Sample preparation for proteomics by MS

Sjef.Boeren@wur.nl, Laboratory of Biochemistry, Wageningen UR

WAGENINGEN UNIVERSITY & RESEARCH
Biochemistry Lab.

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Our next PhD Proteomics **course** (including sample prep) may take place in 2022 in Wageningen, the Netherlands.

Previous 2019 course info:
https://www.vlaggraduateschool.nl/en/courses/course/Prot19.htm

1. General information

1.1 pH stuff and more

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% Sodium DeoxyCholate (DC) can be used to **extract hydrophobic proteins** to replace 4% SDS:
Pasing, Y., S. Colnoe and T. Hansen (2017). "Proteomics of hydrophobic samples: Fast, robust and low-cost workflows for clinical approaches." Proteomics **17**(6).
Schmidt, A., K. Kochanowski, S. Vedelaar, E. Ahrne, B. Volkmer, L. Callipo, K. Knoops, M. Bauer, R. Aebersold and M. Heinemann (2016). "The quantitative and condition-dependent Escherichia coli proteome." Nature Biotechnology **34**(1): 104-110.

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1.2 Abbreviations and solutions

		Find it in:	MW			in
AA	**AcrylAmide (Wear gloves)**	lab 3030	71	20 mM = 1.4 mg/ml	200 mM = 14 mg/ml	water
AcNi	Acetonitril	lab 3030				
ABC	Ammonium BiCarbonate	lab 3030	79	50 mM = 0.2 g	/ 50 ml	water
AmAc	Ammonium Acetate	lab 3030	77	10 mM = 38.5 mg	/ 50 ml	water
Cystein	Fluka 30090, >99%	lab 3030	121	125 mM = 15 mg/ml	200 mM = 24 mg/ml	water
DTT	**Dithiotreitol (4°C)**	FRESH! (refrig)	154	20 mM = 3.1 mg/ml	150 mM = 23 mg/ml	water
IAA	Iodoacetamide (4°C)	FRESH! (refrig)	185	20 mM = 3.7 mg/ml	200 mM = 23 mg/ml	water
TFA	TriFluoro-Acetic acid	lab 3030	Safety cupboard MS stuff		dilute in fume cupboard only	
TCEP	Tris(CarboxyEthyl)Phosphine	-20°C	287		100 mM = 29 mg/ml	water
Tris	Stock 10*. pH 8 with HCl	weighing room	121	1 M = 121 g/l	6.0 g / 50 ml	water
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 unless stated otherwise					
Urea	Make FRESH	weighing room		Do not warm up		Tris
UT	8 M urea (Sigma, U0631)	weighing room	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**		

- 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

1.3 Tips and ways to reduce the amount of Keratins in your samples.

1. **DO:** Use commercial electrophoresis gels. They contain less keratin than your own ones.
   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 large petri dish for staining.
2. Use Nitril gloves, not Latex ones.
3. Wash your hands under running tap water before you start and as often as possible in between handlings.
4. Do not work in a standard flow cabinet intended for microbiological work. Due to the large air flow, 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 of protein.
8. Use new (or only used for proteomics) throw away (polypropylene) plastics.
8. Do not use hand creams when preparing samples for MS.
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.
13. Clean your pipets before use. Especially when you have pipetted detergent (Triton X100, Tween 80 etc) with them.

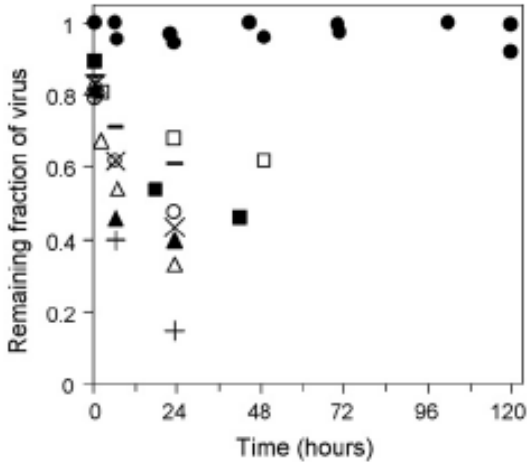

Fig. 5. Virus loss in different containers (20 mM HEPES, 150 mM NaCl, pH 7.8; initial Ad5 concentration was approximately 6×10^{10} p/mL ≈ 0.02 g/L): (●) LoBind™ protein tube (Eppendorf), (□) Fisherbrand 0.5 mL tube, (▬) Glass (National Scientific), (■) Fisherbrand PCR tube, (◯) polycarbonate tube (Eppendorf),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

Eppendorf LoBind tubes bind much less viruses then other brands of 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	**LC-MS/MS by Easy nLC1000 → Q Exactive HF-X**				
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 (Not all software mentioned has been tested by me)	Overview of GO tools (but not complete): http://geneontology.org/ --> Tools. 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 **GO enrichment analysis** can be done within: - Internet resources like DAVID (Database for Annotation, Visualization and Integrated Discovery, https://david.ncifcrf.gov/, 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/ 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.

