

# INSTRUCTION MANUAL

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## SERVA ICPL™ Quadruplex PLUS Kit

(Cat. No. 39233.01)



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## Safety Information

Before starting, please read the provided MSDS and the safety information on the product labels. In case of missing MSDS, please download the corresponding MSDS from our website at [www.serva.de](http://www.serva.de).

**For research use only. Not for use in diagnostic procedures.**

Ensure that a suitable lab coat, disposable gloves and protective goggles are worn, when working with chemicals.

For further information, please contact SERVA by email: [info@serva.de](mailto:info@serva.de) or visit our website.



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**Important additional information about the ICPL™ Quadruplex PLUS protocol listed in section 5. “Important to know”.**

ICPL is a registered trademark by Toplab GmbH, Martinsried, Germany

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.

# 1. Introduction to the ICPL chemistry

## 1.1. Overview

The ICPL technology<sup>1</sup> 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 tissues or body fluids, and is compatible with all currently in proteome studies used separation methods. The ICPL Quadruplex method<sup>2</sup> allows the simultaneous quantitative analysis of four independent proteome samples.

The ICPL™ Quadruplex PLUS Kit contains trypsin and endoproteinase Glu-C for double digestion<sup>3</sup> of labelled proteins. The double digest will even improve the high sequence coverage of the method. The included ICPL™ Standard PLUS is suitable for process validation. The standard contains endoproteinase Glu-C/Trypsin NB treated quadruplex labelled BSA peptides.

To run a control sample a test mixture containing three proteins in four different amounts is provided with the kit.

### Key features:

- Comparative analysis of up to four proteomes
- Labelling of proteins prior to proteolytic digestion or separation steps minimizes experimental errors
- Protein separation and fractionation subsequent to protein labelling reduces the enormous complexity of biological samples within each protein fraction
- Improved sequence coverage due to the use of endoproteinase Glu-C and Trypsin NB as double digest<sup>3,4</sup>
- Accurate and precise (<10 % CV) quantification based on non-isobaric MS quantification<sup>5</sup>
- Enabling quantitative Top-Down proteomics workflows<sup>6,7</sup>
- Analysis of posttranslational modifications, e.g. phosphorylation, glycosylation and isoforms
- Increase of signal intensity in MALDI-TOF-MS
- Quantification with MS (MS/MS not necessary)
- Labelling leads to shift of the isoelectric point (advantage for analysis of basic proteins)
- Labelled BSA Glu-C/trypsin digest reference included for process validation purposes
- ICPL™ *Quant*<sup>8</sup> and ICPL™ *ESIQuant* freeware for data analysis

#### Technical support:

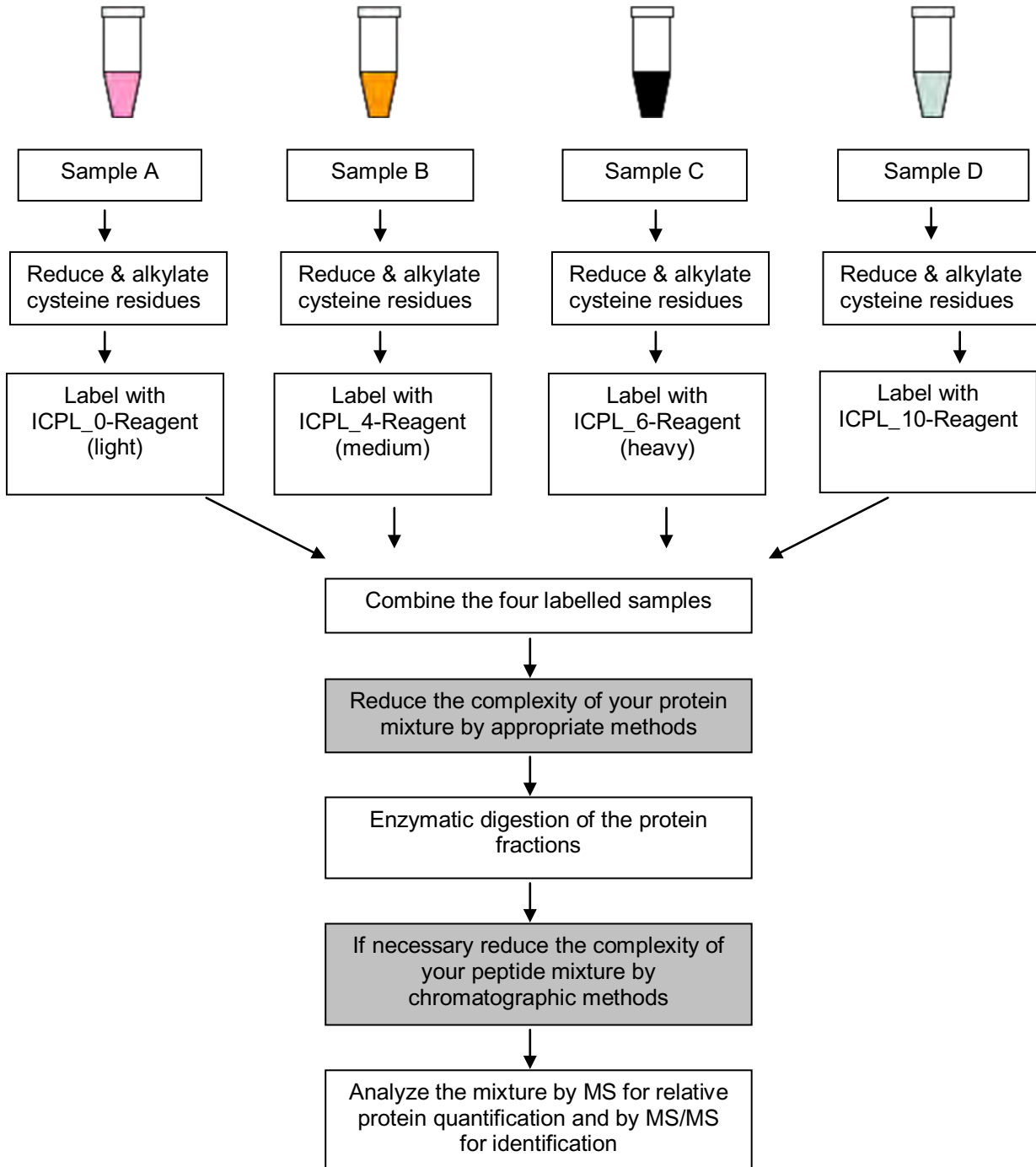
For detailed technical questions, please contact Toplab GmbH:

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

E-Mail: [info@toplab.de](mailto:info@toplab.de)

## 1.2. Workflow

If you are running the ICPL protocol the first time, it is recommended, to analyse the provided protein mixtures through the entire protocol (see section 3). The expected results should be comparable with the results shown at the end of the protocol.



**Fig. 1:** Overview of the ICPL reagent technology.

### 1.3. 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
ICPL Standard PLUS (white)	12 µg lyophilized
Trypsin NB Sequencing Grade, mod. (white)	1 x 25 µg lyophilized
Endoproteinase Glu-C (red)	3 x 25 µg 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:

- 2 M Urea (SERVA Cat. No. 24524)
- 50 mM Tris/HCl, pH 8.0 (SERVA Cat. No. 37180)
- 2 N NaOH
- 2 N HCl
- Argon
- Acetone (ice cold)
- 80 % Acetone (ice cold)
- 50 mM Acetic acid p.a.
- 100 mM Triethylammonium bicarbonate pH 8.3 (TEAB)
- pH-meter equipped with micro-electrode
- Cool centrifuge/ultra centrifuge
- Thermo mixer (up to 60 °C)

#### Storage conditions

The SERVA ICPL™ Quadruplex PLUS Kit will be shipped at room temperature. Upon arrival store the kit immediately at –20 °C. Long term storage at –80 °C is as well possible.

## 2. Protocols

### 2.1. Before you begin

#### 2.1.1. Dissolution of the protein sample

##### Required solutions

- Lysis Buffer (white)

**Important:** 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. If your sample contains any substances which may interfere with the ICPL protocol, perform acetone precipitation to clean up the sample (see chapter 2.2.3.).



##### Procedure

In a first step, the protein 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 and spin down
- Leave sample in thermo mixer at 1000 rpm for 20 min. at 25 °C
- Vortex 2 min and spin down
- Sonicate 4 times for 30 sec in ultrasonic bath (cool sample during pauses in ice water for 2 min)
- Vortex 1 min and spin down
- 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



### 2.1.2. Determination and adjustment of the protein content

- Determine protein concentration by Bradford assay (SERVA Cat. No. 39222)
- Adjust the concentration 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  $\mu$ 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.



#### **If working with the Protein Mixes for ICPL (white, contained in the kit):**

- Dissolve the lyophilized proteins in 20  $\mu$ l Lysis Buffer
- No further determination is necessary.

## 2.2. ICPL labelling of proteins

### 2.2.1. Carbamidomethylation

#### Required solutions

- Reduction Solution (yellow)
- Alkylation Reagent (blue)
- Solution Buffer (white)
- Stop Solution 1 (green)
- Possibly, 2 N NaOH or HCl

#### Procedure

The carbamidomethylation protocol is identical for all four samples (labelling with ICPL\_0, ICPL\_4, ICPL\_6 and ICPL\_10).

