Improved sequence coverage and confident protein identification using a two-step digestion protocol combined with acid-labile detergent

Summary

This application note clearly demonstrates the benefits of using LPI™ FlowCell in a two-step digestion protocol using a shave-and-conquer approach in combination with an acid-labile detergent, PPS Silent™ Surfactant. New peptides are identified in the second digestion step leading to improved sequence coverage, an increased number of confident protein identifications and a completely new set of proteins not detected in the first digestion step. Membrane protein vesicles are immobilized on the surfaces of LPI™ Maxi FlowCell, where the flow cell design allows for easy exchange of solutions containing *e.g.* proteases. This feature is highly suitable for obtaining multiple complementary peptide fractions from a single sample, greatly enhancing the protein sequence information obtainable from a membrane preparation analysis.

Results

We conclusively show that a second trypsin treatment of a previously digested sample gives more protein sequence coverage if compared to a single step digestion protocol, and that using PPS Silent™ Surfactant in the second step potentiates the results. In brief, a membrane protein preparation was immobilized onto LPI™ FlowCell and subjected to a two-step digestion protocol, similar to a shave-and-conquer approach. The first step consisted of a brief trypsin digestion step. The second step consisted of a trypsin digestion either with or without a digestion enhancer in the form of PPS Silent™ Surfactant.

Step 2 increases protein sequence coverage

1248 peptides where identified in Step 1. With PPS Silent™ Surfactant included, Step 2 resulted in the identification of 1428 peptides of which 743 were new when compared to Step 1, bringing the total to 1991. Without detergent 936 peptides were identified of which 325 were new (Figure 1).

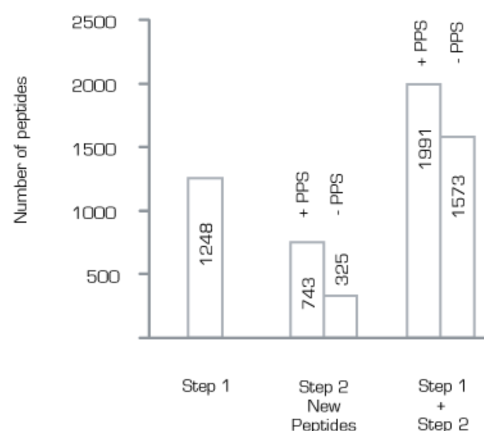

Figure 1. Step 2 increased the total number of peptide identifications by 325 without detergent, and by 743 with PPS Silent™ Surfactant.

The new peptides identified in Step 2 increased the sequence coverage of proteins identified in Step 1. Of the 446 proteins identified in Step 1 (including single-peptide identifications), the sequence coverage of 186 proteins was increased by Step 2 when PPS Silent™ Surfactant was used. Without detergent in Step 2 the sequence coverage of 141 proteins was increased (Figure 2). Using PPS Silent™

Surfactant, it was possible to increase sequence coverage by up to 30% for some proteins.

Increase of sequence coverage (%) due to step 2

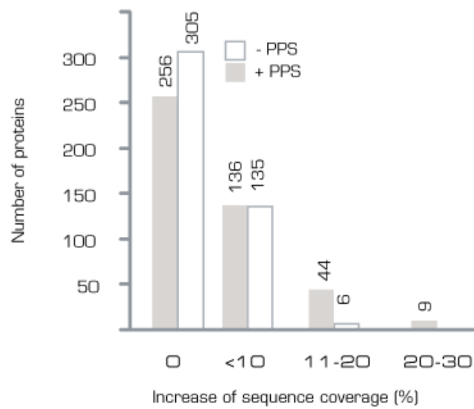

Figure 2. Step 2 increased sequence coverage of proteins identified in Step 1. PPS Silent™ Surfactant potentiated the sequence coverage increase.

Step 2 increases the number of confident protein identifications

236 confident protein identifications (at least two peptide sequences per protein) were made based on peptide data from Step 1. The data from Step 2 identified 63 additional proteins when PPS Silent™ Surfactant was used. In the absence of detergent in Step 2 the number was 15.

Step 2 added complementary peptide sequences to proteins identified in Step 1. With PPS Silent™ Surfactant included, 62 proteins identified by a single peptide in Step 1 received at least another peptide sequence from Step 2. Without detergent the number was 40.

The total increase in number of confident protein identifications due to Step 2 was 125 using PPS Silent™ Surfactant and 55 without (Figure 3).

Total increase of confident protein identifications due to Step 2

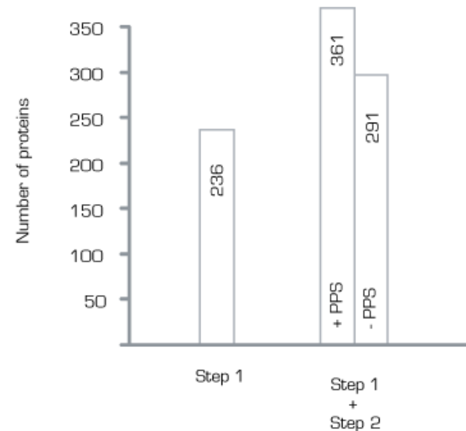

Figure 3. Step 2 increased the total number of confident protein identifications by 55 without detergent, and by 125 with PPS Silent™ Surfactant. Data is based on proteins identified by at least 2 peptide sequences.

