TRUFORMA® Point-of-Care Canine
Free Thyroxine (fT4) Assay

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Key Messages

- Accurate and precise measurement of canine free thyroxine (fT4) levels is needed for the diagnosis of canine thyroid disease.

- The TRUFORMA® platform uses innovative bulk acoustic wave (BAW) technology to provide a non-optical and fluorescence-free detection system for diagnostic use at the point of care (POC) in veterinary clinics.

- The dynamic range of the TRUFORMA canine fT4 assay allows for the quantification of fT4 throughout the range necessary to evaluate canine thyroid function.

- The high precision and correlation to a reference laboratory assay shown for the TRUFORMA canine fT4 assay provides veterinarians with accurate and reliable diagnostic results at the POC, creating opportunities for improved patient treatment and real-time client communication.

Introduction

Accurately diagnosing thyroid dysfunction can be a challenge in veterinary practice. Current immunoassay testing methods performed at reference laboratories are complex and costly, whereas available POC tests deliver variable performance.[1] An accurate, precise fT4 assay at the veterinary POC that allows for faster diagnosis and treatment while eliminating falsely elevated fT4 concentrations due to sample heating during transport to the reference laboratory is needed.[2] The TRUFORMA platform, which uses BAW sensor technology, is the first POC canine fT4 assay that provides rapid, reliable, and accurate measurement of fT4 to aid veterinarians in differentiating healthy dogs from those with thyroid disease.

> The objectives of this study were to:
>
> - Determine analytical performance attributes for the TRUFORMA canine fT4 assay.
> - Describe how the TRUFORMA canine fT4 assay differs from a commercially available assay.
> - Compare TRUFORMA canine fT4 assay performance with an assay routinely used at veterinary diagnostic laboratories to identify dogs with thyroid disease.

Clinical Significance of fT4 Testing

Thyroxine (T4) and triiodothyronine (T3) are synthesized and secreted by the thyroid gland in response to stimulation by thyroid-stimulating hormone (TSH), a glycoprotein produced by thyrotrope cells in the anterior pituitary gland (**Figure 1**). T4 and T3 are involved in the negative feedback regulatory mechanisms, which control release of thyrotropin-releasing hormone (TRH) and TSH from the hypothalamus and pituitary gland, respectively.

Once secreted, >99% of T4 is bound to plasma proteins and <1% is unbound or free T4 (fT4). Total T4 (tT4) testing measures both protein-bound and fT4 levels in the blood, whereas fT4 testing measures unbound hormone only.

Figure 1. Hypothalamic-Pituitary-Thyroid Axis
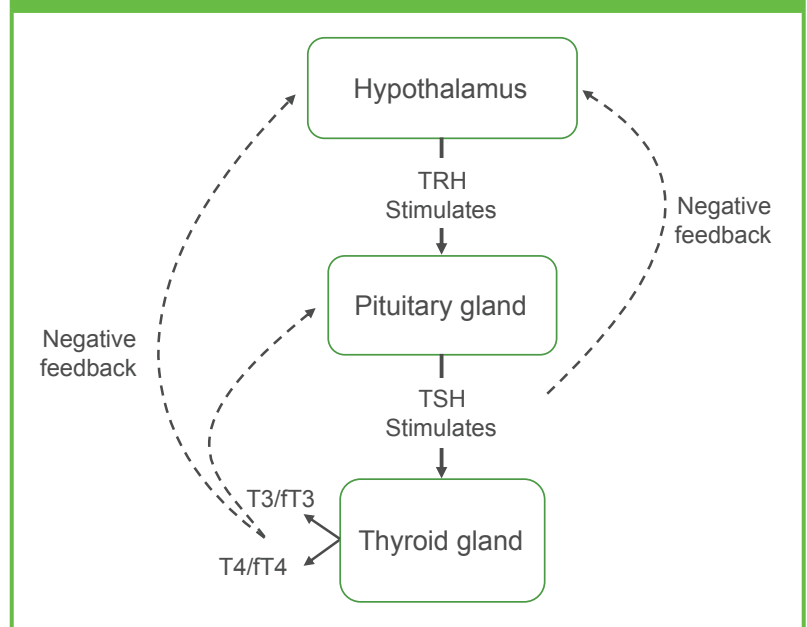
fT3, free triiodothyronine; fT4, free thyroxine; T3, triiodothyronine; T4, thyroxine; TRH, thyrotropin-releasing hormone; TSH, thyroid-stimulating hormone.