1. Check the pH of the sample buffer and if necessary, adjust to  $8.5 \pm 0.1$  by addition of HCl or NaOH using a micro pH-electrode.
2. Add 0.5  $\mu\text{l}$  Reduction Solution (yellow) to 20  $\mu\text{l}$  sample solution (equivalent to 100  $\mu\text{g}$  protein), overlay sample with argon (or equivalent) to exclude oxidation. Vortex and spin down. Incubate proteins for 30 min at 60 °C.
3. Cool sample to 25 °C and spin down sample to remove condensed solution from the lid.
4. Dissolve one vial of Alkylation Reagent (blue) in 200  $\mu\text{l}$  Solution Buffer (white) and wrap in aluminium foil (light protection).
5. Add 0.5  $\mu\text{l}$  of this freshly prepared alkylation solution to each sample, vortex and spin down. Wrap samples quickly in aluminium foil for light protection and leave samples for 30 min at 25 °C.
6. Stop reaction by adding 0.5  $\mu\text{l}$  Stop Solution 1 (green) to each sample, vortex and spin down. Incubate for 15 min at 25 °C.



**Optional break point:** At this point the samples can be stored under argon (or equivalent) at -20°C for a later processing.

## 2.2.2. Isotope labelling of the proteins

### 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

### Procedure

1. **Check pH and adjust to 8.3** before starting the label procedure!
2. Add  
3  $\mu$ l of ICPL\_0 Reagent (purple) to sample A,  
3  $\mu$ l of ICPL\_4 Reagent (orange) to sample B,  
3  $\mu$ l of ICPL\_6 Reagent (black) to sample C,  
3  $\mu$ l of ICPL\_10 Reagent (white) to sample D.
3. Overlay the four samples with argon (or equivalent) to exclude oxidation, vortex (10 s) and sonicate for 1 min in ultrasound bath. Spin down samples.
4. Incubate samples for 2 h at 25 °C.
5. Add 2  $\mu$ l of Stop Solution 2 (red) to each sample, vortex and spin down.
6. Incubate for 20 min at 25 °C to destroy excess reagent.
7. Combine all four ICPL labelled samples, vortex and spin down.
8. **Adjust the pH of the mixture to  $11.9 \pm 0.1$**  by adding 2 N NaOH (about 4  $\mu$ l for 4 x 20  $\mu$ l sample volume) to destroy possible esterification products.
9. After 20 min at 25 °C add the same amount of 2 N HCl to neutralize sample (usually it is not necessary to check the pH).



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### 2.2.3. Sample preparation depending on the following workflow

**Acetone precipitation:** This is an essential step to reduce salt concentration and to prevent SDS precipitation in further protein separation steps.

#### Required solutions

- Acetone cooled to  $-20\text{ }^{\circ}\text{C}$
- 80 % Acetone / 20 % Water (v/v) cooled to  $-20\text{ }^{\circ}\text{C}$

#### Procedure

Add the equal amount of dist. water to your sample, i.e.  $114\text{ }\mu\text{l}$  ( $= 4 \times (20\text{ }\mu\text{l sample} + 1.5\text{ }\mu\text{l reduction} + 3\text{ }\mu\text{l reagent} + 2\text{ }\mu\text{l stop solution}) + 8\text{ }\mu\text{l pH adjustment}$ ). Vortex and spin down.

1. Add 5-fold excess (related to total volume of sample and water) of ice-cold acetone to your sample, i.e.  $1140\text{ }\mu\text{l}$  acetone ( $= 228\text{ }\mu\text{l} \times 5$ ), vortex and spin down. Leave sample at  $-20\text{ }^{\circ}\text{C}$  overnight.
2. Spin down precipitated proteins at  $20.000\text{ g}$  for  $30\text{ min}$  at  $4\text{ }^{\circ}\text{C}$ .
3. Overlay precipitated proteins with  $100 - 200\text{ }\mu\text{l}$  ice-cold 80 % acetone, vortex and spin down again at  $20.000\text{ g}$  for  $5\text{ min}$  at  $4\text{ }^{\circ}\text{C}$ .
4. Discard supernatant and repeat step 4. Once.
5. Remove remaining acetone at room temperature by evaporation (let the cup stand open).
6. Dissolve the depleted samples in appropriate buffers for protein separation (1D or 2D gels, OFFGEL, chromatography etc).



**Optional break point:** The samples can be stored now at  $-20\text{ }^{\circ}\text{C}$ .

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 for ICPL\_0, ICPL\_4, ICPL\_6 and ICPL\_10 can be cleaved directly by trypsin and/or endoproteinase Glu-C. For a better solubility, we recommend to dissolve the acetone precipitate using a buffer containing  $2\text{ M}$  urea. However, before digestion with trypsin and/or endoproteinase Glu-C, the urea content should be diluted to  $1\text{ M}$  urea.

## 2.2.4. Enzymatic digestion of the labelled proteins

### 2.2.4.1. Trypsin digest

#### Required solutions

- 50 mM Tris/HCl/2 M urea, pH 8.0
- 50 mM Tris/HCl pH 8.0
- Trypsin NB
- 50 mM Acetic acid
- 1 % TFA

#### Procedure

1. Dissolve sample in 50 mM Tris/HCl/2 M urea pH 8.0 (final concentration of 5 µg/µl).
2. Dilute sample with an equal volume of 50 mM Tris/HCl, pH 8.0 to get a final urea concentration of 1 M and a protein concentration of 2.5 µg/µl.
3. Reconstitute Trypsin NB (25 µg lyoph.) by adding 100 µl 50 mM acetic acid.
4. Add trypsin solution to the sample to get an enzyme/protein ratio of 1:50 (2 % of the complete protein content).
5. Incubate sample over night at 37 °C.
6. Stop reaction by adding 5 µl of 1 % TFA.



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## 2.2.4.2. Endoproteinase Glu-C digest

### Required solutions

- 50 mM Tris/HCl/2 M urea, pH 8.0
- 50 mM Tris/HCl pH 8.0
- Endoproteinase Glu-C
- Bidist. H<sub>2</sub>O
- 1 % TFA

### Procedure

1. Dissolve sample in 50 mM Tris/HCl/2 M urea pH 8.0 (final concentration: 5 µg/µl).
2. Dilute sample with an equal volume of 50 mM Tris/HCl, pH 8.0 to get a final urea concentration of 1 M and a protein concentration of 2.5 µg/µl.
3. Reconstitute endoproteinase Glu-C (25 µg lyoph.) by adding 100 µl bidist H<sub>2</sub>O.
4. Add endoproteinase Glu-C to the sample to get an enzyme/protein ratio of 1:10.
5. Incubate sample over night at 25 °C.
6. Stop reaction by adding 5 µl of 1 % TFA.



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### 2.2.4.3. Double digest using Glu-C and trypsin

#### Required solutions

- 50 mM Tris/HCl/2 M urea, pH 8.0
- 50 mM Tris/HCl pH 8.0
- Trypsin NB
- 50 mM Acetic acid
- Endoproteinase Glu-C
- Bidist. H<sub>2</sub>O
- 1 % TFA

#### Procedure

1. Dissolve sample in 50 mM Tris/HCl/2 M urea pH 8.0 (final concentration: 5 µg/µl).
2. Dilute sample with an equal volume of 50 mM Tris/HCl, pH 8.0 to get a final urea concentration of 1 M and a protein concentration of 2.5 µg/µl.
3. Reconstitute endoproteinase Glu-C (25 µg lyoph.) by adding 100 µl bidist. H<sub>2</sub>O.
4. Add endoproteinase Glu-C to the sample to get an enzyme/protein ratio of 1:10
5. Incubate sample 4 h at 25 °C.
6. Reconstitute trypsin (25 µg lyoph.) by adding 100 µl 50 mM acetic acid.
7. Add trypsin to the sample to get an enzyme/protein ratio of 1:50 (2 % of the complete protein content).
8. Incubate sample over night at 37 °C.
9. Add 5 µl 1% TFA to stop the reaction, vortex and spin down.



## 2.3. ICPL labelling of peptides

ICPL labelling of peptides following carbamidomethylation and enzymatic digestion of a protein mixture (100 µg protein):

### 2.3.1. Carbamidomethylation

#### Required solutions

- 100 mM Triethylammonium bicarbonate pH 8.3 (TEAB)
- Reduction Solution (yellow)
- Alkylation Reagent (blue)
- Solution Buffer (white)
- Stop Solution 1 (green)
- Possibly, 2 N NaOH or HCl

#### Procedure

1. Dissolve protein in 40 µl 100 mM triethylammonium bicarbonate pH 8.3 (TEAB).
2. Add 1 µl Reduction Solution (yellow), vortex and spin down. Incubate at 60 °C for 30 min (overlay with protective gas i.e. argon).
3. Dissolve one cup of Alkylation Reagent (blue) in 200 µl Solution Buffer (white) and wrap in aluminium foil (light protection).
4. Add 1 µl alkylation solution (prepare fresh every time), vortex and spin down. Incubate 30 min at 25 °C in the dark.
5. Add 1 µl Stop Solution 1 (green), vortex and spin down. Incubate 15 min at 25 °C.





### 2.3.2. Enzymatic digestion

#### Required solutions

- Trypsin NB
- 50 mM Acetic acid
- 1 % TFA

#### Procedure

1. Reconstitute Trypsin NB (25 µg lyoph.) by adding 100 µl 50 mM acetic acid.
2. Add trypsin to the sample to get an enzyme/protein ratio of 1:20 and incubate over night at 37 °C.
3. Stop reaction by adding 5 µl of 1 % TFA.



