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TRUFORMA® Point of Care

eACTH Assay – Equine Applications

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Key Messages

- Accurate and precise measurement of endogenous ACTH (eACTH) levels is needed to diagnose and manage endocrine disease in horses.
- The TRUFORMA platform uses innovative bulk acoustic wave (BAW) technology to provide a nonoptical and fluorescence-free detection system for diagnostic use at the point of care (POC) in veterinary clinics.
- The TRUFORMA eACTH assay is the first endogenous ACTH assay offered at the POC and eliminates reference lab testing workflows, including shipping and additional sample handling, which can result in eACTH degradation.
- The dynamic range of the TRUFORMA eACTH assay allows for 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 pituitary pars intermedia dysfunction (PPID).
- The TRUFORMA eACTH assay is the first assay to measure and report both full length eACTH and the related hormone CLIP, both of which are increased in PPID.
- The high precision and correlation to a reference laboratory assay shown for the TRUFORMA eACTH 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 endocrine dysfunction is challenging in veterinary medicine due to the complexity of current reference laboratory immunoassay methodologies and the unavailability of POC tests. 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 eACTH levels at the POC.

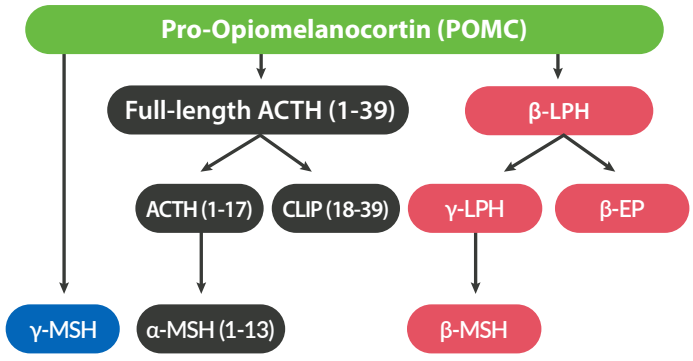
The objectives of this study were to:

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

Clinical Significance of Equine eACTH Testing

Pituitary pars intermedia dysfunction or PPID is the most common endocrine disorder in the geriatric horse population. Roughly 20-25% of horses above the age of 15 years are affected with the disease, though horses as young as 7 years have also been diagnosed.[1] While often referred to as "Equine Cushing's Disease", a different region of the pituitary gland is affected in PPID than in human and canine Cushing's Disease. More specifically, melanotropes in a normal equine pituitary gland are inhibited by dopamine release from hypothalamic neurons. This dopamine-mediated inhibition closely controls the generation of the adrenocorticotropic hormone (ACTH) precursor molecule pro-opiomelanocortin (POMC) in the pars distalis portion of the pituitary gland. ACTH, composed of 39 amino acids, is further cleaved and amino acids 1-13 are processed to become α-melanocyte stimulating hormone (α-MSH) and amino acids 18-39 are processed to become corticotropin-like intermediate peptide (CLIP) in the pars intermedia portion of the pituitary gland as depicted in **Figure 1.**[1] In PPID, degeneration of the dopaminergic neurons leads to disinhibition of the melanotropes and overproduction of many POMC derived pituitary hormones, including ACTH. The gold standard Immulite® 2000 ACTH test, from which equine reference ranges are derived, is a chemiluminescent immunoassay (CLIA) and is known to have a cross reactivity of about 17% with CLIP.[2] The presence of CLIP and CLIP-like peptides in equine samples has been confirmed by liquid chromatography – mass spectrometry

Figure 1. POMC Processing



POMC is processed into full-length ACTH (1-39), which is cleaved to produce α-MSH (1-13) and CLIP (18-39). POMC, pro-opiomelanocortin; ACTH, adrenocorticotropic hormone; α-MSH, α-melanocyte stimulating hormone; CLIP, corticotrophin-like intermediate peptide.

(LCMS).[3] The impact of CLIP levels on ACTH measurements by CLIA is unknown. ACTH and CLIP levels are known to vary throughout the year[4,5] leading to season specific recommendations for diagnosis.[6] The biological significance of full-length ACTH, α-MSH and CLIP in the context of PPID and equine health is unknown, but elevated levels of all these hormones have been reported in PPID.[3,5]

The most identifiable clinical sign in PPID-affected horses is hypertrichosis. While the exact pathophysiologic mechanism behind this change is unknown, a longer, curly haircoat that fails to shed is a hallmark of PPID and has been documented to occur in close to 70% of horses with the disease.[7] Additionally, PPID is associated with a variety of clinical signs including laminitis, muscle atrophy, weight loss, polydipsia, polyuria, lethargy, infertility, persistent lactation, exercise intolerance, dysregulation of sweating, and recurrent infections.

