ZOMEDICA®

# TRUFORMA® Point-of-Care Canine Cortisol Assay

Dannyalle Breeden, Research and Development Scientist, Zomedica

Ian Harmon, Sr. Director of Research and Development, Zomedica

Drew Narwold, Sr. Research and Development Scientist, Zomedica

## Key Messages

- Accurate and precise measurement of cortisol levels in canines is needed to diagnose and manage hypoadrenocorticism and hyperadrenocorticism.
- 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 cortisol assay allows for the quantification of high and low concentrations of cortisol within the same assay, which is vital for accurate diagnosis of hyperadrenocorticism and hypoadrenocorticism in dogs.
- The high precision and correlation to a reference laboratory assay shown for the TRUFORMA canine cortisol 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 and monitoring canine adrenal dysfunction is challenging in veterinary practice due to the complexity of current reference lab methodologies for immunoassays and the variability in performance of the POC tests. There is a need for a POC cortisol assay that has been optimized for canine samples. The TRUFORMA platform uses BAW sensor technology to provide veterinarians rapid, reliable, and accurate measurement of canine cortisol levels at the POC.

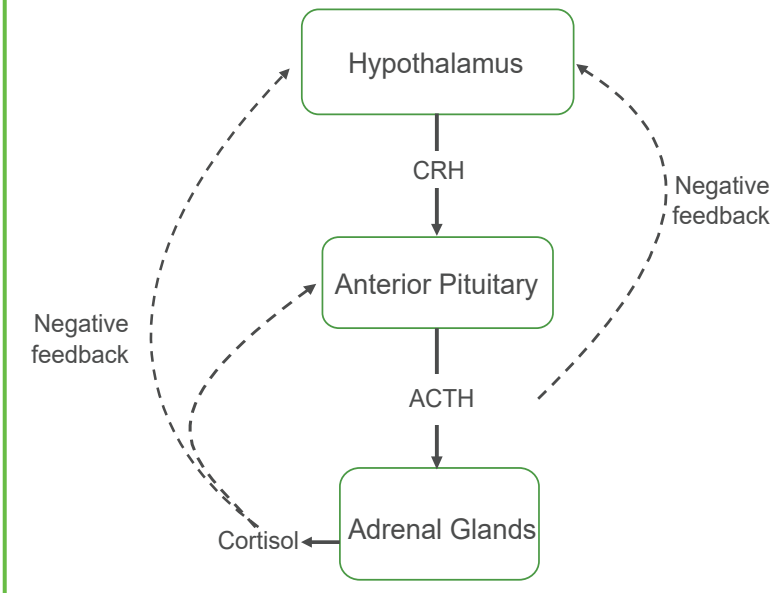
The objectives of this study were to:

- Determine analytical performance attributes for the TRUFORMA canine cortisol assay.
- Describe how the TRUFORMA canine cortisol assay differs from currently available assays.
- Compare TRUFORMA canine cortisol assay performance with an assay used as part of the standard of care at veterinary diagnostic laboratories.

# Clinical Significance of Canine Cortisol Testing

Cortisol is a steroid hormone produced by the adrenal glands in response to adrenocorticotropic hormone (ACTH) secretion from the pituitary gland. Cortisol release occurs in response to stimulation — including stress, hypoglycemia, and physical exercise — and is regulated through a negative feedback loop (**Figure 1**). Two major adrenal disorders common to canines are hyperadrenocorticism, also known as Cushing's disease, and hypoadrenocorticism, also known as Addison's disease. Hyperadrenocorticism is caused by overproduction of adrenal hormones and is most commonly the result of pituitary or adrenal gland tumors or excessive use of steroids. Hypoadrenocorticism is caused by decreased adrenal hormone production and is most often due to immune-mediated destruction of the adrenal glands. Other causes of hypoadrenocorticism include adrenal gland destruction from physical trauma or infection, pituitary or adrenal tumors, abrupt withdrawal from steroid treatment, or excessive use of adrenolytic agents.

