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(54) Title: IMMUNOASSAY FOR QUANTIFICATION OF AN UNSTABLE ANTIGEN SELECTED FROM BNP AND PROBNP

(57) Abstract: The present invention relates to an immunoassay for detection of BNP, proBNP and fragments thereof. Essentially the assay comprises: a) contacting the antigen with a first antibody specific to a fragment corresponding to amino acids 11-22 of BNP, or to a part of this peptide comprising at least three amino acids of said sequence, to obtain a first order immune complex. b) contacting the first order immune complex obtained at step (a) with a second antibody recognizing said first order immune complex, to obtain a second order immune complex, wherein said antibody is unable to recognize free BNP, proBNP or free first antibody; c) Detecting the second order immune complex.

Immunoassay for quantification of an unstable antigen selected from BNP and proBNP

Field of the Invention

The present invention relates to immunoassays, and provides an immunoassay method for detection of unstable antigens. The method is specifically suitable for detection of BNP, proBNP and fragments thereof.

Background of the Invention

BNP and proBNP are reliable markers of heart failure (HF) widely used in clinical practice. Several types of sandwich immunoassays (conventional assays) utilizing two mono- or polyclonal antibodies, specific to different epitopes of BNP or BNP-fragment of proBNP molecule are described in literature.

BNP molecule is known as an extremely unstable molecule rapidly losing its immunological activity in water solutions. This loss of activity is usually associated with proteolytic degradation of the peptide. Sandwich immunoassays commonly used for qualitative or quantitative antigen immunodetection utilize two or more antibodies specific to two or more different epitopes. The longer is the distance between the epitopes, the higher is the probability that sites of proteolysis would be located between the epitopes of the antibodies, thus increasing the sensitivity of the assay to proteolytic degradation of the antigen. And *vice versa*, the closer are the epitopes to each other, the smaller is the probability of the proteolytic cleavage of the molecule between the epitopes.

Immunoassay methods for very small molecules have been described, including the application of so called anti-metatype antibodies. Such methods are disclosed, e.g. for detecting digoxin (Self *et al.*, 1994, Clin. Chem. 40:2035–2041), and angiotensin II (Towbin *et al.*, 1995, J. Immunol. Meth. 181:167–176).

However, it is not an easy task to apply this type of method to different analytes, since very specific monoclonal antibodies are required in such a method.

Description of the Invention

Here we are describing an immunoassay for quantification of BNP and proBNP in human blood. We have named the assay as "unequal sandwich". This assay is applicable to immunodetection of all unstable antigens.

The immunoassay described in the present application utilizes two different monoclonal antibodies. In detection of BNP or proBNP the first monoclonal antibody (MAb 24C5) is specific to the region (or a part of this region) comprising amino acid residues 11–22 ($_{11}$FGRKMDRISSSS$_{22}$) of BNP (which correspond to amino acid residues 87–98 of proBNP) (Fig. 1). The second antibody (namely MAbs Ab-BNP2 and Ab-BNP4), labeled with a signal-producing component, recognizes an immune complex of the first antibody with antigen (BNP, proBNP, or a fragment thereof comprising amino acid residues $_{11}$FGRKMDRISSSS$_{22}$ or a part of this sequence comprising at least three amino acid residues of said sequence). Second antibody does not recognize (or recognizes with very low affinity - 10-fold or less) either free antigen or its fragments, or free MAb 24C5. Thus the primary immune complex comprising MAb 24C5 and BNP (or proBNP, or a fragment thereof) serves as an antigen for the second antibody (MAbs Ab-BNP2 and Ab-BNP4).

Consequently, the general object of the present invention is an immunoassay method for detecting an unstable antigen in a sample, comprising

(a) contacting an antigen of interest with a first antibody specific to a first epitope of the antigen molecule, to obtain a first order immune complex,

(b) contacting the first order immune complex obtained at step (a) with a second antibody, which recognizes said first order immune complex and is specific to a second epitope formed by the antigen of interest and the first antibody, to obtain a second order immune complex, wherein said second antibody is unable to recognize free antigen or a fragment thereof or free first antibody, or recognizes them with significantly lower affinity - 10-fold or less - than they recognize the first order immune complex, and

(c) detecting the second order immune complex formation.

A specific object of the invention is an immunoassay method for detecting an antigen selected from the group consisting of BNP, proBNP and a fragment thereof in a sample, comprising

(a) contacting the antigen with a first antibody specific to the fragment $_{11}$FGRKMDRISSSS$_{22}$ of BNP molecule or to a part of this peptide comprising at least three amino acid residues of said sequence, to obtain a first order immune complex,

(b) contacting the first order immune complex obtained at step (a) with a second antibody recognizing said first order immune complex, to obtain a second order immune complex, wherein said second antibody is unable to recognize free BNP, proBNP or a fragment thereof or free first antibody, or recognizes them with significantly lower affinity - 10-fold or less - than it recognizes the first order immune complex, and

(c) detecting the second order immune complex formation.

We have succeeded in producing specific monoclonal antibodies applicable in the method of the invention. These antibodies are specific objects of the present invention.

Unequal sandwich described herein demonstrates extraordinary insusceptibility to proteolytic degradation of the antigen in comparison with the assays utilizing antibodies specific to distantly located epitopes.

Also such approach could be useful in the cases where the assay is developed for immunodetection of the antigen which is similar to one or more other antigens; has numerous different epitopes on its surface, but has only one (or more, but very limited number) of unique epitopes, that distinguishes that particular antigen from all others.