Canine fT4

Measurement of serum tT4, fT4, and TSH concentrations is currently recommended for the assessment of canine thyroid gland function.[3] Serum tT4 is a useful screening test for hypothyroidism and is commonly used to monitor treatment with thyroid supplementation. However, measurement of fT4 and TSH concentrations are necessary to diagnose canine thyroid disease because total T4 and T3 levels may be affected by nonthyroidal diseases and medications.[3,4] Measurement of fT4 concentration has been shown to have higher sensitivity and specificity than measurement of tT4 concentration in diagnosing canine hypothyroidism.[5]

- Accurate and reliable measurement of canine fT4 levels is needed for the diagnosis of thyroid disease.
- A high-performance veterinary POC immunoassay for fT4, with published performance data, is needed.

TRUFORMA Platform

The TRUFORMA platform uses BAW sensor technology to provide a non-optical and fluorescence-free detection system for diagnostic use at the POC. BAW technology is extremely reliable and precise, and has been well tested in products across industries such as telecommunications and aerospace. Functionalized BAW sensors consist of multiple resonators, each composed of a piezoelectric material subjected to an electrical field. The resonators can be coated with biological detection reagents such as antibodies or nucleic acids for immunoassay and molecular testing, respectively. Whereas most current enzyme-based immunoassays use optical sensors to detect the generation of luminescence or color change, BAW biosensors used as part of TRUFORMA assays measure both binding events and the insoluble product that is generated by the enzymes that accumulate on the sensor surface, thereby creating a frequency shift in resonance proportional to the mass accumulated on the sensor (**Figure 2**). Veterinary medical professionals were the first to use the BAW biosensor technology in a POC diagnostic setting though this technology now has emergency use authorization (EUA) for rapid COVID-19 antigen testing in humans.

