



DIAGNOSTIC AUTOMATION/CORTEZ DIAGNOSTICS, INC.

proANP ELISA
Quantitative Determination of Human proANP(1-98)
In EDTA Plasma, Heparin Plasma, Serum or Cell Culture Supernatants
Cat. No. 2892-14

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1. Introduction

Atrial natriuretic peptide is a peptide hormone synthesized in atrial myocytes as a prohormone and stored in secretory granules as a 126 amino acid prohormone^(1,2). The most important stimulus for the release of the hormone into circulation is stretch of the myocyte fibres^(3,4). On release the prohormone is split into equimolar amounts of the highly biologically active proANP (99-126), also known as α -ANP, and the N-terminal part proANP (1-98)^(5,6). α -ANP binds to specific receptors and therefore is rapidly cleared from the circulation with a half-life of 3-4 minutes. No receptors for proANP (1-98) are known today, therefore this peptide circulates longer which leads to significant higher concentrations in blood compared to α -ANP⁽⁷⁾. Thus, circulating levels of proANP (1-98) are less sensitive to the pulsatile secretion of ANP and may better reflect chronic levels of ANP secretion than the rapidly fluctuating levels of α -ANP.

A comprehensive summary of the atrial peptides is given in a paper of B.C. Stein⁽⁸⁾.

Clinical aspects:

In clinical studies, plasma levels of proANP (1-98) has performed superior to α -ANP in the early diagnosis of cardiac dysfunction⁽⁹⁾. In contrast to many test systems for α -ANP, which need solid phase sample extraction prior to the assay, proANP (1-98) may be determined directly in biological samples. Furthermore, the stability of this

analyte is excellent, it may be stored in plasma samples up to three days at room temperature without affecting the levels ⁽¹⁰⁾.

2. Principle of the assay

The proANP (1-98) test kit is a sandwich enzyme immunoassay designed to determine proANP (1-98) directly in biological fluids.

To provide maximum specificity, the kit incorporates a pair of immunoaffinity purified polyclonal antibodies raised in sheep. The capture antibody, specific for proANP (10-19), is coated onto the microtiter plate. The detection antibody, specific for proANP (85-90), is labeled with biotin. In the first step, sample and detection antibody are added simultaneously to the wells. proANP (1-98), if present in the sample, binds to the precoated capture antibody and forms a sandwich with the detection antibody. After a washing step, which removes all nonspecific bound material, a streptavidin-peroxidase conjugate detects the presence of bound detection antibodies. After removal of unbound conjugate through washing, tetramethylbenzidine (TMB) is added to the wells as a substrate. proANP (1-98) is quantitated by an enzyme catalyzed color change detectable on a standard ELISA reader. The amount of color developed is directly proportional to the amount of proANP (1-98) present in the samples or standards.

3. Contents of the kit

- 12 x 8 well microtiter strips in strip holder, packed in zipped alu bag with desiccant. Wells are coated with a polyclonal sheep anti proANP antibody
- 10x washing buffer
The vial contains 100 ml washing buffer concentrate
- Assay buffer
The bottle contains 120 ml assay buffer, ready to use
- Detection antibody (blue cap)
The vial contains polyclonal anti proANP antibody, labeled with biotin, sufficient for 96 determinations, lyophilized
- 5 standards
The vials contain synthetic human proANP (1-98), lyophilized. The concentrations after reconstitution are stated on the label
- 1 control (yellow cap)
The vials contain synthetic human proANP (1-98) lyophilized in human plasma. The concentrations after reconstitution are stated on the label

- Conjugate
The vial contains 13 ml streptavidin conjugated to horseradish peroxidase (HRPO), ready to use
- Substrate
The vial contains 13 ml TMB solution, ready to use
- Stop solution
The vial contains 7 ml of stop solution, ready to use
- 2 self-adhesive plastic films
- Protocol sheet
- Instructions for use (package insert)

4. Additional material and equipment required

Distilled water

Variable pipettes 10 µl to 100 µl

Multichannel or multipipette

Incubator for 37 °C

Manual or automatic microwell washer

ELISA reader equipped with 450 nm filter

Graph paper or software for calculation of results

5. Reagent and sample preparation - performance of the assay

Freshly collected samples should be stored at 4°C after collection. Serum or plasma must be centrifuged within one day. Samples may be stored at 4°C up to 2 days, for prolonged periods store samples at -20°C. 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, controls and samples on the protocol sheet supplied.
 - Take microtiter strips out of the bag and mark as appropriate. Mark two 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.
1. Dissolve detection antibody (blue cap) in 13 ml of assay buffer and leave at room temperature (18 - 26°C) for 15 min., shake well. Reconstituted antibody is stable at -20°C until expiry date stated on the label. Avoid repeated freeze-thaw cycles!