Figure 1. Hypothalamic-Pituitary Adrenal Axis




CRH, corticotrophin-releasing hormone; ACTH, adrenocorticotropic hormone

## Canine Cortisol

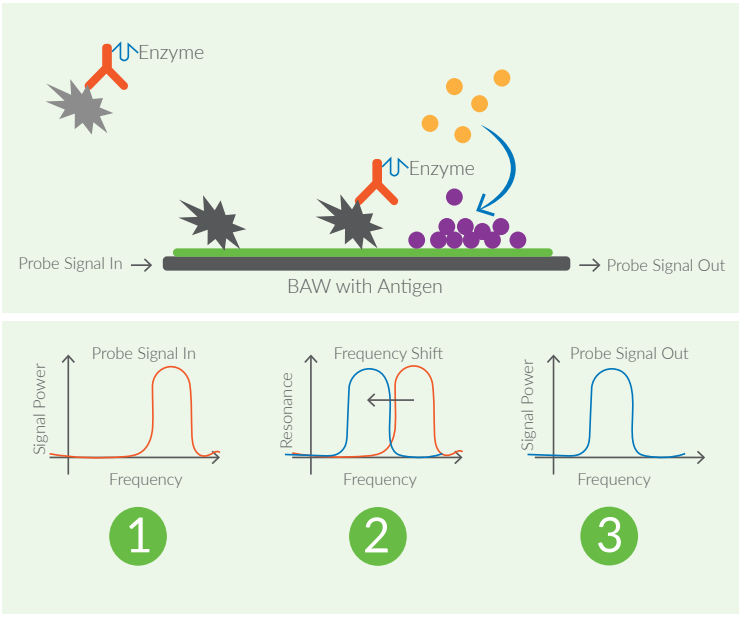
A definitive diagnosis of adrenal disease in canines currently relies on comparing serum cortisol levels at baseline to levels post-dynamic adrenal testing. Dynamic adrenal testing for diagnosing and differentiating hyperadrenocorticism includes ACTH stimulation testing, low-dose dexamethasone suppression testing (LDDST), and/or high-dose dexamethasone suppression testing (HDDST).[1] Measurement of basal cortisol can also be used as a screening test to rule out or exclude the diagnosis of hypoadrenocorticism.[2, 3, 4] Once a diagnosis of hyperadrenocorticism has been reached, a patient's baseline cortisol as well as levels post-ACTH stimulation aid in optimizing treatment dosages and long-term monitoring of patients. This is especially useful for avoiding iatrogenic adrenocortical atrophy in Cushing's patients. There are radioimmunoassay (RIA), chemiluminescent assay, and enzyme-linked immunosorbent assay (ELISA) options available for measuring cortisol levels at reference labs with some having been validated for use in canines.[5] However, the limited dynamic range of many of these cortisol assays, especially suboptimal lower limits of quantification, increases the risk for misdiagnosis of hypoadrenocorticism. A limited number of commercial POC cortisol assays are also available, but validation data from these assays have not been published in peer-reviewed literature.[1] Clearly, there is a need for accurate, reliable quantification of cortisol at the POC.

- Accurate and reliable measurements of cortisol levels are needed for the diagnosis of adrenal dysfunction in canines.
- A high-performance veterinary POC immunoassay for cortisol, 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, for immunoassay testing. 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

