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CG990 Methods in Proteomics

Sample preparation for bottom-up proteomics

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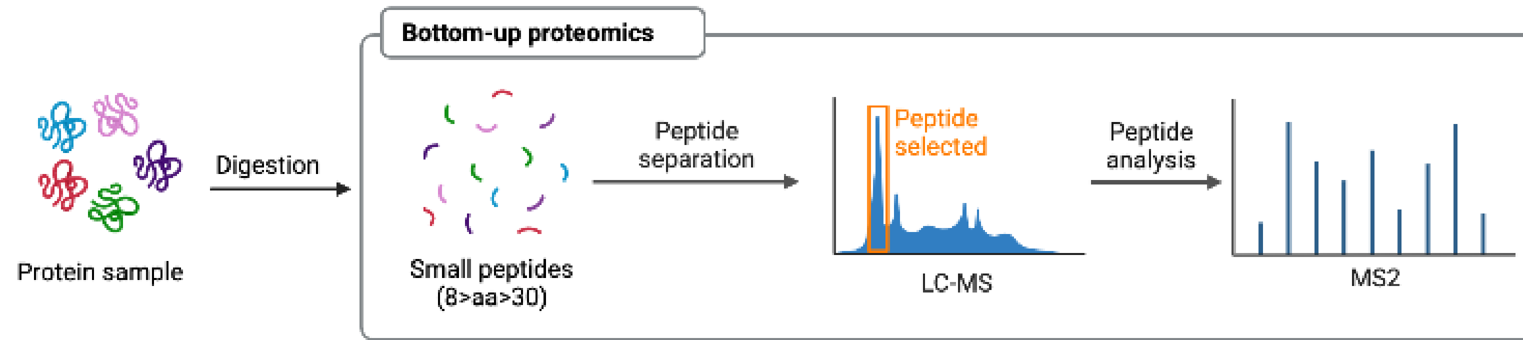
Study materials

Bottom-up proteomics



- Peptides of suitable size for analysis by commonly available MS instrumentation
- Available open-source and commercially developed software tools
- Possible to determine:
  - peptide and inferred protein identity
  - sites of post-translational modifications
  - relative abundances of peptides among samples
- Identification and quantification of thousands of proteins from a single sample (without prior knowledge of the sample composition or reliance on antibodies)

Study materials

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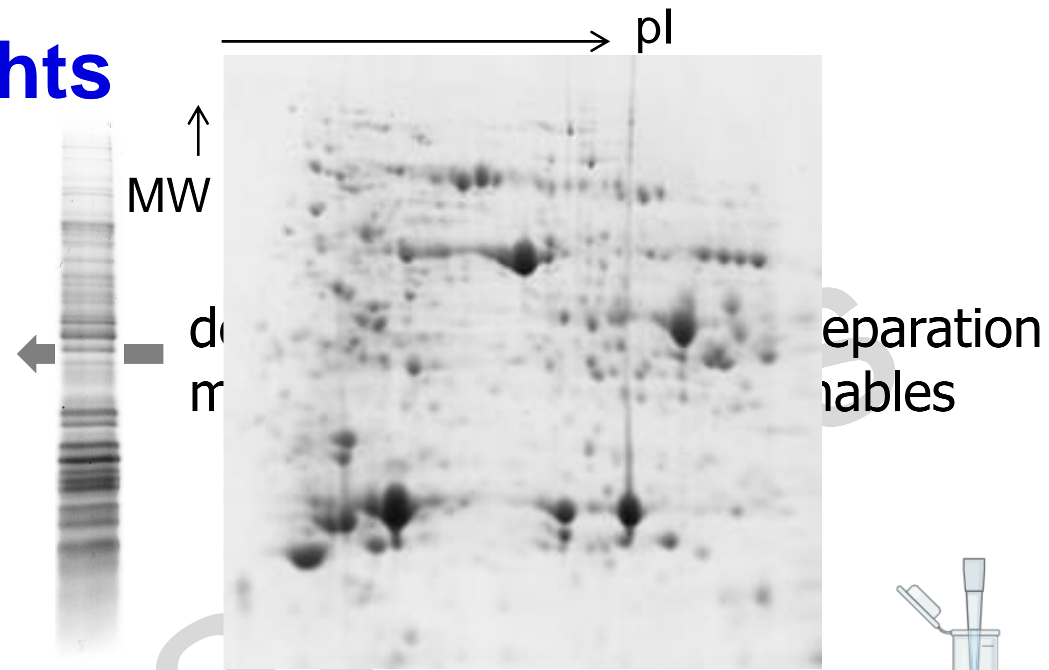
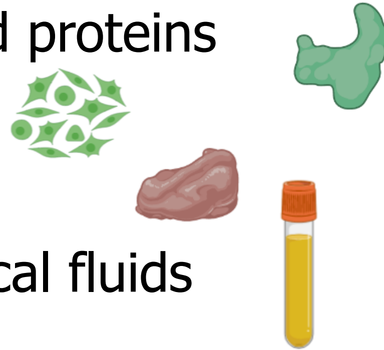
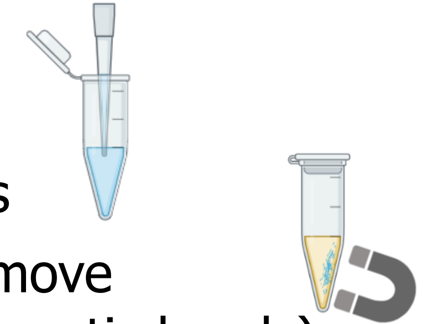
Bottom-up proteomics: Highlights



- Starting material: different types of samples
  - ➢ purified proteins
  - ➢ cells
  - ➢ tissues
  - ➢ biological fluids



- Trends in methodologies:
  - ➢ Gel-free approaches - time-saving, ease, minimized sample loss
  - ➢ Precipitation-free approaches – alternative methods used to remove detergents and other contaminants (suspension trapping, paramagnetic beads)



traditional peptide cleanup methods using reverse-phase chromatography could be used for desalting but not detergent or polymer removal

x

new approaches that use coated magnetic particles enable removal of detergents, polymers, and salts

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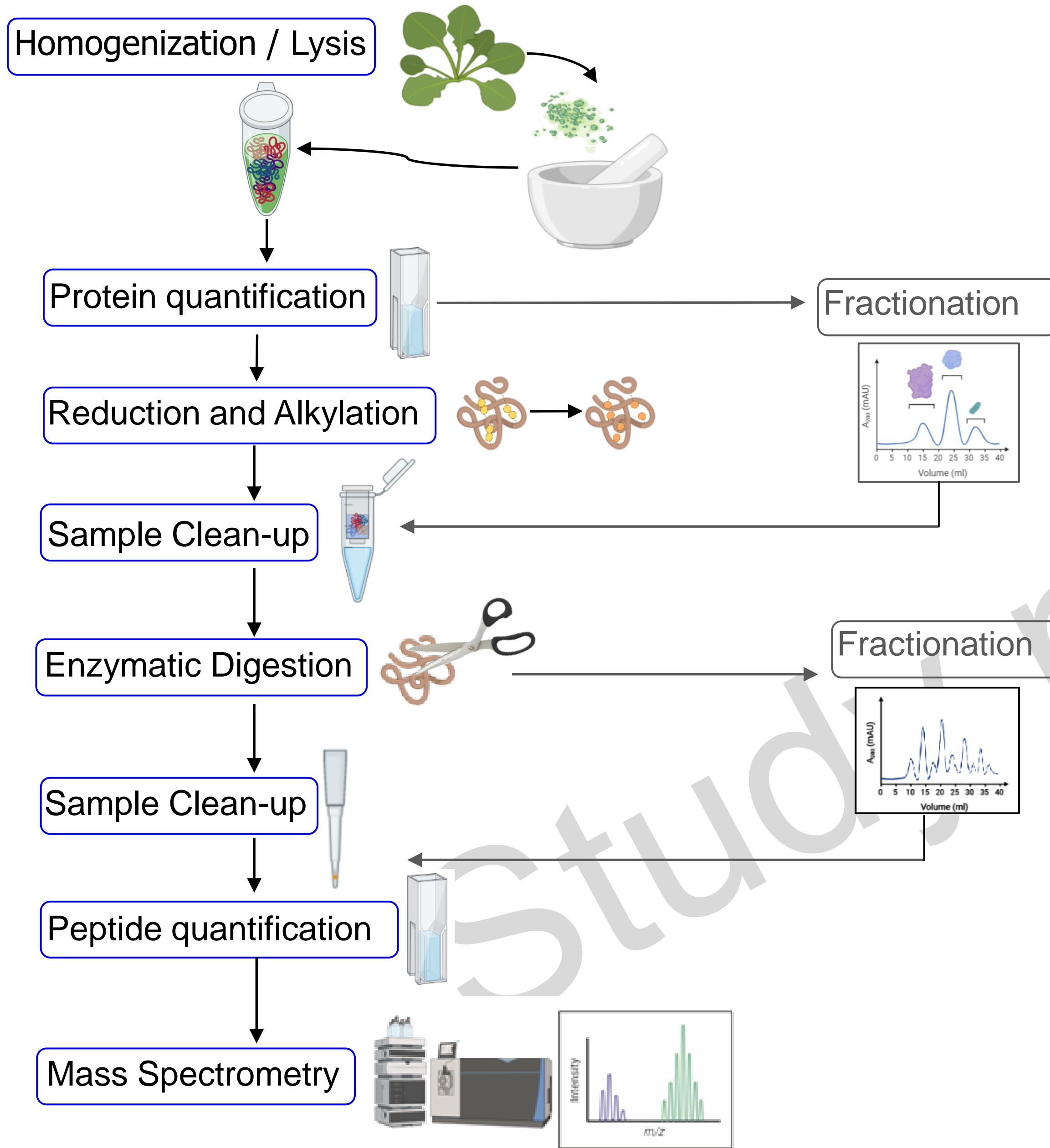
Bottom-up proteomics – Basic pipeline



- Series of steps to digest the protein into peptides through enzymatic digestion, followed by removal of contaminants before analysis by MS.
- The choice of bottom-up workflow depends on the **sample complexity** and the **goals of the experiment**.

Sample complexity – the number of proteins
– dynamic range of protein concentration

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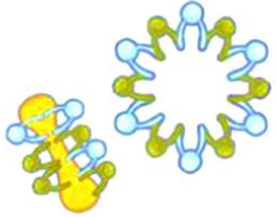
Homogenization / Lysis

- Reagent-based methods



- rapid, gentle, efficient, and reproducible
- extraction of total protein or subcellular fractions

- components non-compatible with MS need to be removed
- suitable for cultured cells but may not be effective for some tissues

- Physical disruption

- lysis of a wide range of cells
- high lysing efficiency

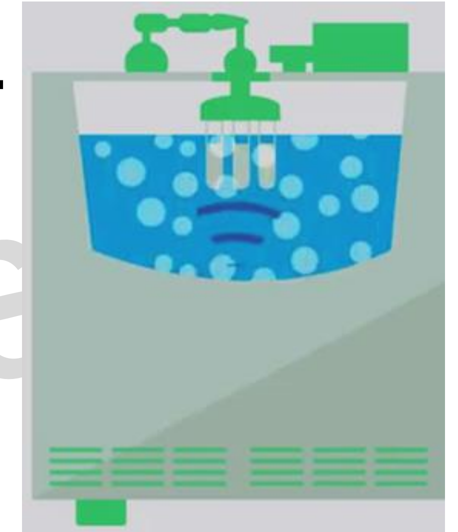
- requires equipment
- limited reproducibility
- protein denaturation and aggregation can occur due to localized heating
- cells disrupt at different times, so subcellular components may be subjected to ongoing disruptive forces

Study materials

Homogenization / Lysis

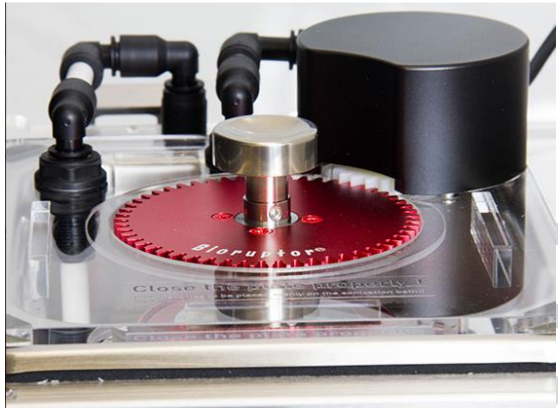
Bioruptor® Pico

Adaptive cavitation Technology **ACT**



Ultrasonic cavitation

- Quick changes of the pressure in a liquid sample during sonication
- Formation of bubbles at local pressure decrease
- Implosion of bubbles at critical size



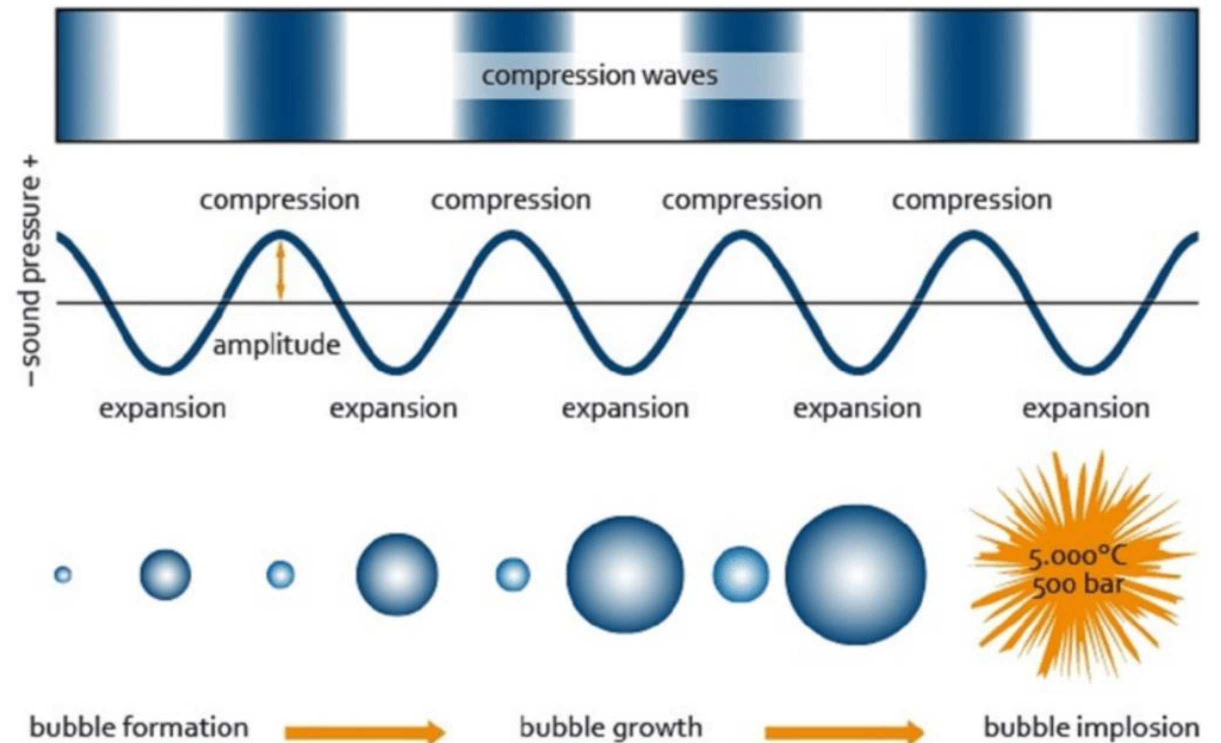
5 microliters to 2 mililiters of sample (based on adaptor type)

Adaptor for 0.2ml tubes for Bioruptor® Pico holder

Adaptor for 0.65ml tubes for Bioruptor® Pico holder

Adaptor for 1.5ml tubes for Bioruptor® Pico holder

15 ml sonication accessories



ry materials

Bottom-up proteomics – Protein / Peptide quantification

- Protein quantification
  – calculating how much enzyme (or chemical) is required for protein digestion
- Peptide quantification
  – control of the yield of the protein cleavage process
  – for determining how much peptide sample should be injected for LC-MS/MS
  – essential in quantitative workflows in which equivalent amounts of total peptide are compared to reveal differences in relative abundance of individual peptides.

