ZOMEDICA®

TRUFORMA® Point-of-Care
Cobalamin and Folate Assay

Zach Butz, PhD, Research & Development Manager, Zomedica

Alexander Naughton, Research & Development Scientist, Zomedica

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

Key Messages

- Accurate and precise measurement of cobalamin and folate levels is needed to diagnose and manage digestive and absorptive disease in dogs and cats.
- 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 Cobalamin and Folate assay is a fully quantitative, POC assay that eliminates reference lab testing workflows, including shipping and additional sample handling.
- The dynamic range of the TRUFORMA Cobalamin and Folate assay allows for the simultaneous quantification of high and low concentrations of serum cobalamin and folate without dilution or additional processing steps, which is vital for accurate and complete diagnosis of digestive and absorptive disease in canines and felines.
- The high precision and correlation to two reference laboratory assays shown for the TRUFORMA Cobalamin and Folate assay provides veterinarians with accurate and reliable diagnostic results at the POC, creating opportunities for improved patient treatment and real-time client communication.

Introduction

Gastrointestinal (GI) issues like vomiting and diarrhea are amongst the most common reasons for canine and feline veterinary visits every year.[1] Thankfully, there are numerous diagnostic tests available to aid general practitioners in working up these cases and ensure their patients receive the appropriate treatment. A subset of GI diagnostic testing focuses on small intestinal and pancreatic function by evaluating two analytes, cobalamin (Vitamin B_{12}) and folate (Vitamin B_9). The serum concentration of these two vitamins has been well studied in relation to small intestinal mucosal diseases, exocrine pancreatic insufficiency (EPI), and small intestinal bacterial overgrowth (SIBO). Current cobalamin and folate immunoassay testing is typically performed at reference laboratories which necessitates sample shipping and longer turnaround times. While reference lab testing can provide accurate results, delayed initiation of treatment may be undesirable when dealing with persistent GI symptoms. The TRUFORMA platform, which uses BAW sensor technology, now offers a fully quantitative multiplexed immunoassay specific for both cobalamin and folate at the POC. Rapid, precise, and accurate measurement of serum cobalamin and folate on a single cartridge consumable will aid veterinarians in assessing small intestine function and other associated disorders.

The objectives of this study were to:

- Determine analytical performance attributes for the TRUFORMA Cobalamin and Folate assay.
- Describe how the TRUFORMA Cobalamin and Folate assay differs from other currently available assays.
- Compare TRUFORMA Cobalamin and Folate assay performance with two separate assays used as part of the standard of care at veterinary reference laboratories to diagnose dogs and cats presenting with GI issues.

Clinical Significance of Cobalamin and Folate Testing

Both cobalamin and folate are absorbed in the small intestine (SI), but the localization and mechanism of their uptake differ slightly. Cobalamin is dependent on the absorptive function of the distal small intestine (ileum) whereas folate is dependent on the absorptive capacity of the proximal small intestine (jejunum).[2] This spatial pattern of nutrient absorption can pinpoint potential small intestinal disease if cobalamin and/or folate serum concentrations fall outside the normal reference range. More specifically, if serum cobalamin or folate levels are below normal, this can indicate ileum or jejunum dysfunction, respectively, while a decrease in both cobalamin and folate levels may denote diffuse small intestinal disorders.[3,4]

EPI is a disease state characterized by insufficient generation of digestive enzymes from pancreatic acinar cells and one of the major impacts stemming from this reduced exocrine pancreatic activity is diminished synthesis of a protein called intrinsic factor (IF). Normally, IF is secreted into the small intestine and binds free cobalamin forming a cobalamin-IF complex that is then absorbed in the ileum via receptor-mediated endocytosis.[5] However, in animals with EPI, intrinsic factor levels are inadequate to support normal absorption of cobalamin via this pathway and upwards of 80% of dogs and 100% of cats may develop hypocobalaminemia.[6,7] This decreased intestinal uptake of cobalamin may also allow bacteria residing in the SI to access additional cobalamin and produce abnormally higher levels of folate (secondary SIBO). Elevated serum folate levels have been documented to occur in just over half of EPI dogs.[6]

Whereas EPI can lead to developing secondary SIBO due to increased cobalamin availability, primary SIBO manifests through abnormal bacterial proliferation and/or alteration of species composition in the small intestine. Ordinarily, gastric acid, intestinal motility, and pancreatic juice antibacterial activity all act to limit the bacterial population in the SI but any alteration to these protective mechanisms can lead to developing SIBO.[8] Just like in secondary SIBO, folate is commonly elevated in primary SIBO while cobalamin serum levels may be depleted due to the competition for cobalamin in the ileum (**Table 1**). Altogether, cobalamin and folate can be very informative biomarkers for diagnostic workups for chronic enteropathies (CEs).

