

ICPL:

A new quantitative mass spectrometric method for proteomics

A new protein labelling strategy for quantitative proteomics, ICPL, overcomes previous limitations in quantification approaches. ICPL allows reduction of proteomes complexity even after labelling of proteins, resulting in higher protein identification rates and dramatic improvements in quantitation errors.

Introduction

Proteome analysis has been carried out routinely for about 10 years. In most cases, the proteins are separated and quantified by 2-dimensional gel-electrophoresis (2D-GE). For identification, peptide mass fingerprints (PMFs), generated with MALDI-TOF mass spectrometry are subsequently applied [1]. This established method is robust, fast and suitable for automation, while the validation of the data is straight forward.

A complementary approach relies on the LC-MS/MS analysis of proteome digests. In the last years it became apparent that the separation power of this approach does not comply with typical proteome complexity – i.e. 30,000 protein / 1,000,000 peptides – and requires additional steps of pre-fractionation on the protein level. Quantification of proteins in such LC-MS/MS approach is achieved with isotopic labelling.

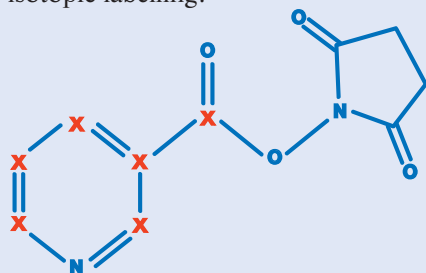


Fig. 1: The ICPL reagent. The mass difference between labeled and unlabeled ($^{12}\text{C}/^{13}\text{C}$) is $\Delta m = 6,0204$ Da (105,0215 vs. 111,0419 Da for every modified amino group: Lys + protein N terminus).

However, the prototype of this technology ICAT [2] as well as the newly introduced iTRAQ labelling technology are not capable for reducing the complexity of the proteomes on the protein level.

This inadequate separation power on the peptide level dramatically restricts the analysis on a true proteomic scale.

ICPL technology

A new technology, isotope coded protein labelling (ICPLTM) was invented by Kellermann & Lottspeich [3]. ICPL allows reduction of complexity using a variety of protein- and peptide separation techniques, after labelling of proteins. Initially, proteins are labelled using a lysine-specific label ($^{12}\text{C}/^{13}\text{C}_6$ -Nicotinic acid-succinimide), resulting in an mass difference of 6.02 Da between the heavy (H)- and light (L)-ICPL label (Fig. 1).

After combination of the labelled proteome samples, the proteins can be digested enzymatically followed by the mass spectrometric quantification in MS mode, while MS/MS analysis of the digested peptides is used for identification. Most interestingly for complex proteome samples, the quantification is performed after further protein pre-fractionation / separation such as 1D-PAGE or LC prior to the