- The colorimetric assays often interfere with substances used for tissue lysis such as detergents or disulfide-bond-reducing agents

  e.g., Bradford assay - not compatible with SDS; BCA assay - not compatible with DTT, β-ME, EDTA.

- UV absorbance methods

  – Proteins contain three different aromatic amino acids carrying benzene, phenol, and indole rings, respectively. Each of these groups can be excited by UV light to fluoresce.

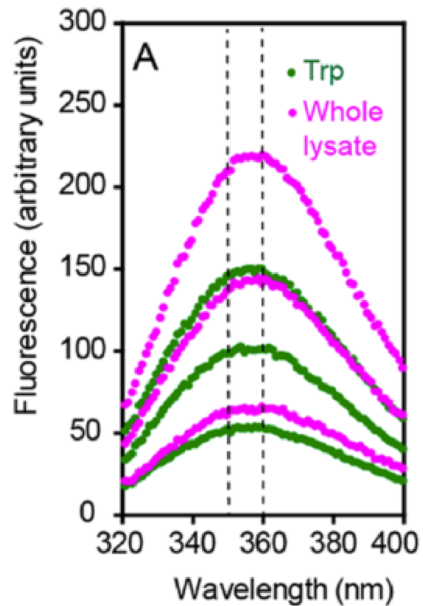
Tryptophan Fluorescence

– highly sensitive to its microenvironment with regard to proteins and to the polarity of the solvent.
– quenched by several amino acids as well as many substances contained in buffers such as detergents
– temperature and pH are influencing the intensity of Trp fluorescence

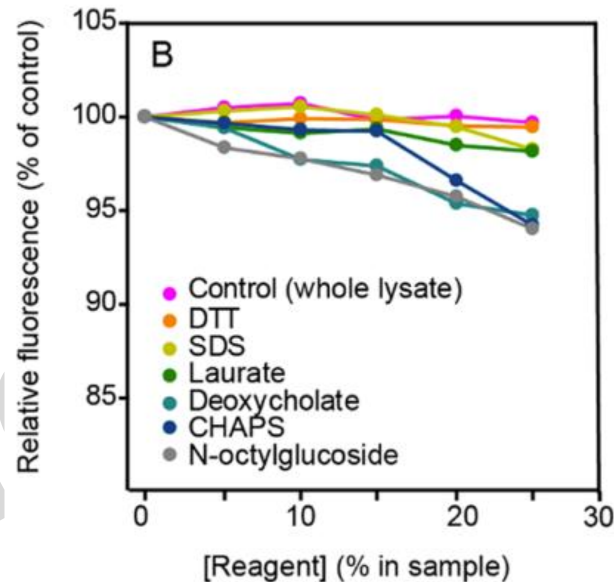
Study materials

Tryptophan Fluorescence (WF) assay

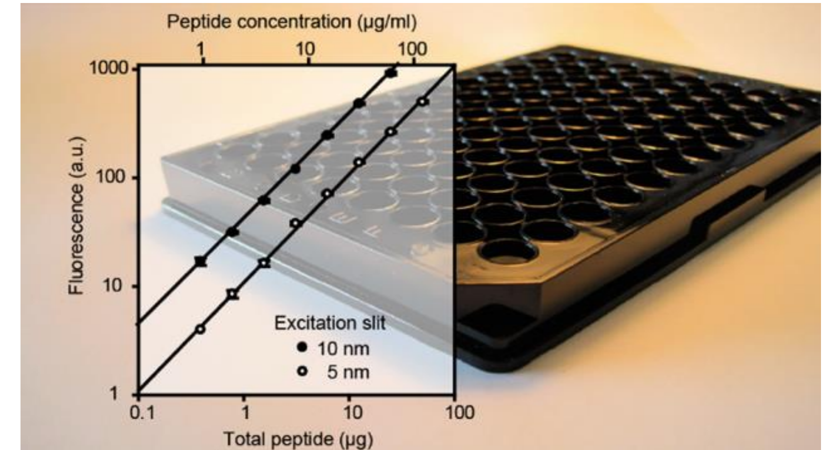
- most detergents quench fluorescence at the concentration used for tissue lysis
- Trp quantification – low interaction of detergents with the proteins in a buffer containing 8 M urea, Trp indole moieties freely exposed to the solvent; Em_{max} = 350 nm
- suitable for high-complex samples but not for protein/peptide fractions
- working concentration of 0.05 - 25.0 μg/mL



Emission spectra of whole cell lysates (6, 12, and 18 μg of total protein) and pure tryptophan (0.05, 0.1, and 0.15 μg) in 2 mL of 8 M urea and 10 mM Tris-HCl, pH 7.8.



Quenching effect of detergents and DTT. Reagent concentration refers to concentration in 2 μL of solution added to 2 mL of 8 M urea.



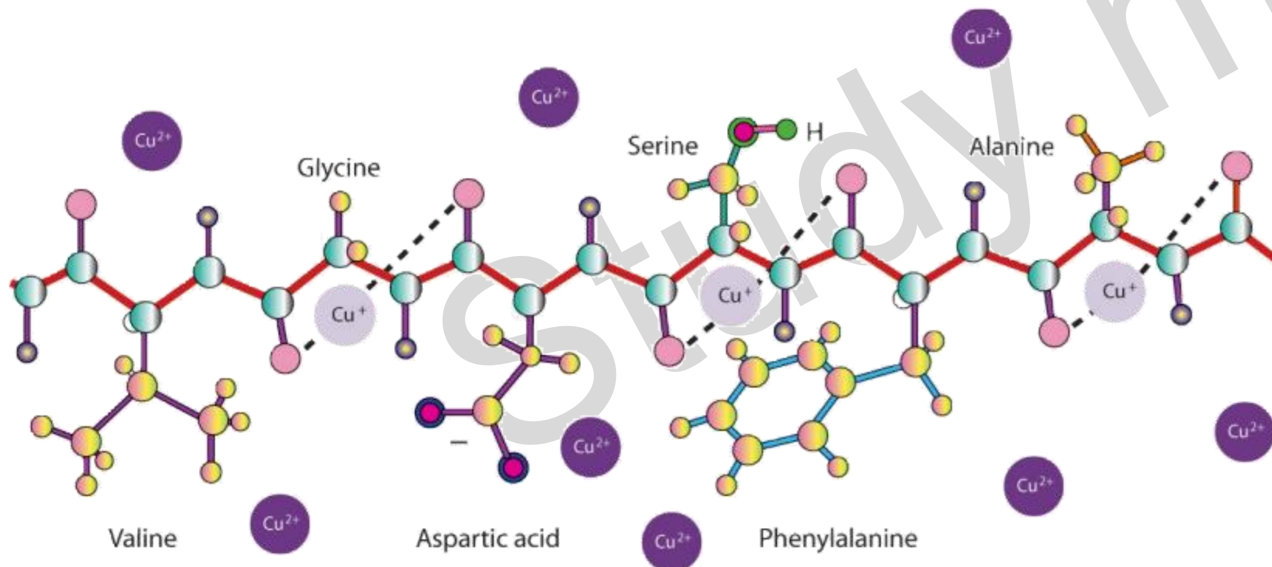
Wisniewski et al., 2015, 87(8):4110-6

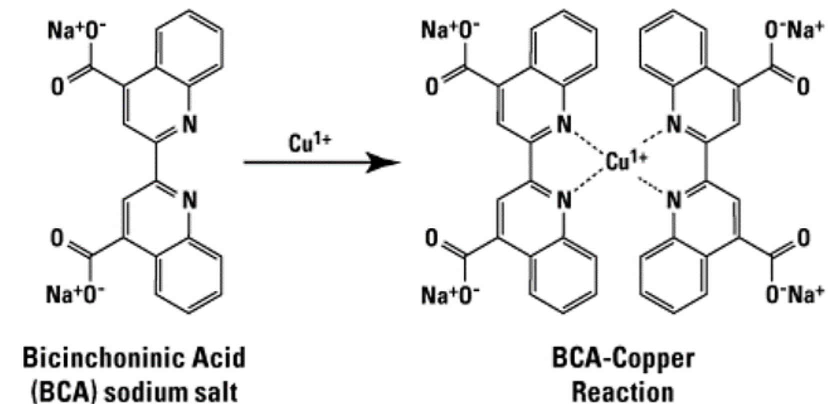
Study materials

MicroBCA assay

- colorimetric detection and quantitation of total protein / peptides
- more suitable for low-complex protein / peptide samples compared to Trp assay
- working concentration of 0.5-20.0 μg/mL
- detergent-compatible
- not compatible with reductants and chelatants (DTT, β-ME, EDTA…)
- the protein solution is mixed with an alkaline solution of cupric ions Cu2+ which chelate with the peptide bonds resulting in cuprous ions (Cu+). Purple-colored reaction product formed by the chelation of two molecules of BCA with one Cu^{I+} ion exhibits absorbance at 562nm

Study materials

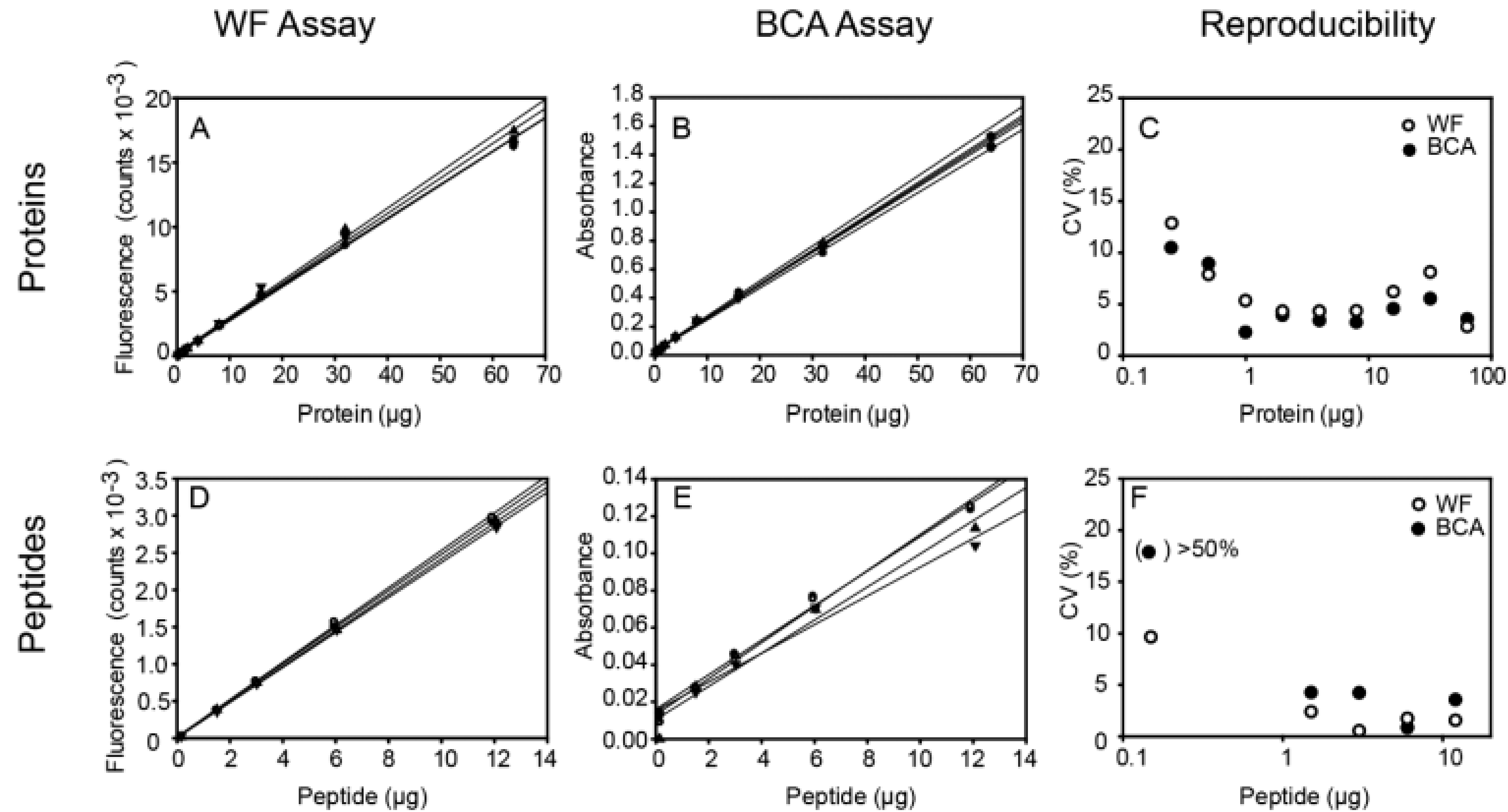




Wisniewski et al., 2015, 87(8):4110-6

WF vs. MicroBCA assay

- both methods have similar sensitivity and reproducibility for protein determination in tissue lysate
- WF assay appears to be more reproducible than the dye-based assay in the determination of peptide contents in protein digests



Wisniewski et al., 2015, 87(8):4110-6

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Reduction and Alkylation of proteins

Denaturation
Reduction

Alkylation

Protein with disulfide bridges

Reduction cleaves disulfide bridges and allows unfolding

- Disulfide bonds of the proteins are irreversibly broken up and the optimal unfolding of the tertiary structure is obtained.
- This chemical modification allows for proteins with a high number of disulfide bonds the successful identification as well as the highest peptide yield and sequence coverage.
- Incomplete reduction and/or alkylation will impair the qualitative and quantitative results.
- Undesired "over-alkylation" = the alkylation of non-thiol moieties:
  N-terminal amino acid>aspartic acid>glutamic acid>histidine>asparagine>lysine>tyrosine

Reduction

DTT

BME

TCEP

Alkylation

IAA

IAC

AA

CAA

DTT– Dithiothreitol
TCEP – tris-(2-carboxyethyl)-phosphine
BME – β-mercaptoethanol

IAA – iodoacetamide
IAC – iodoacetic acid
AA – acrylamide
AA – chloroacetamide

R

disulfide bond in protein

dithiothreitol (DTT)

