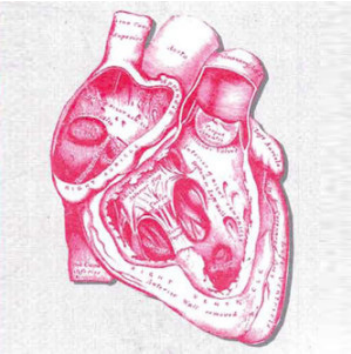
Advanced ImmunoChemical Inc.
Providing for the Advancement of Medical Science

ASSAY NOTES
Product Information

Courtesy of HyTest, Ltd

Human ProBNP and proBNP-derived Peptides (BNP and NT-proNBP)



Brain natriuretic peptide (BNP) is the product of proteolytic processing of the precursor molecule preproBNP (Fig. 1). PreproBNP is composed of 134 a.a.r. and it is synthesized in cardiac myocytes. The removal of signal peptide (a.a.r. 1-26) results in the appearance of the proBNP molecule (a.a.r. 27-134). Then proBNP (108 a.a.r.) is processed in convertase-dependent reaction and forms two peptides, the BNP (a.a.r. 77-108) and the N-terminal part of the proBNP (NT-proBNP, a.a.r. 1-76). Both BNP (biologically active molecule) and NT-proBNP (physiological activity is not clarified) as well as unprocessed proBNP are secreted into the bloodstream and circulate in human blood. The reason(s) for this incomplete proBNP processing were unknown until recently. Amino acid sequences, isoelectric points and molecular weights of all three peptides are presented in Table 1.

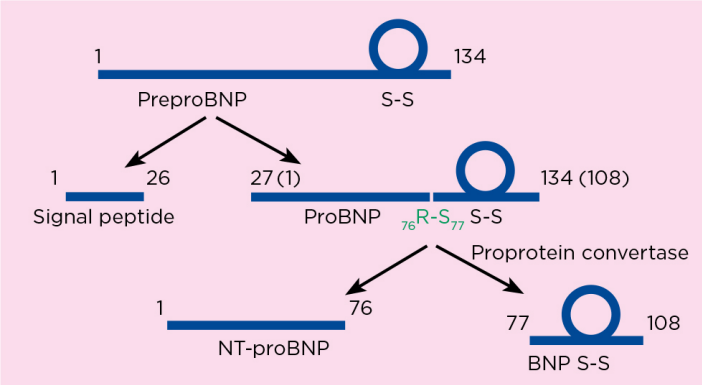


Figure 1. Schematic representation of preproBNP processing. *Removal of the signal peptide (26a.a.r.) from the N-terminus of preproBNP results in the proBNP molecule (108 a.a.r.) which is then processed in a convertase-dependent reaction to form bioactive BNP (32 a.a.r.) and the N-terminal part of the proBNP, namely NT-proBNP (76 a.a.r.).*

BNP
SPKMVQGSGC FGRKMDRISS SSGLGCKVLR RH
Molecular weight: *3466 Da*
pI *10.95*

NT-proBNP
HPLGSPGSAS DLETSGLQEQ RNHLQGKLSE LQVEQTSLEP LQESPRPTGV WKSREVATEG IRGHRKMVLY TLRAPR
Molecular weight: *8457.4 Da*
pI *8.45*

proBNP
HPLGSPGSAS DLETSGLQEQ RNHLQGKLSE LQVEQTSLEP LQESPRPTGV WKSREVATEG IRGHRKMVLY TLRAPRSPKM VQGSGCFGRK MDRISSSSGL GCKVLRRH
Molecular weight: *11905.5 Da*
pI *10.12*

Table 1. Amino acid sequences, isoelectric points and molecular weights of BNP, proBNP and NT-proBNP.

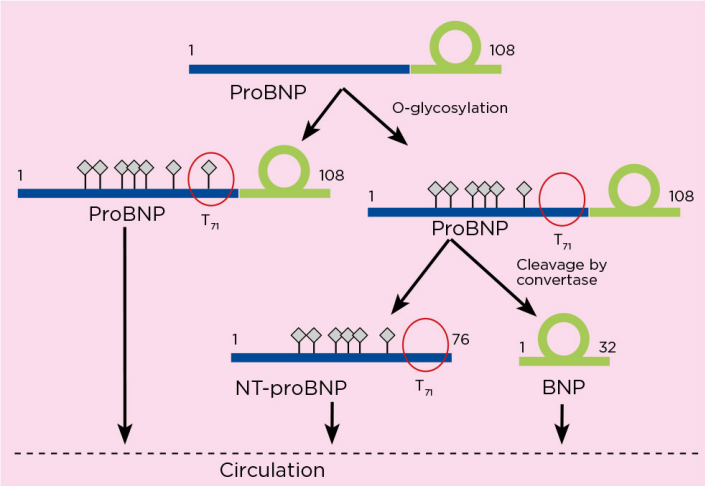


Figure 2. New scheme of proBNP processing. *After translation proBNP is glycosylated at several sites in its N-terminal. Two pools of proBNP different in the status of T71 glycosylation are formed: non- glycosylated at T71 and molecules glycosylated at this site. Glyco- sylation suppress subsequent processing of proBNP. Non-processed proBNP is released in blood. Only proBNP that is not glycosylated at T71 could be effectively processed into BNP and NT-proBNP.*

Recently, some new data regarding proBNP biochemistry has been published. Schellenberger et al. (4) demonstrated that proBNP from HF patients' plasma is an O-linked glycoprotein. Seven sites of O-glycosylation (T36, S37, S44, T48, S53, T58 and T71) were identified for recombinant proBNP expressed in CHO cells. According to the manufacturer's specialists novel findings (5), glycosylation of the region located close to the proBNP cleavage site and especially glycosylation of T71 residue is crucial for further proBNP processing. Glycosylation of T71 suppresses the subsequent processing of proBNP and intact proBNP is released in blood.

ProBNP molecules that are not glycosylated at T71 could be effectively processed into BNP and NT-proBNP (Fig. 2). Consequently, the major part of proBNP molecules in human blood have glycosylated T71 residue, whereas in NT-proBNP T71 it is not glycosylated.

In healthy adults, plasma levels of BNP lie in the range 13.9 - 63.7 pg/ml (BNP Triage assay package insert) while the corresponding values for NT-proBNP are 68-243 pg/ml (Roche proBNPII assay package insert). It was established that proBNP synthesis increases in response to cardiac wall stretch, which leads to increased BNP and proBNP concentrations in blood. An elevated level of both peptides was described for patients with different cardiac pathologies – heart failure (HF), acute coronary syndromes (ACS), left ventricular hypertrophy, cardiomyopathy, valvular heart diseases, atrial fibrillation and cardiac amyloidosis. The BNP concentration in HF patients plasma increases up to several ng/ml, whereas NT-proBNP concentration increases up to several tens of ng/ml.

The blood concentration of both analytes in HF patients correlates with the severity of disease. It has been reported that both peptide concentrations are already elevated in asymptomatic patients during the very early stage of heart failure (NYHA I stageaccordingtotheNewYorkHeartAssociation classification). NYHA classes II and III, and especially class IV patients demonstrate significantly elevated concentrations of BNP and NT-proBNP in their blood. Therefore, peptide measurements in human blood are widely used for the evaluation of patients with suspected HF and when assessing the severity of the disease.

BNP and NT-proBNP measurements are also useful for the risk stratification of the patients with different cardiac pathologies. It was shown that patients who develop complications are characterized by significantly higher BNP and NT-proBNP concentrations than patients without complications. In patients with congestive HF, high BNP (NT-proBNP) levels predict death and are relevant to cardiovascular risk, whereas in patients with ACS elevated levels of both peptides are predictive for mortality and severe heart failure.

At present, both analytes are used in clinical practice as it was demonstrated that their diagnostic and prognostic values are similar (6, 7). Recent data regarding proBNP biochemistry, such as proBNP/NT-proBNP glycosylation or incomplete proBNP processing, could significantly influence the current approach to both BNP and NT-proBNP measurements. As research demonstrated, NT-proBNP glycosylation negatively affects its recognition by some antibodies (8). The central part of the NT-proBNP molecule (a.a.r. 28- 56) is scarcely accessible for antibodies due to O-glycosylation, whereas regions 13-27and61-76arewellrecognizedbyantibodies.The degree of NT-proBNP glycosylation varies significantly from patient to patient and NT-proBNP concentration could be seriously underestimated in the case of the high level of glycosylation of NT-proBNP molecules in human blood. Therefore, we suggest that antibodies specific to the regions that are not affected by glycosylation should be selected for the developmentofNT-proBNPimmunoassays.Advanced ImmunoChemical offers several MAbs specific to unmodified (not glycosylated) regions of NT-proBNP. MAbs were tested with HF patients blood samples and it was shown that antibodies are able to recognize glycosylated protein with high efficiency.

The other important observation concerns proBNP measurements in human blood. Several groups have reported that significant amounts of proBNP can be found in HF patients, blood samples (9, 10, 11). ProBNP is the predominant form displaying BNP immunoreactivity in patients with HF and the proBNP/BNP ratio is not constant and varies from patient to patient, ranging from 1.8 to 32.6 (9, 12).

For this reason, manufacturer specialists suggest that proBNP measurements by assays, utilizing one antibody specific to the BNP and another to the NT-proBNP partofthemolecule,couldbeofthesameclinical value as the BNP measurements.

ProBNP studies in human blood revealed that endogenous proBNP, as well as NT-proBNP, is glycosylated in the region 28-56 and is poorly recognized by antibodies specific to this region. However, in contrast to NT-proBNP, proBNP is also glycosylated in the region 61-76. Therefore, in the development of proBNP immunoassays, specialists recommend using one antibody specific to region 13-27 (which is not occupied with oligosaccharides) and another one that is specific to the BNP portion of proBNP (residues 77-108).

Advanced ImmunoChemical offers a set of high-affinity monoclonal antibodies that are specific to different epitopes of BNP and NT-proBNP molecules. A wide range of MAb combinations is available for the development of sensitive and reliable BNP, NT-proBNP and proBNP immunoassays.

Advanced ImmunoChemical also currently offers monoclonal antibodies for the development of the new type of BNP assay – “Single Epitope Sandwich”. Such an assay utilizes one MAb, 24C511-17 specific to the BNP molecule, and another MAb, Ab-BNP2, which recognizes the immune complex formed by BNP and MAb 24C5 (for additional information see pages 10–13).

