MALDI MS-based biomarker profiling of blood samples

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Non-standard abbreviations: ACN, acetonitrile; CHCA, α-cyano-4-hydroxycinnamic acid; LMW, low molecular weight; MALDI, matrix-assisted laser desorption/ionization; PCS, polypeptide calibrant standard; QC, quality control; SST, system suitability test; TFA, trifluoroacetic acid; TOF, time-of-flight.

Abstract

Differential MS analysis of blood samples from diseased and control subjects is increasingly being employed in the hunt for biomarkers that can detect disease at an early stage. For diagnostic tests, in particular for population screening, robust protocols are required that can offer high-throughput analysis, ideally at high mass spectrometric sensitivity. To achieve this, blood samples need to be collected, prepared and analyzed in a standardized manner that minimizes potential bias. Simple purification methods combined with MALDI MS profiling have so far been championed for providing the best approach.

In this chapter, we describe an adapted and validated protocol based on a simple and fast solid-phase extraction technique using ZipTips®. This protocol facilitates the purification of potential blood biomarkers in a few steps for mass spectral biomarker pattern diagnostics using MALDI. It is suitable for use in an automated high-throughput and potentially clinical environment and has the advantage of only requiring a few microlitres of blood plasma or serum. The presented protocol has been tested over several years in our laboratory and found to be more reproducible and suitable for plasma and serum profiling than similar methodologies based on magnetic bead purification.

1. Introduction

The ultimate goal of biomarker research is to establish straightforward, cost-effective, minimally invasive clinical tests with high sensitivity and specificity for early disease detection. Profiling blood and blood-derived products based on mass spectrometry (MS) is becoming a powerful tool to mine the physiologically informative molecules contained in this biofluid [1-3]. By its nature and availability, blood has the potential to be an ideal medium for elucidating the current state of health. During the last few years many studies, using matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) MS coupled with bioinformatics tools, have been reported as being capable of distinguishing between the polypeptide patterns of healthy individuals and patients diagnosed with various diseases [1, 4-6]. However, none of the biomarkers identified in these studies have so far been progressed towards their clinical use [7, 8].

One of the major obstacles for the direct discovery of new biomarkers in blood is due to its complexity, which includes the presence of salts, lipids, and other biological matrix compounds and the high dynamic range of proteins, making it extremely difficult to detect and analyze disease-specific biomarkers by MS. For example, when untreated plasma or serum is analyzed on a standard MALDI target plate, it does not produce any useful spectra. Thus, to overcome these obstacles there is an obvious need for improved methods to facilitate MALDI MS analysis of blood without losing its diagnostic power. Any such method needs to be employable across many different laboratories and to incorporate high standards and quality control throughout the entire analytical workflow including sample collection, preparation, MS analysis, and data processing and mining [9-11].

As a consequence, detailed studies have been reported with the aim of standardizing protocols and reducing systematic biases. Indeed, it has been shown that many non-

biological variations can be introduced during blood sample collection, preparation, and storage, adding to possible deficiencies in study design and inappropriate data analysis approaches [3, 12-14]. Some of the resulting standardized protocols are now used with more confidence in discovering and validating new biomarkers with the hope of reducing the gap between the discovery of MS-based candidate biomarkers and their clinical application.

The protocol described in this chapter has been developed and refined over a period of more than five years, in particular for the analysis of blood serum. It has emerged from extensive tests that have been undertaken in order to establish a high-throughput protocol that provides both precise and accurate measurements. The protocol is based on MALDI-TOF MS profiling of blood samples that are purified employing commercial ZipTip® technology. As such it is a simple and robust method for providing mass spectral patterns that can be used for sample classification and, thus, potentially for clinical diagnostics and early disease detection.

The overall workflow (see Figure 1) is composed of three main steps which are (i) sample collection, (ii) sample preparation and analyte purification, and (iii) MS data acquisition and analysis. It is suitable for both manual and automated sample preparations and analyses [14]. The automated sample preparation procedures have been developed in our laboratory based on an easy-to-operate and cost-effective robotic platform, a CyBi®-disk robot (CyBio AG, Jena, Germany) equipped with a 96-piston head and modified for the use of ZipTips®. The time needed for the robotic preparation of up to 90 samples is only about 30 minutes. The emphasis of this protocol is on the collection of high-quality data, which can then optionally be combined with other biomarker data or metadata and processed using a number of different complex statistical techniques and bioinformatics. In principle, the MALDI MS profile data files typically undergo a series of data pre-processing steps (e.g. baseline subtraction, smoothing, alignment) before being subjected to various classification and test methods to identify biomarkers (that are differentially expressed in disease), which are in turn

tested either alone or in combination with additional biomarkers (and/or other data) to determine their diagnostic accuracy. Due to the vast variety and complexity of the approaches possible in this field, data processing and mining is beyond the scope of this protocol.

