

Two-Dimensional LC Protein Separation on Monolithic Columns in a Fully Automated Workflow

INTRODUCTION

The complex nature of proteomics samples requires high-resolution separation methods prior to mass-spectrometric (MS) detection. Pathways to protein identification are 2-D PAGE workflows and liquid chromatography (LC) based workflows. Currently, 2-D PAGE is the dominant workflow for the analysis of intact proteins. However, bottlenecks of this approach are poor recovery of low-abundant proteins and the limited degree of automation for subsequent peptide analysis.

To overcome these limitations, an automated multidimensional LC workflow has been developed in which proteins are separated, fractionated, and digested automatically by one LC system. The workflow consists of the following steps:

1. First dimension anion-exchange separation and fractionation of intact proteins
2. Automated reinjection of fractions and applying second dimension reversed-phase (RP) separation of intact proteins
3. Fractionation of the second dimension separation followed by in-well digestion of proteins

The digested proteins are then analyzed by a 1-D RP separation and identified by MS/MS. The complete workflow from protein separation to peptide identification is shown in Figure 1.

This LC based top-down workflow was validated with an *Escherichia coli* protein extract (BioRad) and the Universal Proteomics Standard Set (Sigma).

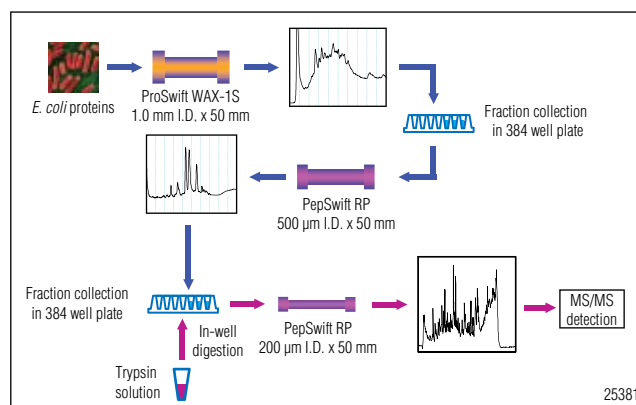


Figure 1. Automated 2-D LC protein separation workflow, including in-well digestion and peptide analysis. The fractions are displayed in the protein separation chromatograms.

AUTOMATED OFF-LINE 2-D LC

An UltiMate® 3000 Proteomics MDLC system with the microfraction collection option (μ FC) was used for the 2-D LC separation of intact proteins based on ion-exchange (IEX) and RP interaction. See Figure 2 for instrument configuration and Table 1 for LC conditions.

Table 1. Experimental Conditions for Automated Off-Line 2-D LC		
	First Dimension (WAX)	Second Dimension (RP)
Column	ProSwift® WAX, 1.0 mm i.d. \times 50 mm	PepSwift™ monolithic RP, 0.5 mm i.d. \times 50 mm
Mobile Phase A	10 mM Tris-HCl (pH 7.6)	Water, 0.05 % TFA
Mobile Phase B	10 mM Tris-HCl, 1000 mM NaCl (pH 7.6)	Water/CH ₃ CN (20/80), 0.04% TFA
Loading Solvent		Water, 0.05% HFBA
Flow Rate	50 μ L/min	15 μ L/min
Gradient	0–1000 mM NaCl in 9 min	15–70% B in 10 min
UV Detection	220 nm, 45 nL flow cell	220 nm, 45 nL flow cell
Sample	<i>E. coli</i> protein lysate 1 μ g/ μ L	Collected fractions
Injection Volume	7 μ L	50 μ L (Complete first dimension fraction)
Fractionation	60 s	60 s
Column Oven Temp.	30 °C	60 °C

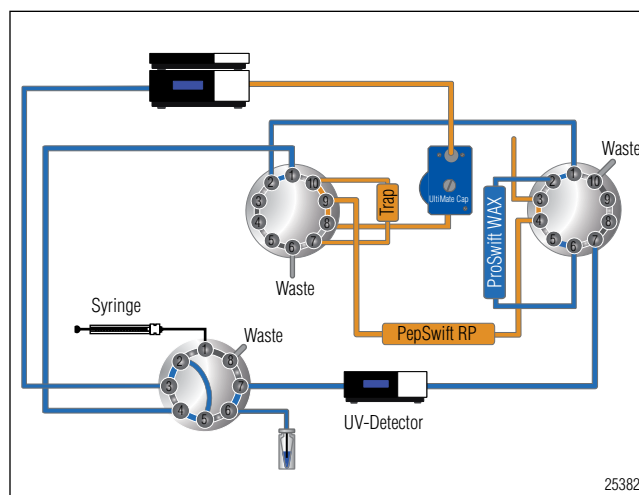


Figure 2. Fluidic set-up of the automated off-line 2-D LC system used for intact protein separation.

Proteins were separated on a monolithic weak anion-exchange (1.0 mm i.d. \times 50 mm ProSwift WAX) column in the first dimension using a 9 min salt gradient. One minute fractions were collected by the autosampler and re-injected onto the monolithic RP (500 μ m i.d. \times 50 mm PepSwift RP) column in the second dimension. The proteins were separated using a 10 min acetonitrile gradient with one minute fraction collection by the autosampler.