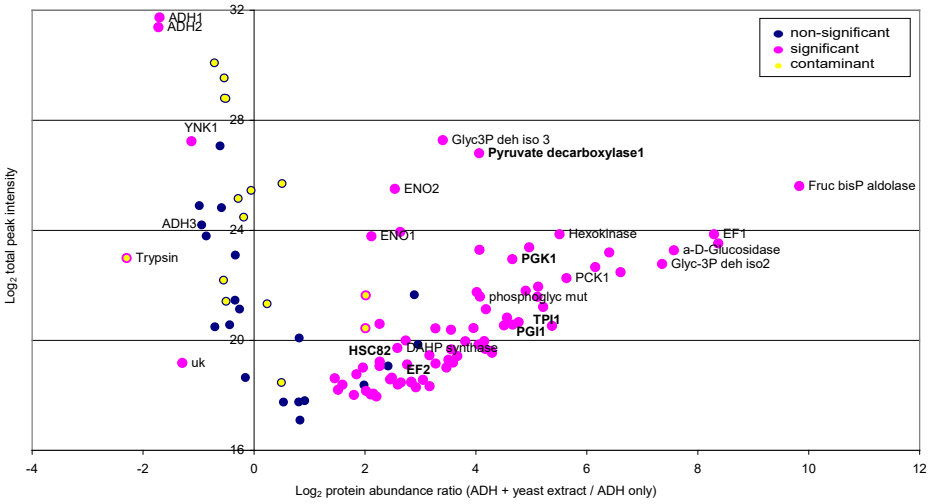

Table 1 | Comparison of spin filter, short SDS-PAGE and TFE methods

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.

Table 1 was 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/PAC	50/60	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)	40 - 80	Yields are rather variable.

1.6 nLC-MSMS sample necessities.

Hand in minimally 25 ul acidic peptide sample (pH 2 – 4) with a concentration of approximately 50 – 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 (3 or 5 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 + Lichrosorb 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). Do this a second time. Transfer the 2 frits 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 lab3030. 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 15 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. Peptide concentrations can be measured e.g. using Pierce Quantitative Colorimetric Peptide Assay (23275) when the concentration is high enough.

Remark 1: 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.
Remark 2: µColumns can be step eluted at pH 10 with 50 ul of a step gradient of AcNi [e.g 5, 8, 11, 18 and 25% AcNi with 20 mM NH_4Formate pH10] to **fractionate** peaks according to their high pH affinities for the C18 column.
Fractions 1 + 4 (5 + 18%) and 2 + 5 (8 + 25%) can be mixed to decrease the number of samples to be injected to 3.
Better: fractionate extensively and mix fractions concatenated: 1+6+11, 2+7+12 etc. (Yang, F., Y. F. Shen, D. G. Camp and R. D. Smith (2012). "High-pH reversed-phase chromatography with fraction concatenation for 2D proteomic analysis." Expert Review of Proteomics 9(2): 129-134).

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 et.al. (2012) Methionine Oxidation Enhances kappa-Casein Amyloid Fibril Formation. Journal of Agricultural and Food Chemistry 60(16), 4144-4155).

Sjef.Boeren@wur.nl, Laboratory of Biochemistry, Wageningen UR

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2. Protein determination (BCA).

by the Bicinchoninic Acid (BCA) method (Pierce:#23225):
(linear sensitivity: 2 – 20 ug protein as measured at 562 nm in a 96 well microplate)

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).

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.

BCA Solutions:

BCA working solution (BCA ws): 12.5 ml reagent A + 0.25 ml reagent B
Standard protein BSA: 2.0 mg/ml (ThermoFisher 23210)

When you made it yourself, measure the exact concentration BSA by measuring the absorbance at 280 nm (blanc is water):

$C_{BSA} = A_{280} * 1.50$ (mg/ml)
$C_{ovalbumin} = A_{280} * 1.30$ (mg/ml)

This measurement is necessary since purified proteins always contain some salts.

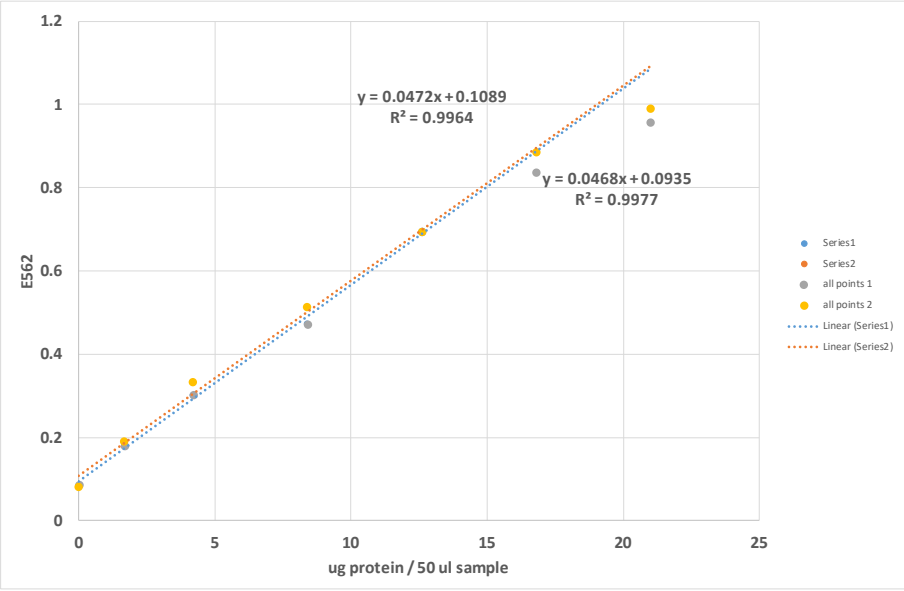
Method:

