Nt-proBNP

Enzyme immunoassay for the quantitative
determination of Nt-proBNP
in biological fluids

Cat. No. BI-20852
12 x 8 tests

Biomedica Medizinprodukte
Gesellschaft mbH & Co KG
A-1210 Wien, Divischgasse 4
Tel. +43/1/291 07 50
Fax +43/1/291 07 71
exp.biomedica@bmgrp.at

www.biomedica.co.at

Contents

1.) Introduction
2.) Principle of the assay
3.) Contents of the kit
4.) Additional material and
equipment required
5.) Reagents and sample preparation -
assay procedure
6.) Calculation of results
7.) Assay characteristics
8.) Technical hints
9.) Precautions
10.) References
11.) Incubation scheme

1.) Introduction

Atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP) are members of a family of hormones secreted from the atrium, ventricle, and vascular endothelial cells (1-3). These natriuretic peptides share a common ring structure of 17 amino acids formed by a disulphide linkage, which is an essential part for their biological activity. Three receptors, type A (specific for ANP and BNP), type B (specific for CNP and ANP) and the clearance receptor, have been characterised.

BNP was first identified in the porcine brain and later isolated from the porcine heart. The mature form of BNP, secreted predominantly by the cardiac ventricle, is formed from a high molecular weight precursor, proBNP (1-108) (4). Bioactive human BNP (BNP-32) consists of the 32 C- terminal amino acid residues, proBNP (77-108).

BNP is stored in the human cardiac tissue mainly as BNP-32, with a lesser amount of the precursor peptide, proBNP (1-108). The circulating plasma forms of BNP are BNP-32, a high molecular weight BNP form that has not been well identified, and the amino-terminal portion proBNP (1-76) (5,6).

BNP elicits a spectrum of diuretic, natriuretic and hypotensive effects similar to those induced by ANP. Recent research suggests that natriuretic peptides are important protectors against fluid overload and high blood pressure (7). Control of fluid and electrolyte balance as well as circulatory homeostasis also involves other regulatory mechanism, including the renin-angiotensin system, vasopressin, and the sympathetic nervous system. The discovery of natriuretic peptides has focused attention on an additional endocrine system that contributes to diuresis and vascular tone. The biology, biochemistry and the pathophysiological role of natriuretic peptides are described in recent reviews (8,9).

Clinical aspects:

Plasma BNP levels are markedly increased in cardiac impairment, renal failure, liver disease and in patients with essential and various forms of secondary hypertension. McDonagh et al. reported raised concentrations of BNP and proANP in studies of patients with left-ventricular dysfunction, especially previous to myocardial infarction. However, they have shown that especially patients with asyptomatic left-ventricular dysfunction, have high circulating concentrations of natriuretic peptides (10). A raised BNP concentration seems to be more accurate than a raised Nt-proANP in detection of left-ventricular dysfunction (11,12,13). In cardiac failure, plasma concentrations of both ANP and BNP are increased proportional to the severity of heart failure. After acute myocardial infarction (AMI), concentrations of natriuretic peptides increase rapidly. In these pathophysiologic states, increased circulating concentrations of natriuretic peptides are considered to be protective against vasoconstricting and sodium-retaining mechanisms. Recent data suggest that circulating proBNP-concentrations raise significantly more than circulating proANP-concentrations after AMI and in chronic heart failure. It was suggested that amino terminal-proBNP may be a more discerning marker of early cardiac dysfunction than BNP-32 (14).

2.) Principle of the assay

This test kit is a competitive EIA (Enzyme Immuno Assay) designed to measure the immunoreactive N-terminal proBNP in diluted human serum, plasma or urine samples. In order to achieve high specificity the kit incorporates an immunoaffinity purified sheep antibody specific for Nt-proBNP (8-29) immobilised to the surface of a microtititer plate well.
The assay is based on the competitive reaction of the unlabelled peptide in the standards or samples and the horse radish peroxidase labelled peptide (tracer) for the limiting binding sites of the Nt-proBNP (8-29)-specific antibody. The concentration of the tracer and the concentration of the capture antibody are constant in all wells. Consequently, the only variable parameters of the system are the concentrations of the unlabelled peptides in the standards and samples. Hence with increasing concentration of the peptide in the standard the binding of the competing tracer is proportionally reduced.
After removal of unbound tracer through washing, substrate (TMB) is added to the wells. The amount of HRP-labelled tracer bound to the microplate well proBNP (8-29) is quantitated by an enzyme catalysed colour change detectable on a standard ELISA reader. The amount of colour developed is inversely proportional to the amount of Nt-proBNP immunreactivity present in the standard or samples. A standard curve is plotted from the values measured and the concentrations of Nt-proBNP in the samples are calculated from this curve.

