Advanced ImmunoChemical Inc.
Providing for the Advancement of Medical Science

ASSAY NOTES
Product Information

Courtesy of HyTest, Ltd

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

Monoclonal antibodies and calibrator from Advanced ImmunoChemical

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. Plasma samples can 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 in-house assays that utilize best MAb combinations.

Anti-canine NT-proBNP monoclonal antibodies

Advanced ImmunoChemical 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.

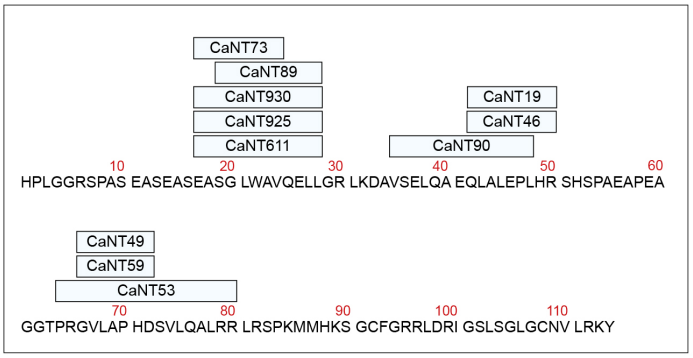

Figure 1. Anti-canine NT-proBNP monoclonal antibodies: Location of epitopes.

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. #8CNT) 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.

Capture	Detection
CaNT90	CaNT89
CaNT19	CaNT89
CaNT930	CaNT49
CaNT90	CaNT53

Table 1. The most sensitive capture-detection pairs.

The sensitivity of these immunoassays for recombinant NT-proBNP was 25 pg/ml. 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.

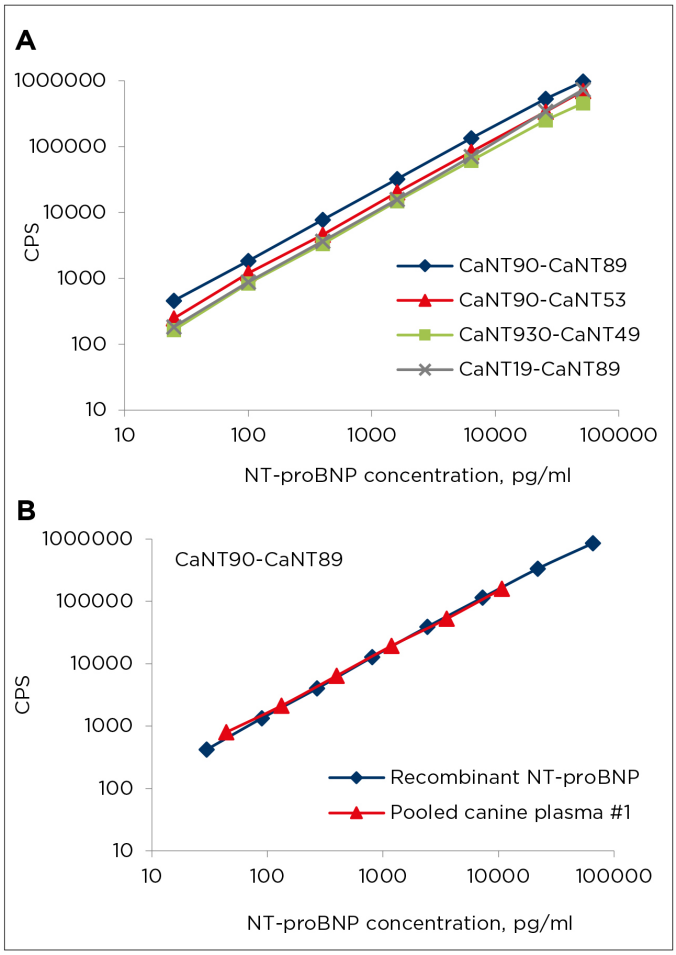

Figure 2. Calibration curves for NT-proBNP sandwich immunoassay.
(A) Calibration curves of the best immunoassays.
(B) Parallelism between the calibration curve and curve of serial dilution of pooled canine plasma sample.
Assay type: Two-step sandwich type fluoroimmunoassays in streptavidin coated plates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

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.