We supply our customers with detailed additional information regarding different MAbs applications – the development of quantitative sandwich BNP, NT-proBNP and proBNP immunoassays, immunodetection of antigens in direct ELISA or Western blotting.

Advanced ImmunoChemical also offers recombinant proBNP and NT-proBNP antigens expressed in E. coli and recombinant glycosylated proBNP expressed in eucaryotic cell line. Recombinant proteins can be used as standards and calibrators in immunoassays.

Also available is our BNP/NT-proBNP/ proBNP free plasma, which could be used as a matrix for standard or calibrator preparation (See page 18).

NT-proBNP

Human recombinant (not glycosylated) proBNP and NT-proBNP expressed in E. coli

Source:	Recombinant protein expressed in E. coli
Purity:	>95% according to Tricine-SDS-PAGE
Application:	NT-proBNP and proBNP calibrators and standards
Storage:	-70°C

Human recombinant NT-proBNP (a.a.r. 1-76) and human recombinant proBNP (a.a.r. 1-108) are expressed in Escherichia coli. Both polypeptides have the same sequence as natural proteins with the only difference being additional Met residue at the N-terminus of the molecule. Antigens are recognized by monoclonal antibodies, specific to different parts of NT-proBNP (Cat. # 2-NT-proBNP). Recombinant proBNP is also recognized by BNP-specific antibodies (Cat. # 2-BNP). Data showing proBNP stability studies (measured by proBNP immunoassay) can be found in Fig. 22).

Both NT-proBNP and proBNP are highly purified, with purity exceeding 95% according to Tricine- SDS-PAGE (Fig. 3) and HPLC studies. Antigens could be used as calibrators or standards in NT-proBNP or proBNP assays. Recombinant proBNP can also be used as a calibrator or standard in BNP assays. The calibration curve for the NT-proBNP-specific assay with recombinant NT-proBNP used as a calibrator is presented in Fig. 16, and with proBNP as a calibrator for ”Single Epitope Sandwich” assay in Fig. 10 and for proBNP immunoassay in Fig. 21.

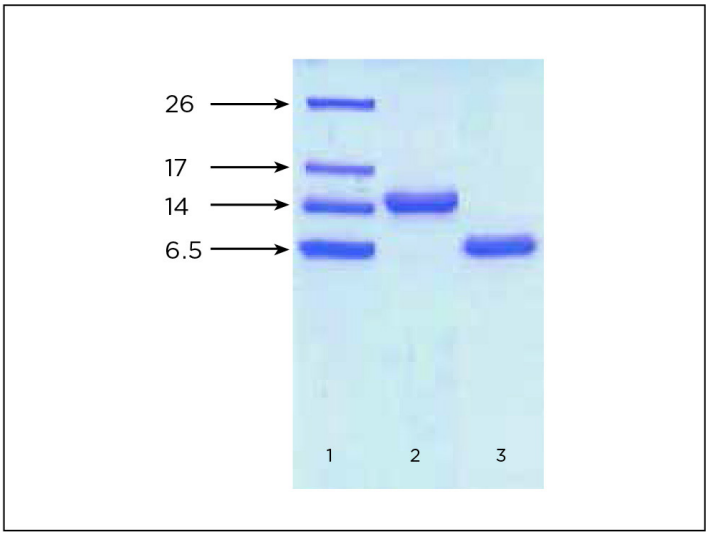


Figure 3. Tricine-SDS-PAGE in reducing conditions of recombinant NT-proBNP and proBNP expressed in E. coli.
Lane 1: low molecular weight standards (Bio-Rad)
Lane 2: proBNP, 3 µg
Lane 3: NT-proBNP, 3 µg
Gel staining: Coomassie brilliant blue R-250

Glycosylated human recombinant proBNP

Source: Recombinant protein expressed in mammalian cell line
Purity: >95% according to Tricine-SDS-PAGE
Application: Calibrator or standard in immunoassays, immunogen for antibody production
Storage: -70°C

As well as endogenous proBNP, also recombinant proBNP expressed in human embryonic kidney epithelial cell line is glycosylated. SDS–PAGE of recombinant peptide shows diffuse bands typical for glycoproteins with an apparent molecular mass of approximately 30 kDa for proBNP (Fig. 4).

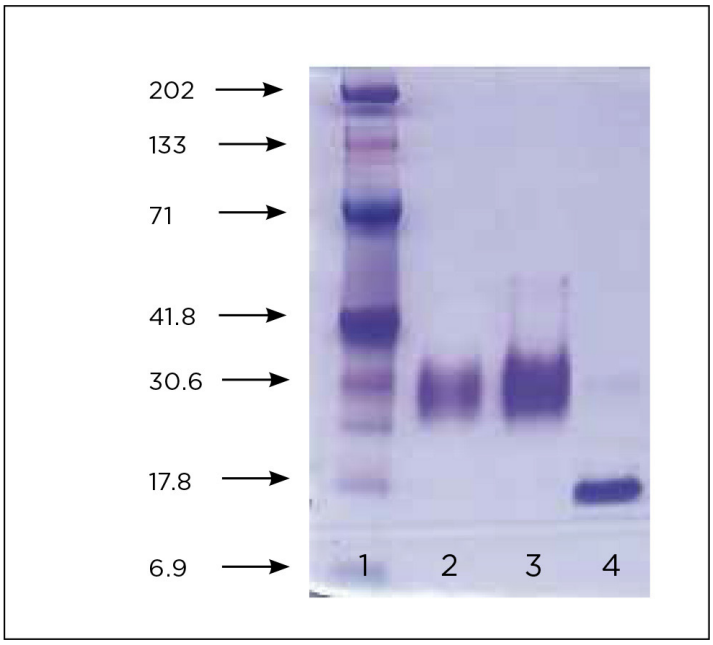


Figure 4. "Tricine-SDS-PAGE" in reducing conditions of glycosylated human recombinant proBNP.
Lane 1: kaleidoscope prestained standards (Bio-Rad).
Lanes 2 and 3: glycosylated proBNP, 3 and 5 µg, respectively.
Lane 4: proBNP from E. coli, 2 µg.
Gel staining: Coomassie brilliant blue R-250.

Fig. 5 shows gel after Tricine-SDS PAGE stained with the GelCode Glycoprotein Staining Kit (Pierce) which specifically detects glycoproteins.

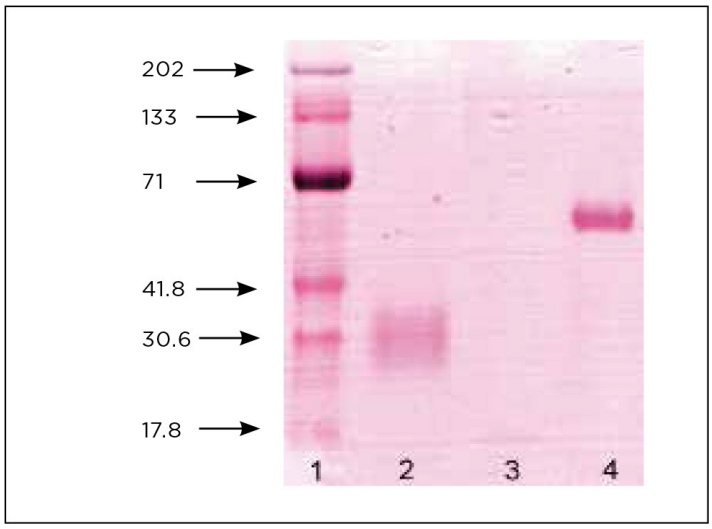


Figure 5. Tricine-SDS-PAGE in reducing conditions of recombinant glycosylated proBNP. Staining of glycoproteins.
Lane 1: molecular weight standards (Bio-Rad)
Lane 2: glycosylated proBNP, 4 µg
Lane 3: proBNP expressed in E. coli, 2 µg
Lane 4: horseradish peroxidase (as positive control), 5 µg
Gel staining: GelCode Glycoprotein Staining Kit (Pierce). As indi- cated in the picture, glycosylated proBNP is stained with the kit in contrast to proBNP expressed in E. coli.

Recombinant glycosylated proBNP was tested in sandwich immunoassays utilizing MAbs, specific to the different regions of proBNP molecule (Cat. # 2-NT-proBNP, 2-BNP). These MAbs are able to recognize recombinant proBNP expressed in E. coli which is non-glycosylated with high sensitivity. It was demonstrated that MAbs specific to the region 1-27 recognized recombinant proBNP expressed in mammalian cell line with high efficiency, whereas MAbs specific to the central region (fragment 28-56) hardly recognized proBNP molecules due to glycosylation. In cases of the antibodies specific to the region 61-76, glycosylated proBNP was almost "invisible" for such antibodies. Whereas BNP part of the proBNP molecule was fully accessible for antibodies.

Recombinant glycosylated proBNP could be used as a calibrator or standard in proBNP and BNP immunoassays.

Glycosylated proBNP as a stable standard for BNP and proBNP immunoassays

Synthetic BNP is currently used as a standard in BNP immunoassays. However, it is known that synthetic BNP demonstrates low stability being reconstituted in plasma or some other proteases-containing matrixes. Due to low stability, the use of synthetic BNP as a standard in BNP assays is limited.

BNP- immunoreactivity in human blood mainly belongs to the proform of BNP – proBNP (9). It was also shown that endogenous proBNP is a glycoprotein (4). Based on these data we suggested using recombinant proBNP as a calibrator in BNP immunoassays.

Stability studies of endogenous proBNP, purified from pooled plasma of HF patients and recombinant glycosylated proBNP (expressed in mammalian cell line), which is the most similar to the endogenous one have revealed that both proBNP forms have comparable stability (Fig. 6). Antigens were reconstituted in pooled normal human plasma and then samples were incubated at room temperature for different time periods up to 24 hours. After that proBNP-immunoreactivity was measured by BNP immunoassay. About 75% of initial proBNP- immunoreactivity was observed after 24 hours of incubation in case of endogenous as well as recombinant proBNP. Detailed description of the used BNP immunoassay see on pages 6-7.