1. thiol deprotonates

2. nucleophilic attack on S in protein

3. nucleophilic attack on other S in DTT

A

Protein

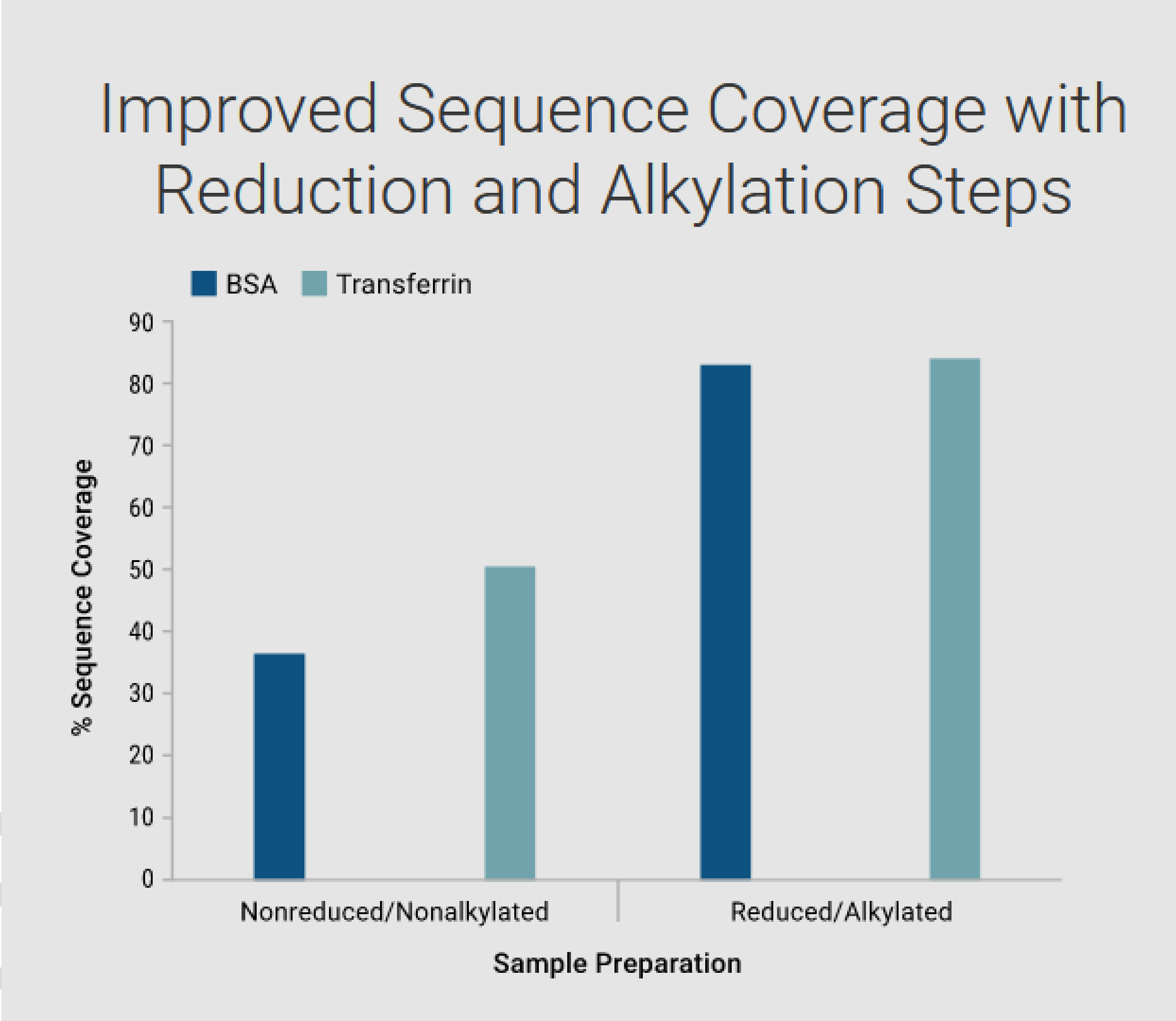
Cysteine

Iodoacetamide (IDAM)

Protein

S-Carbamidomethyl cysteine

Reduction and Alkylation of proteins



Promega datasheet

Enzymatic digestion of proteins

TPCK Trypsin: cleaves the carboxyl side of K and R; autolysis blocked
SOLu-Trypsin: delivered in solution, stable at 4°C for one month
SOLu-Trypsin Dimethylated: delivered in solution, stable at 4°C for one month; autolysis blocked
Rapid Digestion Trypsin: digestion for 1 h at 70°C
Platinum Trypsin: without non-specific proteolytic activity; autolysis-resistent

LysC: cleaves the carboxyl side of K

Glu-C (V-8 Protease)**:** cleaves the carboxyl side of E (in ammonium bicarbonate and ammonium acetate buffers)
cleavage can also occur at both E and D (in phosphate buffers)

Asp-N: cleaves at amino side of D and cysteic acid residues (that result from the oxidization of C)

Chymotrypsin: cleaves at the carboxyl side of aromatic acids - Y, F, W and L.

Thermolysin: cleaves at the N-term of L, F, V, I, A, M at 65–85°C

ProAlanase: cleaves the carboxyl side of P and A

Study materials

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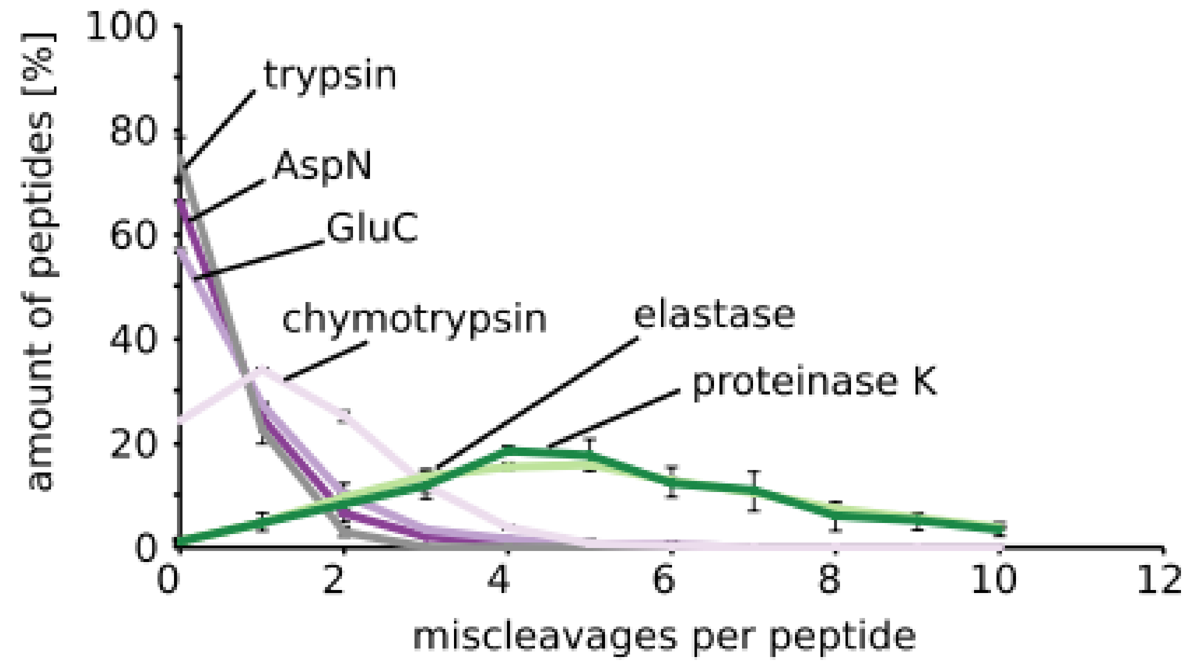
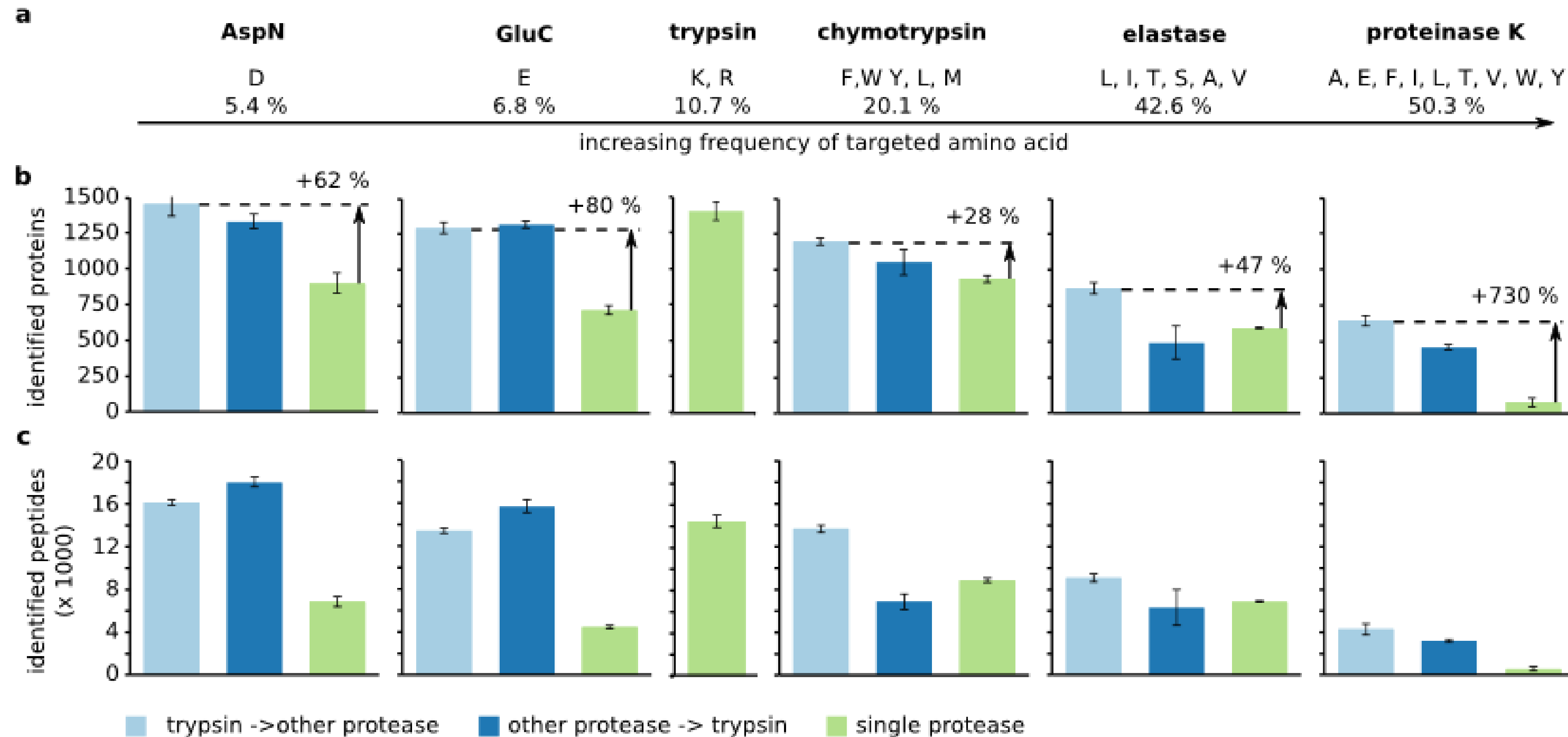
Enzymatic digestion of proteins – alternative proteases



Anal. Chem. 2020, 92, 9523−9527

Study Materials

Sequential digestion



Anal. Chem. 2020, 92, 9523−9527

– trypsin - the protease of first choice in proteomics
– specific studies can benefit from adding a sequential digestion step with trypsin
– specific studies can benefit from usage of alternative protease

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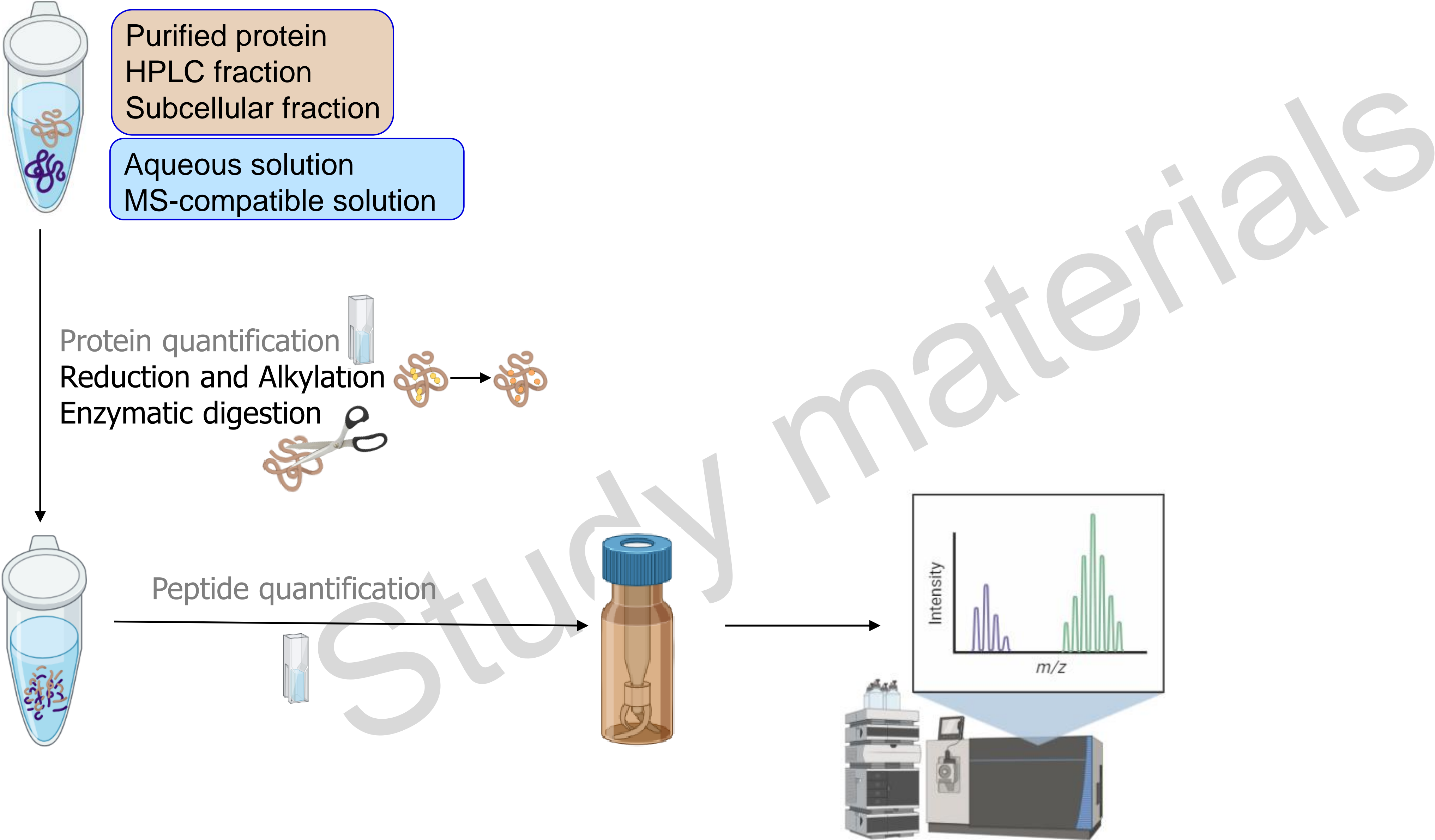
Bottom-up proteomics – Pipeline for low-complex samples



