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TRUFORMA® Point-of-Care Canine eACTH Assay

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Key Messages

- Accurate and precise measurement of endogenous ACTH (eACTH) levels in canines is needed to diagnose and manage adrenal disease in dogs.
- 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, providing reference lab accuracy in house.
- The TRUFORMA canine eACTH assay is the first ACTH assay offered at the POC and eliminates reference lab testing workflows, including shipping and additional sample handling, that can result in eACTH degradation.
- The dynamic range of the TRUFORMA canine eACTH assay allows the quantification of high and low concentrations of plasma eACTH without dilution or additional processing steps, which is vital for accurate and complete diagnosis of hyperadrenocorticism and hypoadrenocorticism in canines.
- The high precision and correlation to a reference laboratory assay shown for the TRUFORMA canine eACTH assay provides veterinarians with accurate and reliable diagnostic results at the POC, creating opportunities for improved patient treatment and real-time client communication.

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Introduction

Accurately diagnosing and monitoring canine adrenal dysfunction is challenging in veterinary medicine due to the complexity of current reference laboratory immunoassay methodologies and the unavailability of tests at the point of care (POC). There is a need for a POC eACTH assay because of the rapid enzymatic degradation this hormone can experience after sample collection. The TRUFORMA® platform uses BAW sensor technology to provide veterinarians rapid, reliable, and accurate measurement of canine eACTH levels at the POC.

The objectives of this study were to:

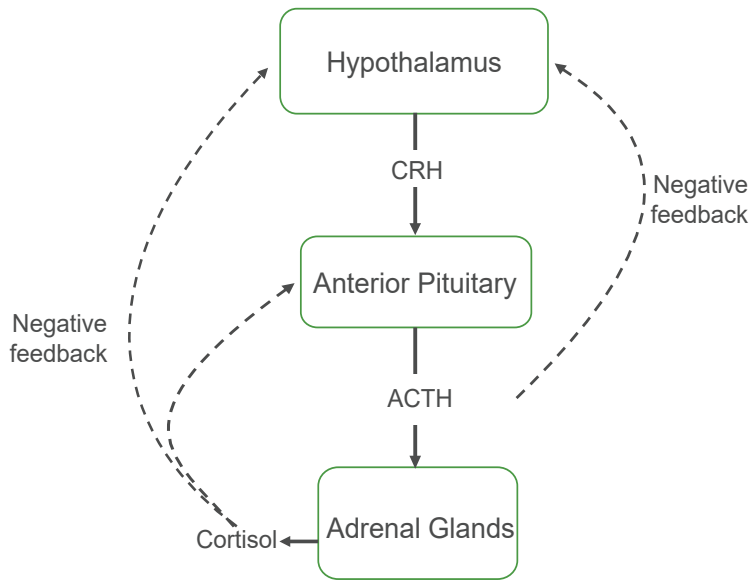
- Determine analytical performance attributes for the TRUFORMA canine eACTH assay.
- Describe how the TRUFORMA canine eACTH assay differs from other currently available assays.
- Compare TRUFORMA canine eACTH assay performance with an assay used as part of the standard of care at veterinary diagnostic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

Clinical Significance of Canine eACTH Testing

Endogenous adrenocorticotropic hormone (eACTH) is produced by the anterior pituitary in response to the release of corticotropin-releasing hormone (CRH) from the hypothalamus. Various types of stress including pain, trauma, and inflammatory mediators stimulate the release of eACTH via CRH. Cortisol, secreted from the adrenal glands, exerts a negative feedback effect on the pituitary gland (**Figure 1**), resulting in decreased eACTH release. Two types of adrenal disorders may affect basal eACTH levels, hyperadrenocorticism (Cushing's disease) and hypoadrenocorticism (Addison's disease). Hyperadrenocorticism is caused by overproduction of cortisol, most commonly as the result of a pituitary tumor (pituitary-dependent hyperadrenocorticism or PDH) while less frequently stemming from an adrenal tumor (adrenal-dependent hyperadrenocorticism or ADH) or excessive use of corticosteroids.

