

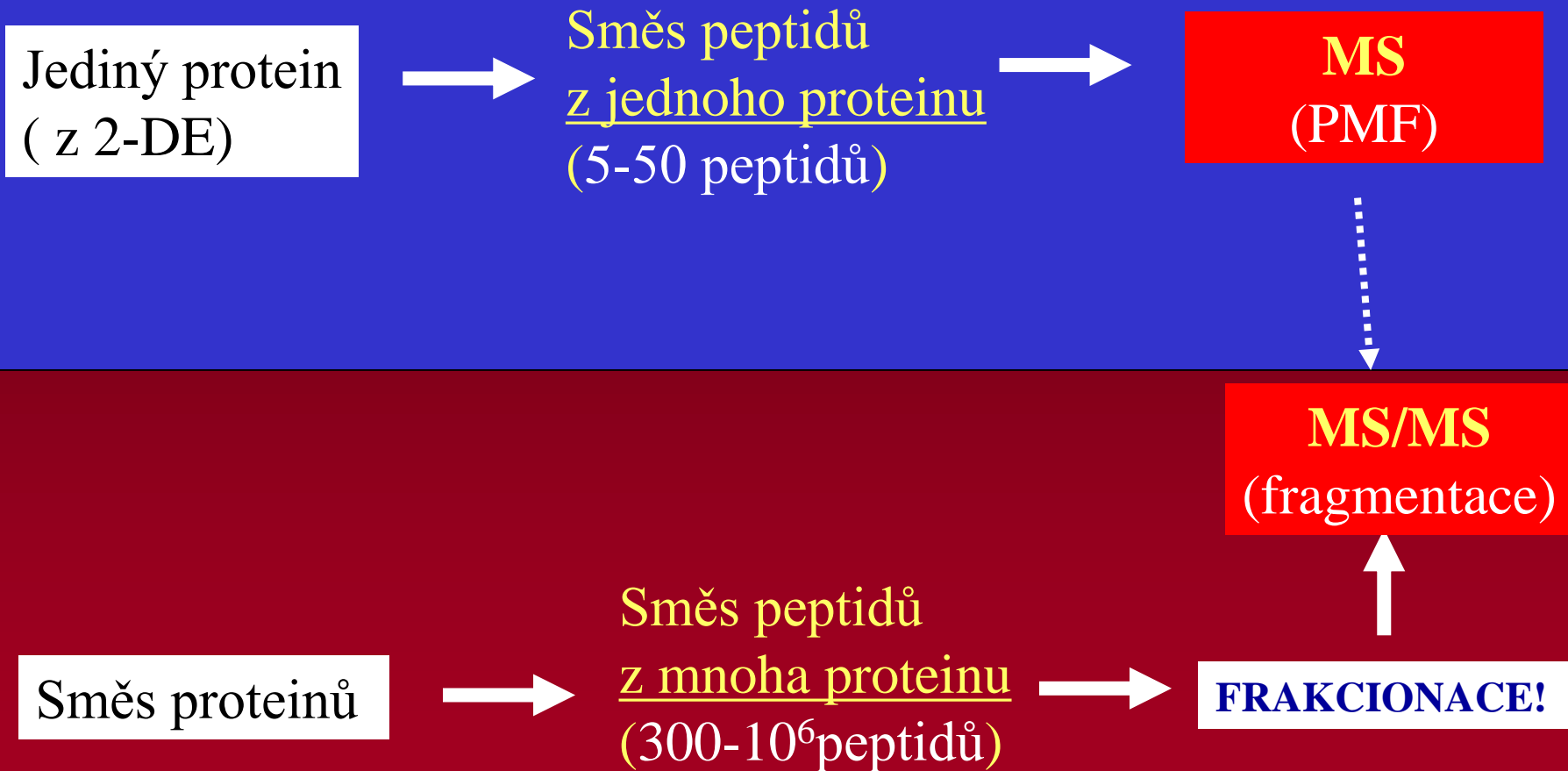
# PROTEOMIKA 2015

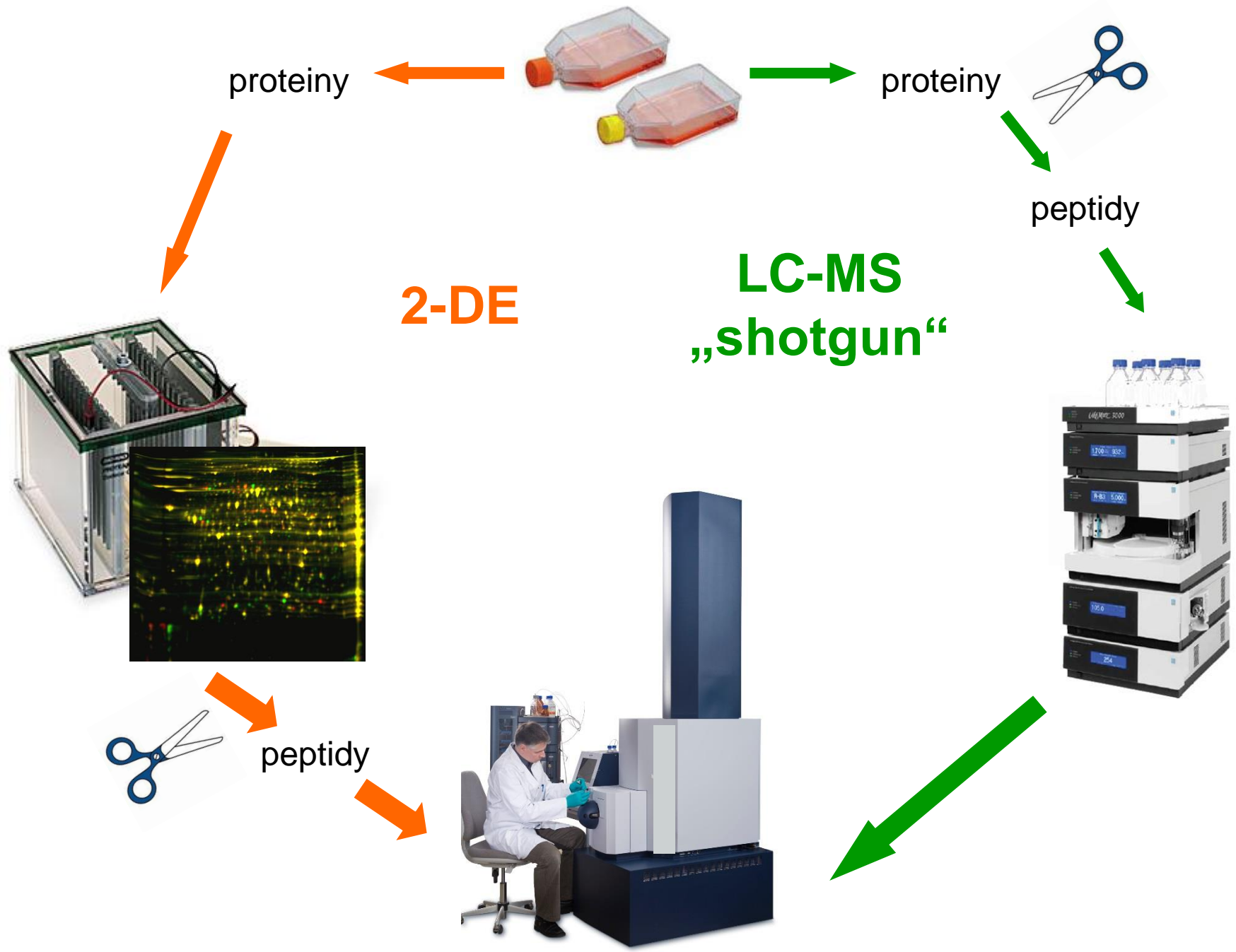
**SHOT-GUN**

**LC, IEF peptidů**

**Kvantitativní přístupy  
iCAT, iTRAQ,  
SILAC, dimetylace,  
AQUA....**

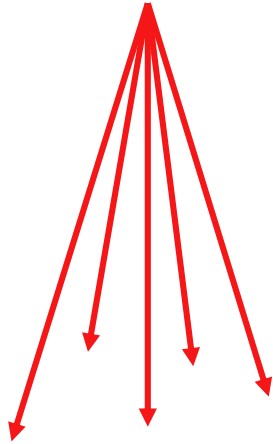
# Kdy použít PMF a kdy musím fragmentovat peptid?





# „Shot-gun“ strategies

10 000 proteins



~ 400 000 peptides

**2D-LC-MS**

**IEF-LC-MS**

**SDS-PAGE-LC-MS (gel-LC)**





# Shotgun postupy v proteomice



DIGESCE

Extrémně komplexní  
směs peptidů  
 $10^5$ - $10^7$  různých

1D SDS-PAGE

„In gel“ digesce  
10-50 „řízků“  
a extrakce

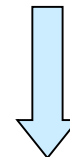
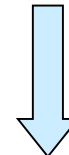
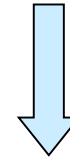
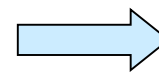
Komplexní směs peptidů  
 $10^2$ - $10^6$  různých

2D LC  
SCX-RP

IEF- RP LC

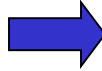
RP LC

ESI -MS/MS  
LC-MALDI MS/MS



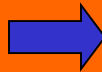
# METODY CHROMATOGRAFICKÉ SEPRACE PEPTIDŮ

**VELIKOST**



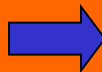
**Gelová filtrace**  
(Size-exclusion chromatography)

**HYDROFOBICITA**



**Chromatografie v reverzní fázi**  
(Reverse phase chromatography, RP-LC)

**AKTUÁLNÍ NÁBOJ**



**Iontoměničová chromatografie**  
(Ion exchange chromatography, SCX/SAX-LC)

**SPECIFICKÉ  
INTERAKCE**



**Afinitní chromatografie**  
(Affinity chromatography)

**A jejich (více-rozměrné) kombinace...**

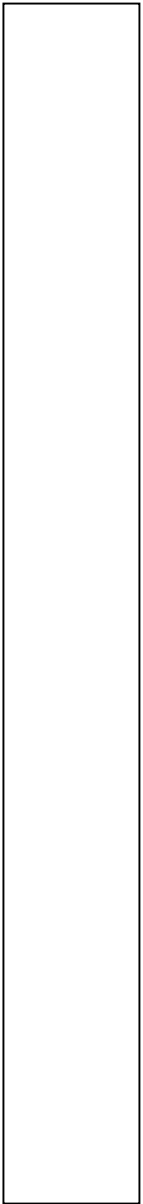
# Identifikace proteinů v shot-gun experimentech

Až stovky tisíc různých peptidů  
kontinuálně eluovaných z (RP) LC

**On-line MS** – přímé spojení LC kolony s **ESI-MS**.

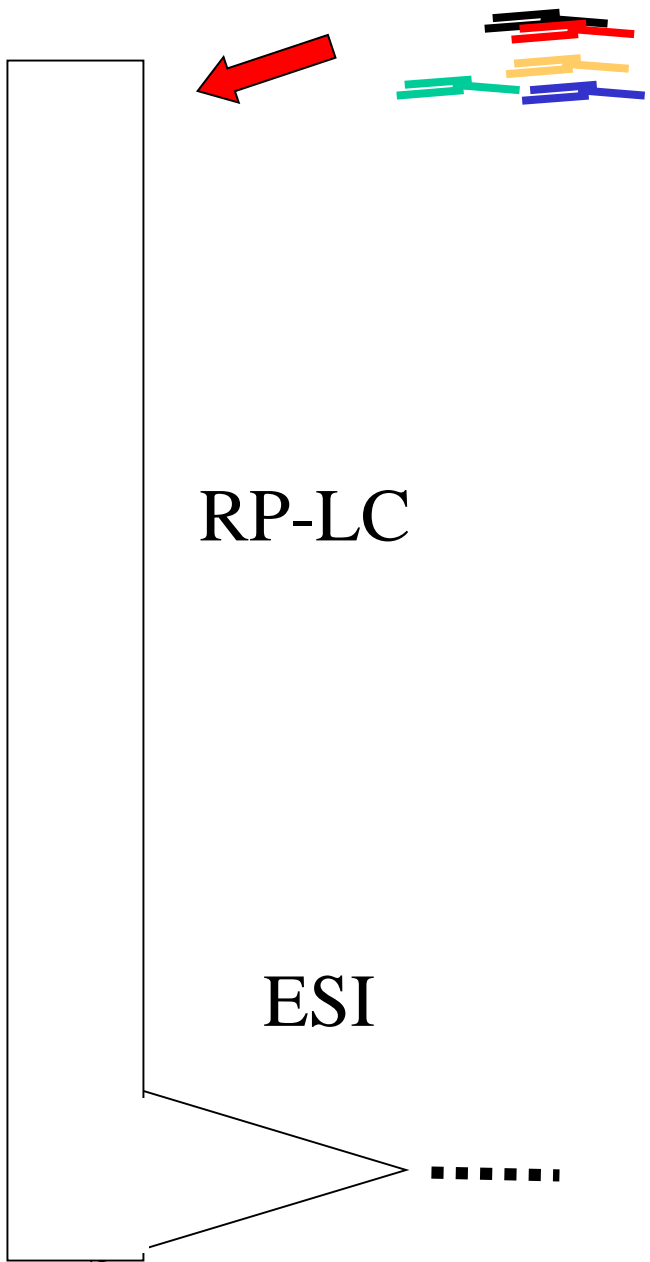
V reálném čase (60-600 minut) jsou eluované peptidy ionizovány. Změřeno MS, izolován prekurzor a provedena fragmentace. Cyklus přepnutí MS-MS/MS až několikrát za sekundu.

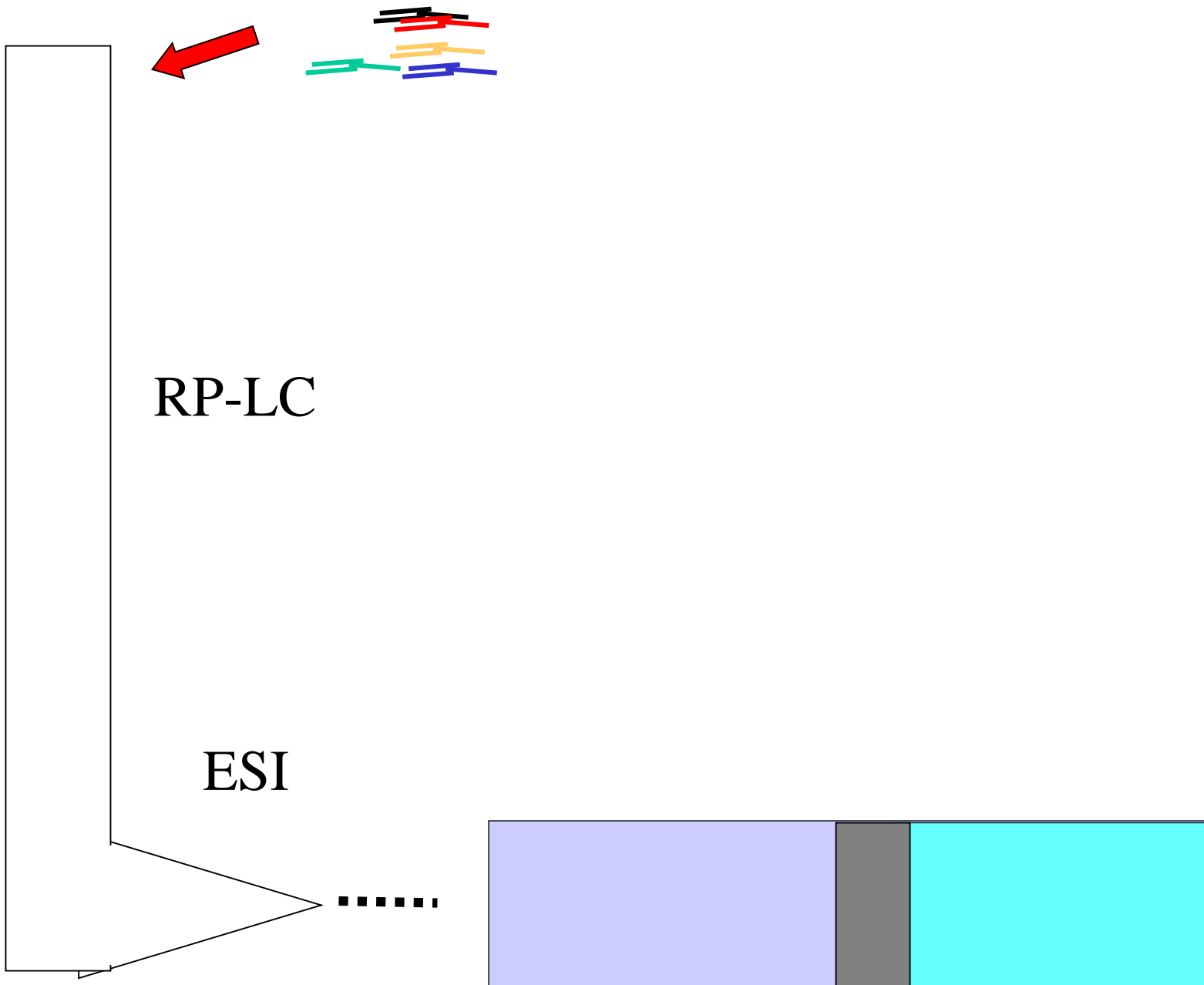
**Off-line MS** – např. **LC-MALDI**. Frakce eluujících peptidů jsou sbírány a MS probíhá až po sběru. Typicky sběr mikrofrakcí (nL) na MALDI destičku s následným měřením MS a MS/MS.

