 RLU} + \text{Well 2 RLU}) / 2$$

Example:

X	X	X	X	X	X	X	X	X	X	X	X
X	X	19228	8478	4458	19453	12527	3720	31919	19009	X	X
X	X	21009	8696	4595	19848	12800	4166	32117	21185	X	X
X	X	18598	7606	30503	11745	8328	3217	15017	17958	X	X
X	X	18903	7161	29344	11984	9001	3522	14856	18456	X	X
X	X	14923	5591	31286	21253	13243	4942	1038	8821	X	X
X	X	13831	5464	30141	20945	13635	5156	959	9639	X	X
X	X	X	X	X	X	X	X	X	X	X	X

$$(19228+21009)/2 = 20119$$

$$(8478+8696)/2 = 8587$$

Calculate the coefficient of variation (CV) % for each test specimen and control using the following equation:

$$\text{CV}\% = ([\text{StDev Test Specimen RLU}] / [\text{Average Test Specimen RLU}]) * 100\%$$

Example:

$$(1259/20119) * 100\% = 6.3\%$$

Calculate the Sample to Reference Ratio (SRR) % using the following equation:

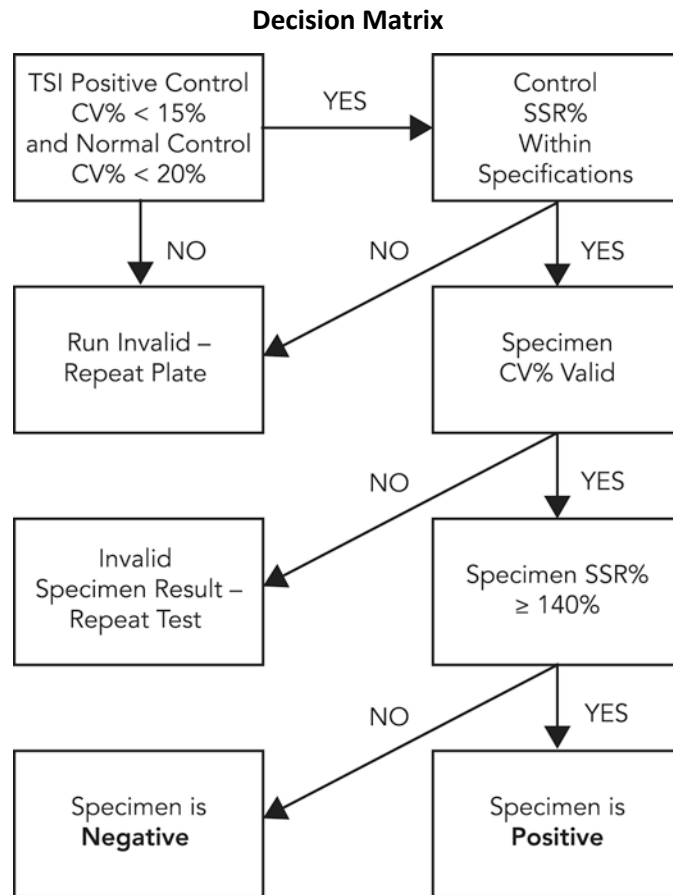
$$\text{SRR}\% = ([\text{Average Test RLU}] / [\text{Average Reference Control RLU}]) * 100\%$$

Example:

$$(20119/8587) * 100\% = 234\%$$

Interpretation of Results (See Decision Matrix below)

- A specimen must have a CV of <15% between the duplicate RLU values to be considered valid. All specimens with an invalid result must be repeated.
- A positive result is one in which the Specimen – Reference Ratio (SRR%) is $\geq 140\%$ of the Reference Control.
- A negative result is one in which the SRR% that is < 140% of the Reference Control.



LIMITATIONS OF PROCEDURE

- This assay requires serum samples only. Use of plasma or whole blood may result in assay failure.
- Serum must be free of particulate matter before analysis can commence. The presence of particulate matter may affect the sensitivity of the assay.
- The testing of serum that is visibly icteric, hemolytic or lipemic may lead to decreased sensitivity in the detection of TSI.
- Incubation times or temperatures other than those cited in the test instructions may give erroneous results.
- Detection of TSI can vary depending upon the specimen quality and subsequent handling. A negative result does not exclude the possibility of the presence of TSI. Results of the test should be interpreted in conjunction with information available from other clinical information, such as physical symptoms and thyroid hormone testing, as recommended by the American Thyroid Association (ATA).
- The TSI Reporter assay is intended for the qualitative detection of TSI. It is not intended for use in monitoring a patient's treatment. The effects of various drug therapies on the performance of this Kit have not been established.
- This is a functional bioassay for the detection of serum TSI. Sample dilutions are not advisable as there is a non-linear relationship between antibody concentration and signal (Relative Light Unit, RLU).