3.) Contents of the kit

- 12 x 8 well microtiter strips in stripholder, packed in alubag with desiccant. Wells are coated with a polyclonal anti Nt-proBNP (8-29) antibody
- 10x washing buffer; concentrated
  The bottle contains 100 ml of washing buffer concentrate
- Assay buffer
  The bottle contains 100 ml of assay buffer, ready to use.
- Nt-proBNP standard (amber vial with white cap). The vial contains synthetic human Nt-proBNP in buffer, lyophilized. The concentration after reconstitution in 3 ml assaypuffer is stated on the label.
- Control (yellow cap)
  The vial contains synthetic Nt-proBNP, lyophilised. The concentration after reconstitution is stated on the label.
- Tracer (plastic vial)
  The vial contains 6 ml HRP-labeled synthetic Nt-proBNP in red stabiliser solution.
- Substrate
  The vial contains 22 ml TMB solution, ready for use.
- Stop solution
  The vial contains 7 ml of stop solution, ready for use.
- 1 self-adhesive plastic film
- Protocol sheet

- Instructions for use (package insert)

4.) Additional material and equipment required

Distilled water

Variable volume pipettes in the range of 50 µl to 1000 µl

Multichannel or multipette

ELISA reader equipped with 450 nm filter

Graph paper or software for calculation of results

5.) Reagents and sample preparation - assay procedure

Freshly collected samples are put on ice immediately after collection. Serum and plasma must be centrifuged within one hour.

Store samples at -20°C if not assayed on the same day. Lipemic or haemolytic samples may give erroneous results. Samples should be mixed well before assaying.

We recommend duplicates for all tests.

- Mark positions for blank, standards, control and samples on the protocol sheet supplied.
- Take microtiter strips out of the bag and mark as appropriate. Mark 2 wells as blank. Store unused strips with the desiccant at 4°C in the alubag. Strips are stable until expiry date stated on the label.
- All reagents and samples must be at room temperature (18 - 26°C) before use in the assay, this usually takes about 30 min.

1. Dilute wash buffer concentrate to 1000 ml (add the 100 ml of concentrate to 900 ml of distilledwater) mix well. Crystals in the buffer concentrate will dissolve at room temperature. This buffer is stable at 4°C until expiry date stated on the label.
2. Dissolve control (clear vial with yellow cap) in 250µl distilled water, leave at room temperature for 30 minutes, mix well.

   Reconstituted control is stable at -20°C until expiry date stated on the label. Avoid repeated freeze-thaw cycles.
3. Dissolve standard (LS) (amber vial with white cap) in 3 ml of assay buffer (PBSB) to obtain a final concentration of 1000fmol/ml after reconstitution. Reconstituted standard is stable at -20°C until expiry date stated on the label. Avoid repeated freeze-thaw cycles! Leave at room temperature for 30 minutes, mix well Prepare a 1+1 dilution series in assay buffer recommendation: 1000/500/250/125/62.5/31.25/0 fmol/ml. Assay buffer is used as 0 fmol/ml standard.
4. Dilute samples and the control 1+5 with assay buffer, mix well (e.g. 100µl plasma + 500µl buffer)
5. Add 200µl of assay buffer into wells marked as blank.
6. Add 200µl of standard, diluted sample or diluted control in the respective wells.
7. Add 50µl of tracer (LT, red) into all wells **EXCEPT BLANK.**

8. Seal plate with plastic film and incubate 24 –25 hrs at 4°C in the dark. No special precaution with respect to protection from ambient light have to be taken during pipetting.
9. Discard the content of the wells and wash 5 times with at least 300µl diluted wash buffer.
10. Add 200µl of substrate (TMB) to all wells, incubate for 20 minutes at RT in the dark.
11. Add 50µl of stop reagent (POT) to all wells and read optical density at 450 nm with reference at 620 nm if available.

