Automation of Cell Surface Protein Isolation Using the Agilent AssayMAP Bravo Platform, and Profiling Using the Agilent 6550 iFunnel Q-TOF LC/MS Systems

Application Note

Authors

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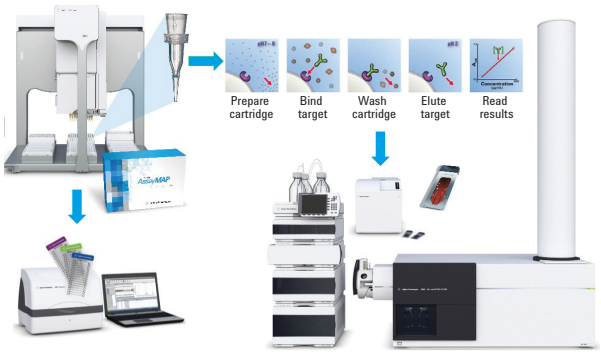
Abstract

This study investigated the feasibility of automating an established cell surface protein isolation protocol using the Agilent AssayMAP Bravo automation platform. Epidermoid cancer cell lines-A431 was used as an *in-vitro* model system, and cell surface proteins were isolated using a combination of AssayMAP Bravo automation and a Pierce Cell Surface Protein Isolation kit. The isolated proteins were then analyzed using the Agilent 2200 TapeStation system in conjunction with the Agilent P200 ScreenTape assay for differential electrophoresis profile. The eluted proteins were then analyzed using the Agilent HPLC-chip coupled to an Agilent 6550 iFunnel Q-TOF with dual stage ion funnel technology. Key cell surface proteins were identified including extracellular matrix proteins, moderately abundant proteins including pancreatic marker protein, plectin-1 along with F-box leucine rich repeat protein-2, *beta*-actin, and PGK-2. This study demonstrates that the AssayMAP Bravo platform can be conveniently used for automated high-throughput sample preparation workflows involving affinity purifications.



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Introduction

Cell surface membrane proteins play a predominant role in cellular signaling processes. Membrane-specific receptor proteins serve as cellular markers and prime drug targets for several pharmaceutical agents. Receptor proteins have been targeted to decipher the molecular mechanisms for several cancers, for example breast, epidermoid, and lung cancers[1]. Enrichment of cell surface proteins by conventional biochemical approaches is a challenging task for various reasons including poor solubility of membrane proteins, sample loss during multiple sample processing steps, and so forth. The primary chemical enrichment strategy involves biotinylating cell surface proteins, and then specifically capturing those proteins using streptavidin. Automation of this workflow provides high-throughput processing, as well as higher precision, ease-of-use, and reproducibility.

The Agilent AssayMAP Bravo is a quantitative, high-throughput sample preparation technology that uses miniaturized, packed-bed cartridges and precise liquid handling. The system comprises a high precision liquid handler with 96 probe syringes that allows precise positive displacement flow control through disposable 5-µL packed-bed cartridges. The AssayMAP Bravo platform includes flexible, customizable protocols with simple user interfaces to enable automated, high-throughput affinity-based enrichment, protein digestion, and peptide cleanup in a microtiter plate format[2]. In this work, the sample preparation using the Pierce Cell Surface Protein Isolation kit was automated using the AssayMAP platform in conjunction with AssayMAP streptavidin cartridges (SA-W). Agilent 2200 TapeStation system with the Agilent P200 ScreenTape assay was used to see differential protein elution after binding and elution. The isolated proteome was then analyzed using the Agilent HPLC-Chip/6550 Q-TOF system for cell surface protein identification.

Materials and Methods

Reagents and kits

Epidermoid carcinoma cell line-A431 was purchased from the National Centre for Cell Science, Pune, India and subcultured as recommended[3]. DMEM media, fetal bovine serum (FBS), and penicillin-streptomycin solution were all purchased from Sigma-Aldrich. The Pierce Cell Surface Protein Isolation kit and Trypsin/Lys-C mix was procured from Thermo Scientific and Promega, respectively. The DC Protein assay kit was purchased from Bio-Rad.

