

INSTRUCTION MANUAL

SERVA ICPL™ Quadruplex-Kit

(Cat. No. 39232.01)



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1. SERVA ICPL™ Quadruplex-Kit

The ICPL™ methodology is based on the isotope labelling of free amino groups of intact proteins by amine specific reagents. Therefore the method is suitable for all kinds of protein samples, inclusive of extracts of tissue or body fluids, and is compatible with all currently in proteome studies used separation methods. The ICPL™-technology is now available with a fourth label. The kit allows for the relative quantification of four different protein samples in one experiment and the efficient, accurate and reproducible quantification of proteins with high sequence coverage. All four labels can be freely combined with each other. By omitting one label you can as well compare only two or three samples.

(Die ICPL™-Quadruplex method was presented on HUPO 2008 in Amsterdam, NL, Halder et al., ICPL (Isotope-Coded Protein Labeling) QUADRUPLEX Technology for quantitative Proteomics.)
The exclusive license for the ICPL™-technology is hold by *TOPLAB GmbH*, Martinsried, Germany (<http://www.toplab.de>). *Serva electrophoresis GmbH* has signed a license agreement for the world wide distribution of the ICPL™ reagent kit.

Technical support:

For detailed technical questions, please contact Toplab GmbH:

Tel.: ++49 (0)89-244 1454 1283

E-Mail: info@toplab.de

Key features:

- **Labelling of proteins** before doing any cleavage or separation steps reduces the possibilities of experimental errors to a minimum.
- **Labelling of proteins** followed by protein separation- and fractionation methods reduces the enormous complexity of biological samples on the protein level.

1.1. Kit components

ICPL_0 reagent (purple)	22 µl
ICPL_4 reagent (orange)	22 µl
ICPL_6 reagent (black)	22 µl
ICPL_10 reagent (white)	22 µl
Stop solution 1 (green)	200 µl
Reduction solution (yellow) in TCEP	200 µl
Alkylation reagent (blue) (Iodoacetamide)	6 x 14.8 mg
Stop solution 2 (red) (Hydroxylamine)	200 µl
Lysis buffer (white) (Guanidine-HCl)	10 ml
Solution buffer (white)	1.5 ml
Protein mix for ICPL_0 (white)	20 µl lyophilized
Protein mix for ICPL_4 (white)	20 µl lyophilized
Protein mix for ICPL_6 (white)	20 µl lyophilized
Protein mix for ICPL_10 (white)	20 µl lyophilized

The kit contains reagents for 4 x 6 reactions. Each reaction may be performed with 100 µg of total protein in a volume of 20 µl.

Additionally required chemicals and equipment not provided in the kit:

- Trypsin or endoproteinase GluC
 - 4M urea
 - 25 mM Tris
 - 2 N NaOH
 - 2 N HCl
 - Argon
 - Acetone (ice cold)
 - 80 % acetone (ice cold)
-
- pH-meter equipped with micro-electrode
 - pH-paper
 - Cool centrifuge/ultra centrifuge
 - Thermo mixer (up to 60 °C)

1.2. Storage conditions

The kit will be shipped at room temperature and is stable for at least 14 days at this temperature. Upon arrival store SERVA ICPL™ Kit immediately at –20 °C. Long term storage at –80 °C is as well possible.

