Sample preparation for proteomics by MS

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1. General information

1.1 pH stuff

You can check the pH of your sample by putting 0.1 ul of sample (or less) on a piece of pH paper.

pH UP from pH 2 to pH 8
1 ml/l HCOOH = formic acid (FA): pH = 2.4
For 1 ml: Make pH 8 – 8.5 by adding 15 – 20 ul 10* diluted conc. NH3 (max 37%).
0.5 ml/l TFA = TriFluoroAcetic acid: pH = 2.1
For 1 ml: Make pH 8 – 8.5 by adding 6- 9 ul 10* diluted conc. NH3 (max 37%).
pH DOWN from pH 8 to pH 2 - 4
50 mM ABC pH 8.0:
For 1 ml: Make pH 3 by adding 35 ul 10* diluted conc. TFA.

1.2 Abbreviations and solutions

AcNi	acetonitril			
ABC	Ammonium BiCarbonate	NH_4HCO_3 (lab 2)	(M=79)	50 mM ABC pH 8.0 = 0.2 g / 50 ml
AmAc	Ammonium Acetate	NH_4CH_3COOH	(M = 77)	10 mM = 38.5 mg / 50 ml
TFE	TriFluoroEthanol (lab 2, safety cupboard MS stuff)			
TFA	TriFluoro-Acetic acid	! FUMING = below fume hood = add in fume cupboard only		
TCEP	reductor Tris(CarboxyEthyl)Phosphine (-20°C)		M=287	100 mM = 28.7 mg / ml
Urea	(always prepare fresh! Do not warm up!) (weighing room)		M=60	8 M = 0.48 g / ml
DTT = Dithiotreitol	(4°C) FRESH! in 50 mM ABC		M=154	50 mM = 7.7 mg/ml, 500 mM = 77 mg/ml
IAA = Iodoacetamide	(4°C) FRESH! in 100 mM Tris pH 8		M=185	100 mM = 19 mg/ml 500mM = 92.5 mg/ml
Cysteine (Fluka 30090, >99%, lab 2)	in 50 mM ABC		M=121	125 mM = 15 mg/ml 500 mM = 60 mg/ml

Stock Tris (10*):	1 M Tris bring to pH 8 with HCl	M=121	1 M = 121 g/l = 6.0 g / 50 ml
ST buffer stock:	4%(w/v) SDS in 100 mM Tris/HCl pH 7.6		100 ul 1M Tris + 0.04 g SDS made up to 1.0 ml
SDT-lysis buffer:	50 mM DTT in ST buffer	M=154	7.7 mg / 1 ml ST buffer
UT:	8 M urea (Sigma, U0631) in 100 mM Tris/HCl pH 8	M=60	100 ul 1M Tris + 0.48 g urea made up to 1.0 ml pH will increase to 8.2 due to the addition of urea.

Trypsin: we have a stock solution of Bovine Sequencing grade Trypsin (Roche 11 047 841 001) of 0.5 ug/ul (500 ng/ul) in 1 mM HCl. Generally dilute 100* in ABC before use.

- 0.5 ml protein LoBind tube:	order#	eppe0030108.094
- 2.0 ml protein LoBind tube:	order#	eppe0030108.132
- Pall 3K or 10K omega filter (20kDa / 50kDa cutoff)	order#	Pall OD003C34 = Sigma-Aldrich Z722049-100EA

Remark: DTT and Iodoacetamide concentrations used are generally between 5 and 100 uM. The concentrations mentioned in the methods described below are still under discussion. Some users claim better results with lower concentrations for proteins having a low number of disulphide bonds.

1.3 Tips to reduce the amount of Keratins in your samples as much as possible

1. **DO:** Use commercial electrophoresis gels from e.g. Pierce or Invitrogen.
   Use Eppendorf low binding tubes to minimize protein losses (see figure below).
   Keep gels covered as much as possible. Put them into a new square petri dish for staining.
2. Wash your hands under running tap water before you start and as often as possible in between handlings.
3. Discharge yourself and your materials (especially the Eppendorf tubes) by using a discharge wire.
4. Do not work in a standard flow cabinet intended for microbiological work. They increase the amount of keratin passing your vials resulting in more keratin in your samples.
5. Do not wear clothes of wool.
6. Try not to lean over the samples too much.
7. Never ever use glass vials for proteins. You will lose a lot protein.
8. Use new (or only used for proteomics) throw away (polypropylene) plastics.
8. Do not use hand creams.
9. Do not use any glassware that has been cleaned with detergent (e.g. in a washing machine).
10. Cheap (non-Eppendorf) micro tubes may contain polymers, mold release agents, plasticizers, etc.
11. Do not use pipet tips that have been sterilized by heating them. The heat may release plasticizer compounds.
12. Use Eppendorf LowBind tubes, not siliconized tubes.

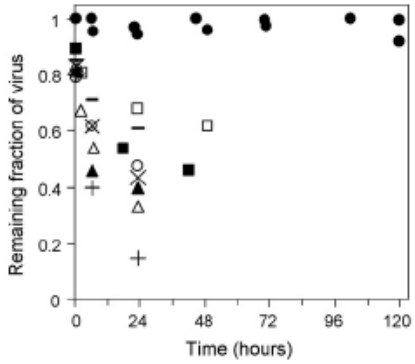

Fig. 5. Virus loss in different containers (20 mM HEPES, 150 mM NaCl, pH 7.8; initial Ad5 concentration was approximately 6×10^{10} p/mL ≈ 0.02 g/L): (●) LoBind™ protein tube (Eppendorf), (□) Fisherbrand 0.5 mL tube, (▬) Glass (National Scientific), (■) Fisherbrand PCR tube, (◯) polycarbonate tube (Eppendorf), (×) prelubricated tube (Costar), (▲) polypropylene tube (ULP), (△) Maxymum Recovery (Axygen), (+) silanized glass (National Scientific).

Eppendorf LoBind tubes bind much less viruses then other brand LoBind tubes.

From:
E.I. Trilisky, A.M. Lenhoff: Sorption processes in ion-exchange chromatography of viruses. J. Chromatogr. A 1142 (2007) 2 - 12.

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1.4 Recommended procedures

	Protein Identification	Label free relative Quantitation	Silac labeled Relative Quantitation	Dimethyl labeled Relative Quantitation	Absolute Quantitation
FASP	+	+	+	+	+
In Gel Digestion (IGD)	+	+	+		
In Stage Tip (iST)	+	+	+	+	+
On column Dimethyl labeling	-	-	-	+	-
Ovalbumin Standard addition	-	**Possible but not advised**	-	-	-
Peptide (labeled) Standard addition	-	-	-	-	+
Peptide fractionation	**+ (always possible e.g. with High pH RP on µColumns)**				
Measurement	**nLC-MS/MS by Proxeon → LTQ – Orbitrap XL**				
Raw Data handling	**MaxQuant** (Protein identification and relative quantitation of peptides larger than 7 AA) **pNOVO+** (deNovoGUI) for peptide de NOVO sequencing (of peptides smaller than 8 AA)				**QualBrowser + MaxQuant**
MQ data handling	**Perseus** (extra filtering of MQ result, statistics and intensity or ratio based clustering of proteins)				
Bioinformatics (Software mentioned in between brackets has not been tested by me)	Overview of GO tools (but not complete): http://geneontology.org/ First **pathway analysis** can be done with eg: Reactome (www.reactome.org), PathVisio (http://www.pathvisio.org/), KEGG (http://www.genome.jp/kegg/tool/map_pathway2.html), Biocyc (Subscription needed! Biocyc.org → e.g. HumanCyc → Change organism and select xxx. Tools → Cellular Overview). **GO enrichment analysis** can be done within: - Internet resources like DAVID (Database for Annotation, Visualization and Integrated Discovery, very easy), or (InterProScan)/PloGO (see below) + WeGO. - Cytoscape (www.cytoscape.org) with plugins like BinGO or ClueGO (or EnrichmentMap). - Use the "R project" environment e.g. PloGO (includes abundance information = more advanced). **Interactome** studies may benefit from database contained information e.g. in STRING-db.org/ (very easy) but also from Cytoscape plugins like Bionetbuilder or Bisogenet. **Alternatives:** commercial software like ProteinCenter, Ingenuity or Metacor (these commercial softwares are unfortunately not available at WUR Biochemistry).				

Silac: Stable Isotope Labeling by Amino acids in Cell culture.
FASP: Filter Aided Sample Preparation
MQ: MaxQuant

MaxQuant label free relative quantitation result after analysis with Perseus statistical software. The Figure was prepared in Excel.