2. Materials

2.1. Blood collection and preparation (in clinical lab)

1. Blood collection tubes (see Note 1):
   - For plasma preparation: Tubes containing an anti-coagulant, such as Vacutainer PST tubes (BD, Franklin Lakes, NJ, USA, cat. no. 367960) or S-Monovette® tubes (Sarstedt, Newton, NC, USA, cat. no. 02.1065) for up to seven mL of blood or Microvette® tubes (Sarstedt, cat. no. 20.1309) for volumes up to 300 µL.
   - For serum preparation: Vacutainer SST tubes (BD, cat. no. 367988) or S-Monovette® tubes (Sarstedt, cat. no. 02.1063) for up to seven mL of blood or Microvette® tubes (Sarstedt, cat. no. 20.1308) for volumes up to 300 µL.
2. Chilled centrifuge capable of spinning blood collection tubes at 13,000 g.
3. Cryovials (e.g. cryostraws) and cryoboxes for sample storage (Fisher Scientific, Loughborough, UK, cat. no. FB71035).
4. Natural microcentrifuge tubes: 0.2 mL and 1.5 mL (Bioquote, York, UK, cat. nos. BA4300F and 16130).

2.2. Sample pre-processing and sub-sampling (in clinical or MS lab)

1. Standard plasma or serum (see Note 2).
2. Cryoboxes for sample storage (Fisher Scientific, cat. no. FB71035).
3. Natural microcentrifuge tubes: 0.2 mL and 1.5 mL (Bioquote, cat. nos. BA4300F and 16130).
4. 96-well microtitre plates with lids (Fisher Scientific, cat. no. TUL-962-060A).
5. Low-profile Thermostrips (ABgene, Epson, UK, cat. no. AB0773).

6. Virkon® disinfection solution (Fisher Scientific, cat. no. HYG-205-030J). See Note 3 for solution preparation.

2.3. Analyte extraction, purification and analysis (in MS lab)

1. Acidification solution (2% TFA, pH ≈ 0.5).
2. Equilibration and washing solution (0.1% TFA, pH ≈ 2).
3. Elution solution (50% ACN/0.1% TFA).
4. α-cyano-4-hydroxycinnamic acid (CHCA) (Bruker Daltonics, Bremen, Germany, cat. no. 201344).
5. Standard plasma or serum (same as in the previous section).
6. Protein and peptide MS calibration standards (Bruker Daltonics, cat. nos. 206355 and 222570).
7. C18 ZipTips® (Millipore, supplied by Fisher Scientific, cat. no. FDR-597-040G).
8. Natural microcentrifuge tubes: 0.2 mL and 1.5mL (Bioquote, cat. nos. BA4300F and 16130).
9. 96-well microtitre plates (Fisher Scientific, cat. no. TUL-962-060A).
10. 30 mL-Teflon® bottle (Fisher Scientific, cat. no. BTK-260-010V).
11. pH 0-6 indicator sticks (Fisher Scientific, cat. no. FB33005).
12. Isofreeze PCR rack (Alpha-Laboratories, Eastleigh, UK, cat. no. LW5990Y).
13. AnchorChip-600 384-spot MALDI target plates (Bruker Daltonics, cat. no.209513).

3. Methods

Users of this protocol should have read and understood all relevant Risk Assessments and Health and Safety rules and regulations and SOPs for the handling of blood and blood-derived samples before starting any work.

The various procedures detailed in the following protocol can be grouped into four independent steps:

(i) Phlebotomy and plasma/serum generation and separation which usually occur in clinical laboratories or health care centres.

(ii) Sub-sampling the plasma/serum samples which can be performed either immediately after blood collection at the collection site or later in the MS laboratory. At the end of this step small, ready-to-analyse sample volumes are stored in microtitre plates.

(iii) Purification of analytes (mainly polypeptides) from plasma/serum: This part of the protocol is critical and involves binding the analytes to a chromatographic phase followed by a washing step at which non-bound compounds are removed. The analytes are then eluted off the chromatographic support before being mixed with the matrix and spotted onto a MALDI target plate. This step is carried out in the MS laboratory (see Figure 2).

(iv) MS data acquisition and analysis: This last step consists of collecting MALDI mass spectra under strict QC procedures from the samples prepared above (see Figure 2). The obtained data are then analyzed using appropriate software and bioinformatics tools.

3.1. Collecting blood samples and preparing plasma/serum

1. Collect blood in the appropriate tube (either for plasma or serum) and label it with the corresponding sample details. Gently mix the blood by inverting the tube 10 times (see Note 4). The following steps are suitable for blood volumes between tens of microlitres and tens of millilitres. For other amounts of blood, this protocol (including consumables and equipment) might need to be adjusted.

2. *(a)* For plasma preparation:

   - Centrifuge the blood sample at 13,000 g for 5 min at 4°C.

   - Decant the plasma (top layer) to appropriately sized and labelled cryovials, taking care not to disturb the middle whitish layer (mononuclear cells and platelets) or the bottom layer (red blood cells).

   (b) For serum preparation:

   - Allow blood to clot for 1 h at room temperature keeping the tube in the upright position (see Note 5).

   - Spin tubes at 1,400 g for 10 min at room temperature.

