ZOMEDICA®

TRUFORMA® Point of Care

Canine Progesterone Assay

Dane Anderson, Sr. Research and Development Scientist, Zomedica

Dannyalle Breeden, Research and Development Scientist, Zomedica

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

Key Messages

- Accurate and precise measurement of endogenous serum Progesterone (P_4) levels is needed to manage timing of breeding, predict parturition in bitches, and identify reproductive disorders.
- 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 Progesterone assay allows for the quantification of high and low concentrations of serum P_4, enabling use during the entire estrus cycle and pregnancy of the patient.
- The high precision and correlation to a reference laboratory assay shown for the TRUFORMA Canine Progesterone assay provides veterinarians with accurate and reliable diagnostic results at the POC, creating opportunities for improved patient care and real-time client communication.

Introduction

Accurately monitoring breeding and parturition is challenging in veterinary medicine due to the time to result of current reference laboratory immunoassay methodologies and the need for semi-invasive examination of the patient. A reliable POC canine progesterone assay alleviates these challenges and ensures the precise timing needed for breeding and parturition. The TRUFORMA platform fits this need by using BAW sensor technology to provide veterinarians with rapid, reliable, and accurate measurement of progesterone levels at the POC.

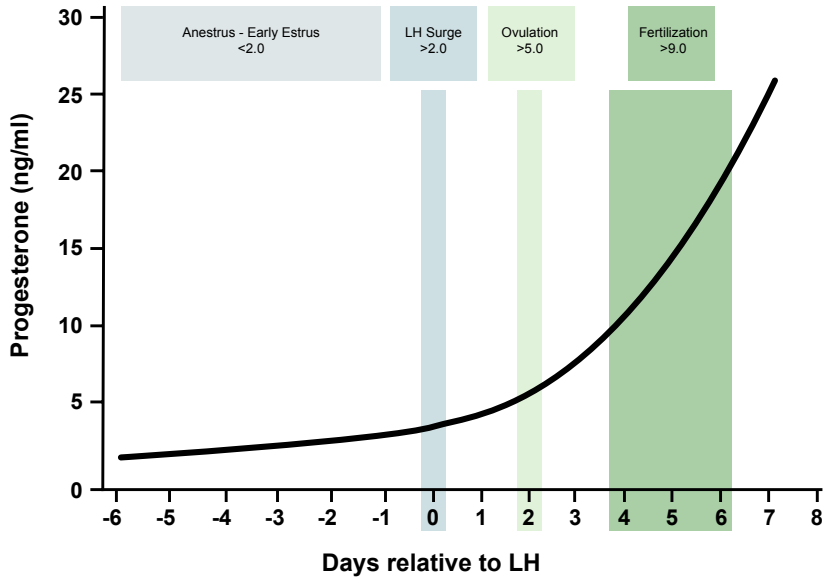
The objectives of this study were to:

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

Clinical Significance of Canine Progesterone

Progesterone is produced by ovarian corpora lutea (CL) and its primary function is the maintenance of pregnancy. Ovulation of ovarian follicles is triggered by a surge of luteinizing hormone (LH) from the pituitary gland. Unlike in other domestic mammals, the LH surge in bitches stimulates preovulatory luteinization of granulosa cells, causing progesterone levels to rise prior to ovulation. Progesterone levels continue to rise after ovulation as the CL matures. Maintenance of pregnancy in bitches is dependent upon ovarian production of progesterone for the entirety of gestation. Gestation length is 63 days, timed from the day of ovulation. Progesterone levels drop precipitously 24 – 48 hours prior to parturition.

Figure 1. Progesterone Profile of Canine Estrus Cycle



LH, Luteinizing Hormone

Canine Progesterone Testing

ZOMEDICA®

Progesterone Analysis for Timing of Breeding

Determination of the optimal breeding time in the bitch is predicated upon detecting the LH surge. Since the LH surge causes preovulatory luteinization of ovarian follicles, timing of the surge can be accomplished by measuring serum progesterone levels.

Serial progesterone measurements, starting at the onset of serosanguinous vulvar discharge and continued until breeding is complete, are recommended. In proestrus/early estrus, measurements taken every 2 – 4 days are usually adequate. After the LH surge, measurements should be taken every 2 days to ensure the expected rise in progesterone has taken place.[1]

A rise in progesterone from baseline to above 2.0 ng/mL indicates that the LH surge has occurred. Ovulation generally occurs 2 days later and is accompanied by a rise in progesterone to above 5.0 ng/mL. Progesterone continues to rise to above 10 ng/mL by day 6 post-LH surge.[2] Typical progesterone values throughout the estrus cycle are given in **Table 1**.

