II. SUMMARY AND EXPLANATION OF THE TEST

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).

III. 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 form 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 -70°C 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.

IV. REAGENTS

A. Thyretain™ TSI Reporter BioAssay Kit Components

1. CHO Mc4 Fresh/Frozen Cells®: Cryovials containing CHO Mc4 cells cryogenically preserved in cryoprotective medium containing DMSO. Reagent is stored at -70°C or lower.
2. Cell Attachment Solution, 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.
3. Growth Medium, 200-mL: Ham’s F-12 cell culture medium containing 10% FBS. Reagent is stored at 2°C to 8°C.
4. Reaction Buffer, 500-mL: A proprietary buffer that augments the reaction of TSI with the TSHR. Reagent is stored at 2°C to 8°C.
5. Control Set:
   a. 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.
   b. Reference Control, 0.5-mL: A bTSH-containing solution against which controls and test samples are compared. Reagent is stored at -70°C or lower.
   c. 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.
6. Luciferase Assay Reagent Set:
   a. Luciferase Substrate, 1 vial, 10-mL: A cell culture lysis buffer.
   b. Luciferase Assay Buffer Solution, 1 vial, 10-mL: A proprietary buffer.

B. Reagent Storage Instructions

<table>
<thead>
<tr>
<th>Table 1: Reagent Storage Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. CHO Mc4 Fresh/Frozen Cells® Store at -70°C or lower</td>
</tr>
<tr>
<td>2. Control Set (Positive, Reference, and Normal) Store at 2°C to 30°C</td>
</tr>
<tr>
<td>3. Cell Attachment Solution Store at 2°C to 30°C</td>
</tr>
<tr>
<td>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.</td>
</tr>
<tr>
<td>4. Growth Medium Store at 2°C to 8°C</td>
</tr>
<tr>
<td>5. Reaction Buffer Store at -70°C or lower</td>
</tr>
<tr>
<td>6. Luciferase Assay Reagent Set Store at -70°C or lower</td>
</tr>
</tbody>
</table>

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. 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. 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, T₃, and T₄, 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.
C. 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.

V. WARNINGS AND PRECAUTIONS
For in vitro diagnostic use

1. 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.

2. 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

a. Cell cultures may have some potential to be hazardous. Personnel working with these cultures must be properly trained in safe handling techniques and have experience with tissue culture before attempting this procedure.

b. All procedures must be conducted in accordance with the CDC 5th edition Biosafety in Microbiological and Biomedical Laboratories, 2007, and CLSI Approved Guideline M2-8A, Protection of Laboratory Workers from Instrument Biohazards and Infectious Disease Transmitted by Blood, Body Fluids, and Tissue.

c. All specimens and materials used to process them should be considered potentially infectious and handled in a manner which prevents infection of laboratory personnel.

i. Biosafety Level 2 or other appropriate biosafety practices should be used when handling these materials.

ii. Decontamination of specimens and cultures is most effectively accomplished using a 1:10 dilution of household bleach.

iii. 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.

iv. Never pipette reagents or clinical samples by mouth; avoid all contact of clinical samples with broken skin.

v. Avoid splashing and the generation of aerosols with clinical samples.

vi. Use aseptic technique and sterile equipment and materials for all cell culture procedures.

vii. 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.

viii. The CHO Mc4 FreshFrozenCells® are single-use only and cannot be re-frozen once thawed.

ix. 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.

x. The handling and preparation of the CHO Mc4 FreshFrozenCells® and cell culture reagents must be performed using aseptic technique, unless otherwise noted.

xi. All reagents should be pre-warmed to ambient temperature before use. This includes Growth Medium, Reaction Buffer, and Luciferase Assay Reagent Set.

xii. The TSI Reporter Controls are supplied at working strength. Any dilution of these reagents will decrease sensitivity.

xiii. Reagents should be used prior to their expiration date.

xiv. Each multi-well plate should be used only once. Do not re-use previously assayed plates.

xv. Microbial contamination of the CHO Mc4 FreshFrozenCells® and cell culture reagents may cause a decrease in sensitivity.

xvi. Use of other reagents than those specified with the components of this Kit, especially those that contain sodium azide, may lead to erroneous results.

xvii. 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.

VI. 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 H5-A6®.

A. Specimen Transport and Storage
Serum specimens should be transported to the laboratory at 2° 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° 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 in a liquid nitrogen Dewar.

i. Repeated freezing and thawing of serum samples should be avoided since this may affect specimen biological activity, leading to erroneous results.

ii. Samples may go through a maximum of three freeze/thaw cycles.

iii. Samples collected for retrospective analysis should be aliquoted upon receipt and immediately frozen.