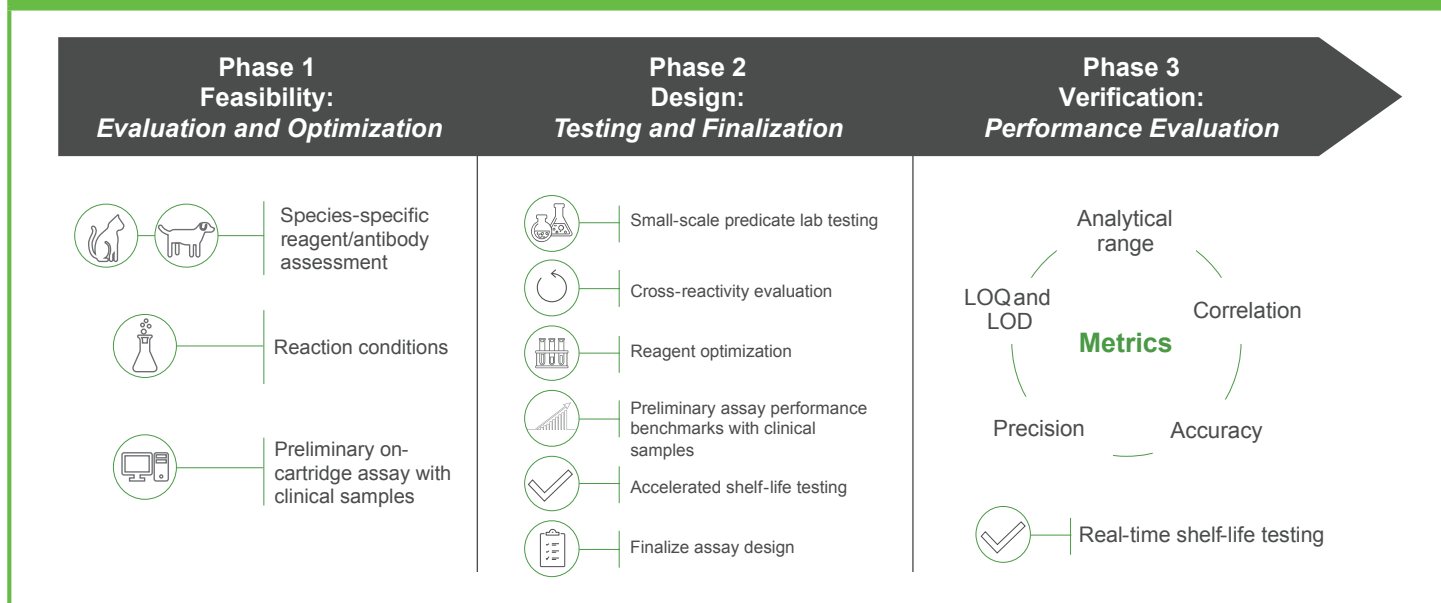**Figure 2.** BAW Technology in the TRUFORMA fT4 Immunoassay
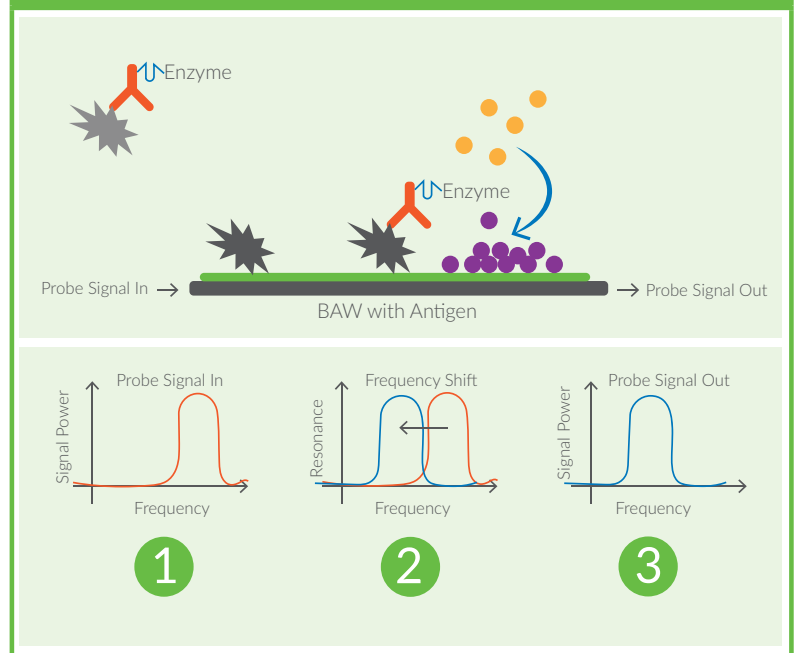
The TRUFORMA fT4 assay is a competitive immunoassay in which the BAW sensor is coated with antigen (dark gray). Antigen present in the sample (light gray) binds to an antibody-enzyme conjugate in solution and prevents the antibody from binding to the antigen-coated biosensor. After several wash steps, an enzyme substrate is exposed to the BAW biosensor surface, and bound enzyme converts the substrate to an insoluble product that accumulates on the BAW biosensor surface. This is measured as a shift in frequency by the BAW biosensor. The signal is inversely proportional to the amount of analyte present in the sample. BAW, bulk acoustic wave; fT4, free thyroxine.

fT4 Assay Development Overview

The TRUFORMA canine fT4 assay is a competitive immunoassay that uses a monoclonal anti-T4 antibody, which was selected for optimal performance in canine testing. Using the industry standard recommendations for bioanalytical method validation and the Clinical and Laboratory Standards Institute (CLSI) guidelines on method comparison and bias estimation (EP09c),[6,7] the TRUFORMA assay performance requirements were chosen to meet or exceed reference laboratory performance to provide unparalleled performance at the POC. The 3 phases of the canine fT4 assay development were designed to create a high-quality and reliable POC assay and included feasibility evaluation and optimization with species-specific assessment, design with testing of preliminary assay performance, and performance verification (**Figure 3**).