Study materials

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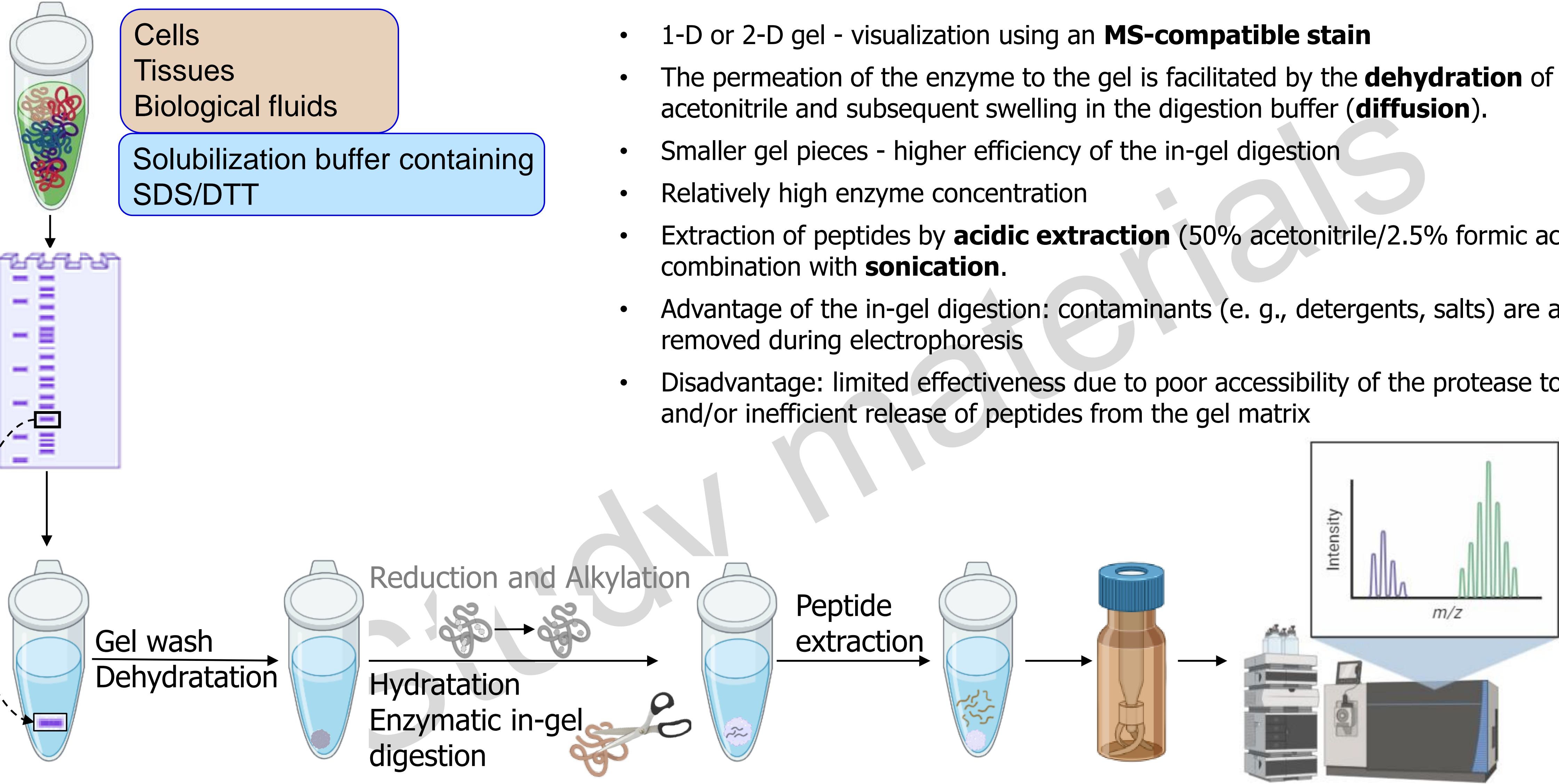
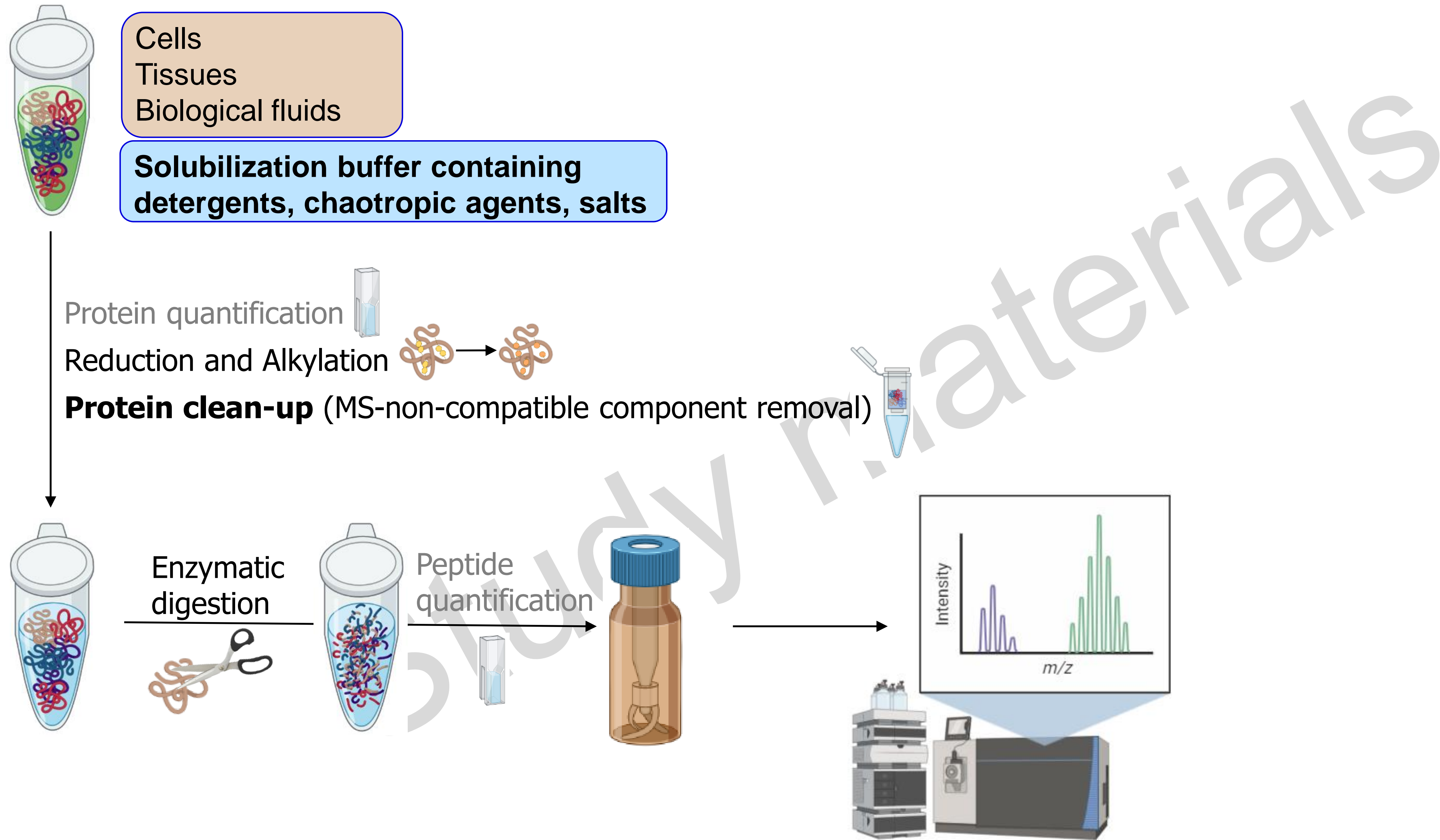
Bottom-up proteomics – Pipeline for in-gel digestion

- 1-D or 2-D gel - visualization using an **MS-compatible stain**
- The permeation of the enzyme to the gel is facilitated by the **dehydration** of the gel with acetonitrile and subsequent swelling in the digestion buffer (**diffusion**).
- Smaller gel pieces - higher efficiency of the in-gel digestion
- Relatively high enzyme concentration
- Extraction of peptides by **acidic extraction** (50% acetonitrile/2.5% formic acid) in combination with **sonication**.
- Advantage of the in-gel digestion: contaminants (e. g., detergents, salts) are already removed during electrophoresis
- Disadvantage: limited effectiveness due to poor accessibility of the protease to proteins and/or inefficient release of peptides from the gel matrix



Study materials

Bottom-up proteomics – Pipeline for high-complex samples



study materials

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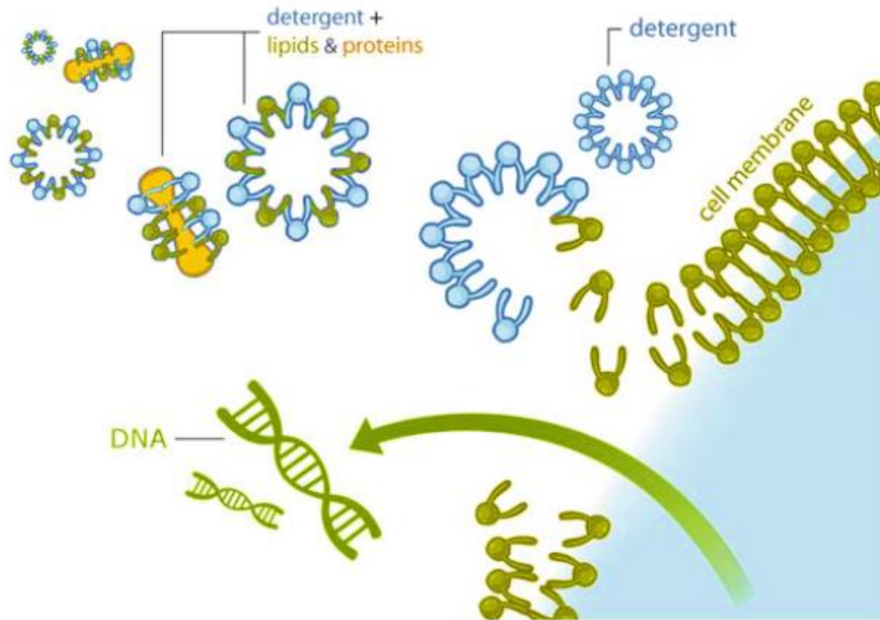
Solubilization of high complex samples and Protein clean-up

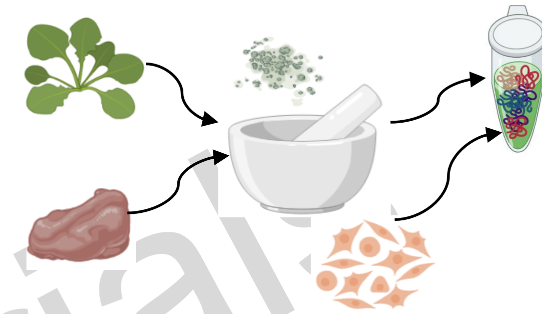
Homogenization in SDT buffer

4% SDS, 0.1M DTT, 0.1M Tris-HCl pH 7.6



Critical Micelle Concentration (CMC)
c < CMC - detergents occur as monomers
c > CMC - detergent molecules organize in micelles which drive solubilization.
Due to their sizes, the SDS and SDS mixed micelles cannot be separated from solubilized proteins by ultra-filtration. In FASP, concentrated urea enables contraction or dissociation of micelles.

Homogenization / Lysis



- Protein powder / Collected cells transfered to the vial with **hot SDT** buffer
- Homogenization supported in Bioruptor, **DNA fragmentation**.
- Complete protein solubilization ensured by incubation at **95°C, 2h**.
- Clean-up and enzymatic digestion:
  Filter-Aided Sample Preparation (**FASP**)
  Single-Pot Solid-Phase-enhanced Sample Preparation (**SP3**)
  Suspension Trapping (**S-Trap**)
- Additional Clean-up:
  Ethylacetate extraction (**EE**)

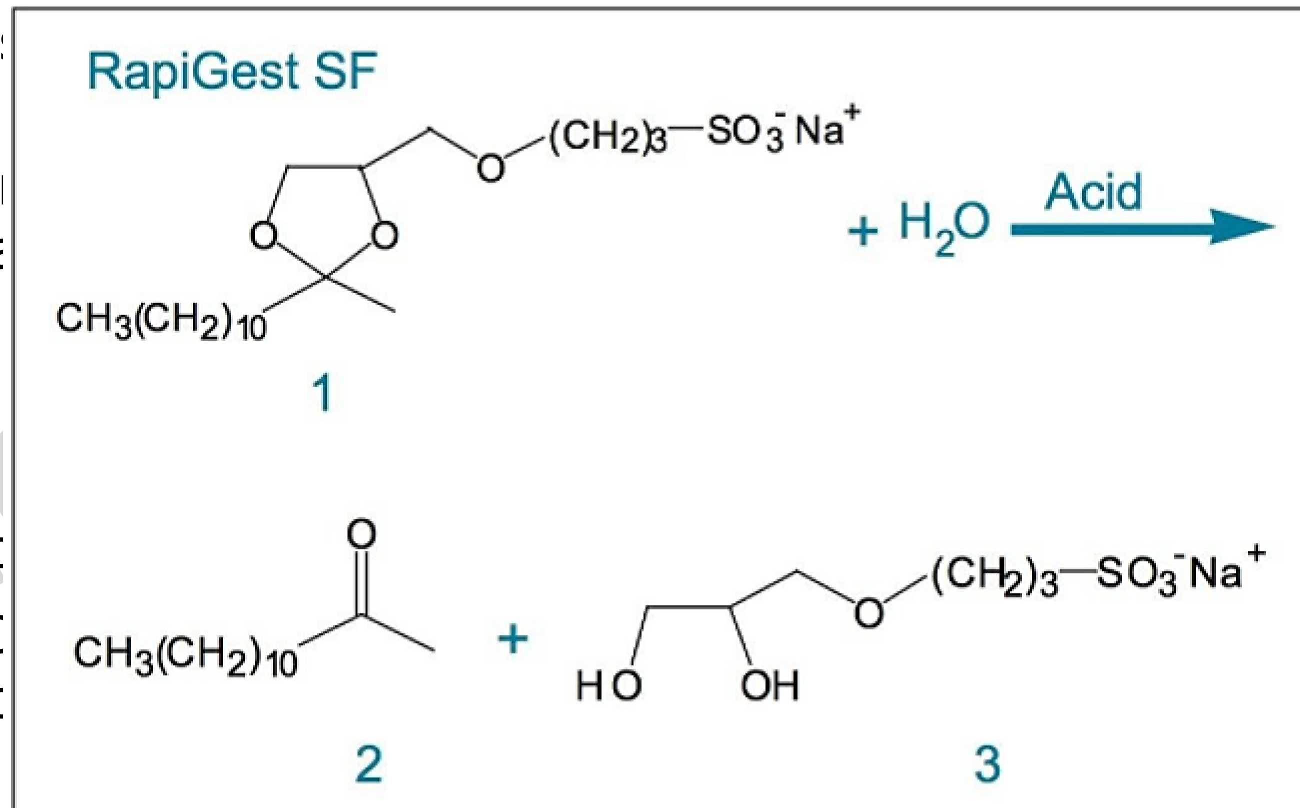
Study material

Impact of SDS in proteomic approaches

- Facilitates protein solubilization
- SDS-assisted protein digestion has been shown to enhance the detection of membrane proteins
- Suppresses enzyme activity during protein digestion
- Affects reversed-phase LC and it… tion of peptides (shift in retention time)
- Suppresses ionionization of othe… SDS dominates due to its ready ioniz… adducts are formed)

Possible solutions:

- SDS removal (protein precipitatic…
- MS-compatible alternatives to SD… PPS Silent Surfactant, octyl β-D-gluc…



Study materials

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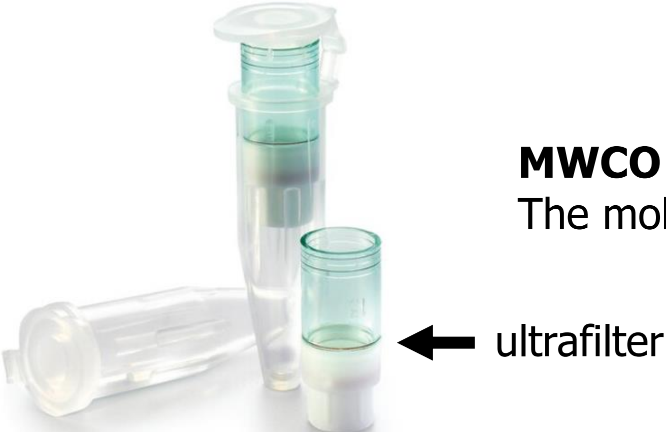
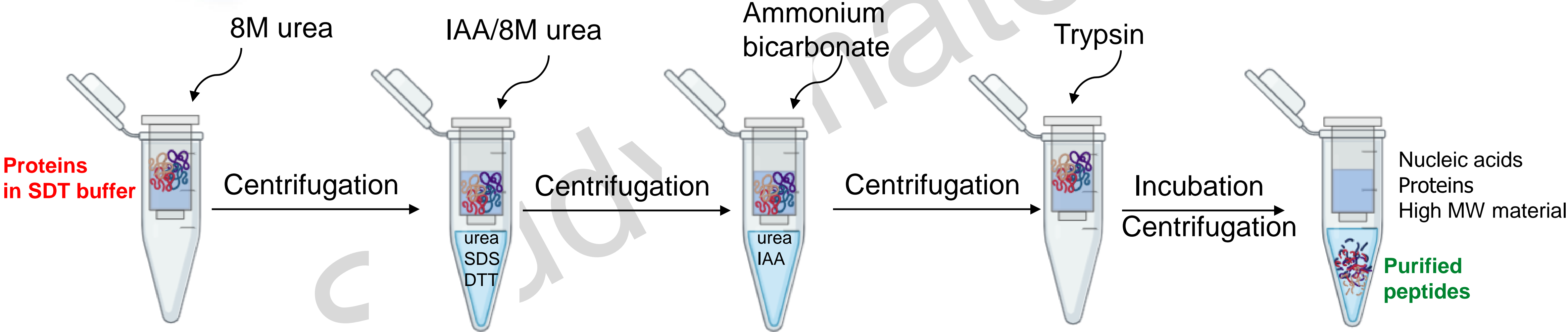
Protein Clean-up: Filter-Aided Sample Preparation (FASP)



