ZOMEDICA®

TRUFORMA® Point of Care

Equine Insulin Assay

Vicci Korman, PhD, Sr. Research and Development Scientist, Zomedica

T. J. Barclay, DVM, Sr. Professional Services Veterinarian, Zomedica

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

Key Messages

- Accurate and precise measurement of insulin levels is needed to diagnose and manage equine endocrine 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 TRUFORMA Equine Insulin assay is one of the first veterinary insulin assays offered at the POC which eliminates reference lab testing workflows, including shipping and additional sample handling, which can result in sample degradation and delayed treatment decisions.
- The TRUFORMA Equine Insulin assay has two different test protocols on the same cartridge. The "Default" protocol is recommend in most cases and has a wide dynamic range (7.5-400 µU/mL). For samples suspected of having high insulin levels the "Auto Dilute" protocol automatically dilutes the sample on the cartridge and reports results up to 1500 µU/mL. The TRUFORMA Equine Insulin assay accurately determines high and low concentrations of plasma insulin without user dilution or additional processing steps, which is vital for accurate and complete diagnosis and monitoring of insulin dysregulation (ID) in horses.
- The high precision and correlation to a reference laboratory assay shown for the TRUFORMA Equine Insulin 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 metabolic 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 insulin assay that has a detection range comparable to radioimmunoassay (RIA) performed at a reference lab. The TRUFORMA platform uses BAW sensor technology to provide veterinarians with rapid, reliable, and accurate measurement of insulin levels at the POC.

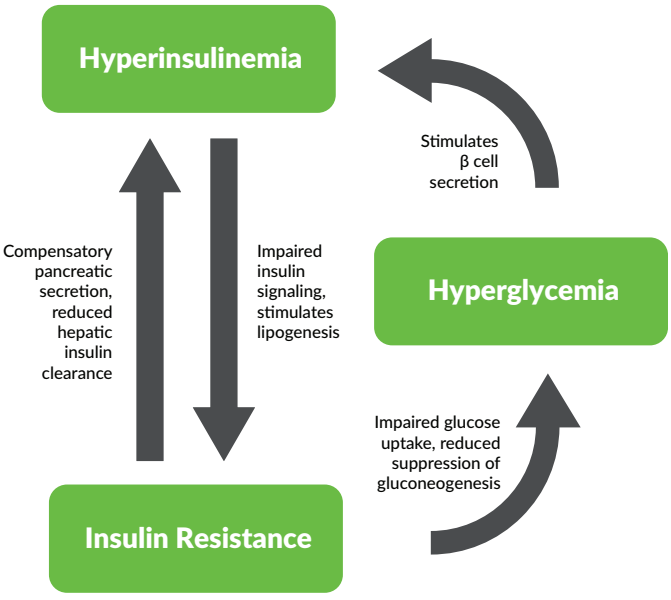
The objectives of this study were to:

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

Clinical Significance of Equine Insulin

Insulin is a small polypeptide hormone produced by the beta cells of the pancreas; it serves as a key regulator of equine metabolism by promoting the transport of glucose from the blood stream to tissue cells. As the name implies, insulin dysregulation occurs when the effectiveness of insulin to control blood glucose levels is impaired. ID can lead to hyperinsulinemia, or high blood insulin levels, which can drive further insulin insensitivity in tissue which in turn leads to higher blood insulin levels creating a vicious cycle.[2] In addition to hyperinsulinemia, ID is a key marker, along with increased adiposity, of EMS which is a collection of risk factors that predispose equids to laminitis.[3]

Figure 1. Mechanisms of Insulin Dysregulation



Modified from Durham *et al.* 2019[1]

Equine Insulin Testing

Diagnosis of ID is based on demonstrating hyperinsulinemia with static and/or dynamic testing. Basal insulin concentration, which is measured in a single plasma sample obtained from a patient in the forage-fed state, is a useful screening tool for ID. Basal insulin values greater than 50 microunits per milliliter (µU/mL) are consistent with ID. Because the basal insulin test has a low sensitivity for ID, mildly increased (20 – 50 µU/mL) or normal (<20 µU/mL) insulin levels cannot reliably rule out the disease. In these cases, the oral sugar test is recommended to confirm the diagnosis.[1]