The data shows that immunoassays using selected MAb combinations are useful for the quantification of NT-proBNP in the plasma of dogs.

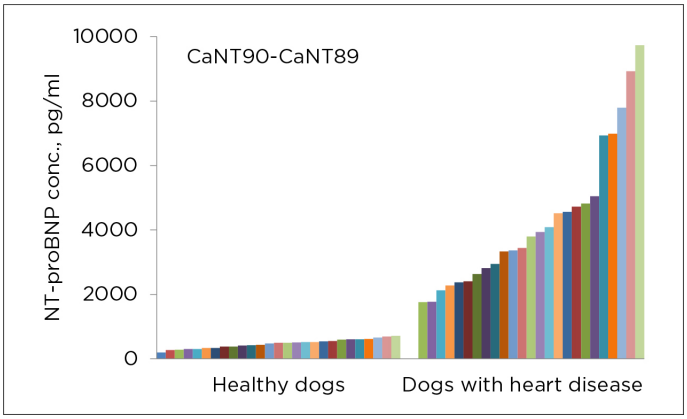

Figure 3. The NT-proBNP concentration in EDTA plasma of healthy dogs and dogs with heart disease.
Assay type: Two-step sandwich type fluoroimmunoassay
Capture MAb CaNT90: 1 µg/well
Detection MAb CaNT89: 200 ng/well, labeled with europium chelate Calibrator: Canine recombinant NT-proBNP
Sample volume: 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 (1-3). 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.

Antibodies 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 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.

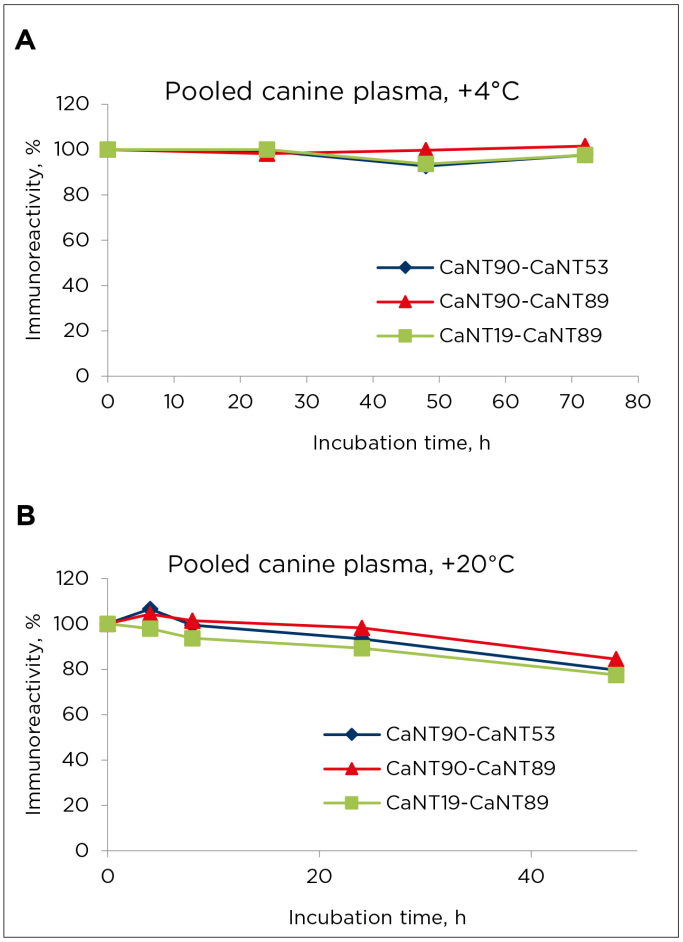

Figure 4. Stability of endogenous canine NT-proBNP in pooled EDTA-plasma. *(A) Immunoreactivity of NT-proBNP in the plasma sample incubated at +4°C for 24, 48 and 72 hours. (B) Immunoreactivity of NT-proBNP in the plasma sample incubated at +20°C for 4, 8, 24, and 48 hours. EDTA plasma was collected 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

