Canine NT-proBNP – A promising marker of heart failure in dogs

NT-proBNP measurement has been introduced into veterinary practice over the last decade. NT-proBNP levels are elevated in dogs with mitral valve disease and dilated cardiomyopathy. The highest concentrations can be observed in dogs that develop congestive heart failure. NT-proBNP measurement helps to distinguish congestive heart failure from primary respiratory tract disease as an underlying cause of respiratory signs in dogs (1). The NT-proBNP concentration in blood correlates with the severity of the disease and reflects the risk of subsequent complications. An increasing number of studies have shown that NT-proBNP can be successfully used for the diagnosis of cardiac disease in dogs, assessing the severity of the disease in dogs with cardiac disease and prognosis in dogs with heart disease (reviewed in 2).

One of the main challenges with canine NT-proBNP measurements is the low stability of the analyte in blood samples. The degradation of NT-proBNP during sample storage and transportation leads to a decrease in the NT-proBNP concentration that is determined in the specimen.

The apparent stability of an analyte depends on the specificity of antibodies used in an immunoassay and can be improved by using antibodies that are specific to a stable part of the molecule. Through selecting antibodies that are less sensitive to the degradation of NT-proBNP this might allow less stringent and complicated instructions for sample handling and storage. Such a robust assay would greatly improve the clinical utility of canine NT-proBNP assay.

Monoclonal antibodies and calibrator from Hytest

We offer both the calibrator and various MAbs with pair recommendations for the development of canine specific NT-proBNP immunoassays. These tools enable the development of highly specific immunoassays for the determination of canine NT-proBNP concentration in blood. In our preliminary studies we observed that plasma samples could be stored for at least 72 hours at +4°C or for 24 hours at +20°C with little to no loss in immunoreactivity of NT-proBNP in the in-house assays that utilized our best MAb combinations.

NT-proBNP and BNP are established biomarkers of heart failure in human beings

B-type natriuretic peptide (BNP) is a cardiac hormone that is involved in the maintenance of blood pressure, water and electrolyte balance. It reduces vascular resistance and increases both diuresis and sodium excretion, thus lowering systemic blood pressure. BNP is synthesized as prohormone and specific cleavage of the precursor molecule results in the formation of active BNP and the N-terminal fragment of proBNP (NT-proBNP). Both BNP and NT-proBNP are secreted to blood in equimolar amounts. Further information can be found in reviews by Goetze (2012) and Potter (2011) (3-4).

In human beings, BNP and NT-proBNP concentrations in blood are increased in different cardiovascular pathologies. However, the most prominent growth is observed in heart failure. Nowadays, both proteins are used as biochemical markers of heart failure. The quantification of either BNP or NT-proBNP in blood improves the diagnostic accuracy compared to standard clinical judgment in the diagnosis of acute heart failure among patients presenting to the emergency department with acute dyspnea. Both BNP and NT-proBNP are also powerful prognostic indicators for patients with heart failure or acute myocardial infarction.

ANTI-CANINE NT-PROBNP MONOCLONAL ANTIBODIES

Hytest offers several monoclonal antibodies that are specific to different regions of canine NT-proBNP (Figure 1). All of the provided antibodies recognize both the recombinant and native NT-proBNP from canine plasma.

Canine NT-proBNP quantitative sandwich immunoassays

A panel of more than sixty monoclonal antibodies was developed against canine NT-proBNP. All of the antibodies were tested as a capture and detection antibody in a sandwich immunoassay to determine the best antibody combinations. Capture antibodies were absorbed onto a 96-well plate while detection antibodies were labeled with stable europium chelate. Recombinant canine NT-proBNP (Cat. #8CNT9) and native canine NT-proBNP from dog plasma were used as antigens for antibody pairs testing. A number of combinations demonstrated high sensitivity in the sandwich immunoassays for detecting both recombinant and endogenous NT-proBNP. The best MAb combinations are given in Table 1.

The sensitivity of these immunoassays for recombinant NT-proBNP was 25 pg/ml [1]. Calibration curves for recommended combinations are provided in Figure 2.

Please note that an immunoassay performance depends on a number of factors. These include the diagnostic platform, the type of label conjugated with the detection antibody and the labeling protocol. Therefore, other combinations of anti-NT-proBNP antibodies with non-overlapping epitopes could demonstrate an improved performance in the immunoassays of our customers than those listed above.