Equine eACTH Testing

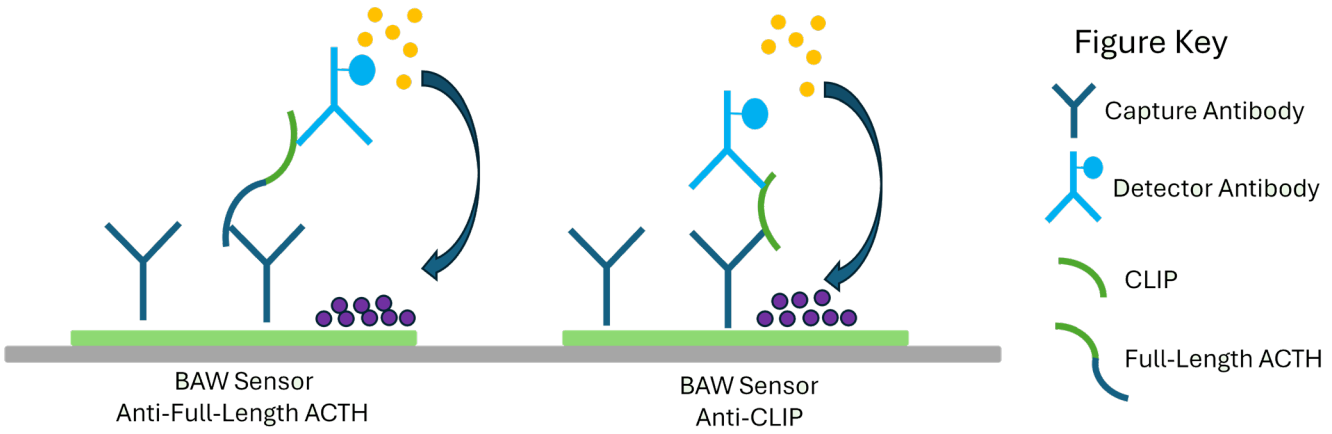
The current testing recommendation for diagnosis of PPID in older horses with some clinical signs consistent with the disease is baseline endogenous ACTH (eACTH). Measurement of eACTH in TRH-stimulated plasma samples is recommended for evaluating early stage PPID.[6] While there is currently a reference laboratory test validated for measurement of equine eACTH, the hormone itself 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.[8,9] With the TRUFORMA eACTH Assay being available at the point of care, these sample handling complications are substantially reduced. **There is a clear need for accurate, reliable quantification of eACTH at the POC.**

TRUFORMA Platform

The TRUFORMA platform uses Bulk Acoustic Wave (BAW) sensor technology to provide a non-optical and fluorescence-free detection system for diagnostic use at the point of care. 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 sensor technology in a POC diagnostic setting though this technology subsequently received emergency use authorization (EUA) for rapid COVID-19 antigen testing in humans.

The TRUFORMA Equine eACTH Test works as a sandwich immunoassay with one BAW sensor solid phase coated with antibodies that capture full-length eACTH (ACTH 1-39), and another BAW sensor coated with antibodies that specifically capture CLIP (ACTH 18-39). During the test, the sample is diluted and flows across the biosensor surface where full-length eACTH and CLIP molecules are captured on their respective sensors (**Figure 2**).

Figure 2. BAW Technology in the TRUFORMA eACTH Immunoassay



The TRUFORMA eACTH assay uses two sandwich immunoassays in which the BAW sensor is coated with a capture antibody (blue). Antigen present in the sample (green and blue) is recognized by the capture antibody (blue) on the sensor surface as well as a detection antibody (light blue) that brings an enzyme to the sensor surface. After several wash steps, an enzyme substrate is added (yellow), 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 directly proportional to the amount of analyte present in the sample. A multiplex biosensor approach is used for simultaneous detection of Full-Length ACTH (blue and green) and CLIP (green) in a single test cartridge. One BAW sensor is specific for Full-Length ACTH and the other is specific for CLIP.

A biotin-labeled detection antibody (dAb) then flows across the surface and binds to any immobilized full-length eACTH and CLIP. Next, a streptavidin-enzyme conjugate flows over the sensor surface, where it is immobilized to the detector antibody via the streptavidin-biotin interaction. After several wash steps, an enzyme substrate is exposed to the BAW biosensor surface.