### 2.3.3. Isotopic labelling of the peptides

#### 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

#### Procedure

1. **Check pH and adjust to 8.3** before starting the label procedure!
2. Add 6 µl (double amount in contrast to protein labelling) of ICPL Reagent (ICPL\_0, ICPL\_4, ICPL\_6 and ICPL\_10) solution to each sample.
3. Overlay the samples with argon (or equivalent) to exclude oxidation, vortex (10 s) and sonicate for 1 min in ultrasound bath. Spin down samples.
4. Incubate samples for 2 h at 25 °C.



5. Add 4  $\mu\text{l}$  of Stop Solution 2 (red) to each sample, vortex and spin down. Incubate for 20 min at 25 °C to destroy excess reagent.
6. Combine all ICPL labelled samples, vortex and spin down.
7. Adjust the pH of the mixture to  $11.9 \pm 0.1$  by adding 2N NaOH (about 1  $\mu\text{l}$  for 20  $\mu\text{l}$  sample volume) to destroy possible esterification products. After 20 min add the same amount of 2N HCl to neutralize sample.
8. Remove excess reagents using RP-C18 Stage Tips, ZipTips or equivalent.

#### **2.3.4. 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)
- Solution A: 0.5 % Trifluoroacetic acid in HPLC grade water
- Solution B: 0.5 % Trifluoroacetic acid in 50 % acetonitrile

##### **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  $\mu\text{l}$  of solution B.
6. Remove the acetonitrile by putting the samples into a Speed-Vac for 10 min.

## 2.4. Mass spectrometry and data analysis

After digestion, the samples can be directly analyzed by mass spectrometry, preferentially by LC-MALDI-TOF-TOF (e.g. Bruker UltrafleXtreme, Applied Biosystems 4700/4800 or 5800) or LC-ESI-MS/MS (e.g. Thermo LTQ Orbitrap, Bruker maXis). Quantification can be performed using ICPL™ *Quant* software (see chapters 3.3. and 4.). ProteinScape 2.1+ (Bruker) is also suitable for quantification.

- Please note: Labels are described differently by Mascot, Unimod and SERVA

Components in Mascot Quantitation Methods	Unimod (and Mascot) modification name	SERVA
Light	ICPL(K)	ICPL_0
Medium	ICPL:2H(4)(K)	ICPL_4
Heavy	ICPL13C(6)(K)	ICPL_6
	ICPL:13C(6)2H(4)(K)	ICPL_10

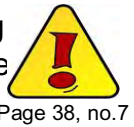


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- The mass difference of ICPL\_0- (<sup>12</sup>C-) and ICPL\_4- (<sup>2</sup>D-) labelled peptides is 4.0251 Da per labelled amino acid, those of ICPL\_0 and ICPL\_6- (<sup>13</sup>C-) labelled 6.0201 Da per labelled amino acid and those of ICPL\_0- and ICPL\_10-(<sup>13</sup>C <sup>2</sup>D-) labelled peptides 10.0452 Da per labelled amino acid.
- The mass differences between labelled (ICPL\_0/ICPL\_4/ICPL\_6/ICPL\_10) and unlabelled peptides are 105.0215Da/109.0466 Da/111.0416 Da/115.0667 Da for each modified amino group.
- The  $\frac{ICPL\_4}{ICPL\_0}$ ,  $\frac{ICPL\_6}{ICPL\_0}$  and  $\frac{ICPL\_10}{ICPL\_0}$  ratios for each protein are calculated as average or median values of the  $\frac{ICPL\_4}{ICPL\_0}$ ,  $\frac{ICPL\_6}{ICPL\_0}$  and  $\frac{ICPL\_10}{ICPL\_0}$  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. As outliers easily skew the distribution of peptide ratios, the use of median based statistics in the informatics package, e.g. in ICPLQuant (MPI) or ProteinScape 2.1 (Bruker) is helpful.

### 2.4.1. Mascot search and quantification

- **Select endoproteinase Arg-C and/or Glu-C as enzyme** in the search dialog  
The alternative selection of “no enzyme” improves protein sequence coverage and the number of 4-plexes that are used for quantification<sup>5</sup>.
- The ICPL modifications (ICPL\_0, ICPL\_4, ICPL\_6 and ICPL\_10, specificity: lysine, protein N-term) were specified as exclusive modifications in a quantitation method. Carbamidomethyl was set as fixed modification.
- The list of modifications used by Mascot is taken directly from the [Unimod](#) database.
- By default, only selected modifications are displayed in the Mascot search form. If you want to see the complete list, you must go to the [search form defaults](#) page and tick the checkbox for 'Show all mods'.
- In Mascot 2.3 and earlier, modification definitions were stored in a configuration file called mod\_file. Mascot now takes its modification definitions directly from an XML representation of the [Unimod](#) database. To update the local definitions, simply download the latest XML file from the [Unimod help](#) page.



### 2.4.2. Mascot Webserver

Available quantification methods including the ICPL modifications:

ICPL duplex (prot/pep) (ICPL\_0 and ICPL\_6)

ICPL triplex (prot/pep) (ICPL\_0, ICPL\_4 and ICPL\_6)

For ICPL quadruplex searches, specify ICPL(K), ICPL:2H(4)(K), ICPL13C(6)(K) and ICPL:13C(6)2H(4)(K) as variable modifications.

### 2.4.3. Local Mascot server

- On your local mascot server you can create a custom quantitation method for ICPL quadruplex for protein identification (and quantification with MASCOT Distiller).
- Create quantitation method for quadruplex search only.
- Log in as admin.
- Open the “Configuration editor” and go to “quantitation methods”.
- Make a copy of the “ICPL triplex pre-digest (MD)” default quantitation method.
- Open “copy of ICPL triplex”.
- Save as: ICPLquadruplex predigest (MD)
- Open the Components tab and add fourth component
  - select “new”
  - component name ICPL\_10
- Add modification group:
  - name: ICPL\_10- mode: exclusive
  - modifications: ICPL:13C(6)2H(4)(K)
- Save changes.

### 3. Examination of the ICPL workflow using the Protein Mixes for ICPL

This section allows validating the entire workflow in the particular laboratory from the labelling step through digestion and LC-MS/MS analysis to the bioinformatics treatment (e.g. ICPL™ *Quant*).

#### 3.1. Protein Mixes for ICPL

The kit contains four Protein Mixes (for ICPL\_0, ICPL\_4, ICPL\_6 and ICPL\_10) each containing different amounts of 3 standard proteins.

Protein	Protein Mix for ICPL_0	Protein Mix for ICPL_4	Protein Mix for ICPL_6	Protein Mix for ICPL_10*	Relative amount
BSA	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	1:0.5:0.25:1
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.

#### 3.2. ICPL labelling of the protein test mixture

1. Dissolve the four Protein Mixes for ICPL in 20 µl of Lysis Buffer (white) each and follow the protocol, starting with step 2.2.1. “Carbamidomethylation”.

Use for each Protein Mix the corresponding ICPL Reagent:

Protein Mix for ICPL\_0 with the ICPL\_0 Reagent,  
Protein Mix for ICPL\_4 with the ICPL\_4 Reagent,  
Protein Mix for ICPL\_6 with the ICPL\_6 Reagent  
Protein Mix for ICPL\_10 with the ICPL\_10 Reagent

Follow the protocol including step. 2.2.4.1. “Trypsin digest” or 2.2.4.3. “Double digest using endoproteinase Glu-C and trypsin”.

2. After trypsin 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.  
In the case of a double digest we recommend an analysis by LC/MS.



Start with step 2.2.1. and follow the protocol

### 3.3. MS analysis of the labelled Protein Mixes

For quantification and analysis of LC/MS data of ICPL-labelled proteins, the ICPL™ *Quant* software was developed by the group of Dr. Friedrich Lottspeich at the MPI for Biochemistry in Martinsried. The software is available free of charge<sup>3</sup>. Please use the following link to the registration page of the software:

<http://www.biochem.mpg.de/en/rg/lottspeich/technologies/ICPL™Quant/index.html>

The software manual and video tutorials for user support are available.

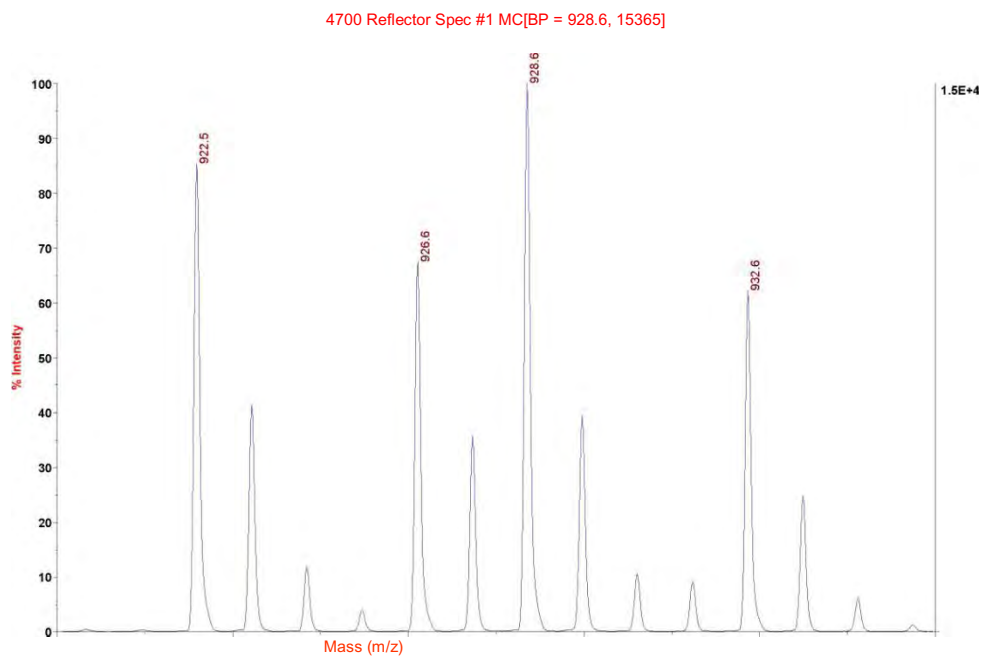
#### 3.3.1. Analysis after a trypsin digest

The following peptides were analyzed **after trypsin** digest by MALDI-TOF-TOF (peptide mass fingerprint).