2. ICPL™ Quadruplex protocol

2.1. Overview of the ICPL™ reagent technology

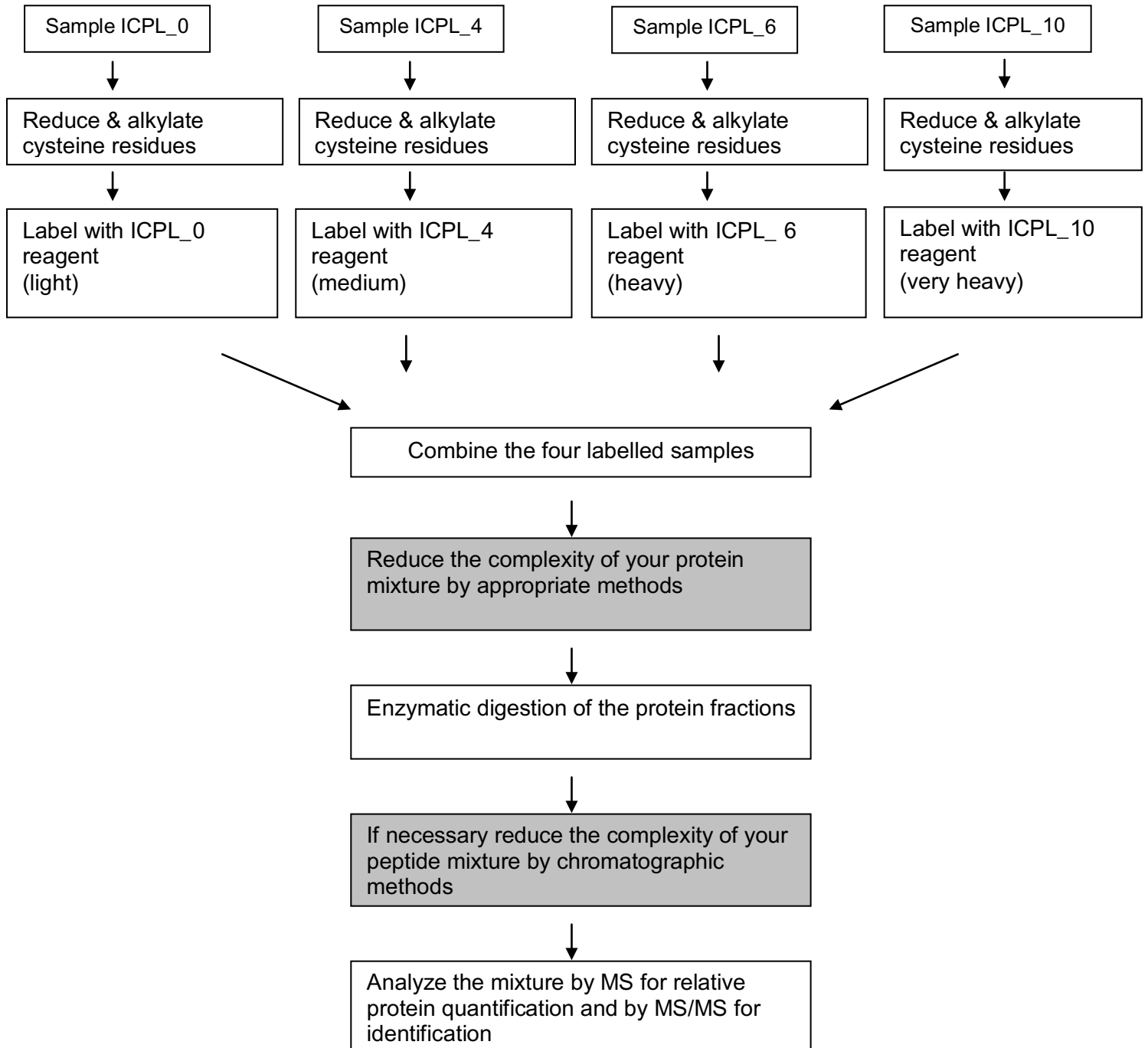


Fig. 1: Overview of the ICPL™ reagent technology

If you are running the ICPL protocol the first time, it is recommended, to analyse the provided protein mixtures through the entire protocol. The expected results should be comparable with the results shown at the end of the protocol.

2.2. Dissolution of the protein sample

2.2.1. Required solutions

- Lysis buffer (white)

Safety information:

The lysis buffer is harmful if swallowed and irritating to eyes and skin. Wear suitable protective clothing and gloves.

2.2.2. Procedure

In a first step, the protein or cell samples (control/sample) will be dissolved in lysis buffer (white).

Important: The protein concentration should be above 5 mg/ml, but must be in any case higher than 2.5 mg/ml.

The following lysis protocol is recommended:

- Vortex 2 min.
- Leave sample in thermo mixer at 1000 rpm for 20 min. at 25 °C
- Vortex 2 min.
- Sonicate 4 times for 30 sec. in ultrasound bath (cool sample during pauses in ice water for 2 min.)
- Vortex 1 min.
- Leave sample in thermo mixer at 300 rpm for 15 min at 25 °C
- Vortex 2 min.
- Spin sample for 30 min. at 75 000 g (ultra centrifuge)
- Use the supernatant directly for the further analysis steps.

Caution! If lysis buffers different from the provided lysis buffer are used, they should be tested with the protein standard mixture before usage for other samples. Use pH 8.5; avoid the presence of primary amino group containing agents. Urea, thiourea, CHAPS can be used, however.