B. Materials Required but Not Provided

1. -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.

2. Humidified, 5% CO2, 35°C to 37°C Incubator

3. Bio-safety Cabinet Class II

4. Luminometer capable of reading a 96 multi-well plate (MWP)

5. Luminometer calibrator plate

6. Microscope, inverted

7. Calibrated Pipettes

   a. Multi-Channel 20 to 200-μL

   b. Single 20 to 200-μL

   c. Single 100 to 1000-μL

   d. Sterile Pipette Tips

8. Luminometer Substrate, 1 vial

9. Luciferase Substrate, 1 vial

10. Luciferase Assay Buffer Solution, 1 vial, 10-mL

11. Sterile Transfer Pipette

12. Pipetman® variable volume pipetters

   a. 5, 10, 25-μLSterile Serological Pipettes

   b. 5, 10, 25-μLSterile Serological Pipettes

13. Water bath, 35°C to 37°C

14. Sterile screw-cap tubes (15- or 50-mL)

15. 13-mm Test Tubes

16. Vortex Mixer

17. Timer

18. Household Bleach

C. Preliminary Comments and Precautions

1. Adhere to the recommended volumes and times in the following procedure to ensure that accurate results are obtained.

2. 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:

3. 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.

4. Aseptic technique must be used throughout the first 15 to 18 hours of culture procedures.

5. 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.

6. 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.

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8. 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.
7. 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.
8. 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 confluenct of the monolayer is assessed prior to use with a microscope at 100X magnification.

**REGARDING CONTROL SET**

9. 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.

D. 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° 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° 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 Fresh Buffer (RB) needed to perform the assay as follows:
   a. Prepare a 1:1 dilution of each specimen as follows:
      i. Label a 13-mm test tube with patient identifier.
      ii. Add 400-μL of RB to each tube.
      iii. Add 75-μL of the diluted controls, reference and specimens (prepared above) to the appropriate RB containing wells (in duplicate) using a 20 to 200-μL pipette.
   b. Store the Luciferase Substrate at 20° to 25°C until used.
   c. Add 100-μL of the diluted controls, reference and specimens (prepared above) to the appropriate RB containing wells (in duplicate) using a 20 to 200-μL pipette.
6. Pour the suspension to a sterile reagent reservoir.
7. Close the tube and mix the cell suspension by inverting several times.
8. Transfer the entire volume of thawed cells to the pre-warmed GM container (i.e., 50-mL centrifuge tube).
9. 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 prior to evaluating control validit.

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

1. Place Reaction Buffer (RB) in a 35° 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 20 to 200x objective for signs of microbial contamination.
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 1:1 dilution of each specimen as follows:
      i. Label a 13-mm test tube with patient identifier.
      ii. Add 400-μL of RB to each tube.
      iii. Add 75-μL of the diluted controls, reference and specimens (prepared above) to the appropriate RB containing wells (in duplicate) using a 20 to 200-μL pipette.
   b. Monolayers must be confluent to be used in the assay. Mark any wells for disqualification based on sub-confluence.
   c. Individual wells containing piled or layered cells should be disqualified from use based on over-confluence.
6. Add 100-μL of 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.
7. Incubate all seeded plates for 18 to 24 hours in a humidified, 5% CO2, 35° 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.
8. 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.

**E. Quality Control**

1. Positive and Normal Controls should be run and calculated with each plate of specimens to confirm the assay performance.
2. 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.
3. The test result will be affected if the assay temperature falls to 19°C or less.

**VIII. RESULTS**

A. Calculation of Results

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

   \[
   \text{Average RLU} = \left( \frac{\sum_{i=1}^{n} \text{RLU}_i}{n} \right) 
   \]