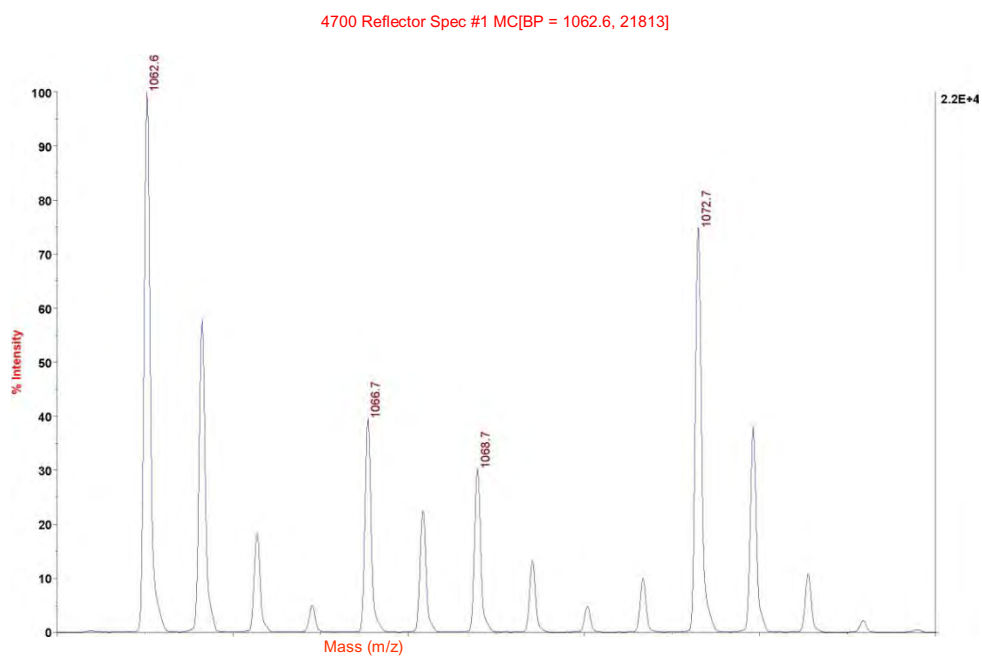
Protein	Mass ICPL_0*	Expected Mass ICPL_0*	Ratio				Peptide sequence
			ICPL_0	ICPL_4	ICPL_6	ICPL_10	
BSA	922.54	922.51	1.00	1.03	0.98	0.89	SLGK*VGTR
BSA	1065.59	1065.57	1.00	0.98	1.06	0.89	EK*VLASSAR
BSA	1106.64	1106.61	1.00	1.09	0.99	1.00	ALK*AWSVAR
BSA	1300.65	1300.61	1.00	0.88	1.01	0.75	CASIQK*FGER
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*VPQVSTPTLVEVSRSLG K*VGTR
BSA	3302.51	3302.57	1.00	0.96	1.04	0.82	LCVLHEK*TPVSEK*VTK*C CTESLVNR
		<b>Mean</b>	1.00	1.00	1.00	0.86	
		<b>StDev[%]</b>	---	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	YPILPEYLCQVK*ELYR
Ovalbumin	2386.22	2386.17	1.00	0.47	0.17	1.04	DILNQITK*PNDVYSFSLAS R
		<b>Mean</b>	1.00	0.49	0.20	1.00	
		<b>StDev [%]</b>	---	5.39	12.15	10.77	
Carbonic Anhydrase II	1791.01	1790.98	1.00	1.21	1.87	0.87	PALK*PLALVYGEATSR
Carbonic Anhydrase II	1994.94	1994.93	1.00	1.03	2.45	0.90	GPEHWHK*DFPIANGER
		<b>Mean</b>	1.00	1.12	2.16	0.88	

Stdev = standard deviation

\*Mass ICPL\_4 increases about 4.0251 Da, mass ICPL\_6 about 6.0201 Da and mass ICPL\_10 about 10.0452 Da per labelled amino acid.