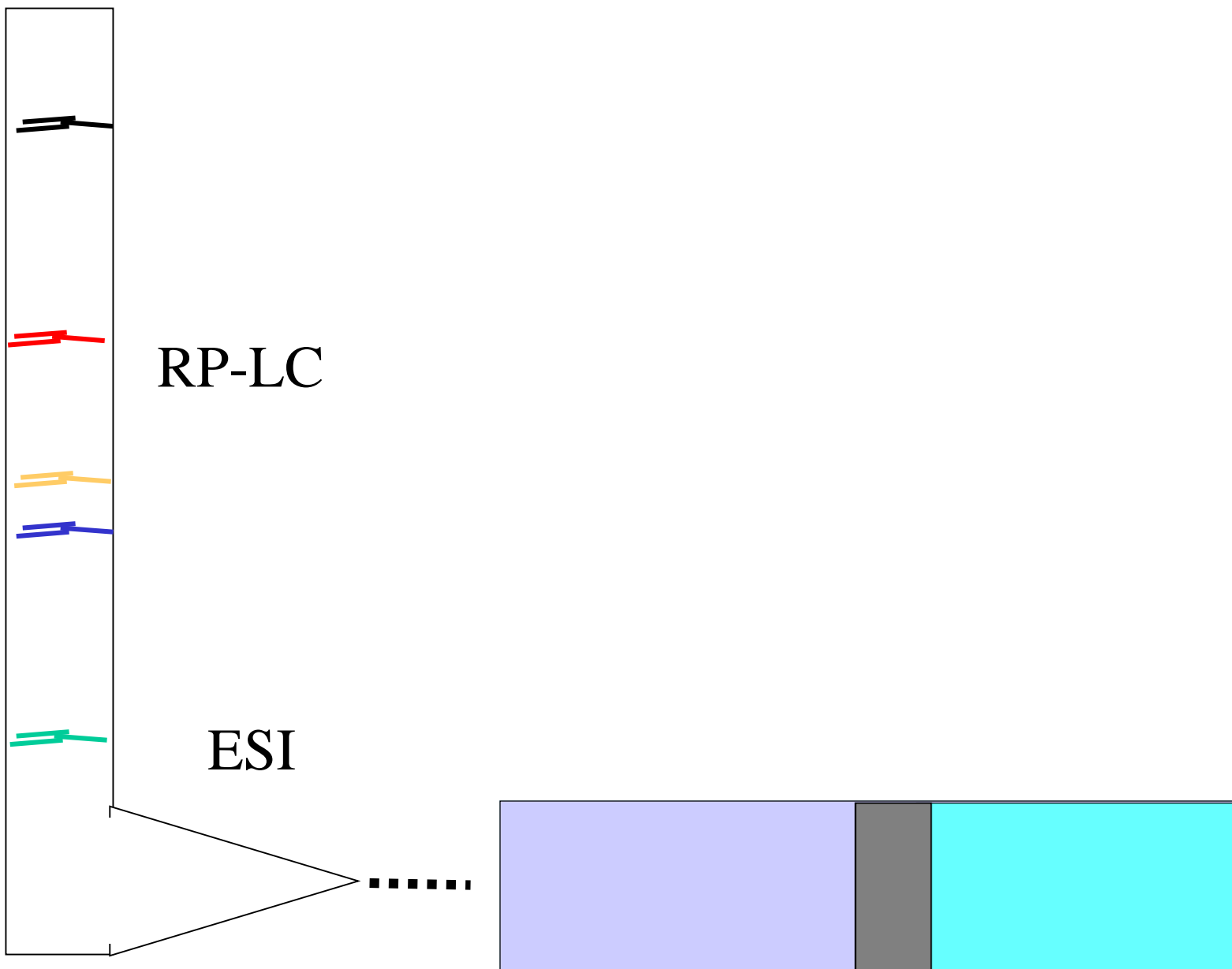


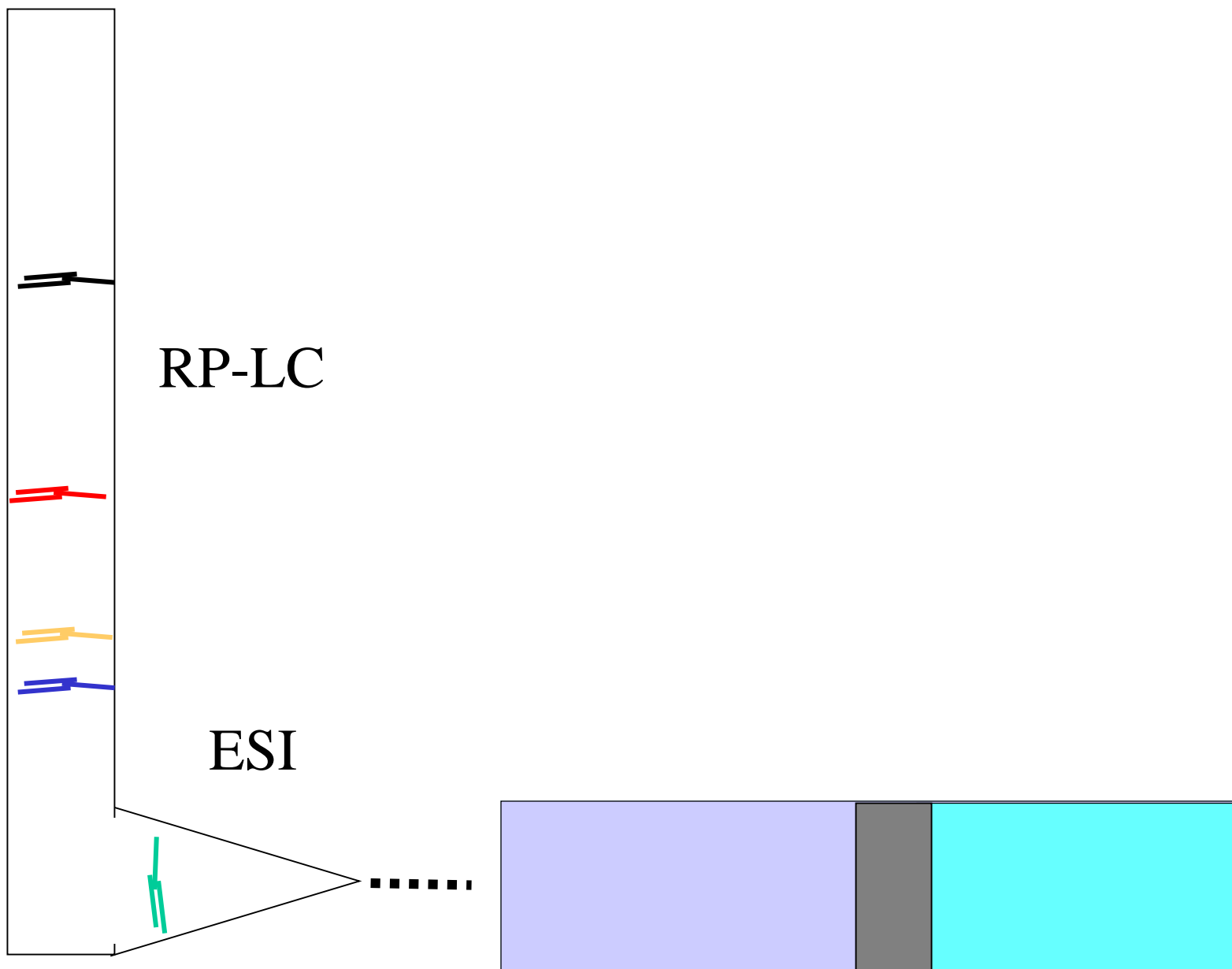
RP-LC





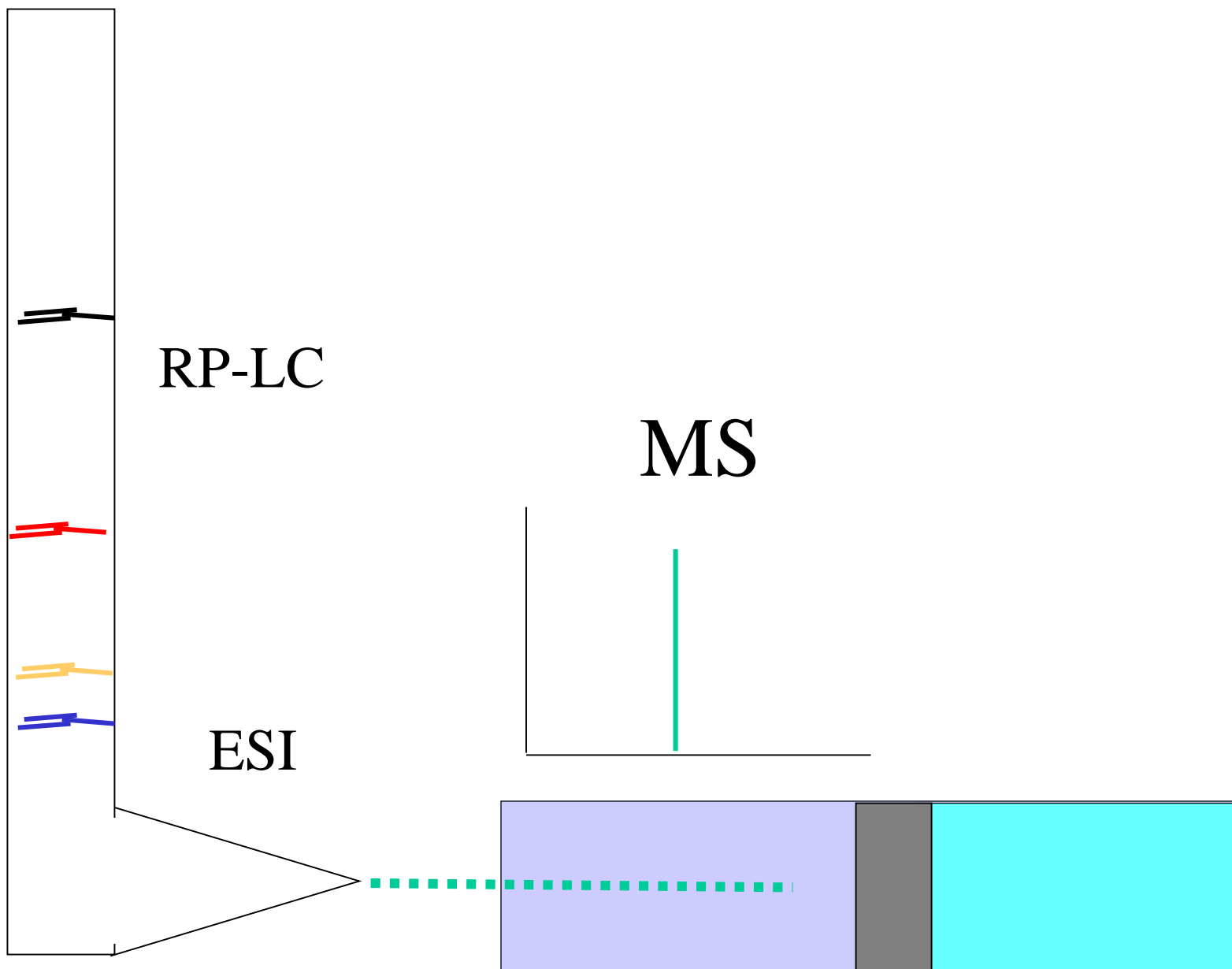


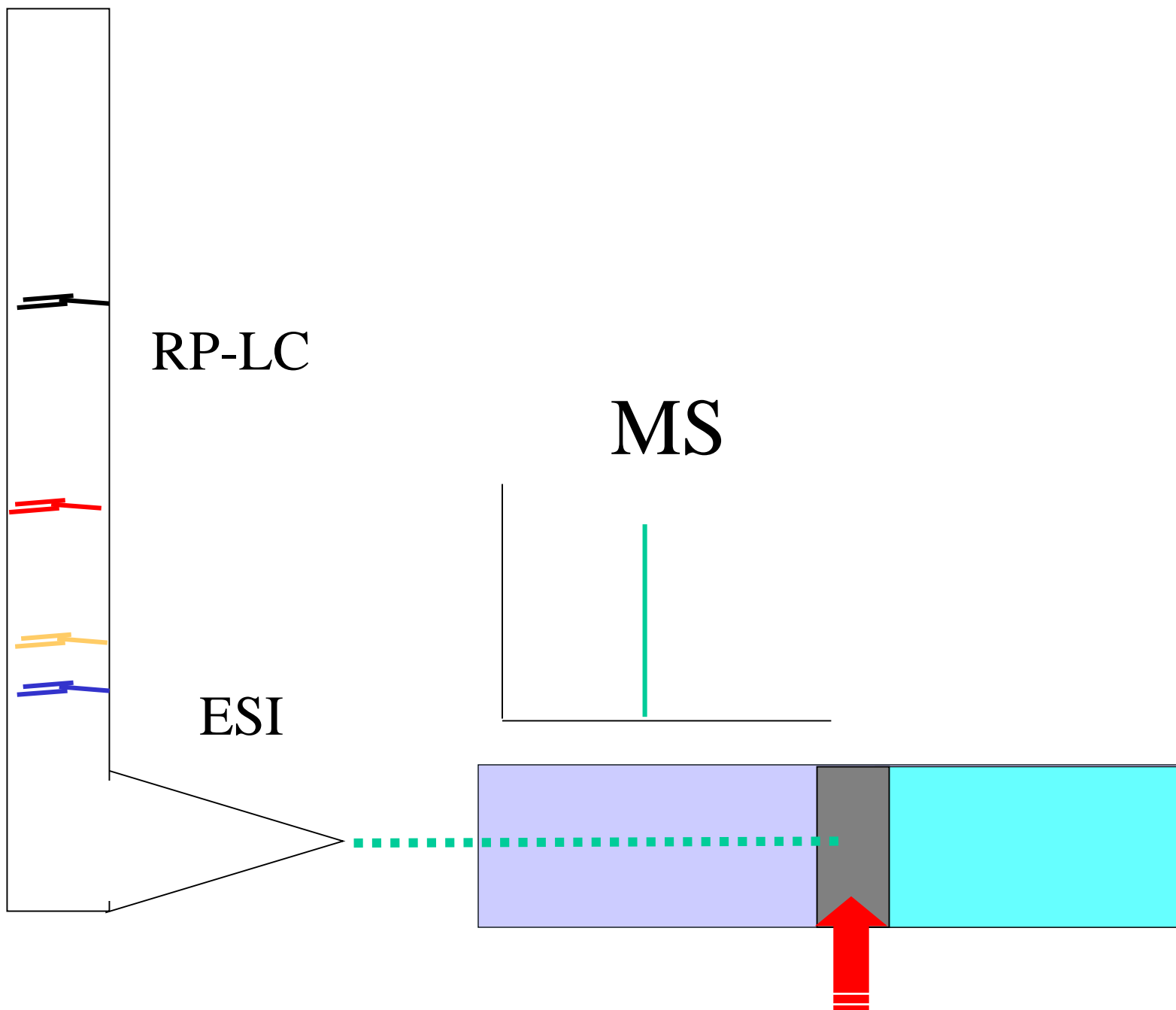


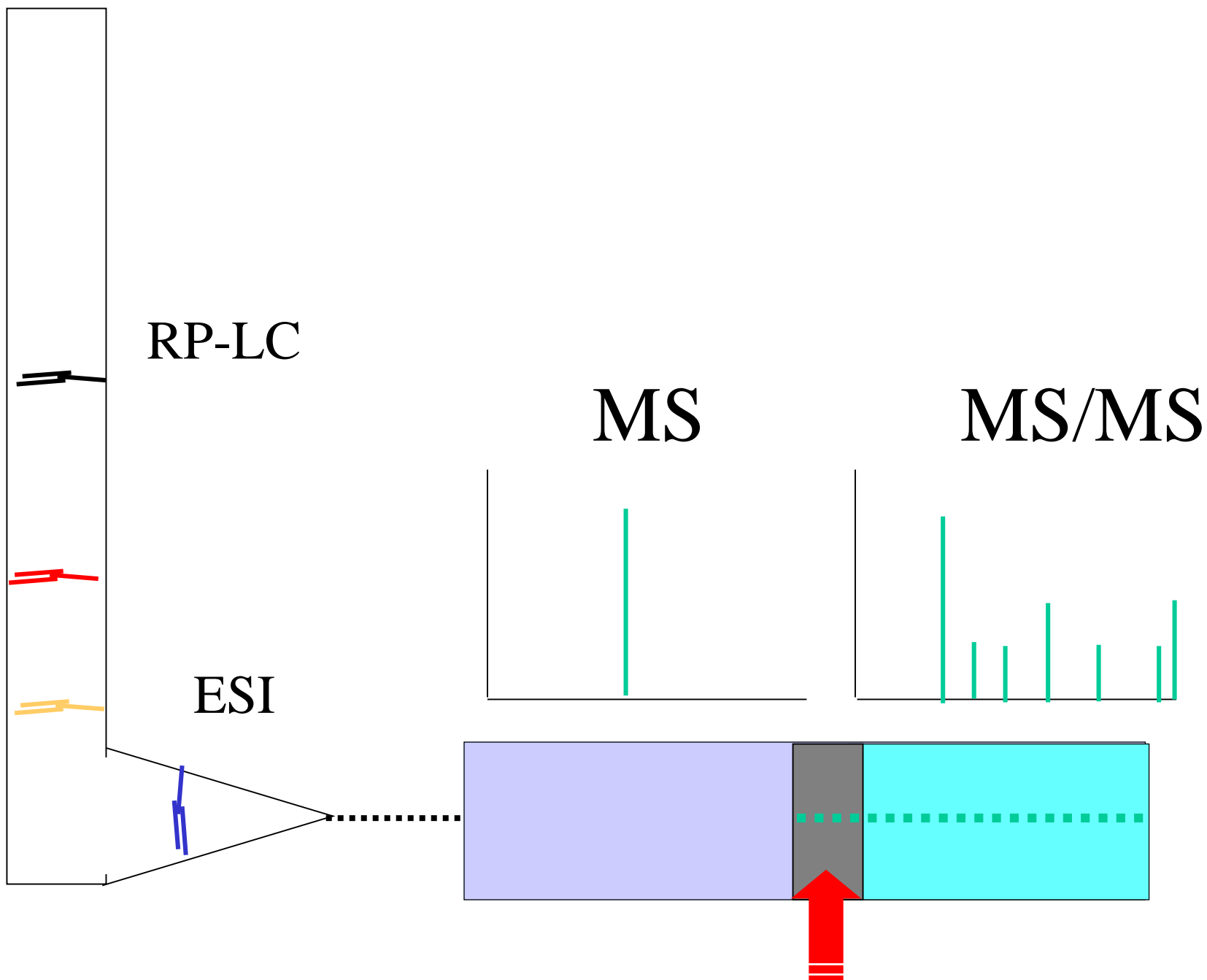


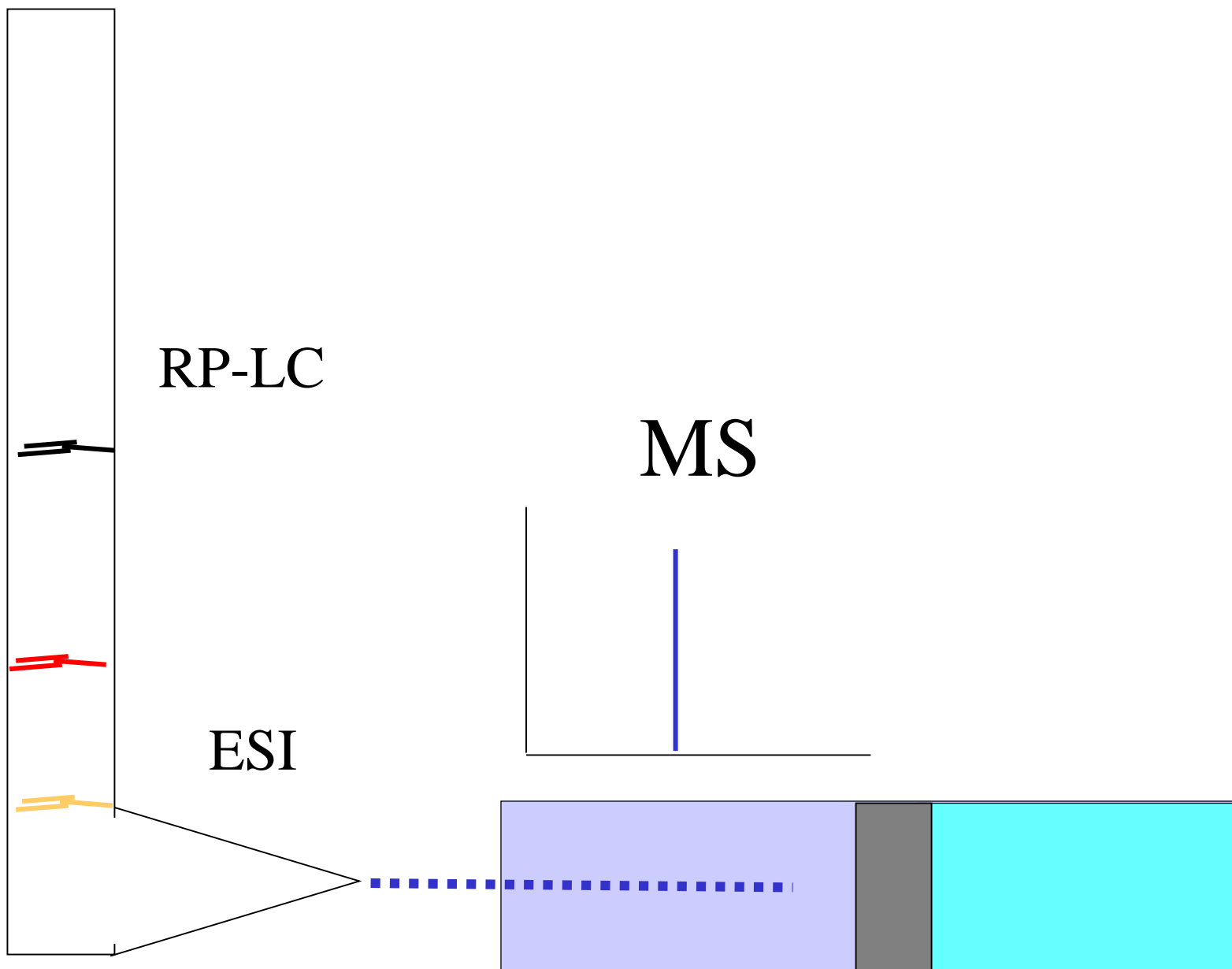
RP-LC

ESI

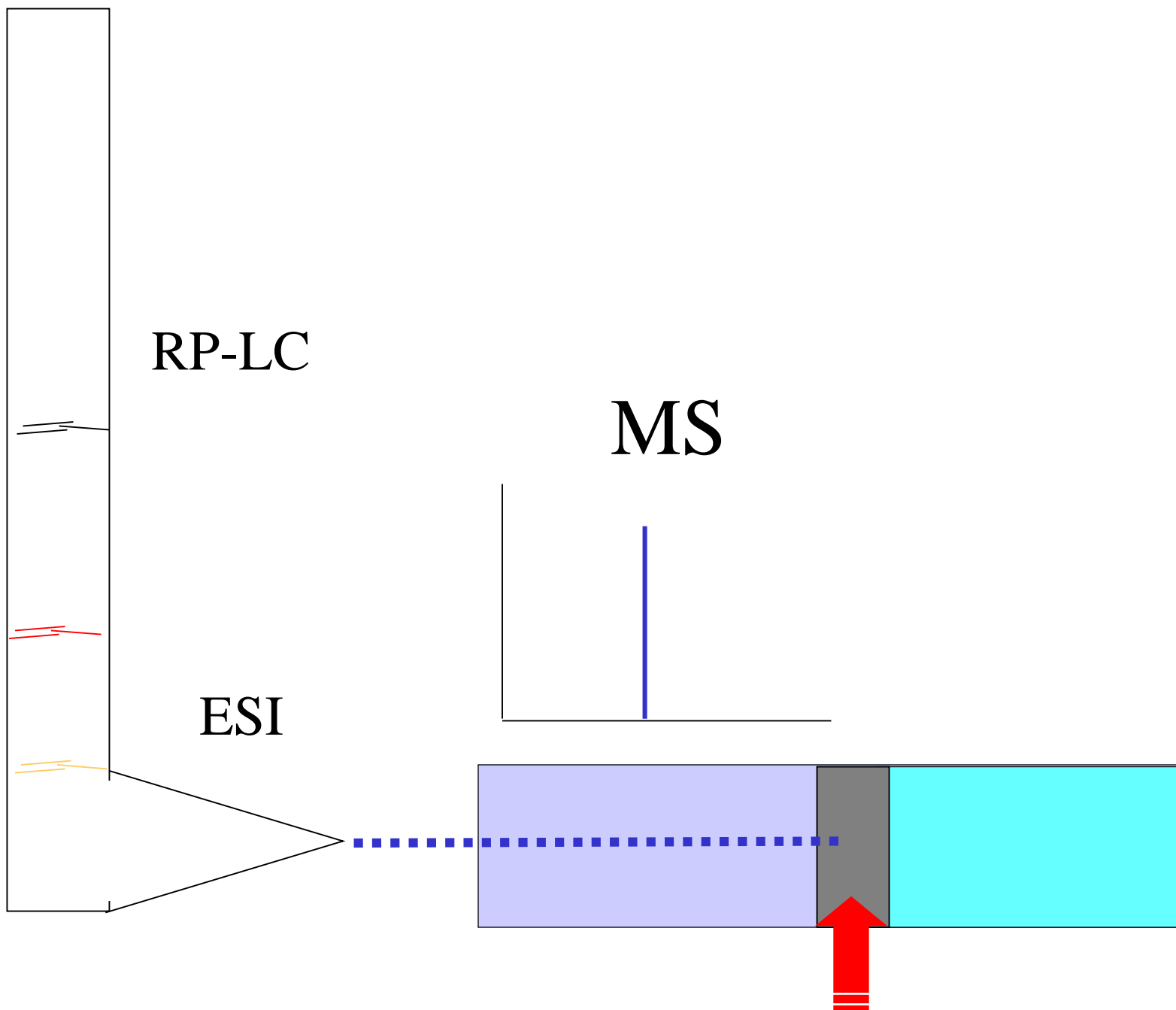


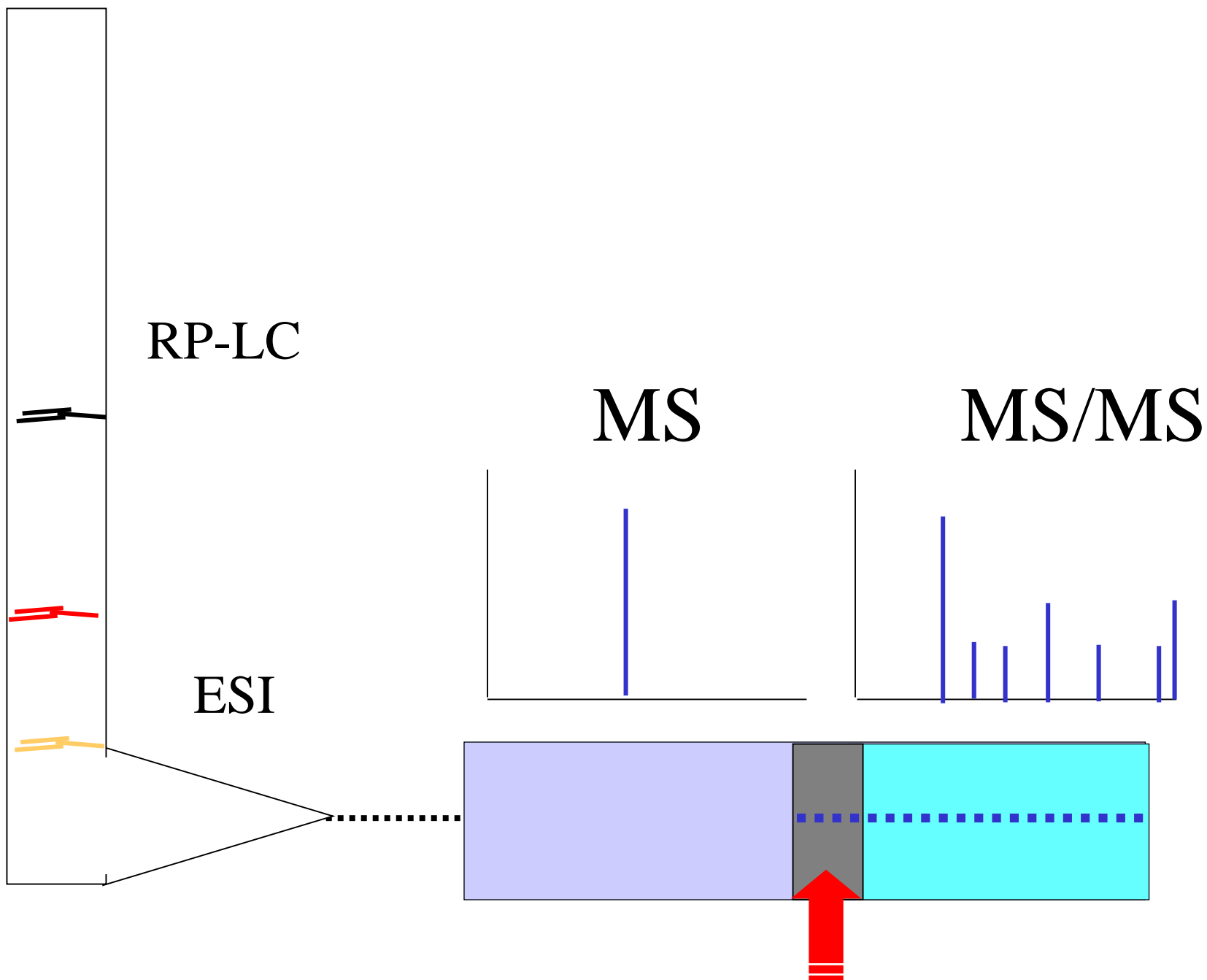








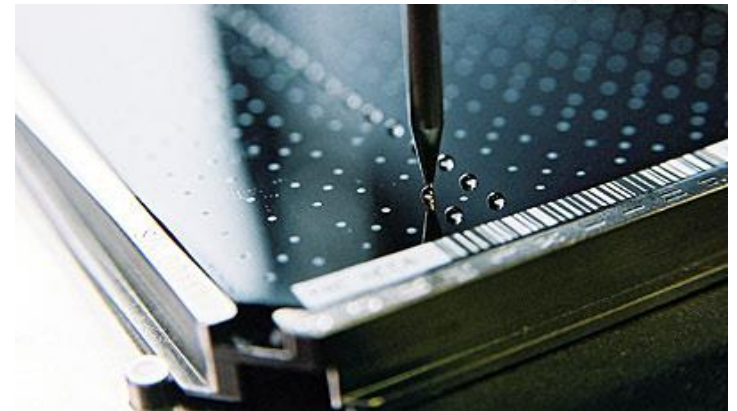
