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AccuBind
ELISA Microwells

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 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.[1] 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 microplate 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 microplate well. Enzyme-T4 conjugate is added, followed by biotinylated x-T4 reagent. After the reactants are mixed, a competition reaction results between the enzyme conjugate and the native thyroxine for a limited number of antibody combining sites that become immobilized on the surface of the wells.

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 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 (Canine T4) – Type 7
The essential reagents required for an enzyme immunoassay include antibody, enzyme-antigen derivative, native antigen and a substrate that produces color.

Upon mixing biotinylated 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 antibody binding sites. The interaction is illustrated by the followed equation:

$$^{Enz}Ag + Ag + Ab_{Btn} \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} AgAb_{Btn} + {}^{Enz}AgAb_{Btn}$$

Ab_{Btn} = Anti-T4-IgG labeled with biotin (Constant Quantity)
Ag = Native Antigen (Variable Quantity)
^{Enz}Ag = Enzyme-antigen Conjugate (Constant Quantity)
$AgAb_{Btn}$ = Antigen Antibody Complex
$^{Enz}AgAb_{Btn}$ = Enzyme-antigen Conjugate -Antibody Complex
k_a = Rate Constant of Association
k_{-a} = Rate Constant of Disassociation
$K = k_a / k_{-a}$ = Equilibrium Constant

A simultaneous reaction between the biotin attached to the antibody and the streptavidin immobilized on the microwell occurs. This effects the separation of the antibody bound fraction after decantation or aspiration.
$AgAb_{Btn} + {}^{Enz}AgAb_{Btn} + \underline{Streptavidin}_{CW} \Rightarrow$ immobilized complex
$\underline{Streptavidin}_{CW}$ = Streptavidin immobilized on well
Immobilized complex = sandwich complex bound to the solid surface

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 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/vial – 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.
For SI units: µg/dl x 12.9 = nmol/L

B. Canine T4 Enzyme Reagent – 1.1 ml/vial – Icon Ⓔ
One (1) vial contains thyroxine-horseradish peroxidase (HRP) conjugates in a bovine albumin-stabilizing matrix. A preservative has been added. Store at 2-8°C.

C. Canine T3-T4 Conjugate Buffer – 11 ml/vial – Icon Ⓑ
One (1) vial contains buffer, dye, preservative, and binding protein inhibitors. Store at 2-8°C.

D. Canine T4 Biotin Reagent – 6 ml/vial – Icon ∇
One 96-well microplate coated with sheep anti-thyroxine serum and packaged in an aluminum bag with a drying agent. Store at 2-8°C.

E. Streptavidin Coated Plate – 96 wells – Icon ⇓
One 96-well microplate coated with Streptavidin and packaged in an aluminum bag with a drying agent. Store at 2-8°C.

F. Wash Solution Concentrate – 20 ml/vial – Icon
One (1) vial contains a surfactant in buffered saline. A preservative has been added. Store at 2-8°C.

G. Substrate Solution – 12 ml/vial – Icon S^N
One (1) vial contains tetramethylbenzidine (TMB) and hydrogen peroxide (H_2O_2) in buffer. Store at 2-8°C.

H. Stop Solution – 8ml/vial – Icon STOP
One (1) vial contains a strong acid (0.5M H_2SO_4). Store at 2-8°C.

I. Product Insert.

Note 1: Do not use reagents beyond the kit expiration date.
Note 2: Opened reagents are stable for sixty (60) days when stored at 2-8°C.
Note 3: Above reagents are for a single 96-well microplate.

4.1 Required But Not Provided:
1. Pipette capable of delivering 0.025 & 0.050 ml (25 & 50 µl) volumes with a precision of better than 1.5%.
2. Dispenser(s) for repetitive deliveries of 0.100 & 0.350 ml (100 & 350 µl) volumes with a precision of better than 1.5%.
3. Adjustable volume (20-200 µl) and (200-1000 µl) dispenser(s) for conjugate and substrate dilutions.
4. Microplate washer or a squeeze bottle (optional).
5. Microplate Reader with 450nm and 620nm wavelength absorbance capability.
6. Test tubes for dilution of enzyme conjugate.
7. Absorbent Paper for blotting the microplate wells.
8. Plastic wrap or microplate covers for incubation steps.
9. Vacuum aspirator (optional) for wash steps.
10. Timer.
11. Quality control materials.

5.0 PRECAUTIONS

For In Vitro Diagnostic Use
Not for Internal or External Use in Humans or Animals

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 by 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) can not 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 specimens to equilibrate to ambient temperature (20°C - 27°C). When assayed in duplicate, 0.050ml (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 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

1. **Working Canine T4 Enzyme Conjugate Solution**
   Dilute the Canine T4-enzyme reagent 1:11 with Canine T3/T4 conjugate buffer in a suitable container. For example, dilute 160 µl of Canine T4-enzyme reagent with 1.6 ml of Canine T3/T4 conjugate buffer for 32 wells (A slight excess of solution is made). This reagent should be used within twenty-four hours for maximum performance of the assay. Store at 2-8°C.
   General Formula:
   Amount of Buffer required = Number of wells ∗ 0.05
   Quantity of Canine T4 Enzyme necessary = # of wells ∗ 0.005
   i.e. = 32 x 0.05 = 1.6 ml for Canine T3/T4 conjugate buffer
   32 x 0.005 = 0.16m (160µL)l for Canine T4 enzyme
2. **Wash Buffer**
   Dilute contents of wash concentrate to 1000ml with distilled or deionized water in a suitable storage container. Store diluted buffer at 2-30°C for up to 60 days.

