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Molecular Differentiation of Ischemic and Valvular Heart Disease by Liquid Chromatography / Fourier Transform Ion Cyclotron Resonance Mass Spectrometry

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Abstract
Proteomic patterns of myocardial tissue in different etiologies of heart failure were investigated using a direct analytical approach with High Performance Liquid Chromatography (HPLC)/Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (FT-ICR MS). Right atrial appendages from 20 patients, 10 with hemodynamically significant isolated aortic valve disease and 10 with symptomatic coronary artery disease were collected during elective cardiac surgery. After preparation of tissue samples and tryptic digestion of proteins, the peptide mixture was HPLC-separated and on-line analyzed by electrospray FT-ICR MS. Data obtained from HPLC / FT-ICR MS runs were compared for classification. To extract the classification features, the selection of best individual features was applied and the "nearest mean classifier" was used for the classification of test samples and the sample projection onto classification patterns. The pattern distribution characteristics of aortic and coronary diseases were clearly different. No interference between samples of both disease categories was registered, even if the distribution of unsupervised classified test samples were closer. Samples representing aortic valve disease showed a closer accumulation pattern of spots compared to the samples representing coronary disease, which indicated a more specific protein classification. Through selective identification of specific peptides and protein patterns with FTMS, valvular and coronary heart disease is for the first time clearly distinguished at molecular level. The described methodology could also be feasible in search for specific biomarkers in plasma or serum for diagnostic purposes.

Key words: Human myocardium, Fourier transform ion cyclotron resonance mass spectrometry, proteome analysis

Introduction

Due to the anticipated growth of the elderly population, the incidence of heart disease will further increase. This trend will continue in spite of improved primary prevention and efficient therapeutic strategies. Heart failure is a syndrome with various causes including ischemic, valvular and congenital disorders, myocardiopathy, myocarditis and hypertension. Although much has been learned about re-modeling processes in heart failure throughout recent years, the basic causes of the cardiac dysfunction at molecular level still remains largely unknown. The majority of these processes are thought to result from underlying alterations in gene expression, which in turn can influence complex interactions among numerous mediators in both signaling and regulatory pathways. While coronary artery disease often leads to myocardial dysfunction with a rapid progression of myocardial ischemia, valvular disease has more chronic characteristics with long lasting adaptive changes in myocardial structure and function.

Being a primary risk factor for left ventricular systolic dysfunction, coronary artery disease initially leads to a reduced perfusion of the myocardial tissue. Influenced by a variety of immunologic cascades, the process of plaque evolution can be accelerated by different effector molecules and activation of inflammation which elicits the acute coronary syndrome leading to myocardial ischemia and subsequent infarction [1]. In contrast, concentric and/or excentric hypertrophic response in the aortic valve disease is a dynamic process in which progressive changes in gene expression first develop adaptive structural and functional changes including cellular hypertrophy and interstitial fibrosis which subsequently depress the left ventricular contractility. Degenerative alteration in cell structure like mitochondrial changes, disruption of sarcomeric units, non-oriented growth of contractile fibers, and disappearance of organelles are the final result [2].

The objective of this study was to evaluate the proteomic patterns of myocardial tissue upon different etiologies of heart failure by using a direct analytical approach with High Performance Liquid Chromatography / Fourier-Transform – Ion Cyclotron Resonance Mass Spectrometry (HPLC / FT-ICR MS) that

has been shown to be a powerful tool for detecting structural alterations of proteins in disease [3-5].

Material and Methods

Analytical Background

Tissue samples containing the protein mixture are digested with trypsin after various preparation steps [3, 6] in order to perform the so-called bottom-up proteomic analysis. The digestion results in a peptide mixture, which needs to be separated by HPLC. Compounds eluting from the HPLC are introduced through an electrospray ionization source [7-9] into the mass spectrometer. The analysis is performed by FT-ICR MS [10] in an ion cyclotron resonance cell that is located in the ultrahigh vacuum system at the center of a superconducting magnet (Fig. 1). The mass-to-charge ratio (m/z) of ions is determined by measuring their cyclotron frequency.

