Canine Total Thyroxine (Canine T4) Test System

Product Code: 12225-300

1.0 INTRODUCTION

Intended Use: The Quantitative Determination of Total Thyroxine Concentration in Canine Serum by a Microparticle Enzyme Immunoassay.

2.0 SUMMARY AND EXPLANATION OF THE TEST

Thyroid disorder in dogs is a common endocrine dysfunction caused by a decrease in thyroid hormone production. Since clinical signs of thyroid deficiency are non-specific, measurement of serum thyroxine concentration is generally regarded as an important in-vitro diagnostic test for assessing thyroid function.

This microparticle enzyme immunoassay methodology provides the technician with optimum sensitivity while requiring few technical manipulations. In this method, serum reference, patient specimen, or control is first added to a microwell plate. Enzyme-T4 conjugate is added, and then the mixtures are incubated. A competition reaction between the enzyme conjugate and the native thyroxine for a limited number of antibody combining sites immobilized on the well.

After the completion of the required incubation period, the antibody bound enzyme-thyroxine conjugate is separated from unbound enzyme-thyroxine conjugate by aspiration or decantation. The activity of the enzyme present on the surface of the well is quantitated by reaction with a suitable substrate to produce color.

The employment of several serum references of known thyroxine concentration permits construction of a graph of activity and concentration. From comparison to the dose response curve, an unknown specimen’s activity can be correlated with thyroxine concentration.

3.0 PRINCIPLE

Competitive Enzyme Immunoassay (TYPE 5)

The essential reagents required for a solid phase enzyme immunoassay included immobilized antibody, enzyme-antigen conjugate and native antigen.

Upon mixing immobilized antibody, enzyme-antigen conjugate and a serum containing the native antigen, a competition reaction results between the native antigen and the enzyme-antigen conjugate for a limited number of insolubilized binding sites. The interaction is illustrated by the equation in the following column.

\[
K_a = \frac{[Ag][A]}{[Ag][B]} = \frac{[Ag Ab]_C}{[Ag][AbC]} = K_a = \text{Rate Constant of Association}
\]

After equilibrium is attained, the antibody-bound fraction is separated from unbound antigen by decantation or aspiration. The enzyme activity in the antibody-bound fraction is inversely proportional to the native antigen concentration. By utilizing several different serum references of known antigen concentration, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

4.0 REAGENTS

A. Canine T4 Calibrators – 0.1 ml/v – Icons A-F

Six (6) vials of serum reference for thyroxine at concentrations of 0 (A), 0.5 (B), 1.0 (C), 2.0 (D), 4.0 (E) and 8.0 (F) µg/dl. Store at 2-8°C. A preservative has been added. Store at 2-8°C.

B. Canine T4 Enzyme Reagent – 1.5 ml/v – Icon C

One (1) vial containing thyroxine-horseradish peroxidase (HRP) conjugate in a bovine albumin-stabilizing matrix. A preservative has been added. Store at 2-8°C.

C. T3/T4 Conjugate Buffer – 13 ml/vial – Icon D

One (1) vial containing a surfactant in buffered saline. A preservative has been added. Store at 2-8°C.

D. T4 Antibody Coated Plate – 96 wells – Icon E

One 96-well microwell coated with sheep anti-thyroxine serum and packaged in an aluminum bag with a drying agent. Store at 2-8°C.

E. Wash Solution Concentrate – 20 ml/vial – Icon F

One (1) vial containing tetramethylbenzidine (TMB) and hydrogen peroxide (H2O2) in buffer. Store at 2-8°C.

F. Substrate Solution – 12 ml/vial – Icon G

One (1) vial containing l-dopa in a PBS buffer. Store at 2-8°C.

G. Stop Solution – 8 ml/vial – Icon H

One (1) vial containing a strong acid (0.5M H2SO4). Store at 2-8°C.

H. Product Insert.

Note 1: Do not use reagents beyond the kit expiration date.

2. Dispenser(s) for repetitive deliveries of 0.100 & 0.350 ml (100 µl).

3. Add 0.100 ml (100 µl) of Working Reagent A, T4 Enzyme conjugate.

4. Swirl the microplate gently for 20-30 seconds to mix and decant (tap and blot) or aspirate. Repeat the decantation or aspiration two (2) times. The activity of the enzyme present on the surface of the well is quantitated by reaction with a suitable substrate to produce color.

The employment of several serum references of known thyroxine concentration permits construction of a graph of activity and concentration. From comparison to the dose response curve, an unknown specimen’s activity can be correlated with thyroxine concentration.

3. Add 0.100 ml (100 µl) of working reagent B, Antibody (patient or control specimen) into the assigned well. A dose response curve is used to ascertain the concentration of thyroxine in unknown specimens.

1. Record the absorbance obtained from the printout of the microplate reader as outlined in Example 1.

2. Plot the absorbance versus the corresponding T4 concentration in µg/dl on linear graph paper. (Do not average the duplicates of the serum specimen.

