

An enhanced automated method for digestion and MALDI spotting

BY GREGORY PORTER AND RICK LUEDKE

SOLID-PHASE extraction (SPE) is a well-known technique used in a wide range of genomics and proteomics applications and remains the method of choice for purifying nucleic acids and proteins for downstream separation and analysis. In genomics, SPE applications include PCR prep, post-PCR, and sequencing reaction purification. SPE is commonly used in protein analytics for peptide cleanup and concentration prior to analysis by mass spectrometry (MS).

In the transition from manual procedures to automation, materials and methods were adapted to vacuum-based protocols that could be more easily integrated onto the decks of robotic liquid handlers. With a capable robotic platform and well-optimized protocols, automation of laborious and time-consuming steps in the SPE process provides a higher measure of efficiency and reproducibility. This automation can lead to improved sensitivities during detection and analysis.

Current automated methods: Protein processing for MALDI and LC-MS

One of the most popular approaches in proteomics for separating, quantifying, identifying, and/or characterizing proteins involves the use of 2-D PAGE (two-dimensional polyacrylamide gel electrophoresis) or HPLC (high-performance liquid chromatography) for separation followed by MALDI (matrix-assisted laser desorption ionization)-MS for detection and analysis. The successful identification and characterization of proteins by MS is highly dependent on the process by which samples are prepared and presented to the mass spectrometer.

In the case of 2-D PAGE samples, a gel plug containing the stained protein is excised and transferred to a microplate for subsequent processing. The key steps that follow can be summarized as: 1) destaining, 2) trypsin enzymatic digestion, 3) extraction of the resulting peptides from the gel plug, 4) desalting and concentration, and 5) dispensing a peptide/ionizing matrix mixture onto the surface of the MALDI target.

To meet the growing demand of today's new proteomic initiatives, laboratories must have the ability to process large numbers of samples in an efficient and effective manner. The ProTeam Digest Workstation (Tecan U.S., Research Triangle Park, NC) enables the complete automation of all 2-D PAGE processing steps without user intervention. Alternatively, the platform can also produce in-solution peptides for presentation to an off-line LC-MS system. In the processing of 2-D PAGE samples, the workstation receives 96-well microplates containing gel plugs as input and provides peptide-spotted MALDI targets ready for MS as output. The ProTeam Digest hosts a number of distinguishing features and capabilities for complete, unattended automation of the MALDI prep procedure.

An integrated robotic manipulator arm (RoMa) enables transport of Society for BioMolecular Standards (SBS)-approved microplates and various on-deck modules (i.e., vacuum manifold) to any location within the workspace area.

Articulated fingers and extended reach capability enable microplates to be inserted and removed from onboard incubators without user intervention. High-performance incubators utilize top and bottom heating elements for precise temperature control and consistency across the plate, minimizing evaporation to lower digestion volumes (3 μ L) and achieve shorter digestion times (2 hr). A separate, fully integrated eight-tip (four fixed and four disposable) liquid handling arm (LiHa) operates independently from the RoMa and serves as a high-precision pipetting tool, capable of aspirating and dispensing low volumes (<1 μ L) into 384-well microplates as well as spotting high-density (i.e., ABI 192 target, Voyager DE [Applied Biosystems, Foster City, CA]) MALDI plates, thereby reducing sample/reagent consumption and enabling higher

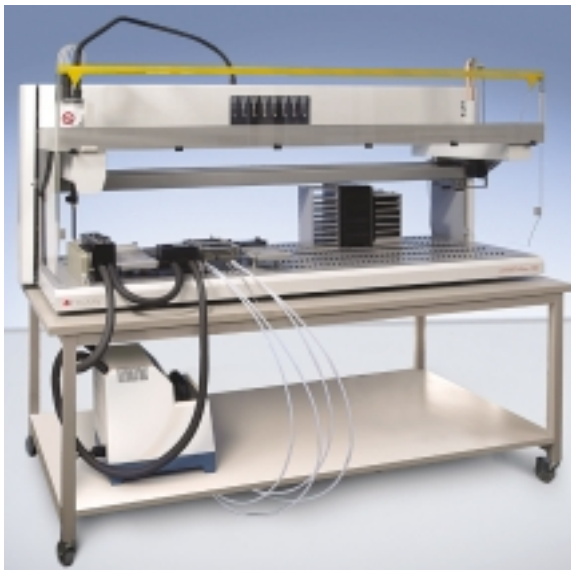


Figure 1 ProTeam Advanced Digest Workstation.