- Excellent performance for samples between 25 and 100 μg of total protein

MWCO molecular weight cut-off
The molecule of a given molecular weight (Da) retained with 90% efficiency by the membrane

← ultrafilter

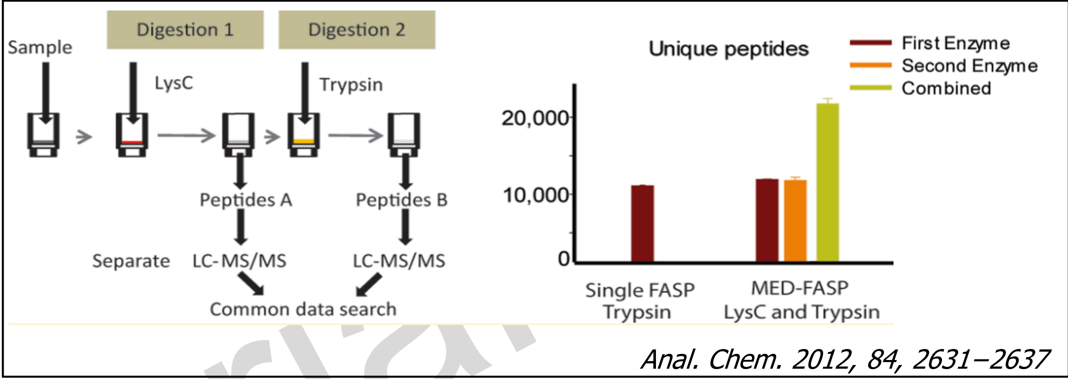


Nature Methods 6, 359 - 362 (2009)

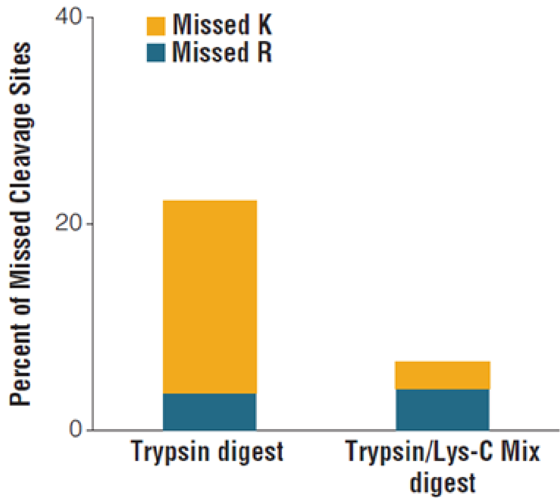
Study materials

Protein Clean-up: Modifications of FASP

- multienzyme digestion (MED) FASP *(Anal. Chem. 2012, 84, 2631−2637)*
  - sequential digestion of protein material with a second or third enzyme
  - increased number of identified proteins and their sequence coverage
  - increased depth of identification of phosphorylation sites



Anal. Chem. 2012, 84, 2631−2637



Trypsin

NNNNNK▼NNNNNR▼NNNNNN

Digested sample	Missed K	Missed R
Yeast extract	18.6%	3.6%
Mouse extract	6.6%	1.1%

Trypsin/LysC

NNNNNK▼NNNNNR▼NNNNNN

Digested sample	Missed K	Missed R
Yeast extract	2.6%	4%
Mouse extract	2.9%	1.5%

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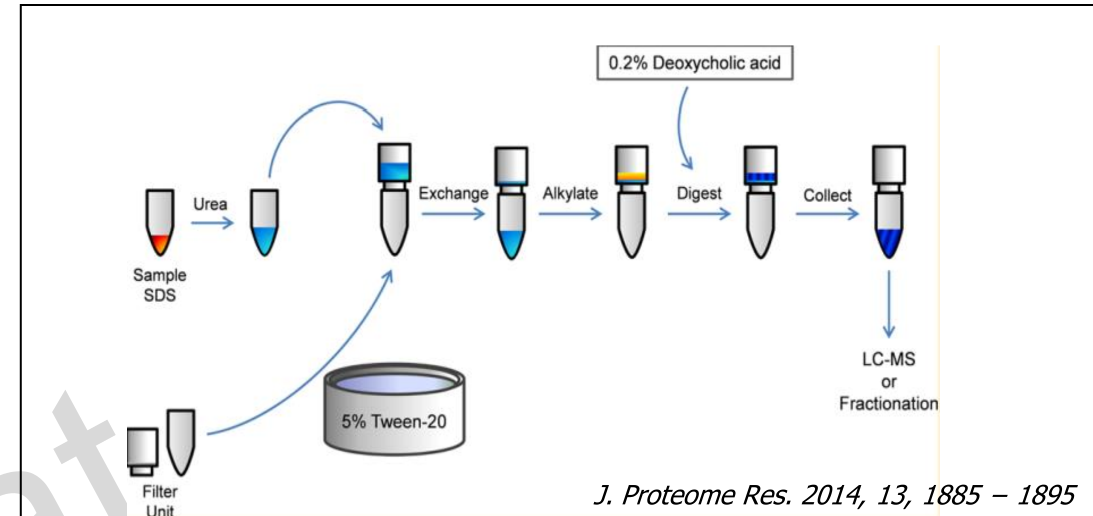
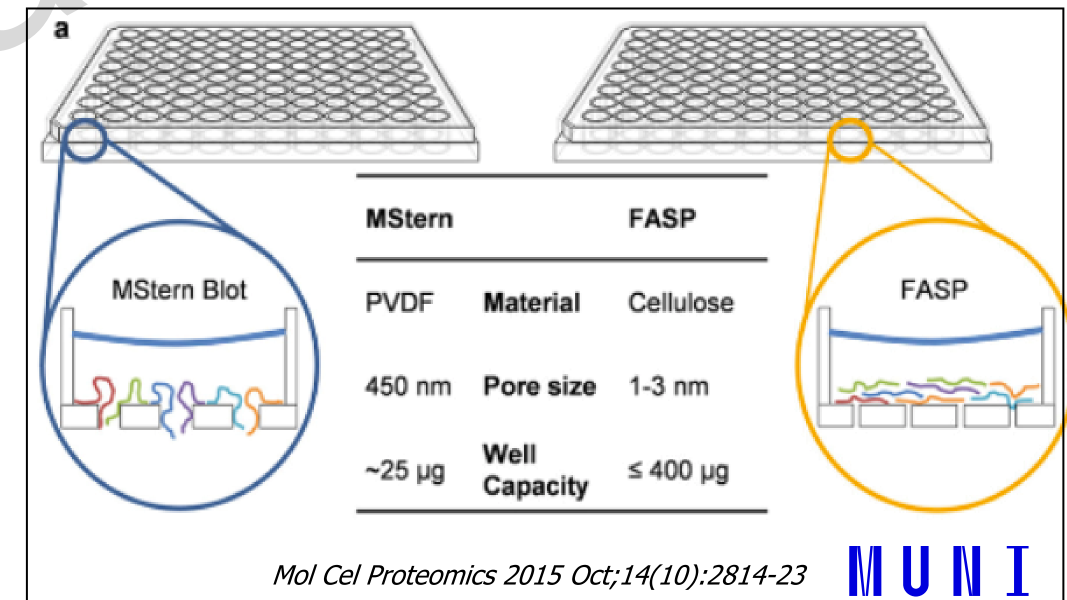
Protein Clean-up: Modifications of FASP

- enhanced FASP (eFASP) *(J. Proteome Res. 2014, 13, 1885 – 1895)*
  - pre-passivation of Microcon filter surfaces with 5% TWEEN-20 to enhance peptide recovery and uses a surfactant (0.2% deoxycholic) during detergent steps and digestion to increase trypsin efficiency



J. Proteome Res. 2014, 13, 1885 – 1895

- 96-well format for high-throughput processing
  - plates with a 10 MWCO membrane; disadvantage: low liquid transfer speeds during centrifugation *(Proteomics 2013, 13, 2980–2983)*
  - MStern-blot (MStern) - plates with large-pore hydrophobic polyvinylidene fluoride (PVDF) membrane which efficiently adsorbs proteins; fast liquid transfer through the membrane using a vacuum manifold. *(Mol Cel Proteomics 2015 Oct;14(10):2814-23)*
  - polyethersulfone (PES) filtration membrane enables to use 10% isopropanol (IPA) as a wetting agent, resulting in a reduction of 50% in the time required for buffer exchange. IPA reduces surface tension between the aqueous layer and the membrane. Reduced critical micelle concentrations of detergents due to presence of alcohol is balanced by urea. *(PLoS ONE 2017, 12(7): e0175967)*

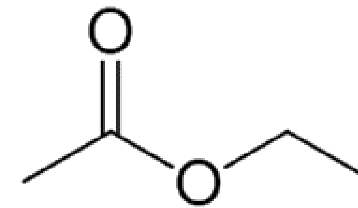


MStern		FASP
PVDF	Material	Cellulose
450 nm	Pore size	1-3 nm
~25 µg	Well Capacity	≤ 400 µg

Mol Cel Proteomics 2015 Oct;14(10):2814-23

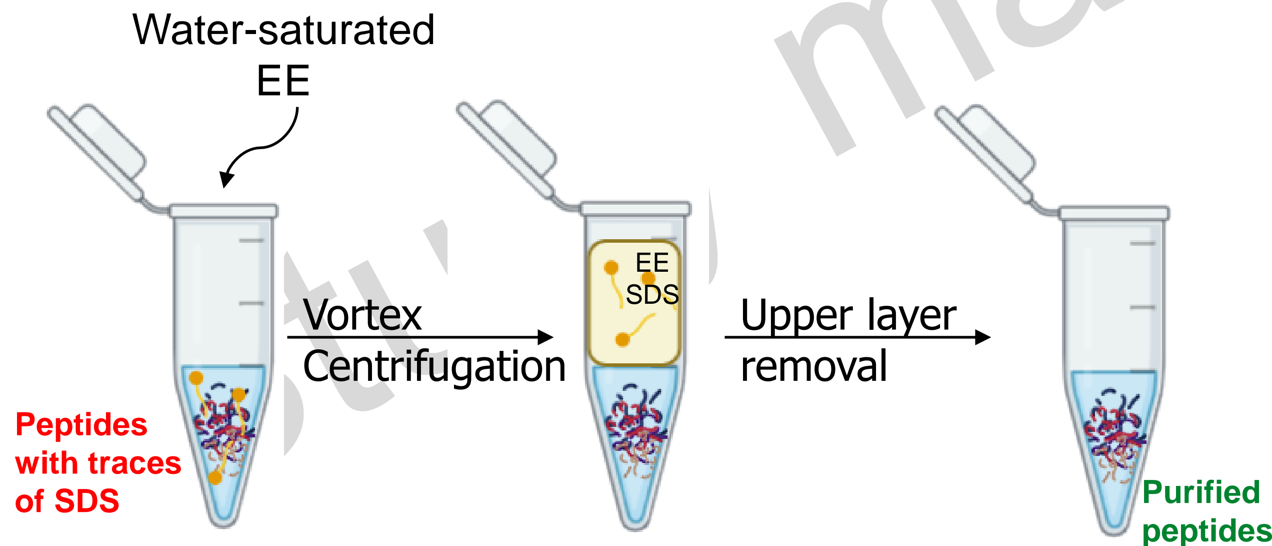
Study mat

Peptide Clean-up: Ethylacetate extraction (EE)



- Ethyl acetate
  - highly volatile
  - low-solubile in water
  - efficient solvent for several detergents (octylglucoside, SDS, Triton X-100, NP-40….)
- EE extraction
  - two-way process : partition of hydrophobic molecules to organic solvent from the aqueous solution, and partition of hydrophilic molecules in the organic solvent to the aqueous phase
  - extraction solvent of highest quality has to be used
  - acid washed glass bottles and pipettes should be used for the storage of EE
  - poly-propylene or poly-ethylene tubes and pipette tips can be used for short term extraction
  - five to ten times the volume of solvent to peptide solution in each extraction
  - loss of particular peptides (e.g., larger peptides)

materials



density (g/mL)	
EE	0.902
water	0.998

Curr Protoc Protein Sci. 2010 February ; CHAPTER: Unit–16.12.

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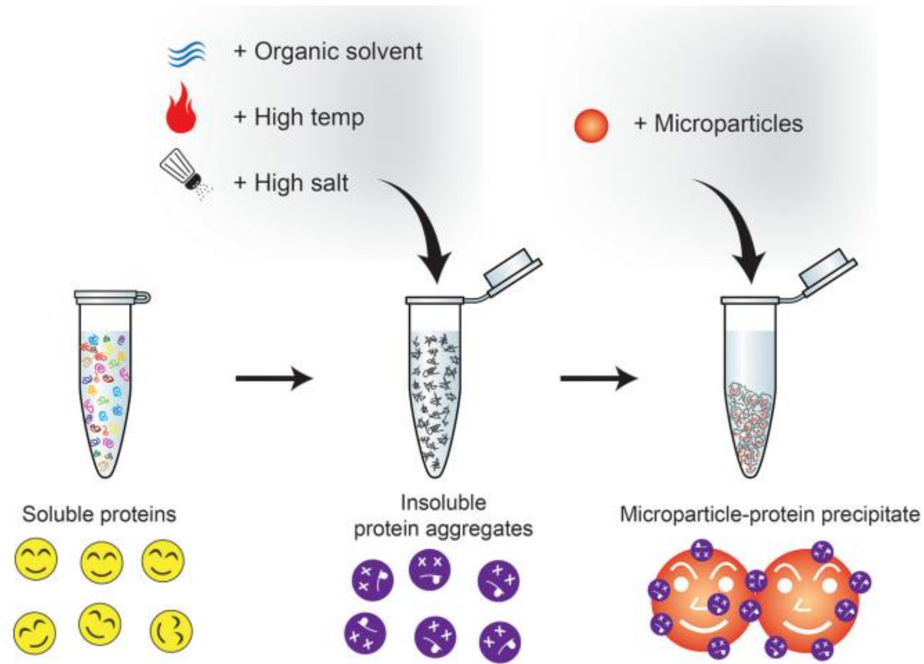
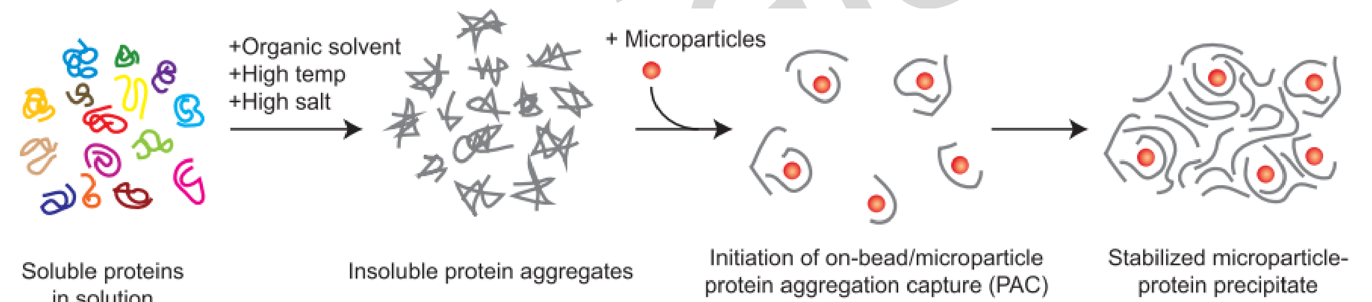
Alternative methods for digestion / clean-up