Figure 2. BAW Technology in the TRUFORMA Canine Cortisol Immunoassay



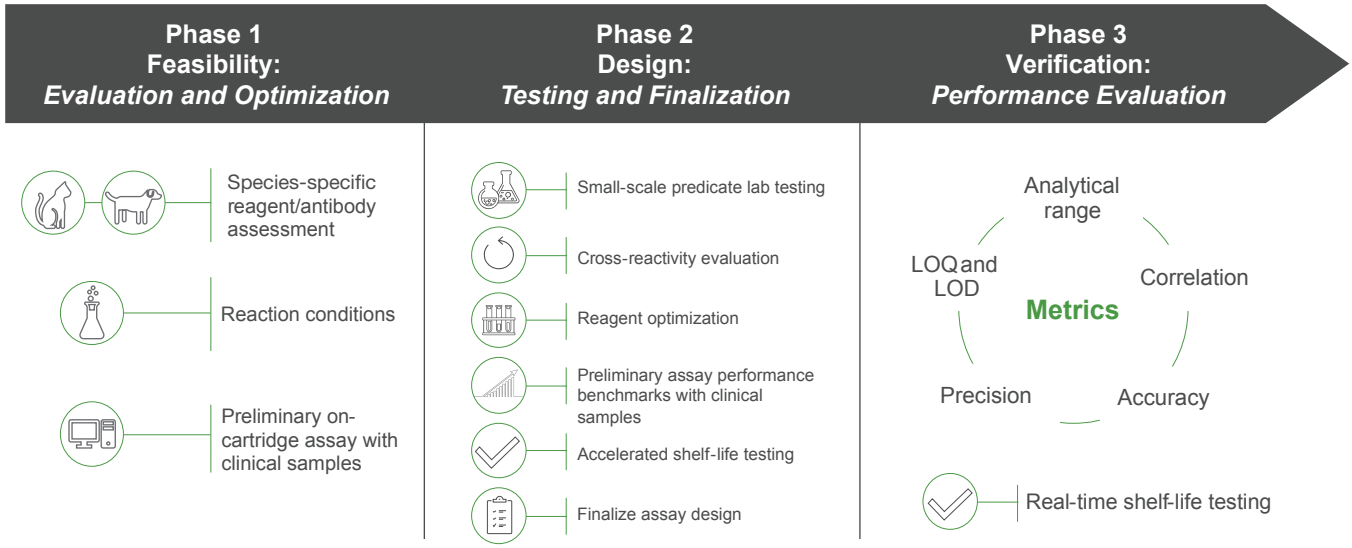

The TRUFORMA cortisol assay is a competitive immunoassay in which the BAW sensor is coated with antigen (dark gray). Antigen present in the sample (light gray) binds to an antibody-enzyme conjugate in solution and prevents the antibody from binding to the antigen-coated biosensor. After several wash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

proportional to the mass accumulated on the sensor (**Figure 2**). Veterinary medical professionals were the first to use the BAW sensor technology in a POC diagnostic setting though this technology subsequently received emergency use authorization (EUA) for rapid COVID-19 antigen testing in humans.

## Cortisol Assay Development Overview

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

Figure 3. Overview of the TRUFORMA Canine Cortisol Assay Development




LOD, limit of detection; LOQ, limit of quantitation

## Assay Verification Results

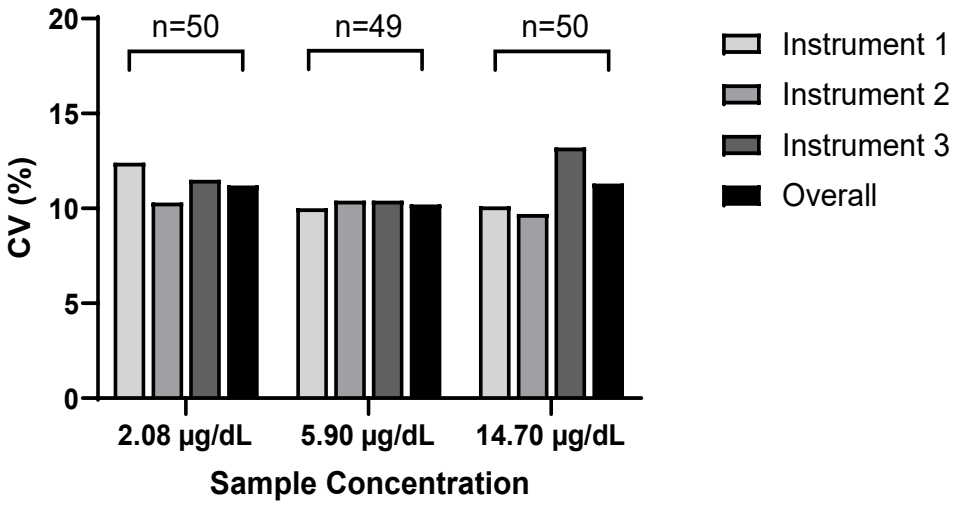
The TRUFORMA canine cortisol assay analytical performance was evaluated and compared with the reference laboratory predicate Siemens IMMULITE® 2000 veterinary cortisol assay, an automated solid-phase chemiluminescent competitive immunoassay. Samples from healthy and diseased canines as well as spiked canine samples were analyzed. Serum samples from baseline blood draws, low-dose dexamethasone suppression tests, and ACTH stimulation tests were also included.