Brief Description of the Drawings

Fig. 1. BNP and pro BNP structures and epitope specificity of MAb 24C5.
MAb 24C5 recognizes fragment of BNP molecule comprising amino acid residues 11–22 and proBNP fragment consisting of amino acid residues 87–98 (marked by dark).

Fig. 2A, 2B and 2C. Antibodies Ab-BNP2 and Ab-BNP4 do not recognize either BNP or proBNP that are not complexed with MAb 24C5.

Eu-labelled MAbs 24C5, Ab-BNP2, Ab-BNP4 (200 ng/well) were incubated in plates coated with:

A. BNP 50 ng/well
B. proBNP 100 ng/well
C. polyclonal anti-BNP antibodies (2 μg/well) preincubated with BNP (0.5 ng/well)

Fig. 3. Antibodies Ab-BNP2 and Ab-BNP4 can recognize immune complex of BNP (or Peptide 11–22) with MAb 24C5

Three-step assay protocol:

First step: plates were precoated with capture MAb 24C5

Second step: After washing the plates were incubated with antigen (BNP or Peptide 11–22);

Third step: After washing the plates were incubated with detection (Eu^{3+}labeled) antibodies (Ab-BNP2, Ab-BNP4 or 57H3).

After washing enhancement solution was added and the signal was measured.

Fig. 4. Antibodies Ab-BNP2 and Ab-BNP4 can recognize proBNP, which forms immune complex with MAb 24C5

Three-step assay protocol:

First step: Plates were precoated with capture MAb 24C5

Second step: After washing the plates were incubated with proBNP (5 ng/ml)

Third step: After washing the plates were incubated with detection antibodies (Ab-BNP2, Ab-BNP4 or 57H3).

After washing enhancement solution was added and the signal was measured.

Fig. 5. Stability of BNP in normal human plasma.

Synthetic BNP was spiked into pooled normal human plasma (2 ng/ml), incubated at +4°C for different periods of time. Immunological activity was tested in three different assays - one conventional and two unequal sandwiches.

Fig. 6. BNP/proBNP measurements in blood of patients with HF and healthy donors. Plasma samples of 6 patients with heart failure (HF 1 - HF 6) and plasma samples of healthy donors (NP1–NP4) were tested in three assays. Synthetic BNP (Bachem) was used as a calibrator in all assays.

Fig. 7A, 7B and 7C. Calibration curves for two unequal sandwiches (24C5 – Ab-BNP2, 24C5 – Ab-BNP4) and one conventional assay (50E1 – 24C5-Eu). Antigen: synthetic BNP (Bachem).

Experimental

Remarks: Antibodies labeled with stable Eu-chelate were used in all experiments as detection antibodies. The monoclonal antibodies 24C5, Ab-BNP2, Ab-BNP4, 57H3 and 50E1 used in the experiments are available from Hytest Ltd, Turku, Finland.

Example 1. Antibodies Ab-BNP2 and Ab-BNP4 do not recognize either BNP or proBNP that are not complexed with MAb 24C5 (Fig. 2)

In the experiment presented in the Fig. 2A and Fig. 2B antigens (BNP and proBNP, respectively) were used for plate coating and Eu-labeled antibodies were tested with the antigen in direct immunoassay. Antibody 24C5 recognizes both forms of the antigen, whereas MAbs Ab-BNP2 and Ab-BNP4 give no response (signal comparable with background) with any of the two antigens.

In the experiment presented in Fig. 2C the plates were coated with polyclonal antibodies specific to different epitopes on BNP molecule. On the second step the plates were incubated with BNP and then with Eu-labeled antibodies. Such approach helps to obtain variable orientation of the antigen against plate surface, insuring that orientation of the molecule on the plate surface does not have influence on the experimental results. In this experiment the same results as described above were obtained: MAbs Ab-BNP2 and Ab-BNP4 were not able to recognize the antigen, which is not complexed with MAb 24C5.

Example 2. Antibodies Ab-BNP2 and Ab-BNP4 can recognize BNP and Peptide 11–22, that are forming immune complex with MAb 24C5 (Fig. 3)

MAb 24C5 is specific to the fragment 11–22 of BNP molecule or to the corresponding region 87–98 of proBNP. To demonstrate that immune complex 24C5 - BNP and 24C5 - peptide 11–22 could be recognized by MAbs Ab-BNP2 and Ab-BNP4 we used MAb 24C5 for plate coating, then incubated the plates with BNP or synthetic peptide corresponding to amino acids 11–22 of BNP sequence (Peptide 11–22). After the immune complex between MAb 24C5 and antigens was formed, the plates were incubated with Eu-labeled antibodies Ab-BNP2, Ab-BNP4 and 57H3, specific to the region 26–32 of the BNP molecule.

Unequal sandwich recognizes BNP and the peptide almost with the same efficiency. Assay utilizing antibodies 24C5 (coating) - 57H3-Eu does not recognize Peptide 11–22 (signal comparable with the background).

Example 3. Antibodies Ab-BNP2 and Ab-BNP4 can recognize proBNP, which forms immune complex with MAb 24C5 (Fig. 4)

Unequal sandwich recognizes proBNP with the same efficiency as a conventional assay. We used MAb 24C5 for plate coating and then incubated plates firstly with recombinant proBNP (5 ng/ml) and secondly with Eu-labeled antibodies Ab-BNP2, Ab-BNP4 and 57H3 specific to the region 26–32 of BNP molecule. The signals obtained in the unequal sandwich and conventional immunoassays are comparable. We concluded that new assays could be used for quantitative immunodetection of proBNP.