3. Connect the points with a best-fit curve.

4. To determine the concentration of T4 for an unknown, locate the nearest absorbance on the best-fit curve for each unknown on the vertical axis of the graph, find the intersecting point on the curve, and read off the concentration (in µg/dl) from the horizontal axis. If the absorbance of the unknown is between two absorbance values, the concentration of the unknown may be averaged as indicated. In the following example, the average absorbance (1.022) intersects the standard curve at (8 µg/dl) T4 concentration (See Figure 1).

*The data presented in Example 1 and Figure 1 is for illustration only and should not be used in lieu of a standard curve prepared with each assay.

Note: Do not use the substrate if it looks blue.

Note: Do not use reagents that are contaminated or have exceeded the shelf life.

**Test procedure should be performed by a skilled individual or a trained professional**

3. Add 0.100 ml (100 µl) of working reagent B, Antibody (patient or control specimen) into the assigned well.

4. Swirl the microplate gently for 20-30 seconds to mix and decant (tap and blot) or aspirate. Repeat the decantation or aspiration two (2) times. The activity of the enzyme present on the surface of the well is quantitated by reaction with a suitable substrate to produce color.

The employment of several serum references of known thyroxine concentration permits construction of a graph of activity and concentration. From comparison to the dose response curve, an unknown specimen’s activity can be correlated with thyroxine concentration.

**Test procedure should be performed by a skilled individual or a trained professional**

1. Format the microplate’s wells for each serum reference calibrator, control and patient specimen to be assayed in duplicate. Replace all used microwell strips back into the aluminum bag, seal and store at 2-8°C.

2. Pipette 0.025 ml (25 µl) of the appropriate serum reference control or specimen into the assigned well.

3. Add 0.100 ml (100 µl) of Working Reagent A, T4 Enzyme conjugate to all wells (see Reagent Preparation Section).

4. Swirl the microplate gently for 20-30 seconds to mix and decant (tap and blot) or aspirate. Repeat the decantation or aspiration two (2) times. The activity of the enzyme present on the surface of the well is quantitated by reaction with a suitable substrate to produce color.

The employment of several serum references of known thyroxine concentration permits construction of a graph of activity and concentration. From comparison to the dose response curve, an unknown specimen’s activity can be correlated with thyroxine concentration.

5. Add 0.350 ml (350 µl) of wash buffer to all wells (see Reagent Preparation Section), decant (tap and blot) or aspirate. Repeat the recess (2) additional times for a total of three (3) washes.

6. Microplate washer or a squeeze bottle (optional).

7. Add 0.100 ml (100 µl) of substrate solution to all wells (see Reagent Preparation Section). Always add reagents in the same order to minimize reaction time differences between wells.

8. Do not shake the plate after substrate addition.

9. Incubate at room temperature for fifteen (15) minutes.

Add 0.050 µl (50 µl) of TMB solution to each well and gently mix for 15-20 seconds. Always add reagents in the same order to minimize reaction time differences between wells.

11. Read the absorbance at 450nm using a reference wavelength of 620-630nm (using a reference wavelength of 620-630nm to minimize well imperfections) in a microplate reader.

The results should be read within thirty (30) minutes of adding the stop solution.

Note: For reassaying specimens with concentrations greater than 25 µg/dl, pipet 0.0125ml (12.5µl) of the specimen and 0.0125ml (12.5µl) of the 0 serum reference into the sample well (this maintains a uniform protein concentration). Multiply the readout value by 2 to obtain the thyroxine concentration.

10. CALCULATION OF RESULTS

A dose response curve is used to ascertain the concentration of thyroxine in unknown specimens.

1. Record the absorbance obtained from the printout of the microplate reader as outlined in Example 1.

2. Plot the absorbance versus the corresponding T4 concentration in µg/dl on linear graph paper (do not average the duplicates of the serum specimen).

3. Connect the points with a best-fit curve.

4. To determine the concentration of T4 for an unknown, locate the nearest absorbance on the best-fit curve for each unknown on the vertical axis of the graph, find the intersecting point on the curve, and read off the concentration (in µg/dl) from the horizontal axis. If the absorbance of the unknown is between two absorbance values, the concentration of the unknown may be averaged as indicated. In the following example, the average absorbance (1.022) intersects the standard curve at (8 µg/dl) T4 concentration (See Figure 1).

*The data presented in Example 1 and Figure 1 is for illustration only and should not be used in lieu of a standard curve prepared with each assay.

Note: Do not use the substrate if it looks blue.

Note: Do not use reagents that are contaminated or have bacteria growth.
11.0 QC. PARAMETERS

In order for the assay results to be considered valid the following criteria should be met:
1. The absorbance (OD) of calibrator 0 µg/dl should be ≥ 1.3.
2. Four out of six quality control pools should be within the expected values established by the Manufacturer only until an in-house range can be determined by the analysts using the method with a population indigenous to the area in which the laboratory is located.