   - Transfer the serum (upper phase) to appropriately sized and labelled cryovials. If resources (time, staff and sample storage) allow, as many aliquots of the appropriate volume as possible should be made in order to minimize freeze-thaw cycles.

3. Immediately store all samples at –80°C.

4. Transport samples to the MS laboratory on dry ice. On arrival they must be immediately stored at –80°C (see Note 6).

3.2. Pre-processing and sub-sampling of plasma/serum samples

This protocol assumes that plasma or serum samples will be sub-sampled into microtitre plates before the analyte extraction procedure. The following steps detail this procedure (see Note 7).

1. Remove the sample tubes from the freezer, and allow the samples to thaw on an ice-bath (see Note 8).

2. Randomize samples before any further steps are taken to avoid any systematic error due to the sample order or history.

3. Transfer the samples from cryostraws to microcentrifuge tubes (this step is only necessary when cryostraws are used such as for long-term, large-scale sample storage):

   - Remove the first straw from the freezer and scan the barcode on the straw or, if no bar codes are used, write down the sample details.

   - Using scissors, cut the straw just below the closure at the top.

   - Invert the straw and place the cut end in a 1.5 mL-tube. Now cut the other end of the straw and dispose of the cut-off pieces in the Virkon® solution. Wash the scissor blades with ethanol.

   - Lift the straw slightly to allow the thawed serum to drain into the tube. Use the pipettor with a tip attached to blow air through the straw from the top down, in order to expel the last few drops of plasma/serum into the tube. Place the empty straw into the Virkon® solution. Use the pipettor with the same tip still attached to mix the content of the tube, ensuring that the whole sample is thoroughly thawed and homogeneous.

4. Sub-sampling from microcentrifuge tubes to microtitre plates:

   - Transfer 5 µL of plasma/serum to the corresponding well in one or more microtitre plates as required, leaving on each microtitre plate 6 wells vacant for the use of control samples as detailed later (see Note 9). Record the position of each sample on a microtitre plate plan (see Note 10).

   - From the remaining volume sub-sample as many aliquots as required into 200 µL-tubes or thermostrips (these can be later used for single point analysis).

   - Pipette 5 µL-aliquots of the standard plasma/serum sample (thawed and homogenized) to 3 of the 6 empty wells on the microtitre plates containing samples and record their position on the microtitre plate plans. This standard sample will be used as initial control for the whole run. The 3 remaining empty wells are later used for post-preparation control samples (see SST solution, section 3.3.1).

5. Cover each microtitre plate with a cover lid and secure the lid in place with a rubber band. Put each plate inside a small clear plastic bag, and place the bags together with a copy of the plate plan in a labelled sample box.

6. Immediately freeze the samples and store them at -80°C until required.

3.3. Extracting analytes from plasma/serum samples

This protocol describes the manual extraction of mainly polypeptidic analytes from blood plasma/serum which is suitable if the number of samples is just a few. It requires the samples to be in a microtitre plate format suitable for the use of an eight-channel pipettor. However,

this protocol can also be applied using single tubes but needs to be adjusted accordingly. For large sample sets and high throughput extraction a liquid handling robot is recommended.

3.3.1. Preparation of solutions (see Note 11)

1. Matrix solution (0.5 mg/mL CHCA in ethanol:acetone; 2:1): Add 1 mL of acetone to an 1.5 mL-tube containing 3 mg of CHCA and vortex until the CHCA is dissolved. Add 4 mL ethanol and 1 mL acetone to a 30 mL-Teflon® bottle, then add the CHCA solution to give a total volume of 6 mL. Mix the solution and keep at 4°C in the dark until needed.

2. Polypeptide Calibrant Standard (PCS) solution: Add 125 µL of the 0.1% TFA solution to a vial of peptide calibration standard (see Materials for details) and vortex thoroughly to solubilize the peptides. Do the same with a second vial for the protein calibration standard (see Materials for details). Transfer 25 µL from the first vial to a 1.5 mL-tube and add all the contents of the second vial plus 600 µL of 0.1% TFA. Mix and aliquot at 4 µL. Store the aliquots at -20°C (see Table 1 for a list of PCS components).

3. System Suitability Test (SST) solution: A 96-well plate containing 5 µL of the standard plasma/serum in each well is prepared in advance using the steps detailed in section 3.3.2. (steps 1.-4.; see below). Sample eluates are pooled, mixed and then aliquotted at 4 µL. Aliquots are stored at -80 °C until required (see Note 12).

3.3.2. Sample clean-up and MALDI sample preparation

1. Preparing the samples

   - Remove the aliquotted samples from the freezer and thaw them on an Isofreeze PCR rack keeping their temperature at no more than 4°C.

   - Immediately after thawing, add 5 µL of 2% TFA to each sample well and aspirate/dispense several times to ensure good mixing.

2. Preparing the ZipTips® (see Note 13).

   - Load 8 ZipTips® onto a multi-channel pipettor.

   - Aspirate 10 µL of ACN onto each ZipTip® and dispense it to waste. Repeat twice.

