

# PROTEOMICS

## Practical Proteomics

### Supporting Information for Practical Review

DOI 10.1002/pmic.200700746

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### **Serum Peptide Profiling using MALDI Mass Spectrometry**

Avoiding the Pitfalls of Coated Magnetic Beads using  
Well-Established ZipTip Technology

### **Experimental Details**

Supplemental Material

## Materials and Reagents

Hydrophobic C8, C18 and weak cation-exchange magnetic beads were part of the purification kits MB-HIC C8 (MB-C8), MB-HIC C18 (MB-C18) and MB-WCX respectively, and were purchased together with  $\alpha$ -cyano-4-hydroxycinnamic acid (CHCA) from Bruker Daltonics (Coventry, UK). RPC18 Dynabeads (DB-C18) were purchased from Invitrogen (Paisley, UK). Pre-packed C4- and C18-ZipTips were purchased from Millipore (Watford, UK), and pre-packed C4- and C18-OMIX tips were kindly supplied by Varian (Oxford, UK). The 96-well magnetic bead separator (MBS) was purchased from Bruker Daltonics.

The 0.2 ml thin-walled PCR thermostrips and 96-well microtitre plates were purchased from ABgene (Epsom, UK), while the 384-well microtitre plates were purchased from Greiner Bio-One (Glos., UK). Microcentrifuge tubes (200  $\mu$ l) were purchased from BioQuote (York, UK). All solvents and water were of HPLC grade and were purchased from Rathburn Chemicals Ltd (Walkerburn, UK). The Fakir Ultrasonic Humidifier LB10 was purchased from Denis Rawlins (Sutton Coldfield, UK). Commercial, pooled serum standards were purchased from Sigma-Aldrich Ltd (Dorset, UK, # S7023, lots 034K8937 and 015K8906) and PromoCell GmbH (Heidelberg, Germany, C-37548, lot C02005-0424).

The set of 30 clinical serum samples used in this study were collected from post-menopausal women as part of the UK Collaborative Trial of Ovarian Cancer Screening (UKCTOCS) (<http://www.ukctocs.org.uk/>). Approval was obtained from the local ethics committees for using these serum samples in this study.

## Serum Purification

Samples were prepared both manually and automatically, using either a robot for pre-packed tips (CyBi<sup>TM</sup>-Disk, CyBio AG, Germany) or a robot for magnetic beads (ClinProt, Bruker Daltonics). The robotic workflows are virtually identical to the workflows performed

manually, apart from the inherent differences due to the switch-over to automation (*e.g.* simultaneous spotting, marginally shorter time frames, *etc.*). Figure 1 of the main article shows the typical workflow diagrams for reversed phase magnetic beads and for reversed phase pre-packed tips.

In order to assess intra-run reproducibility, each sample was prepared in triplicate within the same run. For evaluation of inter-run reproducibility at least 3 sample replicates were also prepared, but in separate, independent runs. Each sample replicate was mixed with matrix and spotted at least 4 times on the MALDI target. A commercial, pooled serum standard was prepared at least once within each batch of samples as a quality control sample. All preparations were performed at room temperature.

## **Protocols using Pre-packed Tips**

### ***Manual Preparation using ZipTips***

Aliquots of 5  $\mu$ l serum were acidified with 5  $\mu$ l of 2% TFA, desalted and cleaned with C4- or C18-ZipTips according to an adapted method from the manufacturer's protocol. An 8-channel pipettor was used to prepare 8 samples simultaneously. Each of the solvents and acids used in the procedure were aliquotted into each well of a series of 8-well thermostrips. ZipTips were fitted to the 8-channel pipettor and wetted using 3 $\times$ 10  $\mu$ l of 100% acetonitrile (ACN), before being equilibrated with 3 $\times$ 10  $\mu$ l of 0.1% TFA. The acidified serum samples were then slowly aspirated and dispensed in the same well (10 cycles) followed by 3 washes with 10  $\mu$ l of 0.1% TFA in water. Finally, peptides were eluted from the ZipTips by aspirating and dispensing (5 times) 7  $\mu$ l of 50% ACN, 0.1% TFA in water.