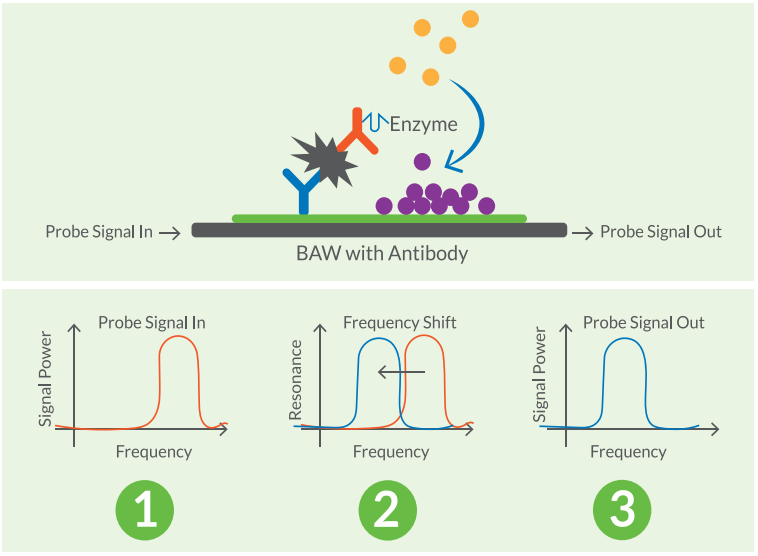
The oral sugar test (OST) is a dynamic test which evaluates the insulin response to an orally administered source of glucose - typically corn syrup. Multiple sugar dosages and insulin cutoff values have been evaluated, and current recommendations are as follows. After the patient has been fasted for 3-6 hours, corn syrup (Karo Light Corn Syrup, ACH Food Companies, Inc.) is administered at a dose of either 0.15 or 0.45 ml/kg. Plasma is collected 60 – 90 minutes later and analyzed. Insulin concentrations above 45 µU/mL (0.15 ml/kg test) or 65 µU/mL (0.45 ml/kg test) are consistent with ID.[4]

Insulin dysregulation and predisposition to laminitis are also characteristics of equine pituitary pars intermedia dysfunction (PPID), and it is possible for EMS and PPID to coexist in the same patient. Therefore, it is recommended that patients with ID also be tested for PPID. If a thyrotropin-releasing hormone (TRH) stimulation test for PPID is to be performed on the same day as an OST, the TRH stimulation test should be performed first.[5]

TRUFORMA Platform

The TRUFORMA platform uses 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.

Figure 2. BAW Technology in the TRUFORMA Equine Insulin Immunoassay



The TRUFORMA Equine Insulin assay is a sandwich immunoassay in which the BAW sensor is coated with a monoclonal capture antibody (blue). Antigen (insulin) present in the sample (gray) binds to a monoclonal 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 (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.