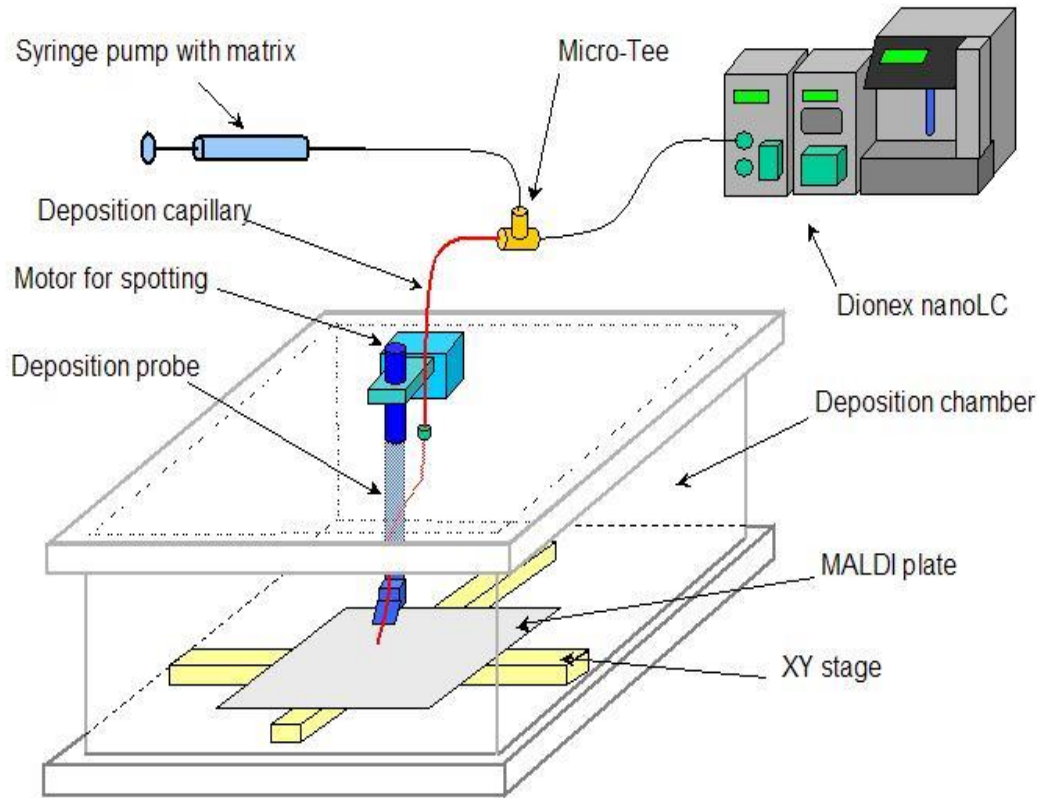




# LC-MALDI MS

Nano LC s robotickým mikrosběračem frakcí.  
Frakce jsou on-line míchány s matricí a spotovány na MALDI destičku

## LC-MALDI MS INTERFACE



# Shotgun postupy v proteomice



DIGESCE

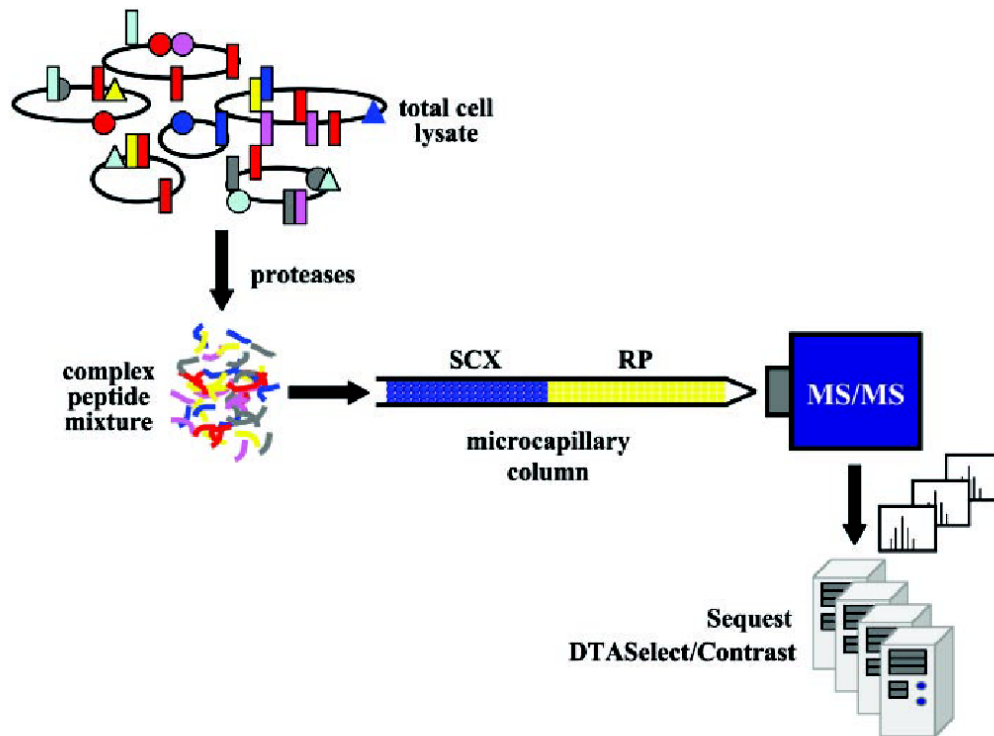
Extremně komplexní  
směs peptidů  
 $10^5$ - $10^7$  různých

2D LC  
SCX-RP

ESI –MS/MS  
LC-MALDI MS/MS

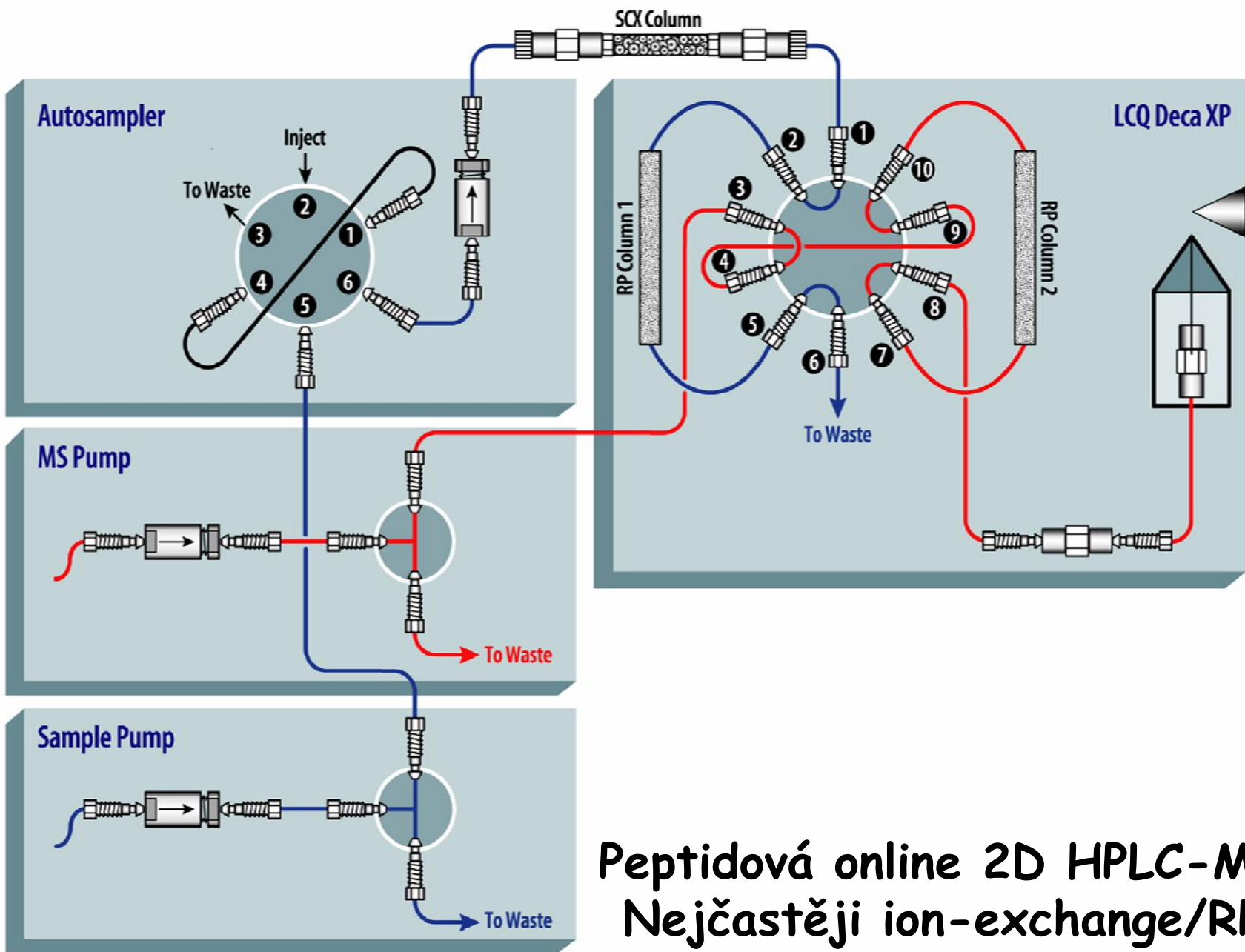
# MudPIT (2002)