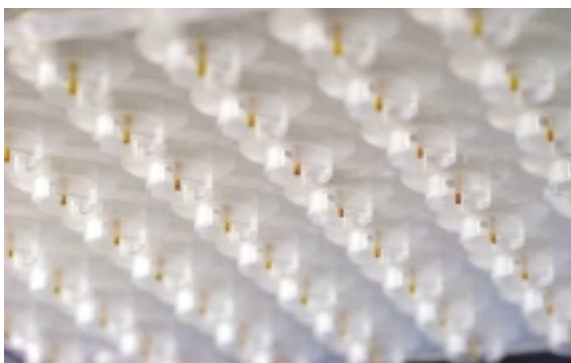


Figure 2 TecPrep solid-phase extraction plate.

MALDI sensitivity downstream. The use of disposable rubber mats sealed over gel plug plates helps to reduce evaporation and prevent inadvertent loss of the gel plug during aspiration steps.

The system can be configured to work with commercially available digestion kits and can use C18-resin disposable tips for postextraction desalting and peptide concentration. The ProTeam Digest is compatible with most MALDI targets supplied by the leading mass spectrometer instrument manufacturers. Throughput is estimated at 200 samples per 8-hr day with downstream sensitivities below 50 fmol.

Advanced automated methods

New automated materials and methods have been developed to further enhance throughput, sensitivity, and reproducibility for protein analytics. In addition to possessing the features described above, the ProTeam ADVANCED Digest Workstation (Tecan) (Figure 1) utilizes DirectSpot[®] technology (Tecan) to provide the latest in fully automated digestion and MALDI target spotting. In this platform, the TecPrep 96 plate (Tecan) (Figure 2), a 96-channel matrix-loaded device, is used in place of individual disposable tips.

The TecPrep 96 plate represents a novel combination of SPE, microplate, and needle spotting technologies that offers significant performance advantages when compared to methods that use disposable tips. Using the TecPrep plate, all processing steps, from destaining and digestion to extraction and spotting, are performed in a single plate, reducing the number of sample handling steps, minimizing peptide losses, and potentially helping gain access to lower abundant species.

Direct spotting of eluted peptides from the TecPrep plate to the target is facilitated by a specially designed