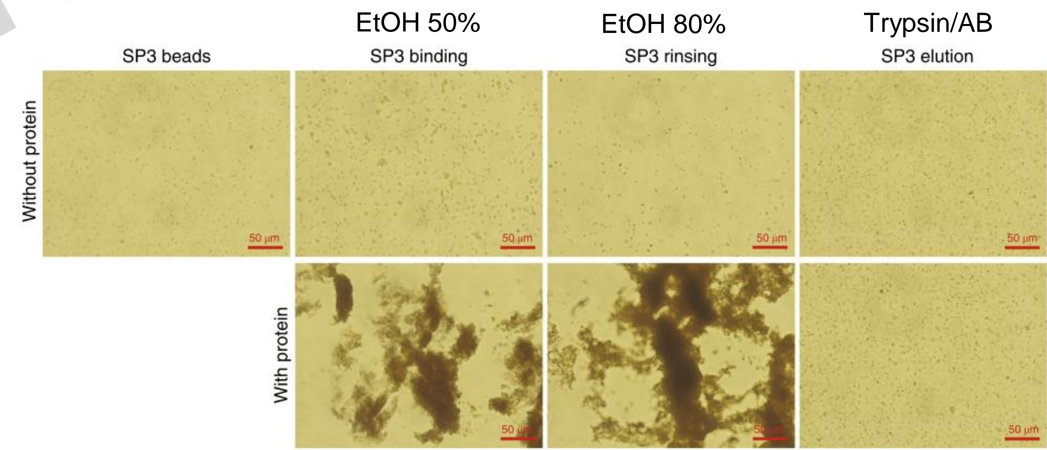
Protein Aggregation Capture (PAC) on microparticles of various surface chemistry

– a mechanism which uses the phenomenon of non-specifically immobilizing precipitated and aggregated proteins on any type of sub-micron particles irrespective of their surface chemistry. *(Mol Cell Proteomics 18: 1027–1035, 2019)*



(Mol Cell Proteomics 18: 1027–1035, 2019)





Nature protocols JANUARY 2019 | 68 – 85

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Study materials

Alternative methods for digestion / clean-up

Single-Pot Solid-Phase-enhanced Sample-Preparation (SP3)

– a paramagnetic bead–based approach

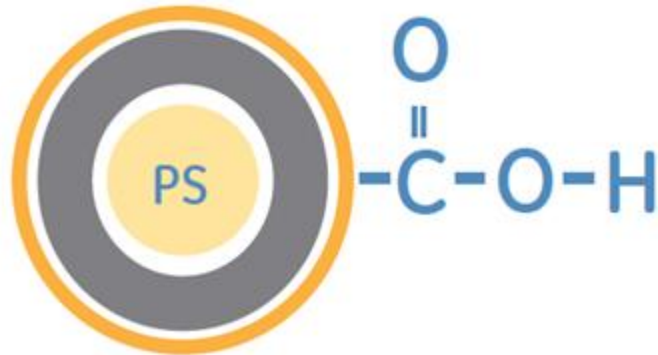
– uses PAC mechanism for exchange or removal of contaminants
(e.g., detergents, chaotropes, salts, buffers, acids, and solvents)

– non-selective protein binding and rinsing steps that are enabled through the use of ethanol-driven solvation capture on the surface of hydrophilic beads

– elution of purified material in aqueous conditions

Study materials

SeraMag beads



- polystyrene core
  made by a free radical emulsion polymerization of styrene and acid monomer
- layer of magnetite
- carboxylated polymer surface
  surface is modified to minimize non-specific binding of proteins

input proteins

PROTEIN LYSATE
0.1 mg - 500 mg

• - Contaminant

CONTAMINANTS
Detergents 0-20%
Chaotropes 0-8M
Salts 0-1M
Solvents 0-50%

add SP3 beads → induce binding → bind to magnet → rinse away contaminants →

Eluted proteins:
- For gel separation
- For affinity enrichment
- For chemical labeling
- For enzyme digestion

Step 18: enzymatic digestion

elute peptides

downstream analysis

ELUTED PEPTIDES

Eluted peptides:
- For offline fractionation
- For affinity enrichment
- For chemical labeling
- For direct MS analysis

(Nature protocols JANUARY 2019 | 68 – 85)

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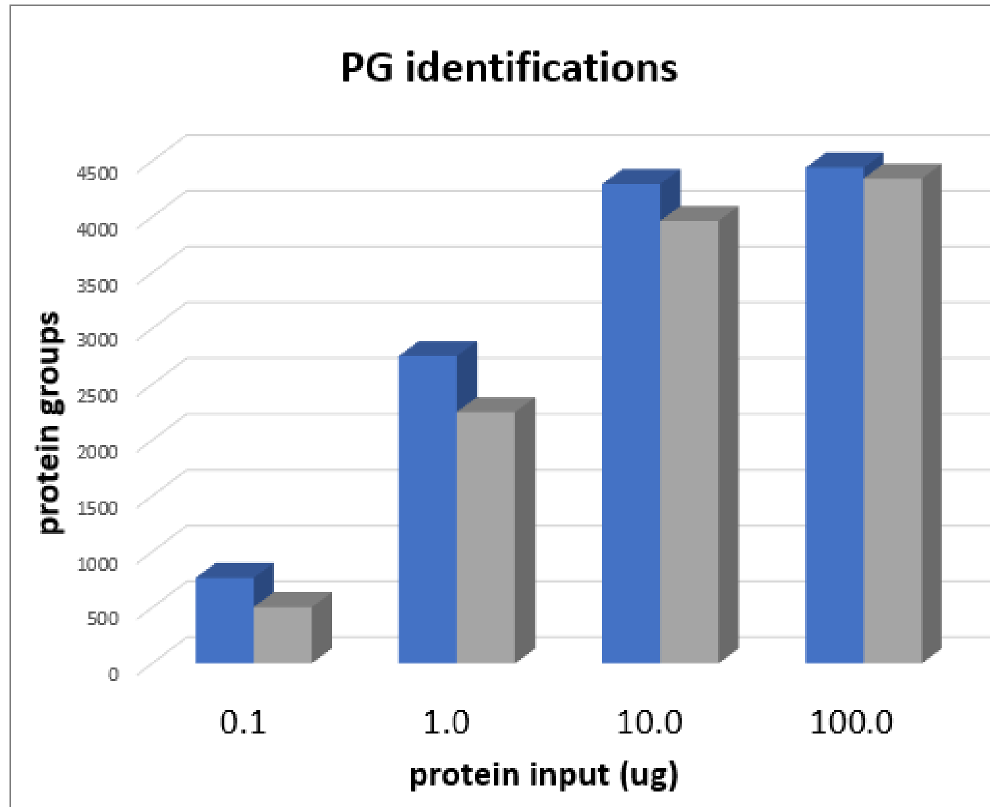
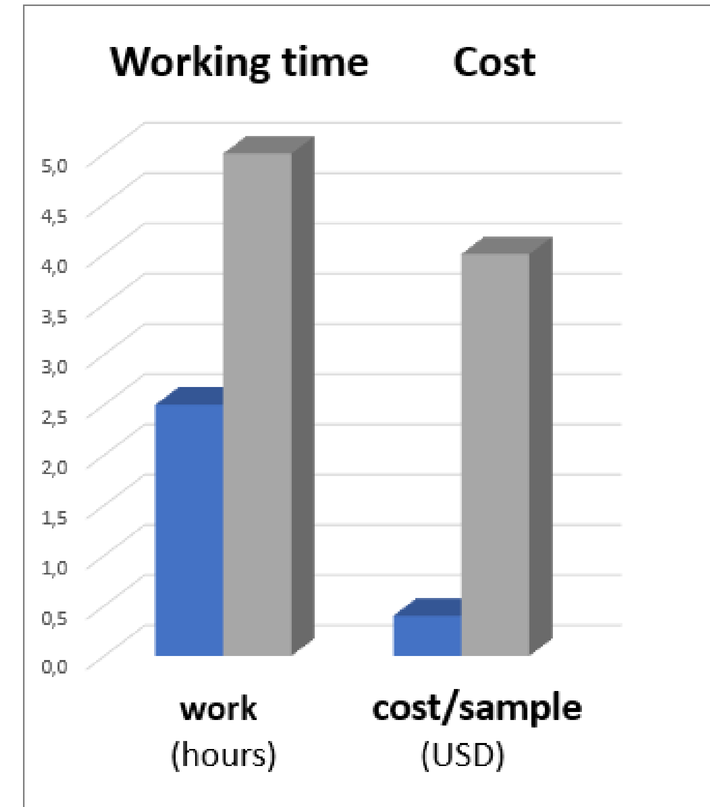
SP3 vs. FASP





Front plant Sci 2021 Mar 10;12:635550

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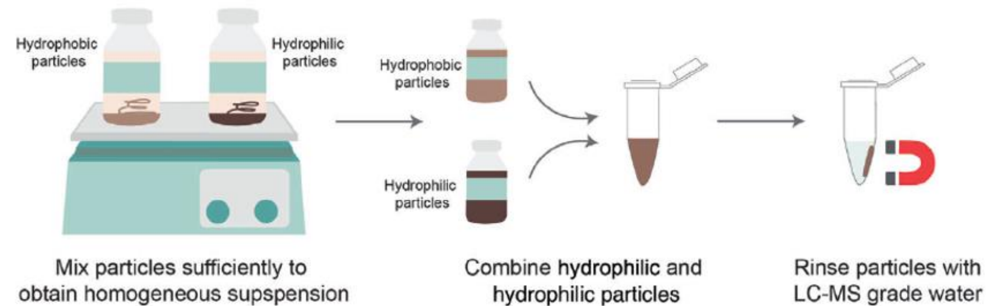
Peptide Clean-up: SP2

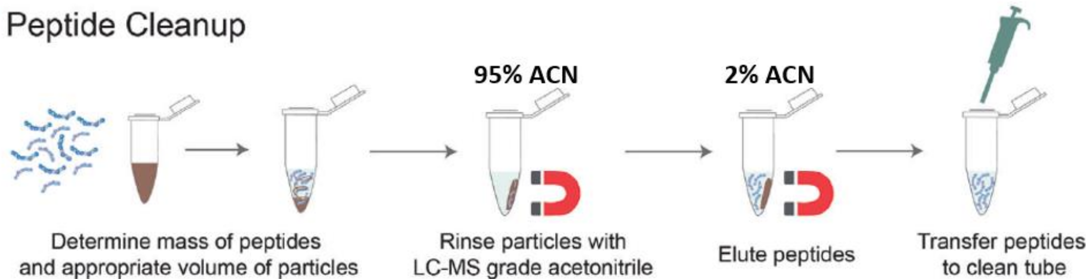
- RP-LC C18 resin – effective for removing salts and concentrating peptides
  – available in a wide variety of easy-to-use formats (e.g., Stage-Tips, Sep-Pak Cartridges, Micro SpinColumns)
  – however, C18 concentrates polymeric species such as polyethylene glycol (PEG) and common detergents (e.g., NP-40, SDS, Triton X).

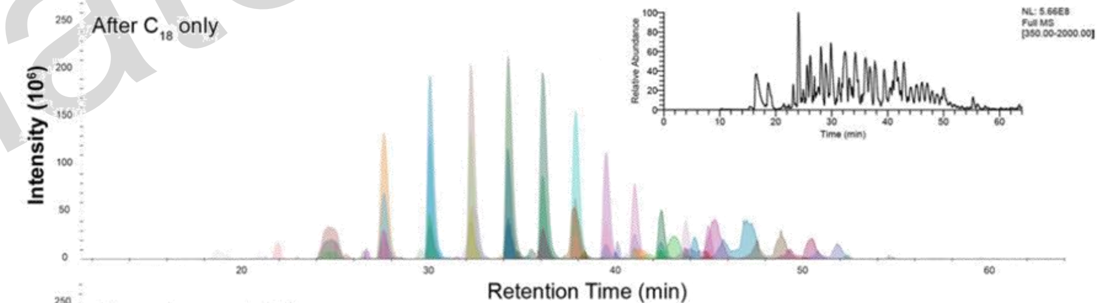
SP2 – lower binding capacity for peptides than for proteins:
50 and 200 ng/ µg for simple and complex peptide mixtures X 100 µg of protein/µg of particle
– suitable for variety of contaminants; (not suitable for Tris removal)
– peptides characterized as long, hydrophobic, or highly negative are more reproducibly processed with SP2 than with C18

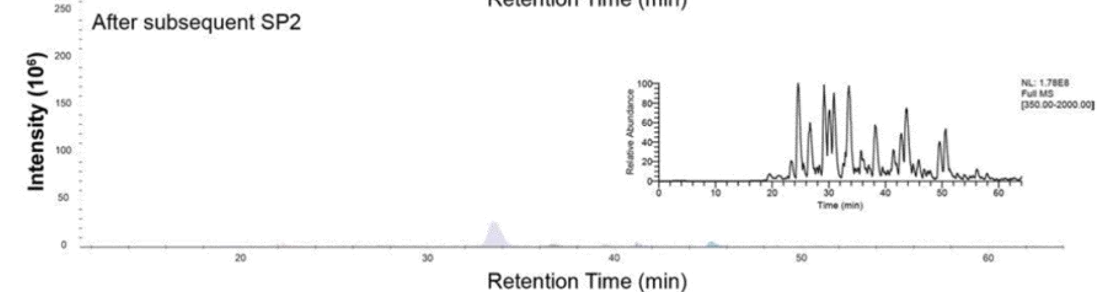
materials

1 Particle Preparation **Sera-Mag SpeedBeads Carboxylate-Modified Particles**









J Proteome Res. 2019 April 05; 18(4): 1644–1656.

Magnetic racks

Alternative methods for digestion / clean-up

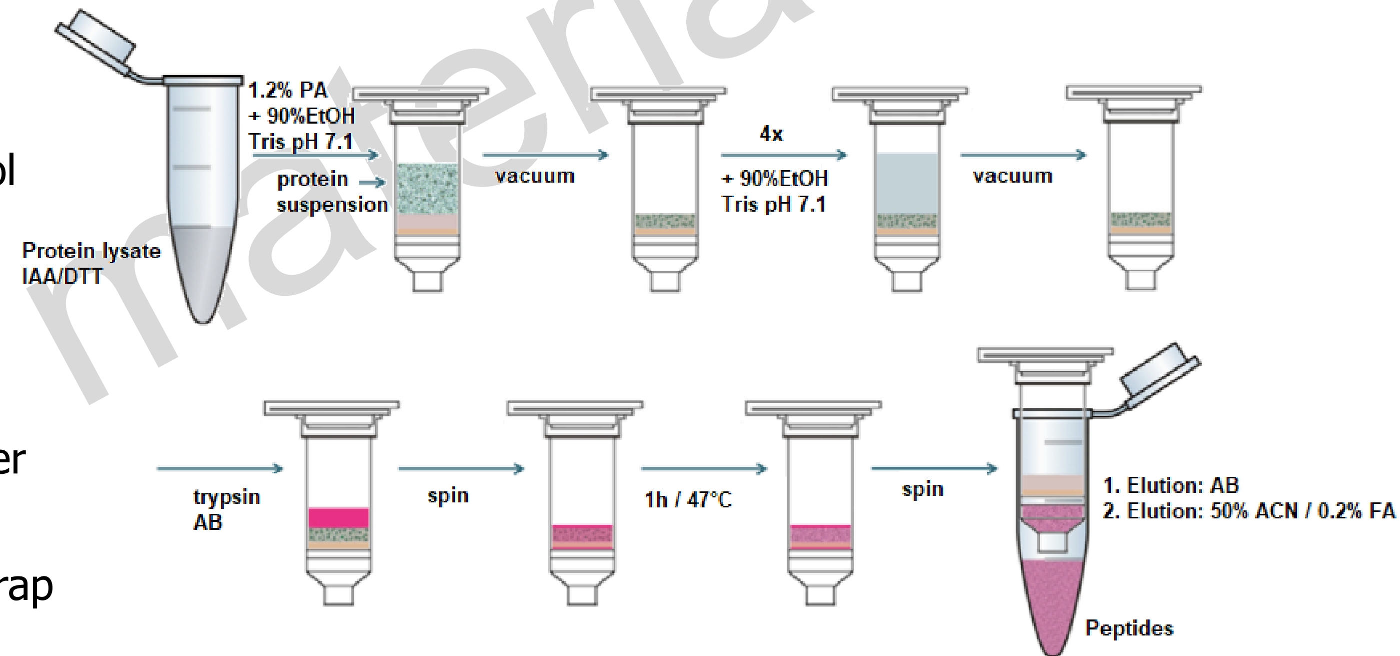
Suspension trapping (STrap)

– an instant creation of a fine protein particulate suspension from an SDS-solubilized protein solution, which can then be trapped by the filter

– aggregation of the suspension minimized by adding the protein–SDS mixture to an ethanolic solution at a near-neutral pH

– SDS monomers are soluble in ethanolic solution and are filtered out together with other contaminants

- sample lysis and solubilization in 5% SDS
- protein denaturation by acidification to pH < 1 and subsequent exposure to a high concentration of ethanol
- such three-stage denaturation ensures complete destruction of undesired enzymatic activity such as proteases and phosphatases
- reduction and alkylation can be performed in 5% SDS, precluding precipitation, or alternatively on-column after the denatured proteins are trapped
- denatured, non-digested proteins are bound to the S-Trap via centrifugation or vacuum



- multiple weak-affinity interactions hold the undigested protein within the pores of the derivatized silica S-Trap
- captured proteins are presented with maximal surface area allowing them to be washed fully free of all contaminants in only minutes: detergents, PEG, glycerol, detergents, salts, Laemmli loading buffer, etc.