As a sandwich immunoassay, a sample containing a high concentration of full-length eACTH and/or CLIP results in a higher amount of dAb-biotin-streptavidin-enzyme complex attached to the solid phase and a larger signal.

The enzyme converts the substrate into an insoluble product that accumulates on the BAW biosensor surface and is measured as a shift in frequency by the BAW biosensor. The signal is proportional to the amount of enzyme present on the BAW biosensor surface. The change in frequency is used to calculate the concentration of full-length eACTH and CLIP present in the sample using stored calibration curves. The full-length eACTH and CLIP biosensors are measured independently, and a concentration is calculated for each biosensor. The full-length eACTH and CLIP doses are added, generating the eACTH Composite value that is reported by the instrument. Full-length eACTH and CLIP values are available on myZomedica.com and are provided for informational use only. The eACTH Composite value is comparable to the immuoreactive ACTH measurement reported by the CLIA, and can be used with existing eACTH guidelines[7] that were established using CLIA results. eACTH Composite values that fall outside of the normal reference range may be indicative of abnormal levels of eACTH as a result of an endocrine disorder.

Specificity of the full-length ACTH and CLIP sensors (**Table 1**) was measured in the TRUFORMA eACTH assay. Spiking of full-length eACTH or CLIP within the dynamic range of the assay did not show any cross reactivity with the other biosensor demonstrating the specificity of each of those sensors. Spiking of higher concentrations above the dynamic range did generate a low level of cross reactivity. High levels of CLIP cross reacted at ~0.2% with the full-length eACTH biosensor and high levels of full-length eACTH cross reacted at ~1.9% with the CLIP sensor.

Table 1. Full-Length eACTH and CLIP Specificity

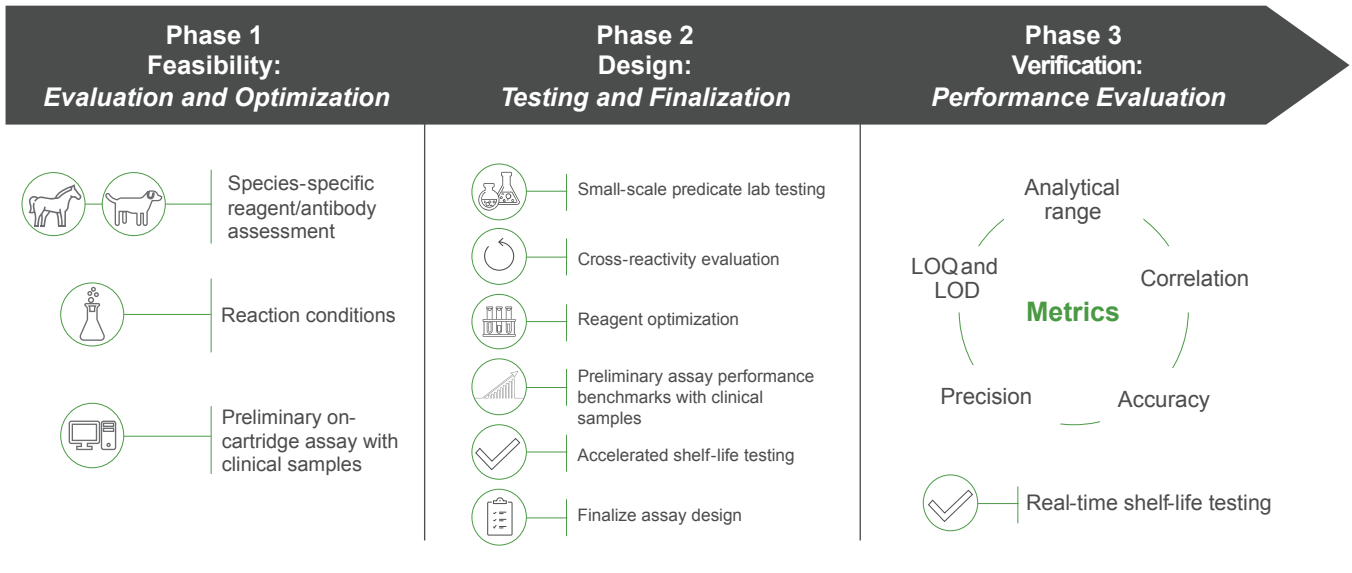
	TRUFORMA Result		% Cross Reactivity
	Full-Length ACTH (pg/mL)	CLIP (pg/mL)	
Low CLIP Spike	<5	457.0	ND
High CLIP Spike	9.8	4570.2*	0.2%
Low Full-Length ACTH Spike	350.1	<12.5	ND
High Full-Length ACTH Spike	3500.5*	68.2	1.9%

