

Determining Protein Concentrations of the Human Ventricular Proteome

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Abstract

Proteomics is mostly used for measurements of relative differences in protein concentrations. Although such analyses are meaningful for comparing differences between two and more conditions, they do not directly provide details on the absolute protein concentrations within a system. Now, proteomics is heading more towards absolute quantitative strategies with results being expressed in copies/cell or ng/mg tissue. In the cardiac context, such quantitative information is crucial for (1) evaluating the feasibility of selecting a certain protein as potential novel drug target, (2) the expected concentration excreted into the circulation when selecting a biomarker, and (3) to build a model of cardiac function at the molecular level. At the same time, by mass spectrometry-based proteomics, a wealth of spectral information is gathered that can be used to evaluate protein levels of a select set of novel disease-altered proteins using, for instance, single reaction monitoring. Here we describe how to build a quantitative map of the human left ventricular proteome using a simple yet effective mass spectrometry-based spectral count method.

Key words Absolute quantitation, Spectral count, Human left ventricle

1 Introduction

With the introduction of stable isotope labels, the large-scale comparison of relative differences in protein abundance has become routine (1). Lately, more and more studies use shotgun mass spectrometry data to evaluate absolute protein concentrations within a biological system in copies/cell or ng/mg (2–7). Further validation has shown that these methods correlate very well with other quantitative measures of absolute abundance, such as single reaction monitoring (6, 8), but also GFP Western blot (9) and mRNA-based readouts (5).

When performing cardiac proteomics in the quest for novel biomarkers, novel therapeutic targets or disease signatures, a quantitative library of the cardiac proteome is a useful resource to serve as reference set. To start this effort, we have recently mapped the complement of the human left ventricular proteome (2) using

the aforementioned mass spectrometry-based spectral count technology. Like the plasma proteome, the cardiac proteome has a challenging dynamic range. This means that a small set of very abundant proteins obscure more low abundant proteins. To increase depth, complementary sample preparation strategies can be used, for instance, using different proteases (10, 11), analysis platforms (e.g., gel-based protein separation, ion exchange-based peptide separation), and the use of multiple ion fragmentation techniques (12). Here we provide a detailed methodological protocol to build a quantitative proteome map of the human left ventricle based on a multifaceted proteomics approach. This methodology is easily transferable to other tissues and samples.

2 Materials

2.1 Tissue Lysis

1. Protease inhibitor cocktail (Complete mini, Roche Diagnostics).
2. Phosphatase inhibitor cocktail (type 1 and 2, Sigma).
3. Phosphate buffered saline (PBS): Prepare 10(×) stock with 1.37 M NaCl, 27 mM KCl, 100 mM Na₂HPO₄, and 18 mM KH₂PO₄, and adjust to pH 7.4 with HCl if necessary. Prepare a working solution by dilution of one part with nine parts Milli-Q water.
4. Lysis buffer: PBS buffer supplemented with 0.1 % v/v Tween 20, protease inhibitor cocktail (one tablet per 15 ml buffer), and 150 µl of phosphatase inhibitor cocktail II (Sigma) (see Notes 1 and 2).

2.2 SDS-PAGE Protein Separation and In-Gel Trypsin Digestion

1. 4–15 % and 10–20 % SDS-PAGE gradient gels (Bio-Rad), standard running buffer, SDS-PAGE loading buffer, and GelCode Coomassie staining solution (Thermo Scientific).
2. Dithiothreitol (DTT, 6.5 mM in 50 mM ABC) and iodoacetamide (54 mM in 50 mM ABC) solution are prepared fresh.
3. Trypsin (Roche Diagnostics), 0.1 µg/µl in 50 mM acetic acid. Stored in single-use aliquots of 10 µl at –80 °C.
4. 50 mM ammonium bicarbonate (ABC) in Milli-Q water and analysis grade acetonitrile.

2.3 In-Solution Digestion with Different Proteases

1. 50 mM ABC in Milli-Q water.
2. 8 M urea in 50 mM ABC.
3. Trypsin, chymotrypsin, and Lys-C (Roche Diagnostics) and Lys-N (from Grifola Frondosa, Seikagaku Corp.) are all dissolved at 0.1 µg/µl in 50 mM acetic acid and stored in single-use aliquots (10 µl) at –80 °C.
4. DTT and iodoacetamide solutions as described above.