- Performance of the Kit can only be assured when components used in the assay are those supplied by Quidel.
- False-positive results may occur when serum TSH levels are > 350 mU/L. Specimens with TSH levels above this range should be disqualified for use.

EXPECTED VALUES

In a study of 118 healthy blood donors, all yielded results below 140% or considered negative by this assay. However, each laboratory should establish its own reference ranges.

SPECIFIC PERFORMANCE CHARACTERISTICS

Studies were performed at two testing sites in the fall of 2008 with a total of 312 specimens evaluated by both the subject (Thyretain TSI Reporter) and comparator (KRONUS TRAb) devices. All specimens were handled in accordance with the procedure in the instructions for use for TSI Reporter and the comparator device product insert.

Tables 1 and 2 show the age and gender distribution for individuals studied at Study Site 1 and 2, respectively.

Table 1. Study Site 1 – Age and Gender Distribution

Age Range ¹	Number Positive/Total Specimens		
	Male	Female	Total
< 18 yrs.	0 / 0	1 / 1	1 / 1
19 to 40 yrs.	0 / 2	7 / 19	7 / 21
41 to 65 yrs.	1 / 4	4 / 13	5 / 17
66 < yrs.	3 / 7	1 / 6	4 / 13
Grand Total	4 / 13	13 / 39	17 / 52

Table 2. Study Site 2 – Age and Gender Distribution

Age Range ¹	Number Positive/Total Specimens		
	Male	Female	Total
< 18 yrs.	0 / 5	5 / 15	5 / 20
19 to 40 yrs.	6 / 11	25 / 65	31 / 76
41 to 65 yrs.	13 / 32	43 / 83	56 / 115
66 < yrs.	5 / 16	15 / 33	20 / 49
Grand Total	24 / 64	88 / 196	112 / 260

One (1) specimen was excluded due to insufficient quantity for testing. Twelve (12) of these specimens were excluded from statistical analysis due to indeterminate results on the comparator device. The remaining 299 specimens were analyzed for positive and negative percent agreement.

Table 3 details summary results from Study Sites 1 and 2 combined information.

¹ <http://www.womenshealth.gov/faq/graves-disease.cfm> web site indicates that GD affects women between the ages 20 to 40 although it can occur in infants, adolescents, <18 years of age and the elderly >65 years of age.

Table 3. Study Sites 1 and 2 Combined Results Summary

299 specimen results		Comparator Device		
		+	-	Indeterminate
Thyretain TSI Reporter	+	120	18	7
	-	8	153	5
	Invalid	0	0	0
		<i>95% Confidence Interval</i>		
<i>Positive Percent Agreement</i>	93.8%	88.2 to 96.8%		
<i>Negative Percent Agreement</i>	89.5%	84.0 to 93.2%		

The ability of the subject device to detect TSI using a cell-based system was compared to the comparator device's radioreceptor assay. The positive percent agreement for Study Sites 1 and 2 combined was 93.8% (95% CI range 88.2% to 96.8%). The negative percent agreement for Study Sites 1 and 2 combined was 89.5% (95% CI range 84.0% to 93.2%). There were no invalid results. The performance data for the two devices are comparable.

An additional study was performed at a third testing (Study Site 3) in the fall of 2008 site using 247 specimens to be evaluated by both the subject and comparator devices. All specimens were handled in accordance with the procedure in the instructions for use for TSI Reporter and the comparator device product insert.

Table 4 shows the age and gender distribution for individuals studied at Study Site 3.

Table 4. Study Site 3 – Age and Gender Distribution

Age Range*	Number Positive/Total Specimens		
	Male	Female	Total
< 18 yrs.	2 / 4	4 / 18	6 / 22
19 to 40 yrs.	5 / 13	20 / 67	25 / 80
41 to 65 yrs.	5 / 23	29 / 96	34 / 119
66 < yrs.	0 / 3	6 / 23	6 / 26
Grand Total	12 / 43	59 / 204	71 / 247

Sixteen (16) of these specimens were excluded from statistical analysis due to indeterminate results on the comparator device. Twenty-four (24) of the remaining specimens were excluded from statistical analysis due to invalid (Null) results using the Thyretain. The remaining 207 specimens were analyzed for positive and negative percent agreement.

The following table (Table 5) details summary results from Study Site 3.