*Theoretical concentration based on dilution. ND – not detected.

eACTH Assay Development Overview

Using industry standard recommendations for bioanalytical method validation[10] and the Clinical and Laboratory Standards Institute (CLSI) guidelines on method comparison and bias estimation (EP09c),[11] 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 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 eACTH Assay Development



LOQ, limit of quantitation; LOD, limit of detection

Assay Verification Results

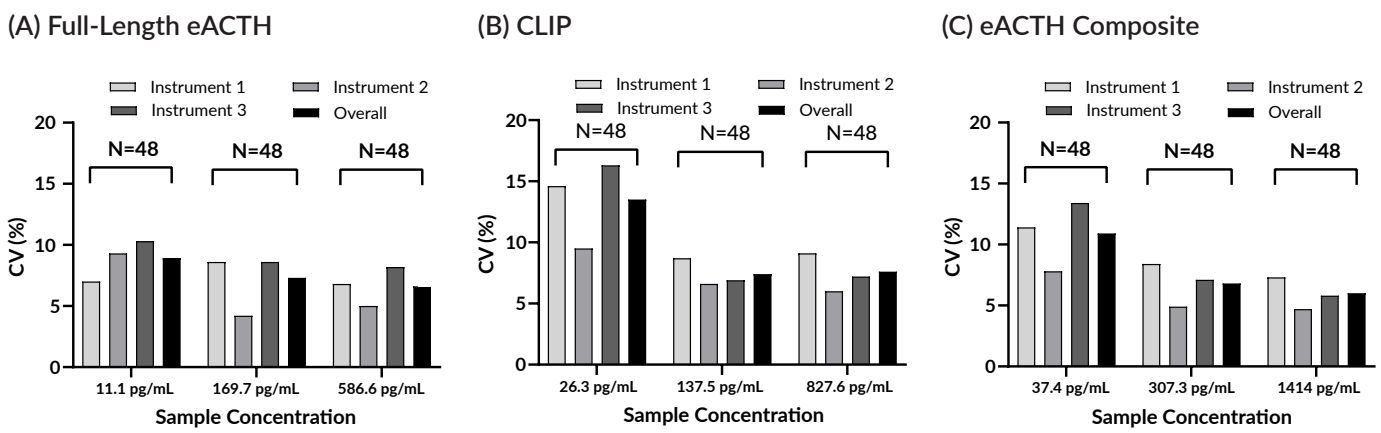
The TRUFORMA eACTH assay's analytical performance was evaluated and compared with the reference laboratory predicate Siemens IMMULITE® 2000 ACTH assay, an automated solid-phase chemiluminescent sandwich immunoassay. Samples were collected throughout the year to confirm performance with known seasonal variability in full-length eACTH and CLIP.[4,5] Additionally, a combination of baseline and TRH-stim samples were tested.

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 samples with varying full-length eACTH and CLIP concentrations. Each sample was tested with sixteen replicates on three different instruments, for a total of 144 results. Observed %CV was calculated for the full-length eACTH, CLIP, and eACTH Composite results.

The TRUFORMA eACTH assay demonstrated an overall %CV <20% for full-length eACTH, CLIP, and eACTH Composite results (**Figure 4 A-C, respectively**). 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%.[10]

Figure 4. Precision of the TRUFORMA Equine eACTH Assay



%CV was calculated for three separate equine plasma samples with varying full-length ACTH and CLIP concentrations over a total of 144 runs. CV, coefficient of variation.

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

Time to Test Results (TTR) refers to the amount of time elapsed from initiating the TRUFORMA 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 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 eACTH assay was calculated to be <5 pg/mL for full-length eACTH and <12.5 pg/mL for CLIP and the lower end of the dynamic range was therefore set as 5 pg/mL and 12.5 pg/mL for full-length eACTH and CLIP respectively. For both full-length eACTH and CLIP, the upper end of the dynamic range was established at 1250 pg/mL based on predefined precision metrics that ensure accurate and reproducible quantitation of plasma samples with the TRUFORMA eACTH assay. The eACTH Composite value uses both the full-length ACTH and CLIP results and reports values from 5-1250 pg/mL. When calculating the eACTH Composite value, results <5 pg/mL for full length ACTH or <12.5 pg/mL for CLIP are treated as a 0 when calculating the eACTH Composite value (**Table 1**).