## 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 three canine samples with varying cortisol concentrations. Each sample was tested with five replicates over five separate days on two different instruments, for a total of 150 results. Observed %CV was calculated.

The TRUFORMA canine cortisol assay demonstrated an overall %CV of <15% (**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 Cortisol Assay




%CV was calculated for three serum samples with varying cortisol concentrations using 150 runs. One statistical outlier was removed from the sample at 5.90 µg/dL following CLSI EP05-A3 guidelines. CV, coefficient of variation.

## Time to Test Results (TTR), Dynamic Range, and Limit of Quantitation

Time to Test Results (TTR) refers to the amount of time elapsed from TRUFORMA initiating the test to generation of the final result. The POC testing of the TRUFORMA platform provides more timely and actionable results compared to the reference lab, 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, and limit of quantitation (LOQ) refers to the lowest (lower LOQ [LLOQ]) and highest (upper LOQ [ULOQ]) analyte concentrations that can be reliably detected with predefined accuracy and precision.

The TRUFORMA canine cortisol assay has an LLOQ that is slightly lower than that of the IMMULITE Veterinary Cortisol assay (**Table 1**). The TRUFORMA canine cortisol assay's dynamic range allows the quantification of both clinically high and clinically low cortisol concentrations. The enhanced dynamic range can improve the ability to diagnose and treat at the POC without the need to send samples to a reference laboratory.

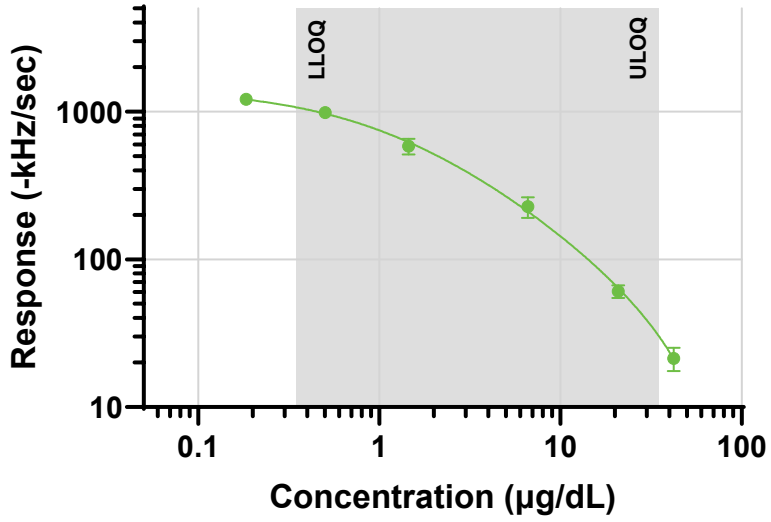
Table 1. Summary of TTR, Dynamic Range and LLOQ for the TRUFORMA Canine Cortisol Assay Compared with the Siemens IMMULITE Veterinary Cortisol Assay

| Test | TRUFORMA | IMMULITE |
|---|---|---|
| | Canine Optimized | Animal Optimized |
| TTR, minutes | <16.0 | >30.0 |
| Dynamic range, µg/dL | 0.349-35.0 | 1.0-50.0 |
| LLOQ, µg/dL | 0.349 | 1.0 |

LLOQ, lower limit of quantitation; TTR, time to test results.

Six calibrators with known concentrations of cortisol were tested using the TRUFORMA cortisol cartridge. Each calibrator was run with nine replicates across three different instruments, and the average value was used to generate a standard curve. The linearity and reportable range of the TRUFORMA cortisol assay illustrates linear performance within the clinically relevant range (**Figure 5**).