2.3. Determination and adjustment of the protein content of the samples

Prior to labelling, the protein concentration of the samples has to be determined by Bradford assay. Subsequently the concentration has to be adjusted to 5 mg/ml by adding Lysis buffer (white).

Note: The protocol is optimized for a protein concentration of 5 mg/ml. However, it works as well at 2.5 mg/ml. As the recovery rate of the protein precipitation step below depends strongly on the total protein concentration, losses are likely when working with lower protein concentrations. Therefore, it is extremely important, to keep the concentrations of the reagents strictly as recommended. If you want to work with increased sample volumes of 40 μ l (for example to facilitate the pH measurement), you rather double the sample amount (if available) than dilute the sample, and you also have to double the volumes of the reagents given in this protocol! Therefore, only half the numbers of samples can then be labelled with the content of this kit.

2.4. Reduction and blocking of cysteine residues (Carbamidomethylation)

2.4.1. Required solutions

- Reduction solution (yellow)
- Alkylation reagent (blue)
- Solution buffer (white)
- Stop solution 1 (green)
- Possibly, 2 N NaOH or HCl
Then, you additionally need a micro pH-meter for adjustment of the pH value

Safety information:

The alkylation reagent (iodoacetamide) is an irritant. Do not breathe dust and wear suitable protective clothing and gloves.

2.4.2. Procedure

Before start:

- Check the pH of the sample buffer and if necessary, adjust to 8.5 ± 0.1 by addition of HCl or NaOH! A micro pH-meter is recommended. Before each measurement, the tip of the electrode is rinsed with distilled water and dried **very carefully** with a dry, dust-free tissue to avoid sample dilution. Alternatively, you can check the pH by pipetting a small volume (1 – 2 μ l) on pH-paper.
 - The carbamidomethylation protocol is identical for all four samples (ICPL_0, ICPL_4, ICPL_6 and sample ICPL_10).
1. Add **0.5 μ l reduction solution (yellow)** to 20 μ l sample solution (equivalent to 100 μ g protein) and **reduce proteins for 30 min at 60 °C**.
 2. **Cool sample to room temperature and spin down sample** to remove condensed solution from the lid.

3. Dissolve **one cup of alkylation reagent (blue)** in **200 µl solution buffer (white)** and wrap in aluminium foil (light protection).
4. **Add 0.5 µl** of this freshly prepared alkylation reagent **to each sample**, wrap samples quickly in aluminium foil for light protection and leave samples **for 30 min at 25 °C**.
5. Stop reaction by adding **0.5 µl stop solution 1 (green)** to each sample and incubate for **15 min at 25 °C**.

2.5. Isotope labelling of the protein samples

2.5.1. Required solutions

- ICPL_0 reagent (purple)
- ICPL_4 reagent (orange)
- ICPL_6 reagent (black)
- ICPL_10 reagent (white)
- Stop solution 2 (red)
- Argon
- 2 N NaOH
- 2 N HCl

Safety information:

The stop solution 2 (hydroxylamine) may cause sensitization by skin contact. Harmful: Danger of serious damage to health by prolonged exposure if swallowed. Avoid contact with skin. Wear suitable protective gloves.

2.5.2. Procedure

1. After carbamidomethylation **add 3 µl of ICPL_0 reagent solution (purple)** to sample ICPL_0, **3 µl of ICPL_4 reagent solution (orange)** to sample ICPL_4, **3 µl of ICPL_6 reagent solution (black)** to sample ICPL_6 and **3 µl of ICPL_10 reagent solution (white)** to sample ICPL_10.
2. **Overlay the four samples with argon** (or equivalent) to exclude oxidation, vortex (10 sec.) and sonicate for 1 min in ultrasound bath. Spin down samples.
3. Incubate samples **for 2 h at 25 °C**.
4. **Add 2 µl of stop solution 2 (red)** to each sample and incubate for **20 min at 25 °C** to destroy excess reagent.
5. **Combine all four ICPL labelled samples** and vortex thoroughly.
6. **Adjust the pH** of the mixture to **11.9 ± 0.1 by adding 2N NaOH** (about 4 µl for 4 x 20 µl sample volume) to destroy possible esterification products. **After 20 min add** the same amount of **2N HCl** to neutralize sample (usually it is not necessary to check the pH).