The collected fractions were digested by adding and mixing a 20 μ L (0.5 ng/ μ L) trypsin solution to the second dimension fractions by the autosampler. After digestion at 30 °C for 3 h, the digestion was quenched by adding 10 μ L 5% HFBA solution, which also enhances trapping efficiency on the monolithic trap column used in the follow-up LC-MS/MS experiments. Peptide fractions were subsequently preconcentrated and desalted onto a monolithic trap column (200 μ m i.d. \times 5 mm PepSwift RP), separated on a monolithic column (200 μ m i.d. \times 50 mm PepSwift RP) using an UltiMate 3000 Capillary LC system and detected by MS/MS using an HCTultra™ ion-trap mass spectrometer (Bruker Daltonics).

2-D LC ANALYSIS OF E. COLI

The 2-D LC separation of intact *E. coli* proteins, including fractionation of both first and second dimension separations, was achieved in four hours. Figure 3A shows a chromatogram of the separation of the intact proteins obtained on a WAX monolithic column applying a salt gradient. The successive RP separation of the protein fractions on a monolithic column are shown in Figure 3B.

From the 2-D LC protein separation, a 2-D retention map was created by the Chromeleon® software. The 2-D retention map was used as a visualization tool to select fractions of interest for subsequent peptide analysis and is shown in Figure 4A. This type of representation is also useful for comparing two different samples in expression proteomics studies.

Figure 4B shows a peptide separation TIC and Figure 4C an MS spectrum of the indicated peak. The absence of protein charge envelopes in the mass spectra indicated a successful tryptic digestion.

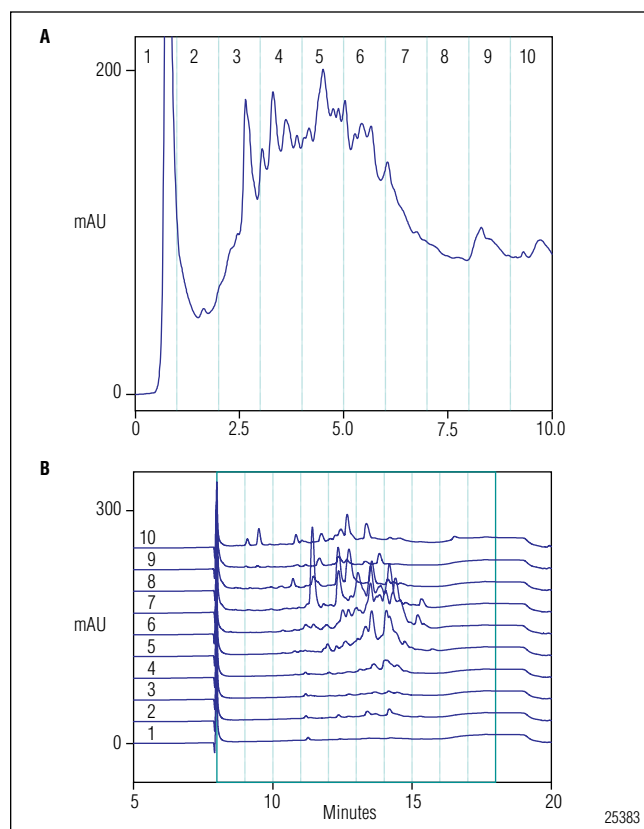


Figure 3. A) First-dimension separation of *E. coli* proteins on the WAX column, B) successive separation of WAX protein fractions on the second-dimension RP column. The fractions are indicated by dashed lines. The numbers displayed vertically in B correspond to the WAX fractions (numbers across the top).

The major benefit of this liquid based workflow is protein prefractionation including automated in-well digestion. Trypsin solution can be added by the autosampler immediately after fractionation, and replaces laborious and time consuming gel spot picking before tryptic digestion.

Another advantage of this liquid-based workflow is the top-down approach, where separation takes place on the protein level. Fractionation of the separation results in less complex fractions where proteins are present in a single fraction. After digestion of the fraction, all peptides from the corresponding proteins will be present in this fraction. This consequently improves confidence in protein identification.

