Chapter 17

Analysis of Serum Proteins by LC-MS/MS

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Abstract

Serum contains a vast array of proteins, some of which are specific to blood whilst others are secreted into blood from tissues and organs. The so-called tissue leakage factors reveal information about the tissue from which they originate and are therefore of great potential importance as disease biomarkers. There are already a number of blood-borne biomarkers in routine clinical use that aid in the diagnosis or management of cancer. However, there is a pressing need for additional markers, and new methods to find them are under development. Here we provide a protocol for serum protein profiling using liquid chromatography tandem mass spectrometry (LC-MS/MS). Included in this procedure, we detail the pre-processing steps of lipid and high-abundance protein removal. These procedures can also be employed up-stream of quantification methods such as isobaric tags for relative and absolute quantification (iTRAQ). **Chapter 12** is devoted to the iTRAQ approach for quantifying proteins, and it is therefore not described in this chapter.

Key words: Serum, serum depletion, strong cation exchange, LC-MS/MS, proteomics.

1. Introduction

Blood is a very attractive source of protein or peptide biomarkers (1). It is minimally invasive and relatively inexpensive to obtain. In addition, tests that can be undertaken routinely by clinical laboratories have been established. This provides the promise that new candidate biomarkers might also be translated to clinical use in the form of assays that are readily amenable to routine widespread use. Serum is the clear liquid obtained after blood has clotted and the clot has been removed. In designing studies aimed at serum biomarker discovery, a number of factors need to be carefully considered. These include decisions as to whether samples from individuals belonging to a distinct disease group

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will be analysed individually or as a pool of several serum samples from that group. The age and gender of individuals should be matched across groups where possible and appropriate disease control groups should be identified and included in the analysis. Finally, the application of standard protocols for the collection and storage of serum samples is necessary for the production of reliable data (2).

In addition to proteins, serum contains lipids which interfere with mass spectrometry-based analysis. Therefore a method that efficiently and selectively removes lipids (3) is required prior to analysis. The serum proteome itself is complex and contains proteins across a large range of concentrations. A small number of highly abundant proteins account for a large proportion of proteins in serum (4). This makes the analysis of less abundant proteins difficult. A variety of methods are available for the removal of high-abundance proteins, including 'Proteoprep 20 Immunodepletion Kit' by Sigma-Aldrich, Gillingham, UK; 'Multiple Affinity Removal System' by Agilent Technologies UK Ltd., Wokingham, UK; and 'Proteomelab IgY' by Beckman Coulter UK Ltd., High Wycombe, UK). Alternatively, the concentration of low-abundance proteins and reduction of the dynamic range of protein concentrations in serum samples can be attempted using 'ProteoMiner Protein Enrichment', Bio-Rad Laboratories, Hemel Hempstedt, UK (5). In the protocol provided below, we will focus on high-abundance protein depletion using the Sigma-Aldrich Proteoprep 20 column. An overview of the entire procedure is provided in **Fig. 17.1**.

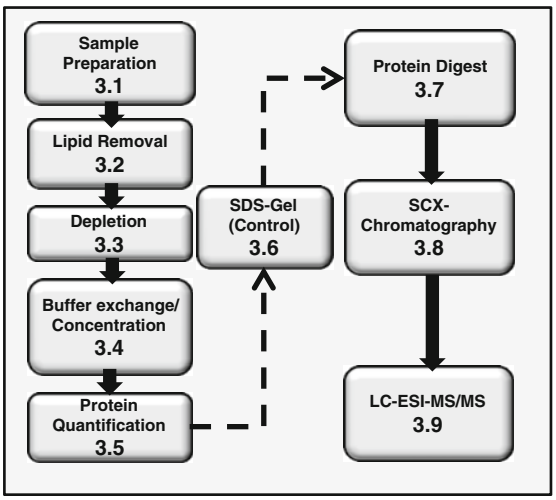

Fig. 17.1 Flow chart providing an overview of the procedure for analysis of serum proteins by LC-MS/MS. The *number* in each box indicates the section in the text, in which the specified step is described.