The general appearance of liquid chromatography / mass spectrometry (LC/MS) data displayed in a m/z vs. time diagram resembles two-dimensional (2D) gel electrophoresis separation results. Using a pattern recognition method, the applied bio-informatics tool is capable of displaying all differences between complete liquid chromatography / mass spectrometry data obtained from different pathologic cases. This allows a thorough comparison of the studied diseases. If effects of two different diseases on the proteomic pattern are investigated, such a comparison leads to classifiers, which in turn can subsequently be analyzed by a liquid chromatography / mass spectrometry run using ion fragmentation with tandem mass spectrometric (MS/MS) methods for obtaining sequence information of the proteins. A selective determination of proteins corresponding to these classifiers helps identify the differences in proteomic appearance of the disease.

Collection and Preparation of the Samples

Right atrial appendages, which are subject to discarding prior to venous cannulation for cardiopulmonary bypass were collected from 20 patients. The patient group (19 male, 1 female) consisted of 10 individuals with hemodynamically significant isolated aortic valve disease and 10 with symptomatic coronary artery disease. The median age was 62 years (45-81) in the aortic group and 66 years (37-83) in the coronary group. All patients in the coronary group were previously treated with beta-blockers and statins and antithrombotic agents were suspended 10-14 days prior to surgery. The preoperative medication in the aortic valve group was less homogenous. Demographic data including preoperative medication are shown in Table 1. Cardiac samples were immediately washed in Krebs-Henseleit solution and fixed on wax plates at room temperature. After separation from the epicardium, trabecular tissues were shock-frozen in liquid nitrogen and stored at -80 °C.

Tissue specimens were kept in Cryotubes (Nunc, Roskilde, Denmark). 4-5 mg were prepared to be homogenized in 300 µL of 8 M urea, 0.4 M NH_4HCO_3 using a Polytron PT 1200 CL mixer (Kinematica AG, Luzern, Switzerland) for 5-15 s until a homogenous solution was obtained. This was followed by the addition of 25 µL of 45 mM dithiothreitol. The mixture was incubated at 95 °C for 25 min and thereafter cooled to ambient temperature. 25 µL of 100 mM iodoacetamide were added, and the mixture was left standing for 1 h in darkness. Then, 100 µL de-ionized water and 100 µg (approx. 2.5% w/w) of trypsin were added. The digestion was performed overnight in the dark at 37 °C.

In order to remove salts and other contaminants, 400 µL of tryptic peptides was acidified with acetic acid to a final acetic acid concentration of 2.5%. The acidified mixture was desalted on a SPE Spec Plus C_{18}

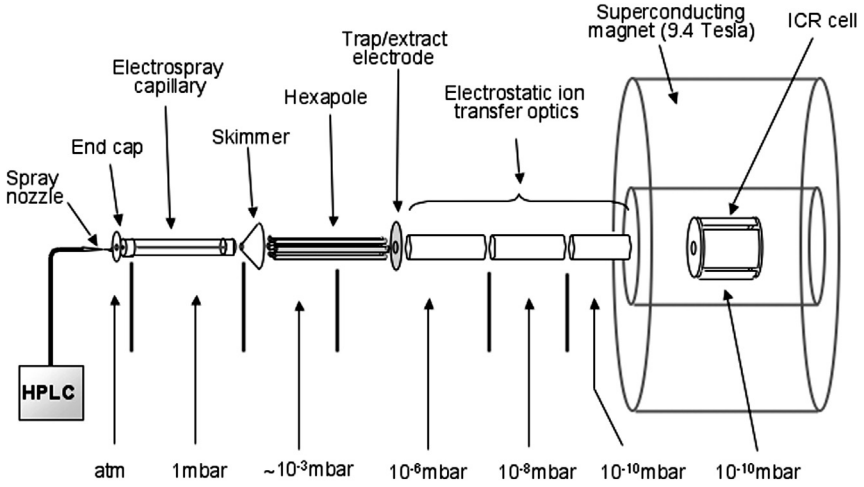

Fig. 1. Non-scaled schematic view of an HPLC / FT-ICR MS system with electrospray ion source. Ions are formed at atmospheric pressure, transferred through a glass capillary with metalized ends and through a skimmer into a hexapole ion trap. The ions are trapped there for a short time (0.6 s) before they are further transferred through the electrostatic ion optics to the ion cyclotron resonance cell. The ICR cell is in the magnetic center of a 9.4T superconducting magnet. The vacuum system, not shown in the figure, consists of pumping stages down to the range of 10^{-10} mbar in the ultra high vacuum chamber of the ICR cell. The numbers shown are approximate pressures in different pumping stages.