2.6. Purification of the labelled proteins by acetone precipitation

2.6.1. Required solutions

- Acetone cooled to $-20\text{ }^{\circ}\text{C}$
- 80 % acetone / 20 % water cooled to $-20\text{ }^{\circ}\text{C}$

2.6.2. Procedure

1. **Add** the equal amount of **dist. water** to your sample, i.e. about **114 μl** (= 4 x (20 μl sample + 1.5 μl reduction + 3 μl reagent + 2 μl stop solution) + 8 μl pH adjustment).
2. **Add** 5-fold excess (related to total volume of sample and water) of **ice-cold acetone** to your sample, i.e. **1140 μl** acetone (= 228 μl x 5) and leave sample at $-20\text{ }^{\circ}\text{C}$ overnight.
3. **Spin down** precipitated proteins at **20 000 g for 30 min at $4\text{ }^{\circ}\text{C}$** .
4. **Overlay** precipitated proteins with ca. 100 – 200 μl **ice-cold 80 % acetone**, shake carefully in your hands and **spin down** again at **20 000 g for 5 min at $4\text{ }^{\circ}\text{C}$** .
5. **Discard supernatant** and remove remaining acetone at room temperature by evaporation (let the cup stand open).
6. The samples can be stored now at $-80\text{ }^{\circ}\text{C}$ or directly dissolved in appropriate buffers for protein separation (1D- or 2D-gels).

The further workflow depends on the complexity of the sample. Complex proteome samples should be separated to a convenient complexity by any protein fractionation method. The protein mixes ICPL_0, ICPL_4, ICPL_6 and ICPL_10 can be cleaved directly by trypsin. For a better solubility, we recommend to dissolve the acetone precipitate using a buffer containing 4 M urea. However, before digestion with trypsin or endoproteinase GluC the urea content should be diluted to 2 M urea.

2.7. Enzymatic digestion of the labelled proteins

2.7.1. Required solutions

- 25 mM Tris/4 M urea
- 25 mM Tris/HCl pH 8.5 resp. 7.8
- Trypsin
- Endoproteinase GluC

2.7.2. Procedure

Enzymatic digestions of the labelled samples are performed according to common protocols. We recommend using trypsin or endoproteinase GluC as enzymes.

1. Dissolve sample in 25 mM Tris, 4 M urea, pH 8.5 for tryptic cleavage or pH 7.8 for cleavage with endoproteinase GluC. For a better solubility, we recommend to dissolve the acetone precipitate using a buffer containing 4 M urea. However, before digestion the urea content should be diluted to 2 M urea.

2. Add enzyme in a protein/enzyme ratio of 50:1 for trypsin or 30:1 for endoproteinase GluC.
3. Incubate sample over night at 37 °C (trypsin) or 25 °C (GluC).

2.8 Peptide Labelling with ICPL

For labelling of peptides with ICPL™ we recommend to directly solve the samples in lysis buffer and then to label according the protocol (see 2.5.). Only if the peptides contain cysteine residues, samples must be reduced and blocked by carbamidomethylation as described in 2.2. to 2.4. Instead by an acetone precipitation, samples are purified by a C18-ZipTip column.

Purification of peptides by RP-C18 material

Required material

- ZipTip C18 (e.g. Millipore or any other distributor, or self packed in gel loader tips)
- 0.5 % Trifluoroacetic acid in HPLC grade water (solution A)
- 0.5 % Trifluoroacetic acid in 50 % acetonitrile (solution B)

Procedure

1. Equilibrate the beads by washing with several column volumes of solution A.
2. Dilute the peptide sample 1:1 with solution A
3. Press the diluted peptide sample through the column
4. Wash the column with several column volumes of solution A
5. Elute from the beads with 2 x 5 µl of solution B
6. Remove the acetonitrile by putting the samples into a Speed-Vac for 10 minutes

2.9. Mass spectrometry and data analysis

After digestion, the samples can be directly analyzed by mass spectrometry, preferentially by LC-MALDI-TOF/TOF or LC-ESI-MS/MS.

2.9.1 Mascot search

- **Select endoproteinase Arg-C as enzyme** in the search dialog in case trypsin digestion was used in the lab.

