Video Article

Optimized Protocol for the Extraction of Proteins from the Human Mitral Valve

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URL: https://www.jove.com/video/55762
DOI: doi:10.3791/55762

Keywords: Biochemistry, Issue 124, human normal mitral valve, protein extraction, tissue homogenization, protein analysis, proteomics, biochemistry

Date Published: 6/14/2017

Citation: Banfi, C., Guarino, A., Brioschi, M., Ghilardi, S., Mastrullo, V., Tremoli, E., Polvani, G. Optimized Protocol for the Extraction of Proteins from the Human Mitral Valve. *J. Vis. Exp.* (124), e55762, doi:10.3791/55762 (2017).

Abstract

Analysis of the cellular proteome can help to elucidate the molecular mechanisms underlying diseases due to the development of technologies that permit the large-scale identification and quantification of the proteins present in complex biological systems.The knowledge gained from a proteomic approach can potentially lead to a better understanding of the pathogenic mechanisms underlying diseases, allowing for the identification of novel diagnostic and prognostic disease markers, and, hopefully, of therapeutic targets. However, the cardiac mitral valve represents a very challenging sample for proteomic analysis because of the low cellularity in proteoglycan and collagen-enriched extracellular matrix. This makes it challenging to extract proteins for a global proteomic analysis. This work describes a protocol that is compatible with subsequent protein analysis, such as quantitative proteomics and immunoblotting. This can allow for the correlation of data concerning protein expression with data on quantitative mRNA expression and non-quantitative immunohistochemical analysis. Indeed, these approaches, when performed together, will lead to a more comprehensive understanding of the molecular mechanisms underlying diseases, from mRNA to post-translational protein modification. Thus, this method can be relevant to researchers interested in the study of cardiac valve physiopathology.

Video Link

The video component of this article can be found at https://www.jove.com/video/55762/

Introduction

Recent evidence has altered the understanding of the roles of the many regulatory mechanisms that occur after mRNA synthesis. Indeed, translational, post-transcriptional, and proteolytic processes can regulate protein abundance and function. The dogma – which says that mRNA concentrations are proxies to those of the corresponding proteins, assuming that transcript levels are the main determinant of protein abundance – has been partially revised.Indeed, transcript levels only partially predict protein abundance, suggesting that post-transcriptional events occur to regulate the proteins within cells[1,2].

Furthermore, proteins ultimately dictate the function of the cell and therefore dictate its phenotype, which can undergo dynamic changes in response to autocrine, paracrine, and endocrine factors; blood-borne mediators; temperature; drug treatment; and disease development. Thus, an expression analysis focused on the protein level is useful to characterize the proteome and to unravel the critical changes that occur to it as part of disease pathogenesis[3].

Therefore, the opportunities that proteomics present to clarify health and disease conditions are formidable, despite the existing technological challenges. The particularly promising areas of research to which proteomics can contributeinclude: the identification of altered protein expression at any level (*i.e.,* whole cells or tissue, subcellular compartments, and biological fluids); the identification, verification, and validation of novel biomarkers useful for the diagnosis and prognosis of disease; and, hopefully, the identification of new protein targets that can be used for therapeutic purposes, as well as for the assessment of drug efficacy and toxicity[4].

Capturing the complexity of the proteome represents a technological challenge. The current proteomic tools offer the opportunity to perform large-scale, high-throughput analysis for the identification, quantification, and validation of altered protein levels. In addition, the introduction of fractionation and enrichment techniques, aimed at avoiding the interference caused by the most abundant proteins, has also improved protein identification by including the least abundant proteins. Finally, proteomics has been complemented by the analysis of post-translational modifications, which progressively emerge as important modulators of protein function.

However, the sample preparation and protein recovery in the biological specimens under analysis still remain the limiting steps in the proteomic workflow and increase the potential for possible pitfalls[5]. Indeed,in most of the molecular biology techniques that must be optimized, the first steps are tissue homogenization and cell lysis, especially during the analysis of low-abundance proteins for which amplification methods do not exist. In addition, the chemical nature of proteins can influence their own recovery. For example, the analysis of highly hydrophobic proteins is

very challenging, because they easily precipitate during isoelectric focusing, while trans-membrane proteins are almost insoluble (reviewed in Reference 5). Furthermore, the tissue composition variability creates a significant barrier to developing a universal extraction method. Finally, because almost all of the clinical specimens are of limited quantity, it is essential to enable protein preparation with maximal recovery and reproducibility from minimal sample amounts[6].

This work describes an optimized protocol for protein extraction from the normal human cardiac mitral valve, which represents a very challenging sample for proteomic analysis. The normal mitral valve is a complex structure lying between the left atrium and the left ventricle of the heart (**Figure 1**). It plays an important role in the control of blood flow from the atrium to the ventricle, preventing backflow and ensuring the proper level of oxygen supply to the whole body, thus maintaining an adequate cardiac output. However, it is often considered to be an "inactive" tissue, with a low cellularity and few components, mainly in the extracellular matrix. This is because, in normal conditions, the resident valvular interstitial cells (VICs) present a quiescent phenotype with a low protein biosynthesis rate[7].

However, it has been demonstrated that, in a pathological state, the number of VICs in the spongiosa increases and their protein synthesis is activated, together with other functional and phenotypical changes[8]. Therefore, it is not surprising that the minimal data available in the literature focus on the analysis of pathological mitral valves[9,10], in which the increased number of activated VICs might explain the relatively high number of identified proteins.

In conclusion, the present protocol may serve to develop the understanding of the pathogenic mechanisms responsible for mitral valve diseases through the study of mitral valve protein components. Indeed, a greater understanding of the underlying pathological processes could help to improve the clinical management of valve diseases, whose current indications for intervention are largely predicated on hemodynamic considerations.