2. Materials

2.1. For Serum Preparation

1. Serum Z/7.5 mL Sarstedt Monovette
2. Cryogenic 2 mL tubes (Nunc GmbH & Co KG., part of Thermo Fisher Scientific, Langenselbold, Germany)

2.2. For Lipid Removal from Serum Samples

1. Diisopropyl ether (DIPE), butan-2-ol, and ethylenediaminetetraacetic acid (EDTA). Beware: DIPE is a sedative that is highly flammable. Precautions should be taken, including work carried out under a fume hood with appropriate ventilation.
2. Solvent Mix: 60:40 mixture of DIPE and butan-2-ol prepared freshly prior to commencing the delipidation process.

2.3. High-Abundance Protein Depletion

1. Proteoprep 20 Plasma Immunodepletion Kit (Sigma-Aldrich).
2. 1 M HEPES solution, pH 7.5: 119.15 g HEPES (free acid) dissolved in 400 mL MilliQ water. Adjust pH with NaOH pellets until pH reaches 6.8. Make the final adjustment with concentrated NaOH. Finally adjust volume with MilliQ water to 500 mL. Filter sterilize and store at 4°C. Attention: NaOH is highly alkaline. Wear safety glasses.
3. Microsep 1 K Centrifugal Devices (Pall Life Science, Portsmouth, UK).

2.4. Buffer Exchange

1. 0.5 M sodium carbonate solution, pH 8.5: Dissolve 2.64 g sodium carbonate in 25 mL MilliQ water. Adjust pH with concentrated HCl, filter sterilize, and store at 4°C. The solution is stable for up to 1 week. Attention: HCl is a strong fuming acid. Use only under a fume hood with ventilation.
2. Microsep 1 K Centrifugal Devices (Pall Life Science).

2.5. Protein Quantification

1. Bio-Rad Protein Assay Solution (Bio-Rad Laboratories)

2.6. Quality Control – SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. 1.5 M Tris–HCl (pH 8.7), 1 M Tris–HCl (pH 6.8), 30% acrylamide/bisacrylamide solution (37.5:1) (beware: non-polymerized acrylamide is neurotoxic), *N*,*N*,*N*,*N*′-tetramethylethylenediamine (TEMED), 10% ammonium persulphate solution (freshly prepared), and 10% sodium dodecyl sulphate (SDS) solution (all chemicals from Sigma-Aldrich).
2. 10X SDS gel running buffer (Laemmli): 30 g Tris-base, 144 g glycine, 10 g SDS (all from Sigma-Aldrich), dissolved in MilliQ water and adjusted to a volume of 1 L.

3. 5X sample buffer (Laemmli): 10% SDS, 50% glycerol, 300 mM Tris–HCl (pH 6.8), 0.05% bromphenol blue. Add dithiothreitol (DTT) to a final concentration of 100 mM prior to use.
4. PageRuler Prestained Protein Ladder (Fermentas UK, York, UK).
5. Silver-Stain Plus Kit (Bio-Rad Laboratories), methanol, acetic acid.

2.7. Trypsin Digest

1. Sequencing grade-modified trypsin (Promega UK Ltd., Southampton, UK).
2. Sodium dodecyl sulphate (SDS), 0.5 M Tris(2-carboxyethyl)phosphine hydrochloride solution (TCEP), and iodoacetamide.

2.8. Strong Cation Exchange Chromatography

1. PolySULFOETHYL A (4.6 × 200 mm inner diameter (i.d.) HPLC column (PolyLC)).
2. Buffer A: 10 mM potassium phosphate (KH_2PO_4), 25% acetonitrile, pH < 3, adjusted with concentrated H_3PO_4.
3. Buffer B: 10 mM potassium phosphate (KH_2PO_4), 1 M potassium chloride, 25% acetonitrile, pH < 3, adjusted with concentrated H_3PO_4.
4. Diluent: 25% acetonitrile, pH < 3, adjusted with 500 mM H_3PO_4.