## Multidimensional protein identification technology

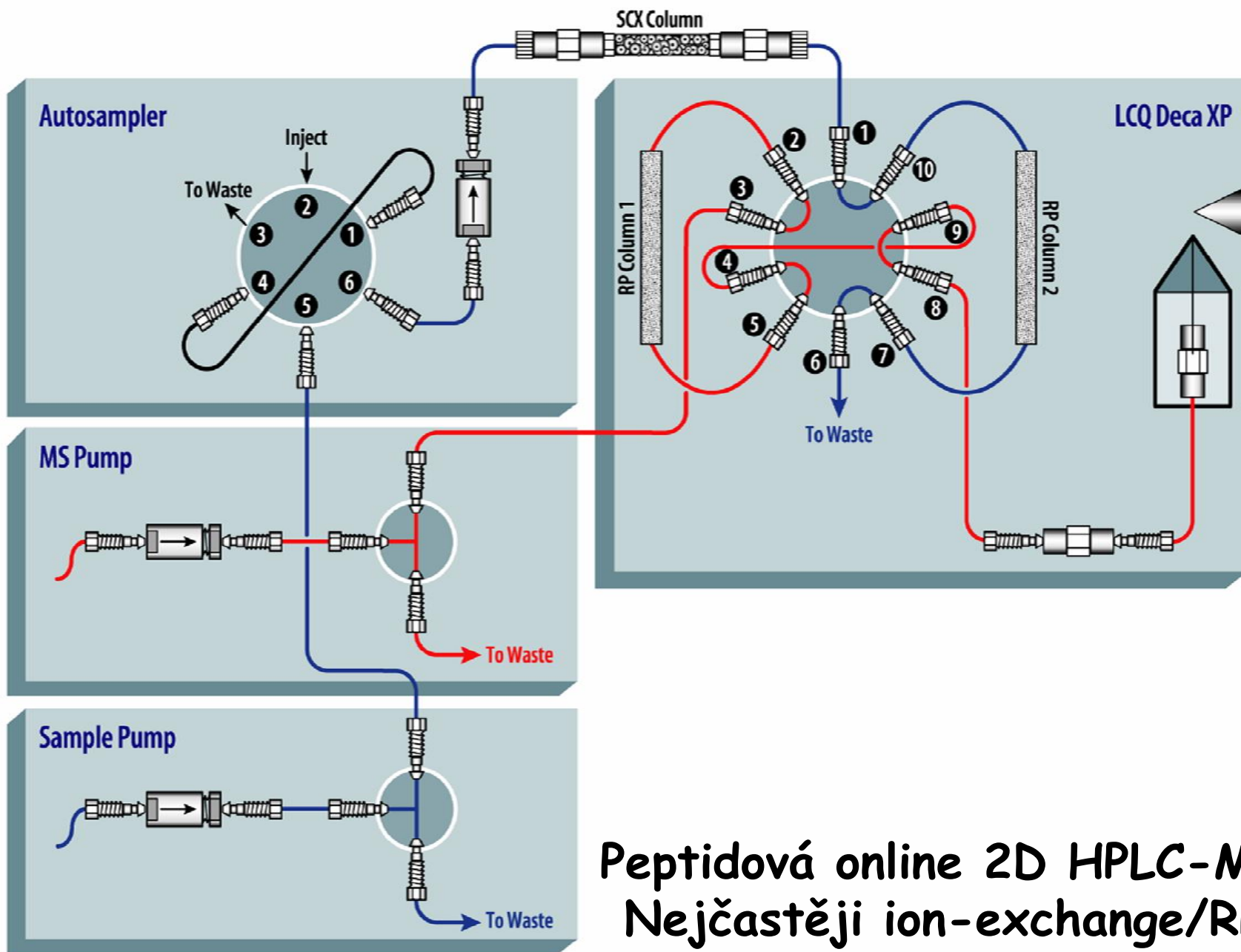


1484 proteinů identifikováno  
(kvasinka - cca 5000 ORF)

**Fig. 1.** Multidimensional Protein Identification Technology (MudPIT). The complex mixture of proteins present in a whole cell lysate is fragmented first with lysine-specific endoproteinase *lysC* in the presence of 8 M urea and then with immobilized trypsin, after dilution to 2 M urea, generating a highly complex mixture. The peptides are collected on a strong cation exchange (SCX) column that is positioned immediately upstream of a reverse-phase (RP) column. Successive peptide fractions are released, depending on their isoelectric point, with salt steps of increasing concentration at low organic solvent concentrations and captured by the second-dimension reverse-phase column. The reverse-phase column is eluted with a gentle gradient of increasing organic solvent concentration between each salt step to displace the peptides, depending on their hydrophobicity, into the mass spectrometer. The ion-trap mass spectrometer (LCQ-DECA, ThermoFinnigan, San Jose, CA) employs data-dependent acquisition software to limit the time spent sequencing any particular peptide, so that as many different peptides as possible are sequenced, regardless of their abundance. SEQUEST software correlates experimental sequence with genomic data (courtesy of Christine Wu, The Scripps Institute, La Jolla, CA).



Peptidová online 2D HPLC-MS  
 Nejčastěji ion-exchange/RP



Peptidová online 2D HPLC-MS  
 Nejčastěji ion-exchange/RP



# Shotgun postupy v proteomice



DIGESCE

Extrémně komplexní  
směs peptidů  
 $10^5$ - $10^7$  různých

2D LC  
SCX-RP

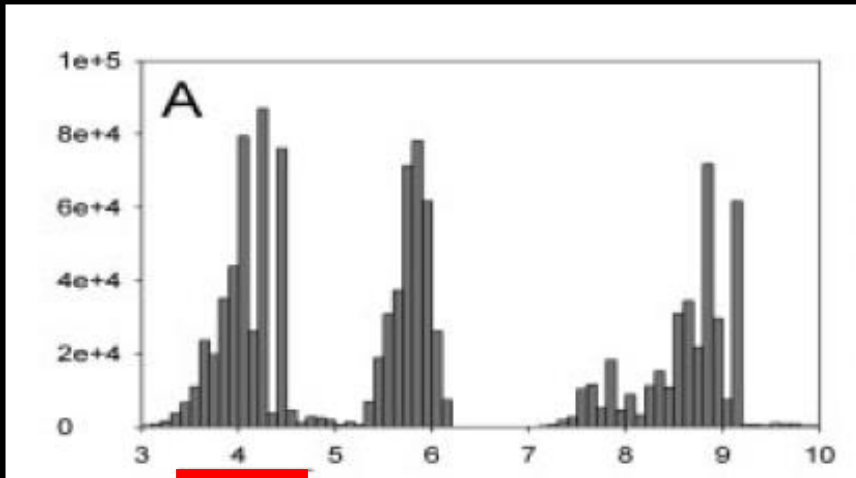
IEF- RP LC

ESI -MS/MS  
LC-MALDI MS/MS



# IEF-IPG peptidů

## Distribuce lidských peptidů dle pI



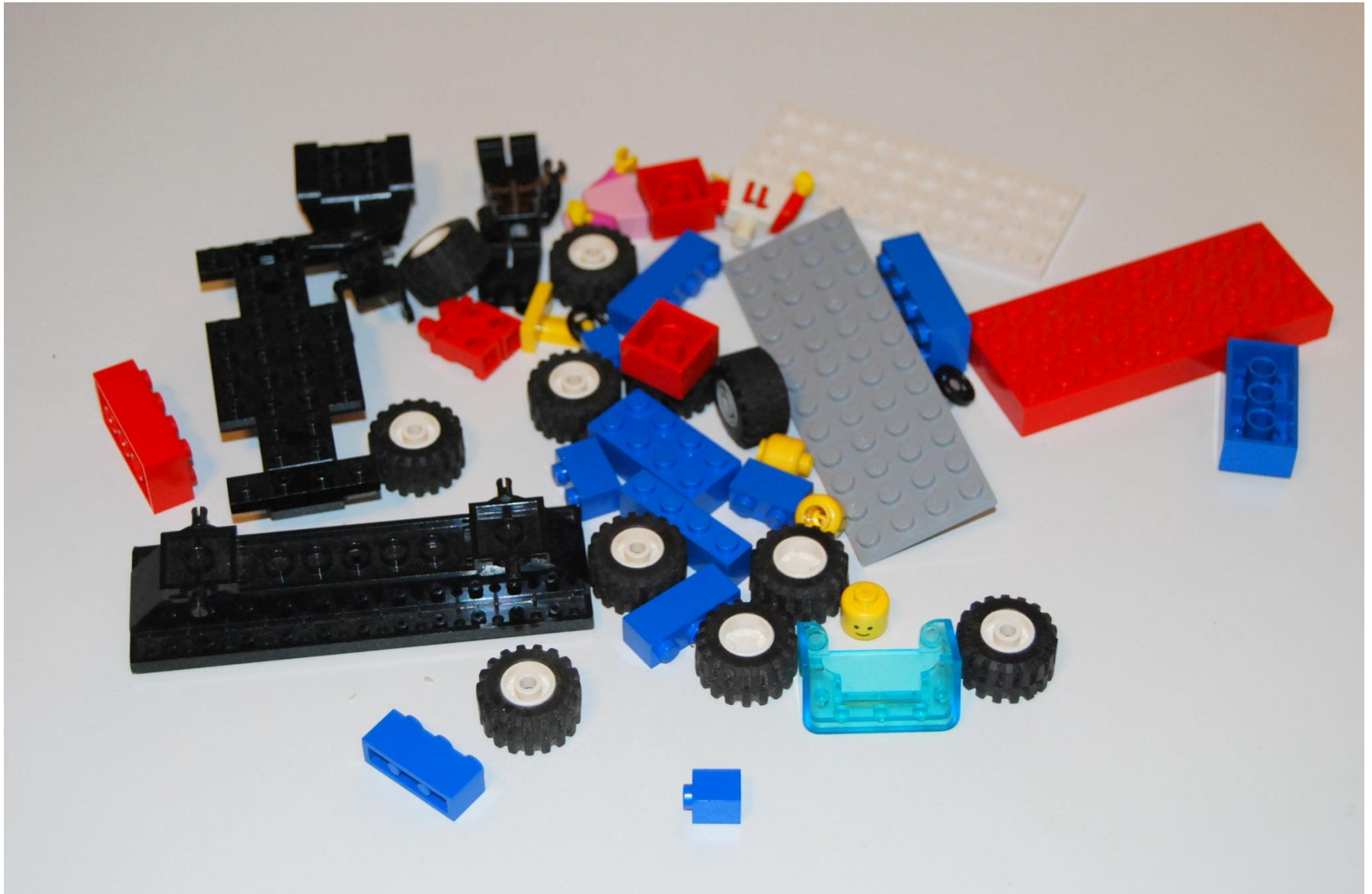
**1/3 lidských peptidů má pI v rozmezí 3,5-4,5  
Je zde zastoupeno 96% známých proteinů**

## Výhody IPG-IEF peptidů

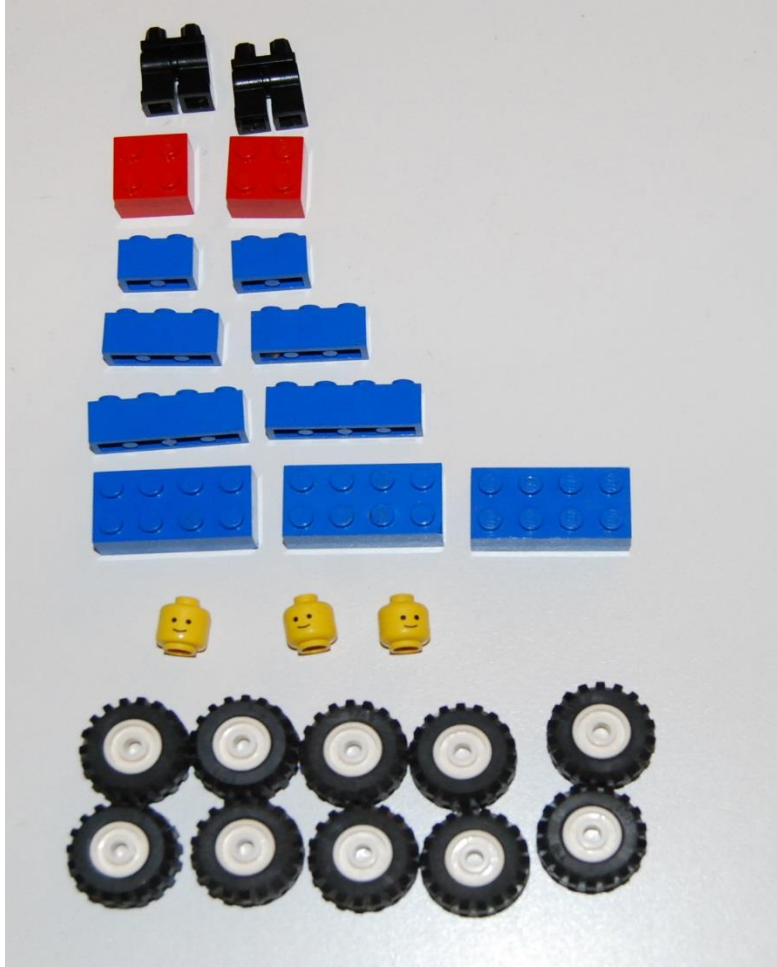
- vysoká separační účinnost
- možnost vyšší nanášky
- informace o pI může sloužit k verifikaci ID
- snížení komplexity vzorku při zachování pokrytí proteomu
- Informace o pI může pomoci při ID
- stejné vybavení jako pro IEF proteinů
- jednotlivé frakce jsou ze stripu extrahovány



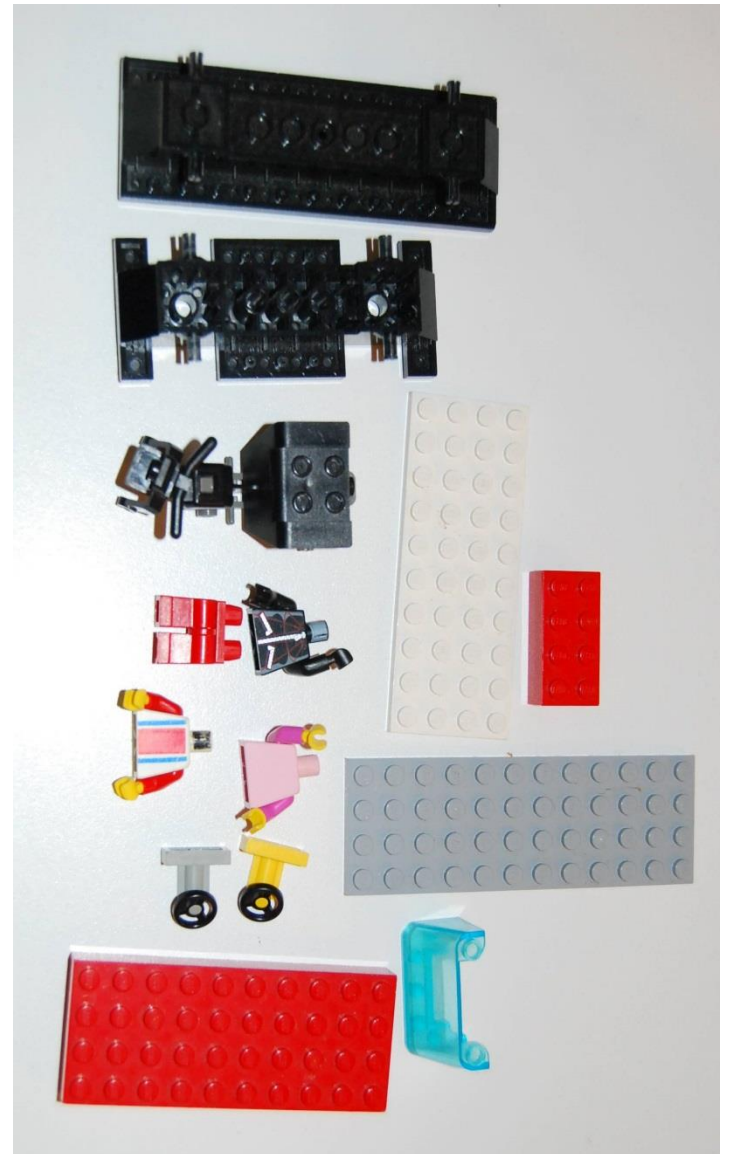
# Protein identification in shot-gun experiments



## Present in more proteins



## Proteotypic



# PROTEOMIKA 2015

**SHOT-GUN**

**LC, IEF peptidů**

**Kvantitativní přístupy  
iCAT, iTRAQ,  
SILAC, dimetylace,  
AQUA....**

**„Label free“**

**MRM**

# Kvاتفیکace pomocí MS ?

**MS není kvantitativní**, z výšky individuálního signálu ve spektru nelze přímo odvozovat abundanci daného peptidu ve vzorku.

Nelze získat (semi) kvantitativní informaci pouhým porovnáním dvou různých spekter.

Relativní kvantifikace může být provedena u dvou chemicky identických látek v jednom spektru.

Tyto látky se mohou lišit v izotopickém složení.