   - Aspirate 10 µl of 0.1 % TFA onto each ZipTip® and dispense it to waste. Repeat twice.

3. Binding and washing the analytes

   - Slowly aspirate and dispense the sample solutions back into the same well. Repeat 9 times.

   - Aspirate 10 µL of 0.1 % TFA onto each ZipTip® and dispense it to waste (or collect if the washing fractions are to be analysed). Repeat twice.

4. Eluting the samples

   - Elute the analytes bound to each ZipTip® with 7 µL of 50 % ACN / 0.1 % TFA by aspirating/dispensing the eluent 5 times through the ZipTips® into the wells of a new microtitre plate and keep it on the Isofreeze PCR rack.

5. Mixing the eluates with the matrix

- Transfer 18 µL of matrix solution into the wells of a new microtitre plate.

- Add 2 µL of each eluate to the 18 µL of matrix solution. Aspirate/dispense at least 5 times to mix.

- Immediately spot 0.8 µL of the mixtures onto the anchors of the MALDI target. Spot each eluate 4 times, leaving 12 random anchors vacant for SST samples (see Notes 12 and 14).

6. MALDI calibrant and control sample preparation

- Transfer 27 µL of the matrix solution to two 200 µL-tubes and close the lids tightly. Take a 4 µL-aliquot of each of the SST and PCS solutions from the freezer and let them thaw at room temperature.

- Vortex the PCS solution and add a 3 µL-aliquot of it to one of the tubes containing matrix solution, and vortex/mix thoroughly. Spot 0.8 µL of this mixture onto each of 10 random calibrant positions on the MALDI target plate. These spots are used for external calibration. The amount of each polypeptide spotted is ca. 10 fmol for peptides and ca. 50 fmol for proteins.

- Vortex the SST solution and add a 3 µL-aliquot of it to the other tube containing matrix solution, and vortex/mix thoroughly. Spot 0.8 µL of this mixture onto their allocated positions (12 per MALDI target plate, see Note 9). These spots will be used to assess the performance of the MALDI sample preparation and MS analysis (see details below in section 3.4.2).

- Wait until all MALDI sample spots are completely dry (see Note 14).

3.4. MALDI MS data acquisition and analysis

3.4.1. Data acquisition

This protocol describes a typical workflow employing an Ultraflex II MALDI-TOF/TOF instrument (Bruker Daltonics) in the linear positive ion mode controlled by the FlexControl software v.3.0 with samples spotted on AnchorChip target plates having 384 anchors of 600 μm diameter.

1. Open the FlexControl software and load optimised FlexControl and AutoXecute methods (see Table 2 and Note 15). These settings must be optimised for each individual instrument.
2. Insert the MALDI target plate into the mass spectrometer. The plate should be analysed within 2 hours of MALDI sample spotting.
3. Calibrate the mass spectrometer using a single calibrant sample spot containing the PCS mixture (see Note 16). Data should be collected automatically by clicking on the tab "Run method on current spot".
4. Collect spectra from an SST sample spot to check that shots are accumulated and the AutoXecute evaluation criteria are met (see Note 17 and Figure 2). If this is not the case, re-adjust the laser power accordingly and collect spectra from another SST spot to fulfil these conditions. Data should be collected automatically by clicking on the Tab "Run method on current spot". A typical mass spectrum obtained from a pooled human serum sample is shown in Figure 3.

5. Load the correct autoXsequence file (containing the names and positions of each sample on the target as well as the file name and path where the spectra will be saved on the hard disk). This file can be generated by using the spreadsheet from the sample plate plan initially prepared.

6. Start the automatic run by clicking on the tab "Start automatic run". Data collection for a full 384-spot plate (spotted from a full 96-well microtitre plate) takes about 6 hours.

3.4.2. Data quality control and filtering/processing

In the following steps FlexAnalysis 3.0 and ClinProTools 2.1 software (Bruker Daltonics) are used.

1. Within the FlexAnalysis program, open the folder containing all the acquired spectra by choosing the "Process" menu, then selecting "Batch check" and clicking on "Folder". Within the next window click on "Browse" and select the batch of sample data folders. Click on "Start Batch Check" and set the expected number of shots to 1,000. By clicking "OK" this will generate a report detailing the spectra with less than 1,000 shots which should then be excluded from further processing.

2. Within the ClinProTools program, load the spectra files of the SST samples by selecting "Open Model Generation Class" under the "File" menu. View the spectra in the pseudo-gel view.

3. Load the spectra of the standard plasma/serum samples, which will be depicted in the pseudo-gel view alongside the spectra of the SST samples. The 2 classes should give virtually identical spectra, in which case the overall preparation of the batch of

samples is deemed acceptable. If the spectra are markedly different or two or more spectra per sample have been excluded further investigation will be needed to determine the cause.

4. Load the spectra of each individual sample as a class into ClinProTools, and inspect them visually to determine any obvious differences within that class. If differences are found it may be necessary to exclude the entire data set for this sample on the basis of poor quality (see Note 18).