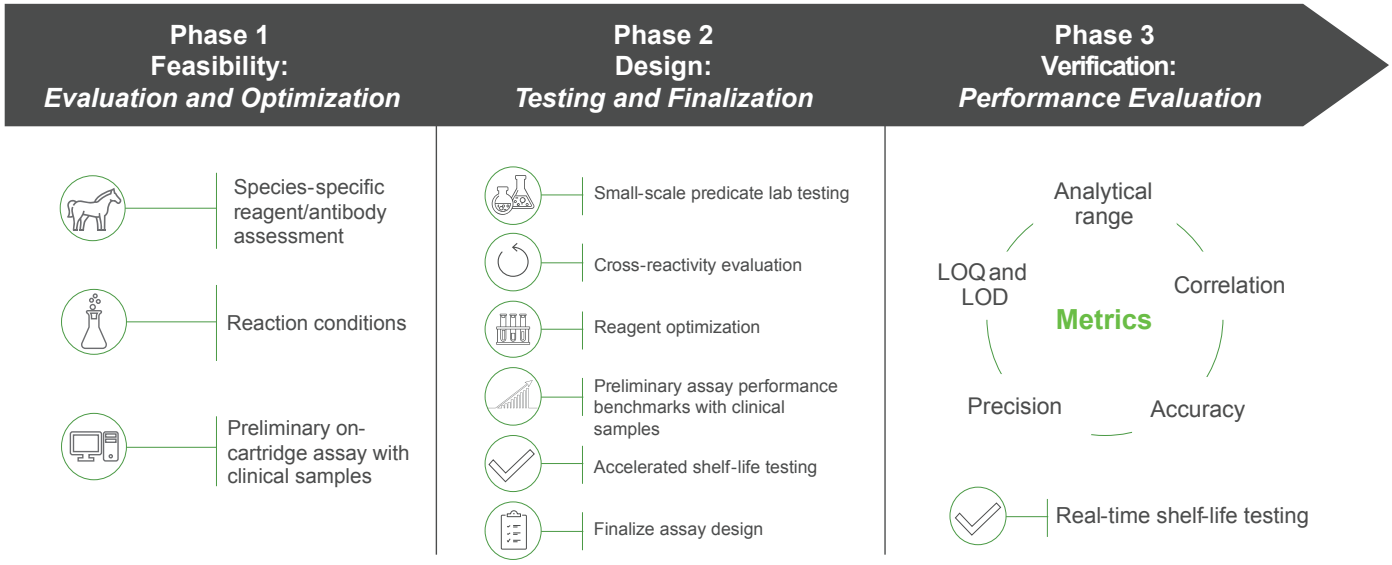
On Cartridge Dilution Capability

This test employs a single-use cartridge containing an assay for the detection of insulin in equine plasma. There are two different run protocols available depending on the anticipated level of the sample. The "Default" protocol is recommended for general screening and testing of samples anticipated to be below 400 µU/mL. The "Auto Dilute" protocol is recommended as a follow up test for samples >400 µU/mL. During the "Auto Dilute" test the sample is automatically diluted on the cartridge to extend the dynamic range to 1500 µU/mL.

Insulin Assay Development Overview

The TRUFORMA Equine Insulin assay was developed to combine the extended range possible with reference lab testing with the convenience of POC testing. Using 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 to provide unparalleled performance at the POC. The three phases of insulin 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 Equine Insulin Assay Development