Example 4. Apparent stability of the antigen (Fig. 5)

Synthetic BNP (Bachem) was spiked into pooled normal human plasma (2 ng/ml), incubated at +4°C for different periods of time and the immunological activity was tested in three different assays - one conventional and two unequal sandwiches.

Apparent stability of the antigen, being determined in unequal sandwiches, described here is significantly higher in comparison with the stability determined by the conventional BNP assays utilizing two MAbs specific to different parts of BNP molecule. As an

example of conventional assay we used assay, utilizing MAb 50E1 specific to the region 26–32 of BNP molecule and MAb 24C5 specific to the region 11–22 of BNP molecule. About 70% of immunological activity was observed after 24 hours of incubation at +4°C (69,8% and 68% for assays utilizing Ab-BNP2 and Ab-BNP4, respectively) in the case the unequal sandwich was used to determine the immunoreactivity, and only 28% in the case of conventional assay. Six days after the beginning of incubation no immunoreactivity was observed in case of conventional assays, whereas about ¼ of initial immunoreactivity was observed in the case of unequal sandwiches.

Example 5. BNP/proBNP measurements in blood of heart failure patients (HF patients) and blood of healthy donors (Fig. 6)

Unequal sandwich, as well as conventional BNP assays are able to detect in human blood both forms of the antigen displaying "BNP immunoreactivity" - i.e. BNP and proBNP. Blood samples from several HF patients and healthy donors were tested in three assays - one conventional, utilizing capture MAb 50E1, specific to the fragment 26–32 of BNP molecule and detection MAb 24C5-Eu and two unequal sandwiches. All assays were calibrated using synthetic BNP. As it follows from Fig. 6, the results of testing in three assays are very similar. In some samples results of testing in conventional assay are lower than in unequal sandwiches. This observation can be explained by the fact that in such samples BNP is partially degraded, but because of the fact that antigen displays better apparent stability in unequal sandwiches the antigen values determined by these assays are higher than in a conventional assay.

Example 6. Calibration curves (Fig. 7)

Calibration curves for two unequal sandwiches and one conventional assay with synthetic BNP used as an antigen are presented in Fig. 7 (A, B and C). Both of the unequal sandwiches demonstrate high sensitivity, comparable with the sensitivity of the conventional assay and could be used for precise detection of BNP and proBNP immunoreactivity in human blood.

Claims

1. An immunoassay method for detecting an unstable antigen selected from the group consisting of BNP, proBNP and a fragment thereof in a sample, comprising

(a) contacting the antigen with a first antibody specific to the fragment $_{11}$FGRKMDRISSSS$_{22}$ (SEQ ID NO:3) of BNP molecule or to a part of this peptide comprising at least three amino acid residues of said sequence, to obtain a first order immune complex,

(b) contacting the first order immune complex obtained at step (a) with a second antibody recognizing said first order immune complex, to obtain a second order immune complex, wherein said second antibody is unable to recognize free BNP, proBNP or a fragment thereof or free first antibody, or recognizes them with significantly lower affinity - 10-fold or less - than it recognizes the first order immune complex, and

(c) detecting the second order immune complex formation.

2. A monoclonal antibody specific to the fragment $_{11}$FGRKMDRISSSS$_{22}$ of BNP molecule or to a part of this peptide comprising at least three amino acid residues of said sequence.

3. A monoclonal antibody specific to a first order immune complex of BNP, proBNP or a fragment thereof with an antibody specific to the fragment $_{11}$FGRKMDRISSSS$_{22}$ of BNP molecule or to a part of this peptide comprising at least three amino acid residues of said sequence.

4. An immunoassay kit for detecting an antigen selected from the group consisting of BNP, proBNP and a fragment thereof in a sample, comprising

(a) a first antibody specific to the fragment $_{11}$FGRKMDRISSSS$_{22}$ (SEQ ID NO:3) of BNP molecule or to a part of this peptide comprising at least three amino acid residues of said sequence, the antibody being able to form a first order immune complex with the antigen, and

(b) a second antibody, which recognizes said first order immune complex obtained at step (a) and is specific to a second epitope of BNP formed by the antigen and the first antibody, and is able to form a second order immune complex with said first order immune complex, wherein said second antibody is unable to

recognize free BNP, proBNP or a fragment thereof, or free first antibody, or recognizes them with significantly lower affinity - 10-fold or less - than it recognizes the first order immune complex.