Oocytes are capable of undergoing fertilization 2 days post-ovulation. Pregnancies may result from natural breeding that take place from 3 days before to 7 – 10 days after the LH surge. However, for critical breedings or those performed via artificial insemination, the optimal time is from days 4 to 6 after the LH surge.[1]

Table 1. Canine Progesterone Levels

Progesterone (ng/mL)	Interpretation	Recommendation
<1.0	Anestrus/Proestrus	Recheck in 3-4 days
1.0 - 1.9	Proestrus/Estrus; Pre-LH surge	Recheck in 2 days
2.0 – 2.9	LH surge	Recheck in 2 days to confirm rise to >5 ng/mL. Plan breeding for 4-6 days after first rise above 2 ng/mL.
3.0 – 4.9	Preovulation	Recheck in 2 days to confirm continued rise. Plan to breed in 3-5 days
5.0 – 9.0	Near ovulation	Recheck in 2 days to confirm continued rise. Plan to breed in 2-4 days.
>9.0	Fertilization period	Breed today and rebreed within the next 2-3 days.

Progesterone Analysis for Predicting Onset of Parturition

When calculated from the day of the LH surge (P_4>2.0 ng/mL), 90% of bitches will whelp at 65±2 days and 100% of bitches will whelp at 65±3 days.[3] Interpretation of progesterone measurements in the prepartum period is given in **Table 2**.

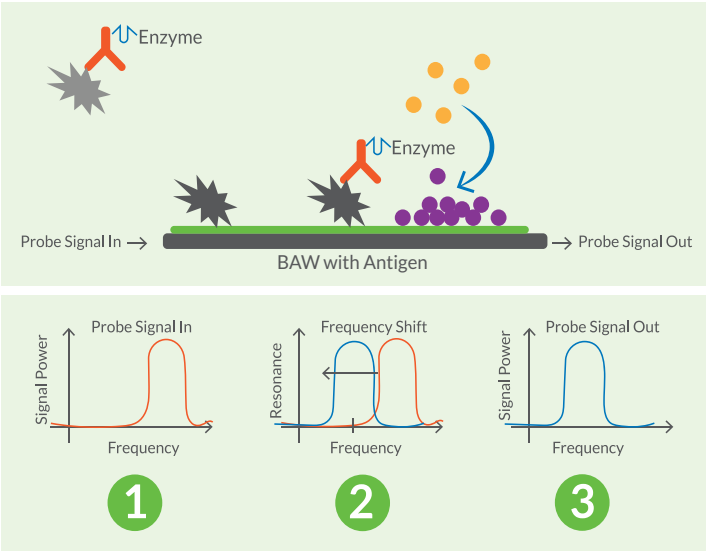
Table 2. Canine Progesterone Interpretation

Progesterone (ng/mL)	Interpretation[4]
> 5.0	Parturition unlikely within next 12 hours (<2% probability)
< 2.7	Parturition likely within 48 hours (99% probability)
< 1.0	Parturition likely within 24 hours (100% probability)

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 Progesterone Immunoassay



The TRUFORMA Canine Progesterone 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 (orange) 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. The signal is inversely proportional to the amount of analyte present in the sample. BAW, bulk acoustic wave.

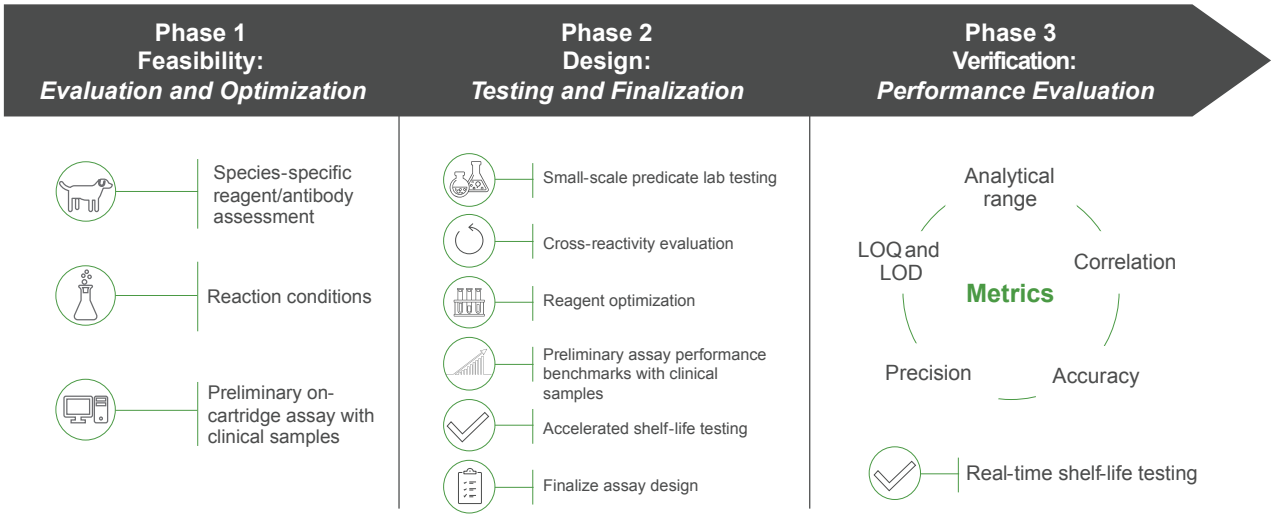
Canine Progesterone Assay Development Overview