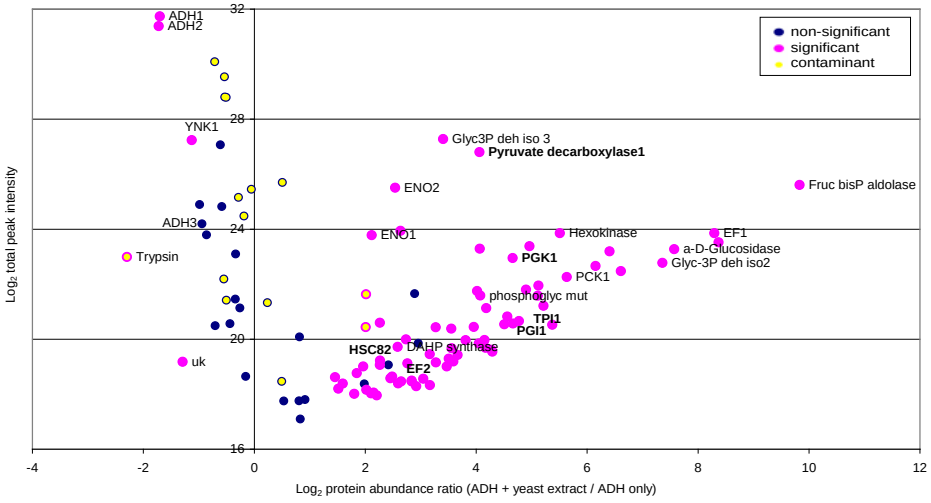

Table 1 | Comparison of spin filter, short SDS-PAGE and TFE methods

Method	Protein load	Peptide identifications	Protein identifications
Spin filter	50 µg	5,369	642
Short SDS-PAGE		4,176	593
TFE		4,663	593
Spin filter	150 ng	86	46
Short SDS-PAGE		298	106
TFE		626	150

Samples of human RKO colon carcinoma cells containing the indicated amounts of protein were prepared in triplicate by the indicated methods and analyzed by reverse phase LC-MS/MS. Peptide identifications are total MS/MS spectrum-to-sequence database matches at 5% false discovery rate; protein identifications are nonredundant identifications with at least two identified peptides and parsimonious protein assembly. Reported values are the means of three technical replicate analyses.

1.5 Sample losses, methods comparison.

Taken from:
Liebler, D. C.; Ham, A. J. L.: Spin filter-based sample preparation for shotgun proteomics. Nature Methods (2009), Volume: 6, Issue: 11, p785-785.

	Yield (%)	Remark
FASP	50	This can be somewhat increased by using multiple digestion steps. (Wisniewski, J.R. and Mann, M. (2012) Consecutive Proteolytic Digestion in an Enzyme Reactor Increases Depth of Proteomic and Phosphoproteomic Analysis. Analytical Chemistry 84(6), 2631-2637)
In-gel digestion (IDG)	20	Purified protein: load a few ug of protein on the gel. Protein extract: more proteins will be identified when you load more protein. Dividing the gel lane into 8 slices means that you should load minimally 16 ug protein on the gel but preferentially (much) more, e.g 50 – 75 ug.
In Stage Tip (iST)	80	

1.6 nLC-MSMS sample necessities.

Hand in minimally 50 ul acidic peptide sample (pH 2 – 4) with a concentration of approximately 10 – 500 ng/ul.

Peptide sample solutions to be measured should not contain detergents (SDS, Tween, Triton, NP etc) or other charged nonvolatile ions like TRIS or phosphate or particles of any kind. (detergent has to be removed by FASP (see section 3.2), by running an SDS gel + doing a In-Gel Digestion or by a specific detergent removal method like with commercial available SDS removal spin columns (e.g. Pierce HiPPR).

Proteins can be transferred into the appropriate (ABC) solution by treatment with a Spin Filter (5 or 10 kDa) filter before doing the Trypsin digestion. Another good and proven method to get rid of impurities is to do FASP (see section 3.2), iST (3.3), an SDS gel-electrophoresis step (see section 4) or at least a C18 uColumn cleanup (see section 1.7). Peptide samples can be desalted/concentrated/transferred by performing the C18 uColumn cleanup as well (see section 1.7).

1.7 General sample cleanup procedures with µColumns.

Peptide solutions can be concentrated or desalted, and beads (from IP/chromatography/SP3) can be removed using µColumns (= C18 Stage tip + C18 column material). With this µColumn, there will be no loss of hydrophilic peptides (in contrast to using commercially available micro tips like Zip Tips or StageTips)

1. Prepare your own µcolumn by:

Use the “cookie cutter” method to cut a small (1.6mm = Gauge 14) piece of a C18 Empore disk (= frit). Transfer the frit to the 200 ul tip with a good fitting plunger and tap it mildly. Add 200 ul methanol to the tip with frit.
Prepare a 50% slurry of LichroprepC18 column material in methanol and add 4 ul of the 50% slurry into the methanol in the tip + frit.
The prepared µColumn can be eluted by hand with a plastic 10 ml syringe, or with the vacuum manifold (connected to a vacuum pump) in lab1. Whatever method you use, do not let the µColumns run dry.
2. Wash the µColumn ones more with 100 ul MeOH.
3. Equilibrate the µColumn ones with 100 ul 1 ml/l HCOOH in water.
4. Dissolve the sample preferentially in 15 – 200 ul of 1 ml/l HCOOH in water (not containing any AcNi) or in any other aqueous buffer.
5. Add sample(s) to the uColumn(s) (wash gel pieces with an extra 50 or 100 ul 1 ml/l HCOOH in water and add that too) and elute through.
6. Wash the uColumn ones with 100 ul 1 ml/l HCOOH in water.
7. Transfer the uColumn to a new 0.5 ml Eppendorf low binding tube.
8. Manually elute peptides from the C18 Stage tip+ uColumn by adding and eluting with 50 ul 50%AcNi + 50% 1 ml/l HCOOH in water directly into the 0.5 ml Eppendorf low binding tube.
9. The sample is now Maldi-tof ready.
10. For LCMS analysis, reduce the AcNi content by putting the samples in a Concentrator (with open cap) at 45 ºC for 2 hours or longer when necessary. The final volume should be below 20 ul. Adjust the sample volume with 1 ml/l HCOOH in water to exactly 50.0 µl. Sonicate (water bath sonicator) for 5 sec in the hot spot when the sample had been dried completely by accident.
The sample now has <5% AcNi, is pH 3, is particle free and therefore is nLCMS ready.

Remark1: The uColumn cleanup step can also be used when you want to concentrate or combine samples. An alternative option to concentrate or combine peptide samples is the Eppendorf concentrator.
Remark2: uColumns can be step eluted at pH 10 with 50 ul of a step gradient of AcNi [e.g 5, 8, 11, 18 and 40% AcNi with 20 mM NH_4Formate pH10], to fractionate peaks according to their high pH affinities for the C18 column.

1.8 About Methionine oxidation

To confirm a peptide sequence containing a Methionine within a known protein, the peptide can be oxidized with 0.1v/v% or 10µM H_2O_2 by incubating at 4 °C overnight. This oxidizes every methionine in the sample and shifts the molecular weight up by 16 Da for each methionine.
(Koudelka, T., Dehle, F.C., Musgrave, I.F., Hoffmann, P. and Carver, J.A. (2012) Methionine Oxidation Enhances kappa-Casein Amyloid Fibril Formation. Journal of Agricultural and Food Chemistry 60(16), 4144-4155).

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2. Protein determination (BCA).

by the Bicinchoninic Acid (BCA) method (Pierce:#23225): (Sensitivity: 5 – 30 ug protein as measured at 562 nm)

The BCA protein concentration determination method is almost independent on the amino acid composition but cannot be used for samples containing reducing agents (like DTT or TCEP) or chelators (like EDTA).

Solutions:

BCA working solution (BCA ws): 25 ml reagent A + 0.5 ml reagent B
Sample : BCA ws = 1 : 20
Ovalbumin or BSA stock = 1 mg/ml in 50 mM NH_4HCO_3.