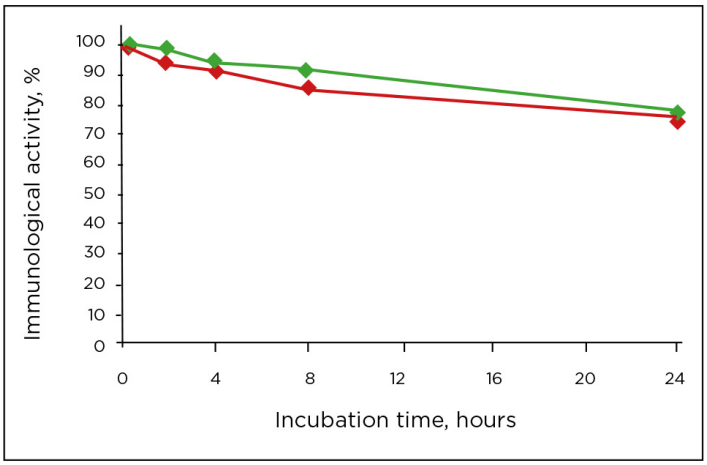


Figure 7. proBNP stability studies. *Recombinant glycosylated proBNP (from mammalian cell line) (-♦-) and endogenous proBNP, purified from pooled HF patients plasma (-♦-) were reconstituted in pooled normal human EDTA-plasma and incubated at RT for different time periods. Immunological activity was measured by immunoassay, utilizing MAb 50E126-32 as capture and MAb 24C511-17 as detection.*

Taking into account the results obtained in stability studies and the prevalence of proBNP in human blood, proBNP expressed in eukaryotic cells is recommended for use as a stable standard or calibrator in BNP immunoassays.

Anti-BNP monoclonal antibodies

Host animal:	Balb/c mice
Cell line used for fusion:	Sp2/0
Antigen:	Human BNP and synthetic peptide 11–22, conjugated to carrier proteins
Specificity:	Human BNP and proBNP
Purification method:	Protein A affinity chromatography
Presentation:	MAb solution in PBS with 0.1% sodium azide
Application:	BNP and proBNP immunoassay, BNP and proBNP immunodetection in Western blotting

Hybridoma cell lines producing MAbs were derived from the hybridization of Sp2/0 myeloma cells with spleen cells of Balb/c mice immunized with human synthetic BNP (whole molecule) or synthetic BNP peptide 11FGRKMDRISSSS22 (for MAbs 24C5 and 26E2) conjugated with carrier protein. Following precise epitope mapping, the exact epitope of MAb 24C5 was determined as a.a.r. 11-17.

All antibodies recognize BNP and proBNP circulating in human blood, as well as synthetic BNP (Bachem, Peptide Institute) and recombinant proBNPs expressed in E. coli (Cat. # 8-pB-rh) and in mammalian cells(Cat.#8-GproNP).

Applications

BNP/proBNP sandwich immunoassay

Several MAb combinations are recommended for sandwich immunoassay. All of the combinations were tested with plasma samples of HF patients and could be used for the development of highly sensitive, rapid sandwich immunoassays suitable for quantitative measurements of BNP and proBNP in human blood.

Recommended pairs for sandwich immunoassay (capture – detection):

$$50E1_{26–32} – 24C5_{11–17}$$
$$50E1_{26–32} – 26E2_{11–22}$$
$$24C5_{11–17} – 50B7_{26–32}$$
$$24C5_{11–17} – 57H3_{26–32}$$

Fig. 7 shows the calibration curve for sandwich fluoroimmunoassay utilizing MAb 50E1 as the capture and MAb 24C5 as the detection antibody. The analytical sensitivity of this immunoassay is better than 0.5 pg/ml (synthetic BNP, Bachem). The 50E1–24C5 immunoassay recognizes three BNP- comprising polypeptides – BNP, recombinant proBNP (non-glycosylated, E. coli), and recombinant proBNP (glycosylated, mammalian cell line) with the same efficiency. A detailed description of this immunoassay has recently been published (9).

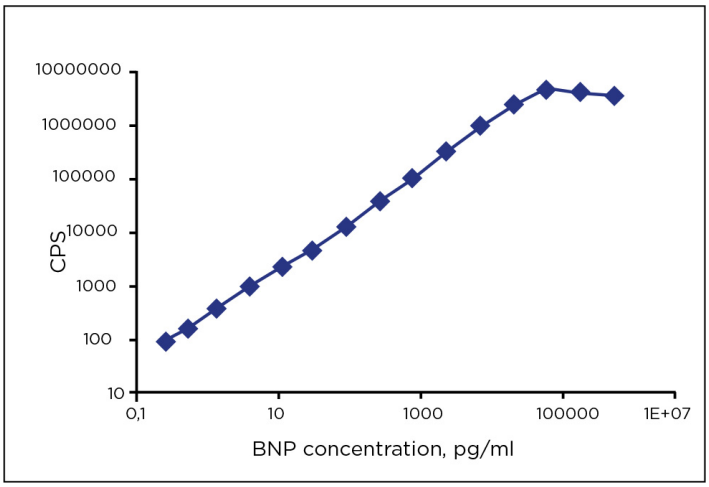


Figure 7. Calibration curve for BNP 50E126–32 – 24C511–17 sandwich fluoroimmunoassay.
Capture antibody: 50E1 (biotinylated)
Detection antibody: 24C5 (Eu3+ - labeled)
Antigen: synthetic BNP (Bachem)
Incubation: mixture of antibodies (50 µl) and antigen (50 µl) is incubated for 30 minutes at room temperature in streptavidin- coated plate.

Analysis of antigens, purified from plasma of HF patients revealed that this immunoassay was able to detect both endogenous BNP and glycosylated proBNP. Moreover, proBNP was the major form contributing to immunological activity measured by this immunoassay (Fig. 8) (9, 12). Fig. 8 shows BNP immunoreactivity measurements in fractions following separation of one representative plasma sample of HF patient on Superdex Peptide gel- filtration column (GE-Healthcare). The BNP assay detected two picks of BNP immunoreactivity, the first corresponding to the proBNP form and the second corresponding to the BNP form.

Results of the stability studies of endogenous proBNP, purified from pooled plasma of HF patients and recombinant glycosylated proBNP (form mammalian cell line), which is the most similar to the endogenous one, being measured by 50E1-24C5 immunoassay see page 6.

For MAb 24C5$_{11\text{-}17}$ application in the “Single Epitope Sandwich” assay (SES assay) see pages 9-12 and reference 12.

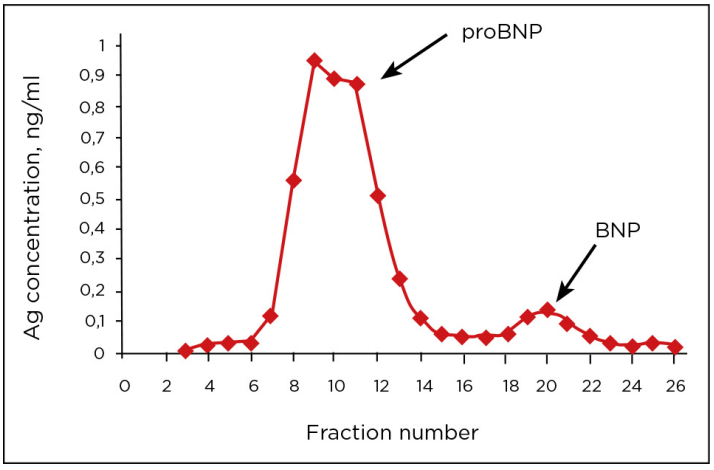


Figure 8. Gel-filtration studies of endogenous proBNP and BNP.
BNP immunoreactivity measurements in fractions after proteins from plasma of HF patients were separated by gel-filtration on Superdex Peptide column (GE-Healthcare). One representative plasma sample. BNP immunoreactivity was measured by 50E1-24C5 assay (-♦-). The first peak corresponds to proBNP, the second corresponds to BNP form.

BNP and proBNP immunodetection in Western blotting

All MAbs recognize synthetic BNP and recombinant proBNP (E. coli, mammalian cell line) in Western blotting following antigen transfer onto nitro- cellulose membrane (Fig. 9).