The TRUFORMA Canine Progesterone assay utilizes a monoclonal murine antibody coated with progesterone on the BAW sensor paired with a highly sensitive monoclonal detection antibody optimized to perform at low concentrations for improved sensitivity and resilience to interference in canine serum. Using the industry standard recommendations for bioanalytical method validation[5] and the Clinical and Laboratory Standards Institute (CLSI) guidelines on method comparison and bias estimation (EP09c),[6] the TRUFORMA assay performance requirements were chosen to meet or exceed reference laboratory performance to provide equal or better results at the POC. The 3 phases of the TRUFORMA Canine Progesterone assay development were designed to create a high-quality and reliable POC assay starting with feasibility evaluation and optimization with species – specific assessment, design testing of preliminary assay performance and finally performance verification and validation (**Figure 3**).

Assay Verification Results

The TRUFORMA Canine Progesterone assay's analytical performance was evaluated and compared with the reference laboratory predicate Siemens IMMULITE® 2000 Progesterone assay, an automated solid-phase chemiluminescent competitive immunoassay. Serum from intact female dogs including breeding, pregnancy, and parturition monitoring samples were analyzed.

Figure 3. Overview of the TRUFORMA Canine Progesterone Assay Development



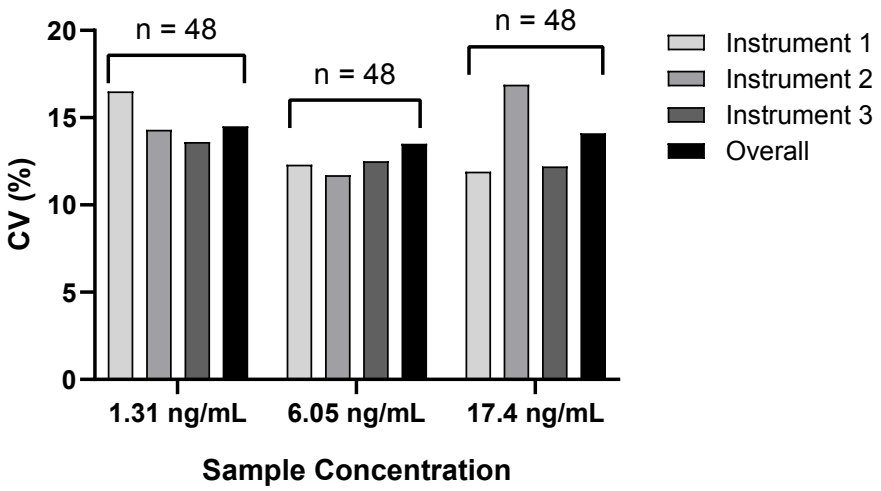
LOD, limit of detection; LOQ, limit of quantitation.

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 samples with varying P_4 concentrations. Each sample was tested with twelve (12) replicates over four (4) separate days on three (3) different instruments, for a total of 48 results per sample. Observed %CV was calculated.

The TRUFORMA Canine Progesterone 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%.[5]

Figure 4. Precision of the TRUFORMA Canine Progesterone Assay



%CV was calculated for three separate canine serum samples with varying progesterone concentrations over a total of 144 runs. CV, coefficient of variation.

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

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 pet owners (**Table 3**).