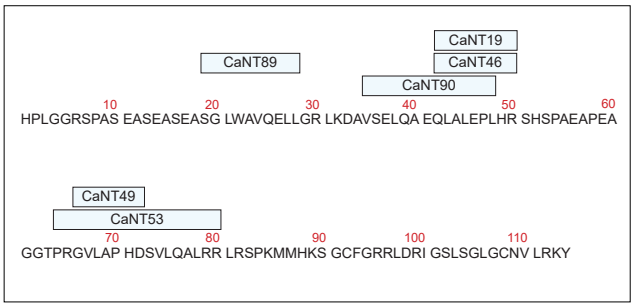

Figure 1.
Anti-canine NT-proBNP monoclonal antibodies: Location of epitopes.

Table 1.
The most sensitive capture-detection pairs.

Capture	Detection
CaNT90	CaNT89
CaNT19	CaNT89
CaNT90	CaNT53

[1] NT-proBNP concentration in pmol/L can be obtained by dividing the concentration in pg/ml by 10.545

Quantification of NT-proBNP in canine plasma of healthy dogs and dogs with heart disease

Selected antibody combinations were tested with plasma samples from healthy dogs and dogs with heart disease. The NT-proBNP concentrations were significantly higher in the group of dogs with heart disease than in control dogs for all immunoassays that were tested in this study. Even for samples with high NT-proBNP concentrations no dilution step was required, which was due to the wide dynamic range of the assays used. Results of NT-proBNP measurements in individual plasma samples using the MAb combination CaNT90-CaNT89 are provided in Figure 3 as an example.

In conclusion, our results demonstrate that immunoassays using selected MAb combinations are useful for the quantification of NT-proBNP in the plasma of dogs.

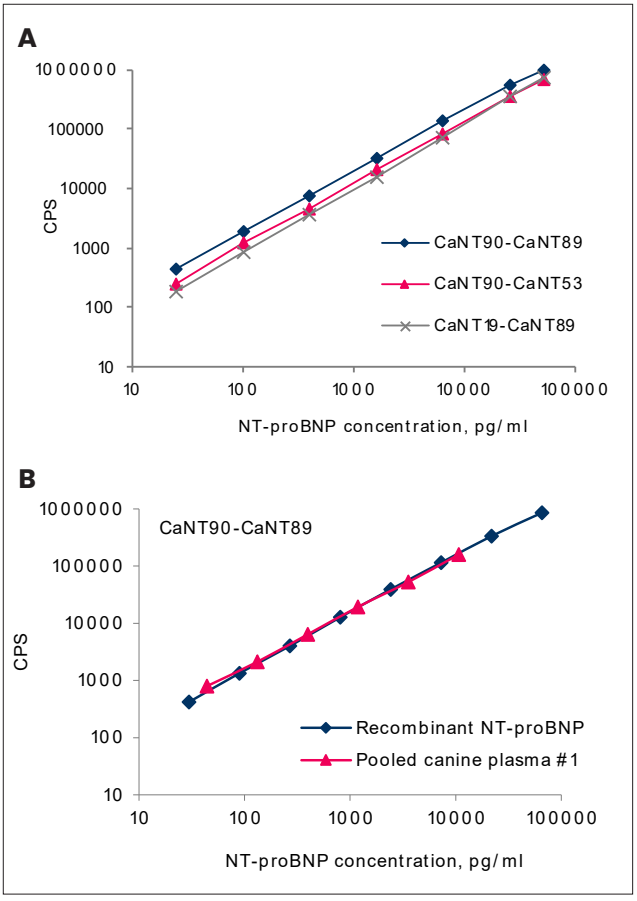

Figure 2.
Calibration curves for NT-proBNP sandwich immuno-assay.
(A) Calibration curves of the best immunoassays.
(B) Parallelism between the calibration curve and curve of serial dilution of pooled canine plasma sample.
Assay type: Two-step sandwich type fluoroimmunoassays in streptavidin coated plates
Capture MAb: 200 ng/well, biotinylated
Detection MAb: 200 ng/well, labeled with europium chelate
Antigen: Canine recombinant NT-proBNP (Cat.# 8CNT9)
Sample volume: 50 µl
Incubation time: 40 minutes at room temperature

Improved apparent stability of endogenous NT-proBNP in plasma samples

One of the main challenges for the reliable measurement of the concentration of NT-proBNP in samples is the degradation of the protein over time (5-7). While proper sample handling and storage are critical in terms of reducing degradation, another important factor is the selection of antibodies in the assay. Analyte immunoreactivity decreases when the epitope of at least one antibody is damaged or a protease cleavage site is located between the epitopes of capture and detection antibodies. Therefore, the apparent stability of an analyte depends on the specificity of antibodies and can be improved by using antibodies specific to the stable part of the molecule. When developing a canine NT-proBNP assay special attention must be paid to the selection of antibodies that should not be affected by the proteolytic degradation of NT-proBNP.