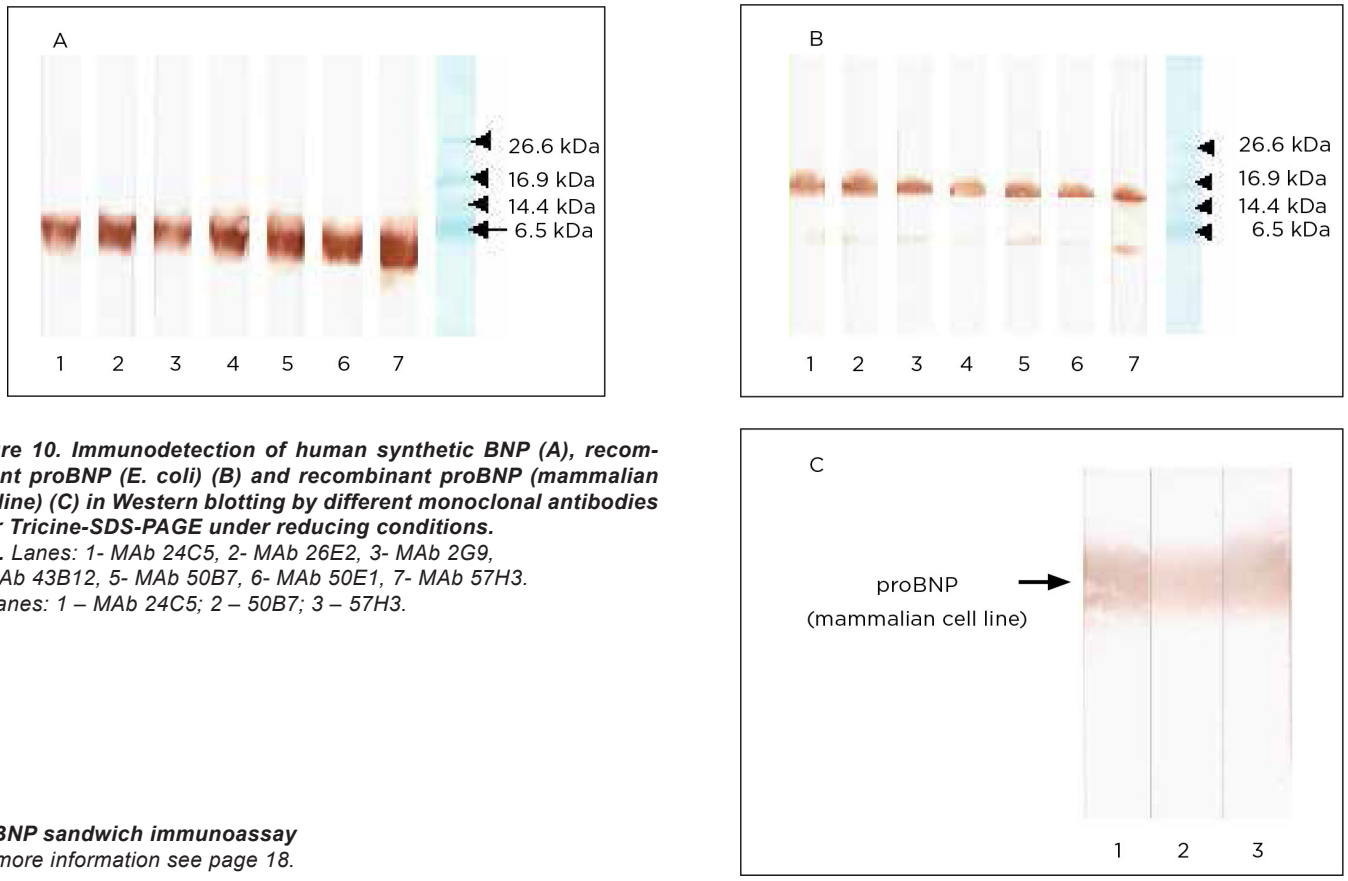


Figure 10. Immunodetection of human synthetic BNP (A), recombinant proBNP (E. coli) (B) and recombinant proBNP (mammalian cell line) (C) in Western blotting by different monoclonal antibodies after Tricine-SDS-PAGE under reducing conditions.
A, B. *Lanes: 1- MAb 24C5, 2- MAb 26E2, 3- MAb 2G9, 4- MAb 43B12, 5- MAb 50B7, 6- MAb 50E1, 7- MAb 57H3.*
C. *Lanes: 1 – MAb 24C5; 2 – 50B7; 3 – 57H3.*

ProBNP sandwich immunoassay
For more information see page 18.

Epitope determination of 24C5 antibodies

To determine the precise epitope of 24C5 antibodies, a set of synthetic peptides within BNP (1-32) sequence was used: 11-22, 11-21, 11-20, 11-19, 11-18, 11-17, 11-16, 14- 22, 13-22 and 11-22. Peptides were covalently linked to a carrier protein (ovalbumin) to enable their direct immobilization on ELISA plates.

Briefly, peptides were diluted in PBS buffer to be directly immobilized on ELISA plates at 5 µg/ mL. After 30 min incubation plates were washed and 24C5 antibodies labeled with stable europium chelate were added at final concentration of 2 µg/ mL. The plates were incubated for 30 min at room temperature with constant shaking. After washing, 200 µL of enhancement solution per well was added and incubated for 3 min at room temperature with gentle shaking. Fluorescence was measured on a Victor 1420 multilabel counter (Wallac-PerkinElmer).

As it follows from Fig. 10, the actual epitope of 24C5 antibodies is 11-17.

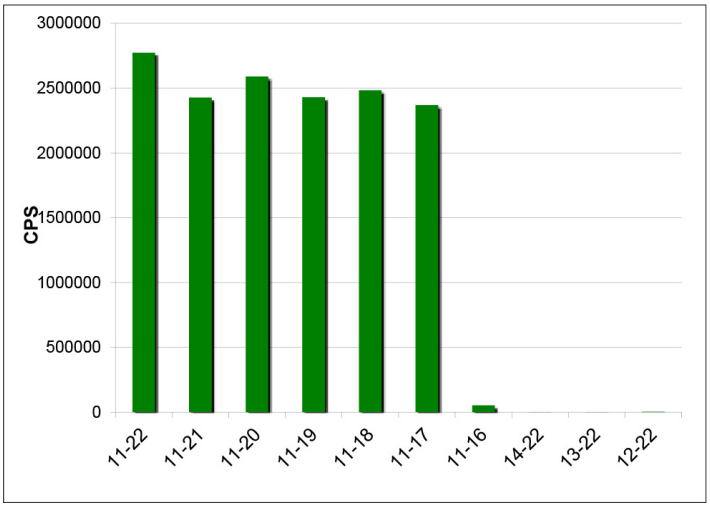


Figure 10. Epitope determination of 24C5 antibodies with a set of synthetic peptides coupled to ovalbumin.

The interaction of 24C5 antibodies with a set of synthetic peptides within BNP (1-32) sequence was tested: 1-10, 5-13, 15-26, 14-21, 14-26, 13-20. Peptides were covalently linked to a carrier protein (ovalbumin) to enable their direct immobilization on ELISA plates. Peptide 11-23, human proBNP 1-108 and BNP 1-32 were used as positive controls.

Briefly, peptides were diluted in PBS buffer to be directly immobilized on ELISA plates at 5 µg/mL. After 30 min incubation plates were washed with PBST and 24C5 antibodies were added at final concentration of 10 µg/mL in PBST. The plates were incubated for 30 min at room temperature with constant shaking. After washing, anti-mouse HRP- conjugate solution in PBST was added to microtiter plates and incubated for 30 min. After incubation plates were washed six times with PBST.

Then 100 µL of OPD solution per well was added and incubated for 5 min at room temperature with gentle shaking. After addition of stop solution, the absorbance was measured at 490 nm (Figure 11).
As it follows from Fig. 12, 24C5 antibodies do not interact with the peptides 1-10, 5-13, 15-26, 14-21, 14- 26, 13-20.

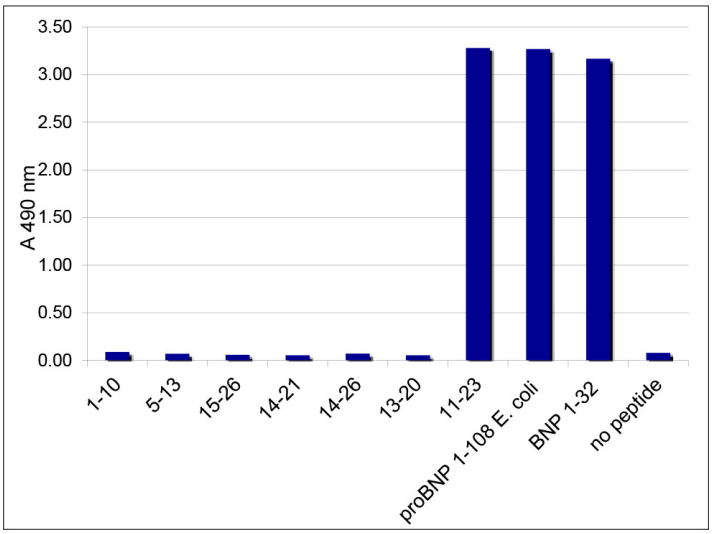


Figure 11. Test of interaction of 24C5 antibodies with a set of synthetic peptides.

Antibodies for New Type of BNP immunoassay – "Single Epitope Sandwich" (SES) assay

MAb 24C5

Antigen:	Synthetic peptide, corresponding to human BNP sequence 11FGRKMDRISSSS22, conjugated with carrier protein.
Host animal:	Balb/c mice
Cell line used for fusion:	Sp2/0
Antigen specificity:	Human BNP or proBNP
Epitope specificity:	Fragment 11-17 of human BNP molecule
Purification method:	Protein A affinity chromatography
Presentation:	MAb solution in PBS with 0.1% sodium azide
Application:	Capture MAb in Single Epitope Sandwich assay

MAb Ab-BNP2

Antigen:	Immune complex consisting of MAb 24C5 and human synthetic BNP
Host animal:	Balb/c mice
Cell line used for fusion:	Sp2/0
Antigen specificity:	Immune complex of BNP-specific MAb 24C5 with human BNP or proBNP
Epitope specificity:	Fragment 11-17 of human BNP molecule
Purification method:	Protein A affinity chromatography
Presentation:	MAb solution in PBS with 0.1% sodium azide
Application:	Detection MAb in Single Epitope Sandwich assay

Brain natriuretic peptide (BNP) is an acknowledged marker of heart failure (HF) that is widely used in clinical practice for HF diagnosis and patient management.

BNP is known as an unstable molecule (13, 14). Several recent studies have revealed that BNP is presented by multiple forms in HF patients' plasma, truncated from both N- and C-termini and only a small portion of BNP circulates as a full-size BNP32 molecule (15). The majority of commercially available BNP assays are designed as sandwich-type immunoassays utilizing two MAbs specific to distantly located epitopes. At least one of these two antibodies is specific to the ring structure, while the other one is usually specific to the C-terminus of the BNP molecule. Recent data regarding BNP instability in circulation suggests that immunoassays utilizing at least one MAb specific to the terminal epitope could underestimate the real BNP content in the blood sample.

Antibodies have been developed for a brand new type of BNP immunoassay – the **"Single Epitope Sandwich" immunoassay (SES assay)** - which differs from all commercially available "conventional"- type sandwich BNP assays (16). In the SES assay the capture antibody (MAb 24C5, epitope 11-17), which is specific to the relatively stable ring part of BNP molecule recognizes antigen. The detection antibody is specific only to the complex of the capture antibody with the BNP (or proBNP) and does not recognize these two molecules, (capture antibody and BNP) separately. Therefore only a single epitope of BNP molecule is needed for this novel type of sandwich BNP immunoassay. This feature provides additional advantages to the SES assay over conventional BNP assays in terms of a higher apparent stability of BNP antigen in the sample or bloodstream.

Sensitivity of the SES assay. The prototype assay, is a one-step assay utilizing biotinylated capture MAb 24C5 and detection MAb Ab-BNP2 labeled with stable Eu3+ chelate. Both MAbs and antigen are simultaneously incubated in streptavidin-coated plates and the assay time is 35 min. The limit of detection of the SES assay is 0.4 pg/ml (human synthetic BNP, Peptide Institute, Japan). This is the highest sensitivity described in literature for all commercial and experimental BNP assays. A detailed description of the prototype SES BNP assay has recently been published (16).