The exact concentration of Ovalbumin or BSA can be directly measured when in 50 mM NH_4HCO_3 at 280 nm:

$C_{ovalbumin}$ = E280 * 1.30 (mg/ml)
C_{BSA} = E280 * 1.50 (mg/ml)

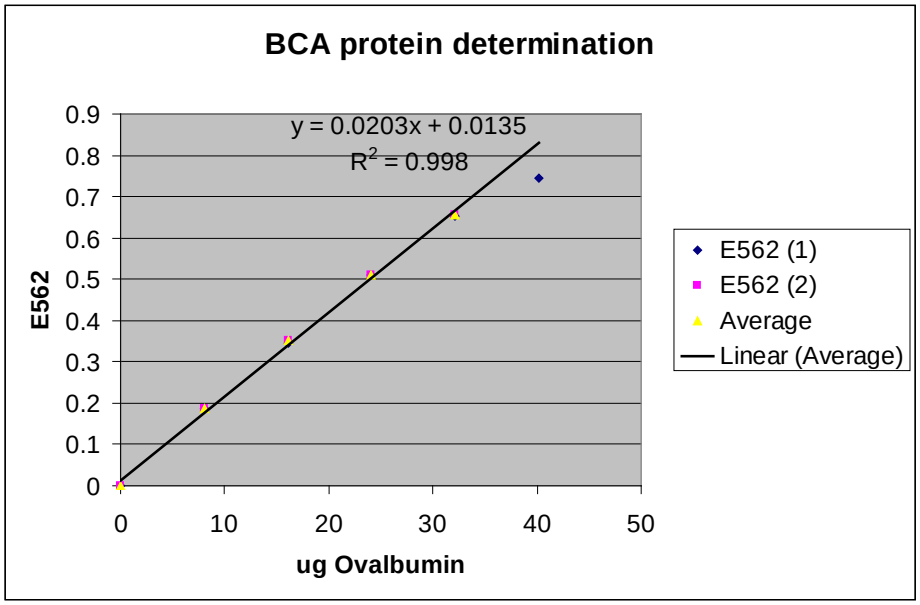
This is necessary since purified proteins always contain some salts.

Pipet the BCA working solution, water and the Ovalbumin or BSA standard or sample directly in a plastic 1 mL cuvette as described in the table below. Seal tightly with parafilm. Mix and incubate at 21 ºC for 60 min or at 37 ºC for 30 min. Mix and measure E562.

Example measurement:

Ovalbumin dissolved at 1 mg/ml a absorbance at 280 nm was measured: E280 = 0.618
Therefore the real concentration was: 0.618 * 1.30 = 0.803 mg/ml

Volume BCA ws (ul)	Volume Ovalbumin Std (ul)	Volume sample (ul)	Volume H2O (ul)	Calc. amount Ovalbumin (ug)	Measured E562 (1)	Measured E562 (2)
950	0	-	50	0	0.000	0.000
950	10	-	40	8.03	0.186	0.186
950	20	-	30	16.1	0.346	0.351
950	30	-	20	24.1	0.510	0.509
950	40	-	10	32.1	0.653	0.655
950	50	-	0	40.2	0.746	0.730
950	-	20	30			



Linear between 0 – 30 ug:

ug protein =
$[E_{562} - 0.0135] / 0.0203$

Note: A convenient incubator + absorbance measurement device using 96 well plates is available in lab 4. Decrease all volumes in the table a factor 4 and you can use it to.

Alternative: Pierce 660 nm Protein Assay (#22660) with the Ionic detergent Compatibility reagent (#22663) can be used in the presence of maximally 5% SDS and/or 500 mM DTT and is linear between 2.5 and 600 ug BSA or 5 to 1200 ug of Ovalbumin. Compared to BSA, the Ovalbumin response is 54%. This strong protein dependence is the main disadvantage of the Pierce 660 nm Protein Assay.

3. Gel free protein digestion methods.

When you have a limited amount of protein then you can use a minimal volume (50 ul) of buffer to do the sonication in and use the iST method (3.1).

3.1 In-StageTip (iST) sample preparation for maximally 5 ug protein without detergent.

Modified from Ref: Kulak, N. A., G. Pichler, I. Paron, N. Nagaraj and M. Mann (2014). "Minimal, encapsulated proteomic-sample processing applied to copy-number estimation in eukaryotic cells." Nature Methods 11(3): 319-324.

Modified (iST) sample prep method:
1. Bring 0.5 mg dried cells or less in a 0.5 ml low binding ep. Wash the cells with 200 ul 0.1 M Tris pH8 (2*) and suspend them in 100 ul 8M urea/0.1 M Tris pH 8 or less. Do NOT use SDS or any other detergent. Prepare a homogeneous suspension e.g. by sonication with water bath sonicator.
2. From the cell suspension, pipette 10ul washed cell suspension into *closed* 200ul stage tips with a double C18 membrane. Manually centrifuge. Sonicate with water bath in hot spot for 1 min. Open tip and flush through.
3. Add 100 ul ABC and flush through.
4. + 100 ul 5% AcNi / ABC and elute through.
5. Reduction: + 20 µL of 50 mM DTT (7.7 mg/ml ABC). 60°C for 30-60 min, elute through.
6. Alkylation: + 20 µL of 50 mM iodoacetamide (9 mg/ml 100mM Tris pH8). 21°C in the dark for 30 min, elute through.

Protein cleanup:
7. + 100 ul ABC, elute through on vacuum station.

8. Do not use the vacuumstation anymore from this point. Put the StageTip into a new 0.5 ml low binding ep. Add 20 µL Trypsin in ABC (100* dil = 5 ng/ul ==> 40 ng) and incubate shaking at rT overnight.
9. Elute through. Add 75 ul 1 ml/l HCOOH in water to the StageTip, elute through. For high protein loads (> 1 ug) stage tips may get obstructed here!
10. Add 5 ul 50% AcNi / 50% 1ml/l HCOOH to the StageTip, elute through, mix low binding ep --> nLCMS ready.

3.2 Filter aided sample preparation (FASP) for maximally 10 ug protein

Modified from ref: Wisniewski, J. R.; Zougman, A.; Nagaraj, N.; Mann, M.: Universal sample preparation method for proteome analysis. Nature Methods (2009) Volume: 6, Issue: 5, Pages: 359-360

1. Sample lysis: Make a concentrated protein sample e.g. by sonicating 20 mg cells in 180 ul ST-lysis buffer in a 1.5 ml low binding Eppendorf tube.
2. Incubate at 95°C (heating block) for 5 min. Cool down and centrifuge at 13 kRPM for 10 min.
3. Measure the protein content of the supernatant using the BCA method described in section 2. Protein determination (BCA) when necessary. Dilute to 1 ug/ul protein when necessary.
4. Pipet 100 ug protein sample (e.g. 100 ul 1 ug/ul) in a new low binding tube, add 0.3 mg of DTT (20 mM) and incubate at 60 C for 1 hour.
5. In a low binding Eppendorf tube (essential!), pipet 450 ul IAA/UT (first) and in the middle add 50 ul of the reduced protein sample in SDT (50 ug protein/500 ul =) and incubate for 1 min while mildly shaking at room temperature.
6. Pipet 10 ug alkylated protein sample (= 100 ul) into a Pall 3K omega filter (10-20kDa cutoff, OD003C34) and centrifuge at 13 kRPM for 30 min. ! Do not centrifuge these filters at a higher speed!
7. Add 100 µl UT solution, gently mix and centrifuge at 13 kRPM for 30 min. Repeat this step 2* to remove all SDS.
8. Add 110 µl of ABC to the filter unit and centrifuge at 13 kRPM for 30 min.
9. Remove the Pall filter cup from its original micro tube and put it into a clean 2 ml low binding Eppendorf tube.
10. Add 100 ul ABC containing 1 ul (0.5 ug) Trypsin. Centrifuge using the “pulse” button up to 3 kRPM (maximum 3 seconds) and incubate gently shaking at room temperature over night (15 h).
11. Centrifuge the filter units at 13 kRPM for 30 min.
12. For 100 ul of sample, acidify to pH 2 to 4 by adding 3.5 ul 10* diluted TFA. The sample is now LCMS ready.

When samples have to be labeled by the Dimethyl labeling method or when you want the absolute maximum amount of peptides in the sample then:
13. Use the acidic ABC eluate for loading onto a C18 µColumn (see section 1.7 General Sample cleanup procedures) and/or Dimethyl labeling (see section 5.1.1 Relative quantitation: on column peptide dimethyl labeling).