1. Pipet indicated volumes shown in bold in the table below into 0.5 ml low binding eppendorf micro tubes. Mix.
2. **Pipet 50 ul of the BSA standard or sample directly in a 96 well plate.**
3. **Add 200 ul of the BCA working solution**. Make sure samples are mixed well in the plate and seal the plate tightly with parafilm. Incubate (at 21 ºC for 60 min or) at 37 ºC for 30 min or more.
4. Measure the A_{562} in the micro plate reader you can find in room 3072.

	Dilute Cal curve into a 0.5 ml LB tube				
	Volume Water (ul)	**Volume BSA (ul)**	BSA amount (ug/50ul)	Measured A562 (1)	Measured A562 (2)
Cal 1	**500**	**0**	0	0.086	0.082
Cal 2	**490**	**10**	2	0.18	0.19
Cal 3	**190**	**10**	5	0.302	0.332
Cal 4	**180**	**20**	10	0.473	0.514
Cal 5	**170**	**30**	15	0.695	0.693
Cal 6	**160**	**40**	20	0.837	0.886
Cal 7	**150**	**50**	25	0.957	0.989
Sample	**40**	**10**		0.626	0.633

Example measurement:

When the BSA was prepared and dissolved at 2 mg/ml. The absorbance at 280 nm was measured to be 1.118



Therefore the real concentration was:
1.118 * 1.50 = 1.64 mg/ml
Adjust x-axis with factor 1.64 / 2

ug protein in the 50 ul well =
$[E_{562} - 0.10] / 0.047$ =

([0.626+0.633]/2-0.10) / 0.047

⟹11.3 ug / 50 ul * 50/10

= 1.13 ug/ul

Sjef.Boeren@wur.nl, Laboratory of Biochemistry, Wageningen UR

3. Gel free protein digestion methods.

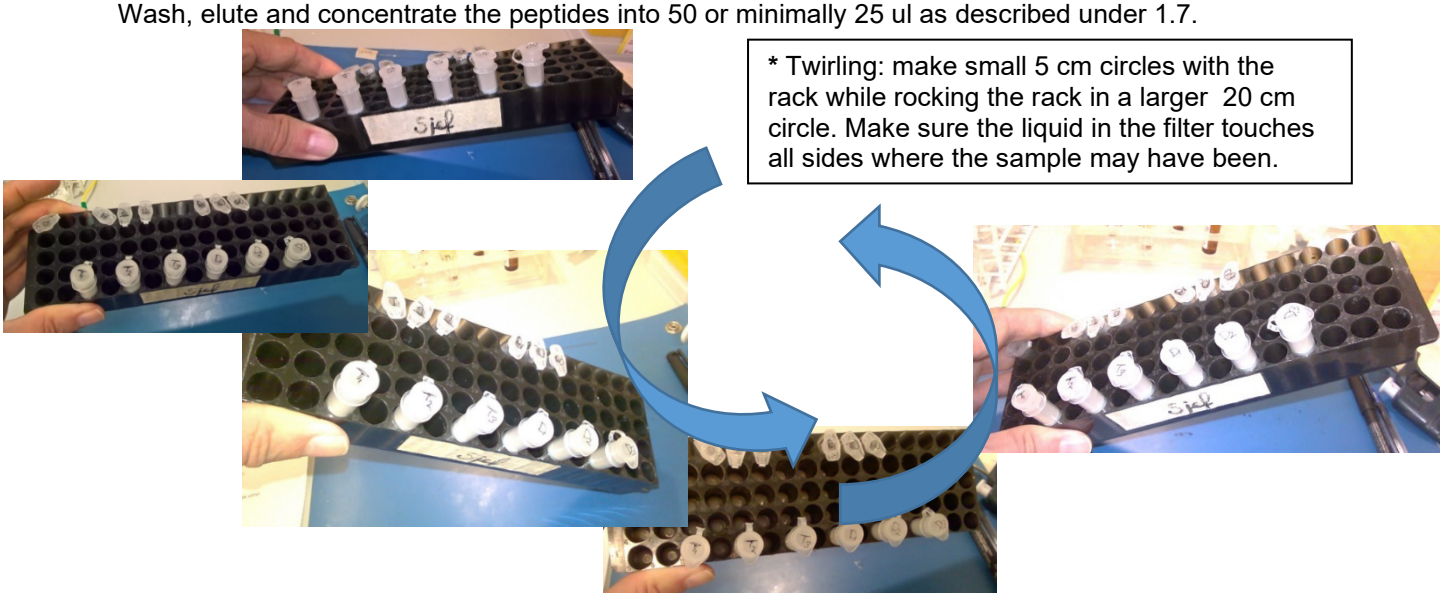
3.1 Filter aided sample preparation (FASP, 40 ug protein[Rem1], easy and reliable)