Interaction with BNP and proBNP forms. According to the recent studies, the major portion of BNP immunoreactivity in the patient's blood is not presented by BNP, but in fact is presented by proBNP (9, 17). To be precise in measurements of BNP immunoreactivity in the sample, assay antibodies should recognize BNP and proBNP with the same efficiency.

The SES assay recognizes three forms displaying BNP immunoreactivity – BNP, non-glycosylated proBNP and glycosylated proBNP – with the same efficiency (Fig. 12). When the SES BNP assay was tested with plasma samples of HF patients, it was shown to be suitable for precise quantification of circulating BNP and proBNP molecules.

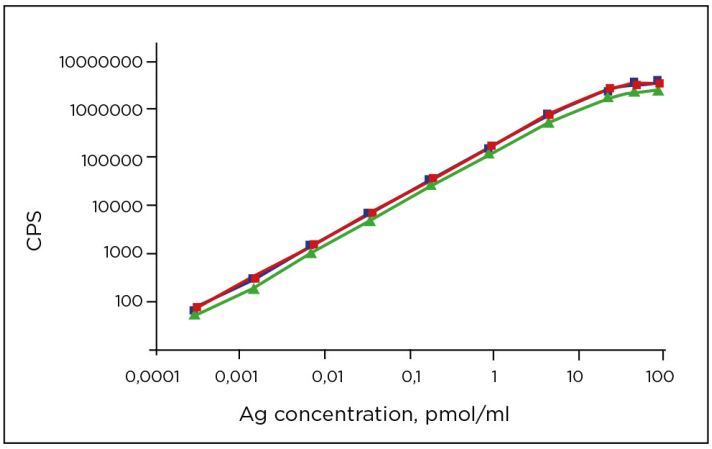
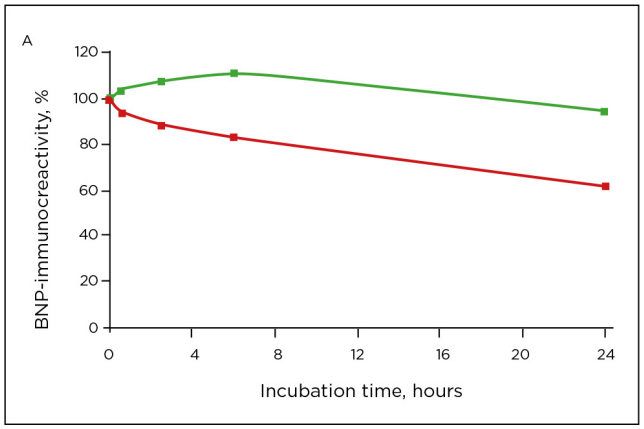


Figure 12. Recognition of different antigen forms displaying BNP-immunoreactivity by SES assay. *Calibration curves for three different antigens: – – synthetic BNP, – – recombinant proBNP (glycosylated, expressed in mammalian cell line; Cat.# 8-GproNP), – – recombinant proBNP (non glycosylated, expressed in E. coli).*

Antigen stability studies. As it was mentioned above, BNP is known as a very unstable molecule that is easily cleaved by endogenous proteases in human blood. However, in SES assay, in which MAbs need the single relatively stable central epitope for sandwich formation, BNP displays significantly higher stability, than when measured in conventional sandwich assays, comprising antibodies with distant epitopes. In Fig. 14 results of stability studies of synthetic BNP and endogenous antigen from plasma of HF patients are presented. Stability was assessed by SES assay and conventional type assay prototype utilizing MAbs with distant epitopes – MAb 50E1 as capture and MAb 24C5 as detection. Samples were incubated at room temperature for different time periods lasting up to 24 hours. Compared to the conventional sandwich assay, the apparent stability of the synthetic antigen is significantly higher when measured by SES assay. While approximately 95% of BNP immunoreactivity was observed with SES assay after 24 hours of incubation, only 62% of initial BNP immunoreactivity was detected when the conventional sandwich immunoassay was used. Furthermore, the apparent stability of the endogenous peptide was also higher when BNP immunoreactivity in individual HF plasma sample incubated for different time periods was measured by the SES assay.



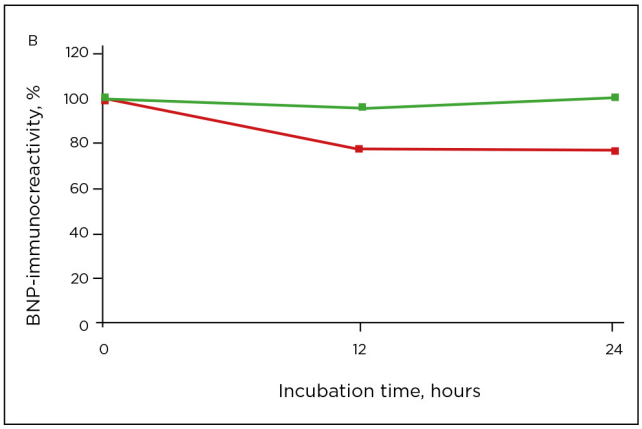


Figure 13. Stability studies of BNP.
(A) *Synthetic BNP (Peptide Institute, Japan) reconstituted in individual normal human EDTA-plasma and* **(B)** *Individual HF patient EDTA- plasma were incubated at RT (24oC) for different time periods. BNP immunoreactivity was tested in the SES assay (-■-) and conventional type BNP assay utilizing two MAbs 50E1 and 24C5 specific to the distant epitopes (-■-).*

BNP measurements in HF plasma. BNP immunoreactivity measurements (BNP and proBNP) in individual EDTA-plasma samples of HF patients performed by two types of BNP assays also revealed the superiority of the single epitope principle over the conventional one. BNP concentration in 94 HF plasma samples was quantified by SES assay prototype and by commercially available conventional type Siemens ADVIA Centaur BNP immunoassay. The Siemens assay utilizes one MAb specific to the C-terminus (epitope 27-32) and another MAb specific to the ring structure of BNP molecule (epitope 14-21) (18). Both assays were calibrated with recombinant proBNP, expressed in E. coli. In all plasma samples, when compared with the results obtained by Siemens BNP immunoassay, SES assay detected significantly more BNP; from 1.2 to 7.2-fold; 2.1±0.9 (mean±SD). BNP concentration in seven plasma samples (7.4% of a total of 94 samples) when measured by SES assay was from 3 to 7.2-fold higher than that measured by Siemens assay. Results of the BNP measurements in 94 HF patients plasma are presented in Fig. 14.

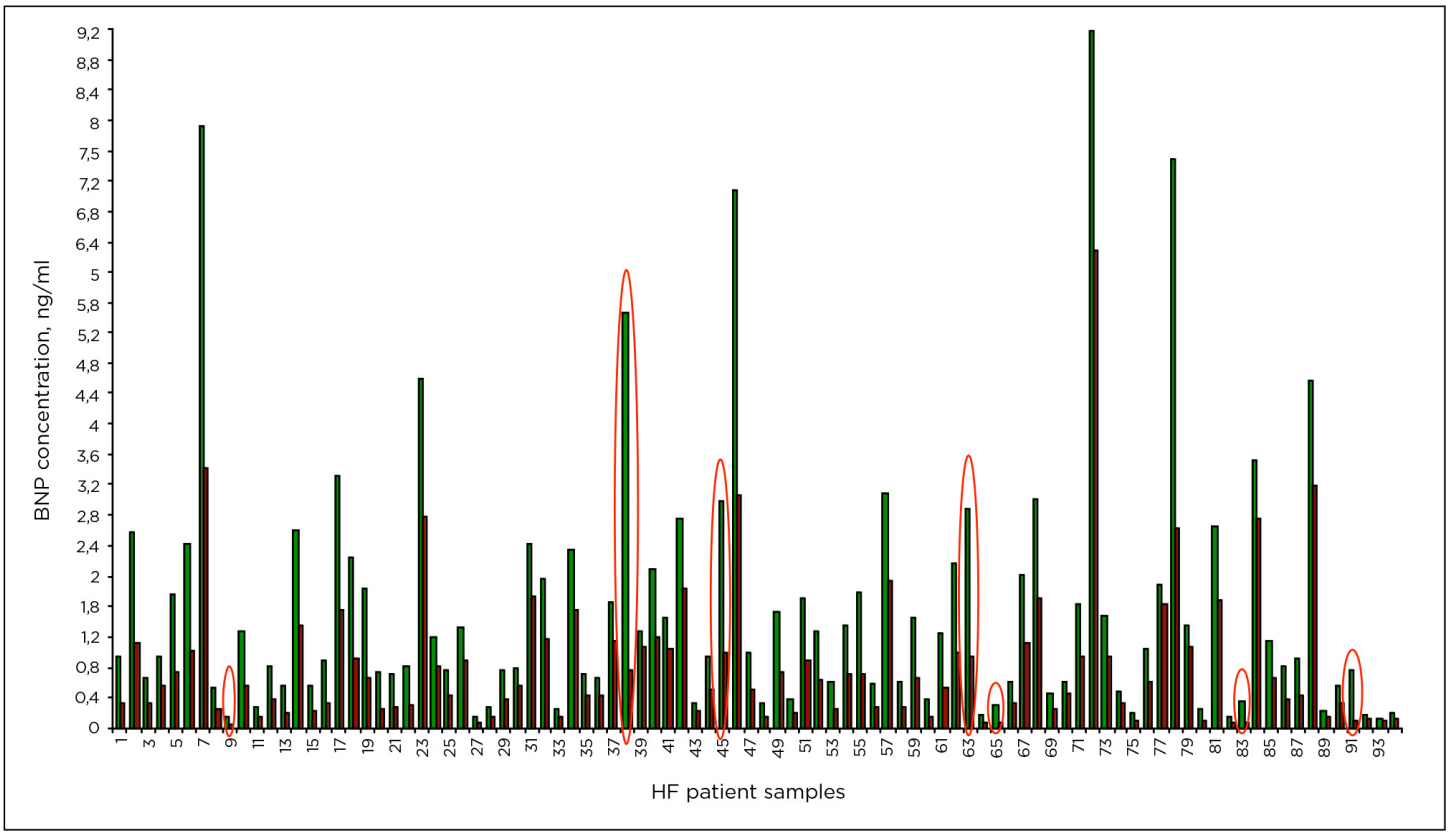


Figure 14. BNP measurements in 94 HF patients' plasma samples. *BNP concentration was measured by the SES (green bars) and Siemens (brown bars) BNP assays. Cases when the concentrations measured by SES assay were 3 to 7.2-fold higher than those measured by Siemens assay are marked by red ovals.*

As can be seen from Fig. 14 Siemens assay underestimates circulating BNP concentrations in HF patients. This observation can at least be explained by the fact that one of the MAbs, utilized in the Siemens assay, is specific to the epitope 27-32 and cannot recognize BNP forms truncated from the C-terminus. The SES assay, being significantly less sensitive to the proteolytic degradation of the antigen, is capable of detecting all forms displaying BNP-immunoreactivity: intact and terminus- truncated antigens. The SES assay appears to be a preferable assay for the absolute BNP quantification in human blood.