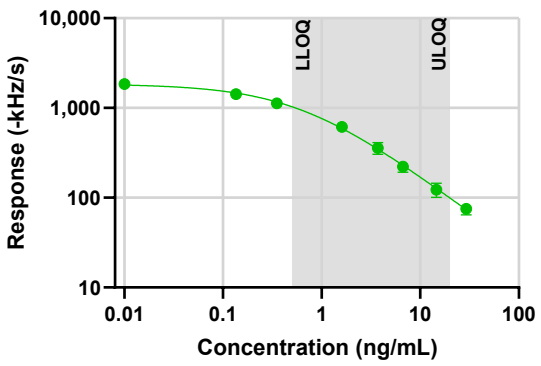
Table 3. Summary of TTR and Dynamic Range of the TRUFORMA Canine Progesterone Assay Compared with the Reference Lab Testing

Method	TRUFORMA	Reference Lab
TTR	<18 minutes	1-2 days*
Dynamic Range	0.5-20.0 ng/mL	0.2-40.0 ng/mL**

TTR, time to test result
*Includes overnight shipping to a reference laboratory
**Range for the Siemens IMMULITE 2000 Progesterone Assay

Dynamic range refers to the span of test result values that can be accurately measured by an assay. The Lower Limit of Quantitation of the TRUFORMA Canine Progesterone assay was calculated to be <0.5 ng/mL for canine serum. The lower end of the dynamic range was therefore set as 0.5 ng/mL. The upper end of the dynamic range was established at 20 ng/mL for canine serum based on predefined precision metrics that ensure accurate and reproducible quantitation of samples with the TRUFORMA Canine Progesterone assay (**Table 3**). Overall, the TRUFORMA Canine Progesterone assay's dynamic range allows the quantification of both clinically high and clinically low P_4 concentrations.

Figure 5. Standard Curve of the TRUFORMA Canine Progesterone Assay



Eight calibrators with known concentrations of progesterone were used to generate a standard curve. The shaded region represents the dynamic range of the TRUFORMA Canine Progesterone Assay. LLOQ, lower limit of quantitation; ULOQ, upper limit of quantitation.

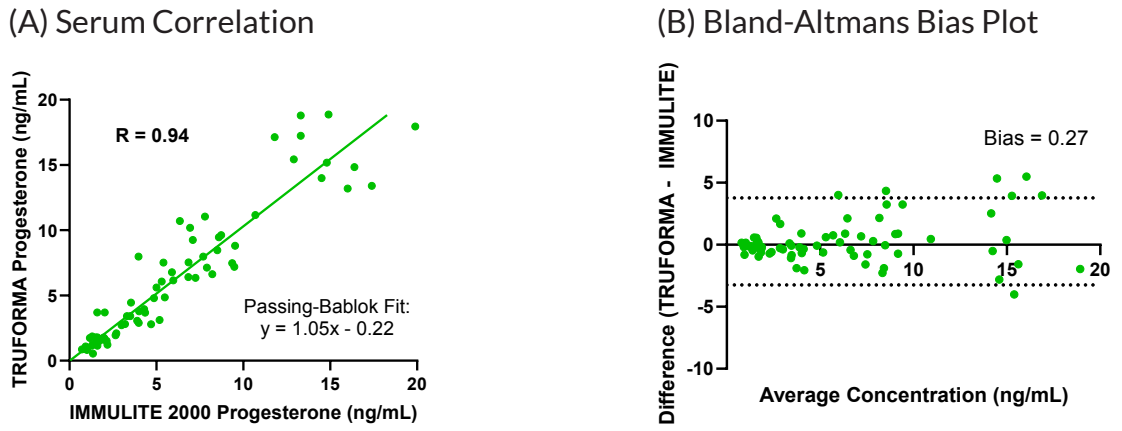
Eight (8) calibrators with known concentrations of P_4 were tested using TRUFORMA Canine Progesterone assay cartridges. Each calibrator was run with nine replicates divided across three different instruments, and the average value was used to generate a standard curve. The reportable ranges of the TRUFORMA Canine Progesterone assay illustrate linear performance within the clinically relevant range as seen below for the canine species type (**Figure 5**).

Correlation Between the TRUFORMA Progesterone Assay and the Siemens IMMULITE® 2000 Progesterone Assay

Assay correlation (Passing Bablok), 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 102 individual canine serum samples were run on the same freeze-thaw cycle on the TRUFORMA and Siemens IMMULITE 2000 devices. Of these, 72 samples were within the dynamic range of the TRUFORMA assay and used for correlation analysis. The instruments report concentrations based on their respective standard curves and these results were used to generate correlation and bias plots.

The TRUFORMA Canine Progesterone assay showed high correlation (R=0.94) with the Siemens IMMULITE 2000 for 72 canine serum samples (**Figure 6A**).