3.3 Normal "In solution" trypsin digestion

1. Dissolve 1-10 ug protein in 100 ul 50 mM ABC (pH 8) (10 ug BSA = 0.15 nmol = 5.3 nmol Cys)
2. Add 10 ul 100 mM DTT* dissolved into 50 mM ABC (pH 8.5 with NH3, 1.0 umol). Incubate at 60 ºC for 1 hour (max 2 hours).
3. Check the pH. Make pH 8.5 with 10* diluted NH3. (Usually not necessary!)
4. Add 15 ul 100 mM Iodoacetamide* dissolved into 50 mM ABC (pH 8.5 with NH3, 1.5 umol). Incubate at 20 ºC in the dark for 1 hour exactly.
5. Add 15 ul 125 mM cysteine dissolved into 50 mM ABC to remove the excess Iodoacetamide (1.9 umol) and, for procedure 3.4 only (below), add another 360 ul ABC to decrease the TFE or MeOH concentration to 10%.
6. Add 5 ul trypsin sequencing grade 20* diluted in ABC to 25 ng/ul (125 ng). Incubate gently shaking at room temperature overnight or at 37 °C for 4 h or at 45 °C for 2 h.
7. After digestion, add 2.5 ul 10* diluted TFA to decrease the pH to 2 - 3. Add more 10* diluted TFA when necessary.
8. Perform the sample cleanup with uColumns as described in: 1.7 General sample cleanup procedures with µColumns. In solution digested samples that have not been cleaned by this procedure cannot be measured.

3.4 Methanol and TriFluoroEthanol (TFE) sample preparation method

With Cysteine reduction and CarboxAmidoMethylation (to prevent auto-oxidation) = +57 Da per cysteine.
Ref. Wang, H. X, Qian, W. J.; Mottaz, H. M.; …. , Smith, R. D.: Development and evaluation of a micro- and nanoscale proteomic sample preparation method. Journal of Proteome Research (2005) 4 (6) p2397-2403

3.5.1. Sonicate in a 2 ml low binding ep 1 mg (or less) of the wet cell sample in 99 ul (or less) of either:

a. 50 mM ABC (pH 8)
b. MeOH/50 mM ABC (60:40 v/v)
c. TFE/50 mM ABC (50:50 v/v)
d. detergent containing buffer (→ detergent has to be removed by FASP (see section 3.2. Filter aided sample preparation), by running an SDS gel (also see chapter 4 In-Gel Digestion method) or by a specific detergent removal method like with commercial available SDS removal spin columns (e.g. Pierce HiPPR).

3.5.2. Continue with the reduction and alkylation as above under 3.3.2 – 3.3.8.

3.5 Other methods:

SP3: Hughes, C. S., S. Foehr, D. A. Garfield, E. E. Furlong, L. M. Steinmetz and J. Krijgsveld (2014). "Ultrasensitive proteome analysis using paramagnetic bead technology." Molecular Systems Biology 10(10).

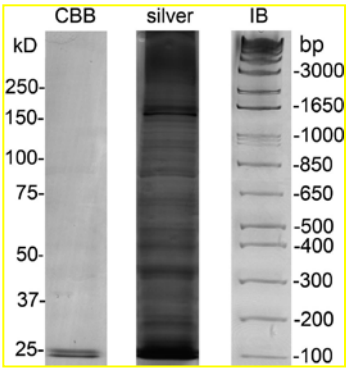
GASP: Fischer, R. and B. M. Kessler (2015). "Gel-aided sample preparation (GASP)-A simplified method for gel-assisted proteomic sample generation from protein extracts and intact cells." Proteomics 15(7): 1224-1229.

Both methods gave poorer results than for iST in my hands ==> Use iST instead.

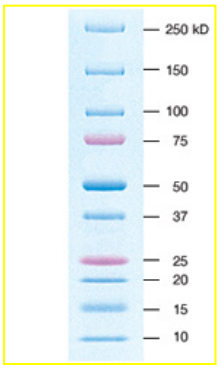
4. In-Gel Digestion method (IGD)

4.1 General info

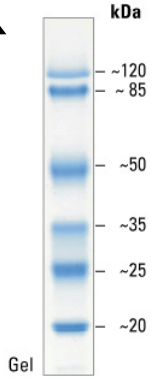
Standard protein gel: 12% bisacrylamide (MW 15 – 200 kDa): e.g. Pierce 12% Precise Tris-HEPES precast gels (10 well) (Fisher PX0025202) or from Invitrogen.



Possible internal markers: 2 ug DNA ladder (1 kb plus, Invitrogen 10488-085) added to the sample. Stain after electrophoresis with "Indoine blue" DNA stain (Sigma R325147) as described by:
Guoan Zhang, David Fenyö, and Thomas A. Neubert: Use of DNA Ladders for Reproducible Protein Fractionation by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) for Quantitative Proteomics.
Journal of Proteome Research 7 (2008) 2, p678-686



Suggested external markers to get an indication of the size of a sample protein:
Protein dual color markers (Bio-Rad Precision plus = Cat.# 161-0374) or
Prestained protein MW marker (Fisher PX0026612) 20, 25, 35, 50, 85, 120 kDa → 10 ul per well



Note: For samples not reduced/alkylated before electrophoresis (as recommended):
Exposure to unpolymerized acrylamide/bis-acrylamide (some of which always remains even in polymerized gels) causes slow alkylation of cysteines (+71.037 adduct)
--> Use commercial gels. Do NOT use freshly prepared gels but at least one day old ones.

Comments (by Andrej Shevchenko):
For highest sensitivity, rinse for 60 minutes or more after the gel has been run and fixed. This helps to keep the background transparent during development.
Do not use glutaraldehyde as the sensitizing agent - it is also a protein cross linking agent!
Ref: Shevchenko A., Wilm, M., Vorm, O. and Mann, M. Anal. Chem. T68T, 850-858 (1996).
Mass spectrometric sequencing of proteins from silver stained polyacrylamide gels.

4.2 Recommended procedure for CCB or Oriole protein gel staining:

1. With the procedure described on the next page, fixation is not necessary. When you still want to fix the proteins, then do so by incubating the entire gel in: 10% acetic acid / 50% water / 40% methanol. Definitely do NOT use cross-linking agents like glutaraldehyde or formaldehyde!
2. Stain either according to the Colloidal Coomassie Staining method (we use the Colloidal Blue Staining Kit which contains Coomassie G-250 (854.02 g/mol), from Invitrogen ordering# LC6025, 150 euro) described below for visible staining or use the Oriole (Bio-Rad 161-0496 ready for use stain) fluorescent stain to observe bands by UV light. Colloidal Coomassie Staining can be used to see more than 50 ng of protein, Oriole can go down to the low ng range.

4.2.1 Colloidal Coomassie Preparing Staining Solution

Shake the Stainer B solution before using it. Prepare the solutions fresh (from top to bottom = keep this order and mix after each addition) as described in the table below in a 50 ml (Greiner) tube. Then directly transfer it to the gel in a new square petri dish.

Solution*	ml per gel
Deionized Water	13.75
Methanol	5
Stainer B	1.25
Stainer A	5

*When Stainer A and Stainer B are combined a precipitate may form which will dissolve within 30 seconds.

4.2.2 Colloidal Coomassie Gel staining procedure

1. Shake gel in 25 ml staining solution in a new square petri dish for 1 - 2 hours.
Note: Staining intensity does not vary significantly if left in stain for 3 hours or 12 hours.
2. Decant staining solution and replace with a minimum of 200 ml of deionized water per gel. Shake gel in water for at least 7 hours. The gel will have a clear background after 7 hours in water.
Note: Gels can be left in water for up to 3 days without significant change in band intensity and background clarity.
3. For long-term storage (over 3 days), keep the gel in a 10% Methanol solution at 4°C.

4.3 In Gel Digestion protocol

4.3.1 Remarks

Proteins visible in a SDS gel after Colloidal Coomassie Staining can be measured by Proxeon nLC-LTQ-Orbitrap MS-MSMS provided you read and follow the remarks concerning Keratin in section 1.3 Reduce the amount of Keratins in your samples as much as possible by… .

4.3.2 Procedure

1. Sample preparation.

a. Make a concentrated protein sample in a suitable buffer e.g. by sonication.
E.g. 50 mg washed cells in 450 ul SDT-lysis buffer pH 8 (= maximally 5 ug protein/ul) or
2 ul 2.0 ug/ul BSA in 398 ul SDT-lysis buffer pH 8 (= 10 ng BSA/ul)
b. Sonicate and incubate at 95°C (heating block) for 10 min. Cool down to room temperature.
Centrifuge at 13 kRPM for 10 min.
e. Pipet 100 ul of supernatant in an ep and add 25 ul of Sample loading buffer (e.g. Pierce 39000 or [50% glycerol/50% water + 1 g/l bromophenol blue]). Incubate shaking at 95°C for 10 min. Centrifuge.
The sample will now have circa 1 - 4 ug protein/ul from 50 mg cells or 8 ng BSA/ul from 2 ug BSA
f. Apply 2 µg (one purified protein) to 60 µg (protein mix) or even **more** protein per sample well.
Each sample well holds maximally 40µL (but then you have to pipet very carefully, 30 ul is more safe).
As a protocol control, use 100 ng of BSA exactly (= 12.5 ul as prepared above).
Also add to one or two wells: 10 ul of (pre-stained) protein Molecular weight marker or 2 ug DNA ladder.