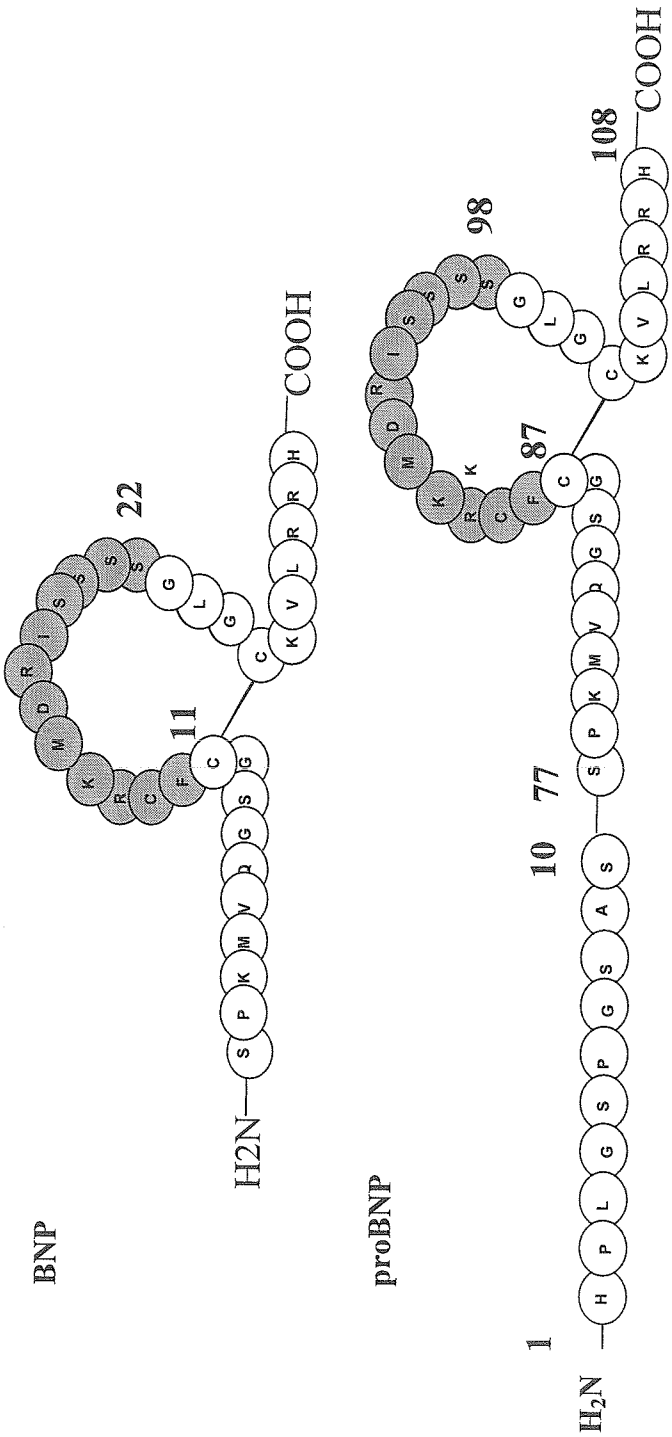
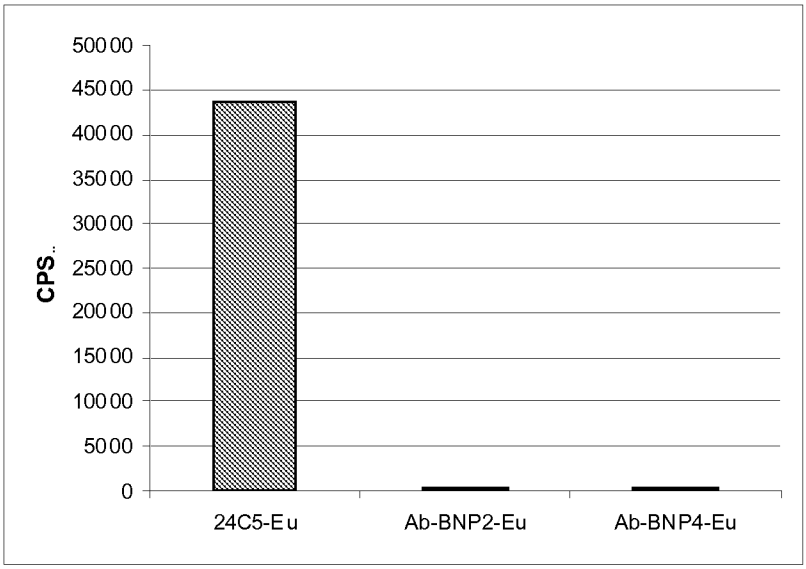


Fig. 1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40000
35000
30000
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24C5-Eu
Ab-BNP2-Eu
Ab-BNP4-Eu