Figure 3. Overview of the TRUFORMA Canine fT4 Assay Development

Phase 1 Feasibility: *Evaluation and Optimization*	Phase 2 Design: *Testing and Finalization*	Phase 3 Verification: *Performance Evaluation*
Species-specific reagent/antibody assessment	Small-scale predicate lab testing	Analytical range
Reaction conditions	Cross-reactivity evaluation	LOQ and LOD / Correlation
Preliminary on-cartridge assay with clinical samples	Reagent optimization	Metrics
	Preliminary assay performance benchmarks with clinical samples	Precision / Accuracy
	Accelerated shelf-life testing	Real-time shelf-life testing
	Finalize assay design	

fT4, free thyroxine; LOD, limit of detection; LOQ, limit of quantitation.

Assay Verification Results

Measurement of fT4 by equilibrium dialysis has historically been considered the reference standard; however, it is time consuming, expensive, and not widely available. An analog chemiluminescent enzyme immunoassay method is also commonly used to measure canine fT4 at reference laboratories because it is faster and less expensive. The TRUFORMA canine fT4 assay performance was compared with the reference laboratory Siemens IMMULITE® 2000 Veterinary Free T4 assay. Normal, diseased, diluted, and spiked canine samples were analyzed.

Analytical Precision

Analytical precision was evaluated by measuring the variability in assay results (between-run percentage coefficient of variation [%CV]) under the normal operating conditions in the laboratory. Precision was evaluated by testing 3 canine samples with varying free T4 concentrations. Each sample was tested with 5 replicates on 5 separate days on 2 different instruments for a total of 150 results. Observed %CV was calculated.

The TRUFORMA canine fT4 assay demonstrated an overall %CV of <20% (**Figure 4**). For each sample, the overall %CV was comparable to the %CV for each instrument, indicating repeatability across instruments. A ± 25% between-runs %CV is recommended and is considered a quality %CV for measuring assay precision in a ligand-binding assay.[6]

Figure 4. Precision of the TRUFORMA Canine fT4 Assay
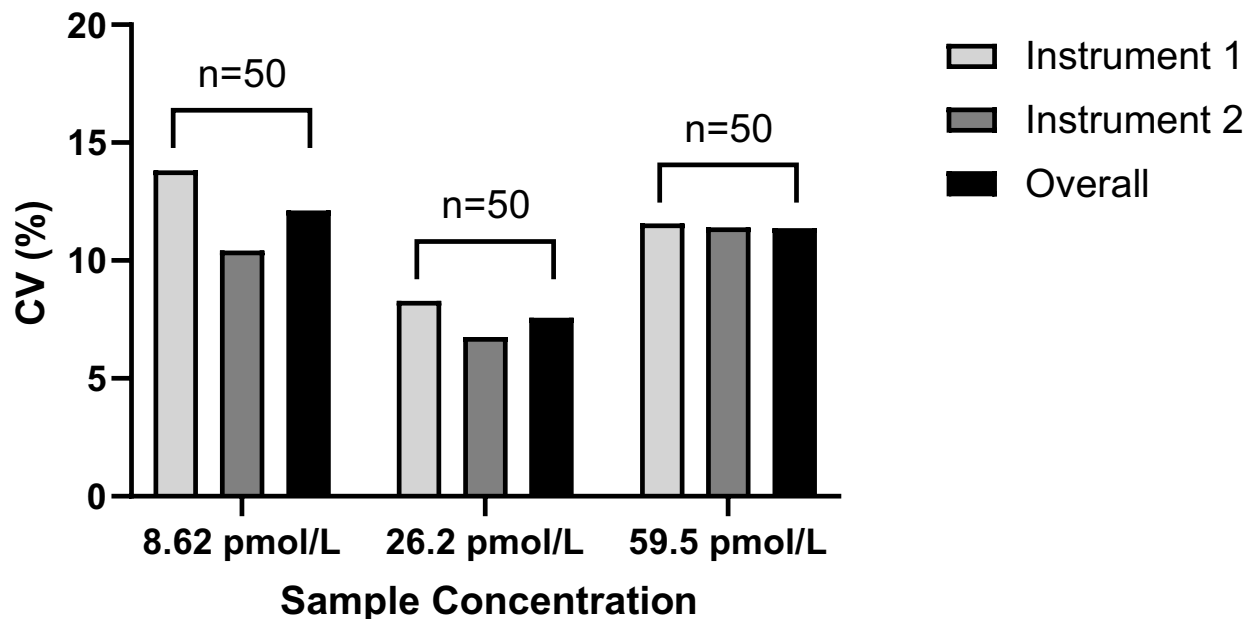
%CV was calculated for 3 serum samples with varying fT4 concentrations using 150 runs. CV, coefficient of variation; fT4, free thyroxine