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

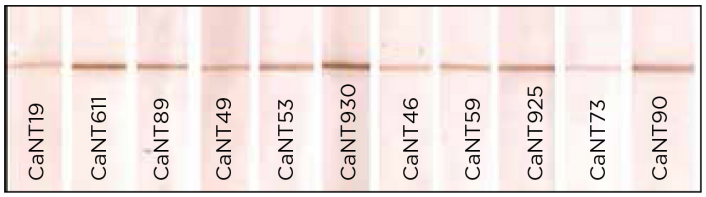

Figure 5. Detection of canine recombinant NT-proBNP (Cat.# 8CNT) in Western blotting by different monoclonal antibodies. *NT-proBNP (0.1 µg/lane) was transferred to nitrocellulose membrane following tricine-SDS-PAGE in reducing conditions and probed by HRP-conjugated monoclonal antibodies (direct detection). In cases of CaNT59 and CaNT73, non-labeled primary antibodies were used for probing and were followed by anti-mouse IgG antibodies conjugated with HRP.*

Canine recombinant NT-proBNP expressed in E. coli

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).

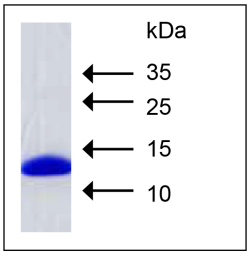

Figure 6. Tricine-SDS-PAGE of canine recombinant NT-proBNP (10 µg) in reducing conditions. *Gel was stained with Coomassie brilliant blue R-250.*

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 provided in 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.

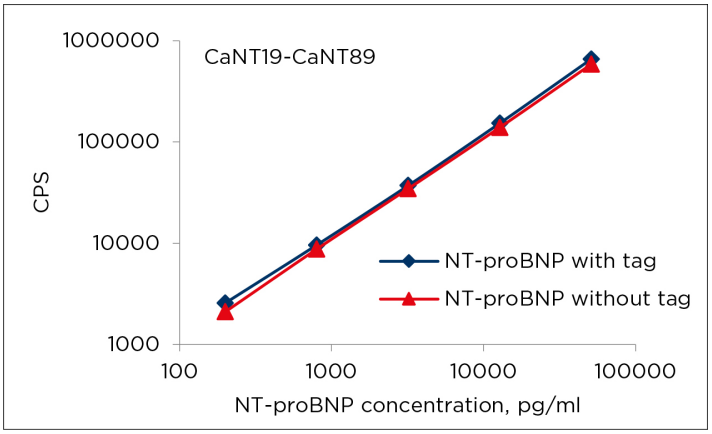

Figure 7. Comparison of immunochemical activity of recombinant canine NT-proBNP with and without an N-terminal proprietary tag.
Assay type: Two-step sandwich type fluoroimmunoassay
Capture MAb CaNT19: 1 µg/well
Detection MAb CaNT89: 200 ng/well, labeled with europium chelate Antigens: canine recombinant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

Ordering Information:
MONOCLONAL ANTIBODIES

Product Name	Cat #	MAb	Subclass	Remarks
NT-proBNP, canine	4CNT	CaNT73	IgG2a	EIA, a.a.r. 17-24
		CaNT925	IgG1	EIA, a.a.r. 17-28
		CaNT930	IgG1	EIA, a.a.r. 17-28
		CaNT611	IgG1	EIA, a.a.r. 17-28
		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
		CaNT59	IgG2b	EIA, a.a.r. 66-72
		CaNT53	IgG1	EIA, a.a.r. 64-80

Ordering Information:
ANTIGEN

Product Name	Cat #	Purity	Source
NT-proBNP, canine	8CNT	>95%	Recombinant

References

1. Collins, S. Measuring NT-proBNP in small animal practice. Royal College of Veterinary Surgeons Diploma in veterinary cardiology. 2013.

2. Collins SA, Patteson MW, Connolly DJ, Brodbelt DC, Torrance AG, Harris JD. Effects of sample handling on serum N-terminal proB-type natriuretic peptide concentration in normal dogs and dogs with heart disease. J Vet Cardiol. 2010 Apr, 12(1):41-8.

3. Hezzell MJ, Boswood A, Lötter N, Elliott J. The effects of storage conditions on measurements of canine N-terminal pro-B-type natriuretic peptide. J Vet Cardiol. 2015, 17(1):34-41.

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