# Kvاتفكافة pomocí MS

**iCAT** – isotope Coded Affinity Tags

**iTRAQ** - isobaric Tags for Relative and Absolute Quantitation

**SILAC** – Stable Isotope Labeling with Aminoacids in Culture

**Dimethylation isotope labeling**

**$^{18}\text{O}$  labeling** – enzymatic digestion in  $^{18}\text{O}$  and  $^{16}\text{O}$  water

**AQUA a QconCAT** – Absolute Quantification of Abundance

# iCAT – Isotope Coded Affinity Tags

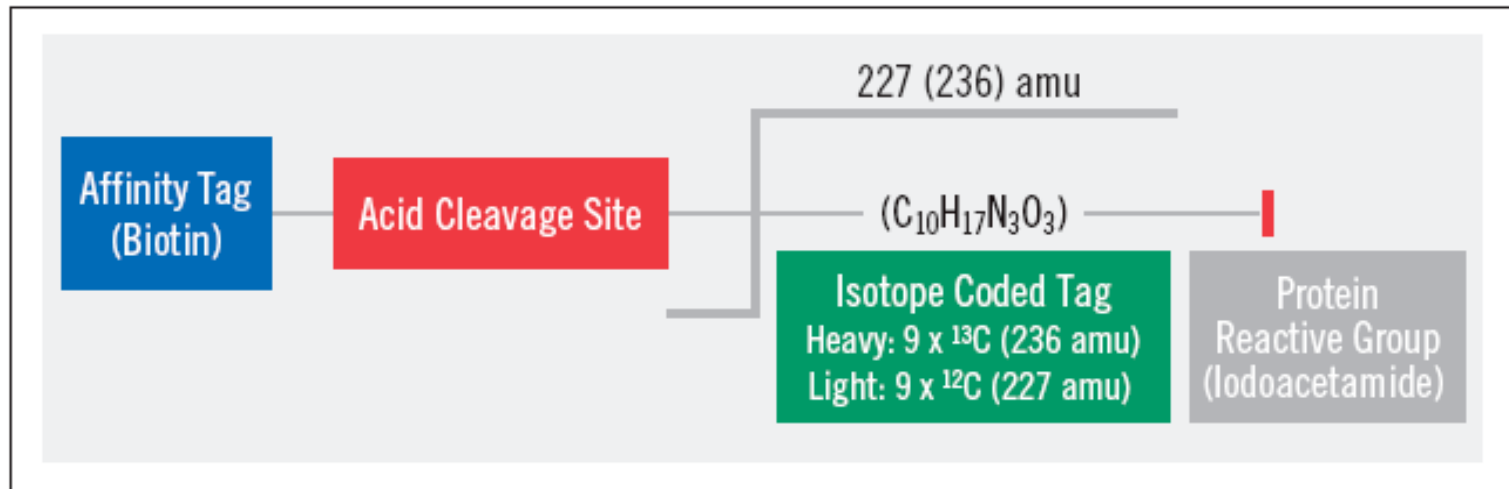


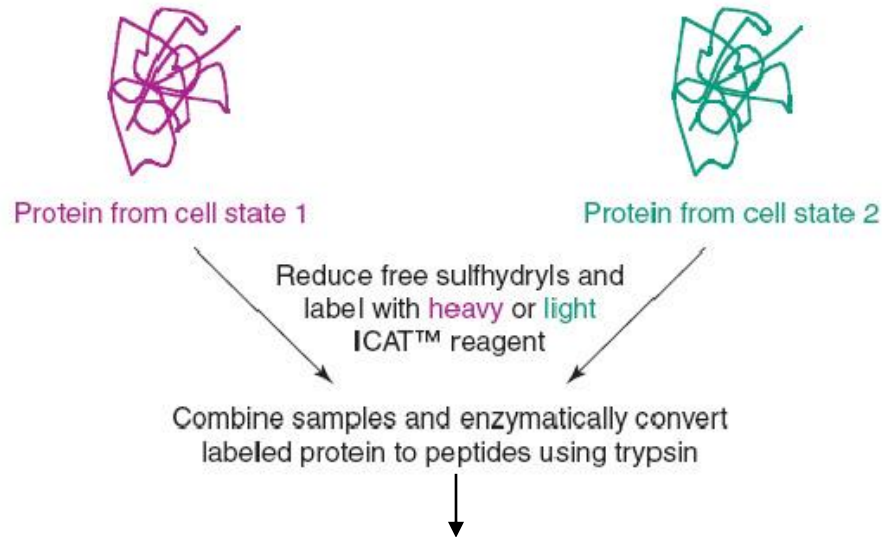
Figure 1. Cleavable ICAT™ Reagent Structure.

*Isotope coded tag:*

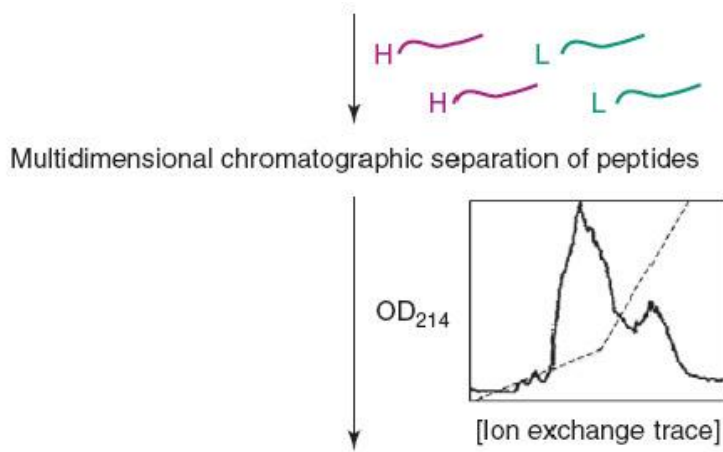
*Obsahuje 9 atomů  $^{13}\text{C}$  nebo 9 atomů  $^{12}\text{C}$*

Označit všechny přítomné bílkoviny na Cys

# iCAT – Isotope Coded Affinity Tags

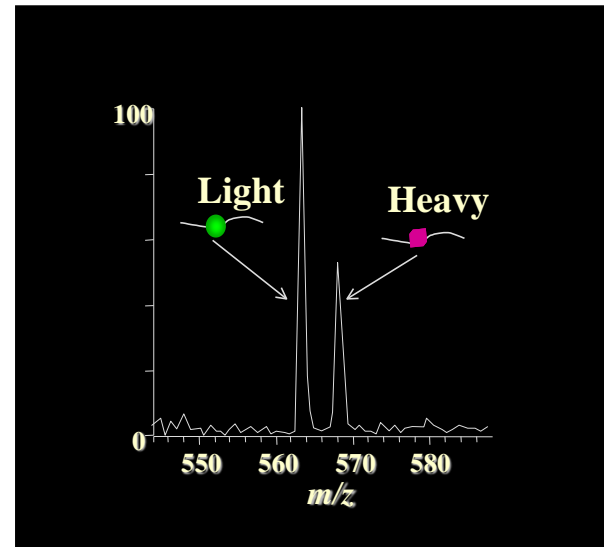


Isolate the biotinylated (iCAT-labeled) peptides



Microcapillary reversed-phase chromatography and tandem mass spectrometry

Kvantifikace MS  
Identifikace MS/MS

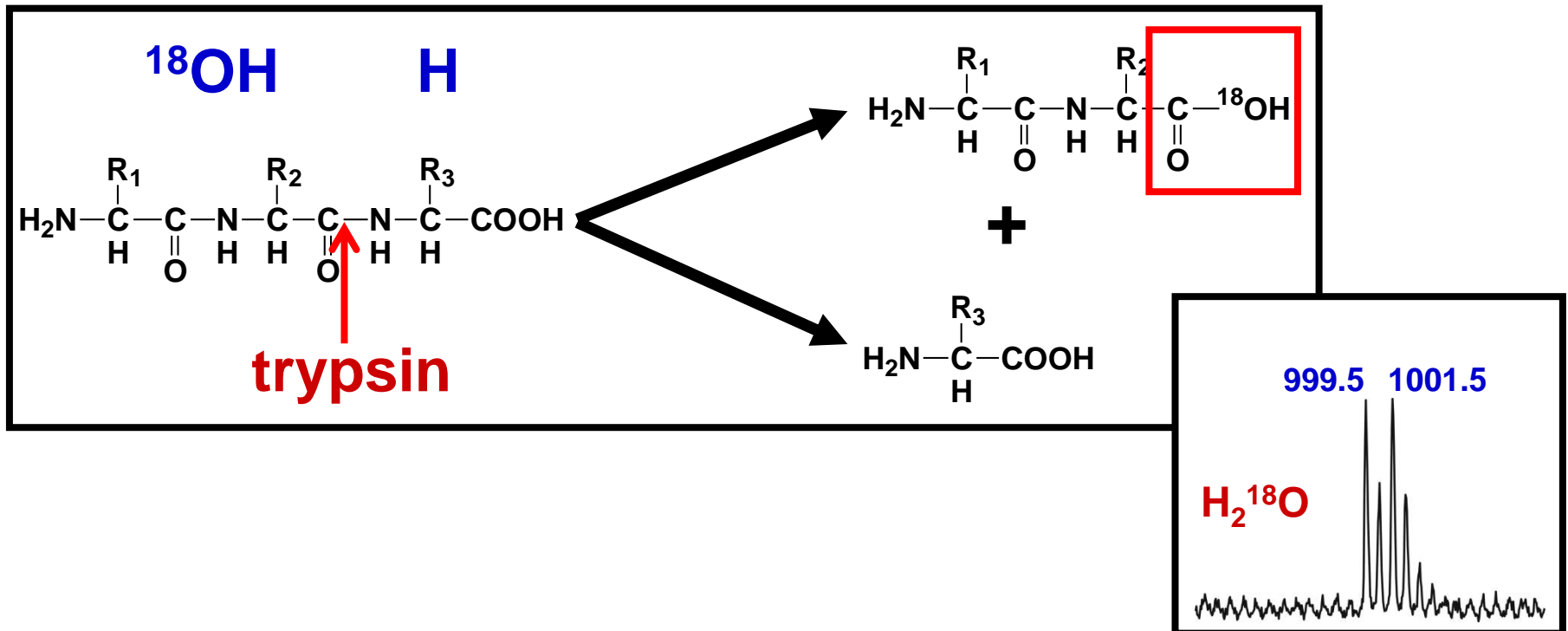




# $^{18}\text{O}$ – enzymatické značení

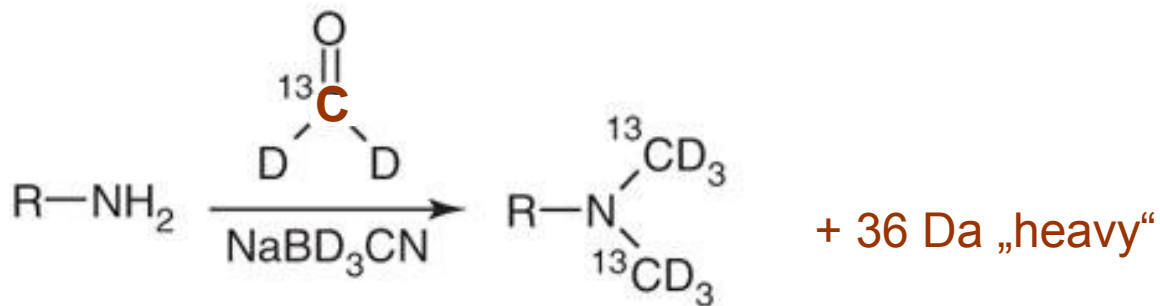
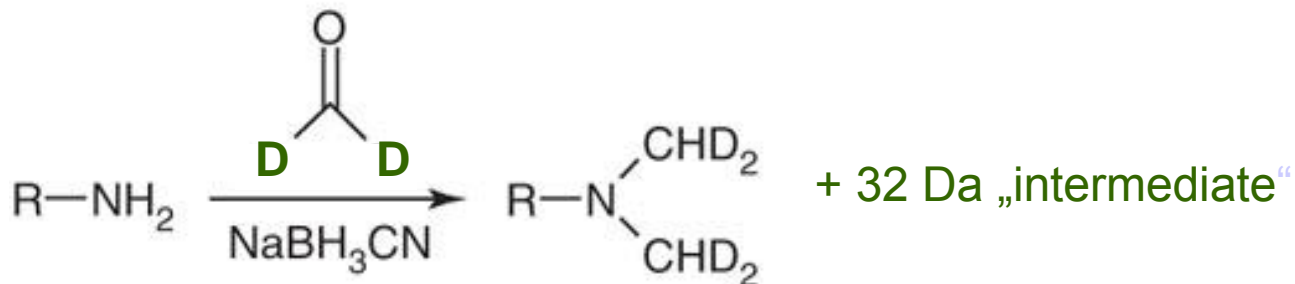
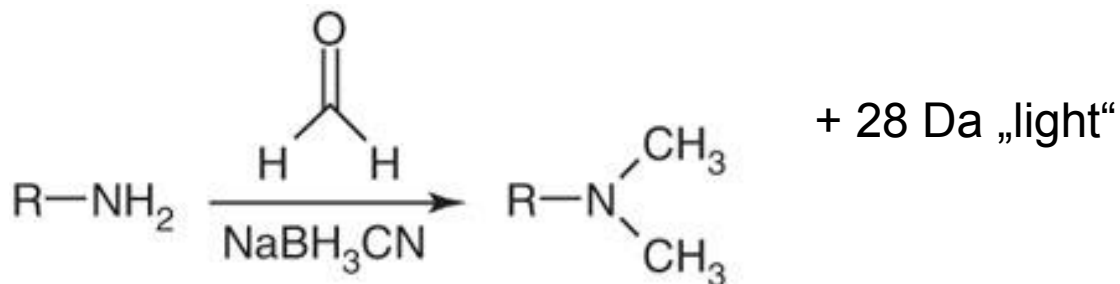
Digeste jednoho vzorku v normální vodě a druhého vzorku ve značené vodě  $^{18}\text{O}$

Dochází k inkorporaci  $^{16}/^{18}\text{O}$  – vzorky se smíchají a analyzují. Podle intenzit píků peptidu s  $^{16}\text{OH}$  a  $^{18}\text{OH}$  určíme relativní kvantitu peptidu.

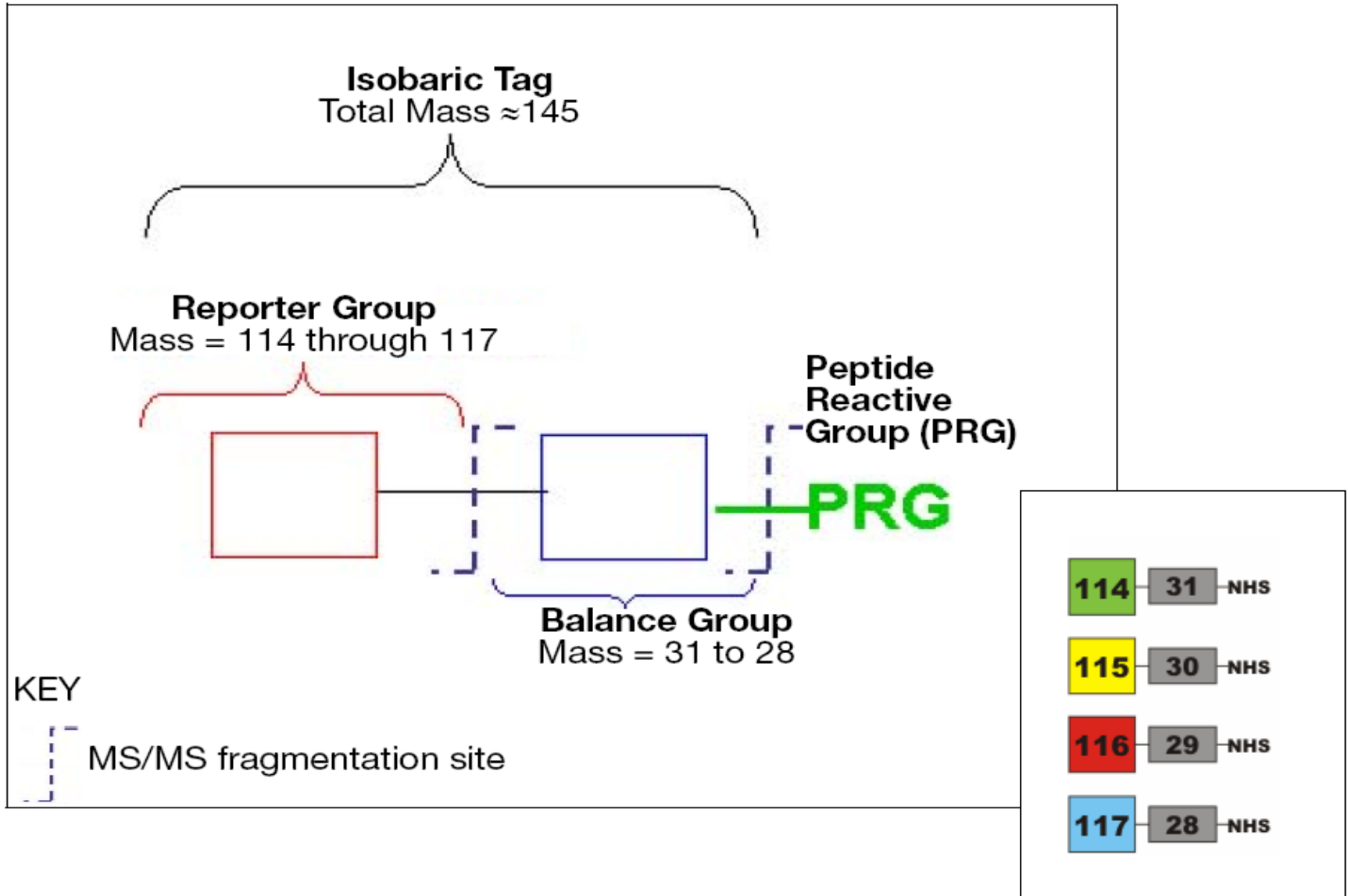


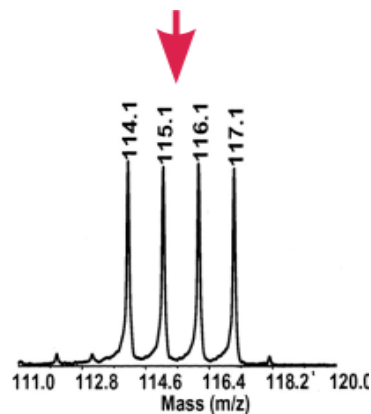
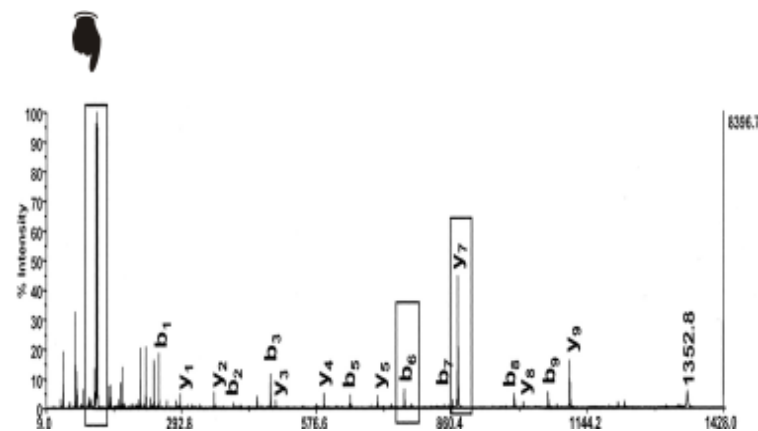
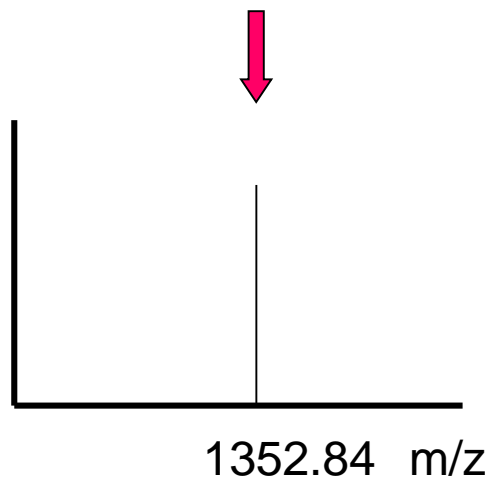
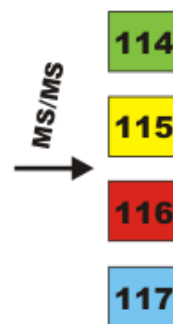
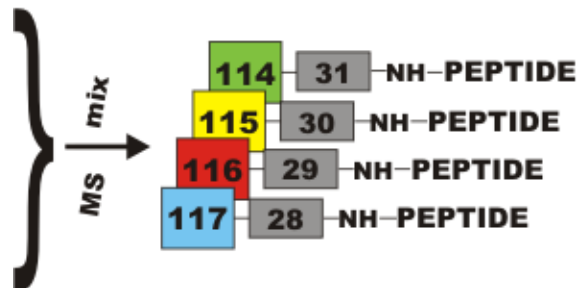
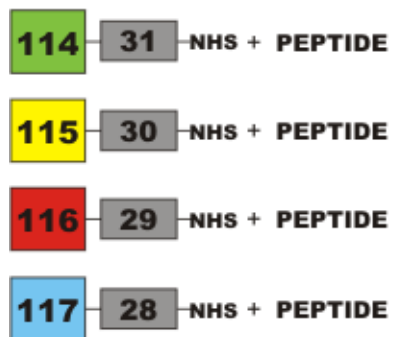
## Dimetylace peptidů (N-konec a ε-aminoskupina Lys)