Study materials

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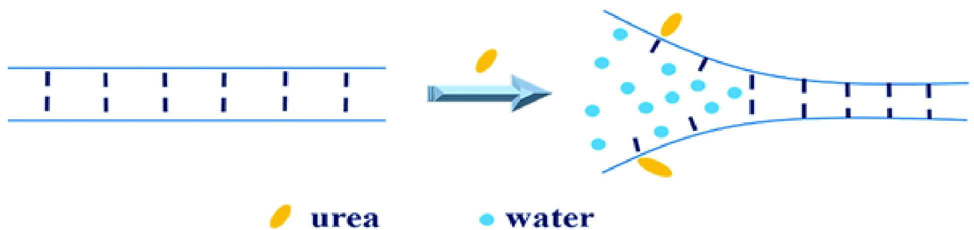
Solubilization of high complex sample for PTM enrichment

Homogenization in Urea buffer

9M Urea, 20mM HEPES pH 8.0



The hydrogen bond interaction between urea and the peptide groups opens the entrance for water, and contributes to the unfolding denaturation of protein.

Zhang et al., 2017, Phys. Chem. Chem. Phys., 19, 32007-32015

Cell count ~1×10E8

PBS wash

Centrifugation

10mL Urea / HEPES

Sonication

Reduction and Alkylation

Dilution (final concentration of urea 2M)

Trypsin

Enzymatic digestion

Clean-up: reversed-phase Solid-Phase Extraction (**SPE**; Sep-Pak® C18 Cartridges)

Digest acidification: pH <3

Elution: 0.1%TFA/**40% ACN**

Lyophilization (TFA removal)

Purified peptides

study materials

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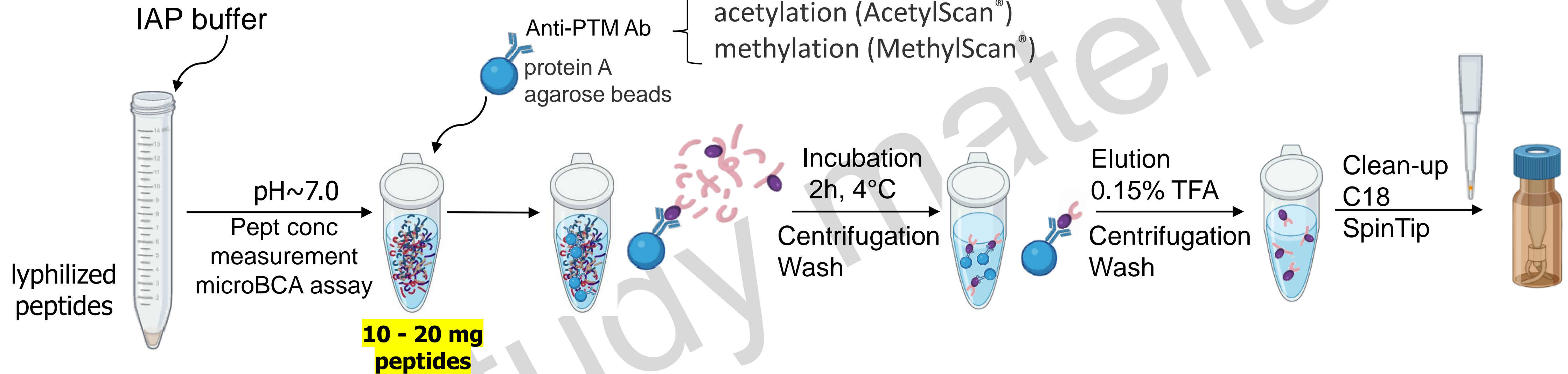
PTM enrichment

PTMScan® Technology (Cell Signaling Technology) - peptide enrichment by immunoprecipitation using a specific bead-conjugated antibody in conjunction with liquid chromatography (LC) tandem mass spectrometry (MS/MS) for quantitative profiling of post-translational modification (PTM) sites in cellular proteins.

ImmunoAffinity Purification (IAP)

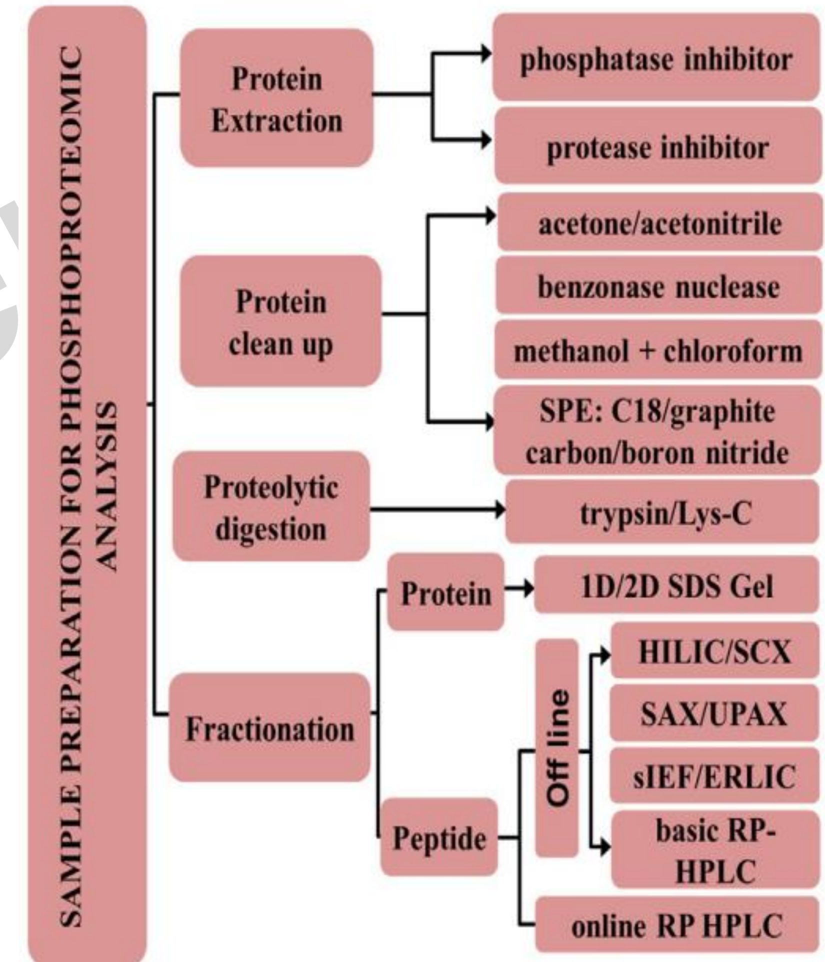


Study materials

Phosphoproteomics

- predominately employs bottom-up mass spectrometry (MS) based techniques
- phosphorylation occurs at single (mono-) or multiple (multi-) sites and can co-occur with other PTM types to generate different „proteoforms“
- phosphopeptides are low abundant relative to non-phosphorylated counterparts
- phosphopeptides tend to have low ionization efficiency due to
  (i) phosphate groups tending to lose protons to carry negative charges
  (ii) background presence of large amounts of unphosphorylated peptides
- phospho-serine/threonine (pSer/pThr) sites using MS techniques has improved, but the determination of tyrosine (pTyr) sites is challenging because the abundance of pTyr is significantly lower than that of pSer/pThr

Study mate



Analytica Chimica Acta 1129 (2020) 158e180

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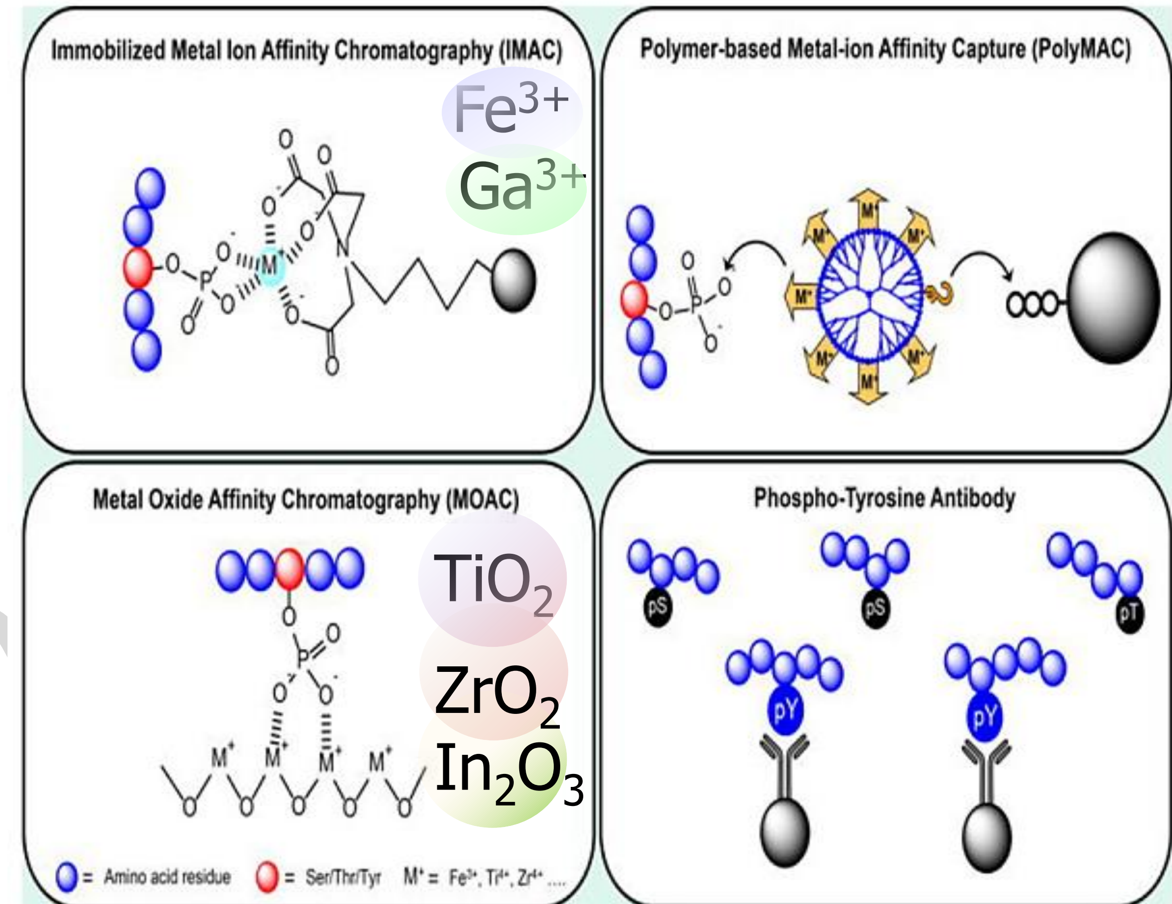
Phosphoproteomics

Affinity-based phosphopeptide enrichment

- selectively binds the negatively charged phosphate groups of the p-peptide to metal ions or metal oxide or employs Ab
- Elution from IMAC and MOAC by displacing the negatively charged phosphate with a basic buffer
- IMAC result in higher detection of multi-p-peptides, while TiO2 enrichment results in a high identification number of mono-p-peptides due to dissociation difficulty (incomplete elution)
- TiO2-based approaches: higher selectivity and specificity, robustness, amphoteric ion-exchange characteristics, tolerance towards many reagents (stable in wide pH ranges)
- Different configurations for operating MOAC-TiO2: spin columns, analytical columns, miniaturized columns, nanoparticles, magnetic beads, …
- pTyr a smaller fraction of the p-proteome; anti-Tyr Ab used for selective enrichment (poor reproducibility, low sensitivity, limited availability/ variability of Ab, limited availibility of bulk starting materials, high costs)



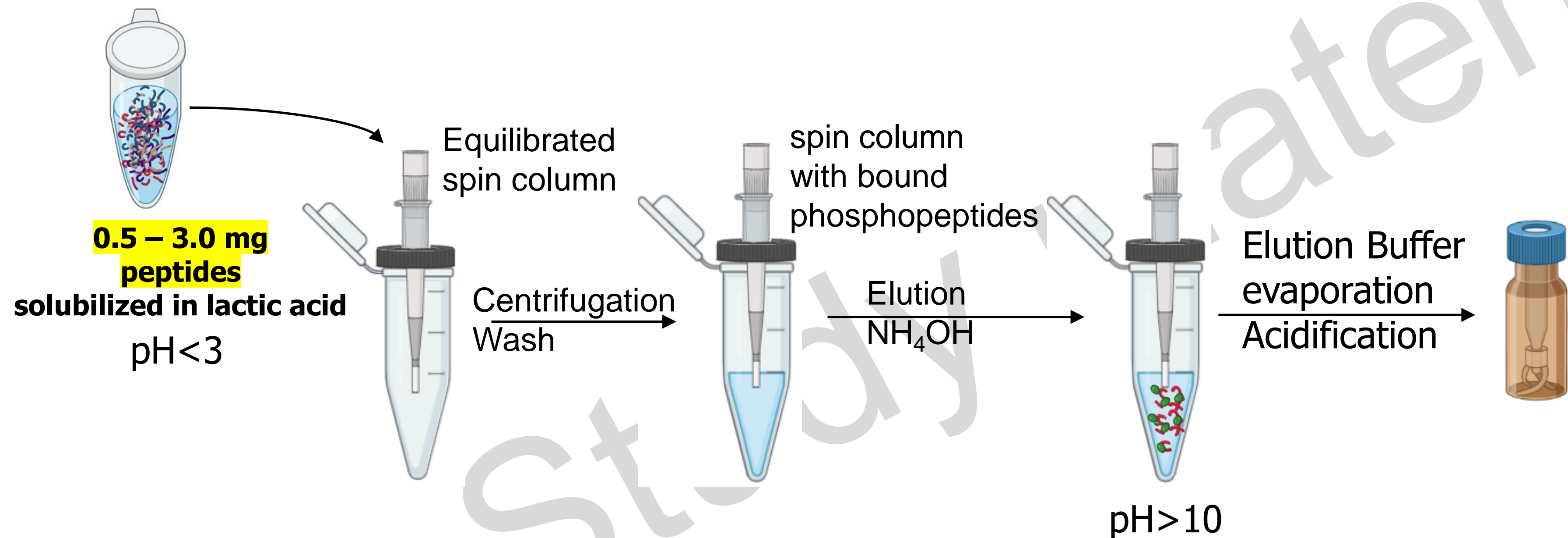
Analytica Chimica Acta 1129 (2020) 158e180