Time to Test Results, Dynamic Range, and Limit of Quantitation

Time to Test Results (TTR) refers to the amount of time elapsed from TRUFORMA initiating the test to generating the final result. The TRUFORMA fT4 assay had a TTR of 16 minutes. Compared with a reference laboratory, the POC testing of the TRUFORMA platform provides more timely results, which can improve patient care through faster clinical decision making and communication with pet owners.

Dynamic range refers to the span of test result values that can be accurately measured by an assay. The analytical sensitivity of the TRUFORMA canine fT4 assay was calculated to be 4.00 pmol/L and this value was set as the lower end of the dynamic range. The upper end of the dynamic range was set at 77.2 pmol/L as predefined precision metrics were met ensuring accurate and reproducible quantitation of canine serum samples (**Table 1**).

The TRUFORMA canine fT4 assay permits the quantification of fT4 throughout the range necessary for evaluating canine thyroid function. The availability of this assay at the POC can improve the ability to diagnose and treat canine thyroid disease without the need to send samples to a reference laboratory.

Table 1. Summary of TTR and Dynamic Range of the TRUFORMA Canine fT4 Assay Compared with the Siemens IMMULITE 2000 Veterinary Free T4 Assay

	TRUFORMA	IMMULITE
TTR, minutes	16	35
Dynamic range, pmol/L	4.0-77.2	3.9-77.2
Analytical Sensitivity, pmol/L	4.0	3.9

fT4, free thyroxine; TTR, time to test results

Eight calibrators with known concentrations of fT4 were tested using TRUFORMA canine fT4 cartridges. Each calibrator was run with 9 replicates across 3 different instruments, and the average value was used to generate a standard curve. The linearity and reportable range of the TRUFORMA canine fT4 assay illustrates linear performance within the clinically relevant range for the TRUFORMA canine fT4 assay (**Figure 5**).

**Figure 5.** Standard Curve for the TRUFORMA Canine fT4 Assay
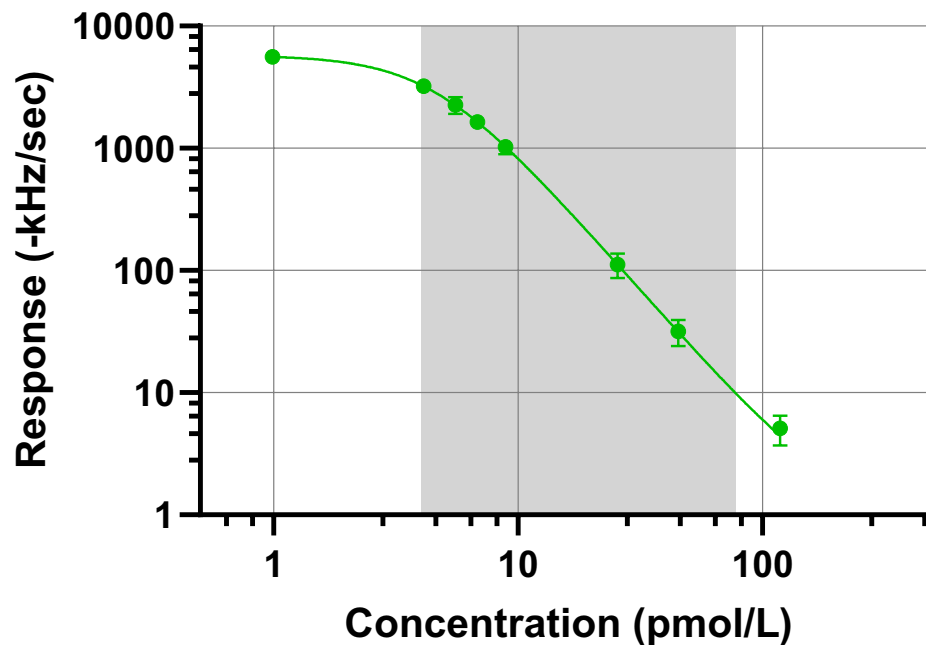
Eight calibrators with known concentrations of fT4 were used to generate a standard curve. The shaded region represents the dynamic range of the TRUFORMA canine fT4 Assay. fT4, free thyroxine.