Table 5. Study Site 3 Results Summary

207 specimen results		Comparator Device		
		+	-	Indeterminate
Thyretain TSI Reporter	+	53	4	2
	-	16	134	12
	Invalid	2	22	2
		<i>95% Confidence Interval</i>		
<i>Positive Percent Agreement</i>	76.8%	65.6% to 85.2%		
<i>Negative Percent Agreement</i>	97.1%	92.8% to 98.9%		

The positive percent agreement (PPA) was 76.8% (95% CI range 65.6% to 85.2%). The negative percent agreement (NPA) was 97.1% (95% CI range 92.8% to 98.9%). There was a total of 26/247 (10.5%) invalid results using the Thyretain that required repeat testing.

An explanation for this decreased positive percent agreement value is the specificity difference between TSI Reporter and the comparator device. The comparator device detects autoantibodies to the TSHR, of which there are two classes, stimulating (TSI-hyperthyroidism) and blocking (TBI-hypothyroidism). The comparator device is unable to distinguish between the two antibody types, as stated in the Assay Limitations of the comparator device's product insert. TSI Reporter detects only stimulating autoantibodies. Thus, during our clinical performance testing, it is likely that some patient sera have TBI and would be positive by the comparator device but negative by the subject device. There currently is no TBI specific cleared device and, as such, it is not possible to further analyze the discordant results. There is no published data indicating the prevalence of TBI in the normal population.

The patient TSH results from the 18 discordant specimens (Comparator Device positive/Subject Device negative) at Study Site 3 were further reviewed. The results indicate that 6 patients meet the ATA definition of hypothyroidism (>3.0mIU/L). If these patients are removed from the dataset from Study Site 3 the PPA increases to 81.5% (95% CI range 70.4% to 89.1%).

Study Site 3 is a reference laboratory that receives specimens from all medical disciplines to be tested as part of a thyroid screening panel. The testing of patients with hypothyroidism is more likely in a screening environment than at Study Sites 1 and 2 that service targeted disciplines (i.e., Endocrinologists and Thyroidologists). ATA guidelines define Hypothyroidism as a high TSH level (>3.0mIU/L). A review of the TSH levels from the three sites indicate a greater percentage of Hypothyroid patients were in fact evaluated at Study Site 3 (24% of specimens) than at Study Sites 1 and 2 (15% and 11%, respectively).

Limit of Detection

The LOD for the TSI Reporter is claimed to be 89.14 %SRR based on the following calculations:

Limit of Blank

$$\text{LOB} = \text{Result at position } [N_B(p/100) + 0.5]$$

Twenty (20) blank measurements were carried out and were ranked 1 through 20. Based on the formula above the LOB is the average of the 19th and 20th ranked measurement

$$(\text{LOB} = 62.75\%).$$

Limit of Detection

$$\text{LOD} = \text{LOB} + c_{\beta} \text{SD}_5$$

Twenty (20) measurements of a low positive were carried out and the standard deviation calculated. Based on the formula above the LOD has been calculated to be 89.14% SRR [62.75+(1.649x16)].

This LOD was verified by performing 25 measurements of a positive specimen diluted to the claimed LOD (5 measurements of 5 blank specimens over 5 days).

Cross-reactivity and Interference by Endogenous Substances:

Cross-reactivity was determined for the following substances by spiking and testing TSI-containing human serum with different concentrations of luteinizing hormone up to 625 mIU/mL, human chorionic gonadotropin up to 40,625 mIU/mL, follicle stimulating hormone up to 2,000 mIU/mL and thyroid stimulating hormone up to 0.35 mIU/mL (Table 6).

Interference was not observed for the following substances by spiking and testing TSI-containing human serum with different concentrations of bilirubin up to 36.6 mg/dL, hemoglobin up to 250 mg/dL and lipids up to 1168 mg/dL.

Table 6. Summary of Cross-reactivity Studies on the Thyretain TSI Reporter

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

Based on testing standards for various reference laboratories and institutions such as LabCorp of America, AACE and Mayo Foundation.

Cross-Reactivity with Other Autoantibodies:

The TSI Reporter was tested on 36 samples with autoimmune diseases other than GD: 16 autoimmune hypothyroidism (Hashimoto's disease [Hm]), 10 Rheumatoid Arthritis (RA) and 10 Systemic Lupus Erythematosus (SLE). One (1) Hm sample tested positive for TSI, however, this was a sample with TSH levels at or near the level of interference reported above. All other samples tested negative.