2. Run the gel as described by the manufacturer. For Pierce 12% Precise gels, run at 120V for maximally 55 min = ca 5.5 cm. This is fine for 8 gel slices. If you want to prepare less slices from one sample, then run the gel shorter (ca 5 min per gel slice).
Use gloves. Open the gel cassette with a thin spatula and put it into a large clean square petri dish. Keep the gel covered with the lid as much as possible to prevent extra keratin contamination.
Stain for 1 - 3 hours (see 5.2) and then de-stain with water for 1 to 20 hours. Refresh the water a few times.

Remark: Some (most) in-gel digestion protocols use a 50% acetonitril to wash away the Coomassie but this is not necessary when the sample is measured by LC-MSMS. The Coomassie stain will be released from the protein during the digestion procedure and will elute from the RP column after the peptides at acetonitril percentages higher than 40% but generally is poorly soluble in 1 ml/l HCOOH in water which is used to dissolve the peptides before injection onto the nLC.

3. Cysteines reduction and alkylation.

a. Add 25 ml 50 mM NH4HCO3 + 0.039 g Dithiotreitol (= 10 mM DTT pH 8 *). Gently shake for 1 h at 60 ºC or (better) overnight at room temperature to reduce all disulfide bridges.
b. Wash with water and add 22.5 ml water + 2.5 ml 1M Tris pH 8 + 0.092 g Iodoacetamide (= 20 mM IAcAm pH 8 *). Incubate at room temperature in the dark while gently shaking for 0.5 to maximally 1 hour. Wash with water thoroughly.

4. Gel cutting. (If the gel gets a bit dry and starts jumping around, than add a small drop of water on top of the gel). Cut out the gel bands or slices (1 – 24) and cut them into small pieces of ca. 1 mm^2. Use a sharp clean scalpel from lab 1 (or a 5 ml white tip or a 1 ml blue tip for higher amounts) on a clean piece of parafilm. Transfer the gel pieces to clean 0.5 ml low binding micro centrifuge tubes. Take a negative (no protein containing gel slice) and positive control (100 ng BSA) as well. A reagent control (containing no gel slice) is not necessary when you use the materials from lab 1 and 2 (rooms 2005 and 2008).

5. Enzymatic digestion.

At this point you may freeze + de-freeze the gel pieces to further increase the Trypsin accessible area.
Add 50 ul cold freshly prepared Trypsin solution (5 ng/ul = 100* diluted into ABC). When there is still some gel piece sticking out of the solution, then add extra ABC (but NO Trypsin) to completely cover the gel pieces.
Preferentially incubate overnight while shaking at room temperature (20 °C) or 4 hours at 37 °C or 2 hours at 45 °C.

6. Extraction of peptides.

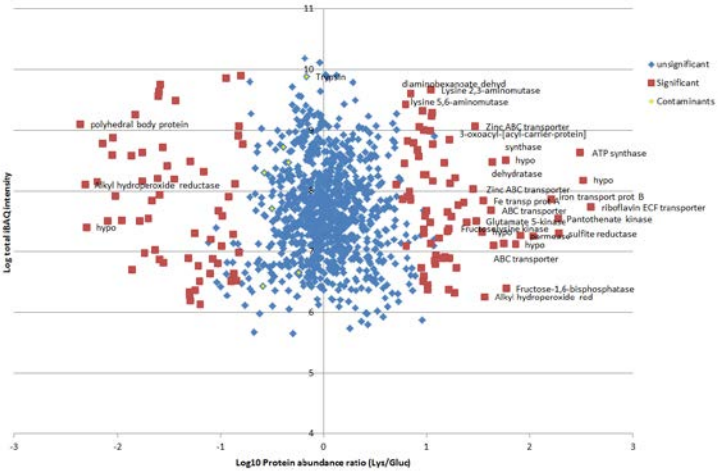
a. Add 10% TFA up to a pH between 2 and 4 (measure the pH with pH paper, ca. 3.5 ul is needed per 50 ul of ABC). Manually sonicate each ep for 1 second in a hot spot in the ultrasonic water bath. When you do not know how to do this, ask someone who does.
b. Perform the µColumn cleanup procedure with a C18 uColumn as described in section 1.7 “General sample cleanup procedures with µColumns”. After loading the first peptide extract, wash the remaining gel with 100 ul 1 ml/l HCOOH in water and add the liquid to the µColumn as well.

The µColumn cleanup step can also be used when you want to concentrate or combine samples. An alternative option to concentrate or combine peptide samples is the Eppendorf concentrator.

5. Quantitation

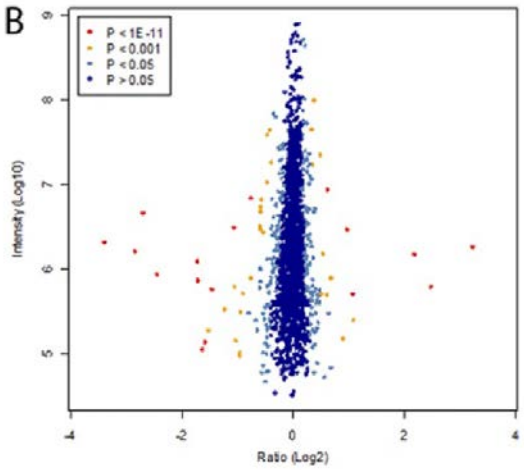
5.1 Relative quantitation (= Sample compared to Control)

Three ways of relative quantitation can be used. The easiest to do is **label free relative quantitation**. In this case, Samples (to be measured at least in triplo, better 4 times or more) and Controls (the same amounts) are measured separately and relative quantitation takes place after calculation of all peak intensities (by MaxQuant) in each chromatogram. This tricky method became feasible due to the high accuracy and low noise of the Orbitrap's MS measurement and release of the MaxQuant software. The statistical program Perseus can be used to find the really significant differences between samples and controls in the MaxQuant result table. The accuracy strongly depends on the sample preparation reproducibility but generally starts from a factor 10 to find significantly different protein concentrations between sample and control.



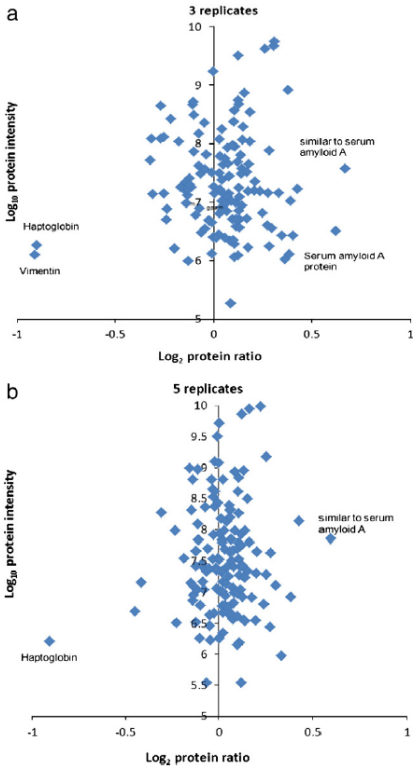
Bui, T. P., J. Ritari, S. Boeren, P. de Waard, C. M. Plugge and W. M. de Vos (2015). "Production of butyrate from lysine and the Amadori product fructoselysine by a human gut commensal." Nat Commun 6: 10062.

SILAC labeling, essential labeled amino acids like arginin and lysin are added in Light, Intermediate and Heavy form, and these labeled amino acids become incorporated during cell growth. Different samples can therefore be mixed in a very early stage, that is directly after cell lysis and protein determination. Here, protein losses occurring during sample preparation will happen for every labeled protein (light/intermediate and heavy), and therefore will not affect the final result. Silac labeling can be regarded as the most accurate relative quantitation method (Accuracy +/- 30%, you may find significantly different protein concentrations between sample and control from a factor 1.5).



Sotoca, A. M., M. D. S. Gelpke, S. Boeren, A. Strom, J. A. Gustafsson, A. J. Murk, I. M. C. M. Rietjens and J. Vervoort (2011). "Quantitative Proteomics and Transcriptomics Addressing the Estrogen Receptor Subtype-mediated Effects in T47D Breast Cancer Cells Exposed to the Phytoestrogen Genistein." Molecular & Cellular Proteomics 10(1).