3.4.3. Preliminary Data Analysis

Curated spectra sets can be analysed using ClinProTools or exported from FlexAnalysis as tab-separated 2-column text files that contain the mass list with the corresponding intensities for use with other software packages for more detailed analysis of the data. For instance, ClinProTools can be used to determine peaks that are significantly different (in intensity or area) between 2 or more classes of spectra, by using the average spectra and clustering feature. It is also possible to use this program to generate other classification models (for example, Genetic Algorithms, Support Vector Machine) based on lists of discriminatory peaks. However, this higher level of data analysis and data mining is beyond the scope of this protocol.

4. Notes

1. Recommendations vary for the best anti-coagulant and whether to use plasma or serum [15]. For plasma analysis, this protocol has been developed using small mouse blood volumes collected with microvette® tubes (Sarstedt, Newton, NC, USA, cat. no. 20.1309) containing heparin as anti-coagulant. For serum analysis, this protocol has been developed using human serum obtained from blood collected with Vacutainer SST tubes (BD, USA, Franklin Lakes, NJ, cat. no. 367988).

2. For plasma analysis, this protocol has been developed with commercial standard mice plasma from C57BL6 mice (Patricell, Nottingham, UK, cat. no. IMS-C57BL6-N-12). For serum analysis, this protocol has been developed with commercial standard human serum (Sigma-Aldrich, Dorset, UK, cat. no. S7023).

3. Virkon® solution: Add 10 g of Virkon® powder to one litre of water. Mix thoroughly until completely dissolved, resulting in a pink coloured solution. Replace the solution when the pink colour has gone (about one week).

4. When collecting blood samples, a proper phlebotomy technique should be applied. Ideally, the same amount of blodd is collected for each sample. Haemolysis should be avoided at this step as well as any other (subsequent) steps.

5. Blood should be allowed to clot for exactly 1 hour, otherwise differences in the mass spectral profiles might arise due to different clotting times of the samples.

6. Avoid freeze-thaw cycles at all steps because they induce proteolysis (in the absence of anti-proteases) and the precipitation of peptides/proteins.

7. All sub-sampling must be carried out in an adequate safety cabinet. Equipment and consumables used for the purpose of sub-sampling should be housed inside the cabinet and should be labelled with biohazard labels (tubes, pipette tips, solutions, etc.). Before starting work, ensure that there are two 1-litre Virkon® solutions available that are less than one week old and still pink. One of the solutions should be placed in a wide-mouthed screw cap jar for disposal of plastic consumables, and the other should be in a screw cap bottle for dealing with accidental spills.

8. The amount of time that individual samples are outside of the -80°C freezer must be kept to a minimum. All sample vials, tubes and plates must be kept on wet ice or Isofreeze PCR racks during the entire sub-sampling procedure. Standard plasma/serum samples should be initially aliquotted at 200 and 500 µL into 500-µL tubes and stored at -80 °C until required.

9. The sample volume available determines the number of replicates that can be prepared as 5 µL is needed for each preparation. All samples with a total volume of less than 5 µL should be excluded. It is preferable to prepare each sample at least 3 times. However, if this is not possible then for each individual sample as many replicates as possible should be made. Each 96-well microtitre plate is used for up to 90 sample aliquots to be run manually or on a liquid handling robot. The remaining 6 wells are vacant for the use of control samples as detailed later. Briefly, 3 vacant wells will be filled with standard plasma/serum samples that will provide controls for the entire sample preparation workflow. The other 3 vacant wells will remain empty in case the microtitre plate is used in an automated workflow. From these wells no samples will be prepared or spotted on the respective MALDI target plate positions, which then can be used for the preparation of SST samples (see Note 12).

10. In an automated workflow for large sample sets, the sub-sampling procedure can be carried out in two steps. First from tubes into parent microtitre plates, then from parent to child (replicate) microtitre plates. For example, in our lab we use a liquid handling CyBi®-Disk robot (CyBio AG) equipped with a 96-piston head for 25 µL-tips and 10 microtitre plate positions, specifically set-up for this task.

11. The matrix solution should be freshly prepared on the day of the experiment. The solutions of 2% TFA, 0.1% TFA and 50% ACN/0.1% TFA should be replaced weekly.

12. SST solutions/samples are used as controls to check both the performance of the mass spectrometer and the quality of the MALDI sample preparation.

13. Ensure that ZipTips® do not dry out or aspirate air during the entire procedure.

14. Ensure that the relative humidity in the lab is between 35–60% during MALDI sample preparation (incl. crystallisation). Very low or very high humidity dramatically affects the crystallization and thus, the MALDI MS ion signal.

15. Data acquisition should be automated. In this protocol, an automated workflow is provided for an Ultraflex II TOF/TOF instrument using the instrument's 'AutoXecute' function. Each spectrum is the sum of 1,000 single laser shots randomized over 10 positions within the same spot (100 shots/position) at a laser frequency of 25 Hz. Before each acquisition cycle of 100 laser shots, the new position on the target spot is pre-irradiated with 10 laser shots at 10% higher laser power to improve spectral quality. Evaluation parameters are set so that only spectra (of 100 shots) containing at least one peak with a resolving power of greater than 300

and a signal-to-noise ratio of more than 10 in the m/z range of 700-10,000 are accumulated.