Assay Correlation Between the TRUFORMA Canine fT4 Assay and Siemens IMMULITE 2000 Veterinary Free T4 Assay

Assay correlation and Bland-Altman bias plot analysis evaluate the agreement and commutability of a new test method with a comparative or reference method. A total of 66 matched serum samples were run on the same freeze-thaw cycle with both the TRUFORMA and Siemens IMMULITE 2000 devices. The instrument reports free T4 concentrations based on the standard curve, and the results for each sample were used to generate correlation and bias plots.

The TRUFORMA canine fT4 assay showed high correlation (R=0.93) with the Siemens IMMULITE Veterinary Free T4 Assay (**Figure 6A**), with no apparent bias (bias, -0.47 pmol/L; non-zero one-sample t-test *p*-value, 0.50) when processing canine serum samples (**Figure 6B**).

Figure 6. Correlation and Bias of TRUFORMA Canine fT4 Assay Compared with the Siemens IMMULITE 2000 Veterinary Free T4 Assay
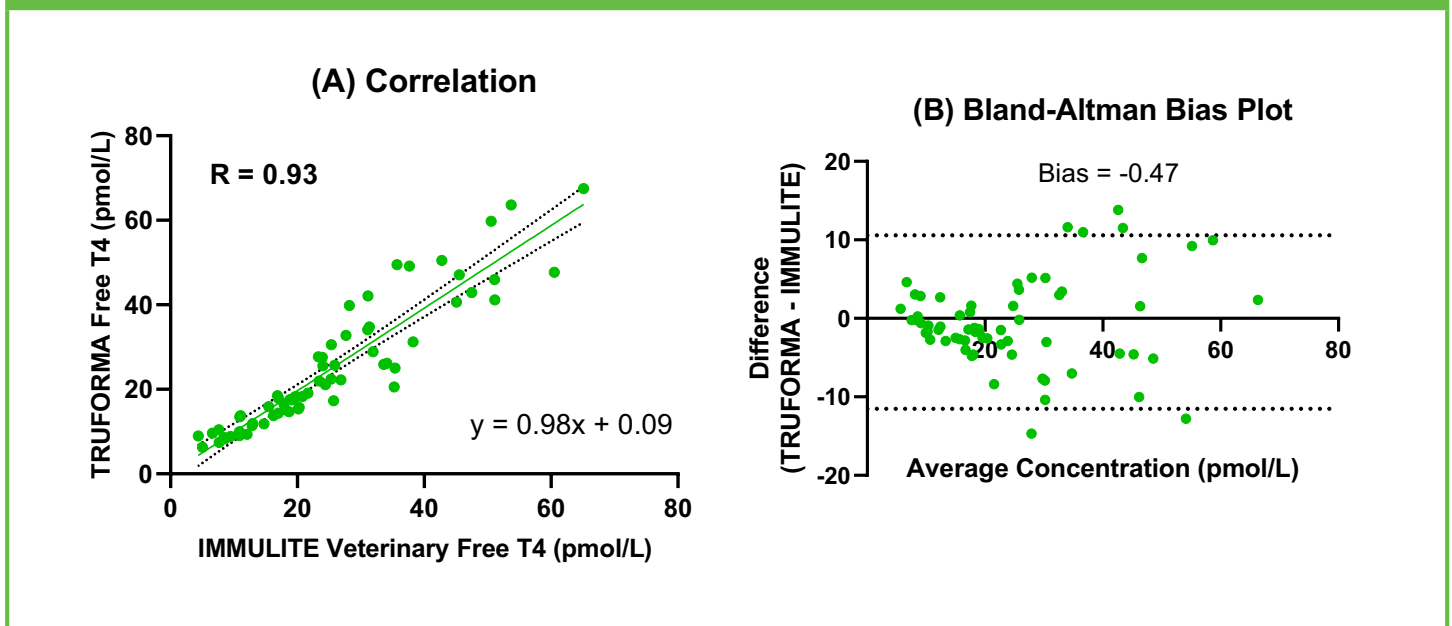
(A) Correlation studies were performed comparing the results from the TRUFORMA canine fT4 and Siemens IMMULITE 2000 Veterinary Free T4 assays using 66 canine samples. Dotted lines represent the 95% CI for the linear regression line. (B) Bland-Altman bias plots were generated by plotting the mean concentration vs the difference (TRUFORMA – IMMULITE). Dotted lines represent 95% limits of agreement. fT4, free thyroxine.