Hypoadrenocorticism is caused by a decrease in cortisol production, most commonly the result of immune-mediated destruction of the adrenal glands. Other causes of hypoadrenocorticism include adrenal gland destruction stemming from infection or trauma, pituitary or adrenal tumors, abrupt withdrawal from corticosteroid treatment, or excessive use of adrenolytic agents.

Figure 1. Hypothalamic-Pituitary Adrenal Axis



CRH, corticotropin-releasing hormone; ACTH, adrenocorticotropic hormone

Canine eACTH

A definitive diagnosis of adrenal disease in canines currently relies on comparing serum cortisol levels at baseline to levels post-dynamic adrenal testing. An ACTH-stimulation test is used to diagnose hypoadrenocorticism, but cannot differentiate between primary and secondary origins of the disease. A low-dose dexamethasone suppression test (LDDST) is the test of choice to diagnose hyperadrenocorticism. However, this test cannot always differentiate between PDH and ADH. Measurement of eACTH is considered the most accurate standalone test for differentiating the root cause of both hyperadrenocorticism and hypoadrenocorticism in dogs.[1]

ACTH-stimulation testing is considered the gold standard for diagnosis of hypoadrenocorticism. Cortisol-to-ACTH ratio (CAR) has been suggested as an alternative to ACTH stimulation in the diagnosis of hypoadrenocorticism. Studies have shown that dogs with hypoadrenocorticism had a significantly lower CAR than either normal dogs or dogs with diseases that mimic hypoadrenocorticism.[2,3,4] Endogenous ACTH is also recommended for etiologic differentiation in dogs with confirmed hypoadrenocorticism and normal electrolyte concentrations.[5]

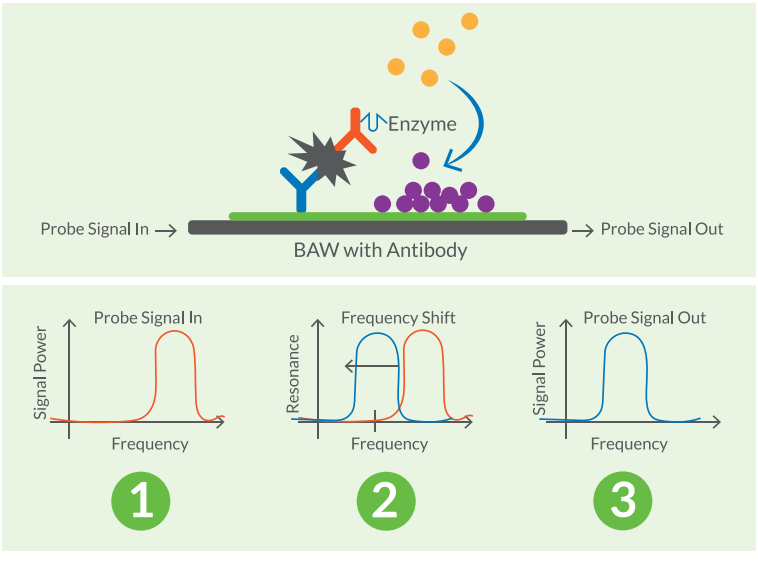
Both a radioimmunoassay and a chemiluminescent assay performed at reference laboratories have been validated for measurement of plasma ACTH in dogs.[6] However, eACTH is very labile and requires adherence to strict guidelines for sample preparation and shipment to a reference lab with any degree of mishandling potentially leading to erroneous values.[1,5] Aprotinin, a protease inhibitor, can be added to prevent eACTH degradation,[7] however it introduces an artificial decrease with certain assays and therefore is not recommended.[1,6]

- There is a clear need for accurate, reliable quantification of eACTH at the POC.