Cell culture and surface protein isolation

A431 cell lines were cultured as an adherent monolayer in DMEM media supplemented with 10 % FBS and antibiotics in T75 cell culture flasks, and maintained in a 5 % CO_2 atmosphere at 37 °C. Isolation of the cell surface proteins was carried out following the Pierce Cell Surface Protein Isolation kit guidelines[4]. In brief, four T75 cm^2 flasks with cell confluency of 90 % were used, and cells were washed twice with ice-cold phosphate buffered saline (PBS) (Pierce). The cells were then treated with 0.25 mg/mL Sulfo-NHS-SS-Biotin probe (Pierce) reconstituted in ice-cold PBS and the flasks were then incubated in the dark at 4 °C with gentle agitation for 30 minutes, to facilitate biotinylation. The reaction was then quenched using 500 µL of supplied Quenching Solution (Pierce). Cells were then scraped from all four flasks, combined, and pelleted by centrifugation at 500 × g for 3 minutes. The pellets were washed with *Tris*-buffered saline (TBS) (Pierce) and pelleted again. Cells were lysed using supplied Lysis Buffer (Pierce) by vortexing for 30 minutes. Cell debris was removed by centrifugation at 10,000 × g for 2 minutes at 4 °C followed by filtration using Agilent 0.2-µm syringe filters.

Affinity purification of the biotinylated proteins was carried out using AssayMAP SA-W cartridges with Antibody Purification protocol following the recommendations[5]. In brief, the cartridges were primed and equilibrated using PBS (Pierce), then clarified lysate supernatant was loaded at four different concentrations, 10, 25, 50, and 75 µg/µL, along with a blank control to optimize binding capacity. The cartridges were then washed using the supplied Wash Buffer (Pierce). The bound proteins were finally eluted using 62.5 mM, *Tris* pH 6.8 containing DTT at a concentration of 50 mM. The AssayMAP-Antibody Purification application interface is presented in Figure 1. The entire workflow of the study is presented as a schematic diagram in Figure 2.

Figure 1. Agilent AssayMAP application interface showing the Antibody Purification protocol adapted for the cell surface protein isolation.

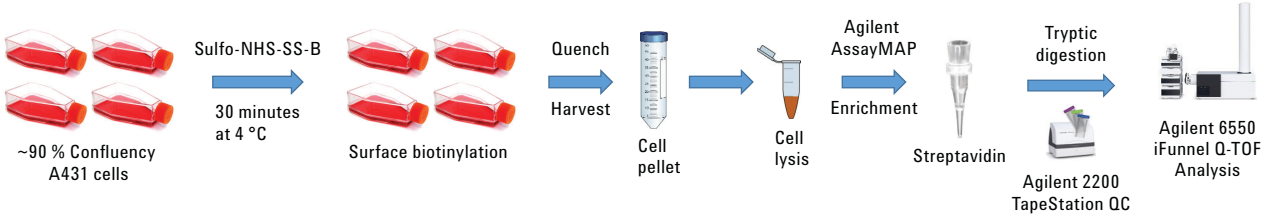

Figure 2. Schematic representation of the cell surface protein isolation workflow.

Agilent 2200 TapeStation system and LC\MS analysis

The samples from the automation process (eluate and flow-through) were analyzed using the Agilent P200 ScreenTape assay with the Agilent 2200 TapeStation system following the manufacturer's guidelines[6]. In brief, 2 µL of sample were labeled with P200 Labeling dye by heating at 75 °C for 7 minutes followed by a denaturation step of 75 °C incubation for 5 minutes. The samples were then mixed with P200 Markers, and placed in the 2200 TapeStation system for analysis.

The eluate fractions from the AssayMAP purification protocol were quantified using the Bio-Rad DC Protein assay[7]. The enriched proteins were digested overnight with trypsin/Lys-C at 37 °C, and the digestions were terminated by the addition of 1 % formic acid[8]. The digested samples were then desalted using custom-made stage-tips with a C-18 column, and peptides were eluted using 0.1 % formic acid in 90 % acetonitrile in water

All LC/MS analyses were performed on the Agilent 1260 Infinity HPLC-Chip/MS system comprised of a micro autosampler, capillary pump, and nanoflow pump interfaced to the Agilent 6550 Accurate Mass Q-TOF with the Chip Cube. The separation was performed on an Agilent Polaris-HR-Chip-3C18, which integrates a 360-nL enrichment column and a 150 mm × 0.075 mm id separation column, both of which are packed with 3-µm Polaris C18A stationary phase. LC/MS conditions used in the study are listed in Table 1.

Results and Discussion

Agilent 2200 TapeStation system analysis

The isolated cell surface proteins using AssayMAP were analyzed using the 2200 TapeStation system before downstream LC/MS analysis. The total cell lysate, flow-through, and eluate from 50 µg/µL load was taken for downstream analysis and analyzed using the P200 ScreenTape assay. A representative gel image is presented in Figure 3. The gel image shows the difference in the electrophoretic profile of the load (L), flow-through (FT) fraction, and eluate (E) protein fraction. The eluate profile shows less protein compared to the load and flow-through, suggesting that a portion of the total proteins that got bound to the streptavidin column and eluted, is biotinylated. The analysis, using the P200 ScreenTape assay, ensures that there are proteins in the elution before carrying out time-consuming LC/MS analysis.