### Reakce formaldehydu s primárními aminy (v přítomnosti kyanoborohydridu)



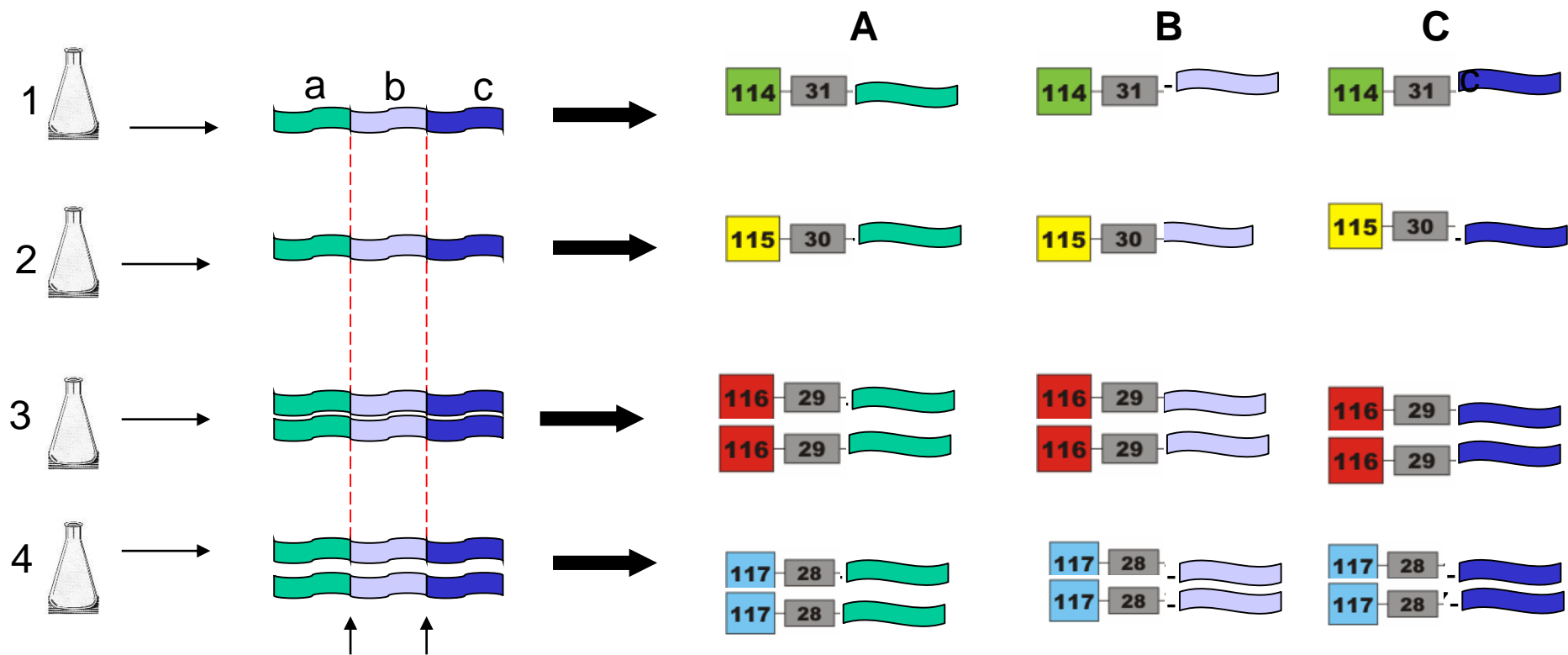
# ITRAQ – Isobaric Tags for Relative and Absolute Quantitation





# iTRAQ experiment

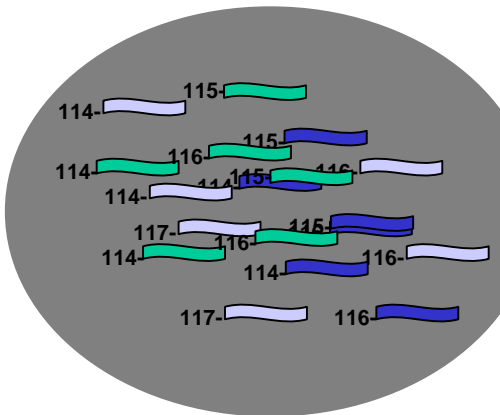
(relativní exprese proteinu ve 4 vzorcích)



Naštěpení trypsinem

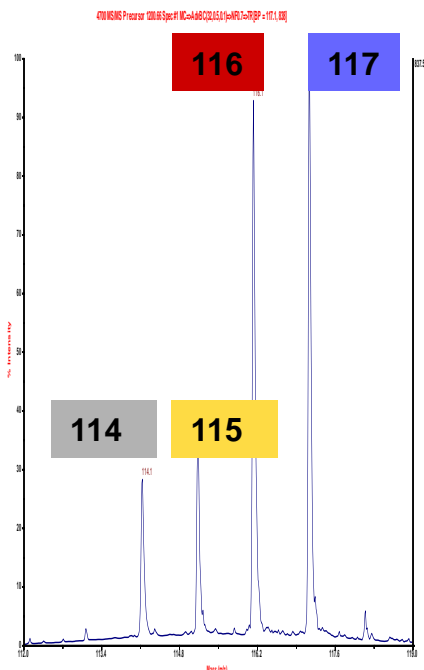
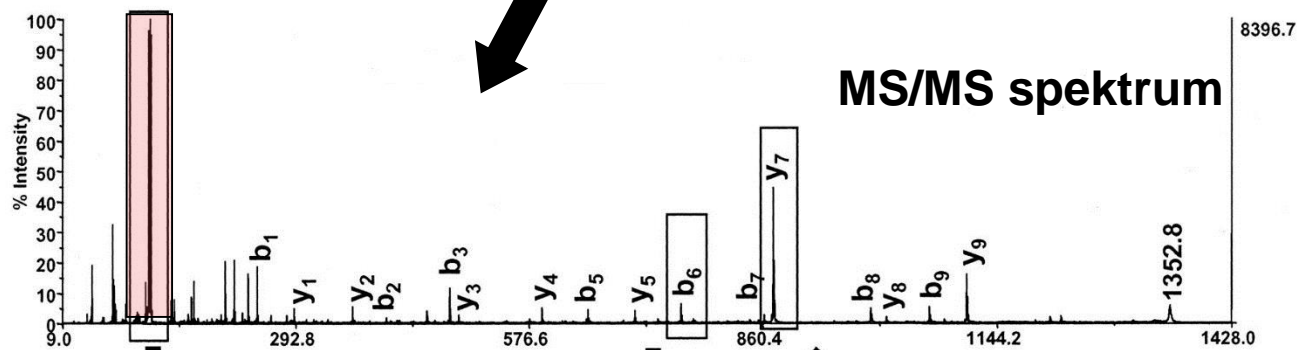
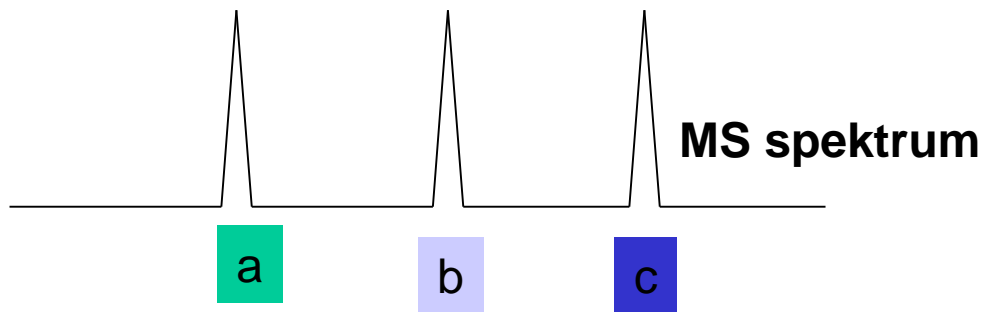
**Všechny peptidy jsou značeny,  
vždy jedním typem značky na vzorek**

# Skupiny smíchaný a peptidy separovány



IEF-HPLC  
nebo  
2D HPLC

# HMOTNOSTNÍ ANALÝZA



Kvantifikace  
Reportérových iontů

**Dvojnásobná  
exprese ve vzorcích  
3 a 4**

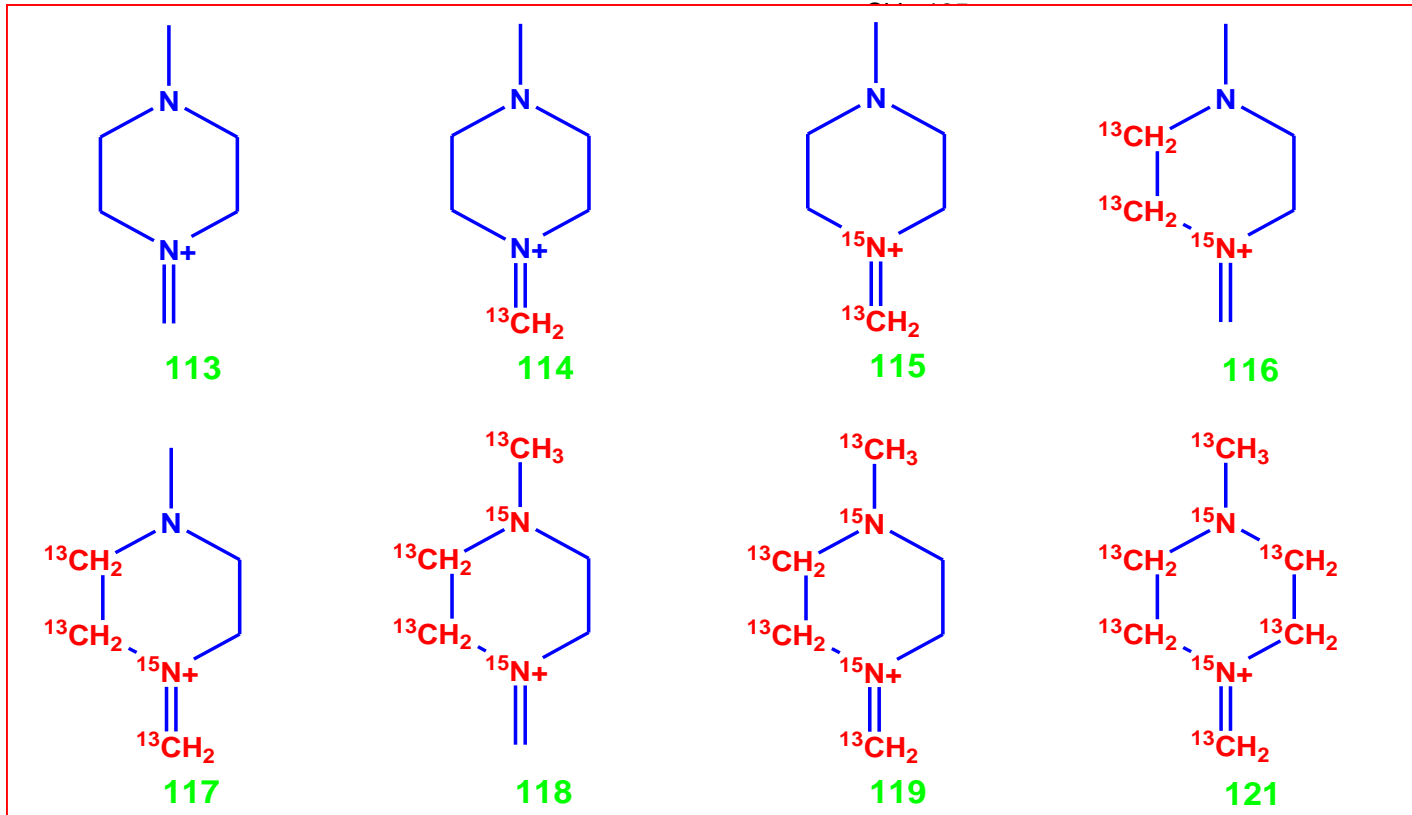
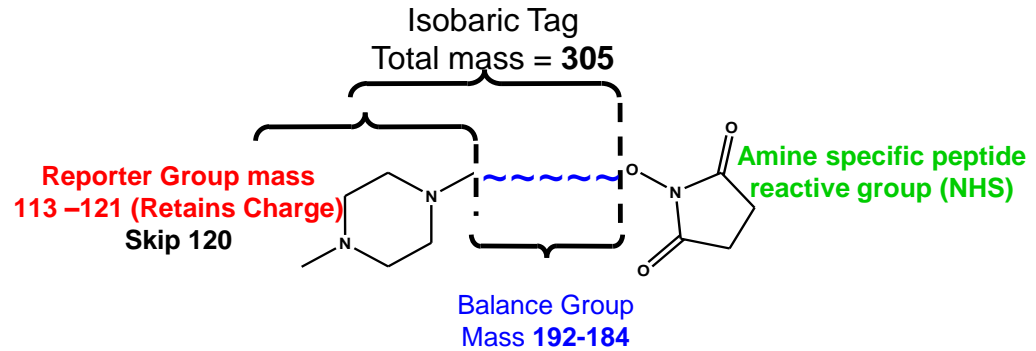
Odečtení sekvence  
peptidu a identifikace  
proteinu

**EPMQTGIK**

**ATP syntáza, H<sup>+</sup> transportující,  
mitochondriální F1 komplex,  
podjednotka alfa**

# iTRAQ – isobaric Tags for Relative and Absolute Quantitation

## 8plex Reagents

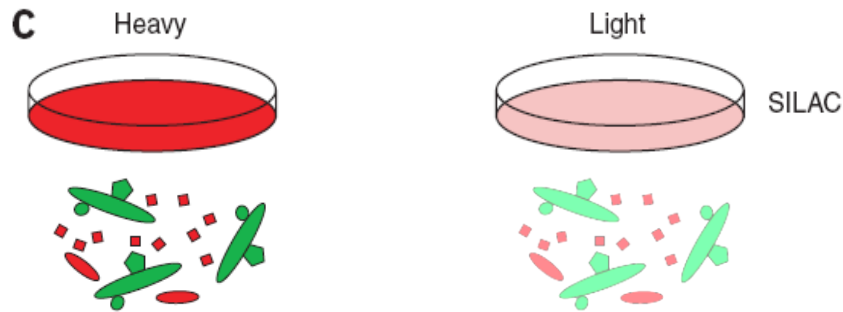


# SILAC – Stable Isotope Labeling with Aminoacids in Culture

**HEAVY**  
1-2 AA značené  
stabilním izotopem

$^{13}\text{C}$ -lysine

$^{13}\text{C}$ -arginine

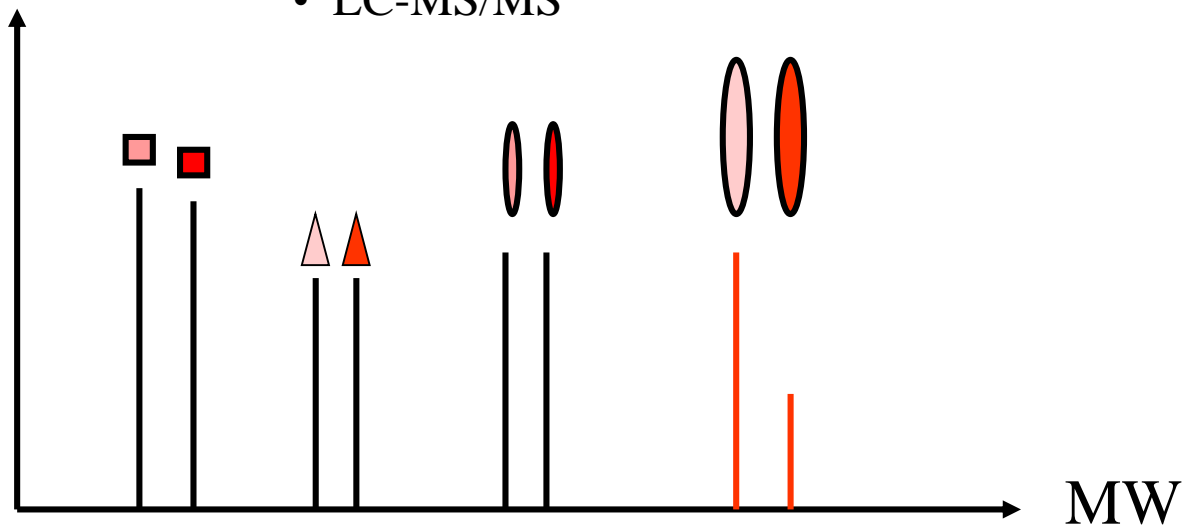


**LIGHT**  
běžné AA

- smíchání
- subcell. frakcionace
- separace
- štěpení trypsinem
- LC-MS/MS

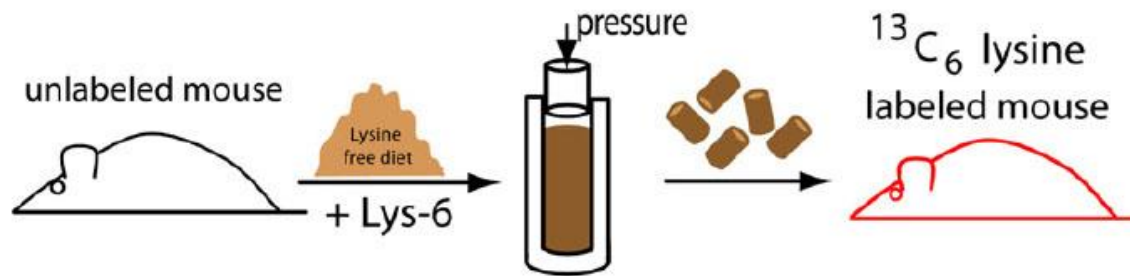
Bezsérové systémy  
nebo dialyzované  
sérum !