Study m

Phosphoenrichment

High-Select ™ TiO2 Phosphopeptide Enrichment Kit

– spherical porous TiO_2, optimized buffers, spin columns
– provide enhanced enrichment and identification of phosphopeptides with minimal nonspecific binding
– phosphopeptide yields are typically ~1-3% of the starting sample
– starting material: lyophilized peptide samples free of detergents and salts



Study materials

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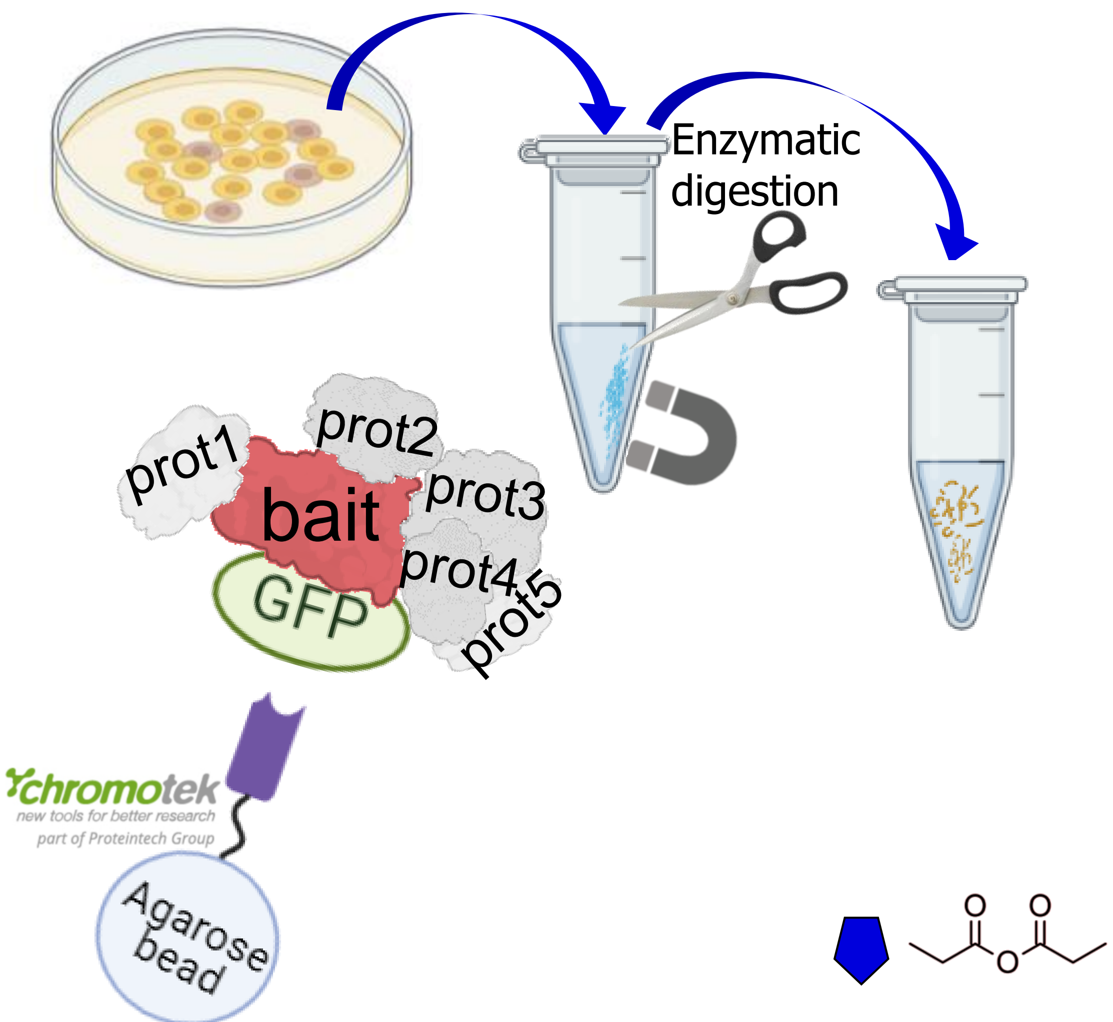
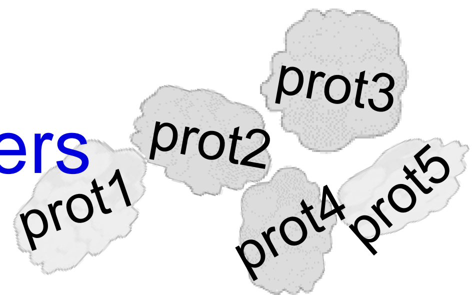
Characterization of enriched (low-abundant) proteins



Identification / Quantification of

- PTMs on bait protein
- Interacting protein partners




```
        10         20         30         40         50
MGKKQNKKKV EEVLEEEEEE YVVEKVLDRR VVKGKVEYLL KWKGFSDEDN
        60         70         80  ac ac ph ph       100
TWEPEENLDC PDLIAEFLQS QKTAHETDKS EGGKRKADSD SEDKGEESKP
       110        120        130        140        150
KKKKEESEKP RGFARGLEPE RIIGATDSSG ELMFLMKWKN SDEADLVPAK
       160  ph ph 170  ph ph 180
EANVKCPQVV ISFYEERLTW HSYPSEDDDK KDDKN
```

```
     ac  0      ac 20         30         40         50
MSGRGKGGKG LGKGGAKRHR KVLRDNIQGI TKPAIRRLAR RGGVKRISGL
                   70      ac            90        100
IYEETRGVLK VFLENVIRDA VTYTEHAKRK TVTAMDVVYA LKRQGRTLYG

FGG
```

Characterization of enriched sequence variants

variant I

```
        10         20         30         40         50
MSSRGGKKKS TKTSRSAKAG VIFPVGRMLR YIKKGHPKYR IGVGAPVYMA
        60         70         80         90        100
AVLEYLTAEI LELAGNAARD NKKGRVTPRH ILLAVANDEE LNQLLKGVTI
       110        120        130        140        150
ASGGVLPNIH PELLAKKRGS KGKLEAIITP PPAKKAKSPS QKKPVSKKAG
       160        170        180        190        200
GKKGARKSKK KQGEVSKAAS ADSTTEGTPA DGFTVLSTKS LFLGQKLNLI
       210        220        230        240        250
HSEISNLAGF EVEAIINPTN ADIDLKDDLG NTLEKKGGKE FVEAVLELRK
       260        270        280        290        300
KNGPLEVAGA AVSAGHGLPA KFVIHCNSPV WGADKCEELL EKTVKNCLAL
       310        320        330        340        350
ADDKKLKSIA FPSIGSGRNG FPKQTAAQLI LKAISSYFVS TMSSSIKTVY
       360        370
FVLFDSESIG IYVQEMAKLD AN
```

variant II

```
        10         20         30         40         50
MSSRGGKKKS TKTSRSAKAG VIFPVGRMLR YIKKGHPKYR IGVGAPVYMA
        60         70         80         90        100
AVLEYLTAEI LELAGNAARD NKKGRVTPRH ILLAVANDEE LNQLLKGVTI
       110        120        130        140        150
ASGGVLPNIH PELLAKKRGS KGKLEAIITP PPAKKAKSPS QKKPVSKKAG
       160        170        180        190        200
GKKGARKSKK KQGEVSKAAS ADSTTEGTPA DGFTVLSTKS LFLGQKLQVV
       210        220        230        240        250
QADIASIDSD AVVHPTNTDF YIGGEVGNTL EKKGGKEFVE AVLELRKKNG
       260        270        280        290        300
PLEVAGAAVS AGHGLPAKFV IHCNSPVWGA DKCEELLEKT VKNCLALADD
       310        320        330        340        350
KKLKSIAFPS IGSGRNGFPK QTAAQLILKA ISSYFVSTMS SSIKTVYFVL
       360        370
FDSESIGIYV QEMAKLDAN
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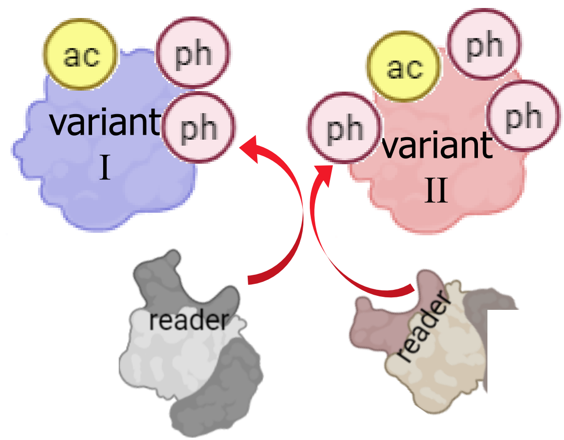
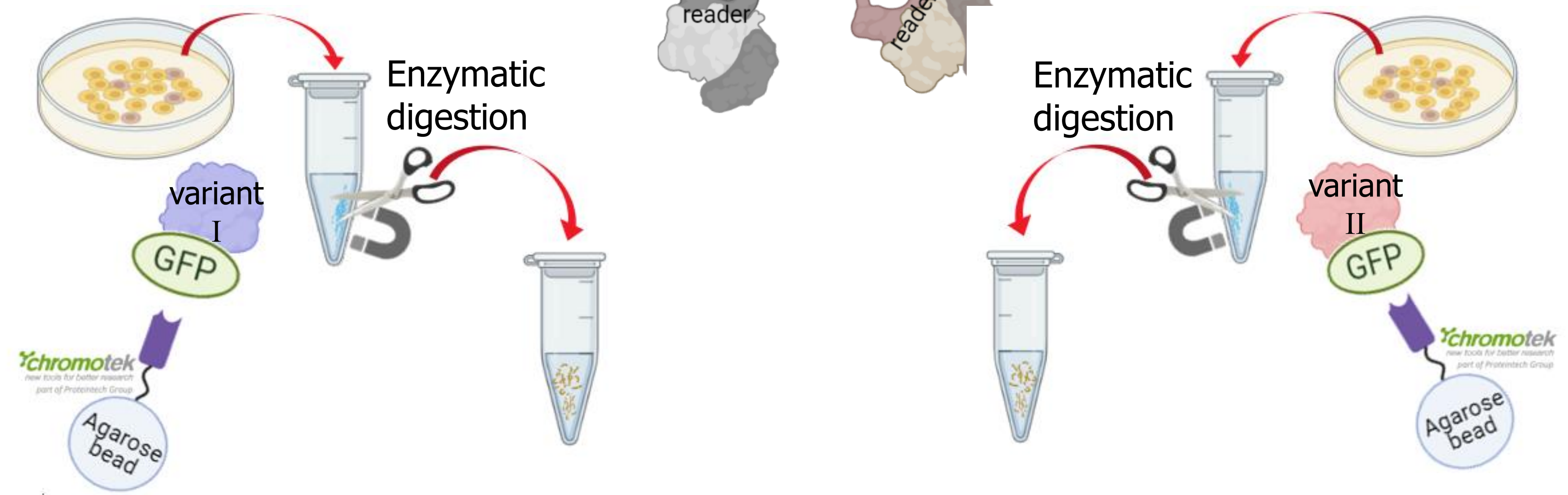
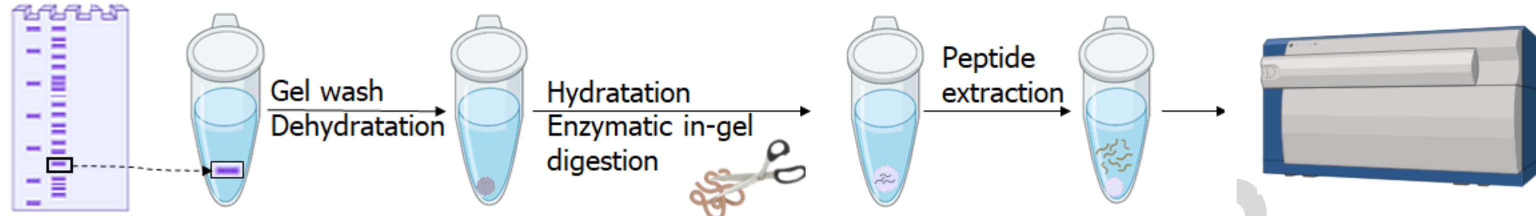


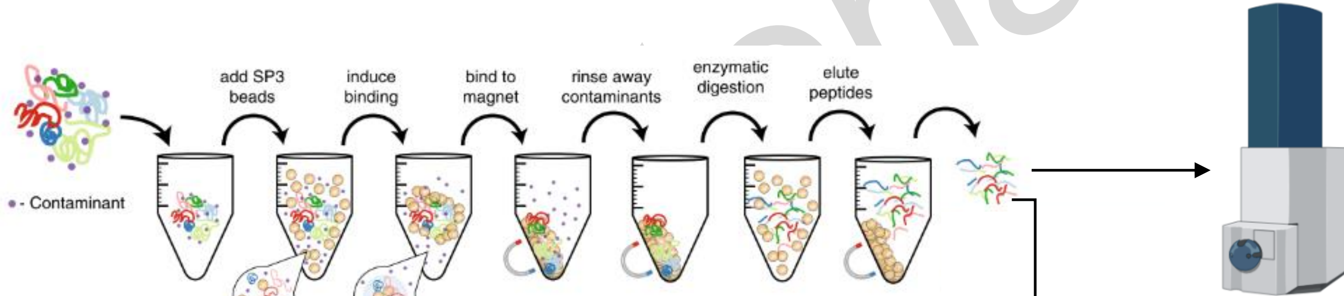


Practical course C8302

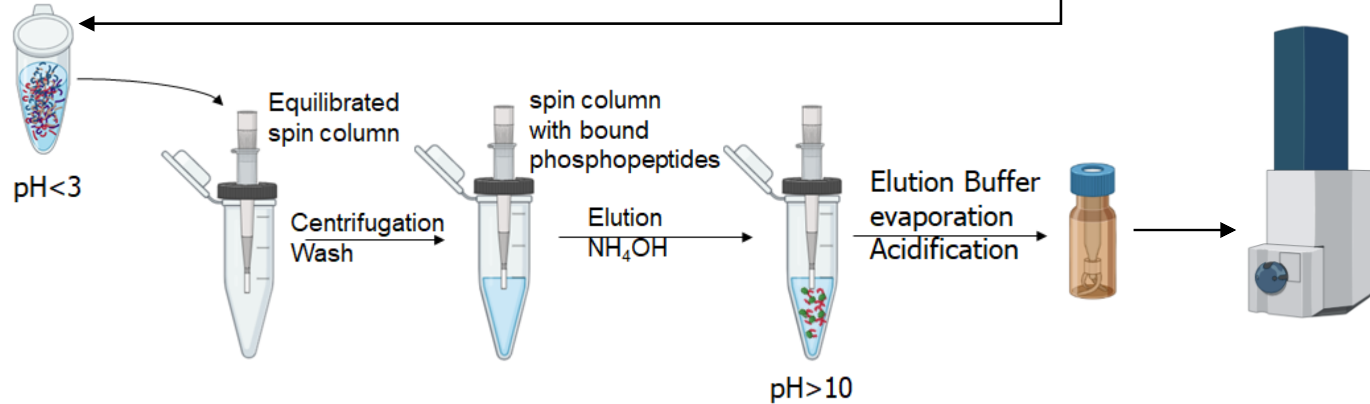
I. In-gel digestion



II. SP3-based digestion / clean-up



III. Phosphoenrichment



Study materials

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Thank you

for your attention!

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Particular figures were created with BioRender.com.

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