Sometimes Silac labeling is impossible though labeling may be desired. Then, samples can also be labeled on the peptide level. So far, we have obtained best results with **Dimethyl labeling** of N-terminal amines and lysines. This is a reductive alkylation method that uses formaldehyde (CH_2O, CD_2O or $^{13}CD_2O$) and cyanoborohydride ($NaBH_3CN$ or $NaBD_3CN$) and is described in detail below. Disadvantage of labeling at the peptide level is that almost the complete sample preparation has to be done for each sample separately. Different samples are mixed after completion of the sample preparation which may result in a large error. Fortunately, not all proteins will be up- or down regulated due to the stimulus so an internal control should normally be possible (Accuracy: +/- 50%, you may find significantly different protein concentrations between sample and control from a factor 2).



Lu, J., S. Boeren, S. C. de Vries, H. J. F. van Valenberg, J. Vervoort and K. Hettinga (2011). "Filter-aided sample preparation with dimethyl labeling to identify and quantify milk fat globule membrane proteins." Journal of Proteomics 75: 340.

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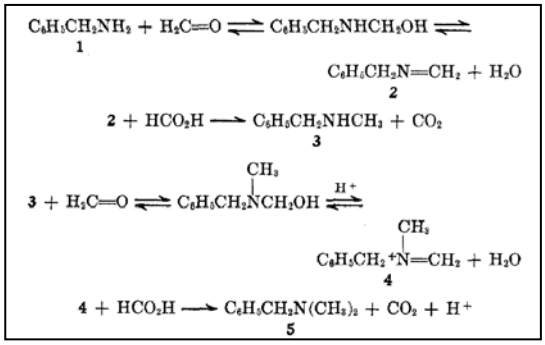
5.1.1 Relative quantitation by on column peptide dimethyl labelling protocol

Ref. Boersema, P.J. et al., Nature Protocols (2009) 4, 4, p484-494: Protocol: Dimethyl labeling for relative quantitation.



Reductive amination of NH2 on N-terminus and lysines with aldehyde:

R-NH2 + 2. H2CO/D2CO + NaBH3CN → R-N[CHD2]2 + CO2 + H3O+

ΔM = + C2H4 = + 28.0313
+ C2D4 = + 32.0564
+ 13C2D6 = + 36.0757

dΔM = 4 / 8 Da per group

Stock solutions

500 mM $NaH_2PO_4.1\ H_2O$	(M= 138)	6.9 g/l	3.45 g / 50 ml
500 mM Na_2HPO_4	(M= 142)	7.1 g/l	3.55 g / 50 ml

35% or 20% (vol/vol) formaldehyde in water (CH_2O, CD_2O or $^{13}CD_2O$)
0.6 M cyanoborohydride in water ($NaBH_3CN$ or $NaBD_3CN$): (M= 64.8) 3.9 mg / 100 ul

Labeling reagent

Prepare per sample/label:
10 ul 500 mM NaH_2PO_4.1 H2O + 35 ul 500 mM Na_2HPO_4 + 425 ul H_2O (= 50 mM pH 7.5)
+ 2.9 ul 35% or 5 ul of 20% (vol/vol) formaldehyde in water (CH_2O, CD_2O or $^{13}CD_2O$).
+ 25 ul of 0.6 M cyanoborohydride in water ($NaBH_3CN$ or $NaBD_3CN$) or 1 mg.
CAUTION Formaldehyde solutions and formaldehyde vapors are toxic, prepare solutions in a fume hood.
CRITICAL Labeling reagent mixtures should be kept at 4 ºC and not stored longer than 24 h to ensure labeling efficiency.

Light:	CH_2O + $NaBH_3CN$
Intermediate:	CD_2O + $NaBH_3CN$
Heavy:	$^{13}CD_2O$ + $NaBD_3CN$

On-column stable isotope dimethyl labeling (TIMING: 1 h):
(i) Make C18+ Stage tips by: Use the “cookie cutter” method to put a small (1.6mm) piece of a C18 Empore disk into a plastic 200 ul tip. Do this by cutting the Empore disk with a large metal needle and transfer it to the 200 ul tip with a good fitting plunger from a syringe. Add 200 ul of methanol to this Stage tip. Add 5 ul of a 50% LichroprepC18 slurry in methanol to the Stage tip to create a C18+ Stage tip.
(ii) Wash the C18+ Stage tip column with 200 ul of methanol.
(iii) Condition the C18+ Stage tip column with 100 ul of 1 ml/l HCOOH in water.
(iv) Load the acidified peptide sample in water (maximally 10 ug peptide, detergent free) on a C18+ Stage tip column.
(v) Wash the C18+ Stage tip column with 100 ul of 1 ml/l HCOOH in water.
(vi) Put 2 ml Eppendorf tubes under the columns to catch reagent that runs through the column.
(vii) In 10 min. time (not faster), flush each of the C18+ Stage tip columns with 100 ul of the respective labeling reagent (light, intermediate or heavy).
CRITICAL STEP To allow for complete labeling, make sure that Step vii takes at least 10 min.
(viii) Wash the C18+ Stage tip columns with 200 ul of 1 ml/l HCOOH in water.
(ix) Add 10 ul 1 M Tris to the eps under the columns to destroy remaining reagent.

Peptide recovery

Manually (= with a syringe) elute and collect the labeled samples in new 0.5 ml low binding eps from the C18 Stage tip columns with 50 ul of 50% AcNi/50% 1 ml/l HCOOH in water.

CRITICAL STEP When performing the protocol for the first time or with a new sample, it is advised to check the labeling efficiency and sample amounts by measuring a fraction of the sample by LC-MS before mixing differentially labeled samples.

For LCMS analysis, reduce the AcNi content by putting the samples in a Concentrator (with open cap) at 45 ºC for 2 hours or longer when necessary. The final volume should be below 20 ul. Adjust the sample volume with 1 ml/l HCOOH in water to exactly 100.0 ul. Sonicate (water bath sonicator) for 5 sec in the hot spot when the sample had been dried completely by accident.
The sample now has <5% AcNi, is pH 3 and is particle free. Mix the samples in the appropriate ratio (light/intermediate/heavy) to make them nLCMS ready.

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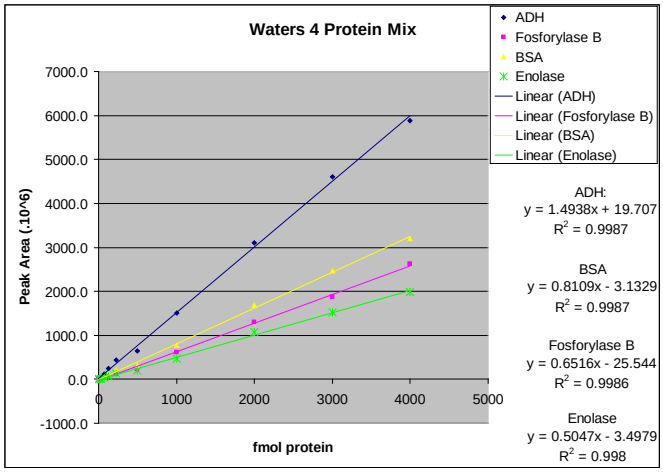
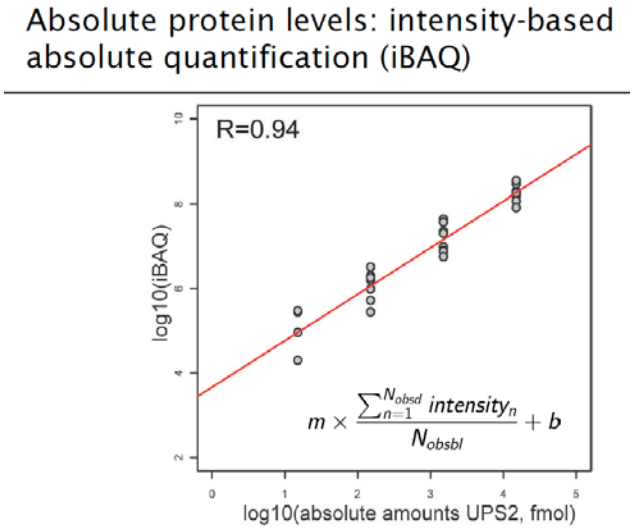
5.2 Absolute quantitation