Note	After the modification of lysine (K) residues by ICPL, lysine is protected against proteolytic digestion. Therefore, trypsin cleaves solely C-terminal of arginine (R), i.e., only the Arg-C activity remains. Endoproteinase Lys-C can not be used at all!
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- For Mascot searches, the ICPL modified residues have to be added to the **modification file (mod_file) on the local Mascot server**. The following 8 entries have to be made with exactly the same name strings to ensure software compatibilities (e.g. with Bruker Daltonics' WARP-LC and BioTools bioinformatics packages):
 1. Title: ICPL:13C(6)
Residues: K 239.13680 239.22440
 2. Title: ICPL
Residues: K 233.11640 233.27000
 3. Title: ICPL:2H(4)
Residues: K 237.14773 237.30197
 4. Title: ICPL:13C(6) (N-term)
ProteinNterm: 112.04970 112.05830
 5. Title: ICPL (N-term)
ProteinNterm: 106.02930 106.10390
 6. Title: ICPL:2H(4) (N-term)
ProteinNterm: 110.13070 110.30197
 7. Title: ICPL:13C(6)2H(4)
Residues: K243.12996 243 243.21396
 8. Title: ICPL:13C(6)2H(4) (N-term)
ProteinNterm: 116.0393 116.1139
- Working with **Mascot 2.1** and below: all 8 modifications have to be selected as variable modifications.
- Working with **Mascot 2.2** the light modifications have to be selected as fixed modifications, heavy as variable modifications.
- **Carbamidomethylation** has to be defined as fixed modification.

2.9.2 Quantification

- The mass difference of ICPL_0- and ICPL_4-labeled peptides is 4.05 Da per labelled lysine group, those of ICPL_0- and ICPL_6-labeled peptides 6.0204 Da per labelled lysine group and those of ICPL_0- and ICPL-10-labeled peptides 10.01 Da per labelled lysine group.
- The mass differences between labelled (ICPL_0/ICPL_4/ICPL_6/ICPL_10) and unlabelled peptides are 105.0215Da/109.0715 Da/111.0419 Da/115.0315 Da for each modified amino group.
- **The ICPL_0/ICPL_4/ICPL_6/ICPL_10 ratios of the proteins are calculated as average or median values of the ICPL_0/ICPL_4/ICPL_6/ICPL_10 ratios of the individual peptides.** In general, the calculation should be done by peak-height of the entire isotopic cluster as enabled in the respective quantification software of the instrument manufacturer. Some instruments may provide superior data if peak areas are used as quantification measure, though.

Note: **Reliable protein quantification requires at least 5 peptide ratios** for a reasonable statistical treatment including outlier detection. If too many proteins provide fewer peptides for quantification, the pre-fractionation efforts prior to LC-MS/MS may need to be enhanced or chromatographic or mass spectrometric methods to be improved. Also repetition of the experiment is a standard approach to improve data reliability.

3. Quantification of the protein test mixture by the ICPL™ method

To test the ICPL™ protocol, the kit contains four protein mixtures ICPL_0, ICPL_4, ICPL_6 and ICPL_10) each containing different amounts of 3 standard proteins.

Protein	Protein mix ICPL_0	Protein mix ICPL_4	Protein mix ICPL_6	Protein mix ICPL_10*	Relative amount
BSA (bovine)	13.5 µg	13.5 µg	13.5 µg	13.5 µg	1:1:1:1
Ovalbumin (chicken)	24 µg	12 µg	6 µg	24 µg	4:2:1:4
Carbonic Anhydrase II (bovine)	12 µg	12 µg	24 µg	12 µg	1:1:2:1

*Protein mix ICPL_10 has the identical composition as protein mix ICPL_0.

1. Dissolve the four protein mixtures in 20 µl of lysis buffer (white) each and label the proteins following the protocol, starting with step 2.4. “Carbamidomethylation” (**e.g. protein mix ICPL_0 with the ICPL_0 reagent, protein mix ICPL_4 with the ICPL_4 reagent, protein mix ICPL_6 with the ICPL_6 reagent and protein mix ICPL_10 with the ICPL_10 reagent**).
2. After labelling and precipitation, the proteins have to be dissolved in 20 µl buffer. For a better solubility, we recommend to dissolve the acetone precipitate using a buffer containing 4 M urea. However, before digestion with trypsin or endoproteinase GluC the urea content should be diluted to 2 M urea.

3. After cleavage, the samples are directly analyzed by MS, preferentially by MALDI-TOF-MS. The ratios of the proteins are calculated by the ratio of the peak areas. The group of Prof. Lottspeich at the MPI for Biochemistry in Martinsried developed the software ICPL™ *Quant* for analysis of LC/MS data of ICPL™-labelled proteins, which will be available free of charge after publication (Brunner et al., ICPL Quant – A New Software Tool for Isotope-labelling Based Proteomics and Biomarker Discovery, HUPO 2008, P-TUE-143).