The true BNP values are required to make a correct diagnosis for patients admitted to emergency department (ED) with symptoms of HF. Specific "rule out" (BNP<100 pg/ml) and "rule in" (BNP>400 pg/ml) values are currently used by cardiologists to make the most accurate diagnosis in ED (19). Fig. 16 represents the difference in BNP content for four selected patients measured by SES and Siemens assays. Being measured by the Siemens BNP assay these patients (especially patient #4) could be misclassified (uncertainty zone: concentration range from 100 pg/ml to 400 pg/ml) and could therefore be mistakenly diagnosed. When measured by the SES assay, the same patients undoubtedly belong to the "rule in" zone. This example confirms the idea that the SES assay approach is a preferable for the precise BNP quantification.

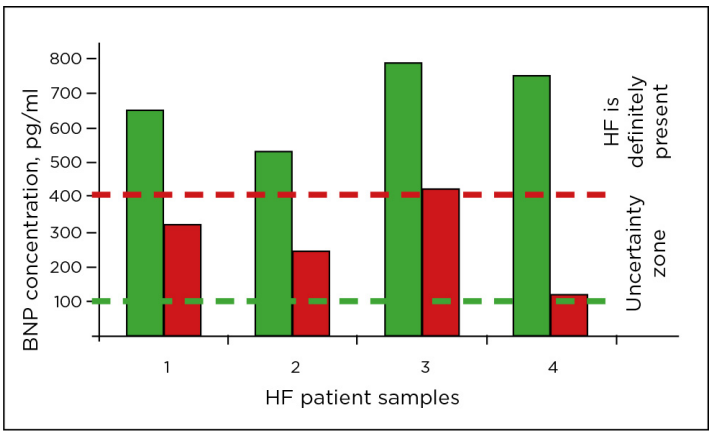


Figure 15. BNP measurements in plasma of four selected HF patients by two BNP assays. *BNP concentration was measured by the SES (green bars) and Siemens (red bars) assays. When measured by the Siemens assay these patients may have unconfirmed HF (BNP concentrations in the range of 100-400 pg/ml), whereas measured by the SES assay - confirmed HF (more than 400 pg/ml). The low- er limit of the uncertainty zone or unconfirmed HF (100 pg/ml) is marked as a green dash line; the lower limit of the zone where HF is definitely present (400 pg/ml) and is marked as red dash line.*

Anti-NT-proBNP monoclonal antibodies

Host animal:	Mice Balb/c
Cell line used for fusion:	Sp2/0
Antigen:	Synthetic peptides, corresponding to different regions of human NT-proBNP, conjugated with carrier protein
Specificity:	Human NT-proBNP and proBNP
Purification method:	Protein A affinity chromatography
Presentation:	MAb solution in PBS with 0.1% sodium azide

Hybridomas producing MAbs were generated after immunization of Balb/c mice with synthetic peptides, corresponding to different parts of NT-proBNP sequence, conjugated to carrier protein. All antibodies were checked on their ability to recognize recombinant NT-proBNP and proBNP expressed in E. coli (Cat.# 8NT2, 8PRO9) in direct ELISA, sandwich immunoassay and Western blotting.

Applications

Direct ELISA

All MAbs recognize recombinant human NT-proBNP and proBNP expressed in E. coli in direct ELISA (Fig. 16).

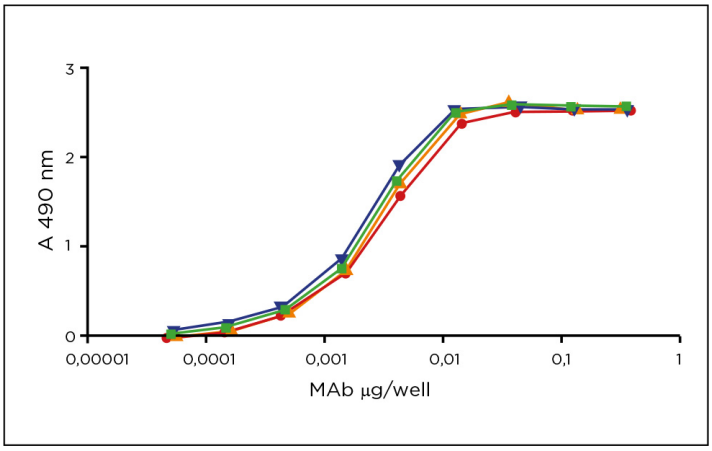


Figure 16. Titration curves of different MAbs in ELISA.
MAbs: 5B6 (▼), 13G12 (▲), 11D1 (●), 15F11(■)
Antigen: human recombinant NT-proBNP expressed in E. coli, 0.01 µg/well

NT-proBNP quantitative sandwich immunoassays

All NT-proBNP monoclonal antibodies specific to different parts of the molecule were tested in sandwich immunoassay as capture and detection antibodies with recombinant NT-proBNP and proBNP expressed in E. coli as well as with serum/plasma samples from HF patients. It was demonstrated that antibody pairs using at least one of the MAbs specific to the very N-terminal region (a.a.r. 1-12) or the mid fragment of NT-proBNP (a.a.r. 28-56) were unable to recognize endogenous NT-proBNP well. In contrast, the same pairs that were not able to recognize endogenous protein detected recombinant NT-proBNP or proBNP ex- pressed in prokaryotic cells (E. coli) with high sensitivity. It was shown that antibodies specific to the very N-terminal part of the molecule were unable to recognize endogenous NT-proBNP due to its proteolytic degradation. Meanwhile glycosylation was the major reason for antibodies specific to the central region of NT-proBNP being unable to recognize endogenous protein. MAb pairs with one of the antibodies specific to region 5-12 or 13- 27 and another antibody specific to region 61-76 demonstrated the highest signal with endogenous NT-proBNP (8).

Therefore, to ensure precise quantitative NT-proBNP measurements in human blood it is recommended to use two-site antibody combinations utilizing antibodies specific to the N- or C-terminal parts of NT-proBNP molecule (Fig. 17). The best recommended pairs for precise NT-proBNP sandwich immunoassay are (capture – detection):

$15C4_{63\text{-}71}$ -$13G12_{13\text{-}20}$
$15C4_{63\text{-}71}$ -$29D12_{5\text{-}12}$
$15F11_{13\text{-}24}$-$24E11_{67\text{-}76}$
$15C4_{63\text{-}71}$ -$18H5_{13\text{-}20}$

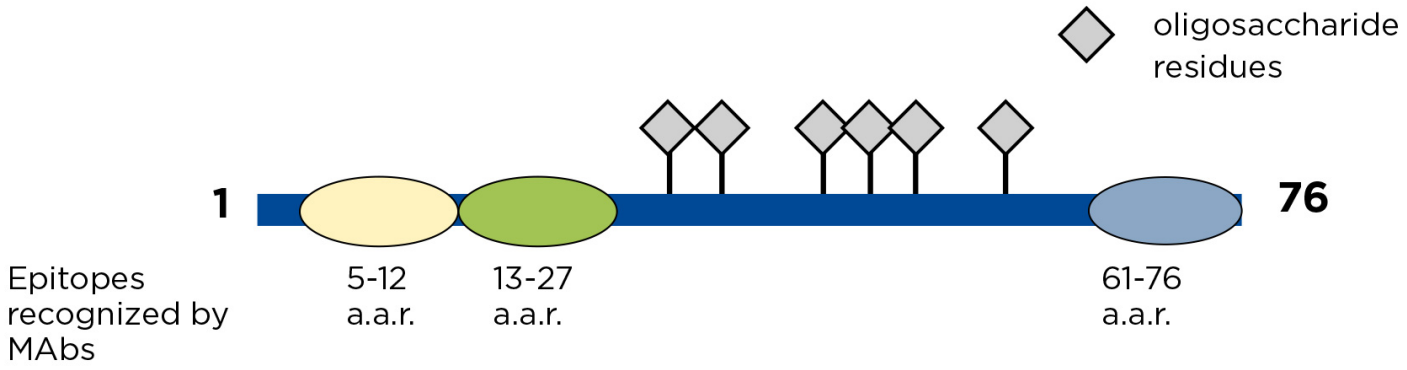


Figure 17. Epitope location of MAbs, which are recommended for the development of NT-proBNP sandwich immunoassays.

All assays utilizing recommended MAb combinations demonstrate high sensitivity (10–15 pg/ml) and good kinetics. A representative calibration curve for the assay 15C4–13G12 is shown in Fig. 18 and a detailed description of this immunoassay has recently been published (9). All of the best MAbs combinations were tested with plasma/serum samples from HF patients to demonstrate the ability of antibodies to recognize the antigen circulating in human blood.

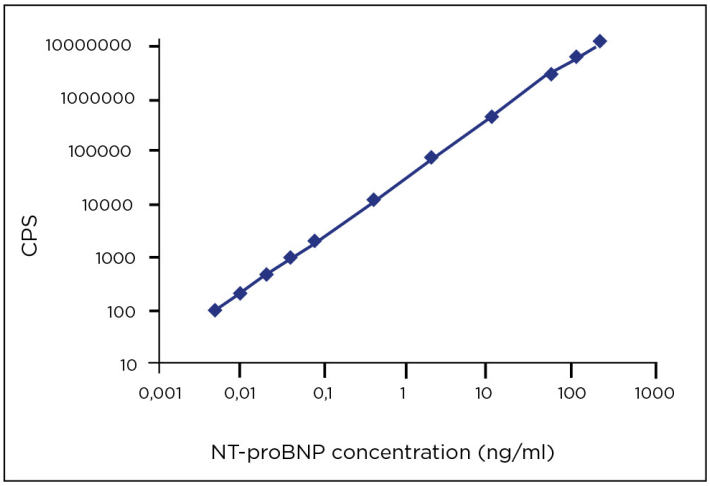


Figure 19. Calibration curve for NT-proBNP 15C4-13G12 assay:
Capture antibody: 15C4 (biotinylated)
Detection antibody: 13G12 (Eu3+ - labeled)
Antigen: human recombinant NT-proBNP expressed in E. coli Incubation: mixture of both antibodies (50 µl) and antigen (50 µl) is incubated for 30 minutes at room temperature in streptavidin- coated plate.