Figure 6. Correlation and Bias of the TRUFORMA Progesterone Assay Compared to the Siemens IMMULITE 2000 Progesterone Assay



Correlation studies were performed comparing the results from the TRUFORMA Canine Progesterone and Siemens IMMULITE 2000 Progesterone assays using 72 canine serum samples. (A) Passing-Bablok fit comparing the two data sets. (B) Bland-Altman bias plot generated by plotting the average progesterone concentration (mean of TRUFORMA and IMMULITE 2000) vs. the difference (TRUFORMA – IMMULITE 2000). Dotted lines represent the 95% limits of agreement.

Cross-Reactivity

Known amounts of P_4 potential cross-reactants were added to a native canine serum and tested in quadruplicate using the TRUFORMA Canine Progesterone assay.

Cross-reactivity was measured in the TRUFORMA Canine Progesterone assay, and the results are reported in **Table 4**.

Table 4. Summary of Cross-Reactivity for TRUFORMA Canine Progesterone Assay

Material	Concentration (ng/mL)	% Cross Reactivity
Altrenogest	10,000	ND
Cortisol	10,000	-0.014%
Danazol	10,000	ND
Estradiol	10,000	ND
Pregnenolone	10,000	ND
Testosterone	10,000	ND
Androstenedione*	1,000	0.292%
Corticosterone	10,000	ND
11-deoxycorticosterone	10,000	0.046%
11-deoxycortisol	10,000	0.034%
17α-hydroxyprogesterone*	1,000	ND
Medroxyprogesterone	10,000	0.112%

* 10,000 ng/mL concentration resulted in observed dose above the analytical measuring range. ND, not detectable within the precision of the TRUFORMA Canine Progesterone assay

Interfering Substances

Known amounts of potential interfering substances were added to a native canine serum and tested in quadruplicate using the TRUFORMA Canine Progesterone assay. The following endogenous interfering substances were tested: 37 mmol/L Triglycerides, 342 µmol/L Bilirubin (unconjugated), 342 µmol/L Bilirubin (conjugated), 2 g/L hemoglobin. No endogenous interfering substances tested had an impact to the results outside the precision of the assay.

Conclusions

The TRUFORMA Canine Progesterone assay demonstrated high precision with wide dynamic ranges, providing confidence in the reliability of P_4 results at the POC. The TRUFORMA Canine Progesterone assay's dynamic range allows the quantification of both high and low P_4 concentrations within the same assay, which is vital for monitoring breeding, timing parturition, and identifying reproductive disorders in dogs. The availability of the TRUFORMA Canine Progesterone assay at the POC allows for immediate testing of samples without significant sample handling and shipping that can result in missed breeding timing and delivery of pups.

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

Abbreviations and Acronyms

BAW	Bulk Acoustic Wave
CLSI	Clinical and Laboratory Standards Institute
CV	Coefficient of Variation
LH	Luteinizing Hormone
LOD	Limit of Detection
LOQ	Limit of Quantitation
LLOQ	Lower Limit of Quantitation
P_4	Progesterone
POC	Point of Care
TTR	Time to Test Results
ULOQ	Upper Limit of Quantitation

References

1. Concannon PW, England G, Verstegen III J, and Linde Forsberg C, eds. Recent advances in small animal reproduction. USA: International Veterinary Information Service; 2008 [updated 2019 May]. Determination of the Optimal Breeding Time in the Bitch: Basic Considerations; [cited 2024 Dec 14]; Available from: https://www.ivis.org/library/recentadvances-small-animal-reproduction/determination-of-optimal-breeding-time-bitch
2. Hollinshead F, Hanlon D. Normal progesterone profiles during estrus in the bitch: A prospective analysis of 1420 estrous cycles. *Theriogenology*. 2019 Feb;125:37-42. doi: 10.1016/j.theriogenology.2018.10.018. Epub 2018 Oct 22. PMID: 30388469.
3. Kutzler MA, Mohammed HO, Lamb SV, Meyers-Wallen VN. Accuracy of canine parturition date prediction from the initial rise in preovulatory progesterone concentration. *Theriogenology*. 2003 Oct 1;60(6):1187-96. doi: 10.1016/s0093-691x(03)00109-2. PMID: 12935856.
4. De Cramer KGM, Nöthling JO. The precision of predicting the time of onset of parturition in the bitch using the level of progesterone in plasma during the preparturient period. *Theriogenology*. 2018 Feb;107:211-218. doi: 10.1016/j.theriogenology.2017.11.018. Epub 2017 Nov 14. PMID: 29175732.
5. US Food and Drug Administration. Bioanalytical method validation. 2018.
6. CLSI. Measurement procedure comparison and bias estimation using patient samples. 3rd ed. CLSI guideline EP09c. Wayne, PA: Clinical and Laboratory Standards Institute; 2018.