2. Dilute wash buffer concentrate to 1000 ml (add the 100 ml of concentrate to 900 ml of distilled water) 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.
3. Dissolve standards in 500 µl of distilled water and leave at room temperature (18 - 26°C) for 15 min., shake well. Reconstituted standard is stable at -20°C until expiry date. Avoid repeated freeze-thaw cycles!
4. Dissolve control (yellow cap) in 500 µl of distilled water and leave at room temperature (18 - 26°C) for 15 min., shake well. Reconstituted control is stable at -20°C until the expiry date stated on the label. Avoid repeated freeze-thaw cycles!
5. Dilute serum or plasma samples 1 + 4 in assay buffer (e.g. 10 µl sample + 40 µl of assay buffer).
6. Add 20 µl of standards, control and diluted samples into the respective wells.
7. Add 100 µl of assay buffer into wells marked as blank.
8. Add 100 µl of detection antibody (blue) to all wells except blank, mix well.
9. Cover strips with plastic film and incubate 150 min. at 37°C. Make sure all wells are sealed well with the film to avoid evaporation.
10. Discard contents of the wells and wash 3x with 350 µl diluted wash buffer.
11. Add 100 µl conjugate (red) to all wells.
12. Cover strips with plastic film and incubate for 1 hour at 37°C in an incubator. Make sure all wells are sealed well with the film to avoid evaporation.
13. Discard contents of the wells and wash 4 x with 350 µl diluted wash buffer.
14. Add 100 µl of substrate to all wells and incubate for 20 min. at room temperature (18 - 26°C) in the dark.
15. Add 50 µl of stop solution to all wells, shake well.
16. Determine absorption immediately with an ELISA reader at 450 nm against ≥ 620 nm as reference.
If no reference wavelength is available, read only at 450 nm.
17. If the extinction of the highest standard exceeds the measuring range of the photometer, absorption must be measured immediately at 405 nm against ≥ 620 nm as reference.

6. Calculation of results

Subtract the blank extinction from all other values.

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-sample results have to be multiplied by 5 to obtain the concentrations of the samples in fmol/ml.

If the concentration of some sample is out of the standard range, these samples must be diluted 1 + 9 with assaybuffer before analysis.

No high dose hook effect is seen in samples with concentrations of up to 30000 fmol/ml proANP (1-98).

The assay has been evaluated using a 4PL logarithm. Different curve fitting methods need to be evaluated by the user. Respective dilution factors have to be considered.

7. Assay characteristics

Reference values from apparently healthy persons:

<1945 fmol/ml
(5th-95th percentile, n=336)

Standard range:

0 to 5000 fmol/ml

Detection limit:

The detection limit is the concentration of proANP (1-98) being 3 SD above zero. For this assay detection limit was determined to be 50 fmol/ml

Cross reactivity:

proANP (1-30)	< 1 %
proANP (31-67)	< 1 %
proANP (79-98)	< 1 %
α -ANP (99-126)	< 1 %
proBNP (8-29)	< 1 %
proBNP (32-57)	< 1 %
proCNP (1-19)	< 1 %
proCNP (30-50)	< 1 %
proCNP (51-79)	< 1 %

Sample volume:

10 μ l of human plasma or serum

Precision:

Intraassay	mean	1 SD	CV	n
	427 fmol/ml	27 fmol/ml	6 %	20
Interassay	mean	1 SD	CV	n
	436 fmol/ml	29 fmol/ml	7 %	5

Recovery:

10 plasma samples were spiked with synthetic proANP (1-98) and measured in the assay.

spiked: 553 fmol/ml

measured: 547 fmol/ml

recovery:99%

Incubation steps: 150 min. / 60 min. / 20 min.

Storage: 4°C

8. Technical hints

- To avoid cross-contamination, change pipette tips between addition of standards, controls, samples, tracer, conjugate, substrate and stop solution. Also use separate reservoirs for each reagent!
- Do not mix stoppers and caps of different reagents - contamination!
- Do not use reagents beyond expiry date.
Protect reagents from direct sunlight
- 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 test components of human source were tested with 3rd generation tests against HIV-Ab and HBsAG; all components were found to be negative. However, standards as well as controls should be handled and disposed as if they were infectious, since no test method can offer complete assurance.

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

- Thimerosal and sodium azide are toxic! Avoid contact with skin and mucous membrane.
- 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 sulphuric acid. Irritation to eyes and skin is possible.
Flush with water after contact!

10. Literature

- 1.de Bold A.J. et al. (1981), Life Sci. 28:89-94
- 2.Miyata A. et al. (1985), Biochem Biophys Res Commun. 129:248-255
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- 7.Wei C.M. et al. (1993), Circulation 88:1004-1009.
- 8.Stein B.C. et al. (1998), Am. Heart J. 135: 914-923
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11. Incubation scheme

	Blank	Std./Contr.	Sample
Standard/Control	-	20 µl	-
Sample	-	-	20 µl
Detection antibody	-	100 µl	100 µl

Mix well, incubate the microwell strips covered with plastic film for 150 min. at 37°C in an incubator.
Discard contents of the wells and wash the wells 3x with 350 µl diluted wash buffer.

Conjugate	100 µl	100 µl	100 µl
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Incubate the microwell strips covered with plastic film for 1 hour at 37°C in an incubator.
Discard liquid and wash the wells 4 times with 350 µl diluted wash buffer.

Substrate	100 µl	100 µl	100 µl
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Incubate microwell strips 20 min. at room temperature (18 - 26°C) in the dark.

Stop solution	50 µl	50 µl	50 µl
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Read absorption with an ELISA microwell reader at 450 nm and 620 nm or 690 nm as a reference.

If the extinction of the highest standard exceeds the measuring range of the photometer, absorption must be measured immediately at 405 nm against 690 nm or 620 nm as reference.