Protocol

In this protocol, the human hearts are collected during multiorgan explantation (cold ischemia time of 4-12 h, mean 6 ± 2 h) from multi-organ donors excluded from organ transplantation for technical or functional reasons, despite normal echocardiographic parameters. They are sent to the Cardiovascular Tissue Bank of Milan, Monzino Cardiologic Center (Milan, Italy) for the banking of the aortic and pulmonary valves. The mitral posterior leaflets are not used for clinical purposes, so they are collected during the aortic and pulmonary valve isolation after informed consent is obtained from the donors' relatives. The tissue for transplantation and research is collected only after parental consent; on the consent sheet, they authorize (or not) the use of the cardiac tissue for research only if it is not suitable for human clinical use (*i.e.,* microbiological, functional, and serological problems), following the guidelines of the ethics committee of Monzino Cardiologic Center.

1. Mitral valve preparation

1. Harvest the human mitral valve as soon as possible after organ explantation (cold ischemia time of 4-12 h).
2. In a clean room, remove the heart from the transport bag containing a cold (4 °C) solution (*i.e.,* saline solution or balanced medium Eurocollins or Wisconsin). Put it into a bucket and place it in a biosafety cabinet (biohazard vertical air flow, class A, Good Manufacturing Practices (GMP) classification) to proceed with the valve preparation.
3. Place the heart on a sterile disposable drape in the cabinet. Using a sterile disposable scalpel, cut the heart completely, perpendicularly to its major axis, on the level of the left and right ventricles, about 4 cm away from the apex.
4. Move the ascending aorta and pulmonary artery to display the left atrial roof.
5. With sterile autoclavable forceps and picks, cut around the left auricle on the left atrial roof, making the mitral valve visible and allowing for the great mitral leaflet (anterior) and the small mitral leaflet (posterior) to be identified.
   NOTE: Antero-lateral and the posterior medial commissures define the border of the anterior leaflet and the posterior area.
6. Using sterile autoclavable scissors and non-traumatic forceps, dissect the left atrium and the ventricle wall thickness around the circumference of the whole mitral valve.
7. Identify the mitro-aortic valve continuity.
   NOTE: The left ventricle contains the whole mitral valve and chords.
8. Separate the anterior mitral valve leaflet from the posterior mitral valve leaflet, cutting the posterior leaflet along the insertion with the ventricle (commissure).
9. **Wash the posterior leaflet in the saline solution. Cut the leaflet into small pieces (<1 cm^2) and individually wrap them in aluminum foil. Snap-freeze them with liquid nitrogen.**
   Caution: Follow organizational safety procedures when using liquid nitrogen.
   1. Sanitize the table of the cabinet with a 70% isopropyl alcohol solution and a 6% hydrogen peroxide solution at the end of the procedure.

2. Protein extraction

1. Use forceps to pick up the sample stored in liquid nitrogen and immediately place it on dry ice while still wrapped in the aluminum foil. Do not leave the sample to thaw during any transfers.
2. Prior to grinding, chill the porcelain/zirconium mortar and pestles of a grinder system (*e.g.,* CryoGrinder), together with the sample, by putting them in a Dewar flask containing liquid nitrogen (~500 mL).
   Caution: Follow organizational safety procedures when using liquid nitrogen.
3. Put the mortar and pestles in a polystyrene box containing dry ice. Remove the sample from the aluminum foil and put it into the mortar.
4. **Grind the sample with the big pestle against the mortar 15-20 times, using the screwdriver to rotate the pestle. Mix the sample with the tip of a pre-chilled spatula during the grinding process.**

1. Repeat with the small pestle.

5. **Transfer the ground sample to a previously weighed tube (*e.g.,* 15 mL centrifuge tube) by inverting the tube, placing it over the mortar, and inverting them together to move the sample to the tube. Use a pre-chilled spatula to recover all material from the mortar.**
   1. Keep the tube with the sample on dry ice to avoid sample thawing during the transfer.
6. Calculate the net weight of the sample.
7. Clean the mortar and the pestles after each sample and decontaminate them by autoclaving or heating them at 200 °C for 2 h.
8. Transfer the powdered sample from the centrifuge tube to the glass tube of a homogenizer by inversion.
9. Add filtered urea buffer (8 M urea, 2 M thiourea, 4% w/v CHAPS, 20 mM Tris, and 55 mM dithiotreitol) to the glass tube, 200 µL of urea buffer for every 10 mg of powdered tissue.
   NOTE: Residual powdered sample left in the centrifuge tube can be recovered using part of the calculated volume of urea buffer.
10. Homogenize the sample using a stirrer equipped with a borosilicate glass mortar and a polytetrafluoroethylene (PTFE) pestle. Slowly press the pestle onto the sample with a twisting motion (1,500 rpm) 10 times.
11. Recover the supernatant and transfer it to a clean 1.7 mL centrifuge tube. Again extract the remaining sample with fresh urea buffer, adding half of the volume used during the first extraction.
12. Repeat step 2.10.
13. Recover the supernatant and combine it with the supernatant from step 2.11. Place the combined supernatant on a tube rotator for 30 min.
14. Centrifuge the tube for 30 min at 13,000 x g and 4 °C.
15. Recover the supernatant and measure the protein concentration using the Bradford protein assay, per the manufacturer's instructions. Store the sample at -80 °C until use.

Representative Results

The extraction and dissolution of proteins in the urea buffer is directly compatible with proteomic methods based on isoelectrofocusing (two-dimensional electrophoresis (2-DE)[11] and liquid-phase isoelectric focusing (IEF)[12]) and with immunoblotting after dilution in Laemmli buffer[13] containing a protease inhibitor cocktail[14].

For gel-free mass spectrometry-based methods (*i.e.,* liquid chromatography coupled to data-independent mass spectrometry analysis (LC/MS^E) and two-dimensional LC/MS^E (2D-LC/MS^E))[15], the samples extracted in the described urea buffer need to be further treated to eliminate the urea and thiourea, which could interfere with subsequent protein digestion and liquid chromatography separation. This desalting step can be accomplished using the commercial protein precipitation kits, following the manufacturer's instructions to precipitate the proteins. The sample can be then dissolved in 25 mM NH_4HCO_3 containing 0.1% cleavable detergents for protein digestion[16]. The precipitation of the proteins can eliminate buffer components, minimally affecting the protein content (protein recovery: >85%), thus rendering the sample suitable for every kind of analysis.