Note: Do not use the substrate if it looks blue.
Note: Do not use reagents that are contaminated or have bacteria growth.

9.0 TEST PROCEDURE

Before proceeding with the assay, bring all reagents, serum reference calibrators and controls to room temperature (20-27 ℃).
***** 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 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 0.050 ml (50 µl) of Working Canine T4 Enzyme Conjugate Solution to all wells (see Reagent Preparation Section).
4. Swirl the microplate gently for 20-30 seconds to mix and cover.
5. Add 0.050 ml (50 µl) of canine T4 Biotin Reagent to each well and mix.
6. Incubate 60 minutes at room temperature.
7. Discard the contents of the microplate by decantation or aspiration. If decanting, blot the plate dry with absorbent paper.
8. Add 0.350 ml (350 µl) of wash buffer (see Reagent Preparation Section), decant (tap and blot) or aspirate. Repeat two (2) additional times for a total of three (3) washes. **An automatic or manual plate washer can be used. Follow the manufacturer's instruction for proper usage. If a squeeze bottle is employed, fill each well by depressing the container (avoiding air bubbles) to dispense the wash. Decant the wash and repeat two (2) additional times.**
9. Add 0.100 ml (100 µl) of substrate solution to all wells. **Always add reagents in the same order to minimize reaction time differences between wells.**
   DO NOT SHAKE THE PLATE AFTER SUBSTRATE ADDITION
10. Incubate at room temperature for fifteen (15) minutes.
11. 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.**
12. 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.**

Note: For reassaying specimens with concentrations greater than 8.0 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.

10.0 CALCULATION OF RESULTS

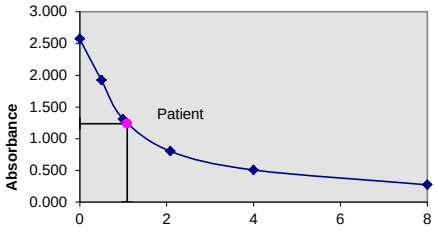
A dose response curve is used to ascertain the concentration of thyroxine in unknown specimens.
1. Record the absorbance obtained from the printout of the microplate reader as outlined in Example 1.
2. Plot the absorbance for each duplicate serum reference versus the corresponding Canine T4 concentration 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 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 (1.236) intersects the standard curve at (1.09 µg/dl) Canine 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

Sample I.D.	Well Number	Abs (A)	Mean Abs (B)	Value (µg/dl)
Cal A	A1	2.567	2.571	0
	B1	2.575		
Cal B	C1	1.896	1.920	0.5
	D1	1.945		
Cal C	E1	1.312	1.309	1
	F1	1.306		
Cal D	G1	0.810	0.801	2
	H1	0.792		
Cal E	A2	0.522	0.507	4
	B2	0.492		
Cal F	C2	0.270	0.273	8
	D2	0.276		
Ctrl 1	E2	0.585	0.593	3.11
	F2	0.601		
Ctrl 2	G2	0.433	0.441	4.58
	H2	0.450		
Patient	A3	1.239	1.236	1.09
	B3	1.234		

Figure 1



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 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.[3,4] Thus, total thyroxine concentration alone is not sufficient to assess clinical status.
3. Canine thyroxine values may be elevated under conditions such as a female dog 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 Canine thyroxine values is found with nonthyroid[2] 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.[3]

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" 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

Within Assay Precision (Values in µg/dl)

Sample	N	X	σ	C.V.
Low	20	1.05	0.049	4.7%
Normal	20	2.21	0.138	6.2%
High	16	4.24	0.18	4.3%

TABLE 3

Between Assay Precision (Values in µg/dl)

Sample	N	X	σ	C.V.
Low	10	3.0	0.25	8.3%
Normal	10	8.7	0.32	3.7%
High	10	16.3	0.69	4.2%

*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 doses of interfering substance to dose of thyroxine needed to displace the same amount of conjugate.

Substance	Cross Reactivity	Concentration
l–Thyroxine	1.0000	-
d-Thyroxine	0.9800	10µg/dl
d-Triiodothyronine	0.0150	100µg/dl
l–Triiodothyronine	0.0300	100µg/dl
Iodothyrosine	0.0001	100µg/ml
Diiodothyrosine	0.0001	100µg/ml
Diiodothyronine	0.0001	100µg/ml

15.0 REFERENCES

1. Peterson M, Melian C and Nichols, R, "Measurement of serum total thyroxine, free thyroxine and thyrotropin concentrations for diagnosis of hypothyroidism in dogs", *JAVMA,* 211, 1396 (1997).
2. Kantrowitz B, Peterson M, Melian C & Nichols R., "Serum total thyroxine, free thyroxine and thyrotropin concentrations in dogs with nonthyroid disease", *JAVMA,* 219, 765 (2001).
3. Young, D.S., Pestaner, L.C., and Gilberman, U., "Effects of Drugs on Clinical Laboratory Tests." *Clinical Chemistry* 21, 3660. (1975)

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MP12225 **Product Code: 12225-300**

Size	96(A)
Reagent (fill)	A) 1ml set
	B) 1 (1.1ml)
	C) 1 (11ml)
	D) 1 (6ml)
	E) 1 (plate)
	F) 1 (20ml)
	G) 1 (12ml)
	H) 1 (8ml)

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