2.9. LC-MS/MS

1. Buffer A: 5% acetonitrile, 0.05% trifluoroacetic acid.
2. Buffer B: 95% acetonitrile, 0.05% trifluoroacetic acid.

3. Methods

3.1. Serum Preparation

1. Place a freshly acquired blood sample (contained in a Sarstedt Monovette) at 4°C for 15 min, until the blood clots.
2. Spin the Sarstedt Monovette at 800 RCF for 10 min at 4°C.
3. In a sterile environment, pipette the clear serum (upper layer) in aliquots into cryotubes.
4. Store the serum at −80°C.

3.2. Lipid Removal from Serum Samples

The removal of lipids is the first step in the preparation of serum samples for LC-MS/MS analysis (*see* **Note 1**). During the following procedure, serum is maintained at 4°C.

1. Place 500 μL of serum on ice and add 50 μg EDTA.
2. In a 1.5 mL microfuge tube, mix 500 μL of the serum containing EDTA with 1 mL of freshly prepared solvent mix (60:40 mixture of DIPE and butan-2-ol) yielding a one-part serum to two-part solvent mix.
3. Rotate the mixture on a blood cell suspension rotor (end-over-end rotation) at 30 rpm for 30 min at 4°C.
4. Centrifuge the mix at 400 RCF for 2 min at 4°C.
5. Carefully remove the aqueous serum phase (at the bottom of the tube) with a syringe, without disturbing the upper lipid layer, and transfer to a new 1.5 mL microfuge tube.
6. Carefully mix the aqueous phase with two parts DIPE and immediately centrifuge again.
7. Carefully remove and discard the upper solvent layer.
8. Remove the residual solvents from the serum phase by vacuum concentration (1 min, 37°C).
9. Aliquot the serum into 100 μL aliquots in 1.5 mL tubes and store at −80°C until proceeding with the depletion of high-abundance proteins.

3.3. High-Abundance Protein Depletion

The Proteoprep 20 Immunodepletion Kit is designed to remove 20 of the most abundant proteins from blood. This effectively results in the removal of 97–98% of protein from serum samples. The removal of these 20 proteins (albumin, IgG, IgA, IgM, IgD, transferrin, fibrinogen, α_2-macroglobulin, α_1-antitrypsin, haptoglobin, α_1-acid glycoprotein, ceruloplasmin, apolipoprotein A-I, apolipoprotein A-II, apolipoprotein B, complement C1q, complement C3, complement C4, plasminogen, prealbumin) is achieved by the use of immobilized antibodies on agarose beads. One single column allows 100 depletion runs, and each run has a capacity of 10 μL of serum (~500 μg). Depletion is performed according to the manufacturer's protocol. We recommend that depletions should be undertaken in a sterile environment, such as a cell culture hood.

1. Dilute the Proteoprep20 Equilibration buffer and Elution buffer with MilliQ Water (*see* **Note 2**).
2. Defrost one aliquot (100 μL) of delipidated serum on ice and dilute 1:11 by adding 1 mL 1X Equilibration buffer.
3. Add 500 μL of this diluted serum onto a Corning Spin X Centrifuge Filter (0.2 μm) and centrifuge at 2000×*g* for 1 min. Repeat with the second 500 μL and then with the remaining 100 μL. If the filter unit becomes blocked, use a new one and apply the diluted serum again. Combine the flow-through fractions in a 1.5 mL microfuge tube.