The application of this protocol for protein extraction from human mitral valves allowed for the identification of a total of 422 proteins, combining four different proteomics approaches previously described in detail[11,15]. Specifically, 169 proteins were identified by 2-DE, 330 proteins by liquid-phase IEF, 96 proteins by LC/MS^E, and 148 proteins by 2D-LC/MS^E (Table 1).

To classify the 422 identified proteins in terms of subcellular localization, a software was used for the gene ontology (GO) analysis (*e.g.,* Cytoscape). The network created with the software and its corresponding plug-in showed that, besides the expected proteins localized in the extracellular region (see the upper-right portion of **Figure 2**), most of the proteins identified by the proteomic approaches were from the intracellular region (*i.e.,* cytoplasm, organelles, vesicles, and cytoskeleton). Cell-surface proteins were also identified (**Figure 2**).

Results were further confirmed in three independent mitral valve samples. Immunoblotting was used to analyze a group of four proteins (*i.e.,* septin-11, four and a half LIM domains protein 1 (FHL-1), dermatopontin, and alpha-crystallin B (CryAB)) that have never been identified in the normal mitral valve (**Figure 3**).

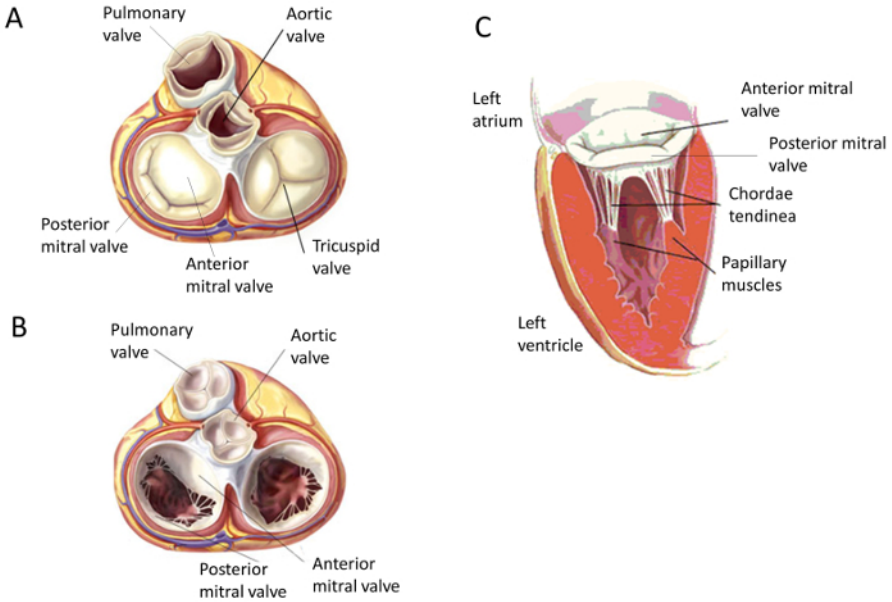

Figure 1: Mitral valve structure. Top view of the human heart showing the closed (**A**) or open (**B**) human mitral valve. Front view of the left ventricle of a human heart (**C**). Please click here to view a larger version of this figure.

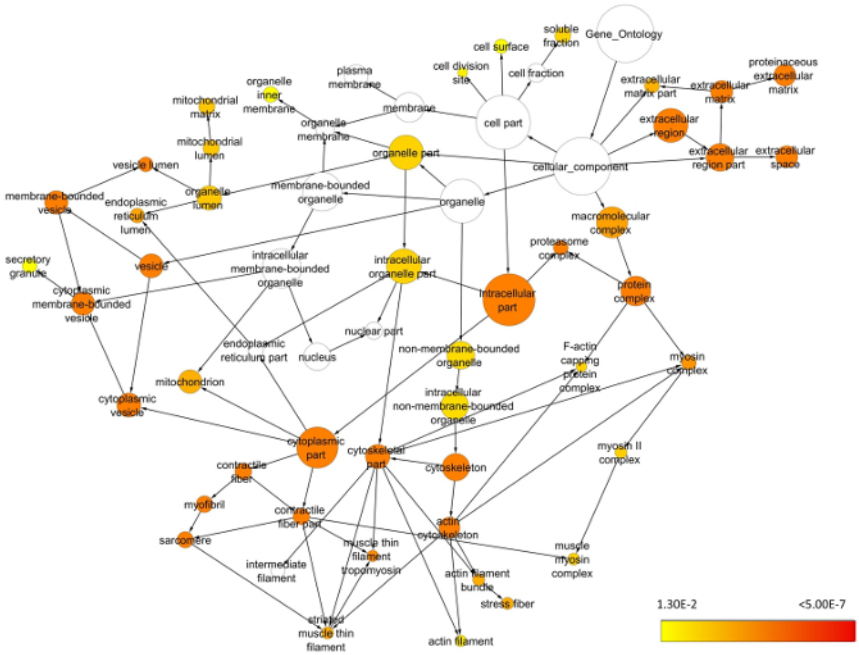

Figure 2: Analysis of the identified mitral valve proteins in term of cellular distribution. Cytoscape and the plugin BiNGO were used to obtain the distribution of gene ontology (GO) terms from the cellular component categories. The circle size is proportional to the number of protein components associated with the selected GO terms, and the color scale for the p-value of over-representation is reported. Please click here to view a larger version of this figure.

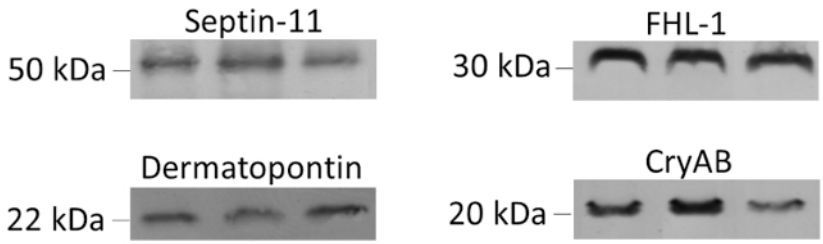

Figure 3: Immunoblotting analysis of septin-11, FHL-1, dermatopontin, and CryAB in whole extract from three human normal mitral valve leaflets. Immunoblotting was performed using mouse monoclonal antibody against CryAB and rabbit polyclonal antibodies against the septin-11, FHL-1, and dermatopontin antibodies. Please click here to view a larger version of this figure.