2.4 Strong Cation Exchange Chromatography

1. Two Zorbax Bio-SCX Series II columns [0.8 mm (i.d.) × 50 mm, 3.5 µm material, Agilent Technologies].
2. A suitable HPLC, e.g., consisting of FAMOS autosampler (Dionex), Shimadzu LC-9A binary pump, and an SPD-6A UV detector.
3. Strong cation exchange (SCX) solvent A: 20 % acetonitrile, 0.05 % formic acid, pH 3.0 in Milli-Q water.
4. SCX solvent B: 500 mM KCl in 20 % acetonitrile and 0.05 % formic acid, pH 3.0 in Milli-Q water.

2.5 LC-MS/MS Analysis

1. Suitable high-resolution mass spectrometer, e.g., LTQ Orbitrap XL equipped with an electrospray ion source and the option to use electron transfer dissociation (ETD) as an additional fragmentation technique. The mass spectrometer is coupled online to a nanoflow HPLC system, e.g., Agilent 1200 series (see Note 3).
2. Trapping column, 20 mm, 100 µm i.d. packed with Aqua C18 reversed phase material (5 µm, Phenomenex).
3. Separation column, 400 mm, 50 µm i.d. packed with Reprosil-Pur C18-AQ reversed phase material (3 µm, Dr. Maisch).
4. Distally coated fused-silica emitter [360 µm (i.d.); 20 µm (i.d.); tip inner diameter, 10 µm, New Objective].
5. HPLC solvent A: 0.1 M acetic acid in Milli-Q water.
6. HPLC solvent B: 0.1 M acetic acid in 80 % acetonitrile in Milli-Q water.

2.6 Data Analysis

1. BioWorks (Thermo Electron) is used to extract mass spectrometric raw data into a searchable format.
2. Proteome Discoverer (Thermo Electron) is used to extract ETD generated data from the raw data files (see Note 4).
3. MASCOT (Matrix Science) is used as search engine for protein identification.
4. Scaffold (Proteome Software) is used to reduce protein redundancy and export FDR filtered datasets (see Note 5).
5. Microsoft Excel is used for combining datasets and calculating the protein abundance.

3 Methods

3.1 Data Gathering

A deep proteome analysis of the human left ventricle requires a multifaceted approach as the dynamic range of the cardiac proteome is challenging due to the presence of a few very abundant proteins. Here we describe how to use complementary chromatographic, mass spectrometric, and sample preparation