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 in clinics. BAW technology is extremely reliable and precise, and has been well tested in products across industries such as telecommunications and aerospace. Functionalized BAW biosensors consist of multiple resonators, each composed of a piezoelectric material subjected to an electrical field. The resonators can be coated with detection reagents, such as antibodies and nucleic acids, for immunoassay and molecular testing. Whereas 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 eACTH Immunoassay



The TRUFORMA eACTH assay is a sandwich immunoassay in which the BAW sensor is coated with a monoclonal capture antibody (blue). Antigen present in the sample (gray) binds to a polyclonal detection antibody in solution (orange) and this complex is recognized by the capture antibody on the sensor surface. After several wash steps, an enzyme substrate is added, 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 and the signal is proportional to the amount of analyte present in the sample.

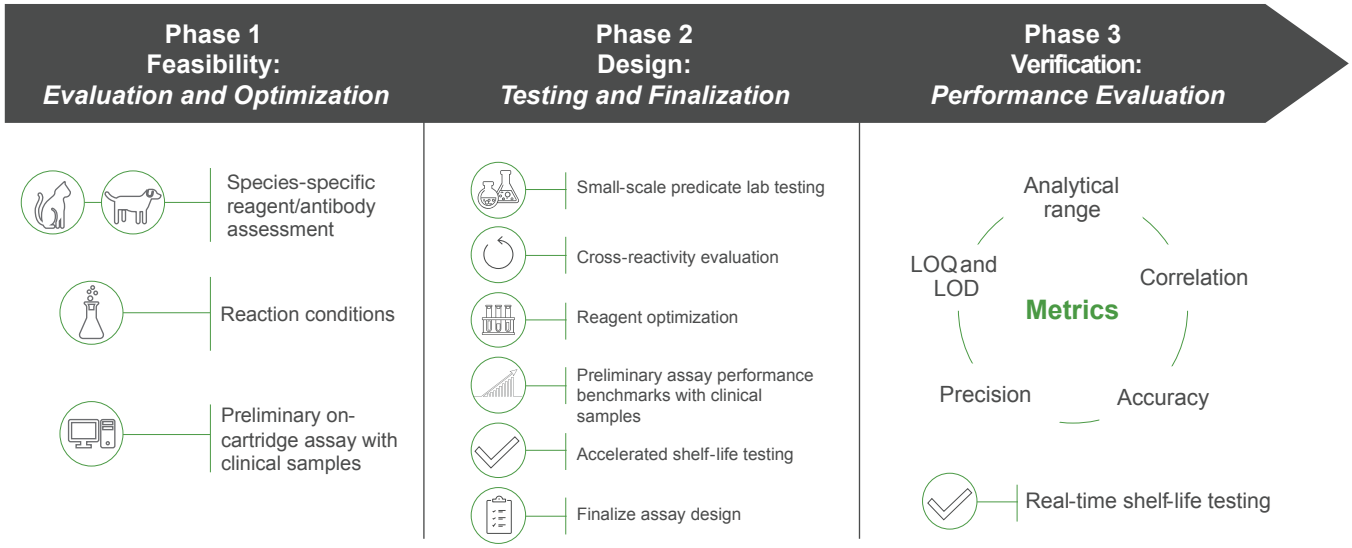
proportional to the mass accumulated on the sensor (**Figure 2**). Veterinary medical professionals were the first to use the BAW sensor technology in a POC diagnostic setting though this technology now has emergency use authorization (EUA) for rapid COVID-19 antigen testing in humans.

eACTH Assay Development Overview

The TRUFORMA® canine eACTH assay uses an antibody pair that was selected to have small, non-overlapping epitopes in order to eliminate interference from corticotropin-like intermediate peptide (CLIP), a proteolytic cleavage product of ACTH. Additionally, because TRUFORMA testing is performed at the point of care, ACTH degradation is less problematic than in comparable reference lab testing.