Accession	Description	2-DE	2D-LC	LC-MS^{E}	liquid phase IEF
A6NMZ7	Collagen alpha VI		x		
O00151	PDZ and LIM domain protein 1				x
O00299	Chloride intracellular channel protein 1				x
O00764	Pyridoxal kinase	x			
O14558	Heat shock protein beta 6	x			
O43399	Tumor protein D54				x
O43488	Aflatoxin B1 aldehyde reductase member 2				x
O43707	Alpha actinin 4		x	x	
O43866	CD5 antigen like precursor				x
O60493	Sorting nexin 3				x
O60701	UDP glucose 6 dehydrogenase				x
O75223	Uncharacterized protein				x
O75368	SH3 domain binding glutamic acid rich like protein	x			
O75390	Citrate synthase mitochondrial precursor				x
O75489	NADH dehydrogenase ubiquinone iron sulfur protein 3 mitochondrial precursor	x			x
O75608	Acyl protein thioesterase 1				x
O75828	Carbonyl reductase NADPH 3				x
O75874	Isocitrate dehydrogenase NADP cytoplasmic				x
O75955	Flotillin				x
O94760	NG NG dimethylarginine dimethylaminohydrolase 1				x
O94788	Retinal dehydrogenase 2	x			
O95865	NG NG dimethylarginine dimethylaminohydrolase 2	x			x
P00325	Alcohol dehydrogenase 1B		x		x
P00338	L lactate dehydrogenase A chain				x
P00352	Retinal dehydrogenase 1				x

P00441	Superoxide dismutase Cu Zn	x		x	
P00450	Ceruloplasmin precursor	x			x
P00488	Coagulation factor XIII A chain precursor				x
P00491	Purine nucleoside phosphorylase				x
P00492	Hypoxanthine guanine phosphoribosyltransferase				x
P00558	Phosphoglycerate kinase 1	x	x		x
P00568	Adenylate kinase isoenzyme 1				x
P00734	Prothrombin		x		x
P00738	Haptoglobin	x	x	x	x
P00739	Haptoglobin related protein precursor	x			
P00751	Complement factor B	x	x		x
P00915	Carbonic anhydrase 1				x
P00918	Carbonic anhydrase 2				x
P01008	Antithrombin III precursor	x		x	x
P01009	Alpha 1 antitrypsin	x	x	x	x
P01011	Alpha 1 antichymotrypsin	x	x	x	x
P01019	Angiotensinogen precursor	x			x
P01023	Alpha 2 macroglobulin		x		x
P01024	Complement C3	x	x	x	x
P01033	Metalloproteinase inhibitor 1 precursor				x
P01042	Kininogen 1 precursor				x
P01593	Ig kappa chain V I region AG				x
P01598	Ig kappa chain V I region EU	x			
P01600	Ig kappa chain V I region Hau	x			x
P01611	Ig kappa chain V I region Wes				x
P01620	Ig kappa chain V III region SIE				x
P01625	Ig kappa chain V IV region Len		x		
P01766	Ig heavy chain V III region BRO		x		x
P01781	Ig heavy chain V III region GAL				x
P01834	Ig kappa chain C region	x	x	x	x
P01842	Ig lambda chain C regions	x		x	x

P01857	Ig gamma 1 chain C region	x	x	x	x
P01859	Ig gamma 2 chain C region	x	x	x	x
P01860	Ig gamma 3 chain C region		x	x	x
P01861	Ig gamma 4 chain C region		x	x	x
P01871	Ig mu chain C region	x	x		x
P01876	Ig alpha 1 chain C region	x	x	x	x
P01877	Ig alpha 2 chain C region				x
P02144	Myoglobin			x	x
P02452	Collagen alpha 1 I chain	x	x	x	x
P02511	Alpha crystallin B chain				x
P02545	Lamin A C 70 kDa lamin	x	x	x	x
P02647	Apolipoprotein A I	x	x	x	x
P02649	Apolipoprotein E	x	x	x	x
P02671	Fibrinogen alpha chain	x	x	x	x
P02675	Fibrinogen beta chain	x	x	x	x
P02679	Fibrinogen gamma chain	x	x	x	x
P02689	Myelin P2 protein	x			
P02735	Serum amyloid A protein precursor	x			
P02741	C reactive protein precursor				x
P02743	Serum amyloid P component	x	x	x	x
P02746	Complement C1q subcomponent subunit B		x		x
P02747	Complement C1q subcomponent subunit C precursor				x
P02748	Complement component C9		x	x	x
P02749	Beta 2 glycoprotein 1	x	x	x	x
P02750	Leucine rich alpha 2 glycoprotein precursor				x
P02751	Fibronectin		x		x
P02760	AMBP protein precursor	x	x	x	x
P02763	Alpha 1 acid glycoprotein 1		x	x	x
P02765	Alpha 2 HS glycoprotein precursor				x
P02766	Transthyretin precursor	x		x	x

P02768	Serum albumin	x	x	x	x
P02774	Vitamin D binding protein precursor				x
P02787	Serotransferrin	x	x	x	x
P02788	Lactotransferrin precursor	x			
P02790	Hemopexin	x	x	x	x
P02792	Ferritin light chain	x			
P04004	Vitronectin	x	x	x	x
P04075	Fructose bisphosphate aldolase A				x
P04083	Annexin A1	x	x	x	x
P04179	Superoxide dismutase Mn mitochondrial precursor				x
P04196	Histidine rich glycoprotein precursor			x	
P04217	Alpha 1B glycoprotein precursor	x			x
P04350	Tubulin beta 4 chain				x
P04406	Glyceraldehyde 3 phosphate dehydrogenase	x	x	x	x
P04792	Heat shock protein beta 1	x	x	x	x
P05091	Aldehyde dehydrogenase mitochondrial precursor	x			x
P05155	Plasma protease C1 inhibitor precursor				x
P05156	Complement factor I precursor				x
P05413	Fatty acid binding protein heart	x			
P05452	Tetranectin precursor TN	x			x
P05787	Keratin type II cytoskeletal 8		x		
P06396	Gelsolin	x	x	x	x
P06576	ATP synthase subunit beta mitochondrial precursor	x			x
P06732	Creatine kinase M type	x			x
P06733	Alpha enolase	x	x	x	x
P06753	Tropomyosin alpha 3 chain	x			x
P07108	Acyl CoA binding protein	x			
P07195	L lactate dehydrogenase B chain	x	x		x
P07196	Neurofilament light polypeptide		x		