Table 1. Demographic data including concomitant disorders and preoperative medication.

	Aortic valve group	**Coronary group**
Number of patients	**10**	**10**
Median age [years] (range)	62 (45-81)	66 (37-83)
Body-Mass-Index [kg/m^2]	26 (21-37)	24 (21-35)
Smoking history	3	8
Hypertension	7	7
Diabetes mellitus	1	3
Dyslipidemia	3	7
Medication		
Beta-blocker	1	10
ACE inhibitors	6	5
Platelet aggregation inhibitors	3	8
Coronary vasodilators	0	4
Statins	2	10

AR, 3 mL column (Ansys Diagnostics Inc, CA). The desalting procedure was conducted in the following way: The column was first wetted in 3 x 500 µL 50% acetonitrile, and then equilibrated with 3 x 500 µL of 1% acetic acid. After equilibration, the sample was adsorbed to the media. After sample loading, the column was washed with 500 µL 1% acetic acid. Finally, the desalted tryptic peptides were eluted with 2 x 100 µL of 50% acetonitrile in 1% acetic acid. The eluate was sampled and vacuum-centrifuged to dryness in a prelubricated microfuge tube (Costar, USA), using a Speedvac ISS1100 (Thermo Savant, Holbrook, NY, USA). After dryness, the samples were stored at -80 °C until further analysis. Before reversed-phase liquid chromatography analysis, the samples were re-dissolved in 50 µL mobile phase A (see below).

Packed Capillary Liquid Chromatography FT-ICR MS for Pattern Recognition

Reversed-phase liquid chromatography was performed using a 10 cm long in-house packed C_{18} column, I.D. 200 µm. The packing material used was ODS-AQ, ∅ 5µm (YMC Europe GmbH, Schermbeck, Germany). Two HPLC-pumps (JASCO 1580, JASCO Japan) delivered mobile phase A: acetonitrile: water: acetic acid (5 : 94.5 : 0.5 volume ratio), and mobile phase B: acetonitrile : water : acetic acid (94.5 : 5 : 0.5 volume ratio). The program of choice was isocratic elution using solvent A for 10 min, followed by the gradients 100-50 % in 54 min and 50-0% A in 6 min. The flow rate through the column was measured before each experiment and adjusted to 2.2 mL/min by changing the total flow rate. A volume of 10 µL of the sample was injected onto the column using a six-port injector valve (Valco Instruments Co. Inc., Schenkon, Switzerland). The separated peptides passed a UV-detector before they were electrosprayed on-line to a Bruker Daltonics BioAPEX-94e Fourier transform ion cyclotron resonance mass spectrometer (Bruker Daltonics, Billerica MA, USA) with a 9.4 Tesla superconducting magnet. In the modified electrospray source of the instrument the samples were sprayed using a Black Dust (polyimide-graphite) sheathless electrospray emitter, with an internal diameter of 50 µm [11]. Data was collected approximately 14 minutes after the injection. In total, 256 mass spectra of 256 K data points were collected in each experiment. Each spectrum was a sum of 10 individual scans, and the total time for collecting one spectrum was 10 s.

Pattern Recognition and Extraction of Classification Features

The pattern recognition and classification strategy have been described in detail by Ramström et al. [5]. A numerical code was developed for the analysis. The first part of the code normalizes the spectrogram intensity, removes the noise and calibrates the individual HPLC / FT-ICR mass chromatograms in time to a common "table" sample. Both the normalization of the spectrogram intensity and removing the liquid chromatography noise are done by applying low-frequency filters in mass and in time. This results in a more accurate time calibration of the patterns by utilizing the positions of common abundant peptides. The second part of the code concerns the calibrated sample classification and extraction of the classification features. The selection of best individual features, followed by the "leave-one-out" verification method, was applied for the extraction of classification features (peaks). The "nearest mean classifier" was used for the classification of test samples and the sample projection onto classification patterns which finally gives just two classification parameters, the Cooman plot [12]. In case of a binary classification, the features are represented by those pattern peaks, which are more abundant in the majority of the samples of one class. These peaks are defined as the characteristic peaks. Thus, two lists of characteristic peaks were generated for

each classification. Finally, the neutral masses were restored for each characteristic peak using the isotope distribution at the corresponding scans.