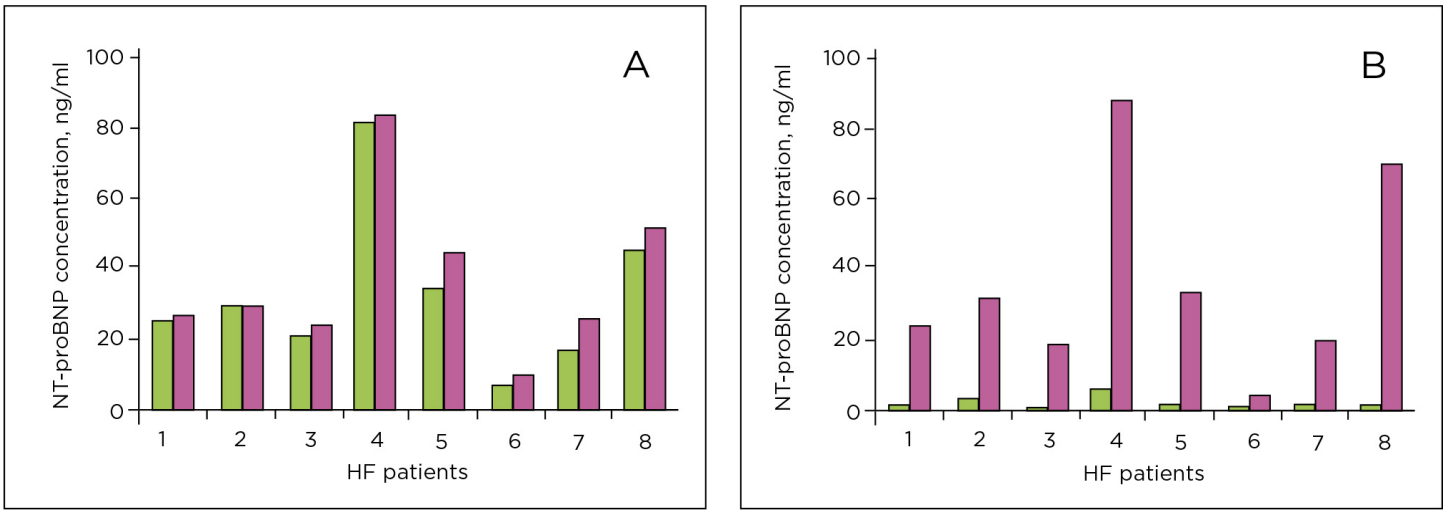


Figure 19. Comparison of endogenous NT-proBNP immunoreactivity before and after deglycosylation in several representative samples. *Concentration of endogenous NT-proBNP before (green columns) and after (violet columns) deglycosylation were measured by sandwich immunoassays: 15C4-13G12 (A), and 11D1-13G12 (B). Monoclonal antibodies used in the former assay are specific to the N- or C-terminal parts of the molecule (not glycosylated). MAb 11D1 utilized in the latter assay as a capture antibody, is specific to the central glycosylated region.*

To elucidate how glycosylation influences measurements of NT-proBNP, assay 15C4-13G12 was tested with NT-proBNP extracted from 52 HF patients plasma samples and then treated by a mixture of enzymes removing O-linked oligosaccharides (8). As a control, the 11D1- 13G12 assay, which is highly susceptible to NT- proBNP glycosylation, was used. Immunoreactivity measured by assay 11D1-13G12 in the samples after deglycosylation in some cases was up to 40 fold higher than in the case of untreated NT-proBNP. It was observed that changes of measurable concentrations after deglycosylation were different in different blood samples. In some cases, measured concentrations increase only 2-3 fold, while in other cases it was 10 fold or even >20 fold. It was concluded that NT-proBNP is glycosylated in all analyzed patients and that the level of NT-proBNP glycosylation is different in the blood of individual patients. The assay 15C4-13G12 using MAbs specific to the terminal parts of the molecule, was not as sensitive to deglycosylation of endogenous protein from individual blood samples. Only a small growth of the signal was observed following deglycosylation (mean 1.37 fold). **It was therefore concluded that antibodies specific to the N- and C-terminal parts of the NT-proBNP molecule (but not to the very N-terminal) are the best choice for the development of the precise NT-proBNP assay.**

Stability studies of endogenous NT-proBNP

Stability of endogenous NT-proBNP after incubation of patients' serum samples at two temperatures (+4°C and +20°C) for different time periods, was analyzed by antigen measurements in sandwich immunoassay 15C463-71-13G1213-20 (Fig. 20). Less than 10% of the initial immunological activity was lost following 72 hours of incubation at +4°C and approximately 10–15% following incubation of the sample after 24 hours at room temperature. Therefore serum samples could be stored in refrigerator (or even at room temperature) for a relatively long period until NT-proBNP concentration is measured by immunoassays, utilizing MAbs specific to the region 13-24 and 61-76.

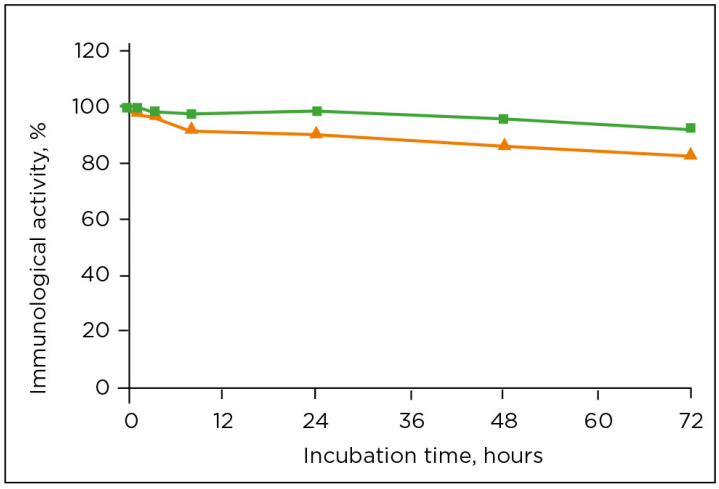


Figure 21. Stability studies of endogenous NT-proBNP being measured in 15C4-13G12 sandwich immunoassay. *Pooled blood serum from patients with HF was incubated at +4oC (■) and at room temperature (▲) for 72 hours.*

NT-proBNP and proBNP immunodetection in Western blotting

All MAbs recognize human NT-proBNP and proBNP expressed in E. coli in Western blotting studies following antigen transfer onto nitrocellulose membrane (Fig. 21). MAbs specific to N-terminal region (1-27) recognize recombinant proBNP expressed in mammalian cell line after transfer onto nitrocellulose membrane (Fig. 22) in contrast to antibodies specific to the region 28-76.

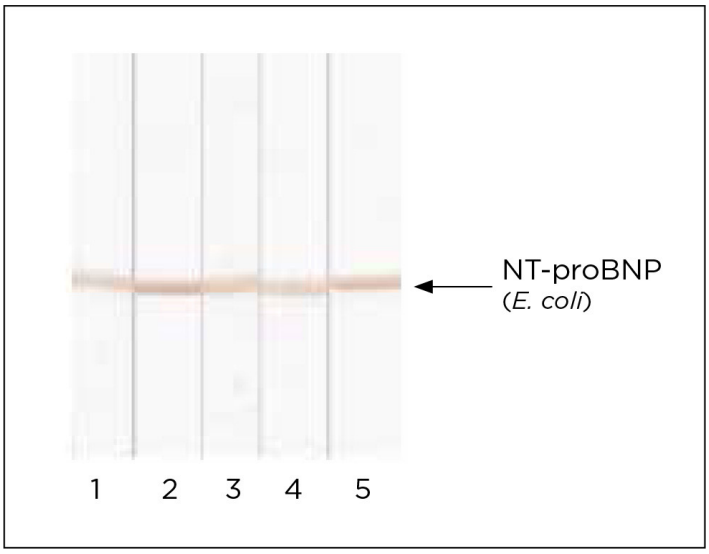


Figure 22. Detection of human recombinant NT-proBNP expressed in E. coli in Western blotting by different monoclonal antibodies after Tricine-SDS gel electrophoresis.
Lanes: 1 – MAb 5B6; 2 – 15F11; 3 – 11D1; 4 – 15D7; 5 – 24E11.
Antigen: recombinant NT-proBNP expressed in E. coli, 2.5 µg/well.

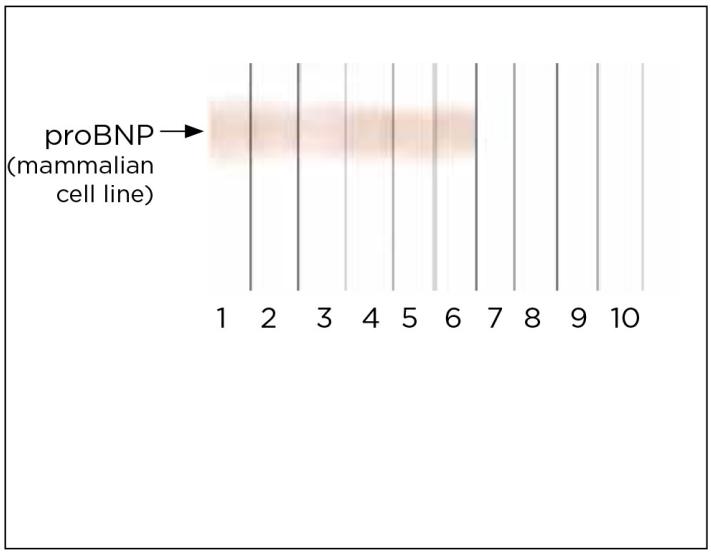


Figure 23. Detection of human recombinant proBNP expressed in mammalian cells in Western blotting by different monoclonal antibodies after Tricine-SDS gel electrophoresis.
Lanes:1–MAb5B6;2–29D12;3–15F11;4–13G12;5–18H5;6–16F3; 7–11D1;8–15D7;9–15C4;10–24E11.
Antigen: recombinant proBNP expressed in mammalian cells (Cat.# 8GOB2), 0.15 µg/line.
MAbs 11D1, 15D7, 15C4 and 24E11 does not recognize glycosylated proBNP expressed in the mammalian cell line.