Table 1. Serum Cobalamin and Folate Levels with Potential Disease Diagnosis

Serum Cobalamin	Serum Folate	Potential Diagnosis
Low	Normal	Ileum-localized disease
Normal	Low	Jejunum-localized disease
Low	Low	Diffuse SI disease
Low	Normal/High	EPI **or** SIBO

Cobalamin and Folate

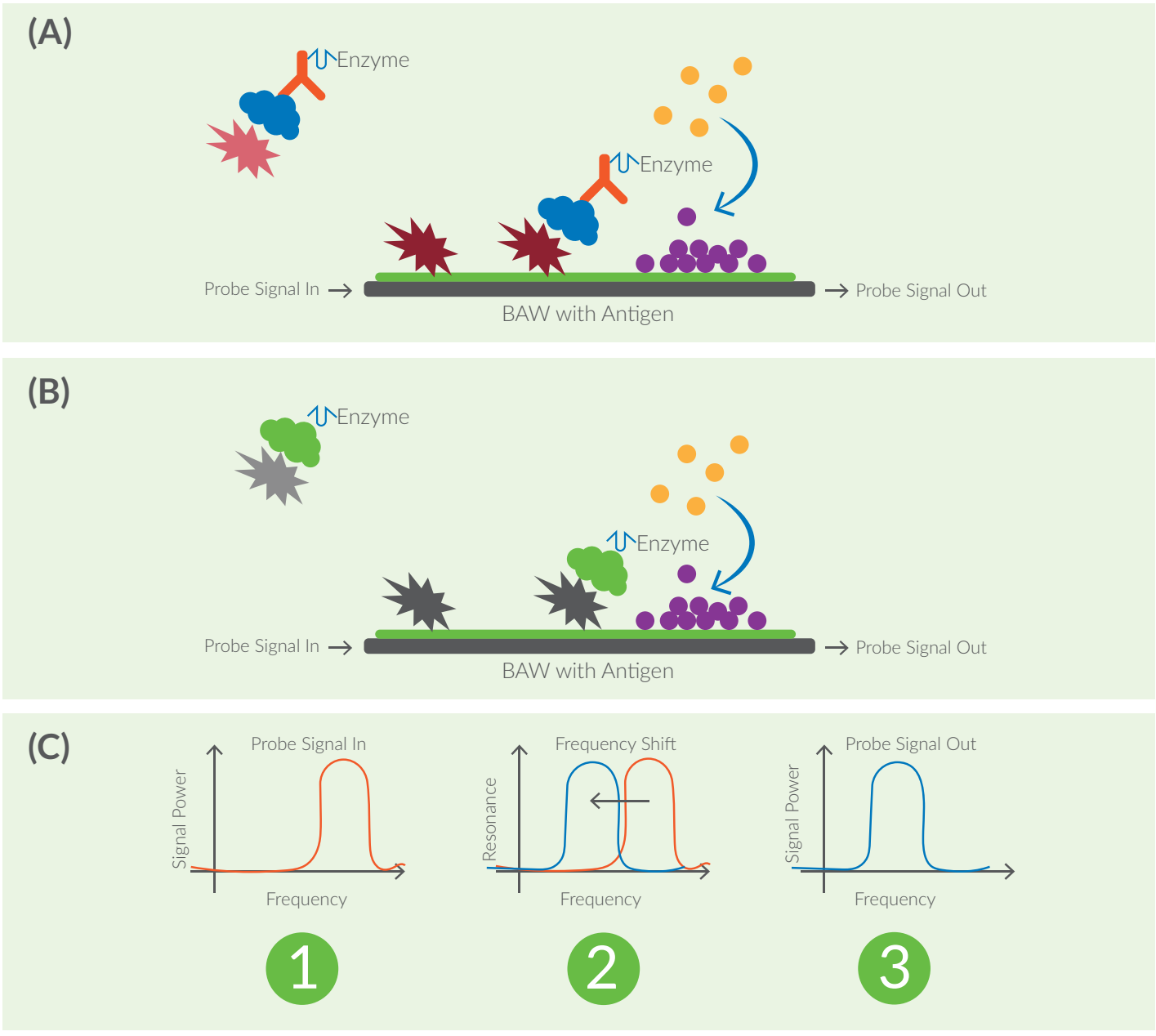
Cobalamin and folate are both water-soluble B vitamins and are involved in proliferative cellular processes like amino acid metabolism, DNA synthesis, and DNA methylation.[9] Both are traditionally abundant in small animal diets and any deficiency is likely linked to a chronic absorptive issue within the small intestine. Should vitamin supplementation be necessary following a diagnostic workup, both folic acid and cobalamin supplements are readily available and well tolerated when the regimen is adapted appropriately to the size of the patient. Failure to initiate an appropriate supplementation protocol, especially for patients with cobalamin deficiency, has been associated with poorer outcomes for dogs with CEs[10] and those undergoing chemotherapy for lymphoma.[11]