16. The calibration can be checked on a second PCS spot and repeated if necessary.

17. Prior to analyzing an entire MALDI target, an SST sample spot should be used for adjusting the laser power and as a general system check. Using this control sample, the resolution and intensity of five major peaks across the mass range 1,800 to 8,200 Da are typically checked against previously obtained data. If necessary, the laser energy is adjusted to keep the intensity and resolution of these peaks within a range of ± 2 standard deviations. In addition, the overall performance of the mass spectrometer should be thoroughly checked every 2-3 weeks using peptide standards.

18. If the number of replicate spectra per sample falls below 3 as a result of spectra exclusion, it may be necessary to repeat the sample preparation, if this is possible.

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Table 1. Polypeptide calibrant list used for external calibration

Polypeptide	Ion type	Average mass m/z
Peptide calibration standard II mixture *(Bruker Daltonics, #222570)*		
Bradykinin Fragment 1-7	$[M+H]^+$	757.858
Angiotensin II	$[M+H]^+$	1047.189
Angiotensin I	$[M+H]^+$	1297.486
Substance P	$[M+H]^+$	1348.642
Bombesin	$[M+H]^+$	1620.860
Renin Substrate Tetradecapeptide Porcine	$[M+H]^+$	1760.026
ACTH clip 1-17	$[M+H]^+$	2094.427
ACTH clip 18-39	$[M+H]^+$	2466.681
Somatostatin 28	$[M+H]^+$	3149.574
Protein calibration standard I mixture *(Bruker Daltonics, #206355)*		
Insulin	$[M+H]^+$	5734.56
Ubiquitin I	$[M+2H]^{2+}$	4283.45
Cytochrome C	$[M+2H]^{2+}$	6181.05
Ubiquitin I	$[M+H]^+$	8565.89

Table 2. Ultraflex instrument settings optimized for the analysis of LMW plasma/serum analytes, using FlexControl software v3.0

Parameters	Values
Laser source	Nitrogen (337nm)
Digitizer	2 GHz
Mass range for data acquisition	700 -10,000 Da
Acquisition mode	Linear, positive ions
Ion acceleration	25 kV
Delayed extraction potential difference	1.6 kV
Lens potential	6 kV
Gating strength for matrix suppression	High (for ions <400Da)
Delayed extraction	100 ns
Detector gain	7.5
Sample rate	0.5 GS/s
Data points per spectrum	32,163

Figure Captions

Figure 1. Workflow showing the major steps required for MS profiling and biomarker discovery from blood.

Figure 2. Workflow diagram showing the various steps of plasma/serum sample preparation using ZipTips®and quality control before MS data acquisition.

Figure 3. MALDI-TOF MS profile of a pooled human blood serum sample obtained from purification of a 5 µL-aliquot using 18-ZipTips®. The mass spectrum was generated by ClinProTools software (V2.1) after baseline-subtraction, smoothing and normalization.

Index

Reversed-phase chromatography

Sample classification

Sample preparation

Serum

ZipTip® technology

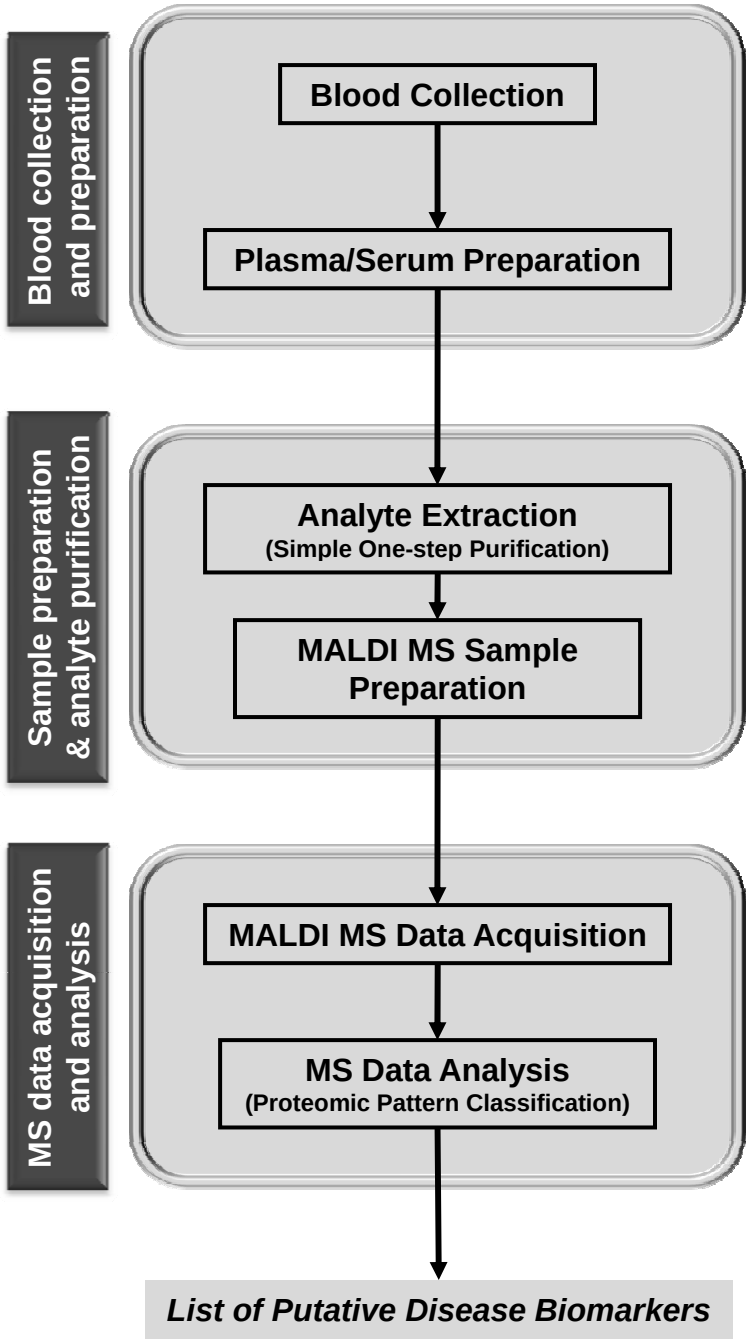
Blood collection and preparation
Blood Collection
Plasma/Serum Preparation
Sample preparation & analyte purification
Analyte Extraction
(Simple One-step Purification)
MALDI MS Sample Preparation
MS data acquisition and analysis
MALDI MS Data Acquisition
MS Data Analysis
(Proteomic Pattern Classification)
List of Putative Disease Biomarkers