ProBNP quantitative sandwich immunoassays

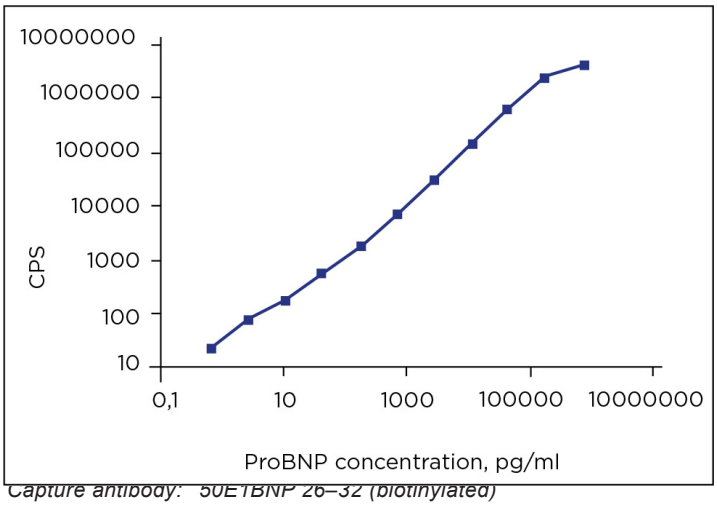
ProBNP is the predominant form displaying BNP immunoreactivity in the blood of HF patients (9). Therefore, proBNP measurements by assays utilizing one antibody specific to the BNP and another specific to the NT-proBNP part of the molecule could be of the same clinical value as BNP measurements by conventional BNP assays, utilizing both MAbs specific to the BNP peptide. For the development of proBNP assays, it is recommended to use one MAb specific to the region 13-27 (which is not occupied with polysaccharide residues) of NT-proBNP (Cat. # 2-NT-proNBP) and another MAb specific to region 11-22 or 26-32 of BNP (Cat. # 2-BNP) (Fig. 25). In contrast to NT-proBNP immunodetection, antibodies specific to the region 61-76 could not be used for the development of proBNP assay. This is because this region is glycosylated in endogenous proBNP molecules, in contrast to NT-proBNP (5).
Recommended pairs for sandwich immunoassay are (capture – detection) :

$$50E1_{BNP\ 26\text{-}32} - 16F3_{NT\text{-}proBNP\ 13\text{-}20}$$
$$50E1_{BNP\ 26\text{-}32} - 18H5_{NT\text{-}proBNP\ 13\text{-}20}$$
$$7B5_{NT\text{-}proBNP\ 13\text{-}20} - 2G9_{BNP}$$

These pairs demonstrate high sensitivity, good kinetics and recognize recombinant proBNP expressed in E. coli and in mammalian cell line, as well as proBNP in HF patients' blood.

Analytical sensitivity of the assay 50E1–16F3 (recombinant proBNP expressed in E. coli used as calibrator) is better than 3 pg/ml (Fig. 23).

As a calibrator or standard for proBNP immunoassays using glycosylated proBNP is recommended, expressed in eukaryotic cells. It is better than antigen expressed in E. coli since it better represents the endogenous protein and has higher stability (Fig. 24).



Capture antibody: 50E1BNP 26–32 (biotinylated)
Detection antibody: 16F3NT-proBNP 13–20 (Eu3+labeled).
Antigen: Recombinant proBNP expressed in E. coli
Incubation: Mixture of antibodies (50 µl) and antigen (50 µl) is incubated for 30 minutes at room temperature in streptavidin-coated plate.

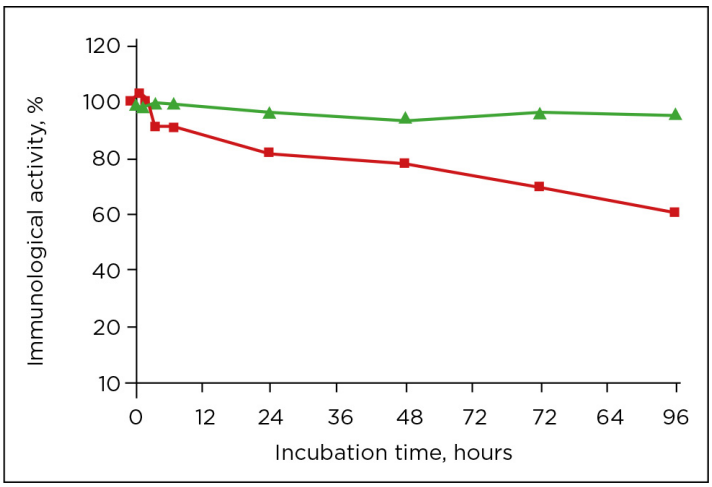


Figure 24. Stability studies of recombinant proBNP expressed in E. coli (-■-) and in mammalian cell line (-▲-). *Antigens were reconstituted in normal human citrate plasma and incubated at +4°C for 96 hours. Immunological activity was measured in sandwich immunoassay utilizing MAb 50E1 as capture and MAb 16F3 as detection antibody.*

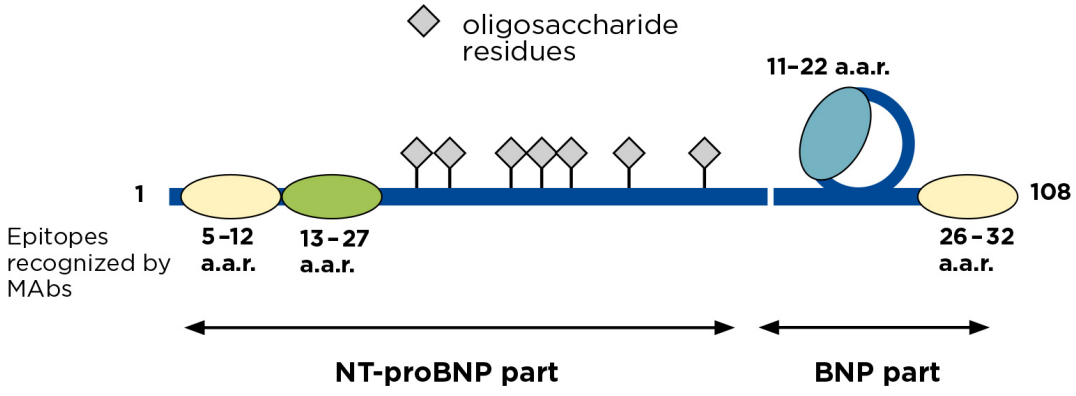


Figure 25: Epitope location of MAbs, which are recommended for the development of proBNP sandwich immunoassays.

BNP and NT-proBNP free plasma

Source:	Prepared from pooled normal human K3-EDTA plasma. Blood samples from donors were tested and found to be negative for HBs Ag, HIV-1 and HIV-2 antibodies, HCV and syphilis
Method of purification:	Immunoaffinity chromatography
Delivery form:	Frozen liquid
Storage:	-20°C

BNP and NT-proBNP free plasma is prepared from pooled normal human K3-EDTA plasma by immunoaffinity chromatography. The affinity sorbent utilizes several MAbs with different epitope specificity to eliminate not only intact proBNP and proBNP-derived molecules from plasma but also proBNP-proteolytic fragments. BNP and NT-proBNP free plasma could be used as a matrix for standard and calibrator preparation.

Ordering Information:

ANTIGEN

Product Name	Cat #	Purity	Source
NT-proBNP, recombinant human	8-NTpB-rh	>95%	Recombinant
ProBNP, recombinant human	8-pB-rh	>95%	Recombinant
ProBNP, glycosylated, recombinant	8-GproNP	>95%	Recombinant

MONOCLONAL ANTIBODIES

Product Name	Cat #	MAb	Subclass	Remarks
BNP	2-BNP	24C5	IgG1	EIA, WB, a.a.r. 11-17
		26E2	IgG1	EIA, WB, a.a.r. 11-22
		43B12	IgG2a	EIA, WB, a.a.r. 26-32
		50E1	IgG1	EIA, WB, a.a.r. 26-32
		50B7	IgG2a	EIA, WB, a.a.r. 26-32
		57H3	IgG2a	EIA, WB, a.a.r. 26-32
		2G9	IgG1	EIA, WB, a.a.r. N/A
Immune complex (24C5-BNP/proBNP)	2-ICBNP	Ab-BNP2	IgG2a	EIA (only as pair with MAb 24c5, Cat# 2-BNP
Immune complex (24C5-BNP/proBNP) - in vitro	2-IC-iv	Ab-BNP2cc	IgG2a	EIA (only as pair with MAb 24c5, Cat# 2-BNP
NT-proBNP	2-NT-proBNP	5B6	IgG1	EIA, WB, a.a.r. 1-12
		29D12	IgG2a	EIA, WB, a.a.r. 5-12
		13G12	IgG2a	EIA, WB, a.a.r. 13-20
		18H5	IgG1	EIA, WB, a.a.r. 13-20
		16F3	IgG1	EIA, WB, a.a.r. 13-20
		15F11	IgG2b	EIA, WB, a.a.r. 13-24
		7B5	IgG1	EIA, WB, a.a.r. 13-24
		11D1	IgG1	EIA, WB, a.a.r. 31-39
		16E6	IgG1	EIA, WB, a.a.r. 34-39
		15D7	IgG1	EIA, WB, a.a.r. 48-56
		15C4	IgG2b	EIA, WB, a.a.r. 63-71
		24E11	IgG2a	EIA, WB, a.a.r. 67-76
		28F8	IgG2a	EIA, WB, a.a.r. 67-76

DEPLETED PLASMA

Product Name	Cat #	Source
BNP and NT-proBNP free plasma	11-BNT-fs	Pooled normal human plasma

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