LOD, limit of detection; LOQ, limit of quantitation

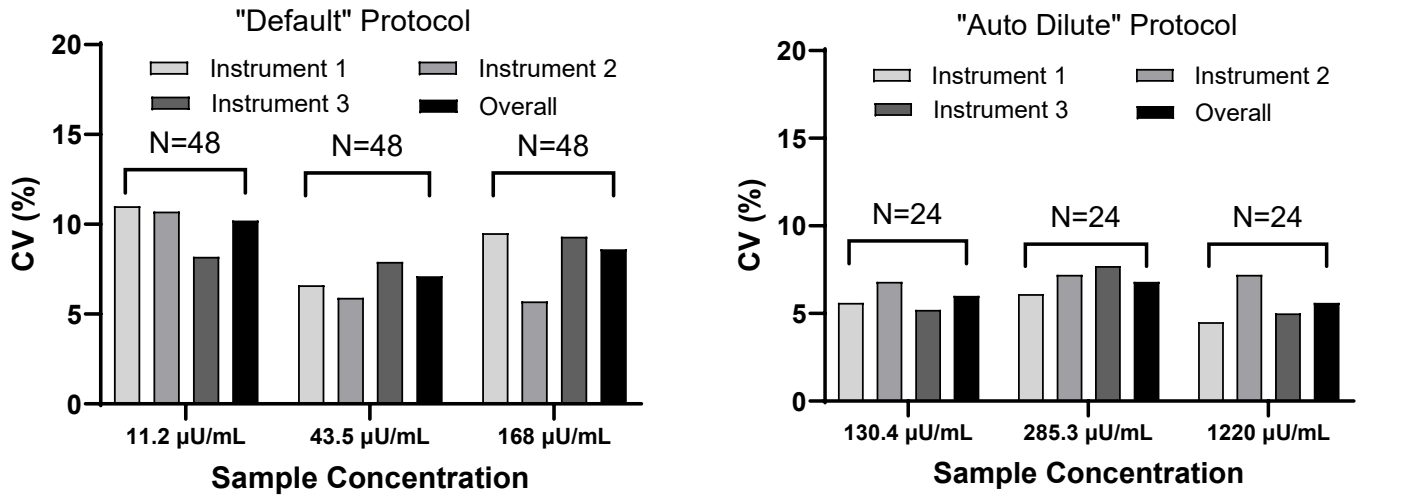
Assay Verification Results

The TRUFORMA Equine Insulin assay's analytical performance was evaluated and compared with the radioimmunoassay (RIA) performed at the Cornell University Animal Health Diagnostic Center. Plasma samples from healthy, suspected diseased and diseased horses were analyzed.

Analytical Precision

Analytical precision was evaluated by measuring the variability (i.e. between-run percentage coefficient of variation [%CV]) under normal operating conditions in the laboratory over several days. Precision was evaluated by testing six equine plasma samples with varying insulin concentrations. Five of the samples were spiked with porcine insulin to produce the mid and high insulin levels. For the "Default" protocol 3 samples were tested with 12 replicates on four separate days across three different instruments for a total of 144 results. For the "Auto Dilute" protocol 3 samples were tested with 12 replicates on two days across three different instruments for a total of 72 results. Observed %CV was calculated. The TRUFORMA Equine Insulin 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. 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%.[6]

Figure 4. Precision of the TRUFORMA Equine Insulin Assay



%CV was calculated for three separate equine plasma samples with varying insulin concentrations over a total of 144 runs for the "Default" protocol and a total of three samples and 72 runs for the "Auto Dilute" protocol. CV, coefficient of variation.

Time to Test Results (TTR) and Dynamic Range

Table 1. Summary of TTR and Dynamic Range for the TRUFORMA Equine Insulin Assay Compared with Reference Lab and other POC Assay.

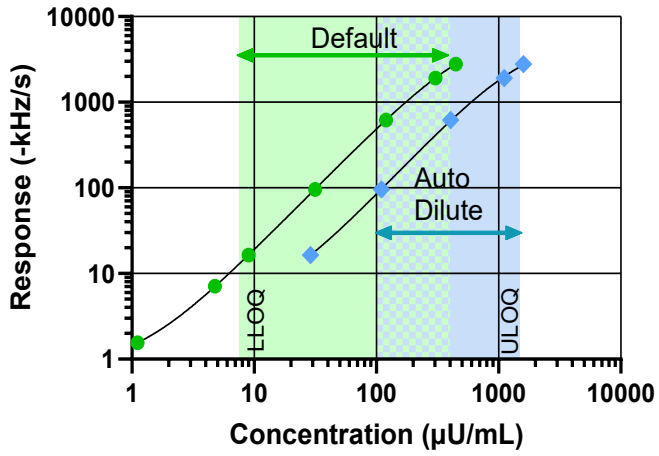
	TRUFORMA (Default)	TRUFORMA (Auto Dilute)	POC-Wellness Ready	Reference Lab - RIA
TTR	< 19 minutes	< 19 minutes	15 minutes	1 to 2 days
Dynamic Range	7.5-400 µU/mL	100-1500 µU/mL	20-99.9 µU/mL	2-200 µU/mL

TTR time to test result.

Dynamic range refers to the span of test result values that can be accurately measured by an assay. The analytical sensitivity of the TRUFORMA Equine Insulin assay was calculated to be <7.5 µU/mL and the lower end of the dynamic range was set as 7.5 µU/mL. The upper end of the dynamic range was established at 400 µU/mL for the "Default" protocol and 1,500 µU/mL for the "Auto Dilute" protocol based on predefined precision metrics that ensure accurate and reproducible quantitation of plasma samples with the TRUFORMA Equine Insulin assay (**Table 1**). Overall, the TRUFORMA Equine Insulin assay's dynamic range allows the quantification of both clinically high and clinically low insulin concentrations, delivering reference lab performance at the POC with POC TTR.