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 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 proportional to the mass accumulated on the sensor (**Figure 1**). 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 1. BAW Technology in the TRUFORMA Cobalamin and Folate Immunoassay



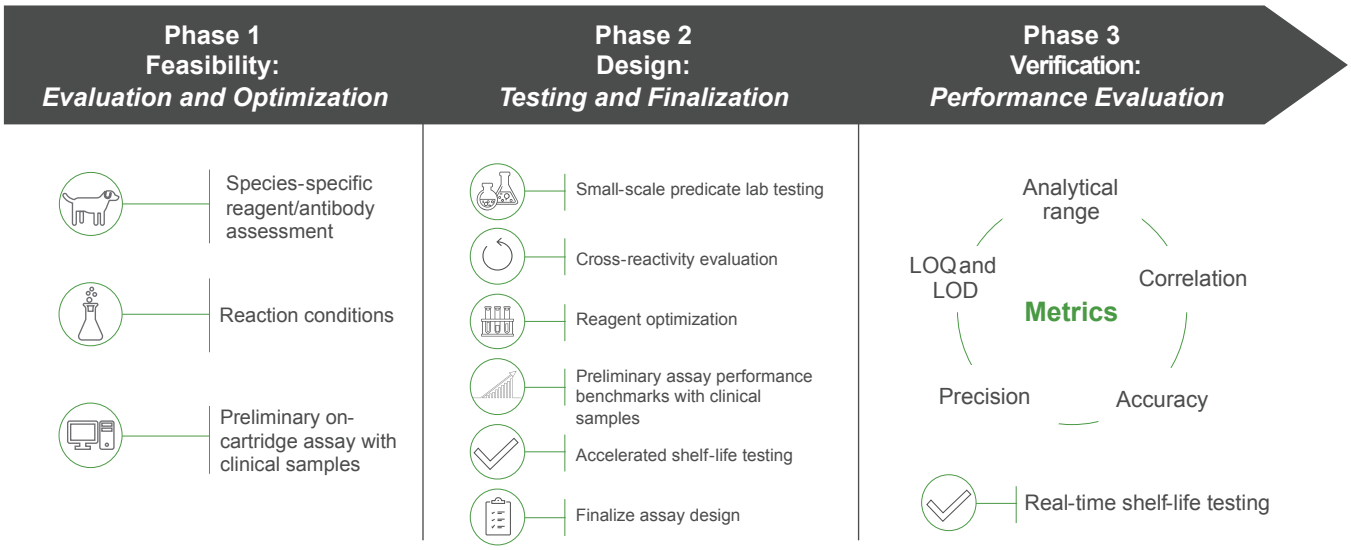
The TRUFORMA Cobalamin and Folate assay is a multiplexed immunoassay with both analytes being detected in a competitive manner. In this design, one BAW biosensor is coated with a cobalamin antigen ([A], dark red molecule) while another is coated with a folate antigen ([B], dark gray molecule). (A) Cobalamin present in the sample (light red) is bound by a specific binding protein (blue) and a conjugated antibody (orange) against the binding protein. This binding event prevents the binding protein-antibody complex from adhering to the cobalamin-coated biosensor. (B) Folate in the sample (light grey) is bound by a conjugated binding protein (green) that prevents the binding protein from adhering to the folate-coated biosensor.

After several wash steps, an enzyme substrate (yellow) is exposed across both BAW biosensor surfaces, and bound enzyme converts the substrate to an insoluble product (purple) that accumulates on the BAW biosensor surfaces. (C) This accumulation is measured as a shift in frequency by the BAW biosensors. The signals generated are specific to each analyte and are inversely proportional to the amount of cobalamin and folate present in the sample. BAW, bulk acoustic wave.

Cobalamin and Folate Assay Development Overview

The TRUFORMA Cobalamin and Folate assay is a multiplexed immunoassay with both analytes being detected in a competitive manner. Specific binding proteins and antibodies were selected for optimal performance. Using the industry standard recommendations for bioanalytical method validation[12] and the Clinical and Laboratory Standards Institute (CLSI) guidelines on method comparison and bias estimation,[13] 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 cobalamin and folate 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 2**).