In the following you find the link to the registration page of the software:
<http://www.biochem.mpg.de/lottspeich/technologies/ICPLQuant/index.html>

The following peptides were analyzed by MALDI-TOF-TOF.

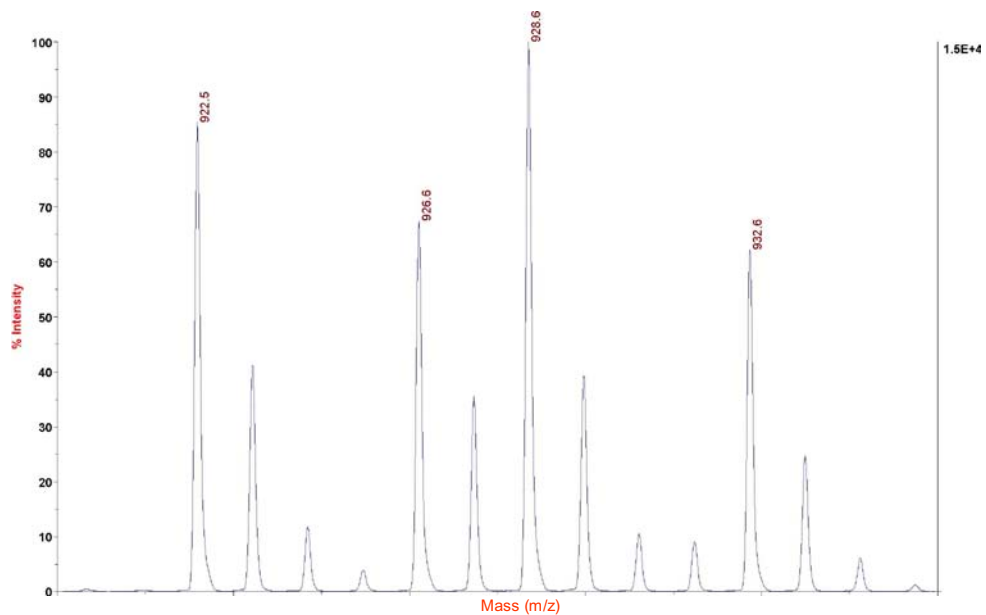
Protein	Mass Light*	Expected Mass Light*	Ratio				Peptide sequence
			ICPL_0 Light	ICPL_4 Medium	ICPL_6 Heavy	ICPL_10 Very heavy	
BSA	922.54	922.51	1.00	1.03	0.98	0.89	SLGK*VGTR
BSA	1065.59	1065.57	1.00	0.98	1.06	0.89	EK*VLASSAR
BSA	1106.64	1106.61	1.00	1.09	0.99	1.00	ALK*AWSVAR
BSA	1300.65	1300.61	1.00	0.88	1.01	0.75	CASIQK*FGER
BSA	1744.95	1744.96	1.00	1.08	0.98	0.88	K*VPQVSTPTLVEVSR
BSA	2648.38	2648.45	1.00	0.97	0.96	0.81	K*VPQVSTPTLVEVSRSLGK*VGTR
BSA	3302.51	3302.57	1.00	0.96	1.04	0.82	LCVLHEK*TPVSEK*VTK*CCTESLVNR
		Mean	1.00	1.00	1.00	0.86	
		StDev[%]	---	7.59	3.53	8.61	
Ovalbumin	1062.63	1062.59	1.00	0.49	0.20	0.95	TQINK*VVR
Ovalbumin	1226.71	1226.70	1.00	0.48	0.23	0.89	K*IK*VYLPR
Ovalbumin	2189.07	2189.11	1.00	0.53	0.21	1.13	YPILPEYLQCVK*ELYR
Ovalbumin	2386.22	2386.17	1.00	0.47	0.17	1.04	DILNQITK*PNDVYSFSLASR
		Mean	1.00	0.49	0.20	1.00	
		StDev [%]	---	5.39	12.15	10.77	
Carbonic Anhydrase	1791.01	1790.98	1.00	1.21	1.87	0.87	PALK*PLALVYGEATSR
Carbonic Anhydrase	1994.94	1994.93	1.00	1.03	2.45	0.90	GPEHWHK*DFPIANGER
		Mean	1.00	1.12	2.16	0.88	
		StDev [%]	---	11.01	18.88	2.52	

Stdev = standard deviation

*Mass medium increases about 4 Da, mass heavy about 6 Da and mass very heavy about 10 Da pro labeled amino acid.