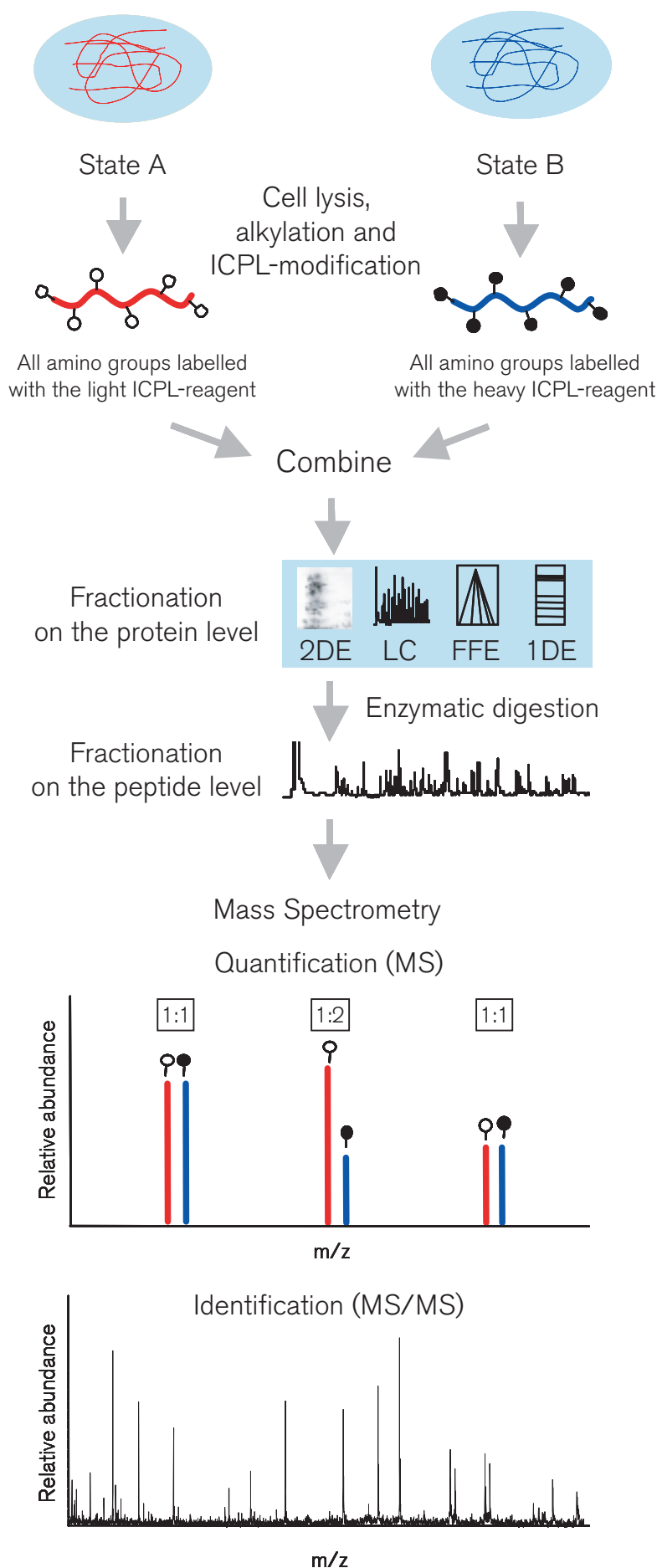
LC-MS/MS analysis (Fig. 2). Even 2D-GE plus PMF analysis can be used for protein separation and quantification from the PMFs.

These optional pre-fractionation steps dramatically increase of the total amount of identified proteins as well as the sequence coverage of proteins by their identified peptides. An increased number of identified peptides per protein is beneficial for the quantification result of the proteins due to an improved statistical basis. In addition such study designs eliminate the undersampling problem that is associated with peptide-only separation strategies and enable a dramatic reduction of manual validation efforts.

LC-MALDI-TOF/TOF analysis

The peptide mixture of the proteolytic digested proteome was separated by capillary- or nano-LC and directly spotted on a Prespotted AnchorChip (PAC) using the PROTEINEER fc robot. Automatic MALDI-MS measurement and data evaluation were under control of (Fig. 3 and 4) the WARP-LC software, which also controlled

Workflow



the automatic selection of peptides for further MS/MS analysis. ICPL-labelled peptides were preferentially selected for MS/MS analysis based on their H/L ratio, dramatically reducing the number of MS/MS spectra and processing time.

In contrast to LC-ESI, MALDI-MS spectra are acquired without any interruption by MS/MS acquisition and thus don't miss out on the provided peptides eluting from the LC-column. MS/MS analyses of all relevant peptides are performed off line well after the LC separation.

The Prespotted AnchorChips (PACs) that we use in these setups are micro-structured disposable plastic chips enabling exact drying of the peptide fractions on defined sample spots [4]. These disposable MALDI targets allow to immobilize whole chromatograms for considerable time (3 months to 1 year is easily possible) which renders them an ideal tool for further re-analysis and archiving of multiple LC-runs eliminating the need to repeat the LC-separation for fraction re-analysis.