Figure 2. Overview of the TRUFORMA Cobalamin and Folate Assay Development



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

Assay Verification Results

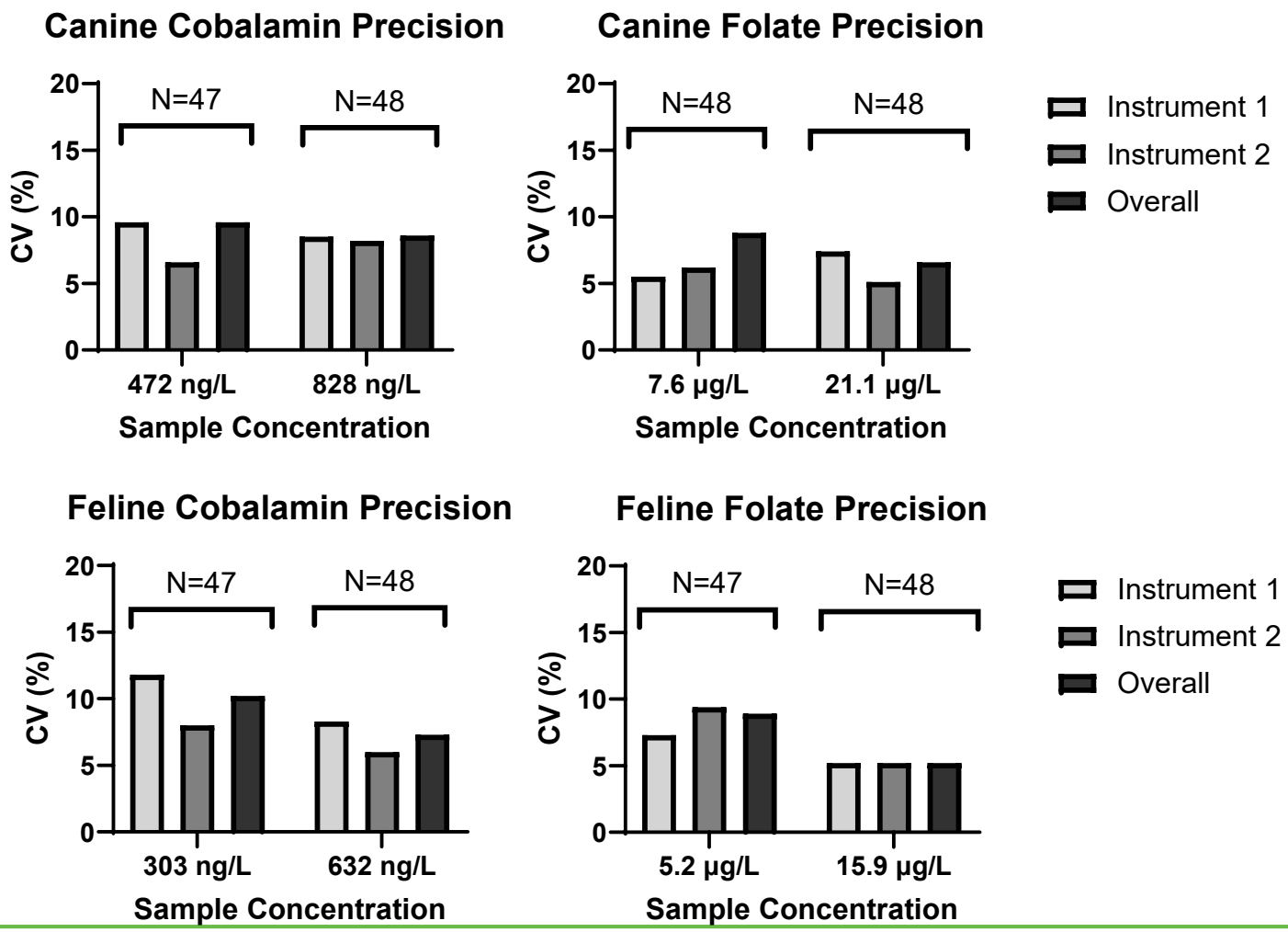
The TRUFORMA Cobalamin and Folate assay's analytical performance was evaluated and compared with the reference laboratory predicate Siemens IMMULITE® 2000's Vitamin B12 and Folic Acid assays, both automated solid-phase chemiluminescent competitive immunoassays. Samples from healthy and diseased canines and felines as well as spiked canine and feline serum samples were analyzed.

Analytical Precision

Analytical precision was evaluated for cobalamin and folate separately 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 two canine samples and two feline samples with varying cobalamin and folate concentrations. Each sample was tested with six replicates over four separate days across two different instruments, for a total of 196 results. Observed %CV was calculated.

The TRUFORMA Cobalamin and Folate assay demonstrated an overall %CV of <15% for cobalamin and <10% for folate (**Figure 3**). 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%.[12]

Figure 3. Precision of the TRUFORMA Cobalamin and Folate Assay



%CV was calculated for two canine and two feline samples with varying cobalamin and folate concentrations over a total of 19 runs. One statistical outlier was removed from the 472 ng/L canine cobalamin sample dataset, one from the 303 ng/L feline data set, and one from the 5.2 µg/L folate sample dataset following CLSI EP05-A3 guidelines. CV, coefficent of variance.