12.0 RISK ANALYSIS

12.1 Assay Performance
1. It is important that the time of reaction in each well is held constant for reproducible results.
2. Pipetting of samples should not extend beyond ten (10) minutes to avoid assay drift.
3. If more than one (1) plate is used, it is recommended to repeat the dose response curve.
4. Addition of the substrate solution initiates a kinetic reaction, which is terminated by the addition of the stop solution. Therefore, the addition of the substrate and the stopping solution should be added in the same sequence to eliminate any time-deviation during reaction.
5. Plate readers measure vertically. Do not touch the bottom of the wells.
6. Failure to remove adhering solution adequately in the aspiration or decantation wash step(s) may result in poor replication and spurious results.
7. Use components from the same lot. No intermixing of reagents from different batches.

12.2 Interpretation
1. If computer controlled data reduction is used to interpret the results of the test, it is imperative that the predicted values for the calibrators fall within 10% of the assigned concentrations.
2. Total serum thyroxine concentration is dependent upon a multiplicity of factors: thyroid gland function and its regulation, thyroid binding globulin (TBG) concentration, and the cross-reactivity of the thyroxine antibody to selected substances. The sensitivity was ascertained by determining the variability of the 0 µg/dl serum calibrator and using the 2σ (95% certainty) statistic to calculate the minimum dose.
3. Total serum thyroxine values may be elevated under conditions such as pregnancy or administration of oral contraceptives. A T3 uptake test may be performed to estimate the relative TBG concentration in order to determine if the elevated T4 is caused by TBG variation.
4. A decrease in total thyroxine values is found with nonthyroid diseases including protein wasting disease, certain liver diseases and administration of testosterone, dihydromidantoin or salicylates. A table of interfering drugs and conditions which affect total thyroxine values has been compiled by the Journal of the American Association of Clinical Chemists. 1,2

13.0 EXPECTED RANGES OF VALUES

The expected values for euthyroid dog population have been established as 1 - 4 µg/dl. 2

It is important to keep in mind that establishment of a range of values, which can be expected to be found by a given method for a population of “normal” persons, is dependent upon a multiplicity of factors: the specificity of the method, the population tested and the precision of the method in the hands of the analyst. For these reasons, each laboratory should depend upon the range of expected values established by the Manufacturer only until an in-house range can be determined by the analysts using the method with a population indigenous to the area in which the laboratory is located.

14.0 PERFORMANCE CHARACTERISTICS

14.1 Precision
The within and between assay precisions of the Canine T4 AccuBind® ELISA test system were determined by analyses on three different levels of pool control sera. The number (N), mean values (X), standard deviation (σ) and coefficient of variation (C.V.) for each of these control sera are presented in Table 2 and Table 3.

### TABLE 2

<table>
<thead>
<tr>
<th>Sample</th>
<th>N</th>
<th>X</th>
<th>σ</th>
<th>C.V.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>20</td>
<td>1.05</td>
<td>0.049</td>
<td>4.7%</td>
</tr>
<tr>
<td>Normal</td>
<td>20</td>
<td>2.21</td>
<td>0.138</td>
<td>6.2%</td>
</tr>
<tr>
<td>High</td>
<td>16</td>
<td>4.24</td>
<td>0.18</td>
<td>4.3%</td>
</tr>
</tbody>
</table>

*As measured in ten experiments in duplicate over a ten day period.

14.2 Sensitivity
The Canine T4 AccuBind® ELISA test system has a sensitivity of 18 pg. This is equivalent to a sample containing a concentration of 0.072 µg/dl. The sensitivity was ascertained by determining the variability of the 0 µg/dl serum calibrator and using the 2σ (95% certainty) statistic to calculate the minimum dose.

14.3 Specificity
The cross-reactivity of the thyroxine antibody to selected substances was evaluated by adding the interfering substance to a serum matrix at various concentrations. The cross-reactivity was calculated by deriving a ratio between dose of interfering substance to dose of thyroxine needed to displace the same amount of conjugate.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Cross Reactivity</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-T3H</td>
<td>1.0000</td>
<td></td>
</tr>
<tr>
<td>d-T3H</td>
<td>0.9800</td>
<td>100µg/dl</td>
</tr>
<tr>
<td>d-T3H3</td>
<td>0.0150</td>
<td>100µg/dl</td>
</tr>
<tr>
<td>l-T3H3</td>
<td>0.0300</td>
<td>100µg/dl</td>
</tr>
<tr>
<td>l-Diiodothyronine</td>
<td>0.0001</td>
<td>100µg/ml</td>
</tr>
<tr>
<td>Diiodothyronine</td>
<td>0.0001</td>
<td>100µg/ml</td>
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</tbody>
</table>

15.0 REFERENCES