**Canine Total Thyroxine (Canine T4) Test System**

**Product Code**: 12225-300

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**1.0 INTRODUCTION**

Intended Use: The Quantitative Determination of Total Thyroxine Concentration in Canine Serum by a Microplate 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 considered an important in-vitro diagnostic test for assessing thyroid function.

This microplate enzyme immunoassay methodology provides the technician with optimum sensitivity while requiring few technical variations. The results should be interpreted in the context of the patient's history and clinical findings.

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

The employment of several serum reference values 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 include 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.

\[
\text{AgAbC.W. + EnzAgAbC.W.} \rightarrow \text{AgAbC.W. + EnzAgAbC.W.}
\]

\[\text{Ag} = \text{Native Antigen (Variable Quantity)}\]

\[\text{EnzAg} = \text{Enzyme-antigen Conjugate (Constant Quantity)}\]

\[\text{AgAbC.W.} = \text{Antigen-Antibody Complex}\]

\[\text{EnzAgAbC.W.} = \text{Enzyme-antigen Conjugate-Antibody Complex}\]

\[K_1 = \text{Rate Constant of Association}\]

\[K_2 = \text{Rate Constant of Dissociation}\]

After equilibrium is attained, the antibody-bound fraction is separated from unbound antigen by decantation or aspiration. The antibody 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**: 1.0 ml/ivial – icons AF

B. **Canine Enzyme Reagent**: 1.5 ml/ivial – Icon

C. **T3/T4 Conjugate Buffer**: 13 ml/ivial – Icon

D. **N-T4 Antibody Coated Plate**: 96 wells – Icon

E. **Wash Solution Concentrate**: 20 ml/ivial – Icon

F. **Substrate Solution**: 12 ml/ivial – Icon SN

G. **Stop Solution**: 8 ml/ivial – Icon

H. **Product Insert**

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

**Note 2:** Reagent is stable for up to 60 days when stored at 2-8°C.

**Note 3:** Collect sample(s) by venipuncture in three (3) ml silicone paper.

**5.0 PRECAUTIONS**

**For In Vitro Diagnostic Use**

Not for Internal or External Use in Humans or Animals

**6.0 SPECIMEN COLLECTION AND PREPARATION**

Collect sample(s) by venipuncture in 3 ml silicone evacuated tubing(s). The usual precautions in the collection of venipuncture samples should be observed. Separate the red blood cells by using serum or plasma for the total T4 procedure. Specimen(s) may be refrigerated at 2-8°C for a maximum period of 48 hours. If the specimen(s) cannot be assayed within 48 hours, the sample(s) may be stored at temperatures of -20°C for up to 30 days. Before assay, allow the specimen(s) to equilibrate to ambient temperature (20°C - 27°C). When assayed in duplicate, 0.500 ml (50µl) of the specimen is required.

**7.0 QUALITY CONTROL**

Each laboratory should assay controls at levels in the hypothyroid, euthyroid and hyperthyroid range for monitoring assay performance. These controls should be treated as unknowns and values determined in every test procedure performed. Quality control charts should be maintained to follow the performance of the supplied reagents. Pertinent statistical methods should be employed to ascertain trends. The individual laboratory should select acceptable assay performance limits. In addition, maximum absorbance should be consistent with past experience. Significant deviation from established performance can indicate unnoticed change in experimental conditions or degradation of kit reagents. Few reagents should be used to determine the reason for the variations.

**8.0 REAGENT PREPARATION**

**Working Reagent A**: Prepare using T4 Enzyme reagent, total T4 conjugate buffer, and hydrogen peroxide (H2O2) in buffer. Store at 2-8°C.

**Working Reagent B**: Dilute contents of wash container to 1000 ml with distilled or deionized water in a suitable storage container. Store diluted reagent at 2-8°C.

**Wash Buffer**: Dilute contents of wash container to 1000 ml with distilled or deionized water in a suitable storage container. Store diluted reagent at 2-8°C.

**9.0 TEST PROCEDURE**

**Before proceeding with the assay**, bring all reagents, serum reference calibrators and controls to room temperature (20 – 27°C). The test procedure should be performed by a skilled individual or a trained professional**.