Figure 5. Standard Curve of the TRUFORMA Canine Cortisol Assay



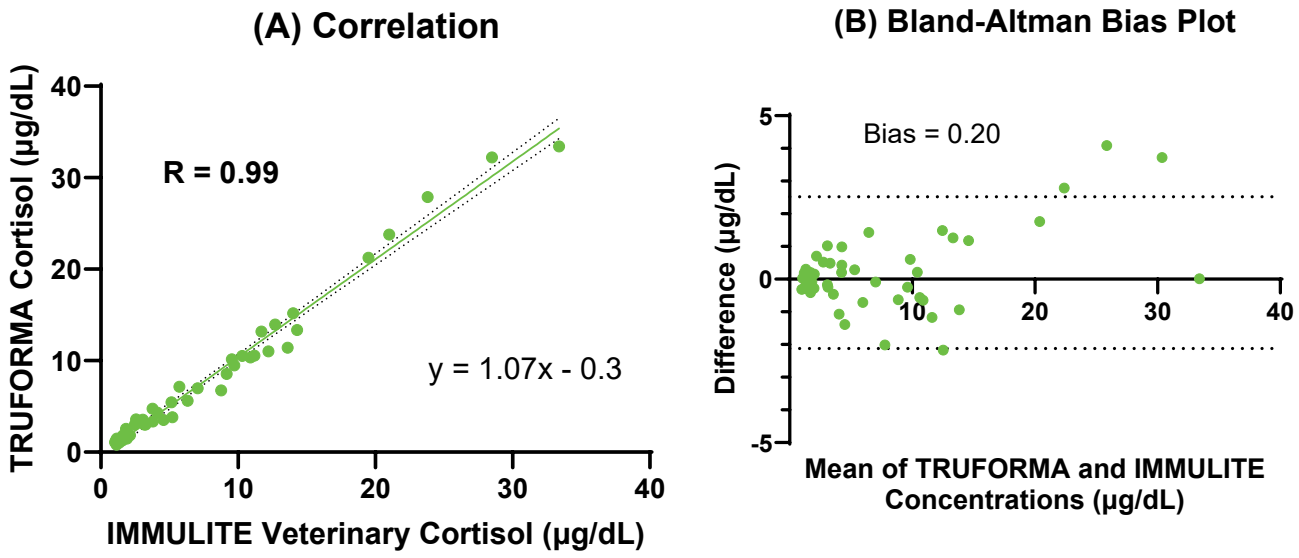

Six calibrators with known concentrations of cortisol were used to generate a standard curve for canine analysis. The shaded region represents the reported dynamic range. LLOQ, lower limit of quantitation; ULOQ, upper limit of quantitation.

## Assay Correlation Between the TRUFORMA Canine Cortisol Assay and Siemens IMMULITE Veterinary Cortisol 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 sixty-four canine serum samples were run on the same freeze-thaw cycle on the TRUFORMA and Siemens IMMULITE devices. The instruments report concentrations based on their respective standard curves and these results were used to generate correlation and bias plots.

Forty-nine of the sixty-four serum samples tested were quantifiable on both platforms while fifteen were <1.0 µg/dL with the IMMULITE veterinary cortisol assay. All fifteen of these samples also fell below 1.0 µg/dL on the TRUFORMA canine cortisol assay with concentration values ranging from <0.349 to 0.94 µg/dL. For the 49 samples that generated valid quantifiable results on both devices, the TRUFORMA canine cortisol assay showed high correlation (R=0.99) with the Siemens IMMULITE veterinary cortisol assay (**Figure 6A**), with no apparent bias (bias, 0.20 µg/dL; 95% CI, -2.1 to 2.5) (**Figure 6B**) across the dynamic range.

Figure 6. Correlation and Bias of TRUFORMA Canine Cortisol Assay Compared with the Siemens IMMULITE Veterinary Cortisol Assay




(A) Correlation studies were performed comparing the results from the TRUFORMA canine cortisol and IMMULITE veterinary cortisol assays using 64 canine serum samples. 49 serum samples provided valid quantifiable results for both assays and were analyzed. Dotted lines represent 95% CI for the linear regression line. (B) Bland-Altman bias plots were generated by plotting the mean concentrations (average of TRUFORMA and IMMULITE) vs. the difference (TRUFORMA – IMMULITE). Dotted lines represent 95% limits of agreement.

## Cross-Reactivity

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

Cross-reactivity was measured in the TRUFORMA canine cortisol assay, and the results are reported in **Table 2**.

