

New Sensitive Sandwich Time-Resolved Immunofluorometric assay for Quantification of BNP in Human Blood

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INTRODUCTION

B-type natriuretic peptide (BNP) levels are reliably elevated in patients with congestive heart failure, acute coronary syndrome. Under pathological conditions the BNP level in patient's blood is higher than 100 pg/ml, whereas in blood of healthy donors it is about 25 pg/ml. BNP analysis proved to be helpful not only in the disease diagnosis but also in risk stratification. Rapid and accurate BNP measurements are essential for timely initiation of treatment. Thus, new generations of sensitive, precise and rapid BNP assays can contribute to better clinical and economic outcomes.

Materials and Methods

Monoclonal antibodies specific to human BNP were produced after Balb/C mice immunization by standard hybridoma techniques.

Antigen: Synthetic human BNP was from Bachem AG (Switzerland). Recombinant human proBNP and BNP peptides were from HyTest (Turku, Finland).

Serum samples: Serum samples from cardiac failure patients were pooled and stored at -70 °C until used.

Western Blotting: After Tricine-SDS electrophoresis BNP and proBNP were transferred onto nitrocellulose membrane. Membrane was incubated for 30 minutes at room temperature in PBST containing 10% dry milk to block sites of the non-specific binding. Incubations with Mabs and with rabbit anti-mouse antibodies conjugated with horseradish peroxidase were performed in the same conditions for 40 min. Antigen-antibody complexes were visualized by reaction with diaminobenzidine.

Results and Discussion

65 hybridoma cell lines producing monoclonal antibodies (Mabs) specific to BNP were generated after immunization of Balb/C mice with synthetic BNP molecule or several synthetic peptides - fragments of BNP molecule conjugated with carrier protein (peptides corresponding to the amino acid residues 1-10, 11-22, 17-23, 26-32). Epitope specificity of Mabs obtained after animal immunization with whole BNP32 molecule were determined in experiments with synthetic peptides conjugated with carrier protein. Out of 65 Mabs obtained, 3 Mabs were specific to the peptide 1-10, 17 Mabs to the peptide 11-22, 1 Mab to the peptide 17-23 and 31 Mabs to the peptide 26-32 (Fig 1). Specificities of 13 Mabs were not identified.

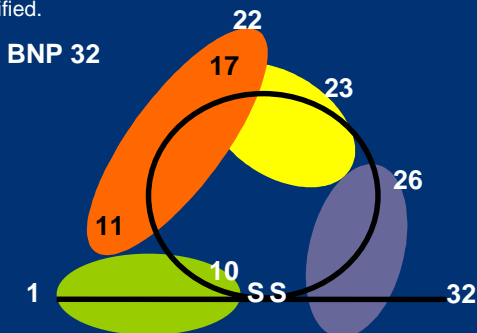


Figure 1: Epitope location of generated monoclonal antibodies.

To confirm specificity of the antibodies and their ability to recognize whole BNP32 molecule and not only synthetic peptides, all Mabs were tested in direct ELISA and also in Western blotting with two antigens - BNP and recombinant proBNP transferred onto nitrocellulose membrane after Tricine-SDS electrophoresis (Fig. 2). Almost all Mabs were able to recognize antigen in Western blotting.

BNP sandwich immunofluorometric assay

All Mabs were tested as capture and detection antibodies in sandwich time-resolved immunofluorometric assay (TR-IFMA). In these studies we also used two antigens - synthetic BNP32 and pooled serum from patients with heart failure - as a source of native antigen circulating in human blood. Detection Mabs were labeled with TEKES - stable europium chelate (Wallac-PerkinElmer, Finland). Fluorescence was measured with Victor 1420 Multilabel Counter (Wallac-PerkinElmer, Finland).

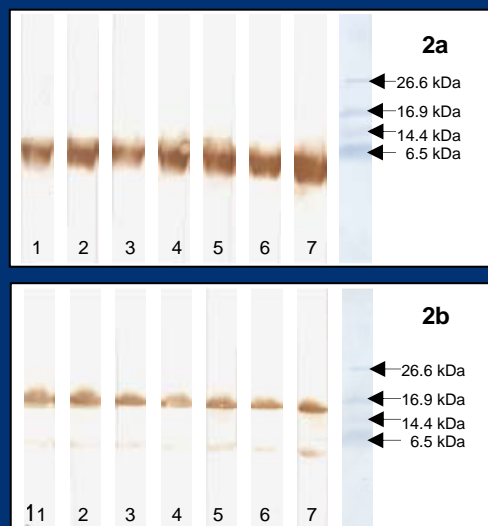


Figure 2: Immunodetection of human synthetic BNP (2a) and human recombinant proBNP (2b) in Western blotting by different monoclonal antibodies after Tricine-SDS gel electrophoresis in reducing conditions. Lane 1: Mab 24C5, lane 2: Mab 26E2, lane 3: Mab 2G9, lane 4: Mab 43B12, lane 5: Mab 50B7, lane 6: Mab 50E1, lane 7: Mab 57H3

Among all possible two-site Mab combinations, only combinations utilizing antibodies specific to the peptides 11-22 and 26-32 demonstrated ability to recognize both synthetic antigen and native antigen from human blood with high sensitivity. Four two-site Mab combinations (50E1₂₆₋₃₂-24C5₁₁₋₂₂, 50E1₂₆₋₃₂-26E2₁₁₋₂₂, 24C5₁₁₋₂₂-50B7₂₆₋₃₂ and 24C5₁₁₋₂₂-57H3₂₆₋₃₂) demonstrated the highest signal level with patient's samples and synthetic antigen and were selected for further evaluations in one-step sandwich immunoassay. Capture Mabs were biotinylated and antibody mixture (200 ng of capture Mab and 200 ng of detection Mab, 0.05 ml) was incubated with the antigen solution (0.05 ml) in the streptavidin coated microtiter plates at room temperature (+22°C) with gentle shaking for 30 minutes. Mab combination utilizing Mab 50E1 as capture (epitope 26-32) and Mab 24C5 as detection (epitope 11-22) demonstrated the best sensitivity with both synthetic and native (patient's blood samples) antigens. The detection limit of such assay was 0.4 ng/L and linear range was 0.3-20000 ng/L (Fig. 3).

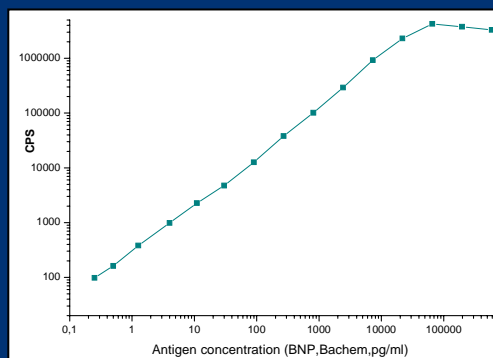


Figure 3: Calibration curve of the sandwich time-resolved immunofluorometric assay for the quantitative determination of human BNP. For capture biotinylated Mab 50E1; epitope 26-32 and for detection Eu3+-labeled Mab 24C5; epitope 11-22.

Kinetic studies revealed that this Mab combination could be used for the development of rapid (5-10 min) sandwich immunoassay for quantitative immunodetection of BNP in human blood.