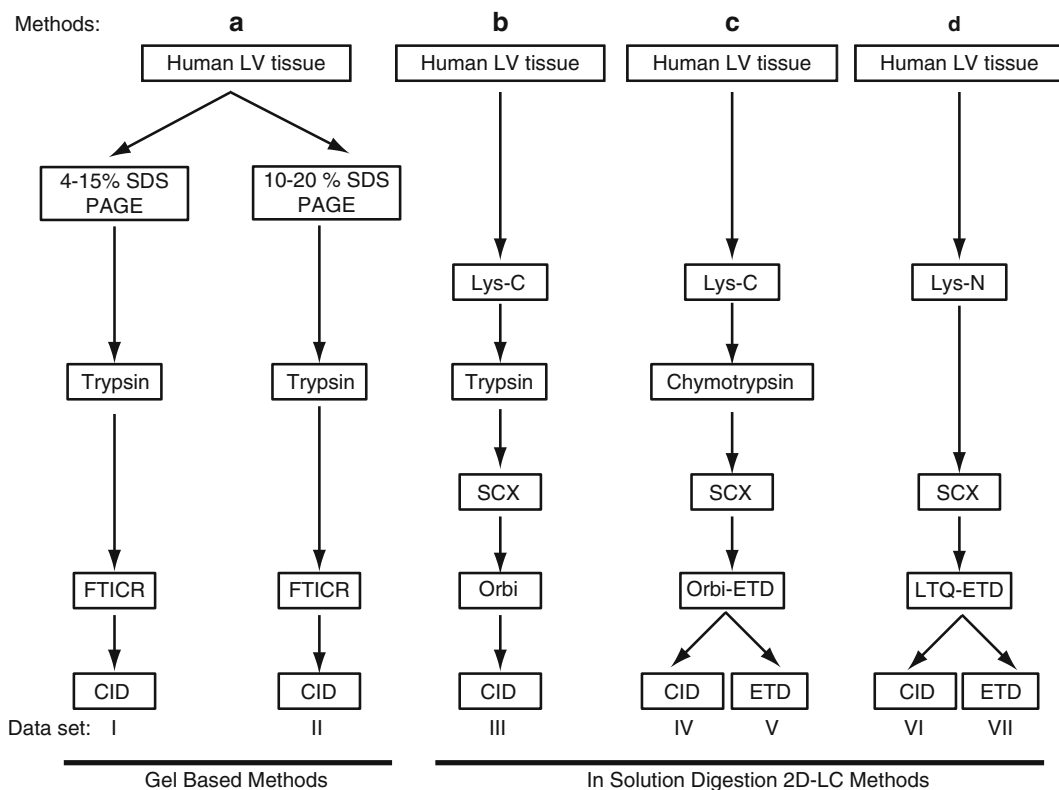


Fig. 1 Multifaceted proteomics approach. The use of four main approaches (Methods A–D) leads to the acquisition of seven LC-MS/MS datasets (I–VII). Method A involves SDS-PAGE separation of the protein extract on two different gradient gels, followed by in-gel trypsin digestion with trypsin and analysis on an LTQ-FTICR mass spectrometer using CID fragmentation. Methods B, C, and D use different digestion regimes with Lys-C and trypsin (B), Lys-C and chymotrypsin (C), and Lys-N (D) followed by two-dimensional peptide separation using SCX in the first and reversed phase in the second dimension. Peptides of Methods C and D are analyzed using both CID and ETD fragmentation (Adapted from Aye et al. (2))

strategies to accomplish this. Both gel-based protein separation and peptide-based strong cation exchange protocols are used to increase depth of coverage. Furthermore, we utilize different digestion protocols involving four different proteases (trypsin, Lys-N, and chymotrypsin) as well as two different peptide fragmentation techniques [collision-induced dissociation (CID) and electron transfer dissociation (ETD)]. The outline of our approach which involves four different methods (A–D) is depicted in Fig. 1.

3.2 Data Mining

To combine all data gathered in the different workflows into the four major (large) datasets and ultimately in one large dataset requires several steps that are outlined below. In addition, for a human cardiac catalogue to be meaningful, a sense of protein concentration would be a valuable addition. Here we describe in detail

a simple spectral count-based approach to achieve this. Such information is invaluable when interpreting the relevance/ expression levels of for instance all members of the protein class of kinases within the human cardiac proteome. In such a way, novel insights can be gained towards the next kinase to target for functional verification within the cardiac context.

3.3 Tissue Lysis

1. Prechill a steel mortar and pestle in liquid nitrogen.
2. Human left ventricular tissue of a male donor with no prior cardiovascular disease ($\sim 1 \text{ cm}^3$) is taken from -80°C and further frozen in liquid nitrogen.
3. Grind the tissue in the mortar and transfer the pulverized tissue with a cold spoon into an Eppendorf tube.
4. Add 1 ml of ice-cold lysis buffer and leave to lyse at room temperature for 5 min and another 10 min on ice.
5. Centrifuge the lysate at $20,000 \times g$ and 4°C . Transfer the supernatant to a cold falcon tube. Extract the insoluble pellet twice more using steps 4 and 5 of Subheading 3.1.
6. Measure the protein concentration of the (combined) supernatant(s).

3.4 In-Gel Protein Separation and Digestion (Method A)

1. Run 50 μg of the heart lysate on two different SDS-PAGE gradient gels (4–15 and 10–20 %, Bio-Rad). Fix and stain with GelCode Coomassie Blue staining. Wash in Milli-Q water.
2. Slice the gel in ~ 70 gel pieces using a Mickle gel slicer (see Note 6).
3. Gel slices are washed (ABC and acetonitrile), reduced (6.5 mM DTT, 30 min, 56°C), washed (ABC and acetonitrile), alkylated (54 mM iodoacetamide in the dark, 30 min), and washed (ABC, acetonitrile).
4. Incubate the gel slices with trypsin (0.1 μg per slice) overnight at 37°C and collect the supernatant and two washes with 5 % formic acid solution.
5. Dry the samples in vacuo.