Table 2. Summary of Cross-Reactivity for the TRUFORMA Canine Cortisol Assay

| Material | Concentration, µg/dL | Cross-Reactivity, % |
|---|---|---|
| Aldosterone | 1000 | ND |
| Triamcinolone | 5000 | ND |
| Spironolactone | 1000 | ND |
| Estriol | 100 | ND |
| Estrone | 500 | ND |
| Dexamethasone | 400 | 5.7 |
| Methylprednisolone | 200 | 4.0 |
| Corticosterone | 400 | 2.3 |
| Cortisone | 400 | 0.4 |
| Progesterone | 400 | 0.2 |
| Betamethasone | 1000 | 0.2 |
| Methotrexate | 100 | 0.2 |
| 17 α-hydroxyprogesterone | 400 | 0.1 |
| Desoxycorticosterone pivalate | 1000 | 0.1 |
| | 100 | 0.1 |
| | 10 | ND |
| Trilostane | 1000 | 0.1 |
| | 100 | ND |
| | 10 | ND |
| Prednisolone | 240 | 13.1 |
| | 8 | ND |
| Prednisone | 5000 | 0.2 |
| | 16 | ND |

ND, not detected

# Conclusions

The TRUFORMA canine cortisol assay demonstrated high precision with a wide dynamic range, providing confidence in the reliability of cortisol results at the POC. The TRUFORMA canine cortisol assay's dynamic range allows the quantification of both high and low cortisol concentrations within the same assay, which is vital for diagnosing adrenal disease in canines. The availability of the TRUFORMA cortisol assay optimized for canine samples at the POC allows for immediate testing of baseline and dynamic adrenal samples, leading to timely diagnosis and treatment initiation. Additionally, the improved LLOQ will increase confidence in the diagnosis of hypoadrenocorticism and interpretation of LDDST and HDDST results compared to the currently available commercial assays.

The TRUFORMA canine cortisol assay provides veterinarians with accurate and reliable diagnostic results at the POC, allowing for a more rapid and informed patient diagnosis, and improved treatment and client communication.

## Acknowledgments

The authors would like to give special thanks to Ashley Wood and Robert Dwyer for invaluable contributions to assay design and paper preparation.


## Abbreviations and Acronyms

| | |
|---|---|
| ACTH | Adrenocorticotropic Hormone |
| BAW | Bulk Acoustic Wave |
| CLSI | Clinical and Laboratory Standards Institute |
| CRH | Corticotrophin-Releasing Hormone |
| CV | Coefficient of Variation |
| ELISA | Enzyme-Linked Immunosorbent Assay |
| EUA | Emergency Use Authorization |
| HDDST | High-Dose Dexamethasone Suppression Test |
| LDDST | Low-Dose Dexamethasone Suppression Test |
| LOD | Limit of Detection |
| LOQ | Limit of Quantitation |
| LLOQ | Lower Limit of Quantitation |
| ND | Not Detected |
| POC | Point of Care |
| RIA | Radioimmunoassay |
| TTR | Time to Test Results |
| ULOQ | Upper Limit of Quantitation |

## References


1. Feldman E, Nelson R, Reusch C, Scott-Moncrieff JC. Canine and Feline Endocrinology. 4th ed. Philadelphia, PA: WB Saunders; 2014.
2. Bovens C, Tennant K, Reeve J, Murphy KF. Basal Serum Cortisol Concentration as a Screening Test for Hypoadrenocorticism in Dogs. J Vet Intern Med. 2014.28(5):1541-5.
3. Gold AJ, Lanlois DK, Refsal KR. Evaluation of basal serum or plasma cortisol concentrations for the diagnosis of hypoadrenocorticism in dogs. J Vet Intern Med. 2016. 30: 1798-1805.
4. Lennon EM, Boyle TE, Hutchins EG, et al. Use of basal serum or plasma cortisol concentrations to rule out a diagnosis of hypoadrenocorticism in dogs: 123 cases (2000-2005). J Am Vet Med Assoc. 2007. 231: 413-416.
5. Russell NJ, Foster S, Clark P, et al. Comparison of radioimmunoassay and chemiluminescent assay methods to estimate canine blood cortisol concentrations. Aust Vet J. 2007. 85:487-94.
6. US Food and Drug Administration. Bioanalytical method validation. 2018.
7. CLSI. Measurement procedure comparison and bias estimation using patient samples. 3rd ed. CLSI guideline EP09c. Wayne, PA: Clinical and Laboratory Standards Institute; 2018.