Conclusions

The LPI™ technology enables immobilization of native membrane protein preparations and provides a tool for exchanging the solution environment around the membrane proteins. This allows for sequential digestion of the sample which increases the number of identified peptides. This approach results in several benefits compared to a single digestion step:

- Improved sequence coverage
- Increased confidence in protein identification
- More protein identifications

Methods

Membrane Preparation

WSS-1 cells were harvested and collected by centrifugation at 600xg for 5 min. Cells were washed by dissolving the pellet in 15 ml ice-cold PBS buffer (137 mM NaCl, 10 mM phosphate, 2,7 mM KCl, pH 7,4) followed by centrifugation (600xg, 5 min). The wash step was repeated twice. The pellet was dissolved in 4 ml 10 mM $NaHCO_3$. Cells were lysed using a Dounce homogenizer (20 strokes). Nuclei and mitochondria were removed by centrifugation (10 000xg, 10 min). The supernatant was washed with 100 mM Na_2CO_3 and bath sonicated on ice for 30 minutes. The membrane material was collected by centrifugation (150 000xg, 1.5 hr).

The pellet was resuspended in LPI™ Ionic (product# N37-67-100), transferred to a glass vial and tip-sonicated on ice for 5 minutes (15% amp, 5s+5s pulses, Vibracell). Particles were removed by centrifugation (500xg, 10 minutes).

Nanoxis LPI™ FlowCell Sample Processing

400 µl sample was injected into four separate LPI™ Maxi FlowCells (product# N37-53-323). Vesicles were immobilized for 1 hour. LPI™ Maxi FlowCells were washed with 2 ml LPI™ Ionic followed by 2 ml LPI™ Basic (product# N37-67-100).

1st digestion step
700 µl trypsin (5 µg/ml in LPI™ Basic) was added to each FlowCell and tryptic digestion for 30 minutes at 37 °C was performed. Peptides were eluted by adding 700 µl LPI™ Basic. Eluate was collected in an Eppendorf tube and further digested overnight at 37 °C.

2nd digestion step
Undigested material in LPI™ Maxi FlowCells was washed with 2 ml LPI™ Basic. 700 µl trypsin (5 µg/ml trypsin in LPI™ Basic) was added to each FlowCell. In two of the four FlowCells the trypsin was supplemented with 1.25 mg/ml acid-labile detergent (PPS Silent™ Surfactant from Protein Discovery). Digestion proceeded for 1 h at 37 °C. Peptides were eluted into an Eppendorf tube by adding 700 µl LPI™ Basic to the FlowCell. Eluates were collected in Eppendorf tubes and further digested overnight at 37 °C..

All samples were filtered through Whatman Anotop 0.02 µm filters and supplemented with 14 µl formic acid. Detergent-containing samples were incubated at room temperature for 1 hour to degrade the detergent. Finally, all samples were frozen at -20 °C.

LC-MS/MS and bioinformatics

The peptide samples from LPI™ Maxi FlowCells were analyzed by LC-MS/MS at the Proteomics Core Facility at Göteborg University.

Prior to analysis, the sample was vacuum centrifuged to dryness and reconstituted in 20 µL 0.1 % formic acid in water. The sample was centrifuged for 15 minutes at 13 000xg and 17 µL was transferred to a sample vial. 3 µl of the sample was diluted 25 times and transferred to the autosampler of the LC-MS/MS system.

For the liquid chromatography, an Agilent 1100 binary pump was used and the tryptic peptides were separated on a 200 x 0.05 mm i.d. fused silica column packed in-house with 3 µm ReproSil-Pur C18 AQ particles (Dr. Maisch, GmbH, Ammerbuch, Germany). 2 µL of the sample was injected and the peptides were first trapped on a precolumn (45 x 0.075 mm i.d.) packed with 3 µm C18-bonded particles. A 40 minute gradient consisting of 10-50% acetonitrile in 0.2 % formic acid was used for separation of the peptides and the flow through the column was reduced by a split to approximately 100 nL/min.

Mass analyses were performed in a 7-Tesla LTQ-FT mass spectrometer (Hybrid Linear Trap Quadrupole – Fourier Transform) (Thermo Electron) equipped with a nanospray source modified in-house. The instrument was operated in the data-dependent mode to automatically switch between MS and MS/MS acquisition. MS spectra were acquired in the FT-ICR while MS/MS spectra were acquired in the LTQ-trap. For each MS scan, the six most intense, doubly or triply charged ions were sequentially fragmented in the linear trap by collision induced dissociation (CID). Fragmented target ions were excluded for MS/MS selection for 6 seconds.

All tandem mass spectra were searched by MASCOT (Matrix Science) against Sprot 55.3 using human taxonomy. Search setting were: enzyme = trypsin; variable modifications = oxidation (M); peptide mass tolerance = 5 ppm; fragment mass tolerance = 0.5 Da; max missed cleavages = 1.

Two replica injections were made per peptide sample. Before comparison of sample groups, MS data from 4 injections per sample group were pooled. The three sample groups were: [Step 1], [Step 2 -detergent], [Step 2 +detergent].