**Cal F**

1. Format the microplate's wells for each serum reference calibrator, control and patient specimen to be assayed in duplicate. Place washed and dried 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 calibrator, control or specimen into the assigned well.

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

4. Swivel the microplate gently for 20-30 seconds to mix and cover.

5. Incubate 60 minutes at 37°C.

6. Discard the contents of the microplate by decantation or aspiration. If decanting, blot the plate dry with absorbent paper.

7. **Assay can be stopped at any time between 30 to 90 minutes.**

8. **Follow the manufacturer’s instructions for proper usage.**

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

**10.0 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 for each duplicate serum reference versus the corresponding T4 concentration in µg/dl on linear graph paper (do not average the duplicates of the serum references before plotting).

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

4. To determine the concentration of T4 for an unknown, locate the average absorbance of the duplicates for each unknown on the vertical axis of the graph, find the intersecting point on the curve, and read the concentration (in µg/dl) from the horizontal axis of the graph (the duplicates 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.

**EXAMPLE 1**

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Well Number</th>
<th>Abs (A)</th>
<th>Mean Abs (B)</th>
<th>Value (µg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cal A</td>
<td>A1</td>
<td>2.648</td>
<td>2.650</td>
<td>0</td>
</tr>
<tr>
<td>Cal B</td>
<td>B1</td>
<td>2.802</td>
<td>2.609</td>
<td>0.5</td>
</tr>
<tr>
<td>Cal C</td>
<td>C1</td>
<td>2.031</td>
<td>1.344</td>
<td>1.355</td>
</tr>
<tr>
<td>Cal D</td>
<td>D1</td>
<td>0.897</td>
<td>0.939</td>
<td>2</td>
</tr>
<tr>
<td>Cal E</td>
<td>E1</td>
<td>0.976</td>
<td>0.668</td>
<td>1</td>
</tr>
<tr>
<td>Cal F</td>
<td>F1</td>
<td>0.468</td>
<td>0.469</td>
<td>8</td>
</tr>
<tr>
<td>Ctrl 1</td>
<td>A1</td>
<td>1.425</td>
<td>1.383</td>
<td>4.6</td>
</tr>
<tr>
<td>Ctrl 2</td>
<td>B1</td>
<td>0.613</td>
<td>0.680</td>
<td>16.3</td>
</tr>
<tr>
<td>Patient</td>
<td>A3</td>
<td>0.984</td>
<td>1.022</td>
<td>8.0</td>
</tr>
</tbody>
</table>

**9.0 INCUBATION**

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

10. Add 0.050 ml (50 µl) of stop 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 in each well at 450nm (using a reference wavelength of 620-630nm to minimize well imperfections) in a microplate reader. The reagent should be read within thirty (30) minutes of adding the stop solution.

**Note**: DO NOT SHAKE THE PLATE AFTER SUBSTRATE ADDITION.
11.0 Q.C. 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 established ranges.

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, thyroxine binding globulin (TBG) concentration, and the concentration alone is not sufficient to assess clinical status.
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 nonthyroidal diseases including protein wasting disease, certain liver diseases and administration of testosterone, dihydroxyandrostenedione 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.

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>
<thead>
<tr>
<th>Substance</th>
<th>Cross Reactivity</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-3-Thyronine</td>
<td>1.0000</td>
<td>10µg/dl</td>
</tr>
<tr>
<td>d-Thyroxine</td>
<td>0.9800</td>
<td>10µg/dl</td>
</tr>
<tr>
<td>d-Triiodothyronine</td>
<td>0.1500</td>
<td>100µg/dl</td>
</tr>
<tr>
<td>I-3-Thyronine</td>
<td>0.0300</td>
<td>100µg/dl</td>
</tr>
<tr>
<td>Iodothyronine</td>
<td>0.0001</td>
<td>100µg/ml</td>
</tr>
<tr>
<td>Diiodothyroine</td>
<td>0.0001</td>
<td>100µg/ml</td>
</tr>
<tr>
<td>Diiodothyronine</td>
<td>0.0001</td>
<td>100µg/ml</td>
</tr>
</tbody>
</table>

15.0 REFERENCES