Kvantifikace MS  
Identifikace MS/MS





# SILAC MYŠ



B



**F1** 93%  $^{13}\text{C}_6$  lysine

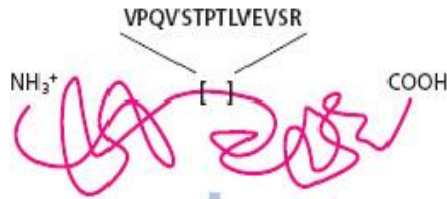
**F2** 97%  $^{13}\text{C}_6$  lysine

**F3-F4** nearly 100 %  $^{13}\text{C}_6$  lysine

# AQUA – Absolute Quantification of Abundance

## AQUA Peptide Selection

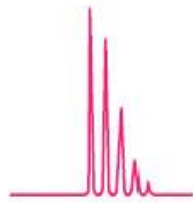
Select an optimal tryptic peptide and stable isotope amino acid from the sequence of your protein of interest



Order synthetic AQUA Peptide from Sigma-Genosys

VPQVSTPTLVEVSR\*

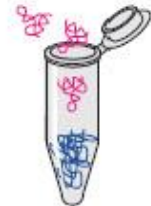
Optimise LC-MS/MS separation protocol for quantitation



Princip: kvantifikace signálu peptidu se signálem identického (ale značeného) peptidu známého množství

## Step 2: Implementation

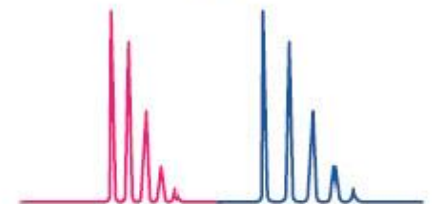
Extract protein from biological samples and add known quantity of AQUA Peptide



Digest



Analyse by LC-MS/MS to quantitate protein of interest



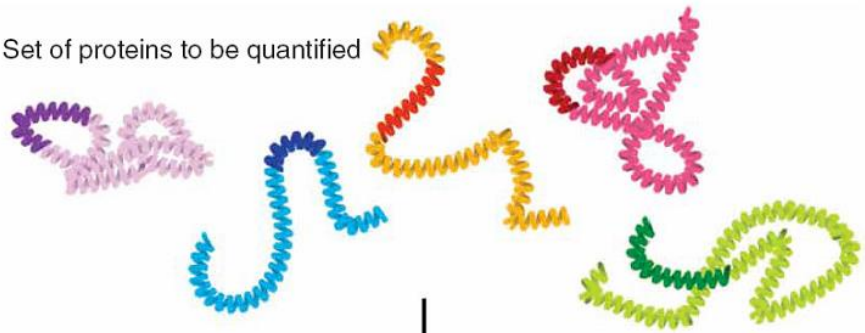
• Použitelné pro kvantifikaci jednoho nebo několika vybraných peptidů..

• Kritický je výběr vhodného peptidu.

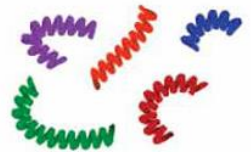
Kvantifikace MS

Identifikace MS/MS

Set of proteins to be quantified



Nominate signature peptides from protein set



Assemble and design gene to encode concatenated signature peptides  
Synthesize gene and insert into expression vector  
Express protein labeled with stable isotopes

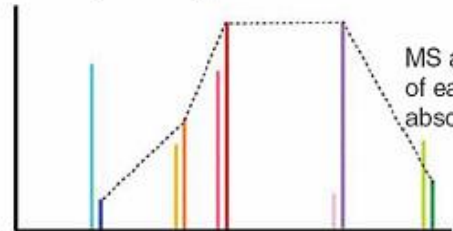


**QconCAT** - absolutní kvantifikace (AQUA) pomocí značených peptidů produkovaných v arteficiálním proteinu složeném z tryptických peptidů z různých bílkovin (konkatemeru) v E.coli



Mix QconCAT and analyte proteins  
Proteolyse to completion

Purify and quantify QconCAT protein



MS analysis of ion intensities of each heavy:light pair yields absolute amount of each protein

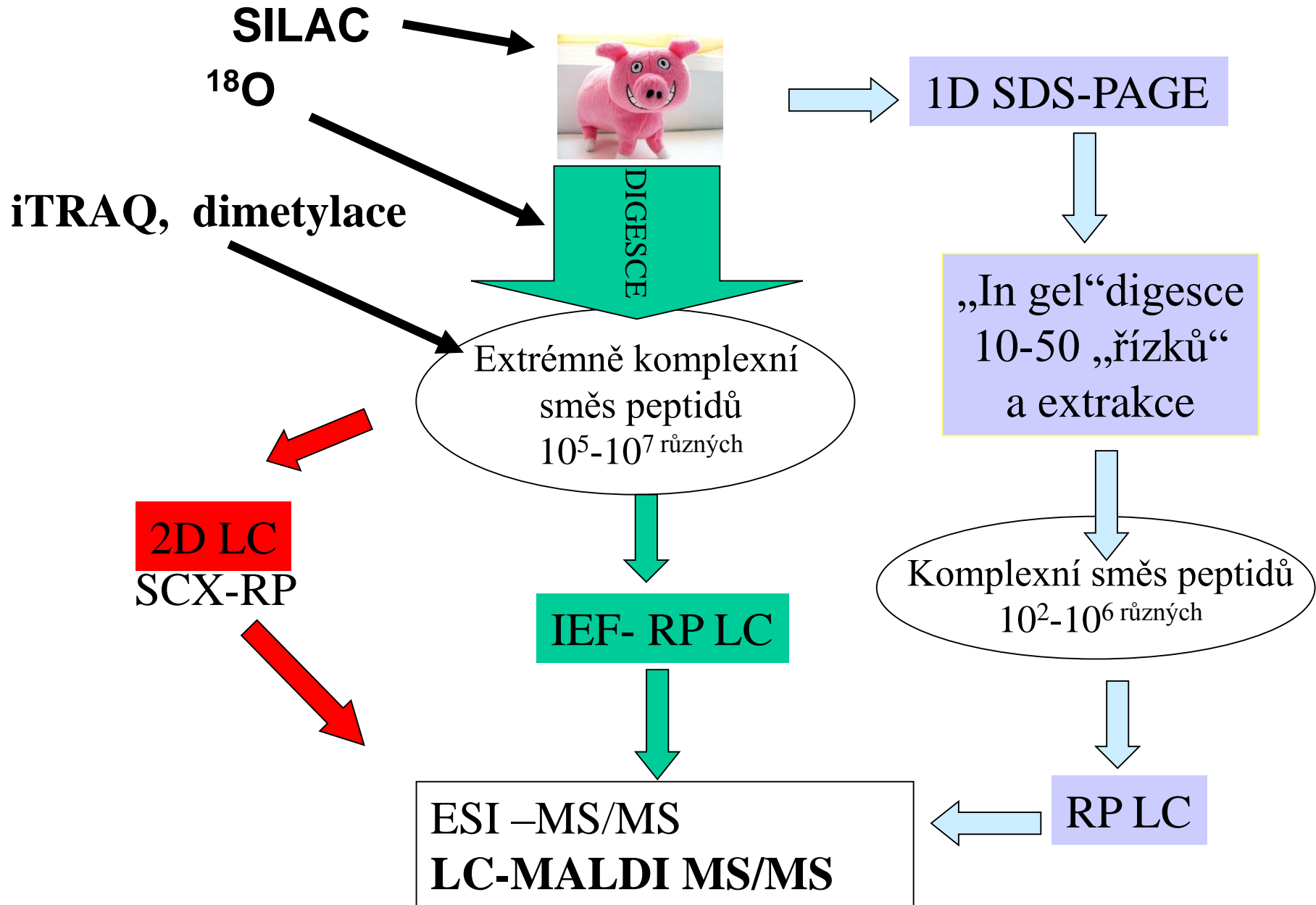
# Kvatifikace pomocí MS

<b>iCAT</b>	<b>značení na úrovni proteinů, jen Cys, kvantifikace-MS snižuje se komplexita</b>
<b>Dimethylace</b>	<b>značí aminoskupiny, značení na úrovni peptidů, kvantifikace MS</b>
<b><sup>18</sup>O digesce</b>	<b>v místě štěpení trypsinem, kvantifikace MS, identifikace při MS/MS</b>
<b>iTRAQ (TMT)</b>	<b>značí aminoskupiny, značení na úrovni peptidů, multiplex, <u>kvantifikace až při MS/MS</u></b>
<b>SILAC</b>	<b>jen živé buňky, metabolické značení, kvantifikace MS</b>

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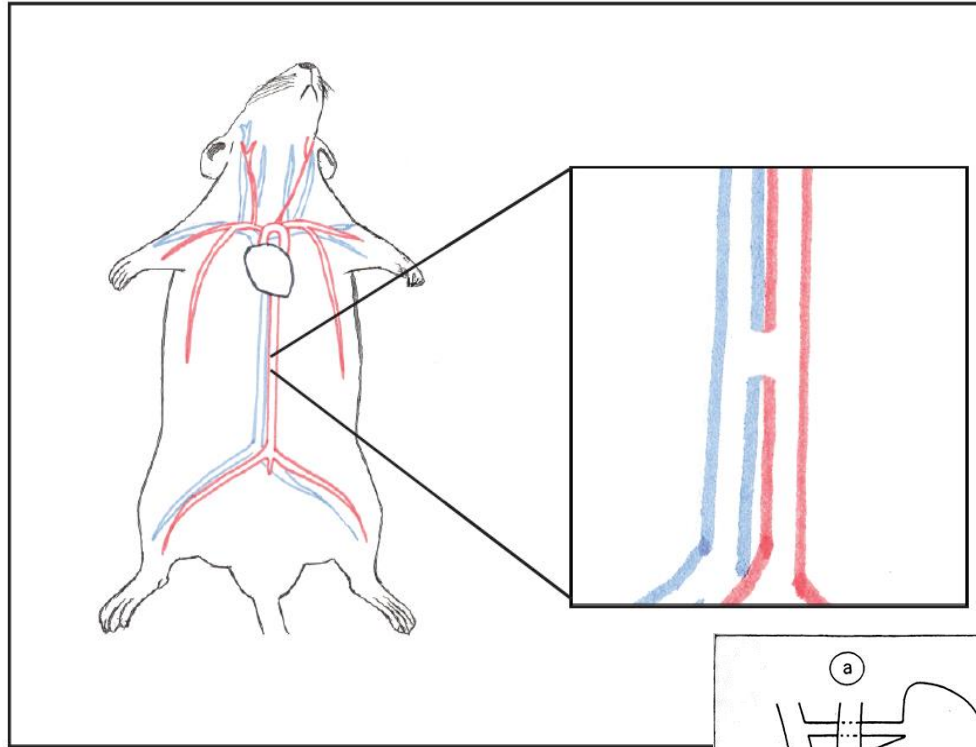
**AQUA**  
**QCONCAT** **absolutní kvantifikace, jen pro předem vybrané  
peptidy, detekce MS**

# SHOT-GUN přístupy v kombinaci se značením

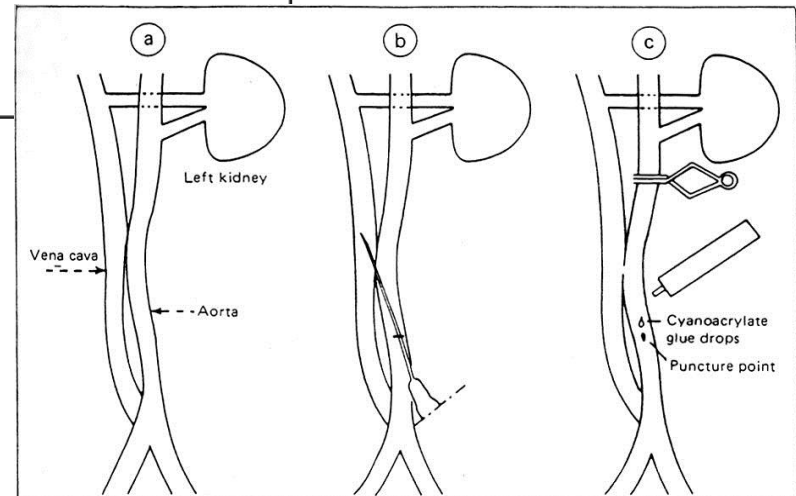


# Rat model of chronic heart failure

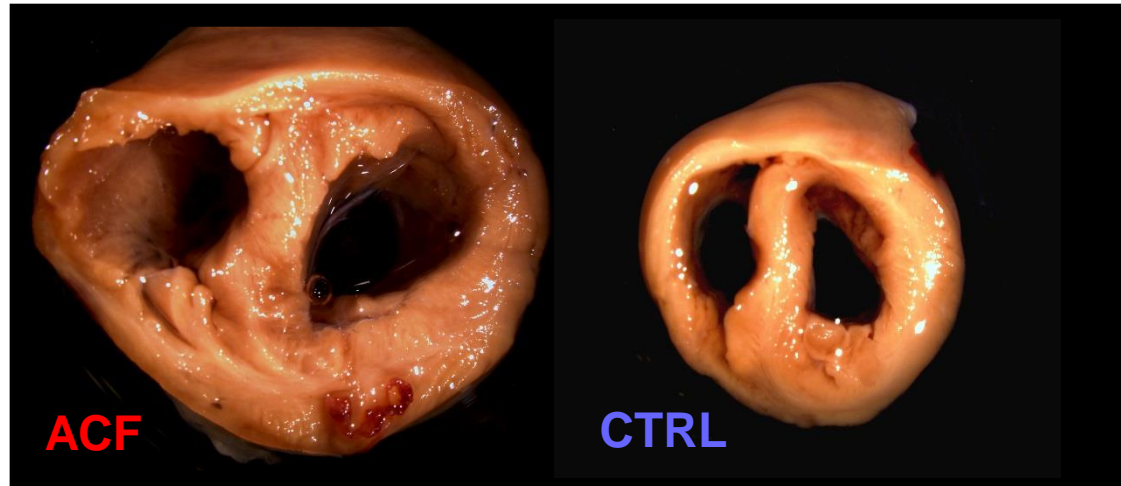
Chronic volume overload induced by **aorto-caval fistula**



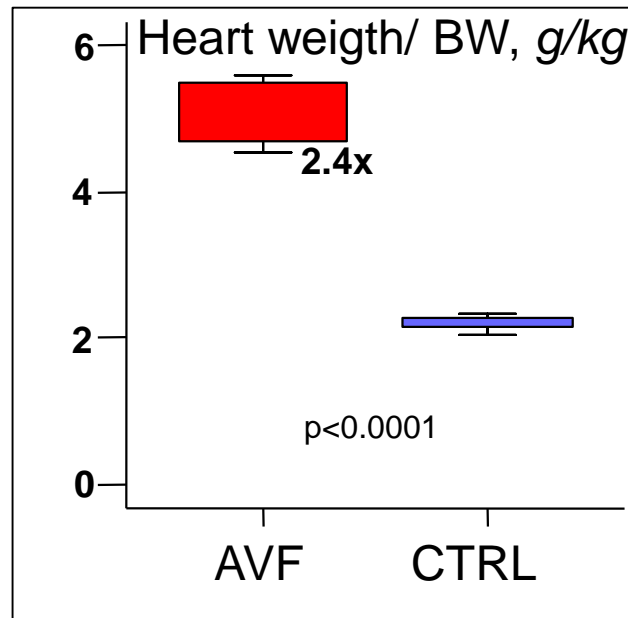
Garcia R, Diebold S: *Cardiovasc Res.* 1990; 24(5):430-2.