P07197	Neurofilament medium polypeptide		x	x	
P07237	Protein disulfide isomerase precursor	x			x
P07339	Cathepsin D precursor	x			x
P07355	Annexin A2	x	x	x	x
P07360	Complement component C8 gamma chain precursor				x
P07437	Tubulin beta chain	x	x	x	x
P07585	Decorin	x	x	x	x
P07737	Profilin 1		x		
P07858	Cathepsin B precursor				x
P07900	Heat shock protein HSP 90 alpha		x		x
P07951	Tropomyosin beta chain	x			
P07954	Fumarate hydratase mitochondrial precursor				x
P07996	Thrombospondin 1	x	x		x
P08107	Heat shock 70 kDa protein 1A 1B	x	x	x	x
P08123	Collagen alpha 2 I chain	x	x	x	x
P08133	Annexin A6	x	x		
P08238	Heat shock protein HSP 90 beta		x		x
P08253	72 kDa type IV collagenase precursor				x
P08294	Extracellular superoxide dismutase Cu Zn precursor	x	x		x
P08590	Myosin light polypeptide 3	x			x
P08603	Complement factor H	x	x	x	x
P08670	Vimentin	x	x	x	x
P08729	Keratin type II cytoskeletal 7		x		
P08758	Annexin A5	x	x	x	x
P09211	Glutathione S transferase P	x	x		x
P09382	Galectin 1	x	x	x	
P09417	Dihydropteridine reductase				x
P09493	Tropomyosin 1 alpha chain	x			x
P09525	Annexin A4	x			x
P09651	Heterogeneous nuclear ribonucleoprotein A1				x

P09871	Complement C1s subcomponent precursor				x
P09936	Ubiquitin carboxyl terminal hydrolase isozyme L1				x
P09972	Fructose bisphosphate aldolase C				x
P0C0L4	Complement C4 A precursor				x
P0CG05	Ig lambda 2 chain C regions		x		
P0CG38	POTE ankyrin domain family member I		x		
P10515	Dihydrolipoyllysine residue acetyltransferase component of pyruvate dehydrogenase complex				x
P10768	S formylglutathione hydrolase				x
P10809	60 kDa heat shock protein mitochondrial precursor				x
P10909	Clusterin	x	x	x	x
P10915	Hyaluronan and proteoglycan link protein 1 precursor	x			x
P11021	78 kDa glucose regulated protein	x	x		x
P11047	Laminin subunit gamma 1 precursor				x
P11142	Heat shock cognate 71 kDa protein	x	x	x	x
P11177	Pyruvate dehydrogenase E1 component subunit beta mitochondrial precursor				x
P11217	Glycogen phosphorylase muscle form	x			
P11310	Medium chain specific acyl CoA dehydrogenase mitochondrial precursor				x
P11413	Glucose 6 phosphate 1 dehydrogenase	x			
P11766	Alcohol dehydrogenase class 3 chi chain				x
P12036	Neurofilament heavy polypeptide		x		
P12109	Collagen alpha 1 VI chain	x	x	x	x

P12110	Collagen alpha 2 VI chain	x	x	x	x
P12111	Collagen alpha 3 VI chain	x	x		x
P12277	Creatine kinase B type				x
P12429	Annexin A3	x			x
P12814	Alpha actinin 1			x	x
P12829	Myosin light polypeptide 4				x
P12882	Myosin 1				x
P12883	Myosin 7		x		x
P12955	Xaa Pro dipeptidase	x			
P13489	Ribonuclease inhibitor				x
P13533	Myosin 6		x		x
P13611	Versican core protein precursor		x		x
P13639	Elongation factor 2				x
P13716	Delta aminolevulinic acid dehydratase				x
P13796	Plastin 2				x
P13804	Electron transfer flavoprotein subunit alpha mitochondrial precursor				x
P13929	Beta enolase			x	x
P14136	Glial fibrillary acidic protein astrocyte			x	
P14314	Glucosidase 2 subunit beta precursor				x
P14550	Alcohol dehydrogenase NADP				x
P14618	Pyruvate kinase isozymes M1 M2		x	x	x
P14625	Endoplasmin precursor	x			x
P15121	Aldose reductase				x
P15259	Phosphoglycerate mutase 2				x
P16152	Carbonyl reductase NADPH 1				x
P17066	Heat shock 70 kDa protein 6	x	x	x	
P17174	Aspartate aminotransferase cytoplasmic				x
P17540	Creatine kinase sarcomeric mitochondrial precursor				x
P17661	Desmin	x	x	x	x
P17980	26S protease regulatory subunit 6A				x

P17987	T complex protein 1 subunit alpha	x			
P18206	Vinculin	x			x
P18428	Lipopolysaccharide binding protein precursor				x
P18669	Phosphoglycerate mutase 1		x		
P19105	Myosin regulatory light chain 2	x			x
P19623	Spermidine synthase				x
P19652	Alpha 1 acid glycoprotein 2 precursor			x	x
P19823	Inter alpha trypsin inhibitor heavy chain H2		x		
P19827	Inter alpha trypsin inhibitor heavy chain H1		x	x	
P20073	Annexin A7	x			
P20618	Proteasome subunit beta type 1 precursor				x
P20774	Mimecan	x	x	x	x
P21266	Glutathione S transferase Mu 3				x
P21333	Filamin A		x		x
P21796	Voltage dependent anion selective channel protein 1				x
P21810	Biglycan	x	x	x	x
P21980	Protein glutamine gamma glutamyltransferase 2		x		x
P22105	Tenascin X		x		x
P22314	Ubiquitin activating enzyme E1				x
P22352	Glutathione peroxidase 3 precursor	x			x
P22626	Heterogeneous nuclear ribonucleoproteins A2 B1				x
P22695	Ubiquinol cytochrome c reductase complex core protein 2 mitochondrial precursor				x
P23141	Liver carboxylesterase 1 precursor	x			
P23142	Fibulin 1	x	x	x	x
P23284	Peptidyl prolyl cis trans isomerase B precursor				x
P23381	Tryptophanyl tRNA synthetase cytoplasmic	x			