Fig. 2A

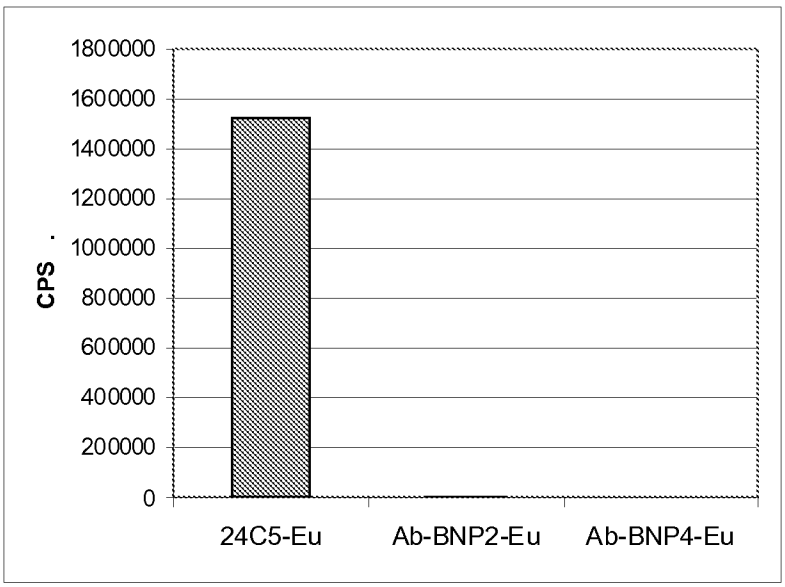
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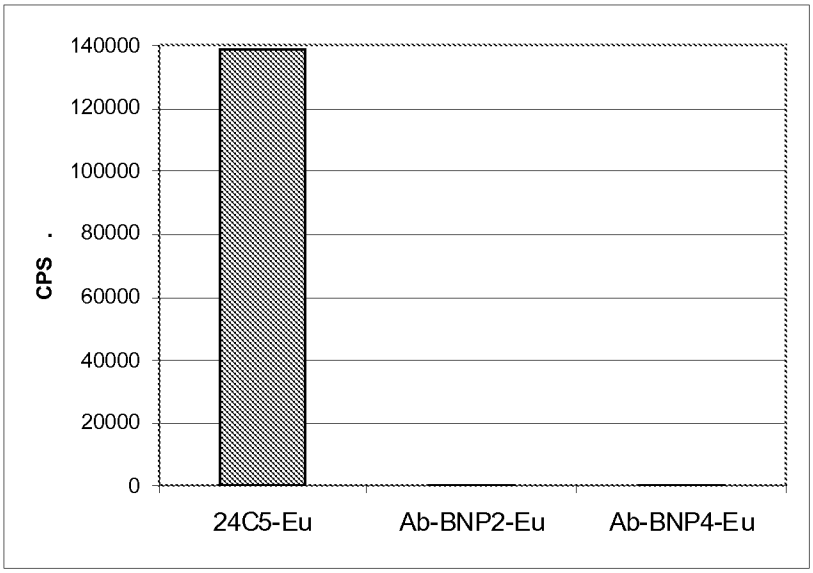
Fig. 2B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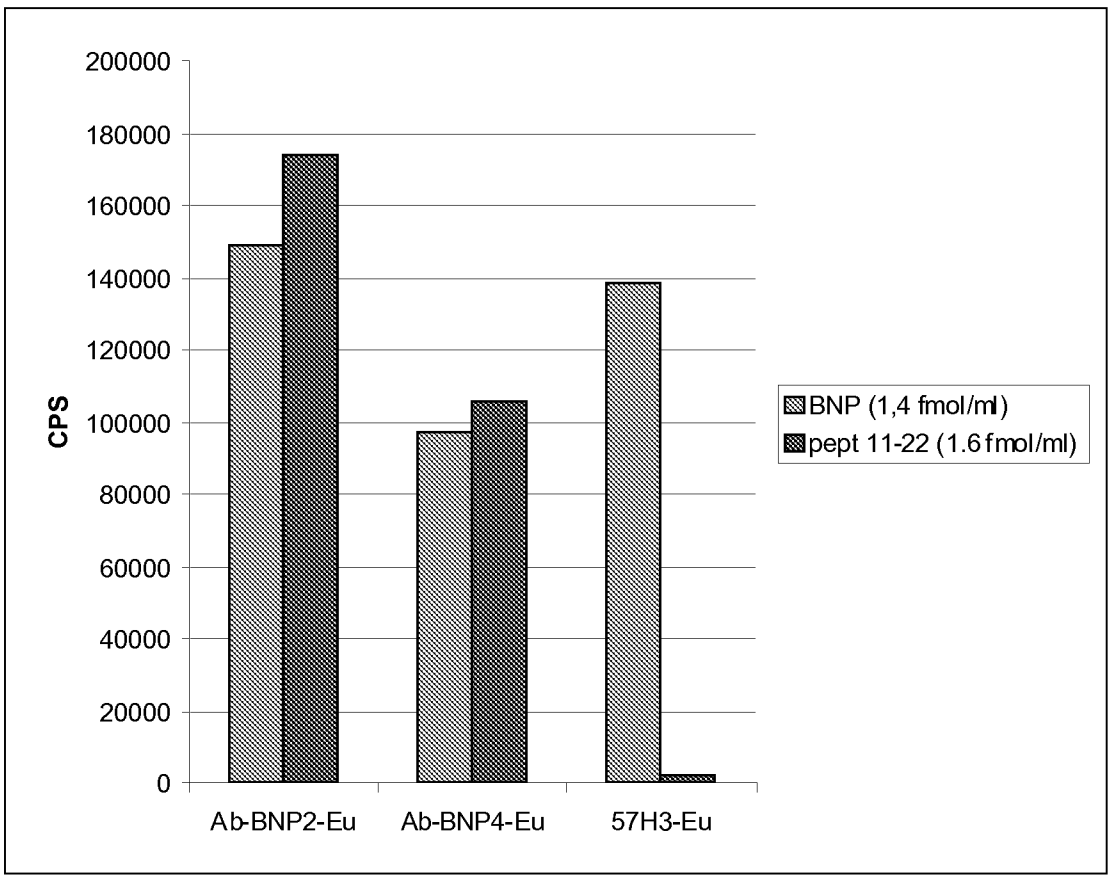
24C5-Eu
Ab-BNP2-Eu
Ab-BNP4-Eu