Seven calibrators with known concentrations of insulin were tested using the TRUFORMA Equine Insulin assay cartridges. The average value was used to generate two standard curves. One standard curve is used with the "Default" protocol and the other curve is used with the "Auto Dilute" portocol. The green area represents the dynamic range of the "Default" protocol, the blue area represents the dynamic range of the "Auto Dilute" protocol, and the checkered blue and green area represents the overlap of the two assay protocols. The reportable range of the TRUFORMA Equine Insulin assay illustrates linear performance within the clinically relevant range (**Figure 5**).

Figure 5. Standard Curve of the TRUFORMA Equine Insulin Assay

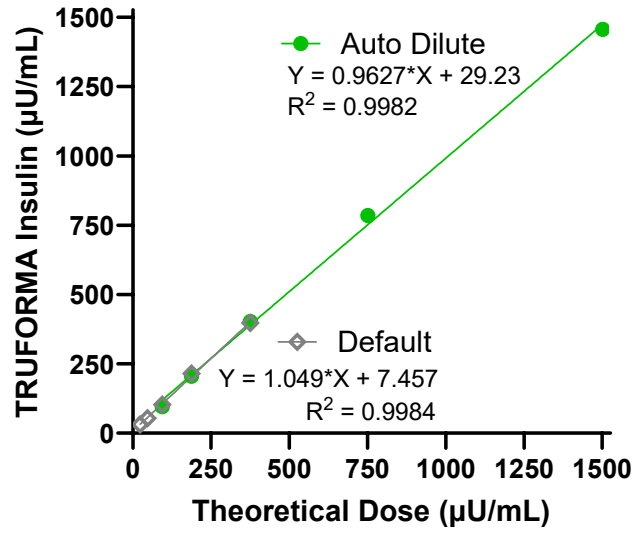


Six calibrators with known concentrations of insulin were used to generate a standard curve. The shaded region represents the dynamic range of the TRUFORMA Insulin Assay for equine plasma. LLOQ, lower limit of quantitation; ULOQ, upper limit of quantitation.

Linearity

Linearity of the TRUFORMA Equine Insulin "Default" and "Auto Dilute" assay protocols was tested with an endogenous insulin sample of 1501 µU/mL as tested by RIA using the dilution protocol at the Cornell University Animal Health Diagnostic Center. This high insulin sample was serially diluted into a plasma sample depleted of endogoneous insulin creating a total of 7 samples ranging from 1501 - 23.5 µU/mL. Samples within the dynamic range of each protocol were graphed against the theoretical dose based upon the dilution. A linear regression was used to evaluate linearity across the dynamic range of both the "Default" and "Auto Dilute" assay protocols. The TRUFORMA Equine Insulin "Default" and "Auto Dilute" assay protocols showed excellent linearity R^2=0.9984 and R^2=0.9982 for the "Default" and "Auto Dilute" assay protocols respectively. (**Figure 6**).