P23526	Adenosylhomocysteinase	x			x
P23528	Cofilin 1				x
P24752	Acetyl CoA acetyltransferase mitochondrial precursor				x
P25311	Zinc alpha 2 glycoprotein precursor				x
P25705	ATP synthase subunit alpha mitochondrial		x		
P25788	Proteasome subunit alpha type 3	x			x
P25789	Proteasome subunit alpha type 4				x
P26447	Protein S100 A4	x			
P27348	14 3 3 protein theta	x			x
P27797	Calreticulin precursor				x
P28066	Proteasome subunit alpha type 5				x
P28070	Proteasome subunit beta type 4 precursor	x			x
P28072	Proteasome subunit beta type 6 precursor				x
P28074	Proteasome subunit beta type 5 precursor				x
P28331	NADH ubiquinone oxidoreductase 75 kDa subunit mitochondrial precursor				x
P28838	Cytosol aminopeptidase				x
P29218	Inositol monophosphatase				x
P29401	Transketolase				x
P29692	Elongation factor 1 delta				x
P29966	Myristoylated alanine rich C kinase substrate				x
P30040	Endoplasmic reticulum protein ERp29 precursor				x
P30041	Peroxiredoxin 6	x			x
P30043	Flavin reductase				x
P30044	Peroxiredoxin 5 mitochondrial precursor				x
P30085	UMP CMP kinase				x
P30086	Phosphatidylethanolamine binding protein 1				x
P30101	Protein disulfide isomerase A3 precursor	x	x		x
P30613	Pyruvate kinase isozymes R L		x		

P30740	Leukocyte elastase inhibitor				x
P31025	Lipocalin 1 precursor	x			
P31937	3 hydroxyisobutyrate dehydrogenase mitochondrial precursor				x
P31942	Heterogeneous nuclear ribonucleoprotein H3				x
P31943	Heterogeneous nuclear ribonucleoprotein H	x			
P31946	14 3 3 protein beta alpha	x	x		
P31948	Stress induced phosphoprotein 1				x
P31949	Protein S100 A11	x			
P32119	Peroxiredoxin 2	x			x
P34931	Heat shock 70 kDa protein 1 like		x	x	
P34932	Heat shock 70 kDa protein 4				x
P35232	Prohibitin				x
P35237	Serpin B6				x
P35443	Thrombospondin 4		x		
P35555	Fibrillin 1		x		x
P35579	Myosin 9		x	x	x
P35580	Myosin 10			x	x
P35609	Alpha actinin 2				x
P35625	Metalloproteinase inhibitor 3		x	x	
P35998	26S protease regulatory subunit 7				x
P36871	Phosphoglucomutase 1	x			x
P36955	Pigment epithelium derived factor		x		x
P37802	Transgelin 2		x		x
P37837	Transaldolase				x
P38117	Electron transfer flavoprotein subunit beta				x
P38646	Stress 70 protein mitochondrial precursor	x			x
P39687	Acidic leucine rich nuclear phosphoprotein 32 family member A				x
P40121	Macrophage capping protein				x
P40925	Malate dehydrogenase cytoplasmic				x

P40926	Malate dehydrogenase mitochondrial precursor				x
P41219	Peripherin		x		
P42330	Aldo keto reductase family 1 member C3				x
P45880	Voltage dependent anion selective channel protein 2				x
P47755	F actin capping protein subunit alpha 2	x			x
P47756	F actin capping protein subunit beta	x			x
P47985	Ubiquinol cytochrome c reductase iron sulfur subunit mitochondrial precursor				x
P48047	ATP synthase O subunit mitochondrial precursor				x
P48637	Glutathione synthetase	x			
P48741	Heat shock 70 kDa protein 7	x	x		
P49189	4 trimethylaminobutyraldehyde dehydrogenase	x			
P49368	T complex protein 1 subunit gamma	x			
P49747	Cartilage oligomeric matrix protein	x	x	x	x
P49748	Very long chain specific acyl CoA dehydrogenase mitochondrial precursor				x
P50395	Rab GDP dissociation inhibitor beta	x			x
P50454	Serpin H1 precursor				x
P50995	Annexin A11				x
P51452	Dual specificity protein phosphatase 3				x
P51884	Lumican	x	x	x	x
P51888	Prolargin precursor	x	x	x	x
P52565	Rho GDP dissociation inhibitor 1				x
P52566	Rho GDP dissociation inhibitor 2				x
P54652	Heat shock related 70 kDa protein 2	x	x	x	x
P55072	Transitional endoplasmic reticulum ATPase				x
P55083	Microfibril associated glycoprotein 4 precursor			x	x
P57053	Histone H2B type F			x	

P60174	Triosephosphate isomerase	x	x		x
P60660	Myosin light polypeptide 6	x	x		
P60709	Actin cytoplasmic 1	x	x	x	x
P60981	Destrin				x
P61086	Ubiquitin conjugating enzyme E2 25 kDa				x
P61088	Ubiquitin conjugating enzyme E2	x			
P61224	Ras related protein Rap 1b precursor				x
P61978	Heterogeneous nuclear ribonucleoprotein K	x			x
P61981	14 3 3 protein gamma	x	x		x
P62258	14 3 3 protein epsilon 14 3 3E	x			
P62491	Ras related protein				x
P62714	Serine threonine protein phosphatase 2A catalytic subunit beta isoform				x
P62736	Actin aortic smooth muscle	x		x	x
P62805	Histone H4		x		
P62826	GTP binding nuclear protein Ran				x
P62873	Guanine nucleotide binding protein G I G S G T subunit beta 1				x
P62879	Guanine nucleotide binding protein G I G S G T subunit beta 2				x
P62937	Peptidyl prolyl cis trans isomerase A		x		x
P62987	Ubiquitin 60S ribosomal protein L40		x		
P63104	14 3 3 protein zeta delta	x	x	x	x
P63241	Eukaryotic translation initiation factor 5A 1				x
P63244	Guanine nucleotide binding protein subunit beta 2 like 1				x
P63267	Actin gamma enteric smooth muscle	x		x	
P67936	Tropomyosin alpha 4 chain	x			
P68032	Actin alpha cardiac muscle 1		x		
P68104	Elongation factor 1 alpha 1		x	x	x
P68133	Actin alpha skeletal muscle			x	