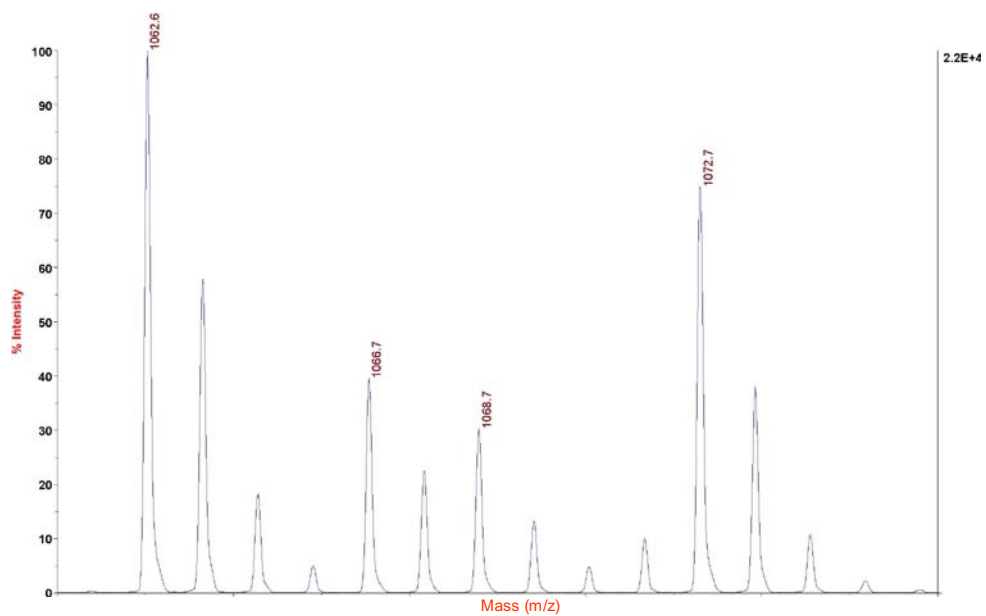
BSA 1:1:1:1
Peptidsequenz: SLGK*VGTR

4700 Reflector Spec #1 MC[BP = 928.6, 15365]



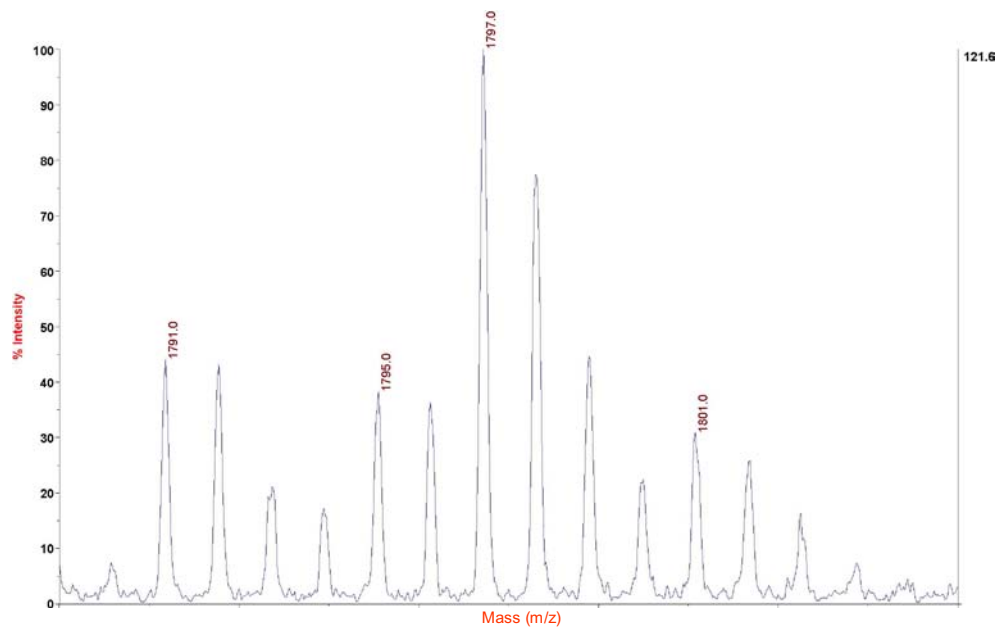
Ovalbumin 4:2:1:4
Peptidsequenz: TQINK*VVR