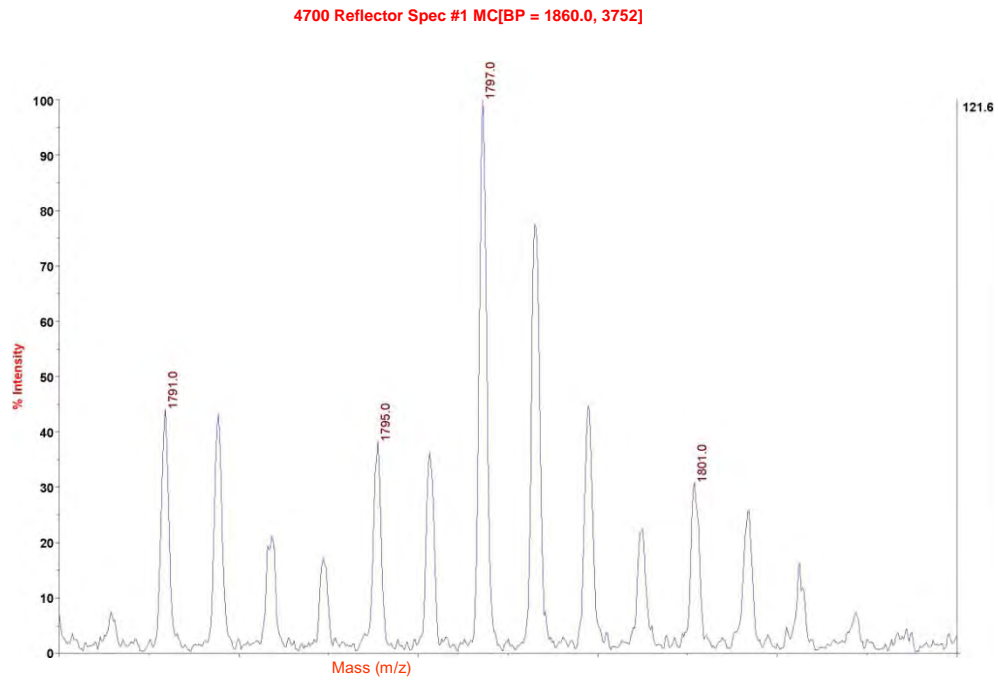


**Fig. 2:** MS analysis BSA 1:1:1:1. Peptide sequence: SLGK\*VGTR



**Fig. 3:** MS analysis Ovalbumin 1: 0,5:0,25:1. Peptide sequence: TQINK\*VVR

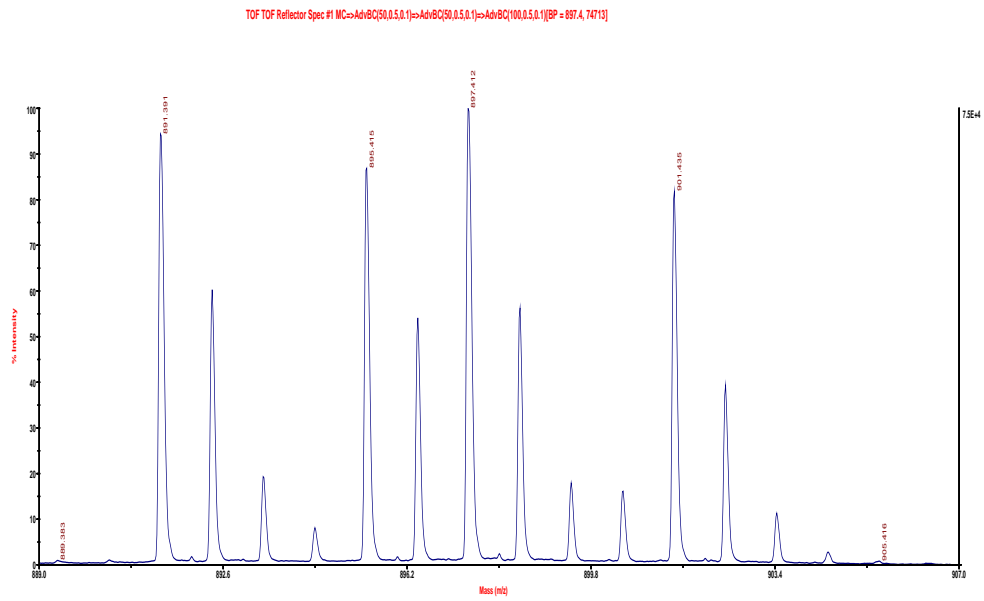




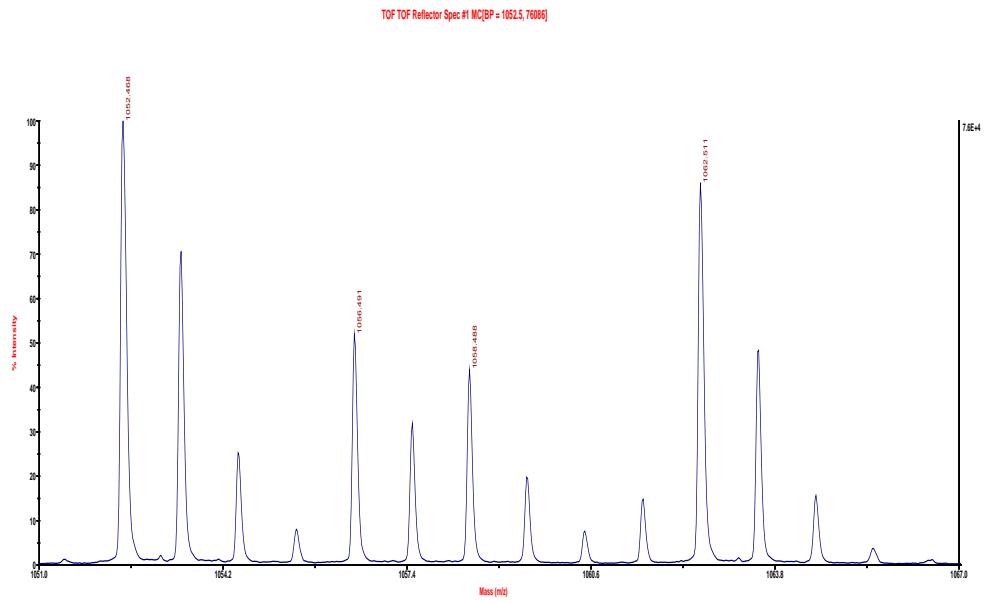
**Fig. 4:** MS analysis Carbonic Anhydrase 1:1:2:1. Peptide sequence: PALK\*PLALVYGEATSR

### 3.3.2 Analysis after Glu-C and trypsin digest

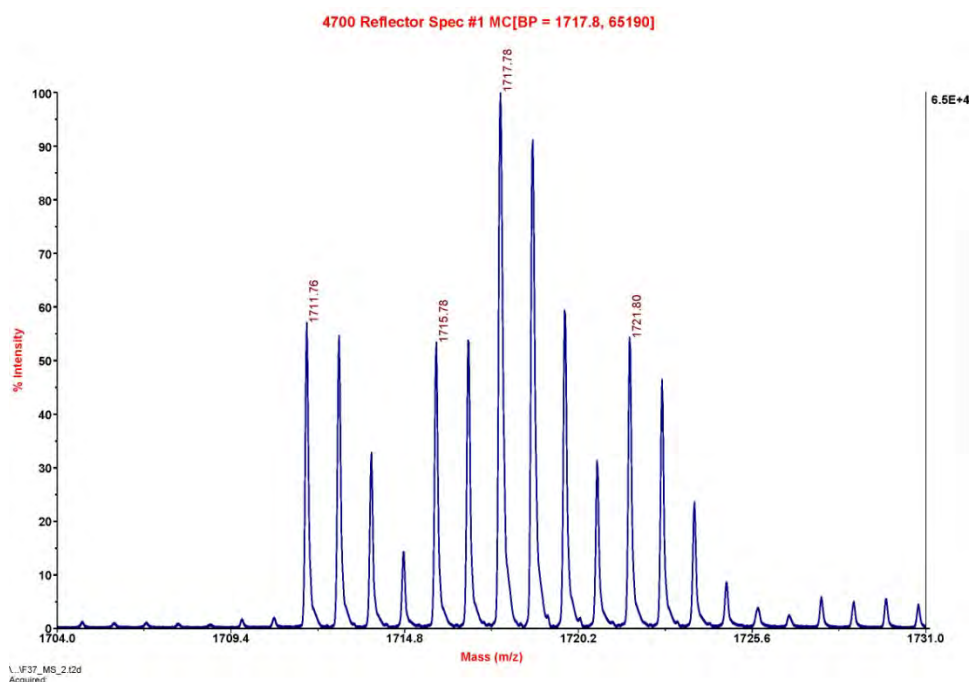
Protein	ICPL 0 mass	ICPL 4 mass	ICPL 6 mass	ICPL 10 mass	Ratio					peptide sequence	Lysin count
					ICPL 0/ICPL 4	ICPL 0/ICPL 6	ICPL 0/ICPL 10	ICPL 4/ICPL 6	ICPL 4/ICPL 10		
BSA	891,39	895,42	897,41	901,43	1,00	0,94	0,97	0,92		K*QEPER	1
BSA	943,44	947,47	949,46	953,49	1,00	1,17	0,99	1,09		HWK*LVNE	1
BSA	966,5	970,53	972,53	976,55	1,00	1,13	0,98	1,06		K*VSQLR	1
BSA	1098,43	1102,46	1104,45	1108,47	1,00	1,13	1,11	1,34		EQLK*TVME	1
BSA	1139,45	1143,47	1145,47	1149,49	1,00	1,08	1,11	1,11		CCDK*PLE	1
BSA	1145,44	1149,46	1151,45	1155,48	1,00	1,05	1,06	1,10		FAK*TCVADE	1
BSA	1180,54	1184,56	1186,56	1190,59	1,00	1,02	1,01	0,93		ELCK*VASLR	1
BSA	1235,46	1243,5	1247,49	1255,54	1,00	1,01	1,05	1,14		K*VTK*CTE	2
BSA	1251,58	1255,6	1257,6	1261,62	1,00	0,99	0,96	0,99		K*SLHTLFGDE	1
BSA	1257,53	1265,59	1269,56	1277,61	1,00	1,04	1,08	0,94		LSOK*FPK*AE	2
BSA	1300,58	1304,61	1306,6	1310,62	1,00	0,96	1,03	0,97		CASIQK*FGER	1
BSA	1389,68	1393,71	1395,7	1399,72	1,00	0,91	0,99	0,80		GPK*LVWSTQTALA	1
BSA	1826,75	1830,77	1832,77	1836,79	1,00	1,14	1,11	1,08		LLYYANK*YNGVFOE	1
				<b>Mean</b>	1,00	1,04	1,04	1,04			
				<b>SD dev [%]</b>	—	6,78	5,01	12,86			
Carbonic Anhydrase II	1711,75	1715,77	1717,77	1721,78	1,00	0,95	2,11	1,25		HWHK*DFPIANGER	1
Carbonic Anhydrase II	2452,08	2460,12	2464,12	2472,16	1,00	1,08	2,16	1,13		YDDSDQK*AVLK*DGPLTGTYSR	2
				<b>Mean</b>	1,00	1,02	2,14	1,19			
Ovalbumin	947,44	955,44	959,44	967,48	1,00	0,55	0,29	1,08		K*AFK*DE	2
Ovalbumin	1226,68	1234,72	1238,71	1246,76	1,00	0,55	0,31	1,17		K*IK*VYLR	2
Ovalbumin	1052,47	1056,49	1059,49	1062,51	1,00	0,54	0,27	1,03		LK*VHHANE	1
Ovalbumin	1566,75	1570,78	1572,78	1576,8	1,00	0,49	0,22	1,01		SLK*TSQAVHAAH-AE	1
Ovalbumin	1062,57	1066,6	1068,6	1072,59	1,00	0,6	0,31	1,05		TQINK*VWR	1
Ovalbumin	1044,43	1048,45	1050,45	1054,47	1,00	0,49	0,27	1,09		YLCQV*K*E	1
				<b>Mean</b>	1	0,54	0,28	1,07			
				<b>SD dev [%]</b>	—	7,79	12,11	5,29			



**Fig. 5:** MS analysis BSA 1:1:1:1; Glu-C and trypsin digested. Peptid sequence: K\*QEPER



**Fig. 6:** MS analysis Ovalbumin 1:0,5:0,25:1; Glu-C and trypsin digested. Peptid sequence: LK\*VHHANE



**Fig. 7:** MS analysis Carbonic Anhydrase II 1:1:2:1; Glu-C and trypsin digested. Peptid sequence: HWHK\*DFPIANGER

### 3.4. ICPL™ Standard PLUS

The ICPL™ Standard PLUS contains 12 µg of trypsin and endoproteinase Glu-C treated BSA (Quadruplex labelled, 3 µg/label).