P68363	Tubulin alpha 1B chain	x	x	x	
P68371	Tubulin beta 2C chain	x	x	x	
P68402	Platelet activating factor acetylhydrolase IB subunit beta	x			
P68871	Hemoglobin subunit beta		x	x	x
P69905	Hemoglobin subunit alpha		x	x	
P78371	T complex protein 1 subunit beta	x			
P78417	Glutathione transferase omega 1	x			x
P80748	Ig lambda chain V III region LOI				x
P98095	Fibulin 2		x		x
Q01082	Spectrin beta chain brain 1				x
Q01449	Myosin regulatory light chain 2 atrial isoform				x
Q01518	Adenylyl cyclase associated protein 1	x			
Q01995	Transgelin Smooth muscle protein 22 alpha				x
Q03252	Lamin B2	x			x
Q03591	Complement factor H related protein 1 precursor				x
Q04917	14 3 3 protein eta	x	x		
Q06323	Proteasome activator complex subunit 1	x			
Q06828	Fibromodulin	x	x	x	x
Q06830	Peroxiredoxin 1			x	x
Q07507	Dermatopontin	x	x	x	x
Q07960	Rho GTPase activating protein 1	x			
Q08257	Quinone oxidoreductase				x
Q08431	Lactadherin		x		x
Q12765	Secernin 1				x
Q13011	Delta 3 5 Delta 2 4 dienoyl CoA isomerase mitochondrial precursor	x			x
Q13228	Selenium binding protein 1	x			x
Q13404	Ubiquitin conjugating enzyme E2 variant 1				x
Q13409	Cytoplasmic dynein 1 intermediate chain 2				x
Q13509	Tubulin beta 3 chain	x	x	x	

Q13642	Four and a half LIM domains protein 1				x
Q13765	Nascent polypeptide associated complex subunit alpha				x
Q13885	N Tubulin beta 2A chain	x			x
Q14194	Dihydropyrimidinase related protein 1		x		
Q14195	Dihydropyrimidinase related protein 3	x	x	x	x
Q14624	Inter alpha trypsin inhibitor heavy chain H4 precursor				x
Q14697	Neutral alpha glucosidase AB precursor	x			
Q14764	Major vault protein				x
Q14767	Latent transforming growth factor beta binding protein 2		x		x
Q14894	Mu crystallin homolog NADP regulated thyroid hormone binding protein				x
Q15063	Periostin precursor	x	x	x	x
Q15084	Protein disulfide isomerase A6 precursor	x			
Q15113	Procollagen C endopeptidase enhancer 1 precursor				x
Q15181	Inorganic pyrophosphatase				x
Q15365	Poly rC binding protein 1				x
Q15366	Poly rC binding protein 2				x
Q15582	Transforming growth factor beta induced protein		x	x	x
Q15819	Ubiquitin conjugating enzyme E2 variant 2				x
Q16352	Alpha internexin			x	
Q16473	Putative tenascin XA		x		
Q16555	Dihydropyrimidinase related protein 2	x	x		x
Q16698	2 4 dienoyl CoA reductase mitochondrial precursor				x
Q16891	Mitochondrial inner membrane				x
Q562R1	Beta actin like protein 2		x		

Q6S8J3	POTE ankyrin domain family member E		x		
Q6UWY5	Olfactomedin like protein 1 precursor		x		x
Q71U36	Tubulin alpha 1A chain	x	x		x
Q7Z7G0	Target of Nesh SH3 precursor		x	x	x
Q8WUM4	Programmed cell death 6 interacting protein				x
Q8WWX9	Thioredoxin like selenoprotein M precursor	x			
Q92597	Protein NDRG1				x
Q92945	Far upstream element binding protein 2				x
Q96CN7	Isochorismatase domain containing protein 1				x
Q96CX2	BTB POZ domain containing protein				x
Q96KK5	Histone H2A type 1 H		x	x	
Q96KP4	Cytosolic nonspecific dipeptidase	x			
Q99426	Tubulin specific chaperone B				x
Q99497	Protein DJ 1	x			x
Q99536	Synaptic vesicle membrane protein	x	x		x
Q99714	3 hydroxyacyl CoA dehydrogenase type 2				x
Q99715	Collagen alpha 1 XII chain		x		x
Q99798	Aconitate hydratase mitochondrial precursor				x
Q9BQE3	Tubulin alpha 6 chain	x			
Q9BUF5	Tubulin beta 6 chain	x	x		
Q9BUT1	3 hydroxybutyrate dehydrogenase type 2				x
Q9BVA1	Tubulin beta 2B chain	x	x	x	
Q9BXN1	Asporin	x	x	x	x
Q9H0W9	Ester hydrolase C11orf54				x
Q9H4B7	Tubulin beta 1 chain		x		
Q9NRN5	Olfactomedin like protein 3 precursor	x	x		
Q9NRV9	Heme binding protein 1				x
Q9NSB2	Keratin type II cuticular Hb4		x	x	
Q9NVA2	Septin 11				x
Q9UBR2	Cathepsin Z precursor				x

Q9UBX5	Fibulin 5		x		x
Q9UK22	F box only protein 2				x
Q9Y277	Voltage dependent anion selective channel protein 3				x
Q9Y281	Cofilin 2				x
Q9Y490	Talin 1	x			
Q9Y696	Chloride intracellular channel protein 4				x
Q9Y6C2	EMILIN 1		x		x

Table 1: List of the proteins identified in the mitral valve tissue extract when four different proteomic approaches were applied: two-dimensional electrophoresis (2-DE), two-dimensional LC-MS^E (2D-LC/MS^E), LC/MS^E, and liquid-phase IEF. The method by which each protein was identified is reported.