3.5 In-Solution Digestion (Methods B, C, and D)

1. Dilute 200 μg tissue lysate into 200 μl ABC supplemented with 96 mg urea (8 M final concentration).
2. Reduce (2 mM DTT, 15 min 56°C) and alkylate (4 mM iodoacetamide, 30 min. room temperature, dark) all cysteine residues.
3. Method B: Digest with Lys-C (4 μg) for 4 h at 37°C and then dilute the entire solution fourfold (2 M urea). Digest with trypsin (4 μg) overnight at 37°C . Desalt the sample over C18 material and dry in vacuo. Reconstitute in SCX solvent A.

4. Method C: Perform lysate dilution, reduction, alkylation and Lys-C digestion and dilution to 2 M urea as described in Subheading 3.3, steps 1–3. Then digest the Lys-C digest further with chymotrypsin (4 µg) overnight followed by C18 desalting, in vacuo drying, and reconstitution into SCX solvent A.
5. Method D: Repeat steps 1 and 2 of Subheading 3.3. Add Lys-N in a 1:85 w/w ratio (so for 200 µg total lysate, this is 2.35 µg Lys-N). Digest overnight at 37 °C. Desalt, dry in vacuo, and reconstitute in SCX solvent A.

3.6 Strong Cation Exchange Fractionation (Methods B, C, and D)

1. These instructions assume the use of Zorbax Bio-SCX Series II columns [0.8 mm (i.d.)×50 mm (l), 3.5 µm]. SCX is performed using two in-line coupled Zorbax Bio-SCX Series II columns, FAMOS autosampler, Shimadzu LC-9A binary pump, and SPD-6A UV detector. SCX is performed at pH = 3 (see Note 7).
2. Inject the equivalent of 200 µg protein lysate by FAMOS autosampler.
3. Load the injected sample to the column with SCX solvent A at a flow rate of 100 µl/min for 5 min.
4. Elute with a 1 %/min linear gradient of SCX solvent B at a flow rate of 50 µl/min for 45 min. Then equilibrate the column with SCX solvent A for 10 min.
5. A total of 50 SCX fractions (1 min each, 50 µl elution volume) are collected by using a suitable fraction collector.
6. Dry all fractions in vacuo and store at –30 °C until LC-MS/MS analysis.

3.7 LC-MS/MS Analysis

1. These instructions assume the use of a nanoflow liquid chromatography setup, directly coupled to, for instance, a LTQ Orbitrap XL mass spectrometer equipped with an electrospray ion source for MS analysis and an ETD source for alternative fragmentation.
2. Resuspend the dried samples from step 6 of Subheading 3.4 in 40 µl 10 % formic acid.
3. Inject 10 µl of the resuspended peptide mixtures individually onto an Agilent 1200 series LC system, equipped with a 20 mm Aqua C18 trapping column [100 µm (i.d.), packed in-house] and a 400 nm Reprosil-Pur C18-AQ analytical column [50 µm (i.d.), packed in-house].
4. Trapping is performed at 5 µl/min for 10 min in HPLC solvent A, and elution is achieved with a gradient of 10–35 % HPLC solvent B in 45 min in a total analysis time of 60 min. The flow rate is passively split to ~100 nl/min for peptide

separation. Nanospray is achieved using a distally coated fused-silica emitter.

5. The LTQ Orbitrap XL mass spectrometer is operated in a data-dependent mode, automatically switching between MS and MS/MS. Full-scan MS spectra (350–1,500 m/z) are acquired in the FT Orbitrap with a resolution of $R = 60,000$ at 350 m/z after accumulation to a target value of 500,000 ions in the linear trap. Parent ions were isolated for a more accurate measurement by performing a single-ion monitoring scan and fragmented by CID and ETD in data-dependent mode (two most intense ions, minimum intensity of 500). Ions were fragmented using CID with normalized collision energy of 35 and an activation time of 30 ms. ETD fragmentation was performed with supplemental activation. Fluoranthene was used as reagent anion, and ion/ion reaction in the linear ion trap was taking place for 100 ms.

