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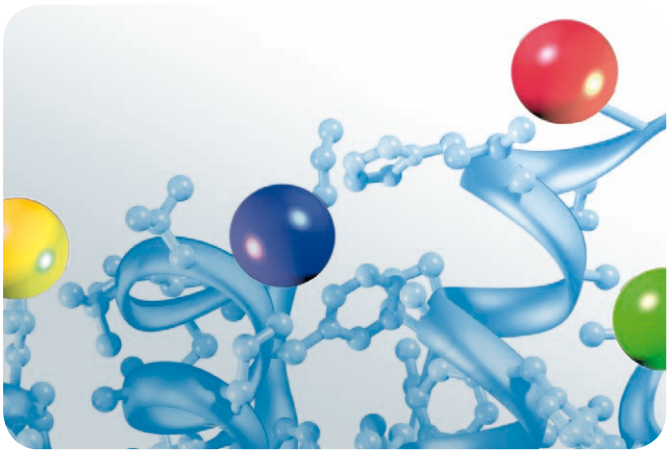


Application Note # MT-106

Isotope-Coded Protein Labeling (ICPL) for Quantitative Proteomics Workflows Compatible with Protein Separation Strategies

Abstract

The new Serva ICPL™ Quadruplex PLUS (ICPL 4-plex) protein quantification kit was used to establish instrument and data analysis methods on the ultrafleXtreme MALDI-TOF/TOF instrument. Resulting data were analyzed using ProteinScape 2.1 that provide good quantification results from ICPL 4-plex measurements. We analysed a protein mixture that was labelled in 4 states with defined quantities to validate the compatibility of the ICPL 4-plex Kit with the bioinformatics suite ProteinScape 2.1, and the MALDI-TOF/TOF.

Introduction

labeling on the protein level, as it occurs with both, the ICPL chemistry and the SILAC metabolic labeling strategy, is uniquely well compatible with protein based separation strategies in contrast to peptide based labels. However, so far only two to three different proteomic states could be processed in one experiment using protein labeling approaches, limiting the throughput. The well established ICPL protein labeling technology [1-4] is now available as a 4-plex kit from SERVA and Bruker. The label reacts with protein amino groups in one of four forms (Fig. 1) that vary in their heavy isotope substitution, and thus by their molecular weight. Stable isotope labeling with ICPL permits a top-down proteome quantification strategy in which the proteins can be separated, e.g., by 2DGE, SDS-PAGE or protein chromatographic techniques [4]. This is a unique advantage as separations downstream of the labeling step do not introduce errors that can affect the quality of quantification. As labeling occurs routinely prior to the enzymatic digestion step in case of SILAC and ICPL workflows, reproducibility issues of enzymatic digestions do not affect quantitative results.

With the new ICPL 4-plex technology it is possible to quantify four different proteome samples in one experiment at maximum depth. An example for such a strategy is the "GeLC" approach, in which an SDS-PAGE separation is followed by LC-MS/MS analysis of each band´s digest eluate [3,4]. These two separation dimensions increase the analysis depth in such experiments and huge datasets are generated that require dedicated software support.

In this Application Note we describe the bioinformatics handling of ICPL 4-plex datasets in ProteinScape 2.1. The efficiency of the quantification approach with the new ICPL 4-plex is demonstrated for protein samples with controlled identical concentrations. We demonstrate that the established ICPL 2-plex quantification errors of approx. 10 % [5] are not compromised using the extended 4-plex chemistry.

Experimentals

ICPL labeling:
Four sets of nine standard proteins (250 pmol each) containing (bovine) serum albumin, serotransferrin, lactoperoxidase, β-lactoglobulin, α-lactoglobulin, ribonuclease; (rabbit) glycogen phosphorylase, glyceraldehyde-3-phosphate dehydrogenase and (horse) myoglobin were dissolved in water. They were labelled with the four forms of the ICPL label with the SERVA ICPL™ Quadruplex PLUS Kit (#258968), resulting in a modification of lysine ε-amino groups in the forms ICPL_0, ICPL_4 [2H(4)], ICPL_6 [13C(6)] and ICPL_10 [2H(4) 13C(6)] (Fig. 1). Proteins in the four samples had a relative abundance of 1:1:1:1. After ICPL labeling they were merged and digested either by trypsin or by sequential double digestion with Glu-C and trypsin following the kit protocol.