Fig. 2C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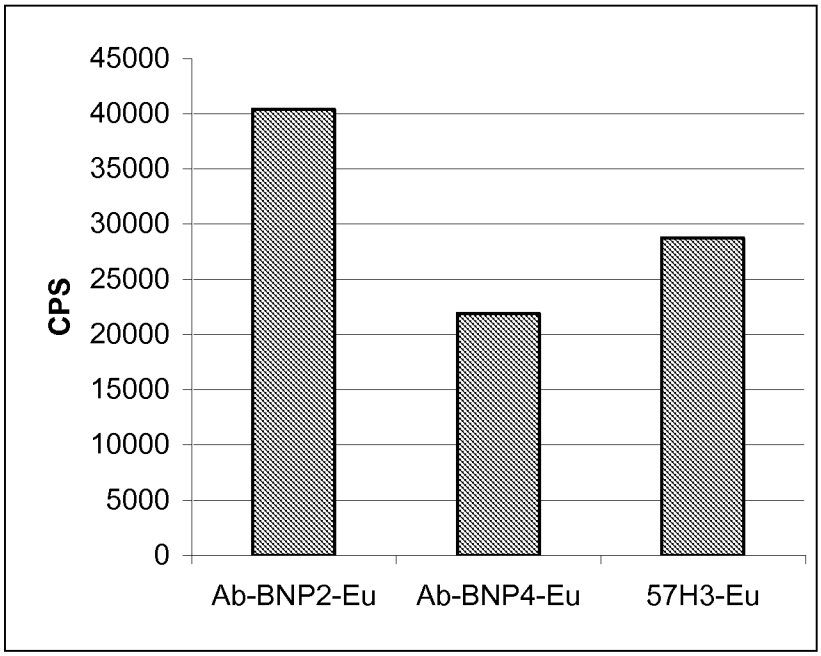
Ab-BNP4-Eu
57H3-Eu
BNP (1,4 fmol/ml)
pept 11-22 (1.6 fmol/ml)

Fig. 3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CPS
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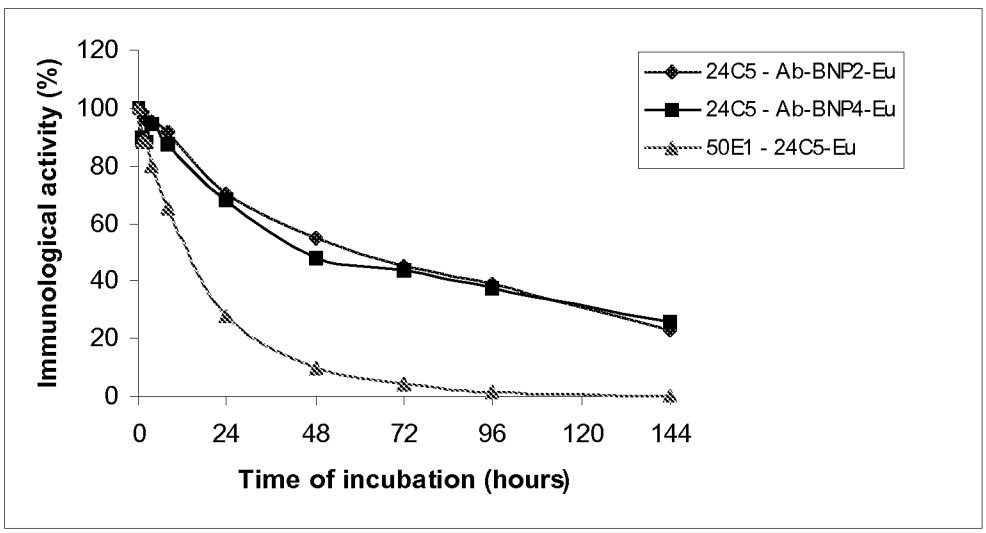
Ab-BNP4-Eu
57H3-Eu

Fig. 4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100
80
60
40
20
0
Immunological activity (%)
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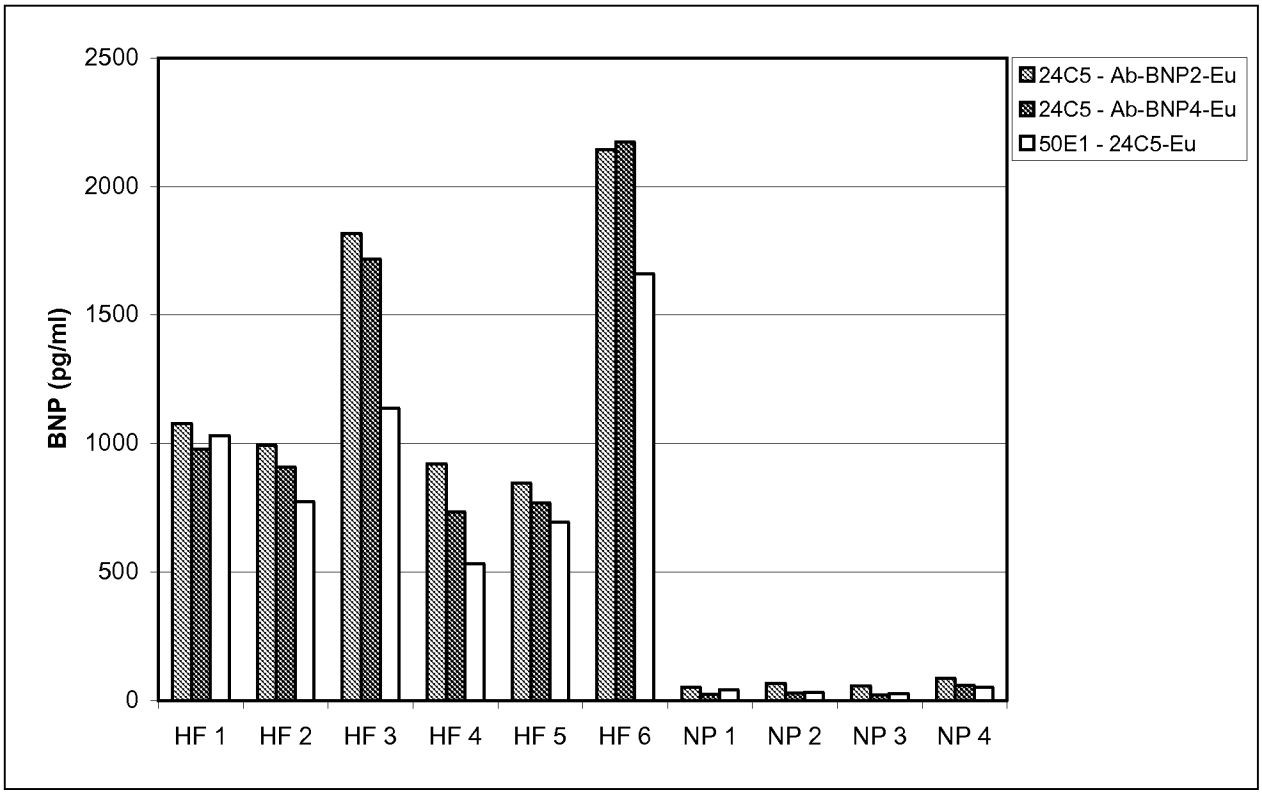
24
48
72
96
120
144
Time of incubation (hours)
24C5 - Ab-BNP2-Eu
24C5 - Ab-BNP4-Eu
50E1 - 24C5-Eu