Protein	ICPL_0	ICPL_4	ICPL_6	ICPL_10	Relative amount
BSA	3 µg	3 µg	3 µg	3 µg	1:1:1:1

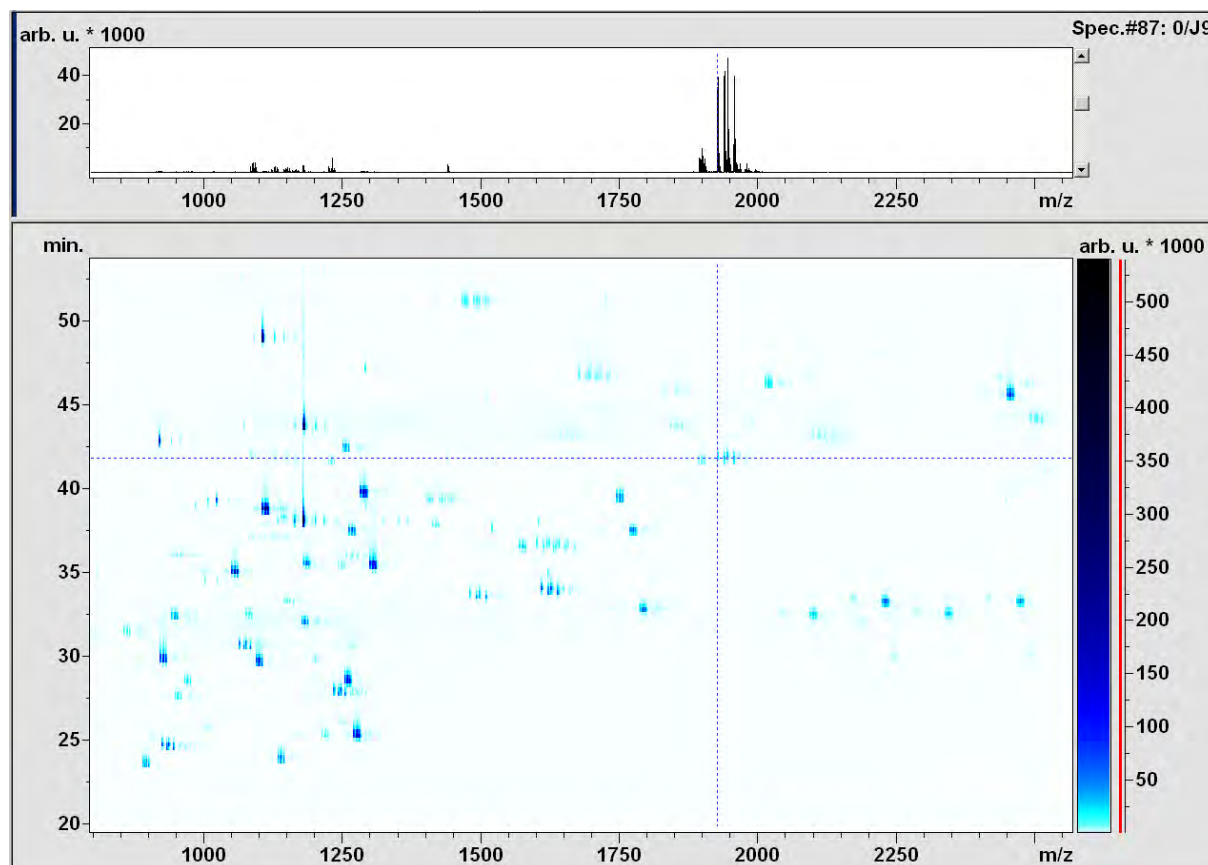
Depending on the sensitivity of your analysis system the lyophilized standard has to be dissolved in an appropriate concentration.

After reconstitution, the peptides are directly analyzed by MS, preferentially by MALDI-TOF-MS. The ratios of the proteins are calculated by the ratio of the peak areas.

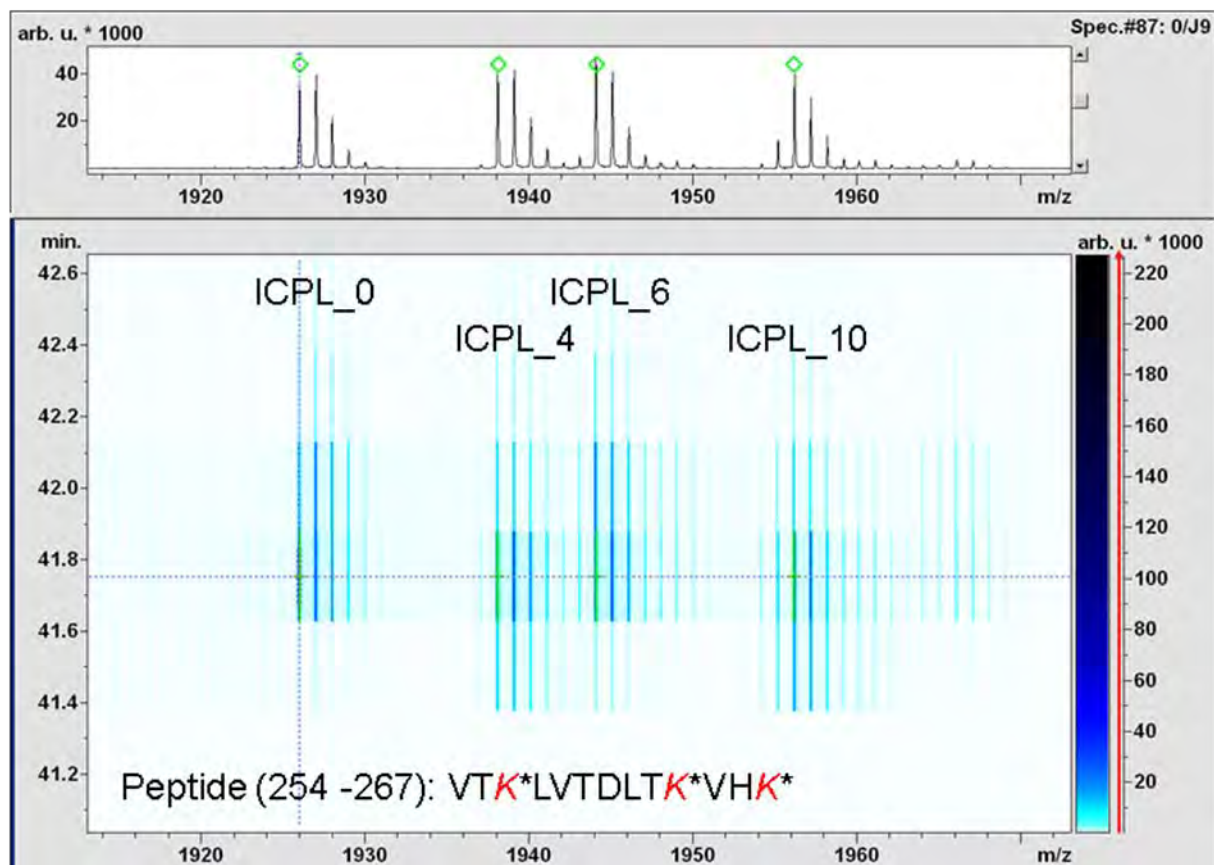
### 3.5. MS analysis of the ICPL™ Standard PLUS

Please find below an example of the ICPL™ Standard PLUS analysis using a Bruker ultrafleXtreme™ and the ProteinScape 2.1+ quantification software.

Data generated by ABI 4700/4800 or 5800 (MALDI) or Thermo Orbitrap (ESI) can also be handled with the new ICPL™ *Quant* software.

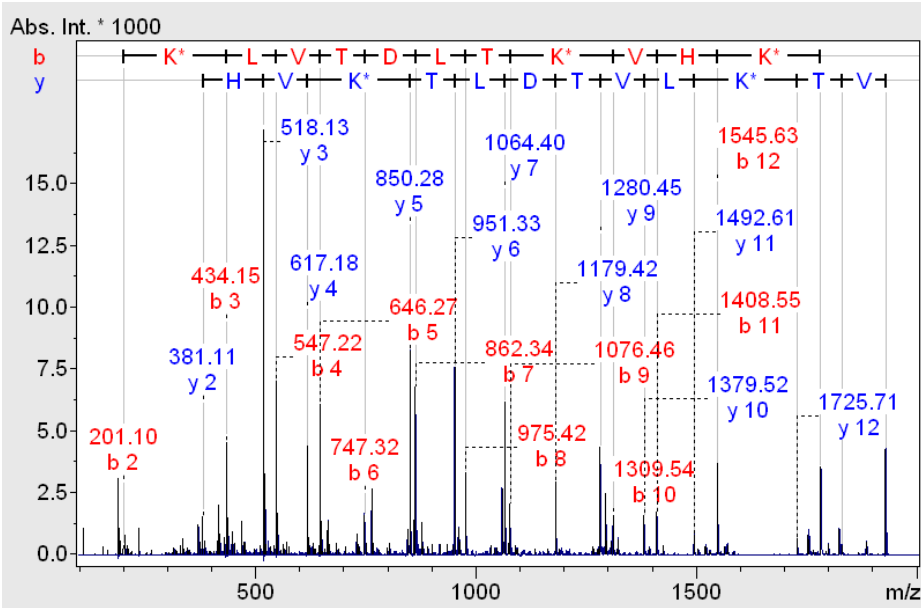


**Fig. 8:** Survey view of the nano-LC-MALDI MS analysis of the digest of ICPL Quadruplex labelled BSA. 35 ng labelled BSA after Glu-C and trypsin digestion were used. The cursors cross point to the Quadruplex pattern that is shown in a close-up view in fig. 9.