4. Now prepare the depletion column by removing the bottom plug (make sure you keep it, as you need it at the end of the procedure) and loosen the upper cap. Place the column in a 2 mL tube. Centrifuge at 2000×*g* for 30 s.
5. Remove the screw cap and attach the Luer Lock cap onto the column. Using a 20 mL syringe filled with Equilibration buffer slowly push 8 mL of Equilibration buffer through the column.
6. Remove the Luer Lock cap, attach the red cap loosely, and place in a 2 mL tube. Centrifuge at 2000×*g* for 30 s.
7. Place the column in a fresh 1.5 mL microfuge tube and add 100 μL from the diluted, filtered serum. Ensure that the serum is absorbed into the agarose matrix and not attached to the plastic of the column. Place the red cap loosely on top.
8. Incubate for 15–20 min at room temperature.
9. Centrifuge the column at 2000×*g* for 30 s. Collect the flow-through in a 1.5 mL tube. (This is the depleted fraction.)
10. Wash the column by applying 100 μL Equilibration buffer and centrifuge immediately at 2000×*g* for 30 s. Collect this fraction separately (*see* **Note 3**).
11. Repeat Step 10.
12. Attach the Luer Lock and apply 2 mL of 1X Elution buffer by using a 20 mL syringe. Collect the flow-through (this is the eluted fraction, containing the high-abundance proteins). Add 0.5 mL of 1 M HEPES solution, pH 7.4, to adjust the pH every five elution steps.
13. Attach the Equilibration buffer syringe and slowly draw 8 mL of 1X Equilibration buffer through the column.
14. Disassemble the Luer Lock and attach the red cap. Centrifuge at 2000×*g* for 30 s.
15. Repeat from Step 7 for the desired number of depletions. It is necessary to undertake 20–30 depletions to obtain approximately 300–500 μg of depleted protein. Always store the flow-through fractions (depleted and eluted) at 4°C.
16. Concentrate the depleted and eluted fractions using Microsep Centrifugal devices (at 7500 g in a fixed angel rotor (34°−45°) for approximately 8 h at 4°C. Concentration should be ceased when material from 10 depletion cycles reaches ~100 μL in volume (*see* **Note 4**).
17. Final depletion step: 100 μL of concentrated depleted serum from ten depletions are subjected to a further

depletion step (*see* Steps 8–15). If more than 100 μL are loaded, no washing step is required, samples are loaded onto the beads, eluted by centrifugation, and the column is directly loaded again.

18. Column storage: For short-term storage, after equilibration centrifuge the column at 2000×*g* for 30 s, apply the bottom plug and add 300 μL of 1X Equilibration buffer. For long-term storage prepare the storage solution: 10 μL Proteoprep Preservative Concentrate in 5 mL of 1X Equilibration Buffer. 300 μL is added as for short-term storage. Columns are stored at 4°C.

3.4. Buffer Exchange

Both concentrated fractions (i.e. the depleted-low-abundance and the bound-high-abundance fractions) require buffer exchange prior to digestion with trypsin. Several buffers are suitable for the trypsin digestion step, such as 50 mM ammonium bicarbonate buffer. We use 0.5 M sodium carbonate solution, pH 8.5.

1. Both concentrated fractions are diluted in 2 mL 0.5 M sodium carbonate solution and concentrated using Microsep 1 K Centrifugal Devices as above.

3.5. Protein Quantification

1. Protein concentration is determined using Bio-Rad Protein Assay Reagent (Bio-Rad). For the standard curve known concentrations of albumin (1–10 μg/μL) are used.
2. The low concentration range assay is used in the test tube format. 2 μL of standard or sample is added to 798 μL of MilliQ water. 200 μL of Bio-Rad reagent is added, mixed, and incubated for 10 min at room temperature.
3. The absorbance at the wavelength of 595 nm is measured in a spectrophotometer.

3.6. Quality Control – SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The protocol is based on the Mini-Protean System (Bio-Rad, Hemel Hempstead, UK).

1. Clean the glass plates to be used and assemble the gel plates in the gel casting unit.
2. Prepare the 10% separating gel by mixing 4 mL MilliQ water, 3.3 mL 30% acrylamide mix, 2.5 mL 1.5 M Tris/HCl (pH 8.8), 100 μL 10% SDS, 100 μL 10% ammonium persulphate, and 4 μL TEMED. Directly after addition of TEMED pour the gel and leave sufficient space for the stacking gel. Overlay the gel carefully with 0.1 % SDS solution. After polymerisation (~30 min) discard the 0.1 % SDS solution, rinse with MilliQ water, and air dry.
3. Prepare the stacking gel by mixing 2.7 mL MilliQ water, 670 μL 30% acrylamide mix, 500 μL 1 M Tris/HCl (pH 6.8), 40 μL 10% SDS, 40 μL 10 % ammonium persulphate,

and 4 μL TEMED. Directly after addition of TEMED pour it on top of the separation gel and insert the comb until polymerized (~30 min).