Overall, the TRUFORMA 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 eACTH Assay Compared with the Siemens IMMULITE ACTH Assay

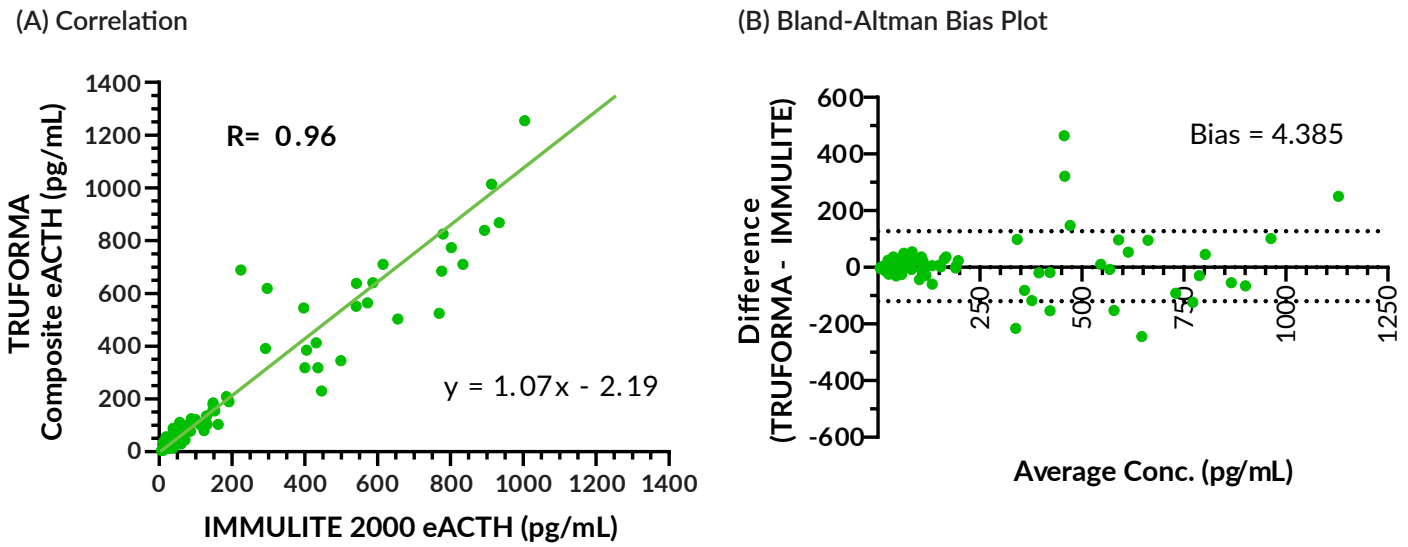
Test	TRUFORMA	IMMULITE
	Equine Optimized	Human Optimized
TTR, minutes	<20.0	>60.0
Dynamic range, pg/mL	5 – 1250	5 – 1250*
Analytical Sensitivity, pg/mL	<5	5

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

Correlation Between TRUFORMA eACTH 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 174 equine plasma samples were run on the same freeze-thaw cycle on the TRUFORMA and Siemens IMMULITE 2000 XPi devices. The instruments report concentrations based on their respective standard curves and these results were used to generate correlation and bias plots. The TRUFORMA eACTH Composite result showed high correlation (R=0.96) with the Siemens IMMULITE for equine plasma samples (**Figure 6A**), while bias analysis depicted scatter with no apparent bias (**Figure 6B**).

Figure 6. Correlation and Bias of the TRUFORMA Equine eACTH Assay Compared with the IMMULITE 2000 ACTH Assay

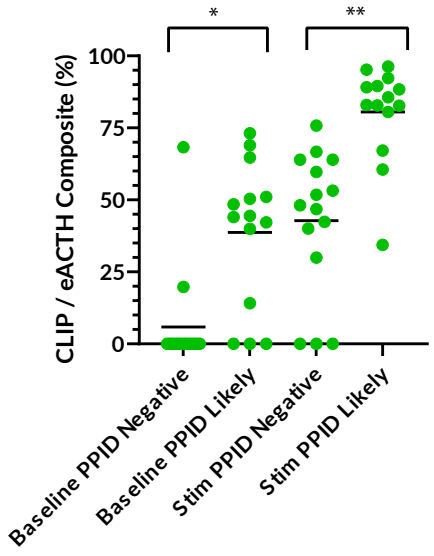


Correlation analysis was performed comparing the results from the TRUFORMA eACTH and IMMULITE ACTH assays for 174 equine plasma samples (A). Bland-Altman bias plots were generated by plotting the mean concentrations vs. the difference (TRUFORMA - IMMULITE) (B) Dotted lines represent the respective 95% limits of agreement.