We tested the ability of our antibodies to detect endogenous canine NT-proBNP during sample storage. Pooled EDTA plasma of dogs with heart disease was incubated at two different temperatures. At +4°C NT-proBNP remained stable for at least 72 hours (95-105% of initial immunoreactivity was detected in samples, Figure 4A). Meanwhile, with the plasma incubated at +20°C, 89-98% of initial immunoreactivity was detected in samples after 24 hours (Figure 4B). This preliminary data indicates that with our recommended antibody pairs plasma could be stored at +4°C for at least 72 hours with little to no loss in the immunoreactivity. When stored at room temperature, the signal decreased - but not dramatically - during the first 24 hours. Please note that the stability of native NT-proBNP in individual plasma samples or in serum samples might differ from the results shown here.

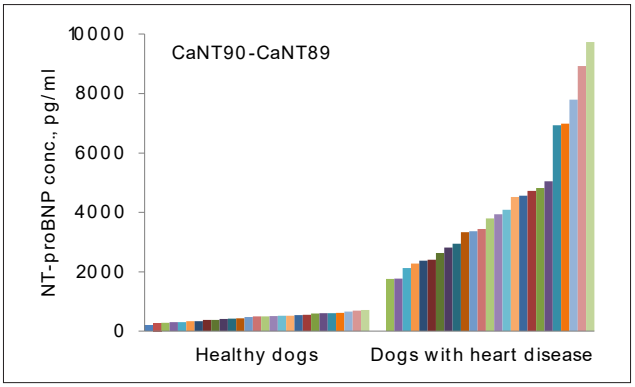

Figure 3.
The NT-proBNP concentration in EDTA plasma of healthy dogs and dogs with heart disease.
Assay type: Two-step sandwich type fluoroimmunoassay
Capture MAb CaNT90: 1 µg/well
Detection MAb CaNT89: 200 ng/well, labeled with europium chelate
Calibrator: Canine recombinant NT-proBNP (Cat.# 8CNT9)
Sample volume: 50 µl
Incubation time: 40 minutes at room temperature

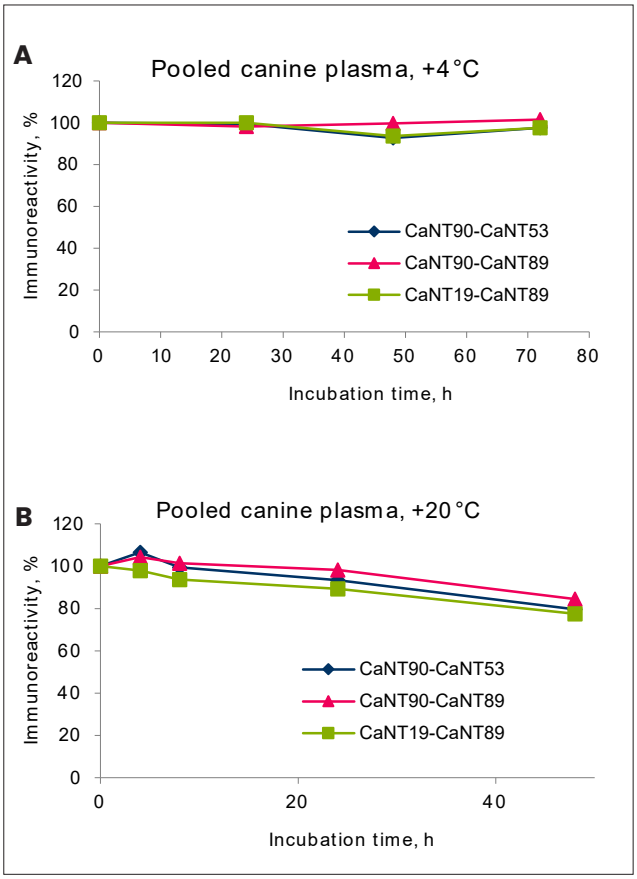

Figure 4.
Stability of endogenous canine NT-proBNP in pooled EDTA-plasma.
(A) Immunoreactivity of NT-proBNP in the plasma sample incubated at +4°C for 24, 48 and 72 hours. (B) Immunoreactivity of NT-proBNP in the plasma sample incubated at +20°C for 4, 8, 24, and 48 hours. EDTA plasma was collected without protease inhibitors; samples were centrifuged, separated and stored frozen at -70°C before use. Pooled EDTA plasma was incubated at +4°C or +20°C with the addition of 0.1% of NaN3 to prevent bacterial growth. Following incubation, samples were stored at -70°C prior to measurements. The NT-proBNP concentration in the pooled plasma was 9 ng/ml (determined by CaNT90- CaNT89 immunoassay).
Assay protocol like described in the caption of Figure 3 using CaNT90 and CaNT19 as capture and CaNT53 and CaNT89 as detection MAbs respectively.