3.8 Data Analysis and Combination of Raw Files

1. Process obtained .RAW files from each LC-MS/MS run with BioWorks. Combine the data of the individual LC-MS/MS runs into a single MASCOT generic file (.mgf) per method (see Note 8).
2. Perform MASCOT searching on the .mgf file against an appropriate database using the following criteria: Carbamidomethylation on cysteine residues as a fixed modification and methionine oxidation, serine/threonine/tyrosine phosphorylation, and N-terminal acetylation (proteins) as variable modifications. Allow two missed cleavages and a peptide mass tolerance of 10 ppm and an MS/MS fragment mass tolerance of 0.9 Da.
3. Filter the resulting data to a false discovery rate (FDR) of <1 % per method dataset to determine the cutoff threshold MASCOT score using standard procedures (see Note 9).
4. Load each .dat file into the Scaffold software package (Proteome Software) and apply the determined MASCOT score cutoff.
5. Export the required protein data (unique peptides, assigned spectra, sequence coverage, etc.) from Scaffold to Excel and combine all obtained datasets using the “vlookup” commands to build a comprehensive protein-oriented table (see Note 10) (Table 1). Further calculations can also be made in Microsoft Excel, e.g., total number of unique peptides and total number of observed spectra.

3.9 Quantitative Mapping of the Left Ventricular Proteome

1. The spectral counts of each complete gel lane or SCX separation (combine CID and ETD data when applicable; see Fig. 1) are turned into absolute quantitative information (ng/mg) with the F_{abb} factor. Calculate the F_{abb} of protein i by dividing

Table 1
Combining multiple datasets

UniProt	Protein description	MW (kDa)	Sum uniq. peps.	Number of identified spectra (per method)					Sum spectral counts	Average conc. (ng/mg)
				A-I	A-II	B	C	D		
P02144	Myoglobin	17,166	127	503	282	453	330	786	2,354	51,154.2
P68871	Hemoglobin subunit beta	15,980	93	335	164	338	380	139	1,356	26,334.6
P69905	Hemoglobin subunit alpha	15,239	69	161	115	323	380	143	1,122	23,677.1
P06732	Creatine kinase M-type	43,083	178	521	267	684	1,101	277	2,850	20,808.7
P05413	Fatty acid-binding protein, heart	14,840	87	205	142	270	370	9	996	19,862.4
P99999	Cytochrome c	11,731	63	82	113	106	12	180	493	16,856.1
P10916	Myosin regulatory light chain 2, ventricular/cardiac muscle isoform	18,771	92	173	156	154	201	109	793	14,428.1
P04406	Glyceraldehyde-3-phosphate dehydrogenase	36,035	120	263	58	504	519	159	1,503	12,342.3
P02768	Serum albumin	69,348	280	713	201	1,129	496	330	2,869	11,812.3
P07195	l-Lactate dehydrogenase B chain	36,620	113	341	59	299	295	178	1,172	9,698.1
P68400	Casein kinase II subunit alpha	45,126.5	6	2	0	3	0	1	6	34.6

Q13557	Calcium/calmodulin-dependent protein kinase type II delta chain	56,352.7	25	32	1	24	0	2	59	228.1
P17612	cAMP-dependent protein kinase catalytic subunit alpha	40,573.3	19	19	0	5	0	4	28	173.2
Q2M3C7	A-kinase anchor protein SPHKAP	186,439	2	0	0	3	0	0	3	3.2
Q8N3K9	Cardiomyopathy-associated protein 5	449,187	2	0	0	4	0	0	4	1.8
P54289	Voltage-dependent calcium channel subunit alpha-2/delta-	123,169	2	0	0	3	0	0	3	4.9

Protein-centric data presentation of the ten most abundant proteins found in the left ventricle sample (based on seven datasets, data aggregated for Methods C and D). Each dataset is individually searched (MASCOT) and filtered for FDR (RockerBox and Scaffold). Subsequently, each dataset is combined in excel using “vlookup” commands. Within this Excel table, the F_{abs} values and subsequently the average concentration in ng/mg can be calculated using the instructions of Subheading 3.7, steps 1–3. Also depicted are the examples within Fig. 2 in the lower abundance levels. MW = molecular weight, SUM Uniq. Pepts. = the sum of the unique peptides observed over all runs, A-I = 4–15 % SDS-PAGE, A-II = 10–20 % SDS-PAGE (see Fig. 1), SUM spectral count = the sum of all observed spectra identified for that particular protein, Conc. = concentration