RESULTS

In the classification using all representative samples, 240 characteristic peaks in the coronary artery disease group and 90 characteristic peaks in the aortic valve disease group were observed. A different distribution pattern of aortic valvular and coronary diseases was found. In Figure 2, the symbols represent both cardiac disorders accumulated on either side of the diagonal line with increased distance from the diagonal related to the specification for particular disease type. Above the diagonal, samples from myocardial tissue with coronary disease and below the diagonal samples from myocardial tissue with aortic valve disease are located. Each filled symbol represents an individual supervised classified training sample while each open symbol represents an individual unsupervised classified test sample. As illustrated in Figure 2, the algorithm led to only two potential misclassifications of the unknown test samples. When the HPLC / FT-ICR MS for these ambiguous samples were manually inspected, large gaps in the total ion chromatograms due to unstable electrospray and sudden changes in elution rates could be observed, resulting in difficulties for the algorithm to correctly calibrate and align the data sets. There were intense and stable representative peaks found in both classes. Having a clear difference in proteomic pattern between both myocardial disorders, no interference between aortic and coronary samples was registered, even if the distribution of open symbols on either sides of the diagonal (unsupervised classified test samples) displayed a closer distance to the diagonal. The aortic valve samples showed a closer accumulation pattern of the spots indicating a more specific classification of the proteins compared to the coronary samples.

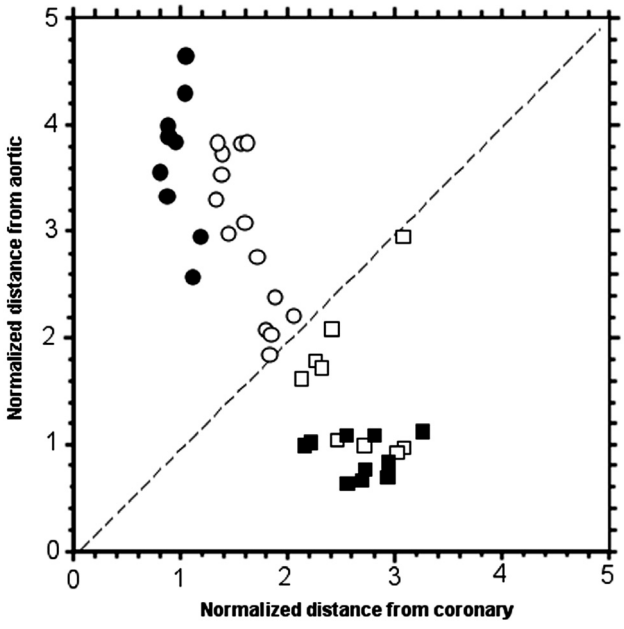

Fig. 2. Pattern for the classification of coronary (circular symbols) vs. aortic valve disease (square symbols) samples. Each point represent an individual sample where filled symbols (● and ■) represent supervised classified training samples while open symbols (O and □) represent unsupervised classified test samples. All samples with exception of two were unambiguously correctly classified.

DISCUSSION

Different attempts to identify myocardial protein patterns at molecular level have been made in recent years, including 2D gel electrophoresis of cardiac tissue and a considerable number of different proteins could be identified and classified [13, 15]. Using high-resolution polyacrylamide 2D gel electrophoresis, Pleissner and co-workers could identify quantitative and qualitative differences between atrial and ventricular protein patterns in human myocardial samples. Furthermore, a protein database for myocardial 2D gel electrophoresis with accessing possibility via world-wide-web was described by the same author [16]. However, a comparative identification of myocardial protein patterns in failing heart, originating from different etiologies of cardiac disease is still not available. Therefore, our investigation on myocardial tissue samples representing valvular versus coronary heart disease has a pioneering character. We showed that, molecular differences in both disease forms could for the first time be distinguished through selective identification of specific peptides and protein patterns by FT-ICR mass spectrometric analysis, which was not used before for this type of comparative study. The material we have applied is unique and the methodology we apply do not have the inherent disadvantages, which are present in the 2D technique including discrimination of hydrophobic proteins, membrane bound proteins, small proteins etc. Also the dynamic range in HPLC / FT-ICR MS is normally better than in ordinary 2D gels. In addition, our methodology is also considerably less time consuming (a few hours compared to several days). Until now FT-ICR MS investigations of digested and HPLC-separated cardiac proteins were limited to studies of individual proteins either for establishing analytical methods or for studying structural properties of a particular cardiac protein [17].