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

Remark 1: 40 ug protein will result in 0.2 ug/ul peptides and is intended to be used with the standard 1 hour gradiënt.
Remark 2: All centrifugation steps should be done at 12 kRPM in the Eppendorf 5424 centrifuge in lab 3030 (≤14000 *g)
Remark 3: Do check whether the filter is liquid free after each centrifugation step. When not, continue centrifugation until the filter is liquid free.

1. Before use, **clean the Pall nanosep filters** (Pall 3K omega (10–20 kDa cut-off, OD003C34) by adding 650 ul ethanol and centrifuge at 12 kRPM for 45'.
2. **Prepare BCA solutions** as shown in Chapter 2 Protein determination on p5.
3. **Sample lysis**: make a concentrated protein sample.
   e.g. by sonicating 10 mg wet weight (washed) cells in 100 ul 100 mM Tris pH8 in a 0.5 ml low binding Eppendorf tube for 30 sec with a probe sonicator with a thoroughly cleaned metal tip. (V=100 ul)
4. **Measure the protein content** *in duplo* using the BCA method (See chapter 2) with e.g. 2 * 10 ul sample.
   Do the BCA incubation (at 37 °C) and measurement during the reduction and alkylation incubation times of the next 2 steps. (V=80 ul)
5. **Reduction:** Add 10% of the volume of 150 mM dithiothreitol (M=154: 150 mM=23 mg/ml water)
   here: + 8 ul --> 14 mM (V=88 ul)
   Incubate at 45 °C for 30 min in a thermomixer. Immediately cool to 20 °C afterwards.
   (When using Deoxycholate, DTT reduction will make the solution slightly turbid because of some DC precipitation.)
6. **Unfold:** Add 2* the volume of fresh prepared and cool 100 mM Tris/HCl pH 8.0 + 8 M urea (0.48g/ml) in a low binding Eppendorf tube. here: + 176 ul (V=264 ul)
7. **Alkylation**: Add 10% of the volume of 200 mM AcrylAmide (M= 71: 14 mg/ml acrylamide in water)
   here: + 26.4 ul --> 18 mM (V=290 ul)
   Incubate for 10 min while mildly shaking at room temperature.
   In the meantime, measure the BCA protein determination 96 well plate.
8. **Transfer** 40 ug alkylated protein sample as found in the BCA protein determination (e.g. 145 ul) to an ethanol washed Pall filter cup.
   Pipet directly on the membrane in the middle without touching the filters poly-propylene side.
   Centrifuge at 12 kRPM for 45 min.
9. **Wash 1**: Take all samples out of the centrifuge. Check filter content. Remove the liquid from the ep and add 150 µL 50 mM NH_4HCO_3 in water (ABC) to the filter unit. **Twirl*** the ABC around over the filter. Centrifuge: 12 kRPM for 45'.
10. **Wash 2**: Take all samples out of the centrifuge. Check filter content. Remove the liquid from the ep and add 100 µL 70% ethanol/30% ABC to the filter unit. **Twirl*** the liquid around over the filter. Centrifuge at 12 kRPM for 30 min.
11. Remove the Pall filter cup from its original micro tube and put it into a clean **2 ml low binding Eppendorf tube**.
12. **Digestion**: Add 100 µL 100* diluted Trypsin/ABC (5 ng/ul) to the filter and incubate overnight while mildly shaking at room temperature on the shaking platform in the fume hood of lab 3030.
13. **Centrifuge** the peptides through the membrane at 12 kRPM for 30min.
14. Add 100 ul 1 ml/l HCOOH in water, twirl* over the filter. Centrifuge at 12 kRPM for 30 min.
15. Add 10% trifluoroacetic acid (ca 3 ul) to the filtrate to adjust the pH of the sample to around pH 3.
16. Store samples in a freezer until they will be measured.

For peptide concentrations below 50 ng/ul: samples can be concentrated 4-8 times without losses:
17. Load all 200 ul sample onto a C18 µColumn (see section 1.7 General Sample cleanup procedures on p4), Wash, elute and concentrate the peptides into 50 or minimally 25 ul as described under 1.7.



***** Twirling: make small 5 cm circles with the rack while rocking the rack in a larger 20 cm circle. Make sure the liquid in the filter touches all sides where the sample may have been.

Sjef.Boeren@wur.nl, Laboratory of Biochemistry, Wageningen UR

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3.2 Protein aggregation capture (PAC, 10 - 100 ug protein)

PAC generally yields more hydrophobic peptides than FASP. This results in more quantified proteins.