Figure 1

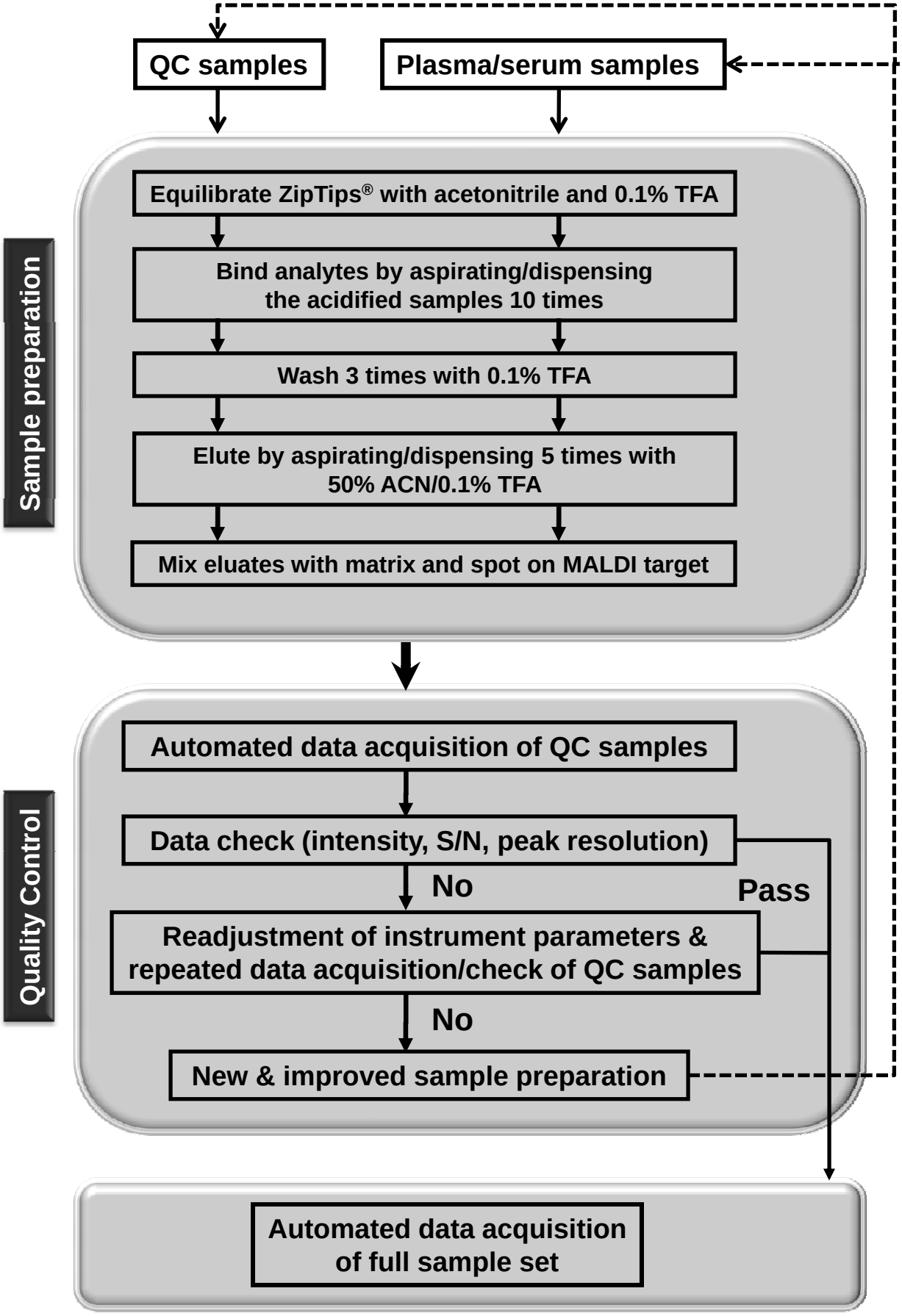
QC samples
Plasma/serum samples
Sample preparation
Equilibrate ZipTips® with acetonitrile and 0.1% TFA
Bind analytes by aspirating/dispensing the acidified samples 10 times
Wash 3 times with 0.1% TFA
Elute by aspirating/dispensing 5 times with 50% ACN/0.1% TFA
Mix eluates with matrix and spot on MALDI target
Quality Control
Automated data acquisition of QC samples
Data check (intensity, S/N, peak resolution)
No
Pass
Readjustment of instrument parameters & repeated data acquisition/check of QC samples
No
New & improved sample preparation
Automated data acquisition of full sample set