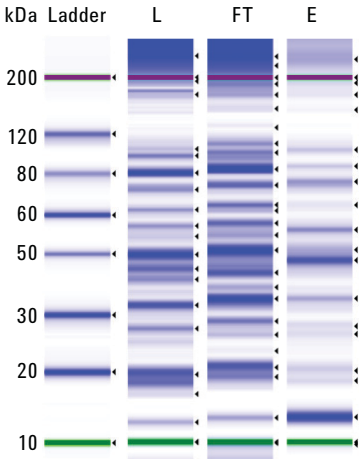

Figure 3. Agilent P200 ScreenTape gel image showing total cell lysate load (L), flow-through (FT), and eluate (E) after Agilent AssayMAP automated isolation.

Table 1. LC/MS parameters used in the study.

HPLC-Chip Conditions	
HPLC-Chip	Agilent Polaris-HR-Chip-3C18
Mobile phase A	0.1 % formic acid in water
Mobile phase B	0.1 % formic acid in 90 % acetonitrile in water
Loading	2 µL/min with 3 %B
Analytical flow rate	300 nL/min
Q-TOF analytical gradient	3 %B at 0 minutes 22 %B at 15 minutes 45 %B at 36 minutes 87 %B at 42 minutes 3 %B at 45 minutes
Injection volume	5 µL volume with 3 µg on-column load
Agilent 6550 Q-TOF MS acquisition parameters	
Instrument mode	Extended dynamic range (2 GHz) with low mass range (1,700 *m/z*)
Drying gas	11 L/min, 250 °C
Acquisition rate	3 (MS) and 8 (MS/MS) spectra/sec
Acquisition range	300–1,700 (MS) and 50–1,700 (MS/MS)
Collision energy	Slope of 3.2 and intercept 1 (+2) or slope of 2 and intercept 3.1 (+3) or slope of 3.6 and intercept –4.8 (+3 and higher)
Isolation	Wide (~9 amu)
Data dependent acquisition	20 precursors per cycle using precursor abundance based acquisition rate with accumulation time limit enabled; active exclusion after one spectrum for 0.5 minutes

LC/MS analysis

The eluted fraction from the AssayMAP protocol was quantified to be 1.5 µg/µL of proteins, which were then digested using the trypsin/Lys-C Mix with a protein:protease ratio of 25:1. The resulting peptides were then desalted using stage-tips, and subjected to LC/MS analysis using the 6550 Q-TOF Mass Spectrometer. Spectra were acquired using data-dependent acquisition. Figure 4 shows the total ion chromatogram of the tryptic-digested cell surface peptide mixtures.