This method has a considerable potential to be used for early verification of heart disease of various etiologies since protein-based transformations responsible for disease may arise far before clinical signs can be identified by today's routine diagnostic techniques. Identified peptides and proteins could act as potential biomarkers but could also be used for verification (by immuno-histochemistry) and validation of the findings. Identities would also allow an elective search for specific biomarkers peripherally in plasma and/or serum and thereby facilitate the development of a more high-throughput immunoassay based diagnostic tool, e.g. ELISA [18] or protein chip assays. A more thorough investigation of representative peptides ("classifiers") as markers of larger proteins that seem to be involved with the pathogenesis of these diseases is currently in progress.

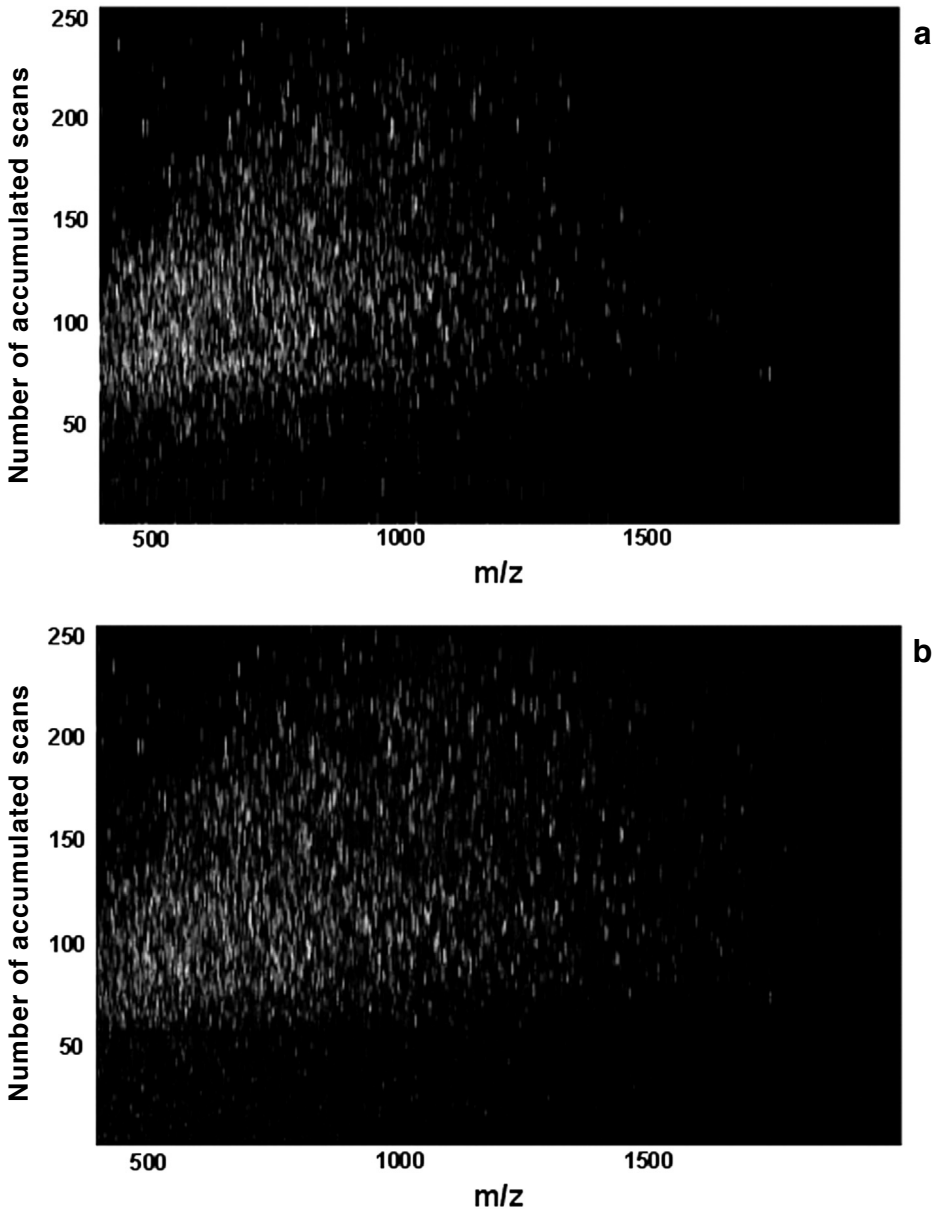

Fig. 3. Examples of data (m/z vs. number of accumulated scans, 10 seconds each) obtained from HPLC/FT-ICR mass spectrometry of aortic (a) and coronary (b) samples. The light spots in the diagrams correspond to individual mass spectral peaks. In the original diagrams the peak intensities are color coded for easy recognition.