The Quality of ICPL Quantification

A lysate from *E. coli* was divided into two identical aliquots and spiked with additional proteins from different species and in various concentrations, followed by either labelling with the light or the heavy ICPL-label (ICPL-kit, Bruker Daltonics). Subsequently, both samples were mixed, digested with trypsin and analysed via LC-MALDI (Fig. 2). The *E. coli* proteins continuously show an intensity ratio of H/L = 1:1 of the ICPL-labelled peptides. The spiked ovalbumin provided a regulation of H/L = 0.53 (theoretical: 0.5). In general all quantification errors in ICPL-experiments were in the 5 % range, if more than 4 clearly identified pairs of peptides were determined. However, for identification of

Fig. 2: Schematic representation of the ICPL work flow. Proteins from different sources are labelled with the heavy (H) or the light (L) form of the ICPL reagent. After merging the samples and an optional protein fractionation, analysis is performed by proteolytic digestion, LC-separation, MS-quantification and MS/MS identification.

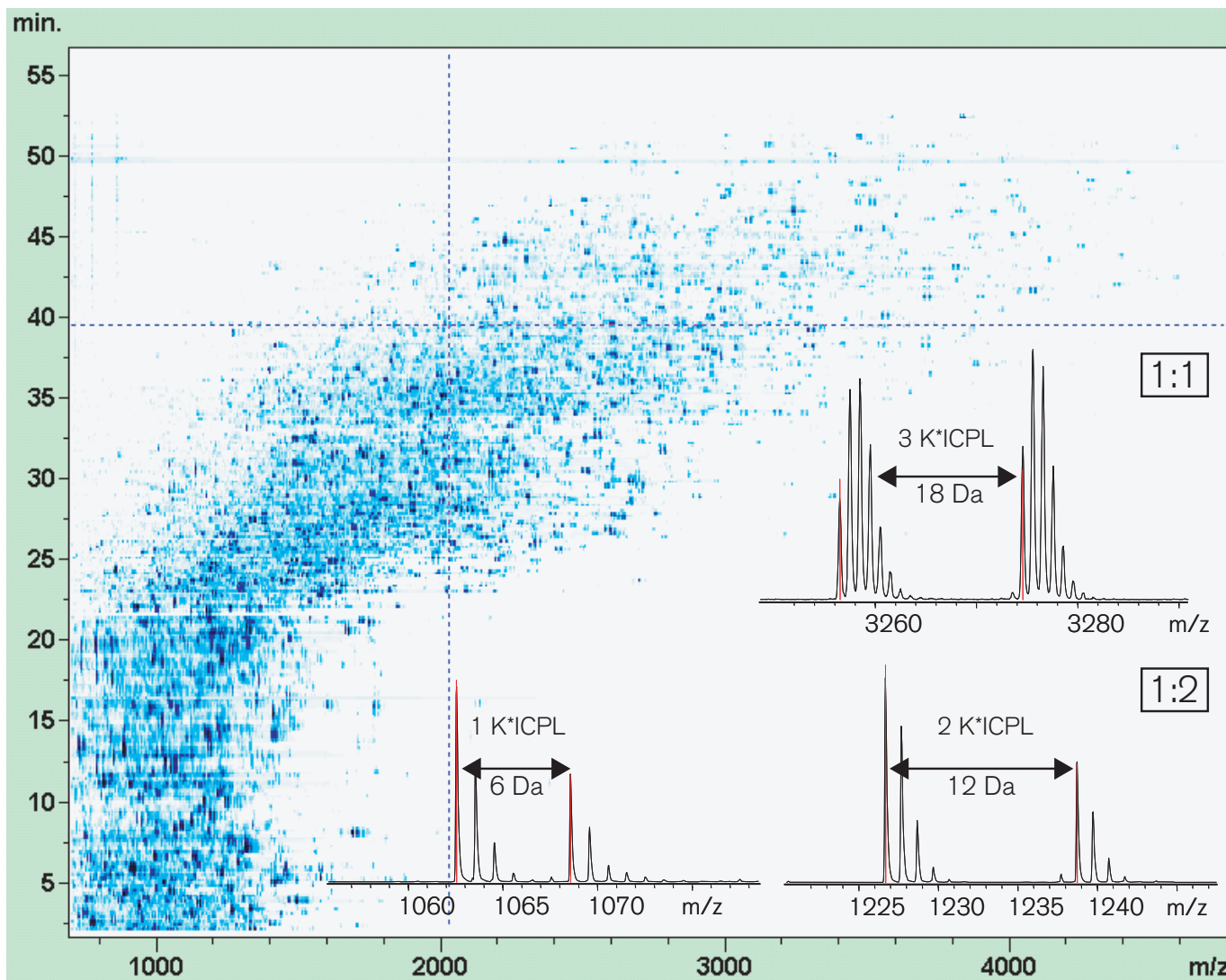


Fig. 3: WARP-LC Survey View of a LC-MALDI run with ICPL-labelled *E. coli* lysat. The retention time (min.) vs. the peptide mass (m/z) is shown. Some peptide pairs, used for quantitation are shown. *E. coli* proteins are in a ratio of 1 : 1. Multiple labelled peptides (e. g. 1 to 3 lysine groups per peptide) are responsible for the distance of pairs of 6, 12, or 18 Da. The two peptides shown to be regulated in a ratio of 1 : 2 derive from the labelled ovalbumin.

a protein even two pairs are already sufficient. In case of ovalbumin, a quantification error of 6% was determined. If only two or three pairs of peptides are given, outliers can not be identified on solid statistical grounds, seriously affecting the quality of the analysis as well as the validation of data.

Thus, the pre-fractionation on the protein level allows reducing the sample complexity prior to the LC-MALDI analyses and to achieve a higher number of peptide pairs per protein. This effect can be demonstrated very impressively with the 2D-GE-PMF analysis of ICPL-labelled proteomes. Here, a quantification error well within the 5%-range was achieved with a protein-sequence-coverage of 30-80% [3].

