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 instrumental diagnostic test for assessing thyroid function.

This microplate enzyme immunoassay methodology provides the diagnostic test for assessing thyroid function. The enzyme activity in the antibody-bound fraction, measured by reaction with luminol, is inversely proportional to the native antigen concentration. By utilizing several different serum reference concentrations 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/vial – ICON

B. Canine T4 Enzyme Reagent – 1.1 ml/vial – ICON

C. Canine T4 Biotin Reagent – 6 ml/vial – ICON

D. Canine T3-T4 Conjugate Buffer – 11 ml/vial – ICON

E. Wash Solution Concentrate – 20 ml/vial – ICON

F. Substrate Solution – 12 ml/vial – ICON

G. Stop Solution – 8 ml/vial – ICON

### 5.0 PRECAUTIONS

#### For In Vitro Diagnostic Use

Safe Disposal of kit components must be according to local regulatory and statutory requirement.

### 6.0 SPECIMEN COLLECTION AND PREPARATION

Collect sample(s) by venipuncture in three (3) ml silicone evacuated tube(s). The usual precautions in the collection of venipuncture samples should be observed. Separate the red blood cells from the centrifugation use serum or plasma for the Canine 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 to equilibrate to ambient temperature (20°C - 27°C) before assay.

When assayed in duplicate, 0.050 ml (50 µl) 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 set 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. Fresh reagents should be used to determine the reason for the variations.

### 8.0 REAGENT PREPARATION

**Note 1:** Above reagents are for a single 96-well microplate.

**Note 2:** 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 3:** For reasaying specimens with concentrations greater than 8.0 µg/dl, pipet 0.025ml (12.5µl) of the specimen versus 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.

### 9.0 TEST PROCEDURE

Before proceeding with the assay, bring all reagents, serum reference calibrators and controls to room temperature (20-27 °C). 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 any unused microwells 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 or specimen into the assigned well.
3. Add 0.050 ml (50 µl) of Working Canine T4 Enzyme Conjugate Solution to all wells (see Reagent Preparation Section). Mix and cover.
4. Incubate at room temperature for fifteen (15) minutes.
5. 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.
6. Read the absorbance in each well at 450nm (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.

### 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 serum reference versus the corresponding Canine T4 concentration in µg/dl on linear graph paper (do not average the duplicates of the serum reference for more precise plots).
3. Connect the points with a best-fit curve.
4. To determine the concentration of Canine 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 (0.426) intersects the standard curve at (1.09 µg/dl) Canine T4 concentration (See Figure 1).

![Graph Image](image-url)
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 following criteria should be met:
   - The absorbance (OD) of calibrator 0 µg/dl should be > 1.3.
   - The absorbance (OD) of calibrator 1.000 µg/dl should be within ± 10% of the assigned concentration.
3. If more than one (1) plate is used, it is recommended to repeat aspiration or decantation wash step(s) may result in poor replication and spurious results.
4. Plate readers measure vertically. Do not touch the bottom of the wells.
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.

12.0 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.
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 Canine thyroxine concentration is dependent upon a multiplicity of factors: thyroid gland function and its regulation, thyroxine binding globulin (TBG) concentration, and the binding of thyroxine to TBG. Thus, total thyroxine concentration alone is not sufficient to assess clinical status.

13.0 EXPECTED RANGES OF VALUES

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

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” 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 on 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

4. A decrease in Canine thyroxine values is found with nonthyroid diseases including protein wasting disease, certain liver diseases and others. 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.

15.0 REFERENCES