Figure 2

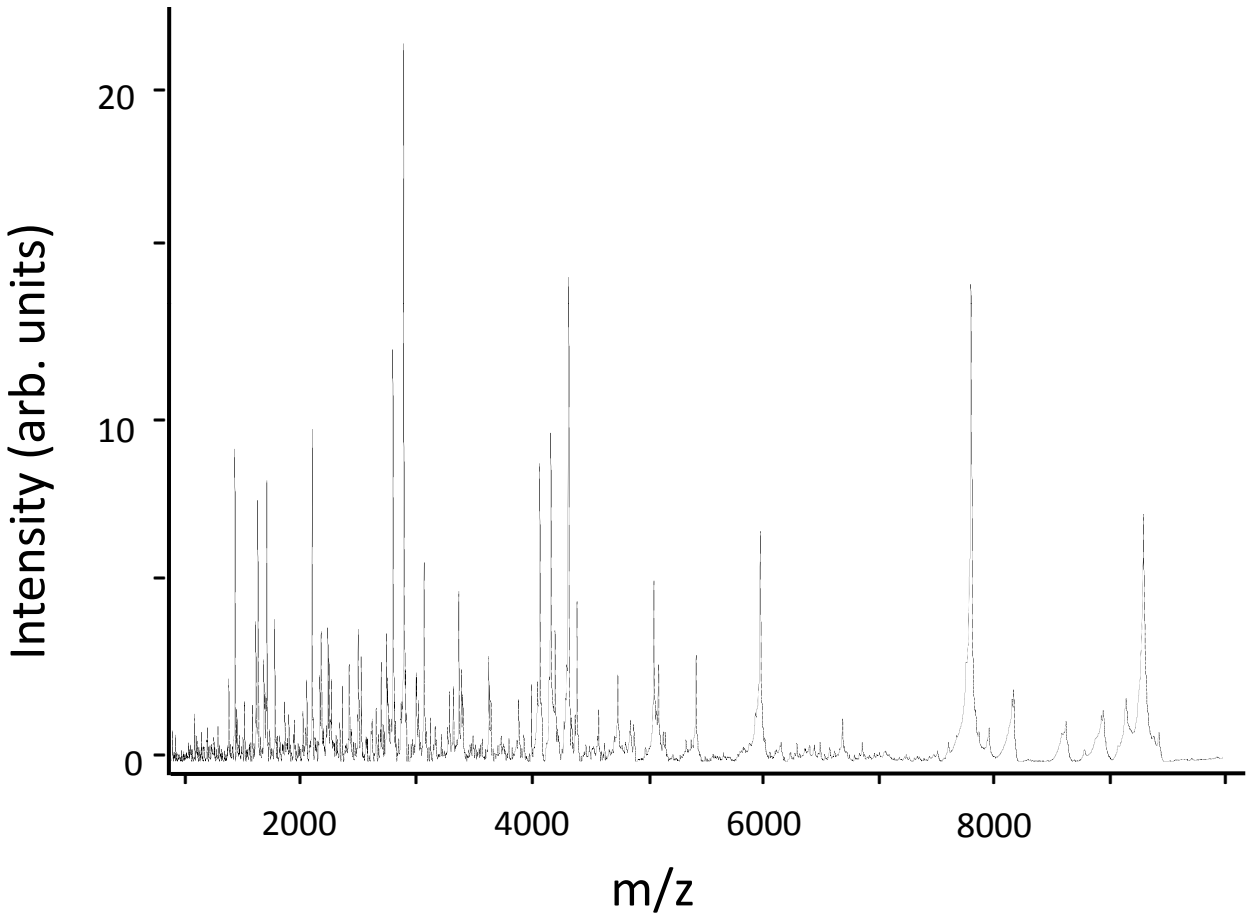

Figure 3