# Hart Failure in 24th week after fistula



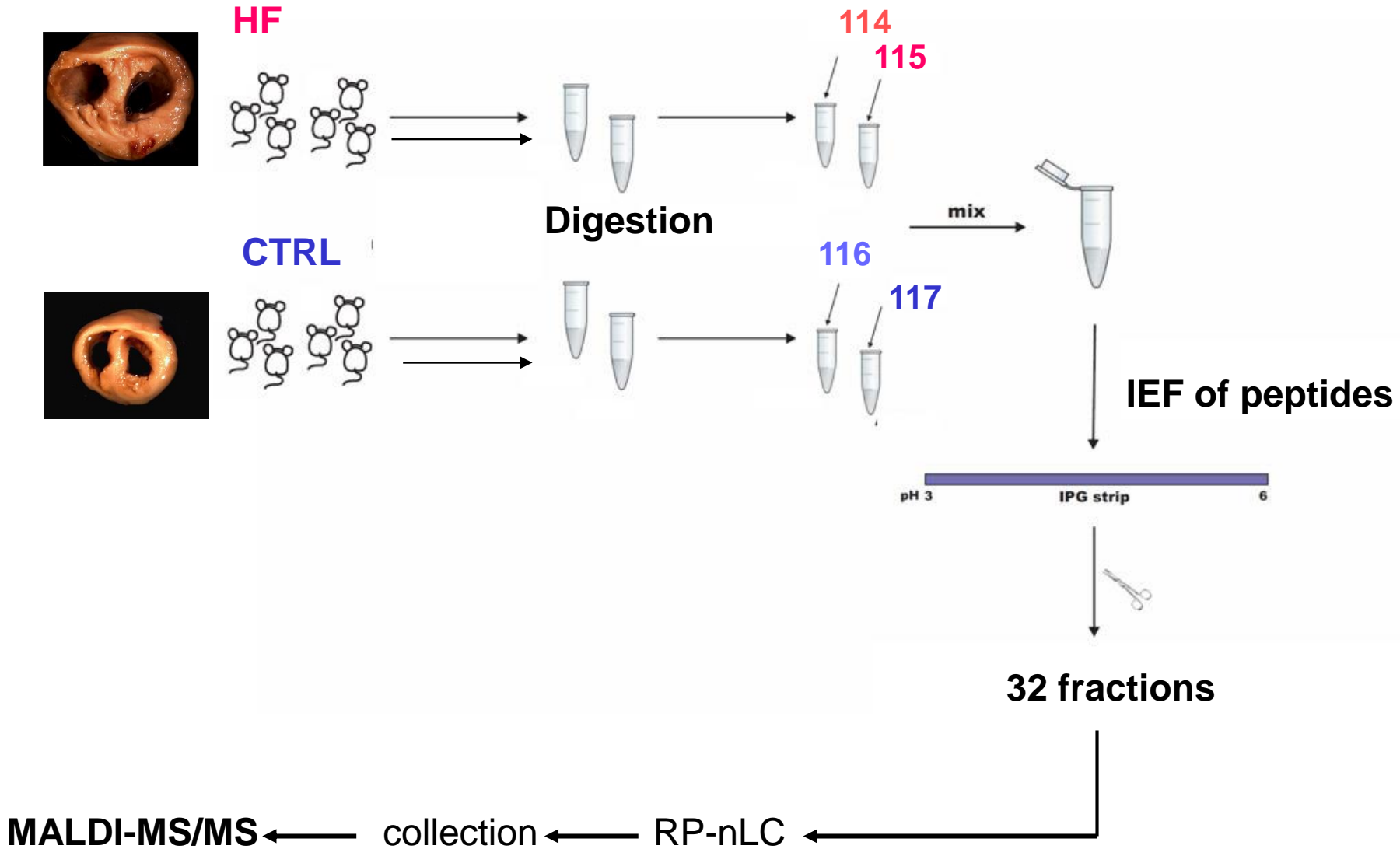
AVF = arterio-venous fistula



## Symptoms:

- Water retention
- Increased LV ED pressure
- Breathlessness
- Lethargy

# IEF-LC-MALDI/MS/MS analysis using iTRAQ chemistry





**DIONEX  
Ultimate® 3000 Proteomics MDLC**



**DIONEX  
Probot Microfraction  
Collector/Spotter**



**Applied Biosystems  
4800 Plus MALDI TOF/TOF™  
Analyzer**



**ProteinPilot™**

**160 nLC runs → ~ 40 000 MALDI spots → ~110 000 MS/MS**



**2030 identified proteins  
+ iTRAQ based quantitation**

**~ 66 differentially expressed proteins**

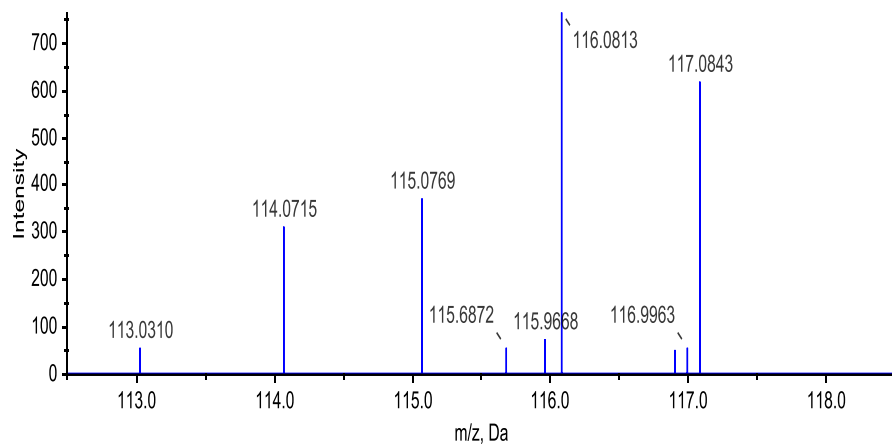
gi|25742739

acyl-CoA synthetase

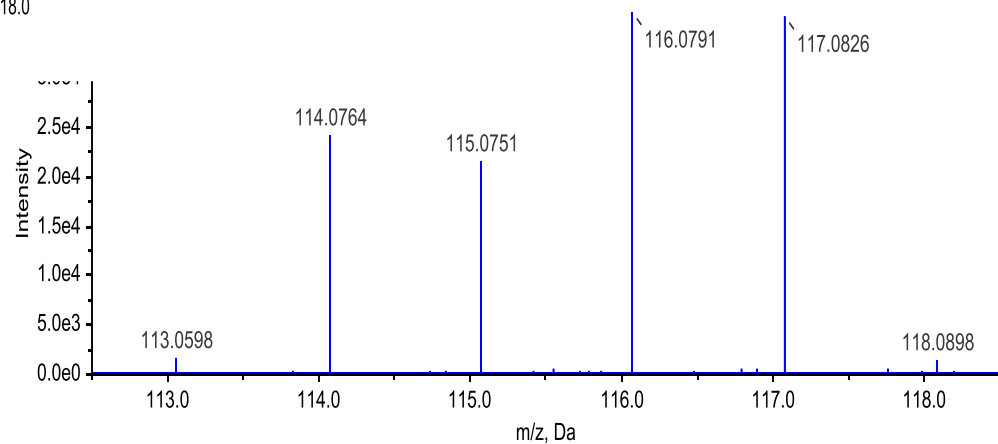
long-chain family member 1

[Rattus norvegicus]

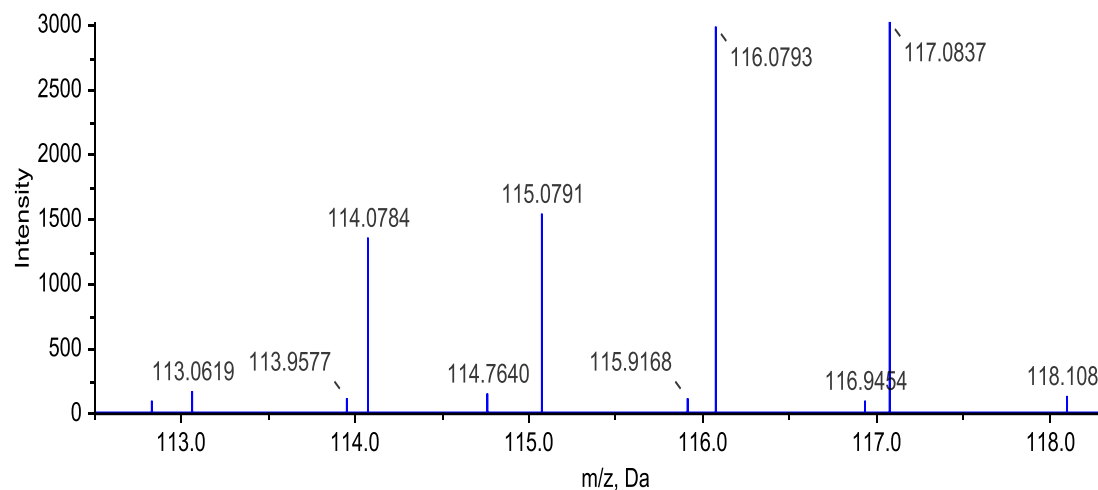
**GFQGSFEELCR**



**LLLYYYDDVR**



**LLLEGVENK**

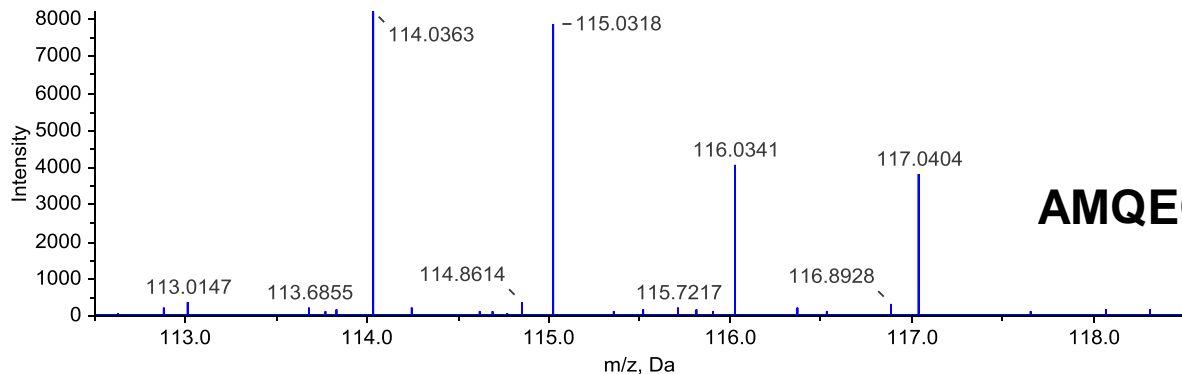
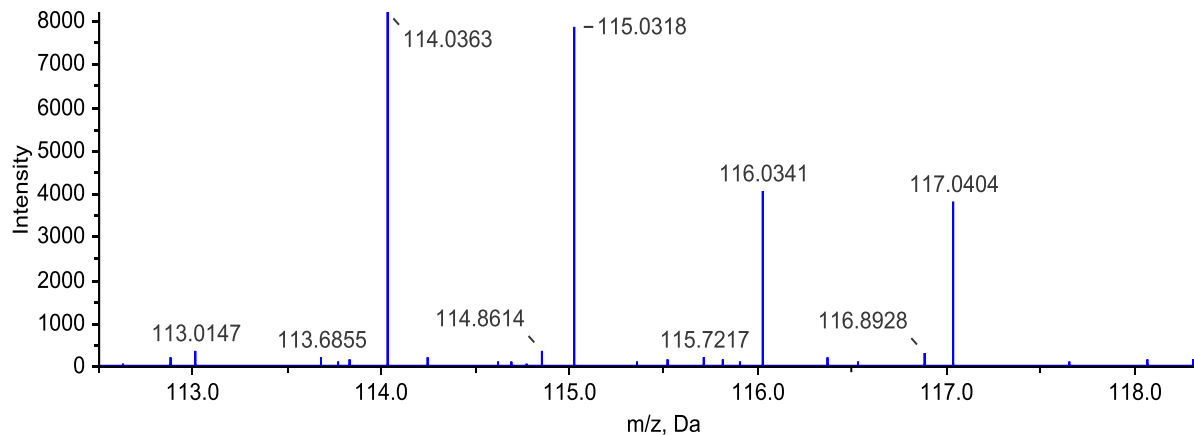


gi|21326447

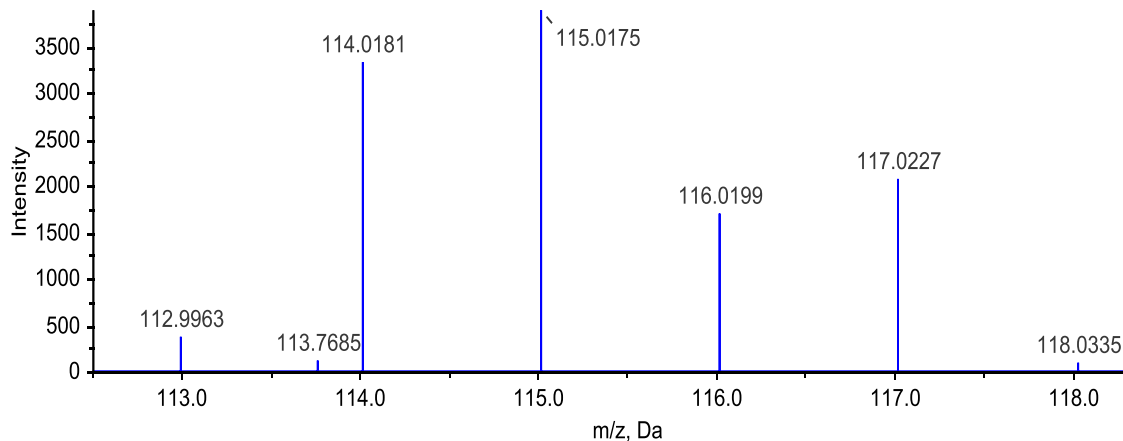
EH-domain containing 4

[*Rattus norvegicus*]

**ADQVDTQQLMR**



**AMQEQLENYDFTK**



**EYQISAGDFPEVK**

# Největší část proteomu (včetně kvantifikace)

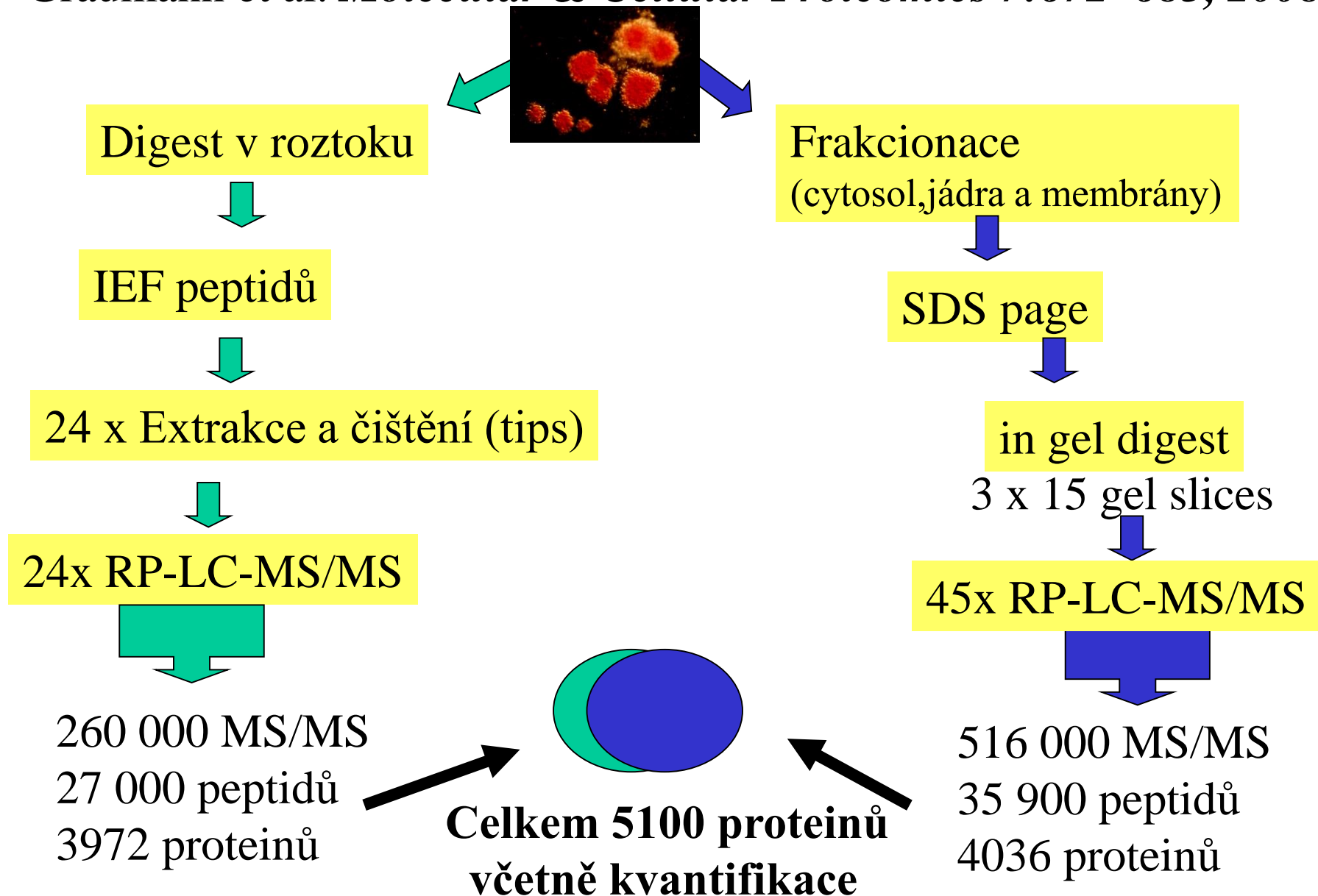
## Stable Isotope Labeling by Amino Acids in Cell Culture (SILAC) and Proteome Quantitation of Mouse Embryonic Stem Cells to a Depth of 5,111 Proteins\*<sup>§</sup>

Johannes Graumann<sup>‡§¶</sup>, Nina C. Hubner<sup>‡§</sup>, Jeong Beom Kim<sup>||\*\*</sup>, Kinarm Ko<sup>||</sup>,  
Markus Moser<sup>‡‡</sup>, Chanchal Kumar<sup>‡</sup>, Jürgen Cox<sup>‡</sup>, Hans Schöler<sup>||</sup>, and  
Matthias Mann<sup>‡§§</sup>

*Molecular & Cellular Proteomics* 7:672–683, 2008.

# Mouse Embryonic Stem Cells (SILAC)

Graumann et al. *Molecular & Cellular Proteomics* 7:672–683, 2008.



Boisvert FM, et al. A quantitative spatial proteomics analysis of proteome turnover in human cells. Mol Cell Proteomics. 2012 Mar;11(3):M111.011429.

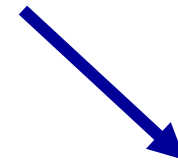
HeLa Cells/SILAC



SDS-PAGE (16 frakcí)



RP-LC



80 000 peptidů



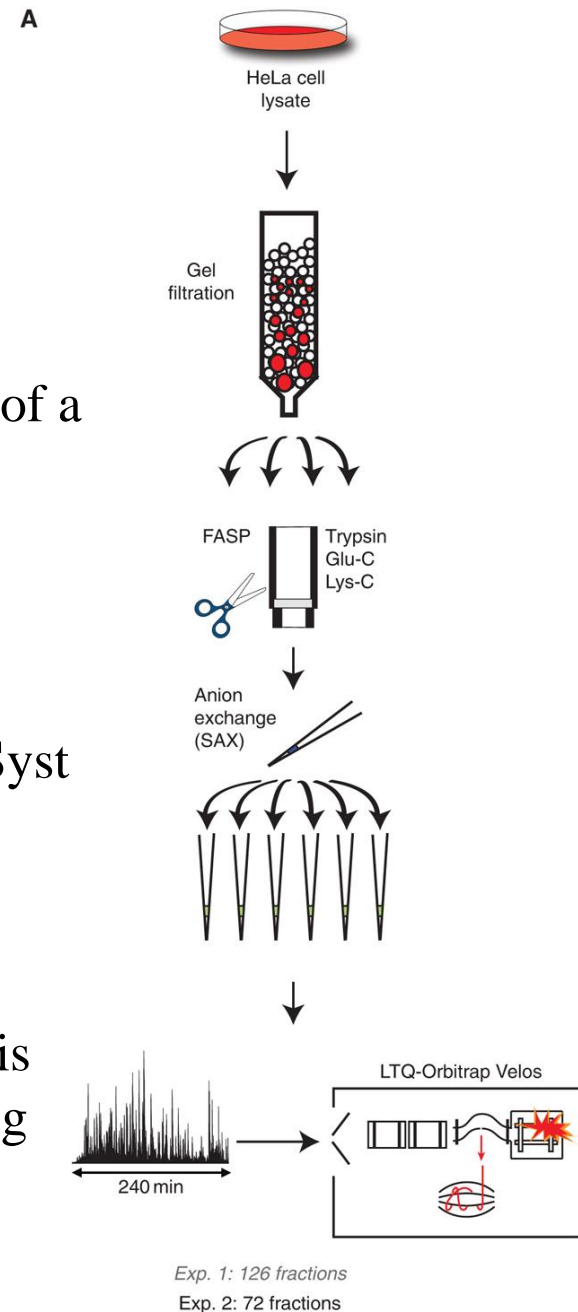
**8041 bílkovin**

>10 000 proteinů  
v jednom experimentu

Beck M, et al. (Aebersold R.) The quantitative proteome of a human cell line. *Mol Syst Biol.* 2011 Nov 8 ,7:549  
(peptide IEF-LC)

Nagaraj N, et al., (Mann M.) Deep proteome and transcriptome mapping of a human cancer cell line. *Mol Syst Biol.* 2011 Nov 8;7:548.  
(SAX-LC)

Geiger T, et al. (Mann M.) Comparative proteomic analysis of eleven common cell lines reveals ubiquitous but varying expression of most proteins. *Mol Cell Proteomics.* 2012 Mar;11(3):M111.014050.  
(SAX-LC)



B Experiment Proteins Peptides MS measurement time

# shot-gun metody

## (pros and cons)

- **až 10000 proteinů v jednom experimentu**
- izotopická nebo label free kvantifikace
- náročnost na instrumentaci a (bio)informatiku
- problém s inferencí proteinu (stejně peptidy v různých proteinech)
- analýza PTM je možná
- **ztráta informace o proteoformách**