Protein Name and Species	Score	MW [Da]	PMS [ppm]	S/N	Exp. No.	[H/L] +/- RMS
ribosomal protein S5 [yeast-like construct]	429.28	20984.8	10.03	8152	11	1.15 +/- 0.22
transcription termination factor Rho: polarity suppressor [Escherichia coli O157:H7 EDL933]	429.45	47024.5	11.41	1465	12	0.87 +/- 0.42
COG0095: F0F1-type ATP synthase, beta subunit [Haemophilus influenzae R294E]	421.79	49931.7	4.75	1322	7	1.17 +/- 0.03
60 kDa chaperonin GroEL [Vibrio fischeri E5114]	420.38	57395.6	14.87	3230	11	1.09 +/- 0.07
malate dehydrogenase	408.77	32417.2	4.93	1673	6	1.12 +/- 0.06
malate dehydrogenase	408.77	29512.8	7.88	1873	6	1.12 +/- 0.06
PHOSPHATE ACETYLTRANSFERASE [EC 2.3.1.8] [PHOSPHOTRANSACETYLASE] [Escherichia coli]	399.04	77325.0	9.44	1243	12	1.02 +/- 0.21
OmpF Porin [Mutant R42c]	397.08	37865.7	11.38	1976	6	1.14 +/- 0.61
pyruvate dehydrogenase (dihydrolipoamide acetyltransferase component) [Escherichia coli O157:H7 EDL933]	396.40	66198.7	11.62	1397	10	1.36 +/- 0.61
ribosomal protein S11 [synthetic construct]	390.16	16952.9	10.95	2604	8	1.20 +/- 0.19
uraminate protein product [Escherichia coli]	388.35	51022.7	6.39	877	7	1.48 +/- 0.57
Chain A, Improved Structural Model For The Catalytic Domain Of E Coli Dihydrolipoamide Succinyltran	387.17	37945.8	11.30	1976	6	1.14 +/- 0.04
35S ribosomal protein L10 [Escherichia coli O157:H7 EDL933]	386.34	26005	11.41	2008	8	1.13 +/- 0.07
ovalbumin	382.09	43964.6	7.04	4442	11	0.53 +/- 0.06
enolase [Escherichia coli O157:H7 EDL933]	273.07	45631.4	12.67	2936	11	1.00 +/- 0.16
phosphoglycerate kinase [Escherichia coli O157:H7 EDL933]	368.70	41275.7	9.54	1967	7	1.06 +/- 0.21
60 kDa chaperonin (Protein Cpn60) [gpfl, protein]	368.25	57397.8	16.21	2996	9	1.06 +/- 0.17
Chain A, Improved Structural Model For The Catalytic Domain Of E Coli Dihydrolipoamide Succinyltran	365.40	26054.7	5.70	1672	11	1.19 +/- 0.11
heat shock protein 60 [Neisseria meningitidis]	359.81	57479.0	13.80	3852	11	0.94 +/- 0.15
ribosomal protein L10	348.76	17782.5	6.00	1329	7	0.89 +/- 0.24
heat shock protein [Candidatus Blochmannia cantaneri]	347.57	57633.1	16.52	3414	11	1.07 +/- 0.09
ribosomal protein S13 [synthetic construct]	345.06	16150.8	13.24	2083	9	1.14 +/- 0.06