Canine NT-proBNP immunodetection in Western blotting

Hytest antibodies can be used for NT-proBNP immunodetection in Western blotting (Figure 5).

CANINE RECOMBINANT NT-PROBNP EXPRESSED IN *E. COLI*

Our recombinant NT-proBNP corresponds to the fragment 1-85 a.a.r. of canine proBNP and contains an additional affinity tag sequence of 16 a.a.r. at the N-terminus. The protein is purified to homogeneity using tag affinity chromatography (Figure 6).

In order to confirm that the N-terminal tag does not interfere with antibody binding, recombinant canine NT-proBNP with and without tag were compared using sandwich type immunoassays. Nine pairs of antibodies specific to different regions of NT-proBNP were used. The results obtained showed that the immunochemical activities of both recombinant proteins were highly similar. Representative calibration curves are providedin Figure 7. This data demonstrates that the recombinant NT-proBNP containing an N-terminal tag is a suitable calibration material for canine NT-proBNP immunoassays.

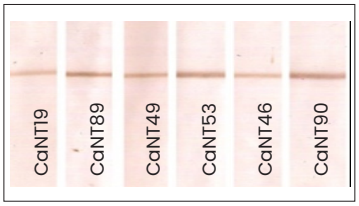

Figure 5.
Detection of canine recombinant NT-proBNP (Cat.# 8CNT9) in Western blotting by different monoclonal antibodies. *NT-proBNP (0.1 µg/lane) was transferred to nitrocellulose membrane following tricine-SDS-PAGE in reducing conditions and probed by HRP-conjugated monoclonal antibodies (direct detection).*

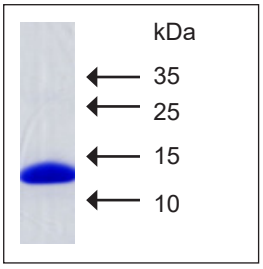

Figure 6.
Tricine-SDS-PAGE of canine recombinant NT-proBNP (10 µg) in reducing conditions. *Gel was stained with Coomassie brilliant blue R-250.*

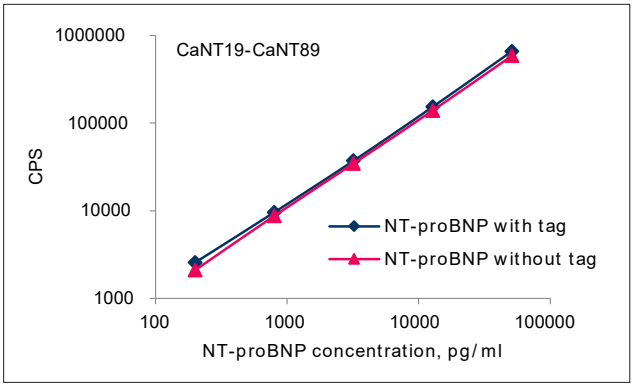

Figure 7.
Comparison of immunochemical activity of recombinant canine NT-proBNP with and without an N-terminal proprietary tag.
Assay type: Two-step sandwich type fluoroimmunoassay
Capture MAb CaNT19: 1 µg/well
Detection MAb CaNT89: 200 ng/well, labeled with europium chelate
Antigens: canine recombinant NT-proBNP with tag (Cat.# 8CNT9) and canine recombinant NT-proBNP without tag
Sample volume: 50 µl. Incubation time: 40 minutes at room temperature.

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ORDERING INFORMATION

MONOCLONAL ANTIBODIES

Product name	Cat. #	MAb	Subclass	Remarks
NT-proBNP, canine	4CNT5	CaNT89	IgG1	EIA, a.a.r. 19-28
		CaNT90	IgG1	EIA, a.a.r. 35-48
		CaNT19	IgG1	EIA, a.a.r. 42-50
		CaNT46	IgG1	EIA, a.a.r. 42-50
		CaNT49	IgG1	EIA, a.a.r. 66-72
		CaNT53	IgG1	EIA, a.a.r. 64-80

These products and some applications in which these products may be used could be covered by patents issued and applicable in certain countries. As the purchase of these products does not include a license to perform any patented application, users of these products may be required to obtain a patent license depending on the specific application and country in which the product is used.

ANTIGEN

Product name	Cat. #	Purity	Source
NT-proBNP, canine	8CNT9	>95%	Recombinant

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