Using the industry standard recommendations for bioanalytical method validation[8] and the Clinical and Laboratory Standards Institute (CLSI) guidelines on method comparison and bias estimation,[9] the TRUFORMA assay performance requirements were chosen to meet or exceed reference laboratory performance in order to provide unparalleled performance at the POC. The three phases of the canine eACTH 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 eACTH Assay Development



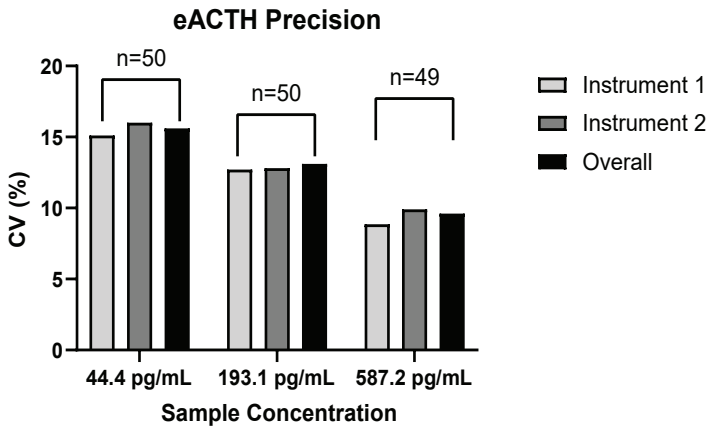
Assay Verification Results

The TRUFORMA canine eACTH assay analytical performance was evaluated and compared with the reference-laboratory predicate Siemens IMMULITE® 2000 ACTH assay, an automated solid-phase chemiluminescent sandwich immunoassay. Samples from healthy and diseased canines as well as spiked canine plasma samples were analyzed.

Analytical Precision

Analytical precision was evaluated by measuring the variability in assay results (between-run percentage coefficient of variation [%CV]) under normal operating conditions in the laboratory. Precision was evaluated by testing three spiked canine samples with varying eACTH 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.

Figure 4. Precision of the TRUFORMA® Canine eACTH Assay



%CV was calculated for three serum samples with varying eACTH concentrations using 150 runs. One statistical outlier was removed from the 587.2 pg/mL sample dataset following CLSI EP05-A3 guidelines. CV, coefficient of variation.

The TRUFORMA® canine eACTH 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. For a ligand-binding assay, a ± 25% between-runs %CV is recommended at the upper and lower limits of the dynamic range while within the range, a quality %CV is ± 20%. [8]

Time to Test Results (TTR), Dynamic Range, and Analyte Stability

Time to Test Results (TTR) refers to the amount of time elapsed from TRUFORMA initiating the test to generating 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 by an assay. The analytical sensitivity of the TRUFORMA canine eACTH assay was calculated to be <5 pg/mL and the lower end of the dynamic range was therefore set as 5 pg/mL. The upper end of the dynamic range was established at 700 pg/mL based on predefined precision metrics that ensure accurate and reproducible quantitation of canine plasma samples with the TRUFORMA canine eACTH assay (**Table 1**).

Overall, the TRUFORMA canine eACTH assay's dynamic range allows the quantification of both clinically high and clinically low eACTH concentrations. Additionally, by being the first eACTH assay offered at the POC, the potential for eACTH degradation in samples is markedly reduced relative to reference lab workflows.