Data Generation and Analysis:
The resulting peptides (0.7 pmol for each protein) were separated by nano-reversed phase HPLC (EASY-nLC) using a linear gradient of 85 minutes from 5 % B to 60 % B (solvent A: water/ 0.05 % TFA, solvent B: 90 % ACN / 9.95 % water / 0.05% TFA) with a flow of 300 nl/min. They were collected onto MTP AnchorChip™ 800/384 MALDI-targets in 10 sec fractions from minute 19 to minute 83 resulting in 384 single fractions using the PROTEINEER fc II spotting robot (all Bruker). A sheath flow of 150 µl/h matrix solution (800 µl of matrix solution containing 748 µl 95 % ACN / water, 36 µl of α-cyanocinnamic acid saturated in 90 % ACN / water, 8 µl 10 % TFA, and 8 µl 100 mM $NH_4H_2PO_4$) provided evenly prepared sample spots. WARP-LC 1.2 was used to control the acquisition using the WARPLC-method "FLEX_ICPL4plex_Default_C12.WarpLCMethod" with the following parameters: 4000 shots were acquired with constant laser power, randomly screening the complete anchor spot. External calibration was performed automatically on Bruker peptide calibration standard II (#222570) spotted onto calibration spots. Spectra were processed in flexAnalysis 3.3 using the LC-MALDI ICPL4plex MSprocessing_C12.FAMethod.

For processing, identification and relative quantification of ICPL 4-plex labelled peptides the ProteinScape 2.1 proteomics suite (Bruker) was used providing access to the ID and quantification data of all peptides/proteins. The software was requested to determine the following intensity ratios between peptides and proteins: (0/4, i.e., ICPL_0/ICPL_4), (0/6) and (0/10) and all ratios were expected to be 1.0. Typical retention time shift settings were 20 sec between the deuterated vs. non-deuterated ICPL forms reflecting the moderate offset between forms (Fig. 2A). Search conditions for MALDI data were: SwissProt_decoy DB, no_enzyme, other mammals, carbamidomethylation (C), 4 variable Protein N-term and 4 ICPL(K) labels, 30 ppm MS Tol, 0.7 Da MS/MS Tol., Min. Mascot ion score 35, Protein FPR < 2 %.

Results

ICPL 4-plex labelled 9 protein mixtures were successfully identified in ProteinScape via automatically triggered Mascot searches and accurately quantified. The 4 peaks of ICPL 4-plex labelled peptides are separated by 4-2-4 Da and the group of deuterated peaks (ICPL_4 and ICPL_10) is chromatographically separated from the non-deuterated peaks (ICPL_0 and ICPL_6) by approx. 10 sec (Fig. 2A, LC-MS SurveyView).