Impact of CLIP on eACTH Results

The TRUFORMA Equine eACTH assay measures both full-length eACTH and CLIP independently and reports the results as an eACTH Composite value, which is the sum of the individual full-length eACTH and CLIP values. The distinct roles of CLIP and full-length ACTH in equine endocrinology are currently being investigated. Preliminary data indicate that the proportion of CLIP relative to full-length eACTH is higher in horses with PPID. This finding is consistent in both baseline (p-value = 0.005) and TRH stimulation samples (p-value <0.0001) (**Figure 7**). Notably, TRH stimulation increases the amount of CLIP present in both PPID affected and unaffected horses as demonstrated by the increase in CLIP-to-eACTH Composite ratio (**Figure 7**). The TRUFORMA instrument displays the eACTH Composite result which should be used with existing clinical guidelines.[7,12] The full-length eACTH and CLIP values are provided via the myZomedica portal for informational use only.

Figure 7. TRH Stim Effect on CLIP and Full-Length ACTH



Percentage of CLIP relative to ACTH Composite result for different patient populations. A total of 29 horses were included (15 in the PPID negative cohort and 14 in the PPID likely cohort). Diagnosis was based on the TRH stim result (PPID Negative ACTH Composite <100 pg/mL, PPID Likely ACTH Composite >200 pg/mL). * p-value= 0.0005, ** p-value<0.0001 using a Mann-Whitney t-test.

Cross-Reactivity

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

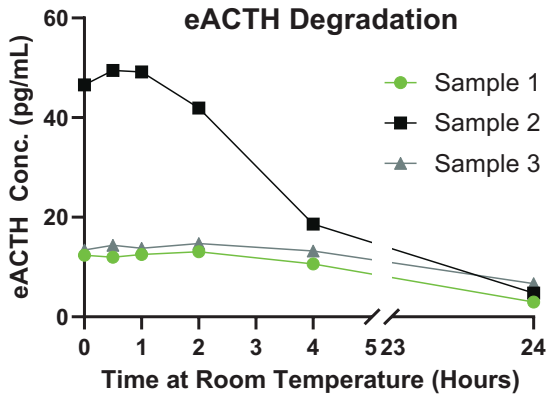
Table 2. Summary of Cross-reactivity for the TRUFORMA Equine eACTH Assay

Cross reactant	Conc (pg/mL)	% Cross-reactivity (ACTH Composite)
ACTH 1-24	500,000	-0.03%
α–MSH	500,000	ND

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 monitored over 24 hours. The rate of degradation varied between samples (**Figure 8**). Sample 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 8. eACTH Sample Instability



Three independent plasma samples were incubated at room temperature and analyzed for levels of eACTH over time.

Conclusions

The TRUFORMA eACTH assay demonstrates high precision with wide dynamic ranges, providing confidence in the reliability of eACTH results at the POC. Additionally, the eACTH Composite results, based upon independent measurement of full-length eACTH and CLIP, show strong agreement with CLIA results that were used to establish current clinical guidelines. The utility of separate full-length eACTH and CLIP measurements in equine disease is an area of active research and these values are being provided to clinicians through the myZomedica data portal for informational use only. The availability of the TRUFORMA eACTH assay optimized for equine samples at the POC allows for immediate testing of samples without significant sample handling and shipping that can result in eACTH degradation. Additionally, having eACTH testing available at the POC for the first time will lead to timely diagnosis and immediate initiation of treatment.

The TRUFORMA 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.

Abbreviations and Acronyms

ACTH Adrenocorticotropic Hormone

BAW Bulk Acoustic Wave

CLIA Chemiluminescent Immunoassay

CLIP Corticotropin-Like Intermediate Peptide

CLSI Clinical and Laboratory Standards Institute

CV Coefficient of Variation

EUA Emergency Use Authorization

LCMS Liquid Chromatography - Mass Spectrometry

LOD Limit of Detection

LOQ Limit of Quantitation

ND Not Detected

POC Point of Care

PPID Pituitary Pars Intermedia Dysfunction

TRH stim Thyrotropin-Releasing Hormone (TRH) Stimulation Test

TTR Time to Test Results

References

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