### ***Manual Preparation using OMIX tips***

This protocol is essentially identical to the previously described ZipTip protocol with the difference of employing 50% instead of 100% ACN for the wetting step and 1% instead of 0.1% TFA for the equilibration step.

### ***Automated Preparation using ZipTips***

A CyBi™-Disk robot equipped with a 96-piston head for 25 µl tips was adapted for automation of the manual ZipTip protocol. The robot was used to aliquot the equilibration and washing solutions from reservoirs into 384-well microtitre plates. All of the various solutions and serum samples used during the protocol were pipetted into 96- or 384-well microtitre plates and, together with the MALDI target, these were placed on the 10 different plate stations of the rotating platform of the robot. To avoid evaporation of solvents, the microtitre plates containing ACN or acetone for equilibration, elution or matrix solutions were kept with their covering lids in place at 4°C until use. During the robotic run a couple of simple manual interventions were needed to replace the standard tip magazine with the ZipTip magazine and *vice versa*.

### **Protocols using Magnetic Beads**

#### ***Sample Preparation using MB-C8 and C18 Hydrophobic Magnetic Beads***

The MB-C8 and C18 beads are paramagnetic non-porous particles modified with hydrophobic C8 (MB-C8) and C18 (MB-C18) reversed phase chromatographic material. The binding and washing solutions were provided as part of the beads' kits and are proprietary. Essentially, the same protocol was used both for the manual and the automated runs, except that in the manual preparation 0.2 ml thin-walled PCR thermostrips were used instead of microtitre plates.

The manufacturer's protocol was employed for preparing serum samples. Briefly, 10 µl of the binding solution and 5 µl of serum were transferred to a 96-well microtitre plate. A 5µl-aliquot of the homogeneous magnetic particle solution was added, mixed, and left for 1 min. The microtitre plate was placed in the MBS for 20 s for migration of the magnetic beads to the well walls. The supernatant was discarded, the microtitre plate was removed from the MBS device, and 100 µl of wash solution was added and carefully mixed with the magnetic beads. The microtitre plate was then moved 20 times back and forth between adjacent wells on each side of the magnetic bar in the MBS device, enhancing the washing of the beads by moving them from wall to wall through the washing solution. After 30 s in the MBS device, the supernatant was removed by aspiration. The entire washing procedure was repeated twice. After the final washing step, bound molecules were eluted from the magnetic beads by mixing and incubating

with 10  $\mu$ l of 50% ACN, 0.1% TFA in water for 1 min. The microtitre plate was placed back onto the MBS device, and after standing for 1 minute the eluate was transferred to a 200  $\mu$ l-microcentrifuge tube.

#### ***Sample Preparation using MB-WCX Weak Cation Exchange Magnetic Beads***

The MB-WCX beads are paramagnetic non-porous particles modified with weak cation-exchange chromatographic material. The binding, washing, elution and stabilization solutions were provided as part of the beads' kits and are proprietary.

As before, the manufacturer's protocol was employed for preparing serum samples. Briefly, 10  $\mu$ l of the binding solution and 10  $\mu$ l of the homogeneous magnetic particle solution were transferred to each well of a thermostrip. A 5 $\mu$ l-aliquot of serum was added and the mixture was mixed up and down with a pipette and left for 5 minutes.

The thermostrip was placed in the MBS for 1 minute for migration of the magnetic beads to the well walls. The supernatant was discarded and the thermostrip was removed from the MBS device and 100  $\mu$ l of wash solution was added and carefully mixed with the magnetic beads. The thermostrip was then moved 10 times back and forth between adjacent wells on each side of the magnetic bar in the MBS device. After 30 s in the MBS device, the supernatant was removed by aspiration. The entire washing procedure was repeated twice. After the final washing step, bound molecules were eluted from the magnetic beads by mixing and incubating with 5  $\mu$ l of elution solution for 1 min. The thermostrip was placed back into the MBS device, and after standing for 1 minute the eluate was transferred to a 200  $\mu$ l-microcentrifuge tube. Finally, 5  $\mu$ l of the manufacturer's proprietary stabilization solution was added to the eluate.