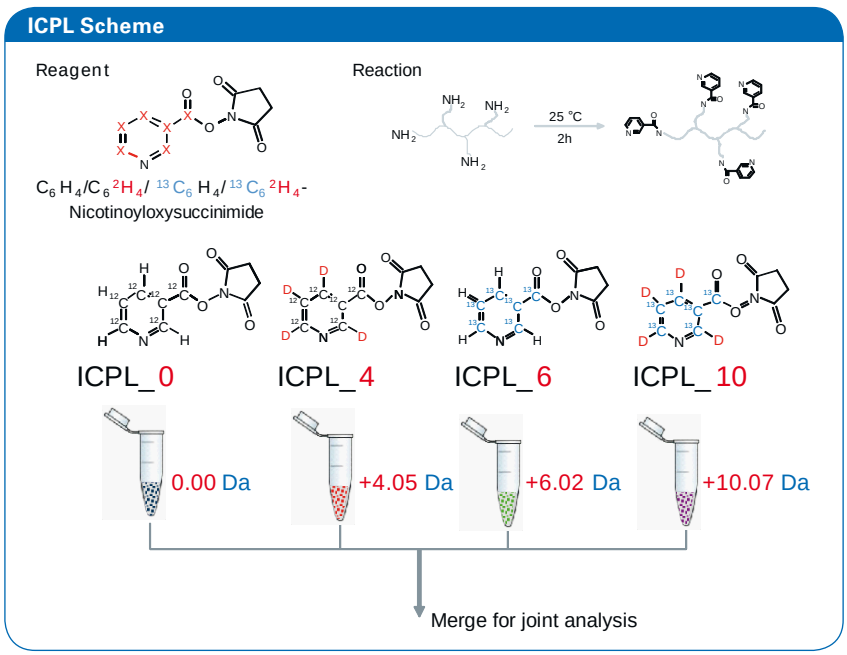


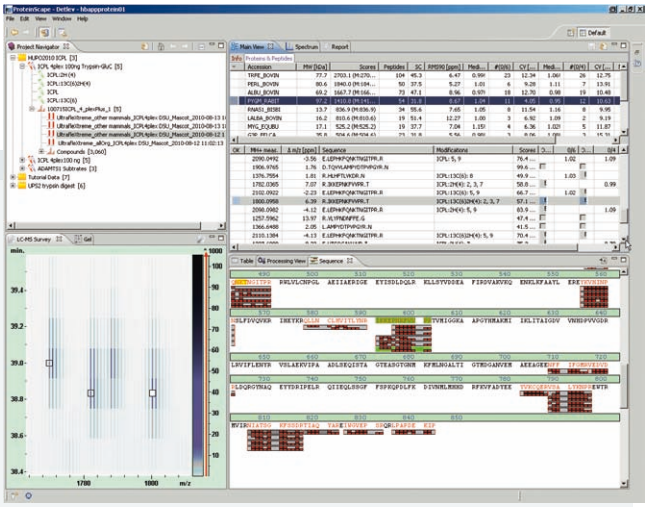
Figure 1: The ICPL chemistry. The basic structure of the nicotinoyloxysuccinimide reagent is shown (top left) as well as the gross reaction scheme with protein N-terminal and Lysin ε-aminogroups. The reagent can be used in 4 isotopically labelled variants, in which substitutions by 4 deuterium atoms ($^2H_4$) and/or 6 ^{13}C atoms ($^{13}C_6$) introduce mass shifts of 4.05, 6.02 and 10.07 Da relative to the native isotopic form. This pattern can be recognized by the analysis software, and is the starting point for the analysis of non-isobarically labelled proteins.

As ICPL-labeling of lysine residues removes a good fraction of tryptic cleavage sites, the standard protocol involves a Glu-C digest followed by a trypsin digest (GT-digest). The additional use of Glu-C generates an increased number of peptides. The trypsin digest provided IDs of 8 out of 9 proteins, myoglobin was not identified. In case of the GT-digest 19 myoglobin peptides were detected, almost all of them were generated by cleavage of at least one glutamic acid (E). In addition, the average sequence coverage (SC, %) of the 8 proteins identified in both MALDI experiments increased from 34 to 40 % and the number of identified peptides from 40 to 46 per protein due to the additional Glu-C digestion step.

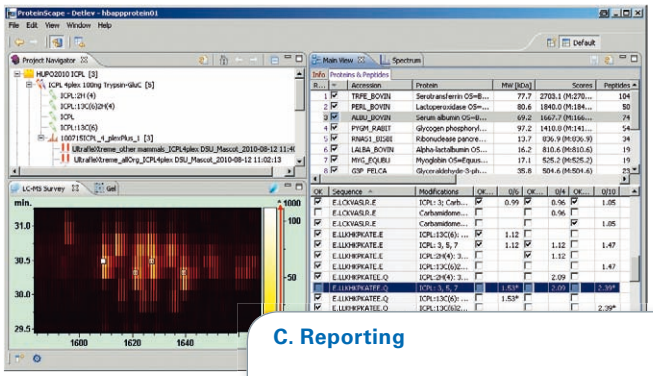
Statistical evaluation of the identified peptides in the GT-digest - searched with "enzyme:none" revealed that the C-terminal amino acid residues consist to 38 % of R residues, 34 % E, 11 % H and 10 % D. The residues N, Y, A, V, M, G and F occurred as C-terminal residues only in 1-2 % of all peptides, C-terminal K was not detected at all, indicating a complete labeling grade.

ProteinScape – data organization

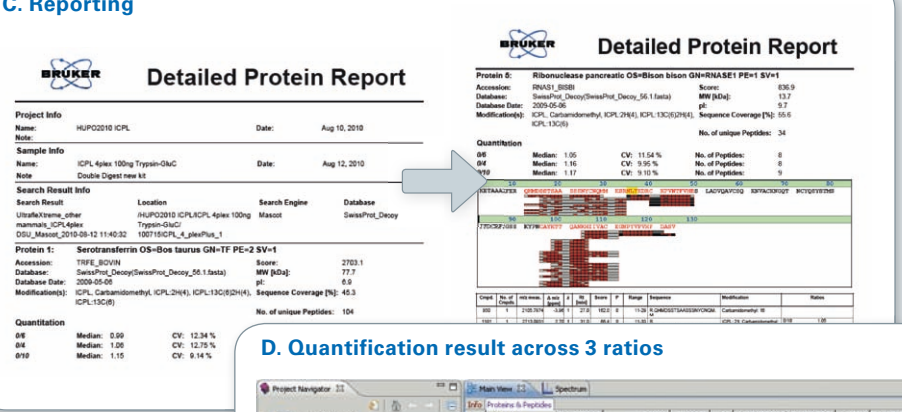
A. Organisation of proteomics quant data in ProteinScape