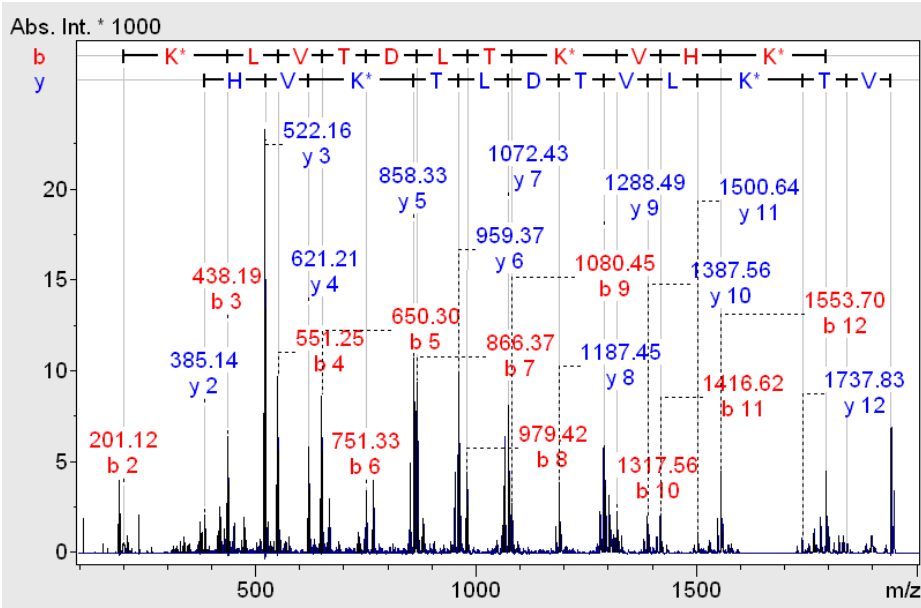


**Fig. 9:** Close-up view of the LC-MS pattern of a peptide that was ICPL Quadruplex labelled at 3 Lys residues. The SurveyView shows the retention times of the 4 label forms of the peptide; top: MS spectrum of the Quadruplex pattern in the spectrum acquired after 41.8 min. Spectra acquired on ultrafleXtreme™ (Bruker). Green diamonds indicate peaks identified by Mascot searches of the MS/MS spectra (see fig. 10).

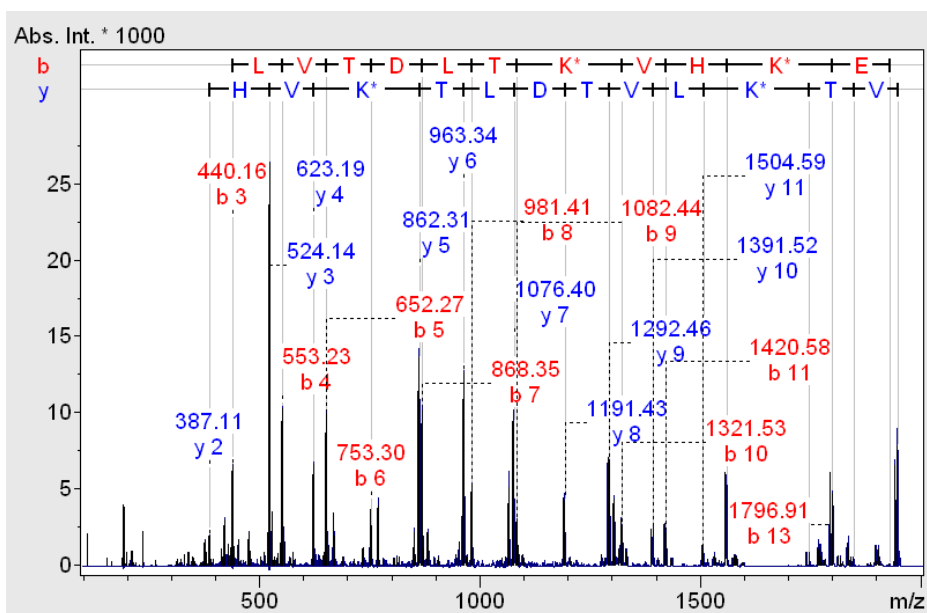
BSA (254-267): 3\* ICPL\_0



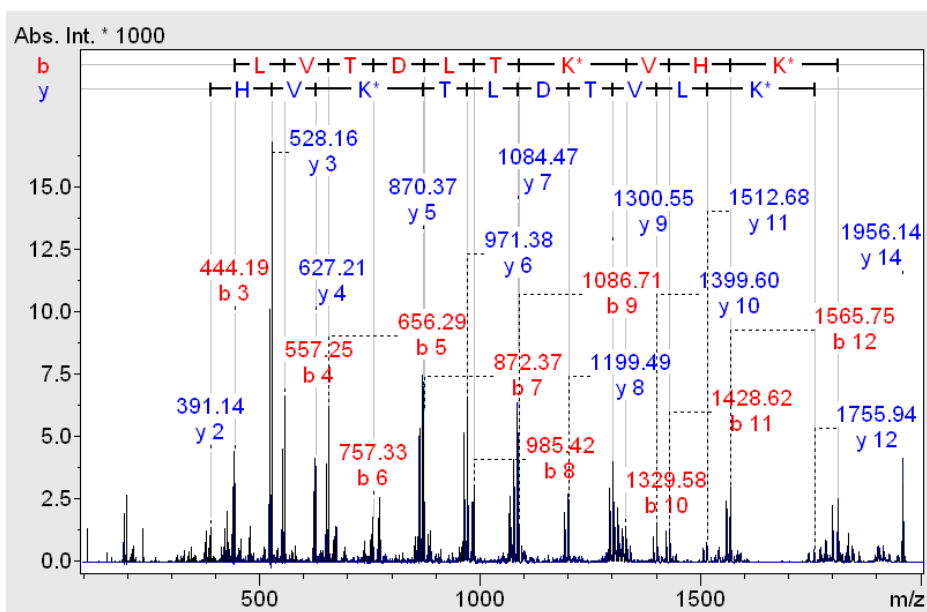
BSA (254-267): 3\* ICPL\_4



BSA (254-267): 3\* ICPL\_6

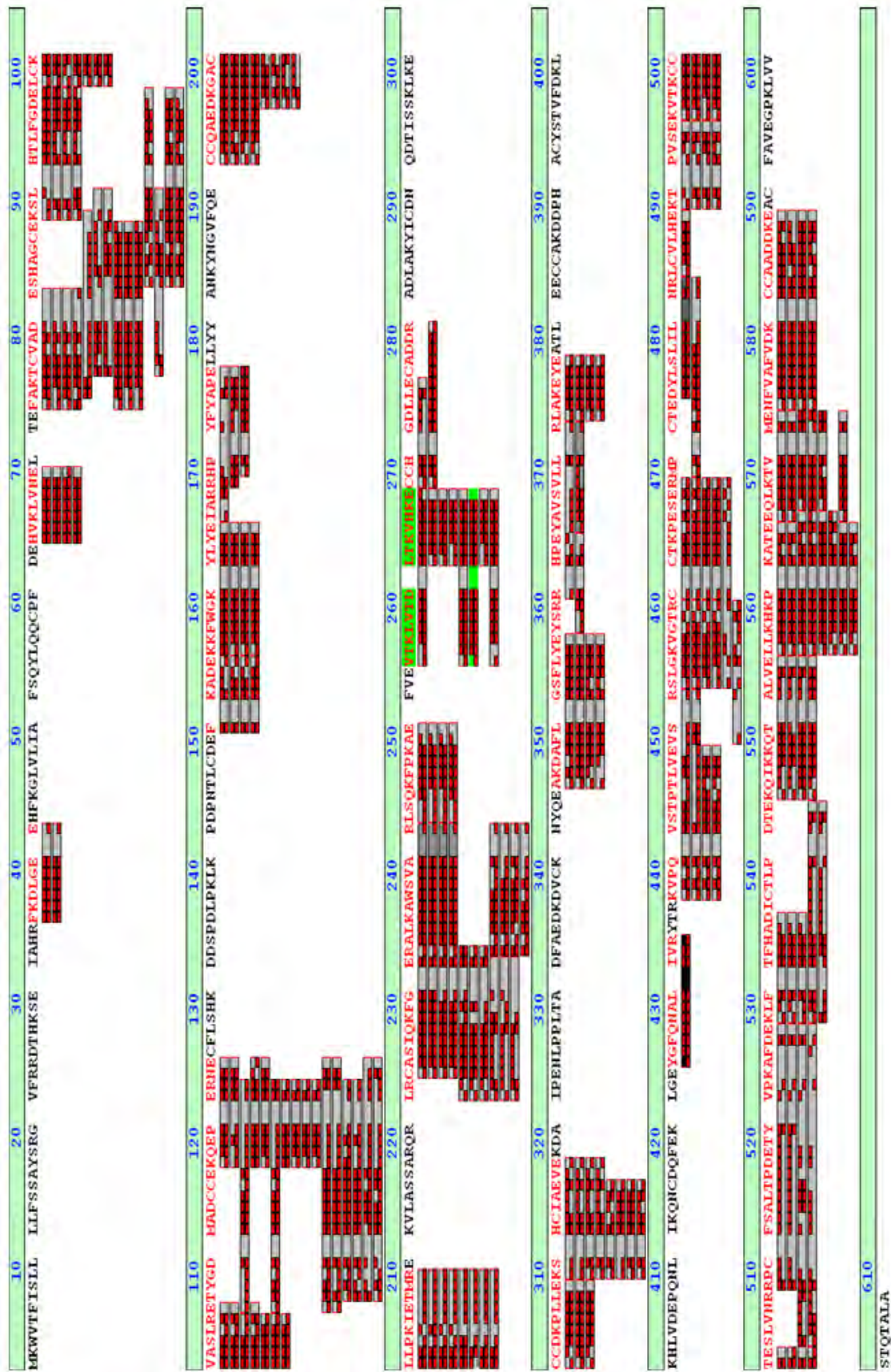


BSA (254-267): 3\* ICPL\_10



**Fig. 10:** MS/MS spectra of the peptide VTKLVTDLTKVHK (see fig. 9) containing the 4 different ICPL labels. Spectra acquired on ultrafleXtreme™ (Bruker).