Figure 6. Linearity of the TRUFORMA Equine Insulin Assay



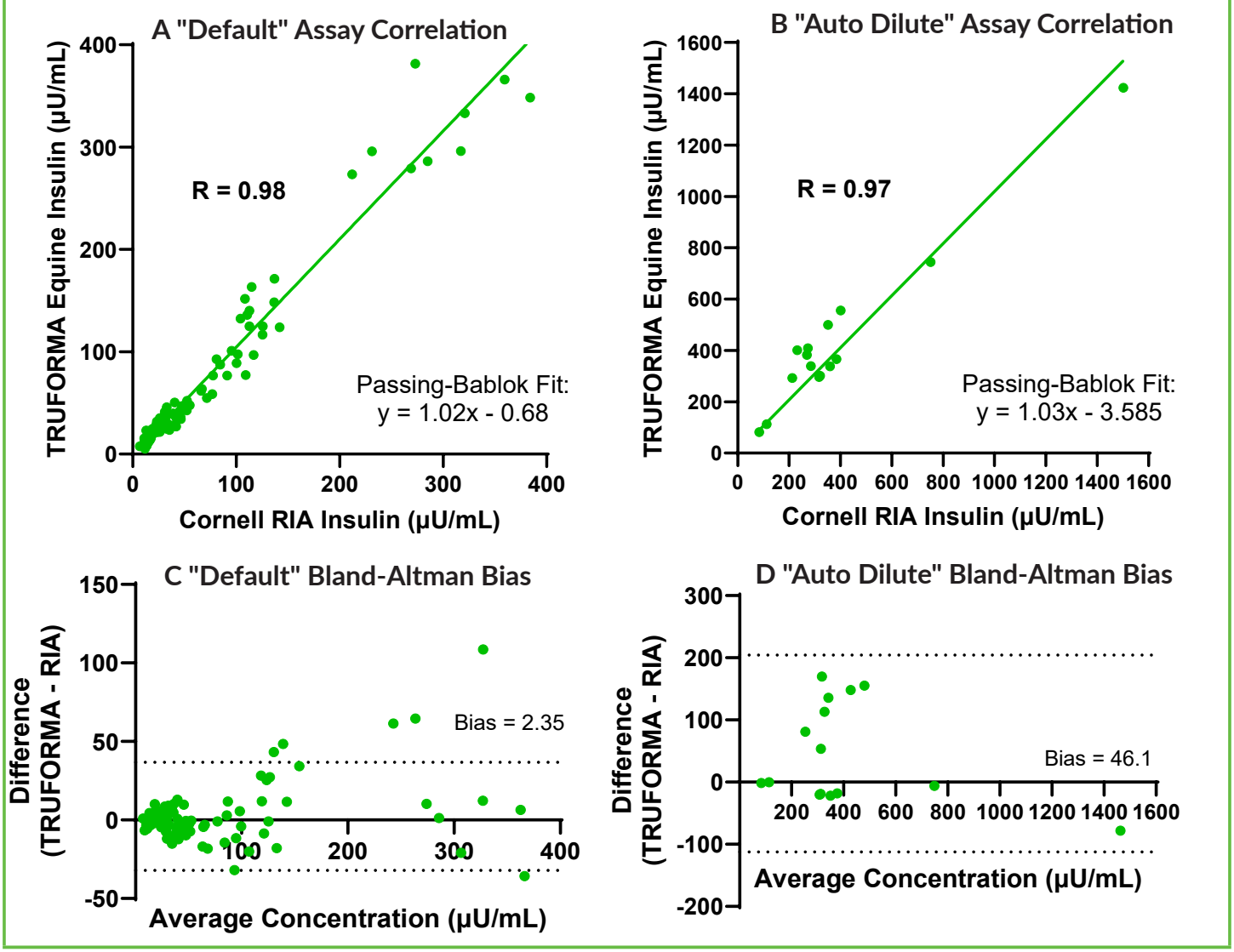
Seven samples ranging from 1501 - 23.5 µU/mL were tested on the TRUFORMA Insulin assay using either the "Default" or "Auto Dilute" protocols. Samples within the dynamic range for each assay shown. Data was fit using a linear regression.

Assay Correlation Between TRUFORMA Insulin Assay and RIA

Passing-Bablok fit 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 114 individual equine plasma samples were tested using the TRUFORMA Equine Insulin “Default” assay protocol and 15 were tested on the “Auto Dilute” protocol as well as insulin testing by RIA at the Cornell University Animal Health Diagnostic Center. The test methods report concentrations based on their respective standard curves and these results were used to generate correlation and bias plots.