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 clients.

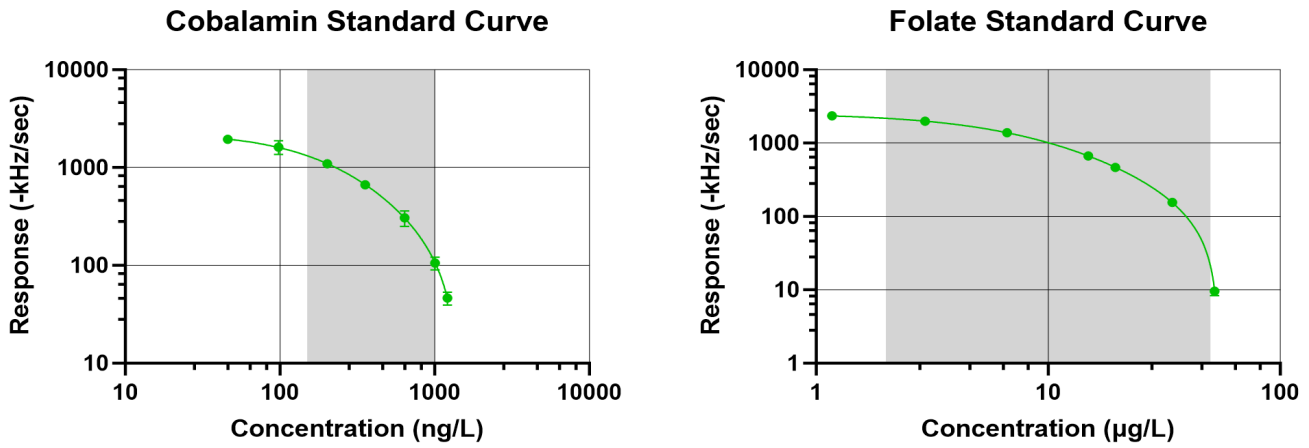
Dynamic range refers to the span of test result values that can be accurately measured by an assay, and limit of quantitation (LOQ) refers to the lowest (lower limit of quantitation [LLOQ]) and highest (upper limit of quantitation [ULOQ]) analyte concentrations that can be reliably detected with predefined accuracy and precision. The LLOQ of the TRUFORMA Cobalamin and Folate assay was determined to be 150 ng/L for cobalamin and 2 µg/L for folate. Meanwhile, the ULOQ was established at 1000 ng/L for cobalamin and 50 µg/L for folate. Overall, the dynamic range of the TRUFORMA Cobalamin and Folate assay allows for the simultaneous quantification of clinically high and low cobalamin and folate concentrations (**Table 2**).

Table 2. Summary of TTR and Dynamic Range of the TRUFORMA Cobalamin and Folate Assay Compared with the Siemens IMMULITE 2000 Vitamin B12 and Folic Acid Assays

	TRUFORMA (multiplex assay)		IMMULITE (separate assays)	
	Cobalamin	Folate	Cobalamin	Folate
TTR	24 mins		90 mins	120 mins
Dynamic Range	150-1000 ng/L	2-50 µg/L	150-1000 ng/L	1 – 24 µg/L

Seven calibrators with known concentrations of cobalamin and folate were tested using TRUFORMA Cobalamin and Folate assay cartridges. Each calibrator was run with nine replicates divided across three different instruments, and the average value was used to generate two separate standard curves, one for cobalamin and another for folate. The reportable ranges of the TRUFORMA Cobalamin and Folate assay illustrate linear performance within the clinically relevant ranges for both cobalamin and folate. (**Figure 4**).