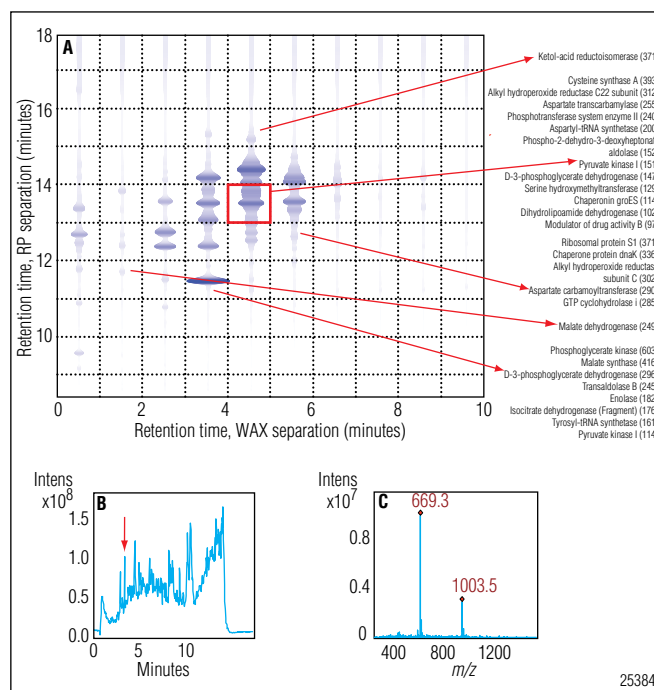


Figure 4. A) Two-dimensional retention map for the separation of intact *E. coli* proteins with protein identification for selected fractions. The grid shown in the figure was created for visualization of the fractions. B) Peptide separation TIC MS for the fraction indicated by the red square. C) MS spectrum for the peptide indicated in B.

To minimize elution of a single protein in multiple fractions, a high separation power is preferred. To evaluate the separation power of the WAX and RP columns, the elution of a protein in multiple fractions was evaluated by mass spectrometric detection of the peptides. For that purpose peptide fragmentation MS spectra were matched with a database. In Figure 5 the protein elution is categorized by the number of WAX-RP fractions in which a protein was identified.

Evaluating the analyzed fractions revealed a highly efficient protein separation with minimal overlap. The majority of the proteins (85%) were found in one fraction. Twelve percent of the proteins eluted in two fractions and 3% eluted in three or more fractions.

The top-down workflow was also evaluated with the recently introduced Universal Proteomics Standard set (Sigma). This challenging, complex mixture contains 48 human proteins (5 pmol of each) ranging in molecular weight from 8 to 80 kDa. Using similar experimental conditions, 83% of the proteins were identified. In a 1-D bottom up separation of the same sample, only 50% could be identified. The separation on the protein level caused this significant increase in protein identification.

CONCLUSION

The automated off-line 2-D LC workflow provides for a fast, highly efficient protein separation using monolithic columns. The 2-D LC workflow shows minimal overlap of proteins found in adjacent fractions; 85% of the proteins are eluted in a single fraction. The liquid-based, top-down workflow allows automated, in-well digestion for superior ease of use. The presented 2-D LC method is an attractive alternative to 2-D PAGE in terms of automation and sample throughput in proteomics.

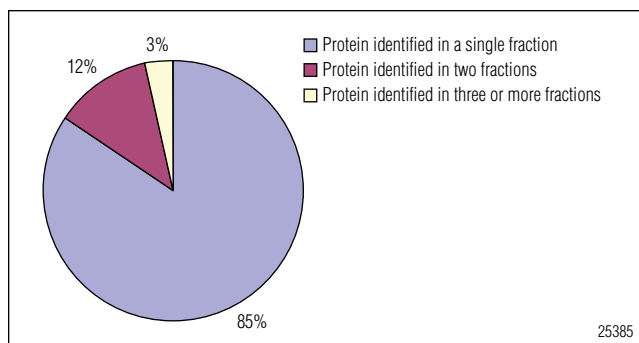


Figure 5. Distribution of proteins over WAX-RP fractions.

MATERIALS FOR AUTOMATED 2-D LC PROTEIN SEPARATION

The products below are recommended for analysts performing the methods outlined in this technical note. In the U.S., call 1-800-346-6390, or contact the Dionex Regional Office nearest you. Outside the U.S., order through your local Dionex office or distributor. Refer to the following part numbers:

PRODUCT DESCRIPTION	PART NUMBER
Materials for Automated Off-Line 2-D Protein Separations	
Off-Line 2-D LC Kit for Proteins, ProSwift WAX 1.0 mm i.d./PepSwift 500 µm i.d.	6720.0103
Off-Line 2-D LC Kit for Proteins, ProSwift WAX 1.0 mm i.d./PepSwift 500 µm i.d., Biocompatible	6721.0103
Splitter Cartridge 1:15 (CAP)	6720.3165
Splitter Cartridge 1:15, biocompatible (CAP)	6721.3165
UZ-View™ Flow Cell for VWD 3x00, 10 mm Path Length, 45 nL Volume	6074.0280
UZ-View Flow Cell for UVD-3000, 10 mm Path Length, 45 nL Volume.....	6073.0003
Materials for 1-D Peptide Analysis	
UltiMate 3000, Monolithic LC, Preconcentration Application Kit, incl. IQ-Kit.....	6720.0049
UltiMate 3000, Monolithic LC, Preconcentration Application Kit, incl. IQ-Kit, inert	6721.0049
Splitter Cartridge 1:100 (CAP)	6720.3150
Splitter Cartridge 1:100, Biocompatible (CAP)	6721.3150
UZ-View Flow Cell for VWD 3x00, 10 mm Path Length, 3 nL Volume	6074.0270
UZ-View Flow Cell for UVD-3000, 10 mm Path Length, 3 nL Volume.....	6073.0002

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LPN 2156 PDF 2/09
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