6.) Calculation of results

A calibration curve is constructed from the standards, and plotted in a spline- or 4PL algorithm system. Commercially available software can be used as well as graph paper. Values for the samples are read from this calibration curve.
The serum- or plasma samples have been diluted 1+5, the result has to be multiplied by 6 to obtain the concentrations of the samples and control in fmol/ml.

7.) Assay characteristics

Reference values from apparently healthy persons:

< 250 fmol/ml negative result

< 250-350 fmol/ml borderline result

> 350 fmol/ml positive result

We recommend for each laboratory to establish own reference values.

Standard range

The dynamic range of the assay covers detection limit to 1000 fmol/ml.

Detection limit:

The detection limit is the concentration of Nt-proBNP
at 95% B/Bo. For this assay the detection limit was determined to be 5 fmol/ml.

Sample volume:

100 µl of human plasma, serum respectively

Precision:

	Intra-Assay CV (%) n = 16	Inter-Assay CV (%) n = 3
320 fmol/ml	6.5	4.4
666 fmol/ml	4.0	3.8

8.) Technical hints

- To avoid cross-contamination, change pipette tips between addition of standards, controls, samples, antibody, conjugate and substrate. Also use separate reservoirs for each reagent.
- When mixing reagents, always avoid foaming.
- Do not mix stoppers and caps of different reagents.
- Do not use reagents beyond expiration date.
- Do not mix or substitute reagents with those from other lots or sources.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- Substrate solution should remain colourless until added to the plate.
- Stop solution should be added to the plate in the same order as the substrate solution.

9.)Precautions

All liquid reagents contain 0.01% thimerosal or 0.095% sodium acide as preservative.

- Thimerosal and sodium acide are toxic! Avoid contact with skin and mucous membrane.
- Do not interchange kit components from different lots.
- Do not use kit components beyond the expiry date.
- Protect reagents from direct sunlight.
- Do not pipette by mouth.
- Do not eat, drink, smoke or apply cosmetics where reagents are used.
  Avoid all contact with the reagents by using gloves.
  Stop solution contains diluted sulfuric acid. Irritation to eyes and skin is possible. Flush with water after contact.

10.) References

1. de Bold A.J. et al. (1981), Life Sci. 28: 89-94
2. Yandle T.G. et al. (1986), Life Sci. 38: 1827-1833
3. Mathisen P. et al. (1991), Scand.J. Clin.Lab. Invest. 53: 41-49
4. Hunt P.J. et al (1995), Biochem Biophys Res Commun 214: 1175-1183
5. Hino et al. (1990), Biochem Biophys Res Commun 167: 693-700
6 Kambayashi et al. (1990), FEBS Letters 259: 341-345
7. Bonow R. et al. (1996), Circulation 93: 1946-1950
8. Nakao K. et al (1996), Current Opinion in Nephrology and Hypertension 5: 4-11
9. Wei CH. et al. (1993), Circulation 88: 1004-1049
10. McDonagh T.A. et al (1998), Lancet 351: 9-13
11. Omland T. et al. (1996), Heart 76: 232-237
12. Yamamoto K. et al. (1996), J. Am. Coll. Cardiol. 27: 69A-70A
13. Davis et al. (1994), Lancet 343: 440-444
14. Hunt et al. (1997), Clin. Endocrinology 47:287-96

11.) Incubation scheme

Sample incubation

	Blank	Standard	Sample	Control
Standard	-	200µl	-	-
Sample	-	-	200µl	-
Control	-	-	-	200µl
Assaybuffer	200µl	-	-	-
Tracer	-	50 µl	50 µl	50 µl

Cover strips with the supplied plastic foil and incubate for **24 – 25 hrs** at 4°C **in the dark**.
Discard the content of the wells and wash 5 times with 300 µl diluted wash buffer.
Remove any remaining fluid by hitting plate against paper towel after the last wash.

Substrate incubation

Substrate	200 µl	200 µl	200 µl	200 µl

Incubate 20 min. at room temperature (18-26°C) in the dark.

Stop and read

Stop solution	50 µl	50 µl	50 µl	50 µl

Mix, and read absorption with an ELISA microwell reader at 450 nm and 620 nm or 690 nm as a reference. If the readings exceed the measuring range of the photometer, absorption can be measured at 405 nm against 690 nm or 620 nm as reference immediately.