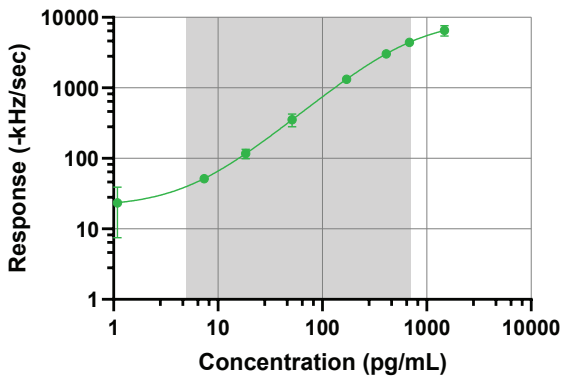
Table 1. Summary of TTR, Dynamic Range and Analytical Sensitivity for the TRUFORMA® Canine eACTH Assay Compared With the Siemens IMMULITE® ACTH Assay

Test	TRUFORMA	IMMULITE
	Canine Optimized	Human Optimized
TTR, minutes	<20.0	>60.0
Dynamic Range, pg/mL	5 – 700	5 – 1250*
Analytical Sensitivity, pg/mL	<5	5

TTR, time to test result; *Documented calibration range as no lower or upper limit of quantification are reported.

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

Figure 5. Standard Curve of the TRUFORMA Canine eACTH Assay



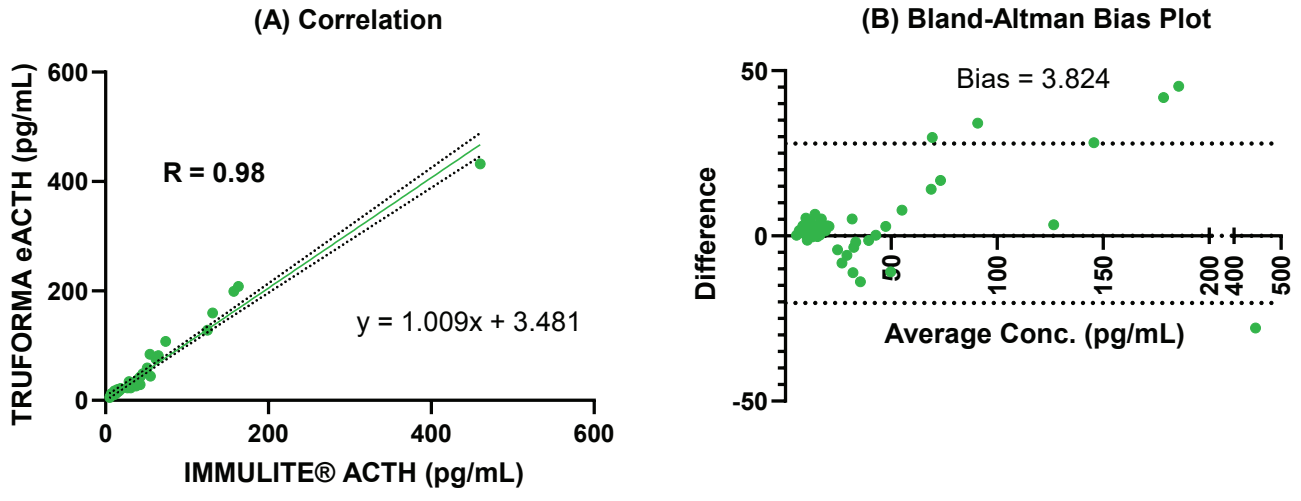
Eight calibrators with known concentrations of eACTH were used to generate a standard curve for canine analysis. The shaded region represents the reported dynamic range of the TRUFORMA eACTH Assay.

Assay Correlation Between TRUFORMA® eACTH Canine Assay and Siemens IMMULITE® ACTH 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 54 individual canine plasma samples were run on the same freeze-thaw cycle on the TRUFORMA® and Siemens IMMULITE® devices. The instrument reports concentrations based on the standard curve, and results were used to generate correlation and bias plots.

The TRUFORMA canine eACTH assay showed high correlation (R=0.98) with the Siemens IMMULITE (**Figure 6A**), while bias analysis depicted scatter with no apparent bias at the lower end of the dynamic range where clinical decisions regarding ADH versus PDH and primary versus secondary hypoadrenocorticism occur (**Figure 6B**).