Assay Cutoff

Patient serum, when tested using the TSI Reporter, will be considered positive for the presence of TSI if the resultant SRR% measures ≥140% of the Reference Control. This is a preliminary cut-off which was established using a "training-set" of samples and was confirmed in the clinical studies. The TSI Reporter prototype device was tested for assay cutoff limits through testing of 30 subjects with diagnosed Graves' disease and 44 normal subjects with no known or clinically diagnosed thyroid disease. The SRR% data obtained for each of these subjects were analyzed using receiver operating characteristic (ROC) curve analysis.

Using a “testing-set” of samples, the preliminary cutoff was verified in pre-clinical testing with an additional 50 GD positive sera obtained from physicians with diagnostic information and 140 normal sera. Additional verification of the cutoff limit was performed through precision testing. A manufactured sample near the cutoff limit ($\approx 146\%$ above the Reference Control) was tested over 15 days ($n=90$) and yielded positive results $\geq 74\%$ of the time and negative results $\leq 26\%$ of the time. CLSI recommendations for assay cutoff verification (Evaluation Protocol (EP) 12-A, Section 7.0) are a 50%/50% positive to negative ratio for samples at the cutoff.

Intra-Assay Precision

Assays using the TSI Reporter were conducted by a single user over a 15-day period in order to evaluate the device for intra-assay precision. Each sample was tested in duplicate wells and the resulting RLU values were averaged per sample. Each plate of cells analyzed per day contained 15 samples. The average variation (CV %) for each sample was calculated across each plate (2 plates per day). The average intra-plate ($n=30$) variation (CV %) was 3.7%.

Inter-Assay Precision (Intra-Day)

The same samples tested during the Precision Study were analyzed for inter-assay precision within each day. Serum samples were manufactured to simulate a patient with high levels, medium levels and low levels of TSI by diluting serum from a known GD subject with highly stimulatory TSI into normal human serum and tested repeatedly. A fourth sample displaying a high normal response was simulated by diluting a TSI-containing sample past the assay cutoff near the LOD and repeatedly tested. Serum from a TSI negative subject, TSI Positive Control and Reference Control were also tested. The total replicates for the types of samples tested per day were 6, 6, 6, 6, 2, and 4 for the high positive, medium positive, low positive, high normal, normal, TSI Positive Control and Reference Control, respectively. The average duplicate was calculated per plate then analyzed for inter-plate variation. For the high TSI, medium TSI and low TSI containing sera, the average inter-assay CV% values calculated on day one of precision testing were 1.9%, 5.0% and 3.4% respectively. The Reference Control, TSI Positive Control, high normal control and normal control were 3.3%, 1.1%, 7.5% and 10.0% respectively, with an overall inter-assay variation within this day calculated to be 4.6%.

Inter-Assay Precision (Inter-Day)

The data collected from the above studies were analyzed for inter-assay precision across a 15-day study. When combined, the average variation of the assay for the high TSI containing serum, medium TSI containing serum, low TSI containing serum, high normal serum, and normal serum ($n=90$, $n=90$, $n=90$ and $n=90$) was 8.9%, 8.6%, 6.8%, 14.6% and 19.2%, respectively. The average assay variation for the TSI Positive Control ($n=30$) and Reference Control ($n=60$) were 9.1% and 6.1%, respectively. The overall average inter-assay variation across 15 days was calculated to be 10.5%.

Table 7 summarizes the precision study results.

Table 7. Intra and Inter-Assay Precision Results

Sample (Intra-Assay)	High	Mid	Low	High Normal	Normal
N	3	3	3	3	3
Mean (%SRR)	362	181	147	106	77
SD	10	7	6	7	5
CV%	2.8	3.6	3.8	6.7	6.3

Sample (Intra-Day)	High	Mid	Low	High Normal	Normal
N	6	6	6	6	6
Mean (%SRR)	361	175	145	102	71
SD	7	9	5	8	7
CV%	1.9	5.0	3.4	7.5	10.0
Sample (Inter-Day)	High	Mid	Low	High Normal	Normal
N	90	90	90	90	90
Mean (%SRR)	345	195	146	86	58
SD	31	17	10	12	11
CV%	8.9	8.6	6.8	14.6	19.2

Assay Reproducibility

To demonstrate competency with and reproducibility of the assay, each trained site performed testing on the panel² described in Table 8 below twice a day over an 8-day span.

Table 8. Reproducibility Panel Variation and Accuracy

Reagent	Volume	Expected Variation	Expected Accuracy
Specimen A	15x500- μ L	+/- 15%	100%
Specimen B	15x500- μ L	+/- 15%	100%
Specimen C	15x500- μ L	+/- 25%	100%
Specimen D	15x500- μ L	+/- 15%	50%

Table 9 reports the results for each of the sites over their proficiency training period. Study Site 3 requested two technicians be trained.