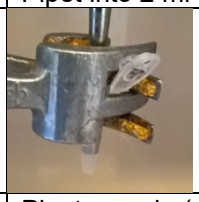
Batth, T. S., M. A. X. Tollenaere, P. Ruther, A. Gonzalez-Franquesa, B. S. Prabhakar, S. Bekker-Jensen, A. S. Deshmukh and J. V. Olsen (2019). "Protein Aggregation Capture on Microparticles Enables Multipurpose Proteomics Sample Preparation." Molecular & Cellular Proteomics 18(5): 1027-1035.

Dagley, L. F., G. Infusini, R. H. Larsen, J. J. Sandow and A. I. Webb (2019). "Universal Solid-Phase Protein Preparation (USP3) for Bottom-up and Top-down Proteomics." Journal of Proteome Research 18(7): 2915-2924.

Modified SP3 from: 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).

Tip: Perform all washes by removing the eppendorf tubes from the magnetic rack and short mixing on a whirlmixer (originally not to be removed). Pulse centrifuge the eps to remove liquid from the cap before removing the liquid from the bottom of the ep.

SpeedBeads (magnetic carboxylate modified particles) = GE Healthcare 45152105050250 + 65152105050250 of 50 ug/uL. For e.g.12 samples, prepare 120 ul taking 60 ul of each type, mix and wash 2* with 1 ml water and re-suspend into 120 ul water.

Step						Total volume
1 Prepare	Pipet into 2 ml low binding ep	5 ul of each speedbead type per sample				
2	 Prepare a concentrated protein extract e.g. by: bring 4 mg wet weight cells (~40 ug protein), from 4 ml culture with an $OD_{600} \geq 0.3$) or more in two 2 ml low binding eps (LB ep). Add 200 ul ice cold 100 mM Tris buffer pH8, mix and centrifuge 1 min at 2 kRPM. Again, add 200 ul ice cold 100 mM Tris buffer pH8, mix and centrifuge 1 min at 2 kRPM. Suspend all of the cells in 50 ul 100 mM Tris pH 8. Sonicate for 15 s at optimal power + frequency. Longer times may melt the ep.					
3	Pipet sample (40 ug protein) In a 2 ml LB tube		µl	20 ul sample	60 ul sample	
4	100 mM Tris pH 8		µl	40	0	Vt = 60 ul
5 **Reduction**	150 mM DTT (C=14 mM)	M=154: 23 mg/ml	µl	10% of the Volume (here 6 ul)		Vt = 66 ul
6	Incubate at 45 °C for 30', mix	In the meantime: prepare fresh 8M urea: 0.48 g/ml in 100 mM Tris pH8				
7 **Unfold**	8 M urea / 100 mM Tris	M=60: 0.48 g/ml	µl	3 * total Volume (here 198 ul)		Fresh, cool Vt=264 ul
8 **Alkylation**	200 mM AcrylAmide (18 mM)	M= 71: 14 mg/ml	µl	10% of the Volume (here 27 ul)		Vt=291 ul
9	Incubate at 21 °C for 30'	In the meantime: wash SpeedBeads 2* in water (see top)				
10	**Check pH, adjust to 7**	+ 10% TFA	µl	ca 1.25 - 1.5% (here 4 ul)		Vt=295 ul
11 **PAC**	SpeedBeads	50 ug/ul	µl	8		Vt=303 ul
12	AcNi		µl	2.5 * total Volume (here 750 ul)		
13	Incubate shaking for 20' at rT					
14	Place tubes on a magnetic rack and allow to separate for 30 seconds. Remove supernatant.					
15 **Wash**	Wash 1 70% ethanol			1000		
16	Wash 2 100% acetonitril			1000		
17 **Digestion**	100* diluted Trypsin / ABC	5 ng/ul in ABC	µl	100		
18	Incubate overnight while shaking at room temperature					
19	Add 10% TFA	(pH = 3)	µl	3 - 4 ul		
20	Prepare C8 filters in 200 ul tips					
21	Pulse centrifuge the samples and put onto magnet. Tilt samples with the liquid towards the magnet for 20 s. Transfer the liquid to a double C8-Filter-Tip. Wash the beads 1* with 100 ul 1 ml/l HCOOH (flush over the beads) without removing the ep from the magnet and add all liquid to the C8 filter as well.					
22 **Filter**	Filter all sample through a double C8-Filter into a 0.5 ml low binding ep. Essential step: Wash/elute the filter with 15 ul 50% AcNi/50% 1ml/l HCOOH in water into the same ep.					
23	Dry of most of the AcNi/water in the concentrator until a volume of 10 to 15 ul					
24	Adjust the final volume to exactly 50.0 µl with 1 ml/l HCOOH (25.0 µl when your protein starting amount was low)					
25	Inject onto nLC-MSMS		µl	Minimally 0.5 - Maximally 5		