The TRUFORMA Equine Insulin assay showed high correlation for the "Default" (R=0.98) and "Auto Dilute" (R=0.97) with RIA for equine plasma samples (**Figure 7A & B**), while bias analysis depicted scatter with low bias for both the "Default" (bias, 2.35 µU/mL; 95% CI -32.04-36.75) and"Auto Dilute" (bias, 46.1 µU/mL; 95% CI -112.2-204.3) protocols (**Figure 7C & D**).

Figure 7. Correlation and Bias of the TRUFORMA Equine Insulin Assay Compared with the RIA Insulin Assay



Correlation analysis was performed comparing the results from the TRUFORMA Insulin and Cornell RIA assays with 114 equine plasma samples using the "Defualt" protocol (A) and 15 equine plasma samples using the "Auto Dilute" protocol (B). Bland-Altman bias plots were generated by plotting the mean concentrations vs.the difference (TRUFORMA - IMMULITE) (C&D) Dotted lines represent the respective 95% limits of agreement.

Cross-Reactivity

Known amounts of pro-Insulin, c-peptide and glucagon were spiked into samples with known amounts of insulin. The samples were then tested using the TRUFORMA Equine Insulin assay (n=7). The cross-reactivity results are reported in **Table 2**, the addition of the potential cross-reactants did not significantly change the assay results.

Table 2. Summary of Cross-Reactivity for the TRUFORMA Equine Insulin Assay

Material	Concentration	Cross-Reactivity, %
Pro-Insulin	5 ng/mL	ND
C-peptide	1 µg/mL	0.5
Glucagon	1 µg/mL	ND

ND, not detected.

Conclusions

The TRUFORMA Insulin assay demonstrated high precision with a wide dynamic range, providing confidence in the reliability of insulin results at the POC. The TRUFORMA Equine Insulin assay's dynamic range allows the quantification of both high and low insulin concentrations within the same assay, which is vital for diagnosing and monitoring EMS in horses. The availability of the TRUFORMA Insulin assay at the POC allows for immediate testing of samples without significant sample handling and shipping that could delay results by days.

The TRUFORMA Equine Insulin 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

BAW	Bulk Acoustic Wave
CLSI	Clinical and Laboratory Standards Institute
CV	Coefficient of Variation
EMS	Equine Metabolic Syndrome
EUA	Emergency Use Authorization
ID	Insulin dysregulation
LLOQ	Lower Limit of Quantitation
LOD	Limit of Detection
LOQ	Limit of Quantitation
ND	Not Detected
POC	Point of Care
PPID	Pituitary Pars Intermedia Dysfunction
TRH	Thyrotropin-Releasing Hormone
TTR	Time to Test Results
RIA	Radioimmunoassay
ULOQ	Upper Limit of Quantitation

References

1. Durham AE, Frank N, McGowan CM, et al. ECEIM consensus statement on equine metabolic syndrome. *J Vet Intern Med*. Mar 2019;33(2):335-349. doi:10.1111/jvim.15423
2. Frank N, Tadros EM. Insulin dysregulation. *Equine Vet J*. Jan 2014;46(1):103-12. doi:10.1111/evj.12169
3. Frank N, Geor RJ, Bailey SR, Durham AE, Johnson PJ, American College of Veterinary Internal M. Equine metabolic syndrome. *J Vet Intern Med*. May-Jun 2010;24(3):467-75. doi:10.1111/j.1939-1676.2010.0503.x
4. Frank NB, S.; Bertin, F.; Burns, T.; de Laat, M.; Durham, A.; Kritchevsky, J.; Menzies-Gow, N. Recommendations for the Diagnosis and Management of Equine Metabolic Syndrome (EMS). *Equine Endocrinology Group 2022*. 2022;
5. Hodge E, Kowalski A, Torcivia C, et al. Effect of thyrotropin-releasing hormone stimulation testing on the oral sugar test in horses when performed as a combined protocol. *J Vet Intern Med*. Sep 2019;33(5):2272-2279. doi:10.1111/jvim.15601
6. US Food and Drug Administration. *Bioanalytical method validation*. 2018.
7. CLSI. *Measurement procedure comparison and bias estimation using patient samples*. . Vol. 3rd ed.