   \[
   \text{Example:} \\
   \begin{array}{cccccccc}
   \hline
   \text{Average RLU} & \text{X} & \text{X} & \text{X} & \text{X} & \text{X} & \text{X} & \text{X} & \text{X} \\
   \text{X} & 12345 & 67890 & 12345 & 67890 & 12345 & 67890 & 12345 & 67890 \\
   \text{X} & 98765 & 43210 & 98765 & 43210 & 98765 & 43210 & 98765 & 43210 \\
   \text{X} & 76543 & 12345 & 76543 & 12345 & 76543 & 12345 & 76543 & 12345 \\
   \text{X} & 54321 & 98765 & 54321 & 98765 & 54321 & 98765 & 54321 & 98765 \\
   \text{X} & 32109 & 45678 & 32109 & 45678 & 32109 & 45678 & 32109 & 45678 \\
   \text{X} & 10987 & 54321 & 10987 & 54321 & 10987 & 54321 & 10987 & 54321 \\
   \text{X} & 87654 & 21098 & 87654 & 21098 & 87654 & 21098 & 87654 & 21098 \\
   \text{X} & 65432 & 10987 & 65432 & 10987 & 65432 & 10987 & 65432 & 10987 \\
   \text{X} & 43210 & 87654 & 43210 & 87654 & 43210 & 87654 & 43210 & 87654 \\
   \text{X} & 21098 & 65432 & 21098 & 65432 & 21098 & 65432 & 21098 & 65432 \\
   \hline
   \end{array}
   \]

   \[
   \text{Note:} \ 
   \text{(19228+21099)/2} = 20119 \\
   (8479+8696)/2 = 8567
   \]

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

   \[
   \text{CV} = \left( \frac{\text{SD}}{\text{Mean}} \right) \times 100
   \]
XI. 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.

Table 2 and 3 show the age and gender distribution for individuals studied at Study Site 1 and 2, respectively.

<table>
<thead>
<tr>
<th>Table 2: Study Site 1 - Age and Gender Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age Range</td>
</tr>
<tr>
<td>&lt; 18 yrs.</td>
</tr>
<tr>
<td>19 to 40 yrs.</td>
</tr>
<tr>
<td>41 to 65 yrs.</td>
</tr>
<tr>
<td>66 &lt; yrs.</td>
</tr>
<tr>
<td>Grand Total</td>
</tr>
</tbody>
</table>

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 4 details summary results from Study Sites 1 and 2 combined.

<table>
<thead>
<tr>
<th>Table 4: Study Sites 1 and 2 Combined Results Summary</th>
</tr>
</thead>
<tbody>
<tr>
<td>299 specimen results</td>
</tr>
<tr>
<td>Comparator Device</td>
</tr>
<tr>
<td>Thyretain™ TSI Reporter</td>
</tr>
<tr>
<td>+</td>
</tr>
<tr>
<td>-</td>
</tr>
<tr>
<td>Null</td>
</tr>
<tr>
<td>95% Confidence Interval</td>
</tr>
<tr>
<td>Positive Percent Agreement</td>
</tr>
<tr>
<td>Negative Percent Agreement</td>
</tr>
</tbody>
</table>

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 96.9%). 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 (Null) 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 using 247 specimens to be evaluated by both the subject 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.

Table 5 shows the age and gender distribution for individuals studied at Study Site 3.

<table>
<thead>
<tr>
<th>Table 5: Study Site 3 - Age and Gender Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age Range</td>
</tr>
<tr>
<td>&lt; 18 yrs.</td>
</tr>
<tr>
<td>19 to 40 yrs.</td>
</tr>
<tr>
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</tr>
<tr>
<td>66 &lt; yrs.</td>
</tr>
<tr>
<td>Grand Total</td>
</tr>
</tbody>
</table>

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 6) details summary results from Study Site 3.

---

X. LIMITATIONS OF PROCEDURE

1. This assay requires serum samples only. Use of plasma or whole blood may result in assay failure.
2. Serum must be free of particulate matter before analysis can commence.
3. The presence of particulate matter may affect the sensitivity of the assay.
4. Incubation times or temperatures other than those cited in the test instructions may give erroneous results.
5. 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).
6. 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.
7. 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).
8. Performance of the Kit can only be assured when components used in the assay are those supplied by Diagnostic Hybrids, Inc.
9. False-positive results may occur when serum TSH levels are greater than (> 350 mU/L). Specimens with TSH levels above this range should be disqualified for use.

IX. LIMITATIONS OF PROCEDURE

1. This assay requires serum samples only. Use of plasma or whole blood may result in assay failure.
2. Serum must be free of particulate matter before analysis can commence.
3. The presence of particulate matter may affect the sensitivity of the assay.
4. Incubation times or temperatures other than those cited in the test instructions may give erroneous results.
5. 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).
6. 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.
7. 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).
8. Performance of the Kit can only be assured when components used in the assay are those supplied by Diagnostic Hybrids, Inc.
9. False-positive results may occur when serum TSH levels are greater than (> 350 mU/L). Specimens with TSH levels above this range should be disqualified for use.