Fig. 5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2000
1500
1000
500
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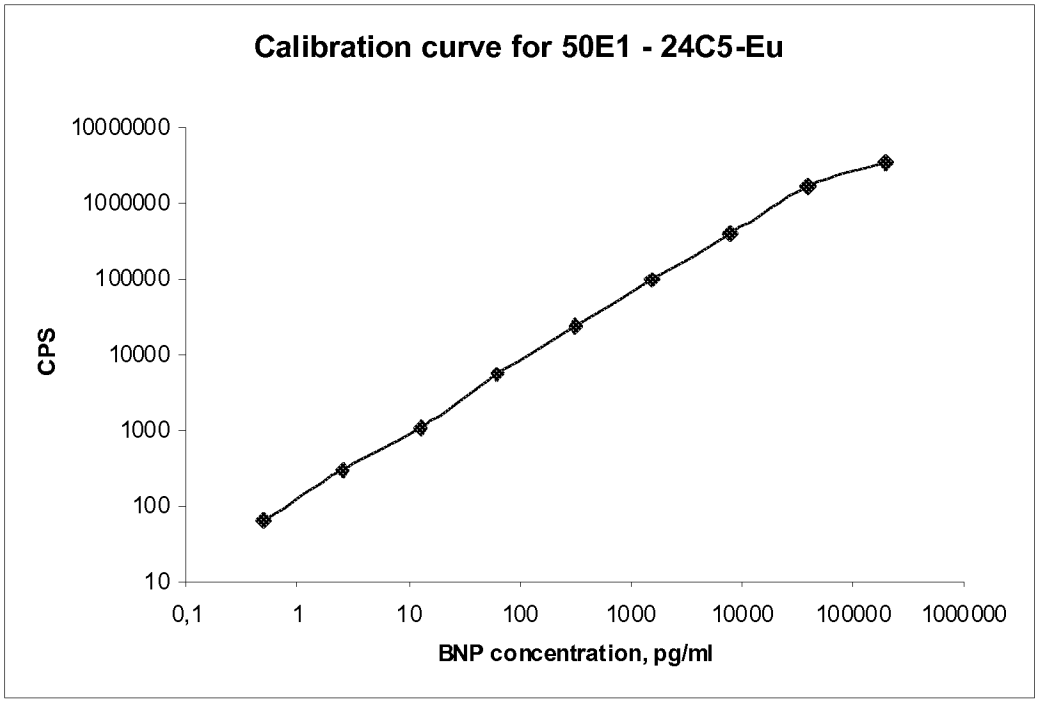
BNP (pg/ml)
HF 1
HF 2
HF 3
HF 4
HF 5
HF 6
NP 1
NP 2
NP 3
NP 4
24C5 - Ab-BNP2-Eu
24C5 - Ab-BNP4-Eu
50E1 - 24C5-Eu

Fig. 6


Calibration curve for 50E1 - 24C5-Eu
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1000000
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10000
1000
100
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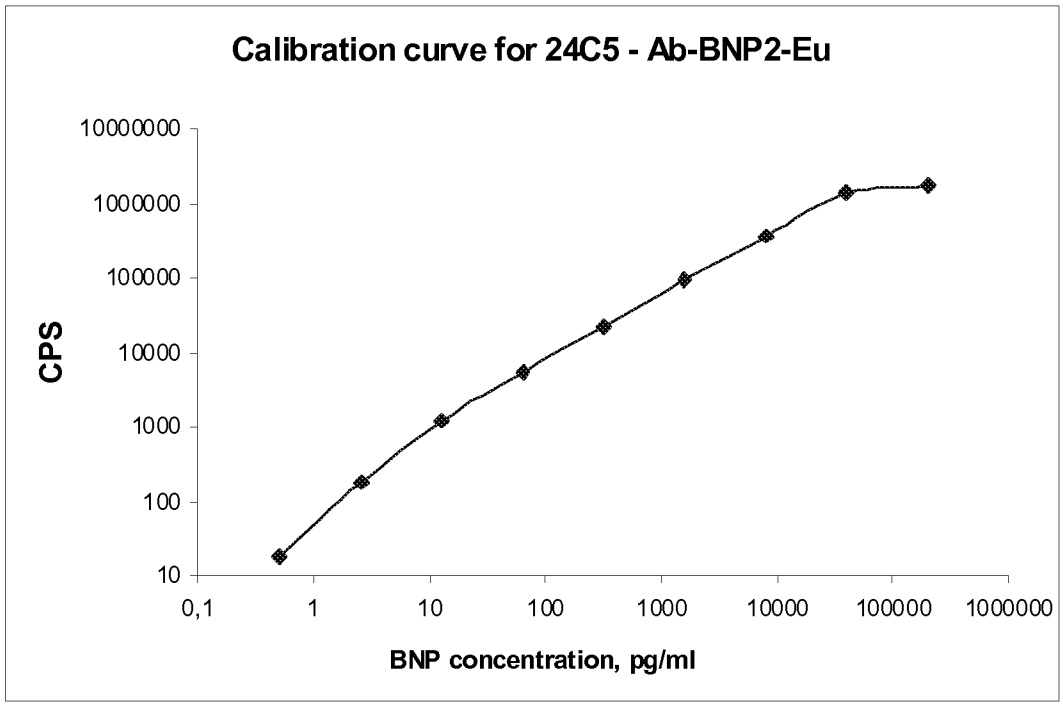
10000
100000
1000000
BNP concentration, pg/ml