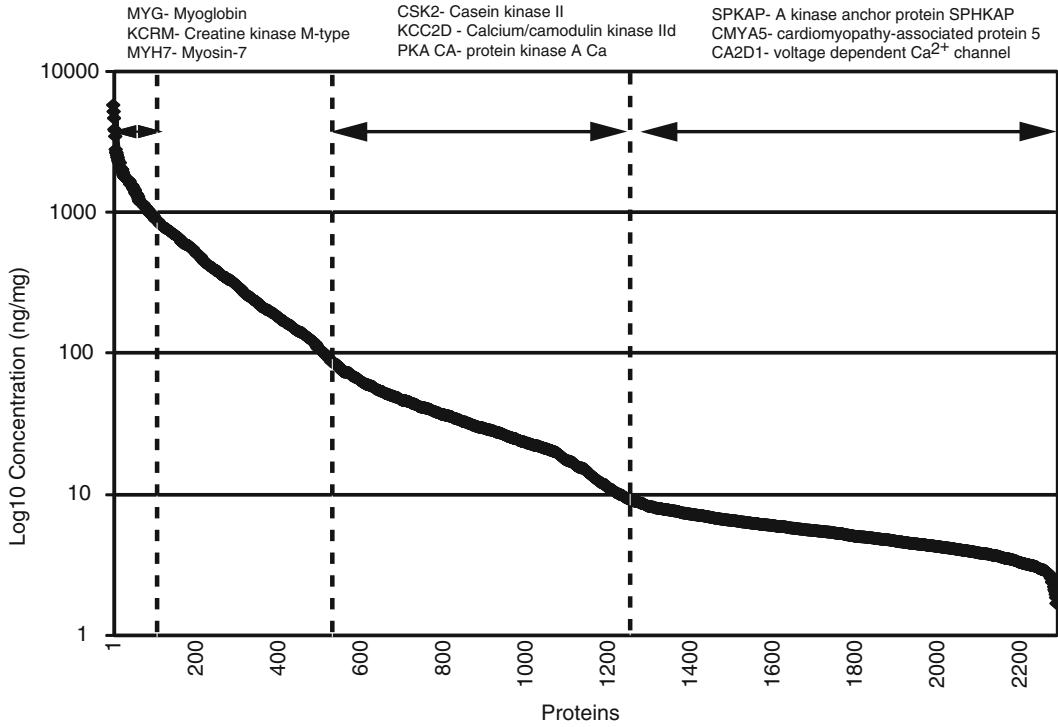


Fig. 2 Protein abundance map of the human left ventricular proteome (Adapted from Aye et al. (2)). The concentration calculation of the left ventricular proteome covers 2,277 proteins (at least two unique peptides) and four orders of magnitude. Depicted are representatives of three different abundance classes (top100, 500–1,250, and 1,250–2,277) to highlight that the first signaling proteins (kinases were chosen here) are at least 100-fold less abundant than the most abundant cardiac proteins. Examples of kinase signaling modulators and ion channels are residing mainly in the lowest part of the abundance map with copy numbers at least 1,000-fold below the most abundant proteins

the number of assigned spectra of protein i (SC_i) by its molecular weight (MW_i): $F_{abb,i} = SC_i / MW_i$.

2. Recalculate the F_{abb} values into absolute numbers by taking the proportion of the protein's F_{abb} towards the sum of all F_{abb} values in a particular dataset, $\sum(SC_k / MW_k)$. This proportion is then multiplied by a constant number, C , to indicate the total input per analysis [in this particular case, the equivalent of 25 μ g total protein was injected (see Note 11)].

$$F_{abb,i} = \frac{SC_i / MW_i}{\sum (SC_k / MW_k)} \cdot C$$

3. Build a quantitative map by ranking the proteins based on their tissue/cellular abundance expressing the $F_{abb,i}$ in ng/mg (Fig. 2).