Figure 6. Correlation and Bias of TRUFORMA Canine eACTH Assay Compared with the Siemens IMMULITE ACTH Assay



(A) Correlation studies were performed comparing the results from the TRUFORMA canine eACTH and IMMULITE ACTH assays using 54 canine samples. Dotted lines represent 95% CI for the linear regression line. (B) Bland-Altman bias plots were generated by plotting the mean concentrations vs. the difference ([TRUFORMA – IMMULITE] vs. mean). Dotted lines represent 95% limits of agreement.

Cross-Reactivity

Known amounts of eACTH proteolytic cleavage products were added to depleted plasma, and potential cross-reactants were added to depleted plasma and tested in triplicate using the TRUFORMA® canine eACTH assay.

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

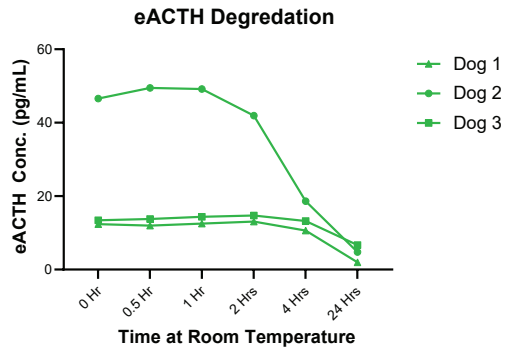
Table 2. Summary of Cross-Reactivity for the TRUFORMA® Canine eACTH Assay

Material	Concentration, pg/mL	Cross-Reactivity, %
Alpha-melanocyte stimulating hormone (alpha-MSH)	500,000	Not Detected
ACTH (18-39)	5,000	0.100
ACTH (1-24)	500,000	0.208
	50,000	0.034
	5,000	0.003
	500	0.001

eACTH Sample Instability

eACTH is an extremely labile molecule and proper sample handling is necessary to ensure clinically accurate measurements. Plasma samples from three different animals were placed at room temperature and eACTH was measured over time (**Figure 7**). The rate of eACTH degradation varied between samples. Dog 2 showed a 10% loss in eACTH concentration at 2 hours and 60% loss at 4 hours. All three samples showed greater than 50% loss in eACTH concentration at 24 hours. This illustrates the necessity of sample temperature control to achieve accurate eACTH measurements and highlights the challenges associated with shipping samples to a reference lab.

Figure 7. eACTH Sample Instability



Plasma samples from 3 dogs were stored at room temperature. eACTH measurements were made at various time points to evaluate sample stability.

Conclusions

The TRUFORMA canine eACTH assay demonstrated high precision with a wide dynamic range, providing confidence in the reliability of eACTH results at the POC. The TRUFORMA canine eACTH assay's dynamic range allows the quantification of both high and low ACTH concentrations within the same assay, which is vital for diagnosing adrenal disease in canines. Rapid degradation of the eACTH molecule at increased temperatures makes reference lab measurements unreliable due to potential complications during shipping. The TRUFORMA eACTH assay provides a point of care test with strong agreement with reference lab methodologies. This allows for immediate and accurate sample analysis alleviating concerns with sample handling. Additionally, having eACTH testing available at the POC for the first time will lead to timely diagnosis and immediate initiation of treatment.

The TRUFORMA canine eACTH 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.

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Acknowledgments

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Abbreviations and Acronyms

ACTH	Adrenocorticotropic Hormone
ADH	Adrenal-Dependent Hyperadrenocorticism
BAW	Bulk Acoustic Wave
CAR	Cortisol-to-ACTH Ratio
CLIP	Corticotropin-Like Intermediate Peptide
CLSI	Clinical and Laboratory Standards Institute
CRH	Corticotrophin-Releasing Hormone
CV	Coefficient of Variation
EUA	Emergency Use Authorization
LDDST	Low-Dose Dexamethasone Suppression Test
LOD	Limit of Detection
LOQ	Limit of Quantitation
POC	Point of Care
TTR	Time to Test Results

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