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 5 ul 150 mM DTT dissolved into 50 mM ABC (pH 8.5 with NH3, 1.0 umol). Incubate at 45 ºC for 1 hour.
3. Check the pH. Make pH 8.5 with 10* diluted NH3. (Usually not necessary!)
4. Add 7.5 ul 200 mM AcrylAmide dissolved into 50 mM ABC (pH 8.5 with NH3, 1.5 umol). Incubate at 20 ºC in the dark for 0.5 hour exactly.
5. Add 8 ul 200 mM cysteine dissolved into 50 mM ABC to remove the excess acrylamide.
6. For procedure 3.4 only (below), add another 360 ul ABC to decrease the TFE or MeOH concentration to 10%.
7. Add 5 ul trypsin sequencing grade 20* diluted in ABC to 25 ng/ul (125 ng).
8. Incubate gently shaking at room temperature overnight or at 37 °C for 4-6 h or at 45 °C for 2-3 h.
9. After digestion, add 2.5 ul 10* diluted TFA to decrease the pH to 2 - 3. Add more 10* diluted TFA when necessary.
10. 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

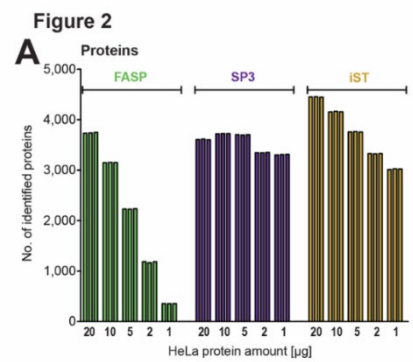
3.4.1. Sonicate in a 2 ml low binding ep 1 mg (or less) of the wet cell sample in 99 ul (or less) of either:

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 3.1. 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).

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

3.5 Other proteomics sample prep 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).
Improved SP3: Moggridge, S., P. H. Sorensen, G. B. Morin and C. S. Hughes (2018). "Extending the Compatibility of the SP3 Paramagnetic Bead Processing Approach for Proteomics." J Proteome Res 17(4): 1730-1740.
Improved SP3: Sielaff (2017). "Evaluation of FASP, SP3, and iST Protocols for Proteomic Sample Preparation in the Low Microgram Range." J Proteome Res 16(11): 4060-4072 with the figure (2A) shown below:



iST: 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.

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 SP3 and GASP gave poorer results than PAC in my hands.

STrap: Ludwig, K. R., M. M. Schroll and A. B. Hummon (2018). "Comparison of In-Solution, FASP, and S-Trap Based Digestion Methods for Bottom-Up Proteomic Studies." J Proteome Res **17**(7): 2480-2490.
Zougman, A., P. J. Selby and R. E. Banks (2014). "Suspension trapping (STrap) sample preparation method for bottom- up proteomics analysis." Proteomics **14**(9): 1006-1010.

SB: STrap uses four C18 plugs + five MK360 quartz plugs = fiber glass. Fibre glass may give a large protein loss but this special pure glass still needs to be tested.

Sjef.Boeren@wur.nl, Laboratory of Biochemistry, Wageningen UR

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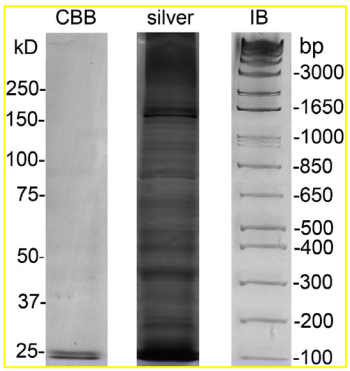
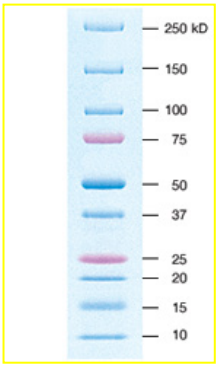
4. In-Gel Digestion method (IGD, 2 µg for 1 purified protein to 60 µg for a complex mix)

4.1 General info

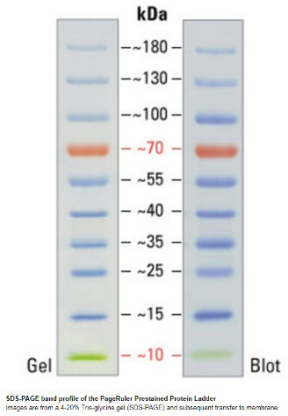
Standard protein gel: 12% bisacrylamide (MW 15 – 200 kDa): e.g. Thermo Bolt 4-12 Bis-Tris gel: NW04120BOX https://www.thermofisher.com/order/catalog/product/NW04120BOX) or from Invitrogen.



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 (ThermoFisher PX0026616).
Use 10 ul per well.



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 XL 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 12 kRPM for 10 min.
e. Pipet 80 ul of sample supernatant in an ep and add 20 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, very carefully, 30 ul is more safe).
As a protocol control, use 100 ng of BSA exactly (= 12.5 ul as prepared above).

2. Run the gel as described by the manufacturer. For 12% gels, run at 120V for about 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 - 2 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 NH_4HCO_3 + 15 mM reductor: 0.057 g DTT.
Gently shake for 1 h or more 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 + 0.036 g AcrylAmide (= 20 mM AA pH 8).
Incubate at room temperature in the dark while gently shaking for 0.5 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 – 8) and cut them into small pieces of ca. 1 mm^2. Use a sharp clean scalpel from lab 3030 on a clean piece of parafilm. Transfer the gel pieces to clean 0.5 ml low binding micro centrifuge tubes.