Figure 4. Standard Curves for the TRUFORMA Cobalamin and Folate Assay

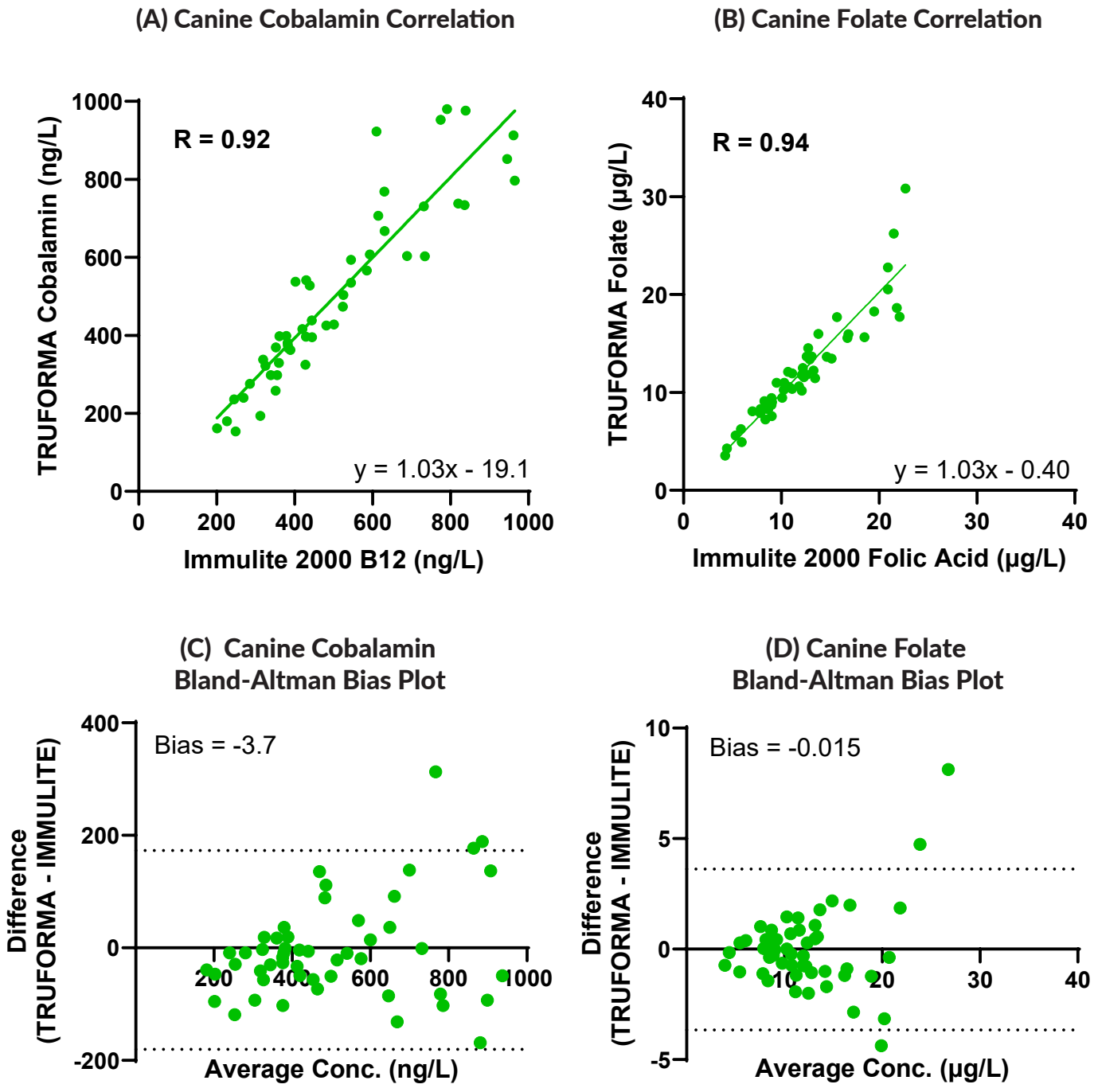


Seven calibrators with known concentrations of cobalamin and folate were used to generate analyte-specific standard curves. The shaded region of each standard curve represents the dynamic range of the TRUFORMA Cobalamin and Folate assay.

Assay Correlation Between the TRUFORMA Cobalamin and Folate Assay and IMMULITE 2000 Vitamin B12 and Folic Acid Assays