#### ***Sample Preparation using DB-C18 Hydrophobic Magnetic Beads***

The DB-C18 beads are paramagnetic non-porous particles modified with hydrophobic C18 reversed phase chromatographic material. No proprietary solutions were supplied with the beads. For both binding and washing solution 0.1% TFA in water was used. The elution solution was 50% ACN, 0.1% TFA in water.

A slightly modified version of the manufacturer's protocol was employed for preparing serum samples. Before using the beads they were pre-washed. An aliquot of 10  $\mu\text{l}$  of the homogeneous magnetic particle solution was transferred to a thermostrip and placed in the MBS for 1 min. The supernatant was discarded, the thermostrip was removed from the MBS device, and 50  $\mu\text{l}$  of washing solution was added and carefully mixed with the magnetic beads. The thermostrip was then moved 20 times back and forth between adjacent wells on each side of the magnetic bar in the MBS. Subsequently, the supernatant was discarded after a rest period of 1 min. The entire pre-washing procedure was repeated twice.

After this pre-washing step, 15  $\mu\text{l}$  of binding solution was added and carefully mixed with the washed magnetic beads using a pipette. An aliquot of 5  $\mu\text{l}$  of serum was acidified with 5  $\mu\text{l}$  of 2% TFA in water, added to the bead suspension, mixed thoroughly and left for 2 min for binding to occur. The thermostrip was placed in the MBS for 1 minute for migration of the magnetic beads to the well walls. The supernatant was discarded and the thermostrip was removed from the MBS device. An aliquot of 50  $\mu\text{l}$  of wash solution was added and carefully mixed with the magnetic beads. The thermostrip was then moved 10 times back and forth between adjacent wells on each side of the magnetic bar in the MBS device. After 30 s in the MBS device, the supernatant was removed by aspiration. The entire washing procedure was repeated twice. After the final washing step, bound molecules were eluted from the magnetic beads by mixing and incubating with 5  $\mu\text{l}$  of elution solution for 2 minutes. The thermostrip was placed back onto the MBS device, and after standing for 1 minute the eluate was transferred to a 200  $\mu\text{l}$ -microcentrifuge tube.

## **Mass Spectrometry**

Ground steel and 600  $\mu\text{m}$ -AnchorChip<sup>TM</sup> target plates (both from Bruker Daltonics) were used for MALDI sample spotting. The AnchorChip<sup>TM</sup> target plates have 384 anchors of 600 $\mu\text{m}$  diameter, which are arranged in groups of 4 anchors around a central calibrant anchor. Ground steel plates have no specific spot positions for calibrants. For sample spotting on AnchorChip<sup>TM</sup> targets, an aliquot of 2  $\mu\text{l}$  of the sample eluate was mixed with 18  $\mu\text{l}$  of freshly prepared matrix solution (0.5 g/l CHCA in ethanol:acetone 2:1) in a 200  $\mu\text{l}$ -microcentrifuge tube. Four volumes of 0.8  $\mu\text{l}$  of this mixture were immediately spotted onto a 600  $\mu\text{m}$ -

AnchorChip™ target plate and allowed to dry. For sample spotting on ground steel targets, an aliquot of 3 µl of the sample eluate was mixed with 3 µl of freshly prepared matrix solution (5 g/l CHCA in ACN:0.1% TFA in water 2:1) in a 200 µl-microcentrifuge tube. Four volumes of 1 µl of this mixture were immediately spotted onto a ground steel target plate and allowed to dry.