Cross-Reactivity

Known amounts of fT4 and potential cross-reactants were added to depleted serum and tested in triplicate using the TRUFORMA canine fT4 assay.

No significant cross-reactivity was observed in the TRUFORMA canine fT4 assay, and no cross-reactants interfered with the reported free T4 concentrations (**Table 2**).

Table 2. Summary of Cross-Reactivity for the TRUFORMA Canine fT4 Assay		
Material	**Concentration**	**Cross-Reactivity, %**
Albumin	5,000,000,000 ng/dL	ND
D-T4	1000 ng/dL	0.020
3,5 Diiodo-L-tyrosine	100,000 ng/dL	ND
L-T3	100,000 ng/dL	0.44
L-T3	1000 ng/dL	0.10
5,5-Diphenylhydantoin	4,000,000 ng/dL	ND
TETRAC	2000 ng/dL	0.01
Methimazole	1,000,000 ng/dL	ND
Phenylbutazone	1,000,000 ng/dL	ND
Oleic acid	10 mmol/L	ND

fT4, free thyroxine; ND, not detected; T3, triiodothyronine; T4, thyroxine; TETRAC, tetraiodothyroacetic acid.

Conclusions

The TRUFORMA canine fT4 assay demonstrated high precision as a POC diagnostic platform, with a dynamic range that permits quantification of fT4 levels throughout the range necessary to evaluate canine thyroid function. Availability of the TRUFORMA canine fT4 immunoassay at the POC will eliminate inaccurate results due to sample heating during shipment to a reference laboratory. Furthermore, when used in combination with the TRUFORMA tT4 and TSH assays, the TRUFORMA canine fT4 assay will provide a more comprehensive thyroid evaluation at the POC than is currently available and this will in turn improve diagnostic confidence.

As the first commercially available POC canine free T4 test, the TRUFORMA canine fT4 assay provides veterinarians with accurate and reliable diagnostic results at the POC, allowing for improved client communication and patient treatment.

Abbreviations and Acronyms

BAW   Bulk Acoustic Wave
CLSI   Clinical and Laboratory Standards Institute
CV   Coefficient of Variation
fT3   Free Triiodothyronine
fT4   Free Thyroxine
LOD   Limit of Detection
LOQ   Limit of Quantitation
ND   Not Detected
POC   Point of Care
T3   Triiodothyronine
T4   Thyroxine
TETRAC   Tetraiodothyroacetic Acid
TRH   Thyrotropin-Releasing Hormone
TSH   Thyroid-Stimulating Hormone
tT4   Total Thyroxine
TTR   Time to Test Results

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