5. Enzymatic digestion.

At this point you may store the samples in a freezer or 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 4-6 hours at 37 °C or 2-3 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). Mix.
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 to the µColumn, add 100 ul 1 ml/l HCOOH in water to the remaining gel pieces, mix, 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.

Sjef.Boeren@wur.nl, Laboratory of Biochemistry, Wageningen UR

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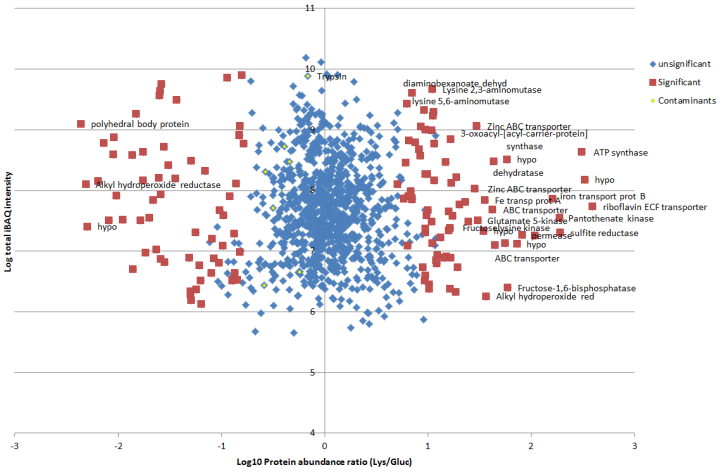
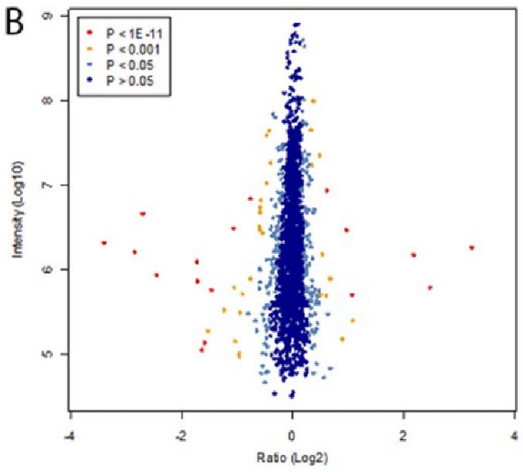
5. Quantitation

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 in 4 fold or more) and Controls (the same amounts) are measured separately. 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.

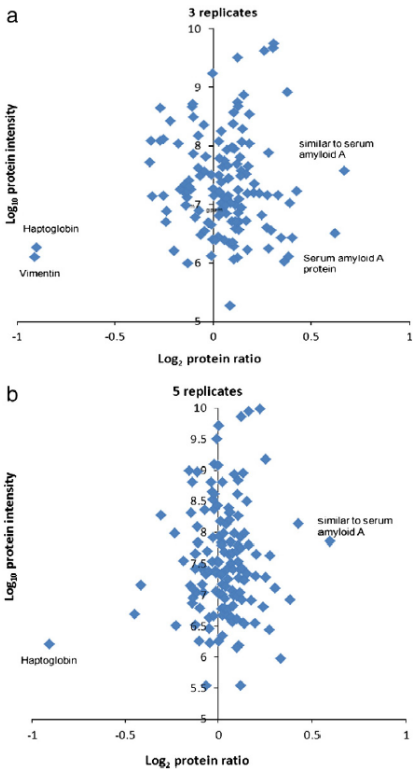


Bui, T. P., J. Ritari, S. Boeren, P. de Waard, C. M. Plugge and W. M. de Vos (2015). "Production of butyrate from lysine and the Amadori product fructoselysine by a human gut commensal." Nat Commun 6: 10062.



SILAC labeling. The essential amino acids 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.

Sjef.Boeren@wur.nl, Laboratory of Biochemistry, Wageningen UR

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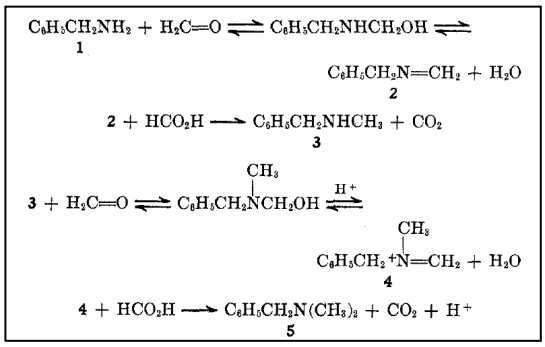
5.1.1 Relative quantitation by on column peptide dimethyl labelling protocol

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.