4. In the meantime prepare the sample by mixing 5–10 μg of depleted fraction with 5X sample buffer (max. loading on a 0.75 mm thick gel is 25 μL). Heat samples at 95°C for 15 min, then cool on ice, and briefly centrifuge (~15 s). The samples are ready for loading.
5. When the stacking gel is polymerized, remove the comb and place the gel into the running chamber. Fill the upper and lower gel chamber with 1X running buffer (10X diluted with MilliQ water) and wash the slots with 1X running buffer using a 1mL syringe with a 25 gauge needle attached.
6. Load the marker (5 μL PageRuler Prestained Protein Ladder) and the samples on the gel. Run the gel at 20 mA through the stacking gel and 25 mA afterwards, until the blue loading dye leaves the separation gel.
7. Stop the run and disassemble the gel unit. Discard the stacking gel and transfer the separation gel carefully into a clean container.
8. Silver staining, using the Silver-Stain Plus Kit, is undertaken according to the Bio-Rad Protocol. The gel is fixed in fixative solution (50% methanol, 10% acetic acid, 10% fixative enhancer, 30% MilliQ water) for 20 min.
9. Rinse the gel several times with MilliQ water, incubating for 10 min each time before replacing with fresh MilliQ water.
10. To stain and develop the gel with developer solution, dissolve 0.5 g development accelerator solution in 10 mL MilliQ Water. Then mix in the following order 7 mL MilliQ water, 1 mL silver complex solution, 1 mL reduction moderator solution, and 1 mL image development reagent. Combine this mix with 10 mL development accelerator solution and immediately apply to the gel after discarding the MilliQ water.
11. When the desired staining is reached, stop the reaction by incubating the gel in 5 % acetic acid solution. The protein pattern from the depleted samples should be similar, but should differ from a normal serum sample containing the highly abundant proteins (*see* **Fig. 17.2**).

3.7. Digestion with Trypsin

1. Add SDS to 200–400 μg of depleted serum in a 1.5 mL microfuge tube to a final concentration of 0.07% using a 2% stock solution (prepared in water and filter sterilized).
2. Add TCEP to a final concentration of 1.78 mM.

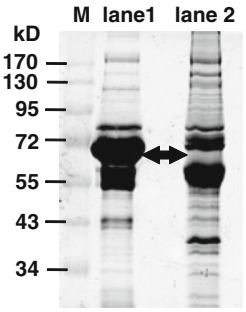

Fig. 17.2 10 μg delipidated serum (*lane 1*) and 10 μg depleted serum (*lane 2*) are separated on a 10 % SDS-Polyacrylamide gel and silver stained using the protocol described in **Section 3.6**. The Fermentas prestained protein ladder is shown (M). The albumin band (~72 kDa) is no longer visible in the depleted fraction (*arrow*). Furthermore, proteins bands not evident in non-depleted serum (*lane 1*) are visible in the depleted fraction. The gel was scanned using a GS800 densitometry scanner (Bio-Rad, Hemel Hempstedt, UK).

3. Mix the sample by vortexing and spin briefly to ensure the sample is at the bottom of the tube.
4. Incubate the sample at 60°C for 1 h and afterwards spin it to the bottom of the tube.
5. Prepare an 84 mM iodoacetamide solution (15.5 mg iodoacetamide in 1 mL MilliQ water; this has to be freshly made) and add it to a final concentration of 2.89 mM.
6. Incubate for 30 min in the dark at room temperature.
7. The trypsin is reconstituted in 20 μL per vial using the manufacturer's supplied buffer. Add 0.2 μL trypsin per μg of serum sample.
8. Mix by vortexing and incubate the serum samples overnight at 37°C.

3.8. Strong Cation Exchange Chromatography

1. 200–400 μg of depleted serum is diluted to 2 mL with diluent and loaded onto the HPLC column.
2. The gradient flow of the HPLC is 1 mL/min. The time gradient is shown in **Table 17.1**.
3. Collect eighty 1 ml fractions using 1.5 mL microfuge tubes.
4. After the run, clean the column with 100 % Buffer B to remove residual proteins and re-equilibrate with 100 % Buffer A.
5. Concentrate the HPLC fractions using vacuum concentration until there are 500 μL. Combine two fractions together (1 min + 2 min, 3 min + 4 min, etc.). Afterwards concentrate the combined fractions until completely dry. Samples may be stored at 4°C until analysed by LC-MS/MS.