Fig. 7A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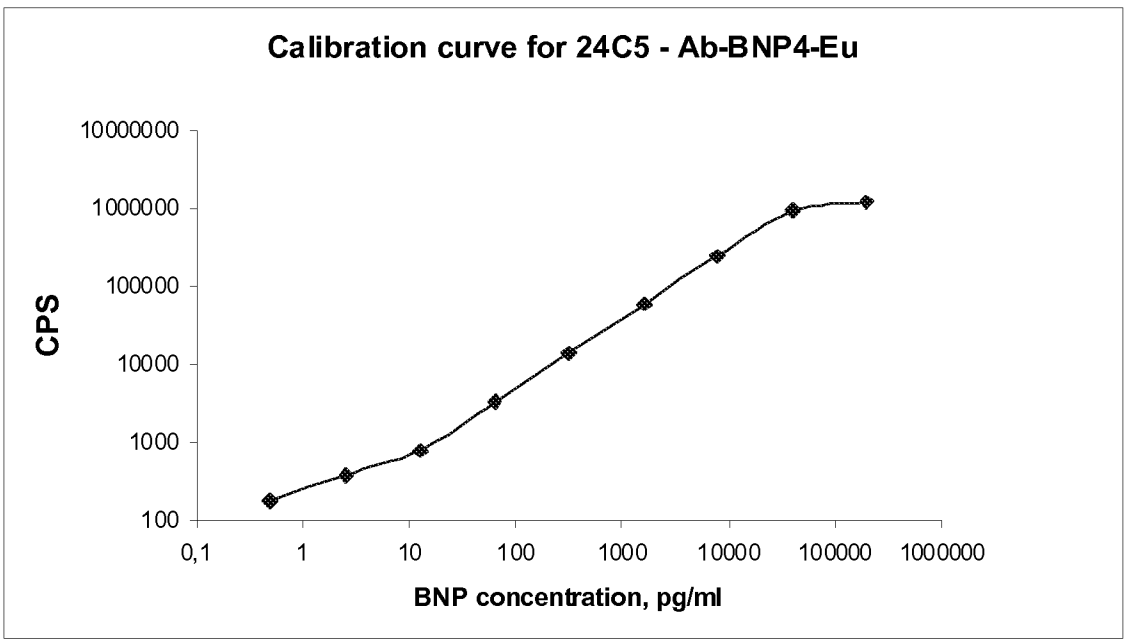
100000
1000000
BNP concentration, pg/ml

Fig. 7B


Calibration curve for 24C5 - Ab-BNP4-Eu
CPS
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10000
1000
100
0,1
1
10
100
1000
10000
100000
1000000
BNP concentration, pg/ml

Fig. 7C

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI2008/050184

A. CLASSIFICATION OF SUBJECT MATTER

IPC: see extra sheet
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC: G01N, C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-INTERNAL, WPI DATA, PAJ, BIOSIS, MEDLINE, EMBASE, EBI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HAMMERER-LERCHER, A ET AL, "Natriuretic Pepetides as Markers of Mild Forms of Left Ventricular Dysfunction: Effects of Assays on Diagnostic Performance of Markers", Clinical Chemistry 2004, Vol. 50 , No 7, p. 1174 - 1183; page 1175, column 2, line 45 - page 1176, column 2, line 2; abstract	2
A	--	1,4
PX	SEFERIAN, R. KARINA ET AL, "The Brain Natriuretic Peptide (BNP) Precursor Is the Major Immunoreactive Form of BNP in Patients with Heart Failure", Clinical Chemistry 2007, Vol. 53, No. 5, p. 866 - 873; page 868, column 1, line 10 - line 27	2
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[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance
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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 August 2008	26 -08- 2008
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Terese Sandström/PR Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI2008/050184

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	NAGATA, S ET AL, "A New Type Sandwich Immunoassay for Microcystin: Production of Monoclonal Antibodies Specific to the Immune Complex Formed by Microcystin and an Anti-microcystin Monoclonal Antibody", Natural Toxins 1999, Vol 7, p. 49 - 55; p. 50, column 1, line 16 - line 48; p. 54, column 2, line 7 - line 22; abstract ---	1,3,4
A	EP 1378242 A1 (BAYER CORP.), 7 January 2004 (07.01.2004), page 2, line 16 - line 32 ---	1-4
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