Table 9. Proficiency Training Period Study Site Results (reported as Average SRR% and Coefficient of Variation)

Site 1	Site 2	Site 3 – Tech 1	Site 3 – Tech 2
<u>Sample A</u>	<u>Sample A</u>	<u>Sample A</u>	<u>Sample A</u>
273% 11.7%	442% 10.8%	282% 13.4%	291% 10.6%
<u>Sample B</u>	<u>Sample B</u>	<u>Sample B</u>	<u>Sample B</u>
294% 14.5%	492% 14.8%	374% 14.5%	344% 12.1%
<u>Sample C</u>	<u>Sample C</u>	<u>Sample C</u>	<u>Sample C</u>
48.0% 14.2%	69.0% 15.6%	44.0% 20.8%	48% 22.6%
<u>Sample D</u>	<u>Sample D</u>	<u>Sample D</u>	<u>Sample D</u>
158% 10.7%	199% 10.9%	145% 14.4%	143% 12.2%

All Study Sites performed the study using manufactured panel samples. Each site's data was analyzed cumulatively to determine the Reproducibility and Repeatability of the panel samples. Samples A and B both had a positive ratio (Number Positive/Total Number Tested) of 180/180, Sample C had a negative ratio

² The panel consisted of four specimens created to meet the requirements for precision as set forth in both CLSI (EP12-A2) and FDA guidance documents [<http://fda.gov/cdrh/ode/odecl051.html>].

(Number Negative/Total Number Tested) of 180/180 and Sample D had a positive ratio (Number Positive/Total Number Tested) of 140/180. The overall coefficient of variation (CV%) for Samples A, B, C and D were 23.6%, 23.5%, 25.4%, and 17.9%, respectively.

An additional smaller study was performed using three samples near the cut off was performed at two sites twice a day for five-days.

Table 10 presents the results for each of the two sites over their proficiency training period.

**Table 10. Reproducibility Results
(reported as Average SRR% and Coefficient of Variation %)**

Study Site 2		Quidel	
<u>Specimen E</u>		<u>Specimen E</u>	
191%	9.4%	172%	18.0%
<u>Specimen F</u>		<u>Specimen F</u>	
104%	20.3%	107%	21.4%
<u>Specimen G</u>		<u>Specimen G</u>	
100%	21.7%	108%	20.0%

Each site's data were analyzed cumulatively to determine the Reproducibility and Repeatability of the panel samples. Sample E had a positive ratio (Number Positive/Total Number Tested) of 55/60 and Samples F and G had negative ratios (Number Negative/Total Number Tested) of 55/60 and 56/60, respectively. The overall coefficient of variation (CV) % for Samples E, F and G were 14.8%, 20.7%, and 21.0%, respectively.

Clinical Sensitivity and Specificity

The clinical sensitivity and specificity for the device was determined by testing 249 characterized specimens. Nineteen (19) of these specimens were excluded from statistical analysis due to invalid (Null) results. The data are presented in the Table 11 below.

Table 11: Clinical Sensitivity and Specificity

		Diagnosis		
		Positives (Graves Disease)	Negative (Other autoimmune diseases and healthy controls)	Totals
Thyretain TSI Reporter	Positive	46	1	47
	Negative	4	179	183
	Null	0	19	19
	Total	50	199	249

Clinical Sensitivity: 92.0% (46/50)

Clinical Specificity: 99.4% (179/180)

Invalid Results (Null): 7.6% (19/249)

CUSTOMER AND TECHNICAL SUPPORT

To place an order or for Technical Support, please contact a Quidel Representative at 800.874-1517 (in the U.S.) or 858.552.1100 (outside the U.S.), Monday through Friday, from 8:00 a.m. to 5:00 p.m., Eastern Time. Orders may also be placed by fax at 740.592.9820. For e-mail support contact customerservice@quidel.com or technicalsupport@quidel.com.

For services outside the U.S., please contact your local distributor. Additional information about Quidel, our products, and our distributors can be found on our website quidel.com.

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40-25000.v2/40-50000.v2 Thyretain TSI Reporter BioAssay





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GLOSSARY

REF

Catalogue number



CE mark of conformity

EC REP

Authorized Representative
in the European Community

LOT

Batch code



Use by



Manufacturer



Temperature limitation



Intended use



Consult e-labeling
instructions for use



Do not reuse

IVD

For *In Vitro* diagnostic use

CONT NaN₃

Contents/Contains