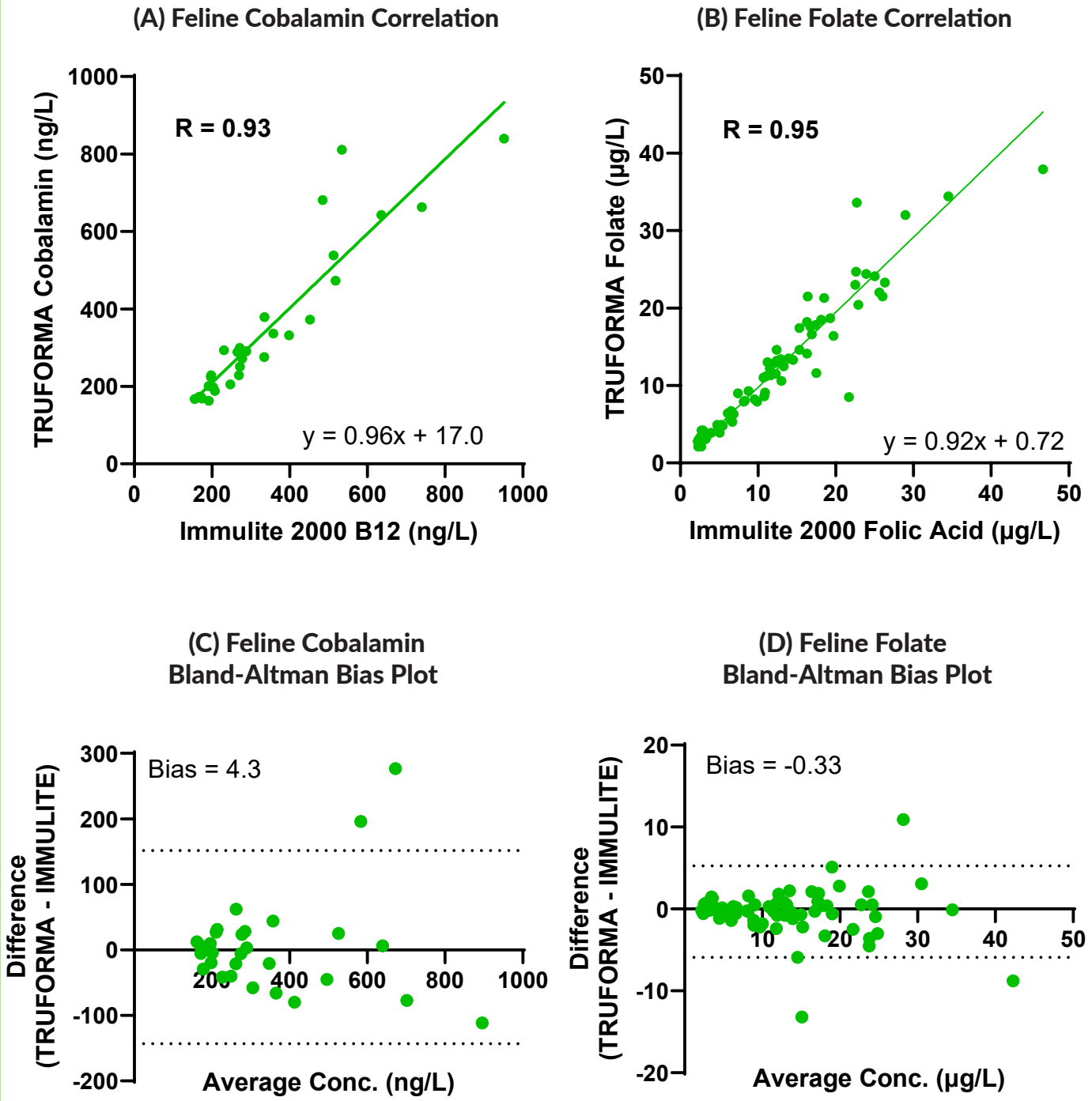
Assay correlation and Bland-Altman bias plot analysis evaluate the agreement and commutability of a new test method with a comparative or reference method. A total of 52 individual canine serum and 77 individual feline samples were run on the same freeze-thaw cycle with the TRUFORMA Cobalamin and Folate Assay and the IMMULITE 2000 B12 and Folic Acid assays. For valid results, each test method reports concentrations based on a standard curve (analyte-specific for the TRUFORMA Cobalamin and Folate assay), and the quantitative results were used to generate correlation and bias plots. The TRUFORMA Cobalamin and Folate assay showed high correlation (R=0.92-0.95) with the IMMULITE 2000 Vitamin B12 and Folic Acid assays (**Figures 5A, 5B, 6A, 6B**), while bias analysis depicted scatter with no apparent bias across the dynamic range for either analyte (**Figures 5C, 5D, 6C, 6D**).

Figure 5. Canine Correlation and Bias of the TRUFORMA Cobalamin and Folate Assay Compared with the IMMULITE 2000 Vitamin B12 and Folic Acid Assays



Correlation studies were performed comparing the results from the TRUFORMA Cobalamin and Folate assay versus the IMMULITE 2000 Vitamin B12 and IMMULITE 2000 Folic Acid assays. Individual canine serum samples were analyzed and produced correlation plots for cobalamin (A) and folate (B). A total of 52 samples were tested. One sample did not have sufficient volume for testing on Immulite for cobalamin and was excluded. All 52 samples were used for folate. Bland-Altman bias plots were generated for each analyte by plotting the mean concentration of either cobalamin (C) or folate (D) vs. the difference (TRUFORMA – IMMULITE 2000 Assay). The dotted lines in (C) and (D) represent 95% limits of agreement. CI, confidence interval.