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REFERENCES

1. Hansson GK (2005) Inflammation, atherosclerosis, and coronary artery disease. N Engl J Med 352: 1685-1695.
2. Maron BJ, Ferrans VJ, Roberts WC (1975) Myocardial ultrastructure in patients with chronic aortic valve disease. Am J Cardiol 35: 725-739.
3. Bergquist J, Palmblad M, Wetterhall M, Håkansson P, Markides K (2002) Peptide mapping of proteins in human body fluids using electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry. Mass Spectrom Rev 21: 2-15
4. Bergquist J (2003) FT ICR mass spectrometry in proteomics. Curr Opin Mol Ther 5: 310-314
5. Ramström M, Ivonin I, Johansson A, Askmark H, Markides KE, Zubarev R, Håkansson P, Aquilonius S-M, Bergquist J (2004) Cerebrospinal fluid protein patterns in neurodegenerative disease revealed by liquid chromatography-Fourier transform ion cyclotron resonance mass spectrometry. Proteomics 4: 4010–4018
6. Kelleher NL, Lin HY, Valaskovic GA, Aaserud DJ, Fridriksson EK, McLafferty, FW (1999) Top down versus bottom up protein characterization by tandem high-resolution spectrometry. J Am Chem Soc 121: 806-812
7. Yamashita M, Fenn JB (1984) Electrospray ion source. Another variation on the free-jet theme. J Phys Chem 88: 4451-4459
8. Kebarle P, Tang L (1993) From ions in solution to ions in the gas phase. The mechanism of electrospray mass spectrometry. Anal Chem 65: 972A-986A
9. Cole RB, editor, (1997) Electrospray ionization mass spectrometry. John Wiley & Sons, New York
10. Marshall AG, Hendrickson CL, Jackson GS (1998) Fourier transform ion cyclotron resonance mass spectrometry: A primer. Mass Spectrom Rev 17: 1-35

11. Nilsson S, Wetterhall M, Bergquist J, Nyholm L, Markides KE (2001) A simple and robust conductive graphite coating for sheathless electrospray emitters used in capillary electrophoresis/mass spectrometry. Rapid Commun Mass Spectrom 15: 1997–2000
12. Ozen BF, Weiss I, Maurer LJ (2003) Dietary supplement oil classification and detection of adulteration using Fourier transform infrared spectroscopy. J Agric Food Chem 51: 5871-5876
13. Jungblut P, Otto A, Zeindl-Eberhart E, Pleissner K-P, Knecht M, Regitz-Zagrosek V, Fleck E, Wittmann-Liebold B (1994) Protein Composition of the human heart: The construction of a myocardial two dimensional electrophoresis database. Electrophoresis 15: 685-707.
14. Corbett JM, Wheeler CH, Baker CS, Yacoub MH, Dunn MJ (1994) The human myocardial two-dimensional gel protein database. Update 1994. Electrophoresis 15: 1459-1465
15. Pleissner K-P, Regitz-Zagrosek V, Weise C, Neuß M, Krüdewagen B, Söding P, Buchner K, Hucho F, Hildebrandt A, Fleck E (1995) Chamber specific expression of human myocardial proteins detected by two-dimensional gel electrophoresis. Electrophoresis 16: 841-850.
16. Pleissner K-P, Sander S, Oswald H, regitz-Zagrosek V, Fleck E (1996) The construction of the world wide web-accessible myocardial two dimensional gel electrophoresis protein database "HEART -2DPAGE": A practical approach. Electrophoresis 17: 1386-1392
17. Wang F, Li W, Emmett MR, Marshall AG, Corson D, Sykes BD (1999) Fourier transform ion cyclotron resonance mass spectrometric detection of small Ca2+-induced conformational changes in the regulatory domain of human cardiac troponin C. J Am Soc Mass Spectrom 10: 703-710
18. Engvall E, Perlmann P (1972) Enzyme-linked immunosorbent assay, ELISA. 3. Quantitation of specific antibodies by enzyme-labeled anti-immunoglobulin in antigen-coated tubes. J Immunol 109:129-35.

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