Sjef.Boeren@wur.nl, Laboratory of Biochemistry, Wageningen UR

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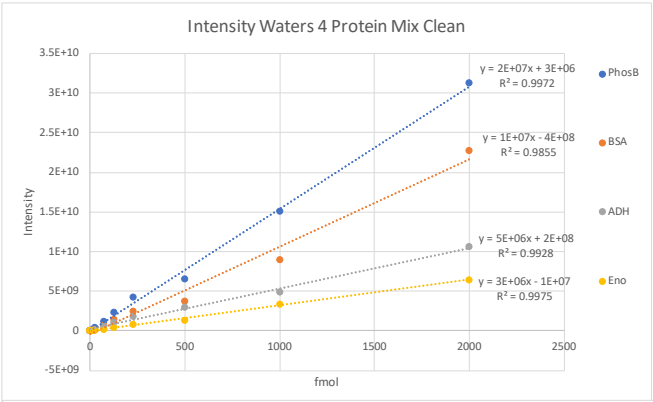
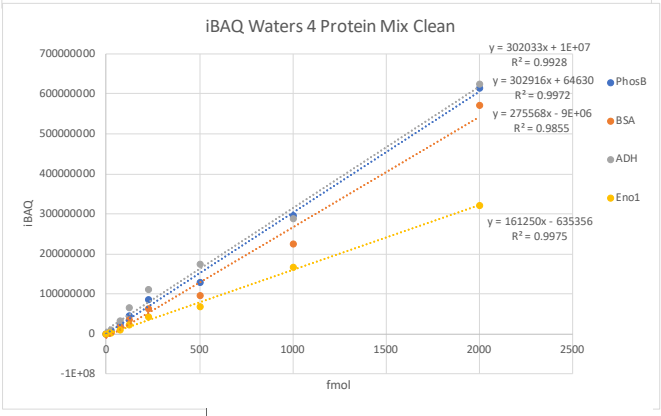
5.2 Absolute quantitation

a. Absolute quantitation on a limited number of 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%).





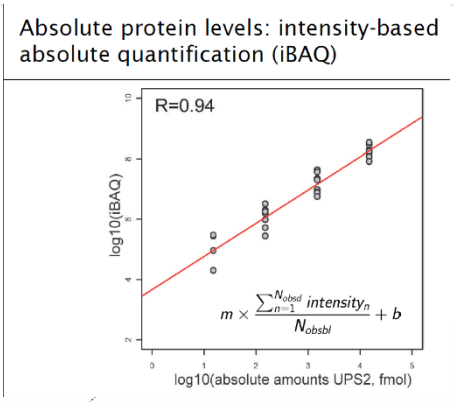
b. Less stringent but more practical when more proteins have to be quantitated, is the method of quantitation with respect to a single added internal standard like BSA (e.g. Pierce PI23210 2.0 mg/ml) or Ovalbumin. This rough absolute quantitation "**relative to an internal standard**" is accurate within a factor of 4.

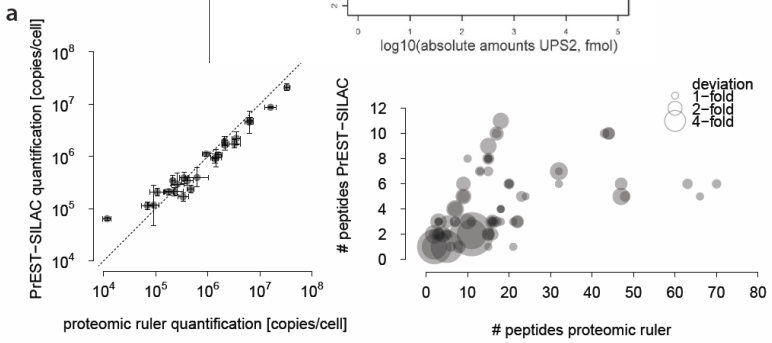
BSA can be replaced by Waters MassPREP Digestion Standard Mix 1 [186002865] (a 4 protein mix, €200/set) or Sigma UPS2, a quantified set of 48 human proteins at different concentrations (€900/set).

Lahtvee, P.J.: Absolute quantification of protein and mRNA abundances demonstrate variability in gene-specific translation efficiency in yeast. Cell Syst. 4, 495–504.e5 (2017).

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).

The error in the iBAQ method is between a factor 2 (as shown to the left) and 10 as shown in Log10 iBAQ vs Log [Protein] plot. The Log-Log figure comes from a presentation by Selbach during the MaxQuant summerschool 2011. It has been published as Fig. S8 in Schwanhausser 2011 mentioned above.





d. For very large datasets obtained by a 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. Additon of labeled peptides produced by either Cell free expression systems (Takemori: MEERCAT Multiplexed Efficient Cell Free Expression of Recombinant QconCATs For Large Scale Absolute Proteome Quantification. Mol Cell Proteomics. 2017, 16(12):2169-2183. SB ca €5000) or Ecoli (Beynon, R. J., Doherty, M. K., Pratt, J. M., and Gaskell, S. J. (2005) Multiplexed absolute quantification in proteomics using artificial QCAT proteins of concatenated signature peptides. Nat. Methods 2, 587–589 SB ca €3000).