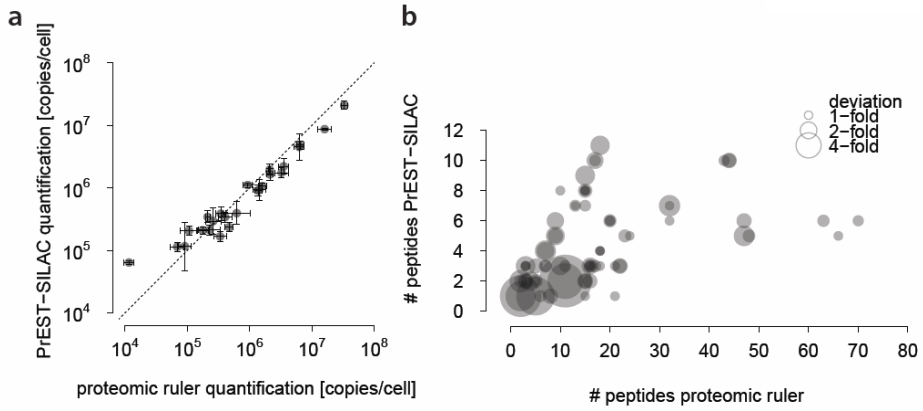
a. Absolute quantitation on 1 or a few proteins can be done by making **calibration curves with synthetic peptides that preferentially contain 1 ^{13}C labeled amino acid**, e.g. the C-terminal K or R in case of a tryptic peptide . To do this, the (HPLC of NMR quantified) labeled peptide has to be added to the sample to generate the calibration curve under exactly the same conditions as the sample measurements. Later, the labeled peptide can also serve as an internal standard. This way of absolute quantitation generally gives a good accuracy (+/- 30%).



b. Less stringent but more practical when more proteins have to be quantitated, is the method of quantitation with respect to a single added internal standard like Ovalbumin (better then BSA). This rough absolute quantitation "**relative to an internal standard**" is accurate within a factor of 4.

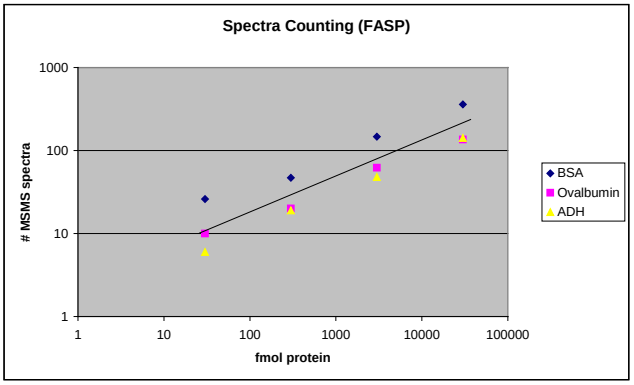
c. Somewhat less accurate but very easy to implement is the "**intensity based absolute quantification (iBAQ)**" method (Schwanhausser et al. Nature 2011, 473, 7347, P337-342) that uses the Total peak intensity as determined by MaxQuant for each protein and corrects that for the number of measurable peptides (=number of tryptic peptides of 7-30 amino acids long without missed cleavages). This method has an error of about a factor 10 as shown in the graph on the right.
This figure comes from a presentation by Selbach during the MaxQuant summerschool 2011. It has not been published.





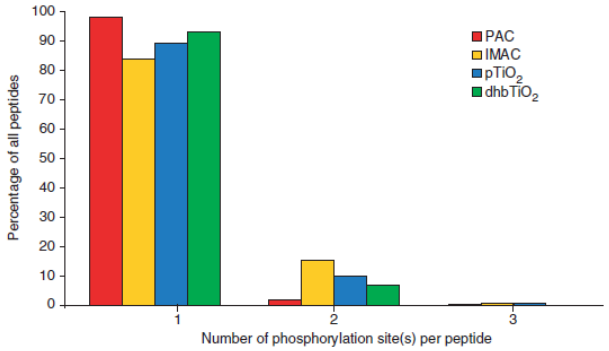
d. For very large datasets obtained by Q-exactive or Fusion (>12.000 peptides), the absolute amount of a protein per cell can be calculated by relating the MS signal intensity from a protein to the total MS signal intensities of all histones observed. Error = a factor 2 only (?).
Wisniewski (2014): A 'proteomic ruler' for protein copy number and concentration estimation without spike-in standards. MCP

e. Less accurate but also very easy is the "**Spectral Counting**" method. It can be applied but has a very large error (upto a factor 20) and is therefore **not advised**. Even the iBAQ method mentioned under c gives better results.

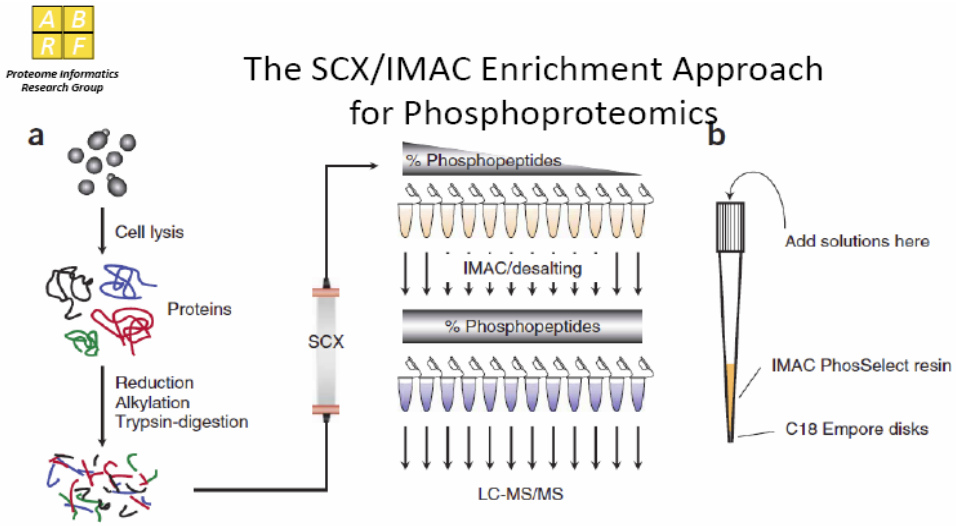


6. Phosphopeptide enrichment methods (S and T only).

Serine, Threonine: TiO2 will yield more mono phosphorylated S + T peptides than IMAC.
IMAC will yield relatively more doubly phosphorylated S + T peptides than TiO2
Tyrosine: Use specific anti-bodies (e.g. Cell Signaling PhosphoScan kit P-Tyr-1000 #8803S)
RP LC coupled to electrospray ionization will mainly show singly and doubly phosphorylated peptides.
Maldi ionization may give more multiply phosphorylated peptides but also Maldi is less sensitive to multiple phosphorylated peptides.



B. Bodenmiller, L. N. Mueller, M. Mueller, B. Domon and R. Aebersold Reproducible isolation of distinct, overlapping segments of the phosphoproteome.
Nature Methods (2007) 4, 3, p231-237.



Sample: 7.5x10e7 human K562 human chronic myelogenous leukemia cells, 4mg lysate
Protocol: Villen, J, and Gygi, SP, Nat Prot, 2208, 3, 1630-1638.
Lysis: 8M urea, 75mM NaCl, 50 mM Tris pH 8.2, phosphatase inhibitors
SCX: PolyLC - Polysulfoethyl A 9.4 mm X 200mm, elute: 0-105mM KCl , 30% Acn .
IMAC: Sigma - PhosSelect Fe IMAC beads, bind: 40% Acn, 0.1% formic acid, elute: 500 mM K_2HPO_4 pH 7
MS/MS: Thermo Fisher Orbitrap XL, high-res MS1 scans in the Orbitrap (60k), Top-8 fragmented in LTQ, exclude +1 and precursors w/ unassigned charges, 20s exclusion time, precursor mass error +/- 10 ppm

A protocol often used:

B. Macek, M. Mann and J. V. Olsen: Global and Site-Specific Quantitative Phosphoproteomics: Principles and applications. Annual Review of Pharmacology and Toxicology (2009) 49, p199-221.

6.1 Phosphopeptides (S, T) enrichment by Titanium Dioxide (TiO2) Chromatography.

6.1.1. DHB free phosphopeptide enrichment with TiO2.

Aryal, U. K. and A. R. S. Ross (2010). "Enrichment and analysis of phosphopeptides under different experimental conditions using titanium dioxide affinity chromatography and mass spectrometry." Rapid Communications in Mass Spectrometry 24(2): 219-231.
Zhou, H. J., T. Y. Low, M. L. Hennrich, H. van der Toorn, T. Schwend, H. F. Zou, S. Mohammed and A. J. R. Heck (2011). "Enhancing the Identification of Phosphopeptides from Putative Basophilic Kinase Substrates Using Ti (IV) Based IMAC Enrichment." Molecular & Cellular Proteomics 10(10).
Humphrey, S. J., S. B. Azimifar and M. Mann (2015). "High-throughput phosphoproteomics reveals in vivo insulin signaling dynamics." Nat Biotechnol 33(9): 990-995.