3.10 Conclusion

Here we describe a simple method to absolutely quantify the human left ventricular proteome. The basis for the quantitation relies on the use of spectral counts and the F_{abb} , a correction factor that normalizes the spectral counts of a protein to its molecular weight. This makes it a very simple yet effective approach as the only software tool required to calculate the protein concentrations is a spreadsheet software package. Although simple by nature, the F_{abb} factor correlates very well with other much more complex absolutely quantitative methods, such as APEX. The only prerequisite to make the F_{abb} -based absolute quantitation proteome-wide is to achieve a very in-depth analysis, i.e., aim for at least 100,000 high-quality spectra. As shown by us and others earlier, the use of multiple proteases, separation platforms, and peptide fragmentation techniques considerably adds protein coverage and peptide diversity (2, 11–18). This is however a requirement if we want to conquer the immense dynamic range challenge of the cardiac proteome to gain access to the interesting low abundant cardiac proteome with all its diversity in disease-relevant signaling proteins (see Note 12).

4 Notes

1. PBS buffer with 0.1 % Tween 20 can be prepared for multiple uses and stored at 4 °C. Buffer should be cold when used. Full lysis buffer should be prepared fresh only very prior to use to retain full efficiency of the protease and phosphatase inhibitors.
2. The described lysis buffer could be replaced by stronger buffers. For example, addition of 8 M urea to the lysis buffer increases lysis efficiency of more insoluble proteins. Sonication of the sample can also increase protein yield.
3. Alternatively, when one has access to an Orbitrap Velos, a decision tree using three fragmentation (CID, ETD, and HCD) methods could be used (12).
4. The Proteome Discoverer software package (v2.0 and up) is now also suitable to combine data extraction of different fragmentation regimes, which makes the use of BioWorks no longer required.
5. Proteome Discoverer (v2 and up) also allows data organization and multiple dataset comparisons. In addition, it has a built-in protein grouping algorithm to further reduce protein identification redundancy.
6. A Mickle gel slicer can cut small and consistently sized gel pieces of approximately 1 mm. Keep the gel wet with Milli-Q water to prevent sticking of gel pieces to the razor blade that is used to slice.

7. At pH=3, all acidic residues (Asp and Glu) are uncharged, allowing all peptides to bind to the column.
8. Several software tools can be used here: MGFcombiner (MSQuant (19), RockerBox (20)), or Proteome Discoverer (Thermo). In addition, when using MSQuant, all individual files can be recalibrated for mass accuracy prior to combining the files, allowing a smaller mass cutoff when searching a more ideal false discovery rate filtering options (19).
9. Alternatively use RockerBox here to do the filtering automatically on your resulting MASCOT .dat file (20).
10. Combining datasets is not arbitrary as typical SCX-LC-MS/MS experiments tend to be extremely large and demand a lot of computing power. We use individual Scaffold outputs and combine them in Excel. Combining in Scaffold is an option but poses high demands in computing power. Another option is to combine different analysis files using Proteome Discoverer (v2.0 and up). Advantage of the latter is that the built-in protein grouping algorithm can be used to reduce protein redundancy automatically. Here computational power is also required. Combining 7 SCX-LC-MS/MS analyses typically generates file sizes easily exceeding 10 GB and requires a 32- or 64-core machine to be opened and manipulated. Combining at .dat file level and using protein grouping and annotation in MASCOT (v2.3 and up) is also an option. Browsing these data in the web-based MASCOT environment is however cumbersome when .dat file sizes approach 1 GB and up.
11. To ensure comparable results between different runs, experiments, and analysis platforms, load equal amounts of total protein onto the LC-MS/MS system. In this case, 50 μ g protein was loaded per SDS-PAGE gel lane. Therefore, to use $C=25\text{ }\mu$ g, load 50 % of the peptide mixture obtained from each gel band onto the LC-MS/MS system (Method A). SCX separation is performed using the peptide mixture of the equivalent of 200 μ g protein. Therefore, to retain quantitative information from each SCX fraction, 12.5 % should be injected.
12. Further curation of the dataset can be performed when assuming that tissue is always contaminated with blood, which means plasma and red blood cell proteins are also identified (hemoglobins, serum albumin, *see* Table 1). Before annotating a protein as present in the myocardium, careful cross-correlation with plasma and other circulating proteomes is advised. For this, published datasets, if they provide spectral counts or unique spectral counts, can also be quantitatively ranked using the F_{abb} factor, before comparison with the obtained proteome.

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