Figure 6. Feline Correlation and Bias of the TRUFORMA Cobalamin and Folate Assay Compared with the IMMULITE 2000 Vitamin B12 and Folic Acid Assays



Correlation studies were performed comparing the results from the TRUFORMA Cobalamin and Folate assay versus the IMMULITE 2000 Vitamin B12 and IMMULITE 2000 Folic Acid assays. Individual feline serum samples were analyzed and produced correlation plots for cobalamin (A) and folate (B). A total of 77 samples were tested. A total of 30 samples were within the dynamic range for both Immulite and TRUFORMA for cobalamin, and a total of 71 samples were within the dynamic range for both Immulite and TRUFORMA for folate Bland-Altman bias plots were generated for each analyte by plotting the mean concentration of either cobalamin (C) or folate (D) vs. the difference (TRUFORMA – IMMULITE 2000 Assay). The dotted lines in (C) and (D) represent 95% limits of agreement. CI, confidence interval.

Cross-Reactivity

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

No significant cross-reactivity was observed in the TRUFORMA Cobalamin and Folate assay, and no cross-reactants interfered with the reported concentrations (**Table 3**).

Table 3. Summary of Cross-Reactivity for the TRUFORMA Cobalamin and Folate Assay

Material	Concentration, pg/mL	Cobalamin Cross-Reactivity, %	Folate Cross-Reactivity, %
Cobinamide	1000	0.03	0.27
Methotrexate	100	ND	1.78
Folinic Acid	1000	ND	0.46

ND, not detected

Conclusions

The multiplexed TRUFORMA Cobalamin and Folate assay demonstrated high precision as a POC diagnostic offering, with dynamic ranges that permit quantification of serum cobalamin and folate levels throughout the range necessary to evaluate canine and feline digestive and absorptive disease. The TRUFORMA Cobalamin and Folate assay provides veterinarians with accurate and reliable diagnostic results at the POC, allowing for improved client communication and patient treatment.

Acknowledgements

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Abbreviations and Acronyms

BAW	Bulk Acoustic Wave
CE	Chronic Enteropathy
CI	Confidence Interval
CLSI	Clinical and Laboratory Standards Institute
CV	Coefficient of Variation
EPI	Exocrine Pancreatic Insufficiency
EUA	Emergency Use Authorization
GI	Gastrointestinal
IF	Intrinsic Factor
LLOQ	Lower Limit of Quantitation
LOD	Limit of Detection
LOQ	Limit of Quantitation
POC	Point of Care
SIBO	Small Intestinal Bacterial Overgrowth
SI	Small Intestine
TTR	Time to Test Results
ULOQ	Upper Limit of Quantitation

References

1. Nationwide.com. Skin allergies, gastrointestinal disease among most common conditions that prompt veterinary visits. Published online April 24, 2024.
2. Dossin O. Laboratory tests for diagnosis of gastrointestinal and pancreatic diseases. *Top Companion Anim Med*. 2011;26(2):86-97. doi:10.1053/j.tcam.2011.02.005.
3. Ruaux C. The "GI Panel": Use, Abuse, and Interpretation. In: *DVM360*; 2015.
4. Steiner J. New Developments in Gastrointestinal Function Testing in Small Animals. In: World Small Animal Veterinary Association World Congress Proceedings, 2003. VIN.
5. Kather S, Grützner N, Kook PH, Dengler F, Heilmann RM. Review of cobalamin status and disorders of cobalamin metabolism in dogs. *J Vet Intern Med*. 2020;34(1):13-28. doi:10.1111/jvim.15638
6. Batchelor DJ, Noble PM, Taylor RH, Cripps PJ, German AJ. Prognostic factors in canine exocrine pancreatic insufficiency: prolonged survival is likely if clinical remission is achieved. *J Vet Intern Med*. 2007;21(1):54-60.
7. Siani G, Mercaldo B, Alterisio MC, Di Loria A. Vitamin B12 in Cats: Nutrition, Metabolism, and Disease. Animals (Basel). 2023 Apr 26;13(9):1474
8. Steiner J. NAVC - How I Treat Small Intestinal Dysbiosis. In: World Small Animal Veterinary Association World Congress Proceedings, 2014. VIN.
9. Lyon P, Strippoli V, Fang B, Cimmino L. B vitamins and one-carbon metabolism: implications in human health and disease. *Nutrients*. 2020;12:2867.
10. Allenspach K, Wieland B, Gröne A, Gaschen F. Chronic enteropathies in dogs: evaluation of risk factors for negative outcome. *J Vet Intern Med*. 2007;21(4):700-708.
11. Cook AK, Wright ZM, Suchodolski JS, Brown MR, Steiner JM. Prevalence and prognostic impact of hypocobalaminemia in dogs with lymphoma. *JAVMA*. 2009;235(12):1437-1441.
12. US Food and Drug Administration. Bioanalytical method validation. 2018.
13. CLSI. Measurement procedure comparison and bias estimation using patient samples. 3rd ed. CLSI guideline EP09c. Wayne, PA: Clinical and Laboratory. Standards Institute; 2018.