B. Reliable outlier detection and handling



C. Reporting



D. Quantification result across 3 ratios

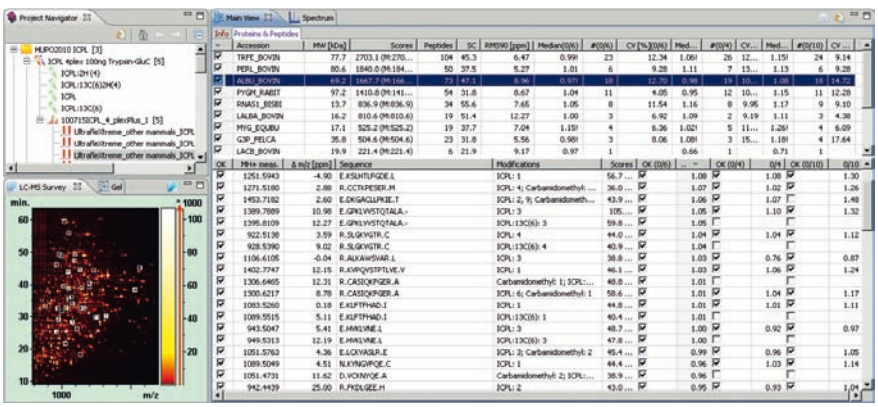


Figure 2: Organisation of proteomics data in ProteinScape. After selection of a search result in the Project Navigator, the Proteins and Peptides list provides access to information in the table and allows to navigate the LC-MS dataset. In addition, all MS/MS data are mapped to the protein sequence to permit evaluation of sequence coverage, digest completeness, and specificity, etc.

In ProteinScape the data are organized to enable fast browsing for validation purposes (Fig. 2A). Fig. 2B shows an inaccurate quantification of one 4-plex pattern of peptide BSA-(555-565) "LLKHKPKATEE" in which ratios between 1.53 (0/6) and 2.39 (0/10) were obtained automatically by the software. Close investigation of this 4-plex in the LC-MS SurveyView (Fig. 2B) indicated a strong overlap with about 3-4 further 4-plex patterns that obscured automatic quantification of this peptide pattern. In the peptide table, though, one can clearly see that BSA-(555-564) "LLKHKPKATE" – which is only one amino acid residue shorter – provides a much better quantification of the relative protein abundances near 1:1:1:1. This highlights the need for manual investigation of quantitative proteomic data and "outliers", as true regulations on the single peptide level could be biologically extremely interesting, e.g., in case of phosphorylation or other modification events. ProteinScape facilitates this kind of validation approaches and accelerates the generation of high quality data that can be exported in various ways including HUPO/PSI compliant pdf files of the ID and quantification summary (Fig. 2C).

The quantification results across the 3 ratios (0/4, 0/6 and 0/10) relative to state ICPL_0 for the GT-digest are shown in Fig. 2D. The protein table (top) summarizes the quantification results and statistical key figures like Protein Scores, Sequence Coverage (SC %), Median Values of Regulation or CV Values for the quantification across all labelled peptides of this protein.

For the selected protein, all detected peptides and their regulation are listed in the peptide table. Outliers are automatically recognized and labelled by the software but left in the table to facilitate manual reinspection. Both tables are freely configurable, sorting and filtering is supported to provide the matching visualization for various tasks. The typical CV values of the quantification results of the GT-digests are in the 10 % and the quantification errors in the 5 % range, in agreement with previous studies using the ICPL duplex [6]. Clearly the additional Glu-C digestion step in the GT-digests improved the ICPL quantification quality and allowed a successful quantification of all smaller proteins - including myoglobin.

Conclusion

The ICPL 4-plex technology for the quantification of protein samples was analyzed by LC-MS/MS and provided high accuracy (5 % error) and precision (CV 10 %) results in a controlled study. The proteomics software ProteinScape allows to summarize the qualitative and quantitative results with regard to outlier detection, decoy validation in a single non-redundant quantitative protein list, as well as visual validation tools. ProteinScape directly supports the HUPO/PSI reporting standards in providing properly formated ID+quantification reports.

References

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Keywords
Protein quantification
Protein labeling
ICPL 4-plex
LC-MALDI
GeLC

Instrumentation & Software
ultrafleXtreme
EASY-nLC
ProteinScape
PROTEINEER sp
SERVA ICPL™ Quadruplex PLUS Kit (#258968)

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