X. 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.

---

**Example:** (1259/2019)*100% = 6.3%

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

\[
SRR\% = \left( \frac{\text{Average Test RLU}}{\text{Average Reference Control RLU}} \right) \times 100
\]

**Example:** (2019/8587)*100% = 234%

---

**Decision Matrix**

- **TSI Positive Control CV% less-than (<) 15%**
  - YES
- **Normal Control CV% less-than (<) 20%**
  - NO
  - Run Invalid – Repeat Plate
- **Specimen CV% valid**
  - YES
- **Specimen SRR% Greater-than or equal to (≥) 140%**
  - NO
  - Null Specimen Result – Repeat Test
- **Specimen is Negative**
  - YES
  - Specimen is Positive

---
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 were a total of 26/247 (10.5%) invalid (Null) 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 (TSH-hyperthyroidism) and blocking (TBII-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 (≥40 mIU/mL).

Limit of Detection

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

- **Limit of Blank**
  
  \[ \text{LoB} = \text{Result at position } N_0 \times (p/100) + 0.5 \]

- Twenty (20) blank measurements were carried out and 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**
  
  \[ \text{LoD} = \text{LoB} + c_0 \times \text{SDLoB} \]

- 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% + (1649x16)).

- 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 7).

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

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 7).

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

Cross-Reactivity with Other Autoantibodies:

The TSI Reporter was tested on 36 samples with autoimmune diseases other than GD: 18 autoimmune hypothyroidism (Hashimoto’s disease [Hm]), 10 Rheumotoid Arthritis (RA) and 10 Systemic Lupus Erythematosus (SLE). One (1) sample 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 greater than or equal to (≥) 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 (<145% above the Reference Control) was tested over 15 days (n=90) and yielded positive results ≥74% of the time and negative results ≤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 tested repeatedly. 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, 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 8 summarizes the precision study results.

<table>
<thead>
<tr>
<th>Sample (Intra-Assay)</th>
<th>High</th>
<th>Mid</th>
<th>Low</th>
<th>Normal</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Mean (%SRR)</td>
<td>362</td>
<td>181</td>
<td>147</td>
<td>106</td>
<td>77</td>
</tr>
<tr>
<td>SD</td>
<td>10</td>
<td>7</td>
<td>6</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>CV%</td>
<td>2.8</td>
<td>3.6</td>
<td>3.6</td>
<td>6.7</td>
<td>6.3</td>
</tr>
</tbody>
</table>

To demonstrate competency with and reproducibility of the assay, each trained site performed testing on the panel described in Table 9 below twice a day over the proficiency training period. Study Site 3 requested two technicians be trained.

Table 10 reports the results for each of the sites over their proficiency training period. Study Site 3 requested two technicians to be trained.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
<th>Expected Variation</th>
<th>Expected Accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specimen A</td>
<td>15x500-µL</td>
<td>+/- 15%</td>
<td>100%</td>
</tr>
<tr>
<td>Specimen B</td>
<td>15x500-µL</td>
<td>+/- 15%</td>
<td>100%</td>
</tr>
<tr>
<td>Specimen C</td>
<td>15x500-µL</td>
<td>+/- 25%</td>
<td>100%</td>
</tr>
<tr>
<td>Specimen D</td>
<td>15x500-µL</td>
<td>+/- 15%</td>
<td>50%</td>
</tr>
</tbody>
</table>

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 and Sample C had a negative ratio (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 11 presents the results for each of the two sites over their proficiency training period.

Table 12: Clinical Sensitivity and Specificity

<table>
<thead>
<tr>
<th>TSH Reporter</th>
<th>Positives (Graves Disease)</th>
<th>Negatives (Other autoimmune diseases and healthy controls)</th>
<th>Totals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thyretain™</td>
<td>46</td>
<td>1</td>
<td>47</td>
</tr>
<tr>
<td>Negative</td>
<td>4</td>
<td>179</td>
<td>183</td>
</tr>
<tr>
<td>Null</td>
<td>0</td>
<td>19</td>
<td>19</td>
</tr>
<tr>
<td>Total</td>
<td>50</td>
<td>199</td>
<td>249</td>
</tr>
</tbody>
</table>

Clinical Sensitivity: 92.0% (46/50)
Clinical Specificity: 99.4% (179/180)
Invalid Results (Null): 7.8% (19/249)

XII. BIBLIOGRAPHY

1. AARDA, American Autoimmune Related Diseases Association; NWHIC, National Women’s Health Information Center.

WARRANTY STATEMENT

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