4700 Reflector Spec #1 MC[BP = 1062.6, 21813]



Carbonic Anhydrase 1:1:2:1
Peptide sequence: PALK*PLALVYGEATSR

4700 Reflector Spec #1 MC[BP = 1860.0, 3752]



4. ICPL™*Quant* Software

ICPL™ *Quant* and ICPL™ *_ESIQuant* are proteomics software tools for analyzing large mass spectrometric datasets acquired from ICPL™ based proteomics experiments quantitatively. Together, they are able to process mass spectrometric data from various vendors and uses results from the MASCOT search engine to generate protein and peptide result tables. The group of Dr. Lottspeich at the MPI for Biochemistry in Martinsried developed the software tools ICPL™ *Quant* and ICPL™ *_ESIQuant* for the analysis of LC/MS data generated from ICPL™-labeled proteins. Both tools are available free of charge.

The following instruments and versions are tested:

- Applied Biosystems 4700/4800 Proteomic Analyzer
Quantification: deisotoped txt files (Data Explorer)
Identification: Mascot dat file
- Bruker Ultraflex2 MS
Quantification: deisotoped peaklist xml files (in the Bruker file structure)
Identification: Mascot dat file
- Thermo Orbitrap
Quantification: deisotoped mzXML file
Identification: Mascot dat file

In the following you find the link to the download page of the software tools:

<https://sourceforge.net/projects/icplquant/files/>

4.1. General information about the ICPL™*Quant* software

ICPL *Quant* has been developed to quantify ICPL labelled peptides on the MS level during LC-MALDI and peptide mass fingerprint experiments accurately. The tool is able to generate a list of differentially regulated peptide precursors for subsequent MS/MS experiments, minimizing time consuming acquisition and interpretation of MS/MS data. ICPL *Quant* is based on two independent units.

Unit 1 performs ICPL multiplex detection and quantification. It proposes peptides to be identified by MS/MS.

Unit 2 combines MASCOT MS/MS protein identification with the quantitative data and produces a protein/peptide list with all relevant information accessible for further data mining.

4.2. General information about the ICPL™-*ESIQuant* software

ICPL™ *_ESIQuant* has been developed to quantify ICPL™-labeled peptides on the MS level during LC-ESI experiments accurately. The ICPL™ *_ESIQuant* software package is one of the key components of the ICPL™-ESI workflow, covering data processing steps like LC-MS feature detection, ICPL™ doublet/ triplet/quadruplet quantification as well as a merging step of LC-MS features and MASCOT search results. As unique features, the software performs isotope pattern overlap corrections and utilizes additional chemical knowledge, e.g. the physico-chemical properties of the ICPL™ labels, to discard false positive isotope patterns, which significantly improves the quality of the final peptide and protein results.

ICPL™_ESIQuant is the first freeware tool on the market, which supports both the shotgun proteomics strategy using Data Dependent Acquisition (DDA) and the directed proteomics strategy using mass inclusion lists for precursor ion selection.

ICPL™_ESIQuant 3.0 (32 and 64 bit version) can be downloaded from <https://sourceforge.net/projects/icplquant/files/>

The main features are:

- Peak picking and deisotoping of mzXML LC-MS raw data
- Recognition of ICPL multiplets (even incomplete ones) in medium complex spectra
- Quantification of all multiplets correcting for isotopic impurities of reagents.
- Preparation of a precursor list containing peptides with user defined regulation factors for MS/MS analysis.
- MASCOT .dat file parser
- Merging of quantification information and identification information
- Protein and peptide output lists in open formats, easily accessible for data mining programs
- Visual display of LC profiles
- 3D plots of all detected ICPL multiplets and singlets
- Computation of protein quantitation ratios (with standard deviation estimation)
- Export of results to files, Excel and to databases

References

- Brunner et al., ICPL™ Quant – A software for non-isobaric Isotope-labelling proteomics, *Proteomics* 10, 2, (2010)
- Brunner A., Kellerman J., Lottspeich F. *A practical guide to the ICPL_ESIQuant software for ICPL-based quantitative proteomics*. *Biochim Biophys Acta*. 2013 Feb 21.pii. S1570-9639 (13) 00082-4. doi: 10.1016/j.bbapap.2013.02.19 [Epub ahead of print]
- Brunner A., Kellerman J., Lottspeich F. *ICPL_ESIQuant – a powerful freeware tool for handling shotgun and directed LC-ESI-MS² experiments*. *J Proteomics Bioinform* 5 (2012): 279-282.