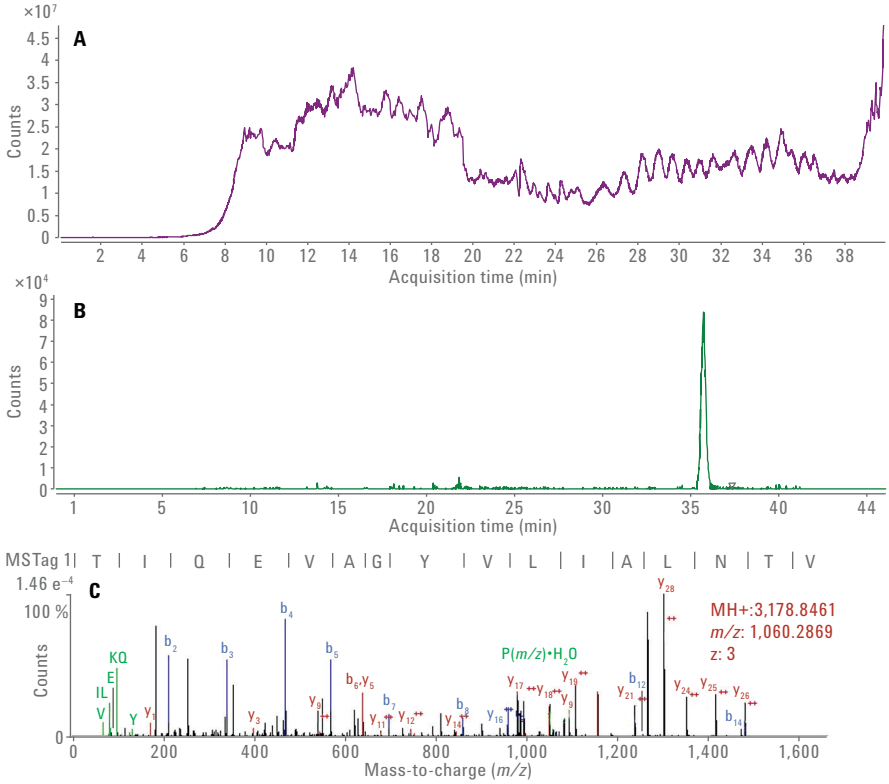

Figure 4. A) Total ion chromatogram (TIC) of tryptic-digested cell surface proteome. B) Extracted ion chromatogram (EIC) of *m/z* 1,060.2869 for TIQEVAGYVLIALNTV peptide from EGFR. C) Spectrum Mill output: MS/MS spectrum of *m/z* 1,060.2869 for TIQEVAGYVLIALNTV peptide from EGFR.

A protein database search of the LC/MS/MS data using SpectrumMill resulted in the confident identification of 300 protein candidates (Figure 5). A stringent validation criterion of 1 % FDR at the spectral level was applied. After manual curation of the identified proteins, about 100 cell surface proteins were found, including platelet-derived growth factor/vascular endothelial growth factor receptor (EGFR), integrin adhesion molecules involved in cell-cell interactions, and membrane transporter proteins such as b-subunits 1 and 2 of sodium/potassium transporting ATase. Several key proteins including pancreatic marker protein, plectin-1, F-box leucine-rich repeat protein-2, *beta*-actin, PGK-2, Interleukin-10 receptor subunit *beta*, Renin receptor, EGFR, 40S ribosomal protein SA, and Prostaglandin F2-*alpha* receptor were also observed.

The Agilent AssayMAP with streptavidin cartridges (SA-W) was successfully used for the purification of the biotinylated proteins, of which key cell surface proteins were identified using LC/MS analysis.

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Total Protein Spectral Intensity	Database Accession #	Protein Name
1	32	11	159.27	29.4	2.32e+008	P10809	60 kDa heat shock protein, mitochondrial
2	24	10	155.68	13.8	9.21e+007	P00533	Epidermal growth factor receptor
3	28	8	135.93	18.1	1.20e+008	P13639	Elongation factor 2
4	32	6	125.90	26.6	1.53e+008	P06576	ATP synthase subunit beta, mitochondrial
5	20	7	124.96	36	1.67e+008	P63261	Actin, cytoplasmic 2
6	11	8	124.85	6.6	2.24e+007	P49327	Fatty acid synthase
7	21	7	123.44	35.2	1.29e+008	P04406	Glyceraldehyde-3-phosphate dehydrogenase
8	40	7	115.82	19.3	3.49e+008	P08195	4F2 cell-surface antigen heavy chain
9	16	6	113.37	32.4	5.73e+007	Q5VTE0	Elongation factor 1-alpha 1
10	17	6	109.75	13.9	7.03e+007	P05556	Integrin beta-1
11	14	6	92.00	17.8	4.04e+007	P14625	Endoplasmin
12	33	7	91.87	29.9	1.70e+008	P07437	Tubulin beta chain
13	22	6	90.60	25.4	6.94e+007	P22626	Heterogeneous nuclear ribonucleoproteins A2/B1
14	34	6	88.83	15	1.14e+008	P40939	Trifunctional enzyme subunit alpha, mitochondrial
15	15	4	83.84	8.1	5.43e+007	Q00839	Heterogeneous nuclear ribonucleoprotein U
16	14	5	75.91	7.5	4.21e+007	P16144	Integrin beta-4
17	19	5	74.47	8.5	6.31e+007	O43707	Alpha-actinin-4
18	30	6	71.14	12.3	1.26e+008	P38646	Stress-70 protein, mitochondrial
19	7	3	68.79	8.8	2.67e+007	P14923	Junction plakoglobin
20	20	4	65.81	14.6	1.71e+008	P68363	Tubulin alpha-1B chain
21	8	4	65.16	7.1	4.12e+007	P05023	Sodium/potassium-transporting ATPase subunit alpha-1

Figure 5. Screenshot of Spectrum Mill protein database search showing partial protein list.

Conclusions

This Application Note demonstrates the successful automation of an established cell surface isolation protocol to capture cell surface proteins using epidermoid cancer cell lines. The automation was achieved using the user-friendly interface of the Agilent AssayMAP application software interface. LC/MS/MS analysis of the isolated proteins also confirmed that the cell surface proteins were captured using the described approach.

Our results demonstrate the ability of the Agilent AssayMAP Bravo automation platform and the HPLC-Chip/MS system to capture and analyze the cell surface proteome.

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