Discussion

One critical step of this protocol is the use of liquid nitrogen to freeze the sample and to chill the grinder system. The use of liquid nitrogen prevents biological degradation and allows for efficient powdering, but it requires specific training for safe handling.

In this protocol features a grinder system for sample grinding because small samples are difficult to recover from standard mortar and pestles. In this case, small samples spread as a fine powder over the mortar surface, rendering collection difficult. Another advantage is that the grinder is motorized, which allows for a greater number of samples to be processed in a reproducible manner and without added fatigue. One limitation on the use of a grinder is that the size of the sample which must be small (100 mg or less) for the pestle to be pressed effectively against the mortar. Furthermore, the grinder components must be warmed to room temperature between uses for cleaning. Consequently, the procedure is time consuming and, if many samples are processed daily, many sets are needed.

An additional critical step is the preparation of the extraction buffer. The salt concentrations, especially for the urea (8 M) and thiourea (2 M), are quite high; thus, the volume of salt is almost half the total volume of the solution. Furthermore, the dissolution is not easy considering that heat must be avoided because, at more than 37 °C, urea can lead to protein carbamylation at the N termini of proteins/peptides and at the side-chain amino groups of lysine and arginine residues[17]. Once dissolved, the urea buffer must be filtered with 0.22-µm filters and can be stored at -80 °C for 4 weeks without affecting its extraction efficacy, but it must be warmed to more than 15 °C before use to allow for complete dissolution.

Modifications and troubleshooting

In this protocol, protein extraction is performed using the described urea buffer because it is one of the most commonly used protein extraction solutions for proteomic studies due to its compatibility with isoelectrofocusing and its efficiency in solubilizing sparingly soluble proteins[18]. It has been demonstrated that this buffer can very efficiently solubilize sparingly soluble proteins, such as integral membrane proteins[19], or proteins that are highly prone to aggregation, such as tubulin[18]. Furthermore, this buffer is fully compatible with the Bradford assay to determine protein concentration, and it can be used directly in 2-DE and liquid-phase IEF analyses.

However, this buffer is not ideal for the solubilization of all proteins present in a sample. It is possible that different extraction buffers could reveal proteins undetectable by this protocol. For example, it is well known that ribosomal and nuclear proteins could be better extracted with acid extraction or trichloroacetic acid/acetone precipitation[20], while alkaline pH levels are more suitable for membrane proteins[21,22]. Therefore, the use of alternative buffers might require additional steps for protein precipitation to eliminate salts that interfere with 2-DE or liquid-phase IEF.

Limitations of the technique

The number of proteins identified by this protocol is relatively low, but the number of identifications and the coverage of the proteomic analysis can be further increased through the use of more modern instruments, whose mass accuracy and sequencing speed have increased dramatically in the last few years[23].It is possible to cover a large part of the proteome, without any prefractionation steps, by employing a long gradient for liquid chromatography separations coupled with a high-resolution MS instrument that has a fast sequencing speed[24].

Significance of the technique with respect to existing/alternative methods

The ability to identify and quantify proteins in the human cardiac valves, such as the mitral valve, is an important and challenging task that will help to elucidate the mechanisms of physiological/pathological processes in valve diseases. Defining the changes of the mitral valve proteome will greatly increase the understanding of the nature of the biological processes that are associated with the disease state of the tissue.

The current knowledge on the physiopathology of the mitral valve is limited and generally obtained through the analysis of individual proteins involved in specific processes, such as extracellular matrix remodeling, hemostasis, inflammation, or oxidative stress[25], mainly studied at the tissue level by immunohistochemistry. As anticipated in the Introduction, the minimal proteomic data available in the literature focuses on the analysis of pathological mitral valves[9,10].

The lack of comprehensive proteomic studies can be ascribed to the complexity of this low-cellularity tissue that is highly rich in extracellular matrix proteins (*i.e.,* proteoglycans, collagen, and elastin). These proteins make up around 80% of the total amount, hampering the analysis of low-abundance proteins[26].

Thus, it was necessary to establish an efficient extraction protocol to maximize protein solubilization in order to describe the proteome of this tissue. This protocol allowed for the extraction of ~50 µg of protein from 1 mg of tissue. This is is a relatively low yield in comparison with "soft" tissue, such as liver (~135 µg from 1 mg of tissue), but it is sufficient to perform a protein analysis on individual samples. This is particularly relevant when defining the intra-individual variability of a phenomenon.

Furthermore, this method has the advantage of being compatible with many analytical applications. The mitral valve proteins dissolved in the proposed extraction buffer can be directly used for immunoblotting and proteomic analysis based on two-dimensional electrophoresis and liquid-phase isoelectrophoresis[11,12] or, after protein precipitation to eliminate buffer component interference, for other assays, such as gel-free mass spectrometry[15].

With the application of this extraction protocol, a more exhaustive characterization of the protein components of normal mitral valve tissue has been obtained through the identification of many intracellular proteins. These proteins are localized in the cytosol or in organelles, not only in the extracellular matrix, and have different molecular and biological functions. Other interesting proteins (*i.e.,* CryAB, septin-11, FHL-1, and dermatopontin) were also identified. These proteins have unknown functions in the mitral valve, but their biological properties suggest a possible role in valve diseases.

Future applications or directions after mastering this technique

With this protocol, it is possible to correlate data concerning protein expression with data on quantitative mRNA expression and non-quantitative immunohistochemical analyses. Indeed, when used together, these approaches will lead to a more comprehensive understanding of the molecular mechanisms underlying disease, from mRNA to post-translational protein modification. Thus, this method can be of interest to researchers focused on cardiac valve physiopathology. Finally, this protocol can also be applied to the porcine mitral valve, which bears a close resemblance to the human valve[27] and is used as an experimental model for valve function evaluation.

Disclosures

The authors have nothing to disclose.

Acknowledgements

The Italian Ministry of Health supported this study (RC 2013-BIO 15). We thank Barbara Micheli for her excellent technical assistance.

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