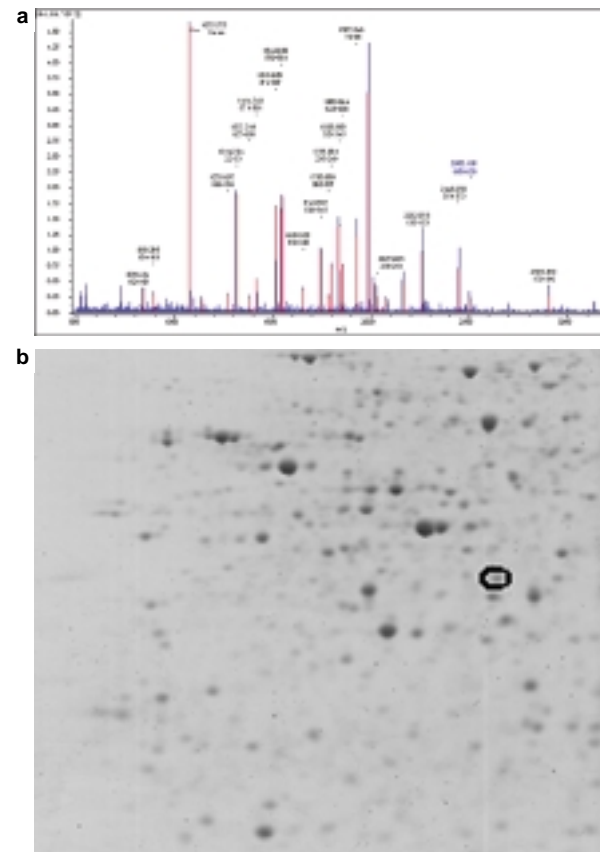


Figure 3 a) Spectrum Analysis Report—catechol O-methyltransferase (membrane-bound form) isolated from rat liver on ProTeam Advanced Digest, 151 score, 20 peptides, 52% coverage. b) 2-D PAGE gel showing corresponding weak intensity spot excised for processing.

vacuum manifold working in conjunction with a MALDI plate x-y directional indexing module. All 96 samples may be processing in parallel for superior reproducibility and throughput (up to 3500 samples per day). For greater flexibility and lower-throughput requirements, the TecPrep 96 plate is also capable of processing individual samples. The ProTeam ADVANCED Digest Workstation, using the DirectSpot technology and TecPrep 96 plate, yields excellent peptide recoveries to bring high sensitivity to the very low-fmol range.

Experimental method and results data

The following method demonstrates in-gel protein processing on the Tecan ProTeam ADVANCED Digest Workstation using the TecPrep 96 plate and subsequent analysis by MS. Proteins were isolated from rat liver, subjected to 2-D PAGE, stained with Coomassie brilliant blue (CBB); 1.4-mm-diam gel plugs were excised and transferred to the TecPrep 96 plate. The plate was pre-equilibrated with the addition of 80% acetonitrile (ACN), 1% trifluoroacetic acid (TFA), and 1% TFA before processing.

Destaining, digestion, extraction, purification, concentration, and spotting

Destaining was performed by the addition of a 50- μ L (30% ACN, 50 mM ammonium bicarbonate) aliquot dispensed into each well followed by a 10-min incubation at 37 $^{\circ}$ C; solution was removed by onboard vacuum filtration ($\times 3$). Shrinking of the gel plugs was performed in 80% ACN, with a 10-min incubation at room temperature; solution removed by the vacuum. Digestion was performed by the addition of 2 μ L trypsin (Roche, Basel, Switzerland; 50 ng dissolved in digestion buffer); 2 μ L digestion buffer

(5 mM TRIS-HCl, pH 8.0) was added to each well of the TecPrep plate. The plate was incubated for 2 hr at 37 °C. To deactivate trypsin and solubilize peptides, a 5- μ L aliquot of 1% TFA was dispensed into each well and the TecPrep plate was incubated for 5 min at room temperature. Solution was removed by the vacuum. Washing was performed using 5 μ L 0.1% TFA ($\times 2$). The AnchorChip 400- μ m target (**Bruker Daltonics**, Billerica, MA) prespotted with 1- μ L aliquots of matrix (5 mg/mL 2,5-dihydroxybenzoic [DHB] acid per milliliter in ACN: 0.1% TFA 1:2). TecPrep-bound peptides were eluted by the addition of 1 μ L (80% ACN, 0.1% TFA) elution solution and were directly spotted to the AnchorChip.

MALDI-MS

MALDI-MS was performed on the AutoFlex (reflector mode) (**Bruker**) (*Figure 3*). The calibrated and annotated spectra was subjected to a database search (SwissProt) using BioTools 2.0 (**Bruker**) and Mascot 1.8 search engine.

Conclusion

The demand for automated protein processing methods will continue as companies try to enhance throughput, re-

producibility, and sensitivity for increased access to low-abundant proteins, an area of particular interest in drug discovery. According to internal studies by **Tecan**, between 10–50% of protein (peptides) can be lost per handling step. Consideration should be given to keeping manipulations of proteins and peptides during processing to a minimum. Selecting an automated protein preparation method and platform that can process over 3000 samples per day in parallel fashion and that is capable of dispensing low volumes with high positional accuracy is a wise choice for enhancing throughput, reproducibility, and sensitivity.

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