Modified procedure:
1. To 90 ul of the centrifuged digested peptide solution (Mix after each addition):
2. Add 10 µL TriFluoroEthanol
3. Add: 114 µL Acetonitril → samples may become slightly turbid
4. Add: 14 µL TriFluoroAcetic acid → 50% ACN, 6% TFA
5. Mix peptide solutions at room temperature for 1 min in a ThermoMixer at 300 rpm.
6. Centrifuge at high speed (≥16,000 xg for 15 min).

7. Prepare ca 5 mm uColumns with 10 ul TiO2 beads slurry (50% beads in 100% MeOH, sonicated, GL Sciences #5010-21315) pipetted into 200 ul AcNi using a 200 ul tip fitted with a C8 filter. With 5 mg TiO2 beads you can use 25 to 50 ug of peptide solution te be enriched.
8. Wash the TiO2 column with 100 ul ultrapure AcNi (e.g. HPLC gradient grade).
9. Equilibrate the TiO2 column with 200 ul Loading buffer (80% ACN, 6% TFA).
10. Add sample to the TiO2 column and slowly elute in 5 min at 18 bar Hg.
11. Non-specifically bound peptides are washed from the TiO2 beads with:
12. 1* 200 ul Loading buffer (80% ACN, 6% TFA) and
13. 2* 200 µL Wash buffer (60% ACN, 1% TFA). In the l**ast step, elute until the column runs just dry.**

14. Elute Phosphopeptides into low-binding tubes slowly with 50 µL Elution buffer (40% ACN, 15% NH_4OH (25%, HPLC grade) added, prepare immediately before use). Pulsed (20 ul, 30 ul) with 5' on shaker in between pulses.
15. Concentrate samples in a concentrator for 30 min at 45°C.
16. Add 10 µL 10% TFA or more until the samples are acidic.
17. Perform the general C18 uColumn cleanup as in 1.7.

6.1.2. Phosphopeptide enrichment with TiO2 with DHB.

Larsen, M. R., T. E. Thingholm, O. N. Jensen, P. Roepstorff and T. J. D. Jorgensen (2005). "Highly selective enrichment of phosphorylated peptides from peptide mixtures using titanium dioxide microcolumns." Molecular & Cellular Proteomics 4(7): 873-886.

25DHB = 2,5-diHydroxy Benzoic acid, MeOH = methanol, AcNi = acetonitril

1. Dry tryptic peptide samples in a concentrator.
2. Prepare ca 5 mm uColumns with 10 ul TiO2 beads slurry (50% beads in 100% MeOH, sonicated, GL Sciences #5020-75010 or better 5010-21315) packed in a 200 ul tip fitted with a C8 filter. With 5 mg TiO2 beads you can use 25 to 50 ug of peptide solution te be enriched.
3. Wash column with 100 ul ultrapure AcNi (e.g. HPLC gradient grade).
4. Equilibrate with 100 ul loading buffer which is 50 mg/ml 25DHB (slightly yellow powder, Fluka, or D33 in Willems lab) in 80% acetonitrile, 1% TFA.
5. The column will turn orange in this step.

6. Dissolve dried samples in: 100 ul loading buffer (50 mg/ml 25DHB in 80% acetonitrile, 1% TFA).
7. Load samples onto the equilibrated TiO2 microColumn.
8. Wash with 100 ul of loading buffer (to remove non-phospho peptides).
9. Wash with 200 ul of wash buffer (80% acetonitrile, 1% TFA without DHB) to remove all 25DHB.
10. Elute with 50 ul Elution buffer (40% ACN, 15% NH4OH (25%, HPLC grade) added, prepare immediately before use). Pulsed (20 ul, 30 ul) with 5'on shaker in between pulses.
11. Concentrate samples in a concentrator for 30 min at 45°C.
12. Add 10 µL 10% TFA or more until the samples are acidic
13. Perform the general C18 uColumn cleanup as in 1.7

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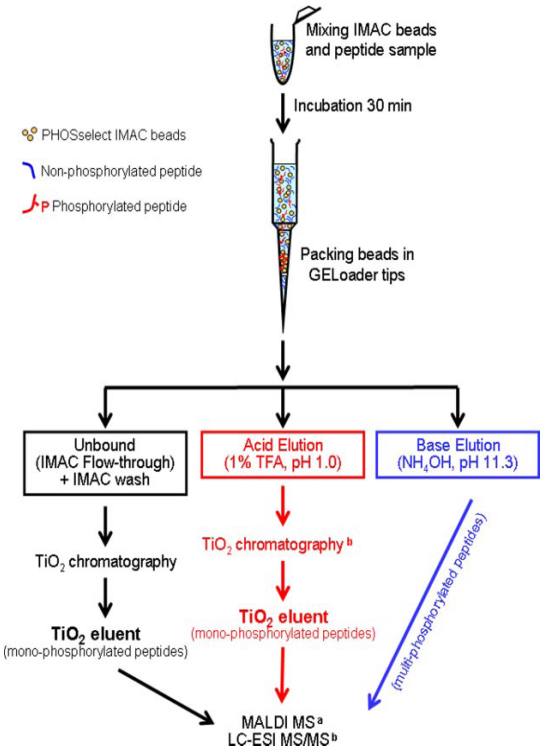
6.2 Phosphopeptide (S,T) sample preparation by sequential elution from IMAC.

Uses the Pierce Fe-NTA Phosphopeptide Enrichment Kit: PX0088300



Ref: Thingholm, T.E., Jensen, O.N., Robinson, P.J. and Larsen, M.R. (2008) SIMAC (sequential elution from IMAC), a phosphoproteomics strategy for the rapid separation of mono phosphorylated from multiply phosphorylated peptides.
Molecular & Cellular Proteomics 7(4), 661-671.

This protocol uses a step elution procedure to separate multi-phosphorylated peptides from mono- and di-phosphorylated peptides. Mono- and di- phosphorylated peptides are made free of non-phosphorylated peptides by using TiO2 columns.

A. Sample Preparation
1. Perform a tryptic digestion, preferentially using the FASP protocol (see 3.2. Filter aided sample preparation (FASP).
2. Quick dry the peptide samples in a rotary vacuum concentrator.
3. Resuspend the dried sample to a concentration of 1-10 ug/ul in Loading Buffer (= 0.1% TFA, 50% AcNi).
4. Wash the Fe-NTA spin column (which contains 200 ul slurry) with 2 * 500 ul of Loading Buffer.
5. Add maximally 5 mg peptides to a washed Fe-NTA spin column and incubate for 20 minutes at room temperature with end-over-end rotation. Remove bottom tab of the column. Place column in a microcentrifuge tube.
6. Centrifuge column at 2,000 rpm for 2 minutes Collect the flow-through for analysis = **non-bound**. Transfer the column to a new collection tube.

B. Wash and elute from IMAC

Wash with 150 ul Loading buffer	= 0.1% TFA, 50% AcNi	Flow through = Sample **A**
Wash 2* with 150 ul Wash buffer	= 1 % TFA, 20% AcNi	Flow through = Sample **B**
Wash with water.		

C. Elute with 100 ul ammonia water, pH 11.3 (10 ul of 25% ammonia solution in 490 ml of MilliQ water) and decrease the pH to around 3 with 10% TFA. Flow through = Sample **C**

Samples non-bound, A and B contain non-phosphorylated peptides and abundant mono-phosphorylated peptides. Sample C contains by far most mono and multi-phosphorylated peptides.

Dry sample C in a vacuum evaporator at 45 °C for 2 - 4 h. Sample C can be dissolved into 100 ul 1 ml/l HCOOH to be analyzed by LCMS.

When you want to see everything: Titanium Dioxide (TiO2) Chromatography for Samples unbound, A and B.
Dry all samples in vacuum evaporator at 45 °C for 2 - 4 h. Unbound, A and B.

Perform the TiO2 chromatography step to samples A and B obtained at step 7.2C to get rid of non-phosphorylated peptides as described under 7.1 Phosphopeptides enrichment by Titanium Dioxide (TiO2) Chromatography.

Dissolve dried samples (unbound, A and B) in 100 (A, B) or 1000 (unbound) ul 1 ml/l HCOOH.
Samples are now nLCMS ready.