Serum mass spectrometric profiles were obtained using an Ultraflex MALDI-TOF mass spectrometer (Bruker Daltonics) controlled by FlexControl software v.2.4 (Bruker Daltonics) and equipped with a pulsed ion extraction ion source, a 337 nm nitrogen laser and a 2 GHz digitiser. Spectra were acquired for the mass-to-charge ( $m/z$ ) range of 700 -10,000 using instrument settings optimized for serum analysis. Spectra were acquired in linear positive mode with 25 kV of ion acceleration (with a DE potential difference of 1.3kV), 6 kV lens potential, and high gating strength to deflect ions with a mass below 400  $m/z$ . Delayed extraction was applied at 100 ns to give appropriate time-lag focusing after each laser shot. For signal detection, the detector gain was set to 7.5 and the sample rate to 0.5 GS/s which gives 32,163 points per spectrum.

Data acquisition was automated using the instrument's 'AutoXecute' function. Each spectrum was the sum of 1,000 single laser shots randomised 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 was pre-irradiated with 10 laser shots at 10% higher laser power to improve spectral quality. Evaluation parameters were 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 2,000-6,000 were accumulated.

The mass spectrometer was externally calibrated in the linear positive mode between 750 and 8,600 Da using a single calibrant spot containing 13 fmoles of the peptide calibrant standard II (#222570, Bruker Daltonics) and 67 fmoles of the protein calibrant standard I (#206355, Bruker Daltonics).

The overall performance of the mass spectrometer was thoroughly checked every 2-3 weeks using peptide standards and a tryptic digest of bovine serum albumin. In addition, a commercial human serum standard (previously prepared several times using the ZipTip protocol, aliquotted and frozen) was used prior to each run for adjusting the laser power and a general system check. Using this standard serum sample, the resolution and intensity of five major peaks across the mass range of 1,800 to 8,200 Da were checked against previously obtained data and if necessary the laser energy was adjusted to keep the intensity and resolution of these peaks within their predetermined range of values of  $\pm 2$  SD.

## Data Processing

Each MALDI-TOF MS spectrum was analysed using FlexAnalysis and ClinProTools software V2.0 (both from Bruker Daltonics). FlexAnalysis was used to compare absolute profiles of spectra, and was also used to check the intensity and resolution of the five selected peaks of the serum standard sample (see above). Spectra loaded into ClinProTools were first normalised before baseline subtraction, peak detection, realignment, and peak-area calculation. The optimal settings were found to be a signal-to-noise ratio of 5, a Top Hat baseline subtraction with 10% as minimal baseline width and a 3-cycle Savitsky-Golay smoothing with a 10 Da-peak width filter.

ClinProTools automatically generated a table ranking the mass spectral peaks in order of their *p*-value. The different classes of mass spectra were then plotted as 2D-peak area distribution views using the two most discriminating peaks found from the peak statistics (see Figure 7 of the main article). The coefficient of variation (CV) of each of the individual peak areas (more than 100 peaks per spectrum) was calculated, and the mean CV for all of the peaks from the same replicate sample was calculated to provide a measure of intra- and inter-run reproducibility.

In addition to pre-acquisition quality control measures, various data quality checks were applied after acquisition of the mass spectra. The spectra obtained from the commercial serum standards allowed regular checks on the quality of the preparation and the MALDI spotting. In

this way, samples that had been affected, for instance, by experimental conditions such as humidity could be identified.

The evaluation settings of the AutoXecute function defined the criteria under which laser shots were accumulated. If for any reason it was not possible to accumulate the required number of shots (usually 1,000) per spectrum, the corresponding spectra were generally of low quality or even zero-line spectra. A low number of satisfactory shots was usually related to either the sample preparation or the crystallisation with the matrix. If all four spots from a particular sample preparation resulted in zero-line spectra the sample preparation was defined as a failure. All spectra with less than 1,000 shots were rejected. Spot replicates that gave significantly different spectra by a quick visual examination were also rejected.