**Fig. 11:** Sequence coverage of the ICPL Quadruple labelled BSA obtained by nano-LC-MALDI analysis. Grey bars represent IDed peptides, the red bricks indicate observed MS/MS fragments.

**Identification:**

Protein	Accession	Score	SC [%]	# Peptides	RMS [ppm]
Serum albumin, Bos taurus	ALBU_BOVIN	2647	55.19	133	5.47

**Quantification:**

# Peptides (0/4)	Median (0/4)	CV [%] (0/4)	# Peptides (0/6)	Median (0/6)	CV [%] (0/6)	# Peptides (0/10)	Median (0/10)	CV [%] (0/10)
24	1.01	9.39	28	0.96	8.54	28	1.06	9.65

**Selection of identified peptides of the ICPL-Quadruplex labelled BSA**

Meas. m/z	Calc. MH+	Dev.(ppm)	MascotScore	Rt(min)	Range	Sequence	Modification
857.41	857.40	6.1	33	31.5	372-377	LAKYE	3: ICPL (K)
861.44	861.43	7.3	29	31.5	372-377	LAKYE	3: ICPL:2H(4) (K)
863.43	863.42	9.8	25	31.5	372-377	LAKYE	3: ICPL:13C(6) (K)
867.46	867.45	8.1	22	31.5	372-377	LAKYE	3: ICPL:13C(6):2H(4) (K)
1235.52	1235.52	2.1	36	28.0	495-502	KVTRCCCTE	1: ICPL (K) 4: ICPL (K) 5: Carba mido methyl (C) 6: Carba mido methyl (C)
1243.58	1243.57	6.7	34	27.8	495-502	KVTRCCCTE	1: ICPL:2H(4) (K) 4: ICPL:2H(4) (K) 5: Carba mido methyl (C) 6: Carba mido methyl (C)
1247.57	1247.56	7.9	29	28.0	495-502	KVTRCCCTE	1: ICPL:13C(6) (K) 4: ICPL:13C(6) (K) 5: Carba mido methyl (C) 6: Carba mido methyl (C)
1255.62	1255.61	10.1	33	27.8	495-502	KVTRCCCTE	1: ICPL:13C(6):2H(4) (K) 4: ICPL:13C(6) 2H(4) (K) 5: Carba mido methyl (C) 6: Carba mido methyl (C)
1841.90	1841.90	-0.7	74	43.8	196-209	DKGACLLPKIETMR	2: ICPL (K) 5: Carba mido methyl (C) 9: ICPL (K)
1849.95	1849.95	0.0	44	43.8	196-209	DKGACLLPKIETMR	2: ICPL:2H(4) (K) 5: Carba mido methyl (C) 9: ICPL:2H(4) (K)
1853.92	1853.94	-12.2	85	43.8	196-209	DKGACLLPKIETMR	2: ICPL:13C(6) (K) 5: Carba mido methyl (C) 9: ICPL:13C(6) (K)
1861.99	1861.99	0.0	42	43.8	196-209	DKGACLLPKIETMR	2: ICPL:13C(6):2H(4) (K) 5: Carba mido methyl (C) 9: ICPL:13C(6) 2H(4) (K)
1926.00	1926.01	-5.4	86	42.0	254-267	VTKLVDTLTKVHKE	3: ICPL (K) 10: ICPL (K) 13: ICPL (K)
1938.09	1938.09	-0.2	54	41.5	254-267	VTKLVDTLTKVHKE	3: ICPL:2H(4) (K) 10: ICPL:2H(4) (K) 13: ICPL:2H(4) (K)
1944.07	1944.07	-3.8	42	42.0	254-267	VTKLVDTLTKVHKE	3: ICPL:13C(6) (K) 10: ICPL:13C(6) (K) 13: ICPL:13C(6) (K)
1956.15	1956.15	1.6	58	41.5	254-267	VTKLVDTLTKVHKE	3: ICPL:13C(6):2H(4) (K) 10: ICPL:13C(6) 2H(4) (K) 13: ICPL:13C(6) 2H(4) (K)

**Tab. 1:** Summary of the ID and quantification of the BSA reference sample. More than 100 peptides can be identified and used for quantification with ICPL™ Quadruplex. Here a sequence coverage (SC) of 55 % was obtained. Quantification correctness was in the 5 % range and CVs were 10 % or better. A list of 4 example peptides (bottom) provides reference information across the mass range for some peptides that can be identified and quantified from the reference sample. Median statistics was used for quantification in the Bruker Compass software.

## 4. ICPL™*Quant* and ICPL™\_*ESIQuant* 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

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- 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-MS<sup>2</sup> experiments*. J Proteomics Bioinform 5 (2012): 279-282.

## 5. Important to know

For further information, please see also the ICPL download center on the SERVA website:

[http://www.serva.de//www\\_root/ar03/templates/Ar03DownloadDetails.jsp?language=En&organisation=001&navSeq=18&categorie=5](http://www.serva.de//www_root/ar03/templates/Ar03DownloadDetails.jsp?language=En&organisation=001&navSeq=18&categorie=5)

1. 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 like tris buffers, ethanolamine, prim. ammonium salts, AMP [2-amino-2-methyl-1-propanol], AMPD [2-amino-2-methyl-1,3-propanediol]. They will interfere with the ICPL reagents.  
Detergents like urea, thiourea, CHAPS can be used, however.
2. The protein concentration should be above 5 mg/ml, but must be in any case higher than 2.5 mg/ml.
3. 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 depends strongly on the total protein concentration, losses are likely when working with lower protein concentrations. Therefore, it is extremely important, to keep strictly the concentrations of the reagents as recommended. If you want to work with increased sample volumes of 40  $\mu$ l (for example to facilitate the pH measurement), you rather double the sample amount (if feasible) 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.
4. A pH-meter with a micro electrode 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.
5. For further storage overlay all ICPL Reagents with argon.
6. Please note for subsequent analysis that the ICPL labelling influences both the protein mass as well as the isoelectric point. Therefore it is recommended to use for 2D analysis IPG strips with a pH range from 3.5 to 4.5 instead of 3 to 4.
7. 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!

8. The dissolved enzymes are stable for at least 4 weeks at -20 °C.
9. Avoid the use of agents with primary amino group in buffers (e.g. TRIS). Otherwise they also will be ICPL labelled (see also pt. 1).
10. **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.

## 6. References

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4. Dorsch et al., Improved Sequence Coverage During Proteome Analyses with Protein Based Isotopic Labelling Methods (ICPL), ASCB meeting 2009, San Diego, USA, Poster Link:  
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5. Paradela, A., et. al., Evaluation of isotope-coded protein labeling (ICPL) in the quantitative analysis of complex proteomes, *Talanta*. 2010 Feb 15;80 (4): 1496-502. Epub 2009 Jul 9.
6. Burker Application Note MT-106, MT-83: Isotope-Coded Protein Labeling (ICPL) for Quantitative Proteomics Workflows Compatible with Protein Separation Strategies
7. Burker Application Note LC-56: ICPL Labeling in Functional Proteomics Experiments: Substrate Identification of the Extracellular Protease ADAMTS1 using SDS-PAGE LC-MS/MS
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## 7. For the experienced user: Short version of the protocol

**Important! Please follow the detailed manual for the complete ICPL workflow (start with 2.3).**

**Each reaction may be performed with 100 µg of total protein in a volume of 20 µl (protein concentration 5 mg/ml). The protocol is identical for all samples in parallel.**

1. Dissolve your samples including control and/or provided Protein Mixes for ICPL in ICPL Lysis Buffer (white). Adjust protein concentration to **5 mg/ml**.
2. Check the pH of your samples and adjust if necessary to **8.5 +/- 0.1** with NaOH or HCl.
3. Add 0.5 µl Reduction Solution (yellow), overlay samples with argon (or equivalent), vortex and spin down.
4. Incubate 30 min at 60 °C.
5. Cool to 25 °C and spin down condensate from lid.
6. Prepare alkylation solution by solving the Alkylation Reagent (blue) in 200 µl Solution Buffer (white).
7. Add 0.5 µl freshly prepared alkylation solution, vortex, spin down and incubate 30 min at 25 °C in the dark.
8. Add 0.5 µl Stop Solution 1 (green), vortex and spin down.
9. Incubate 15 min at 25 °C.

### **Optional break point:**

**Store the samples at -20 °C for later processing.**

10. Check pH and adjust if necessary to **8.3 (!)** with NaOH or HCl.
11. Add 3 µl of ICPL Reagent (one label per sample).
12. Overlay the samples with argon (or equivalent).
13. Vortex, sonicate for 1 min and spin down.
14. Incubate samples for 2 h at 25 °C.
15. Add 2 µl of Stop Solution 2 (red), vortex and spin down.
16. Incubate 20 min at 25 °C.
17. Combine all ICPL labelled samples, vortex and spin down.
18. Adjust the pH of the mixture to **11.9 +/- 0.1** by adding 2 N NaOH (approx. 1 µl per 20 µl sample volume).
19. Incubate 20 min at 25 °C.
20. Add the same amount of 2 N HCl to neutralize sample.
21. Acetone precipitation (Please continue with section 2.2.4. in the detailed manual).