Table 17.1
Buffer gradient for strong cation exchange chromatography using a flow rate of 1 mL/min

Time (min)	Buffer A (%)	Buffer B (%)
0.00	100	0
15.00	100	0
60.00	85	15
75.00	50	50
90.00	50	50
90.05	100	0
95.00	100	0

3.9. LC-MS/MS

1. For LC-MS/MS, analysis fractions are re-suspended in 180 μL Buffer A (5% acetonitrile, 0.05% trifluoroacetic acid).
2. The MS/MS system used is dependent on the instrument. This protocol is based on the use of a QSTAR Pulsar i hybrid mass spectrometer (Applied Biosystems, Warrington, UK). Samples were delivered into the instrument by an automated in-line LC (integrated LCPackings System, 5 mm C18 nano-precolumn and 75 mm × 15 cm C18 PepMap column (Dionex, CA, USA)) via a nanoelectrospray source head and 10 μm inner diameter PicoTip (New Objective, Massachusetts, USA).
3. One quarter of the sample (the ideal amount must be determined) is delivered into the QSTAR.
4. A gradient from 5% Buffer A to 48% Buffer B is applied at a flow rate of 300 nL/min for 60 min.
5. MS and MS/MS spectra are obtained using information-dependent acquisition consisting of a 1 s scan for m/z 400–2000 and the three most intense ions are selected for 1 s MS/MS scans (IDA, Analyst Software). Software such as ProteinPilot (Applied Biosystems) or other appropriate software is used to analyse the MS data.

4. Notes

1. For delipidation, a quantity of 500 μL serum is ideal, but the quantity can be scaled up or down as appropriate. As this is the first step of serum processing for LC-MS/MS analysis,

single samples or pooled serum samples can be used. Ideally, Eppendorf Lo-bind protein tubes (Eppendorf UK Limited, Histon, UK) should be used throughout the whole protocol.

2. It is best to aliquot the 10X buffers (5 mL for Equilibration and 2.5 mL for elution buffer) into 50 mL Falcon tubes under sterile conditions. The elution buffer may be cloudy, so warming it up for a couple of minutes as 37°C resolves the precipitate. After diluting the 10X to 1X we recommend a filter-sterilization step, using a 0.2 μm syringe filter (Minisart Plus, Satorius) and 50 mL syringe. Further we recommend performing all of the depletion steps under sterile conditions to avoid contamination.
3. In our studies we collect the wash fraction separately and concentrate it. We do not add this fraction to the depleted fraction, as it dilutes it and does not contribute significantly to the protein amount. If desired, the wash fraction may be added to the depleted fraction.
4. In our laboratory, several methods were tested to concentrate the depleted protein fraction. We found that the use of centrifugal filter concentration yielded minimal protein loss and optimum protein quality. However, the alternative approaches of precipitation, different types of centrifugal filter devices, and dialysis can be used.

References

1. Hanash, S. M., Pitteri, S. J., and Faca, V. M. (2008). Mining the plasma proteome for cancer biomarkers. *Nature* **452**, 571–579.
2. Schrohl, A. S., Wurtz, S., Kohn, E., Banks, R. E., Nielsen, H. J., Sweep, F. C., and Brunner, N. (2008). Banking of biological fluids for studies of disease-associated protein biomarkers. *Mol. Cell Proteomics* **7**, 2061–2066.
3. Cham, B. E., and Knowles, B. R. (1976). A solvent system for delipidation of plasma or serum without protein precipitation. *J. Lipid Res.* **17**, 176–181.
4. Anderson, N. L., and Anderson, N. G. (2002). The human plasma proteome: history, character, and diagnostic prospects. *Mol. Cell. Proteomics* **1**, 845–867.
5. Sennels, L., Salek, M., Lomas, L., Boschetti, E., Righetti, P. G., and Rappsilber, J. (2007). Proteomic analysis of human blood serum using peptide library beads. *J. Proteome Res.* **6**, 4055–4062.