Ret. #	RT [min]	MW [kDa]	Δm [Da]	z	Ions Score	S/N	Ret. Area	Inst. Type	H/L	Sequence	Variable Modifications
946	8.29	1062.6059	-0.0045	1	48.90	1163.3	6271	Tot/Tot	0.57	TQNNKVR	ICPL_light (K)
947	8.24	1068.6263	-0.0041	1	29.23	658.8	3593	Tot/Tot	0.57	TQNNKVR	ICPL_heavy (K)
9587	25.60	1226.7049	-0.0007	1	37.20	508.7	2369	Tot/Tot	0.50	KIKVLPRI	2ICPL_light (K)
9588	25.59	1238.7457	0.0019	1	33.67	232.4	1176	Tot/Tot	0.50	KIKVLPRI	2ICPL_heavy (K)
5166	33.33	1858.9662	-0.0269	1	26.73	41.4	152	Tot/Tot	0.54	ELNSWVESGDTNGIR	
6592	40.33	2189.1104	-0.0206	1	94.29	670.6	1981	Tot/Tot	0.54	YRILPEYLQGVKELVR	ICPL_light (K)
6593	40.31	2195.1308	-0.0238	1	60.54	352.8	821	Tot/Tot	0.54	YRILPEYLQGVKELVR	ICPL_heavy (K)
5908	36.53	2386.2042	0.0143	1	97.32	113.3	950	Tot/Tot	0.58	DILNGIKFKNDAVSPSLASR	ICPL_light (K)
5909	36.48	2392.2246	0.0110	1	66.97	55.9	294	Tot/Tot	0.58	DILNGIKFKNDAVSPSLASR	ICPL_heavy (K)
7217	45.06	2679.3657	0.0117	1	77.57	400.1	1455	Tot/Tot	0.45	ADHPFLFKDHATNALPFGSR	ICPL_light (K)
7218	45.09	2685.3861	0.0114	1	34.03	244.4	699	Tot/Tot	0.45	ADHPFLFKDHATNALPFGSR	ICPL_heavy (K)

Fig. 4: Summarization of the LC-MALDI analysis shown in Fig. 2. *E. coli* proteins show a H/L ratio of 1; ovalbumin a H/L ratio of 0.5.

Further promising features of the ICPL-technology

In contrast to technologies on the basis of peptide labelling (e.g. iTRAQ), ICPL enables the detection of protein isoforms, posttranslational modifications and variants of protein-splicing that can only be distinguished if at least one of the separation parameters of the workflow is based on molecular properties of intact proteins, such as their molecular weight or their isoelectric point.

Furthermore, the signal intensities of labelled peptides is 10-20 times higher than those of unlabelled ones and thus simplifies MS/MS fragmentation. During the MALDI process, the nicotinic-acids of the ICPL-label serve as MALDI-matrix and thus ease the ionisation of the peptides.

References

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Ordering Info for the ICPL-kit:

Product	Cat. No.	Size
SERVA ICPL™-Kit	#234017	1 Kit (2 x 6 samples)

The SERVA ICPL-Kit is worldwide available through Bruker Daltonics care:
www.bdal.de/care.

The ICPL technology is fully supported by Bruker Daltonics instruments and solutions in PROTEINEER-LC 1.1.

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