### 1. General information

#### 1.1 pH stuff

<table>
<thead>
<tr>
<th>pH UP from pH 2 to pH 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 ml/l HCOOH = formic acid (FA): pH = 2.4</td>
</tr>
<tr>
<td>For 1 ml: Make pH 8 – 8.5 by adding 15 – 20 ul 10* diluted conc. NH3 (max 37%).</td>
</tr>
<tr>
<td>0.5 ml/l TFA = TriFluoroAcetic acid: pH = 2.1</td>
</tr>
<tr>
<td>For 1 ml: Make pH 8 – 8.5 by adding 6- 9 ul 10* diluted conc. NH3 (max 37%).</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>pH DOWN from pH 8 to pH 2 - 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mM ABC pH 8.0: For 1 ml: Make pH 3 by adding 35 ul 10* diluted conc. TFA.</td>
</tr>
</tbody>
</table>

#### 1.2 Abbreviations and some common solutions

- **BCA**: Bicinchoninic Acid
- **IMAC**: Immobilized Metal Affinity Chromatography
- **CCB**: Conjugated Cross-Linked Biotin
- **Oriole**: Oriole antibodies
- **ESI-tof**: Electrospray Ionization Time-of-Flight
- **Maldi-tof**: Matrix-Assisted Laser Desorption/Ionization-Time of Flight
- **FASP**: Filter Aided Sample Preparation
- **MS**: Mass Spectrometry
- **NH3**: Ammonia
- **TFA**: TriFluoroAcetic Acid
- **ABC**: Ammonium Bicarbonate

### 2. Protein determination (BCA)

- **50 mM ABC pH 8.0**

### 3. Protein Digestion methods

#### 3.1 Trypsin digestion tips

- **Filter aided sample preparation (FASP)** for 10 µg protein.
  - **3.2.1. Solutions and Reagents**
  - **3.2.2. Sample processing**
  - **3.2.3. When you have a limited amount of protein**

#### 3.2 Coupling of antibody to CNBr-activated Sepharose beads

- **4.2**

#### 3.3 Normal "in solution" trypsin digestion

- **4.3**

#### 3.4 Methanol and TriFluoroEthanol (TFE) sample preparation method

- **4.4**

#### 3.5 In-Gel Digestion procedures

- **5.1 General info**
- **5.2 Recommended procedure for CCB or Oriole protein gel staining:**
  - **5.2.1. Colloidal Coomassie Preparing Staining Solution**
  - **5.2.2. Colloidal Coomassie Gel staining procedure**
- **5.3 In Gel Digestion protocol**
  - **5.3.1. Important remarks**
  - **5.3.2. Procedure**

### 4. Pull down procedure for Mass Spectrometry

#### 4.1 Pull down general tips:

- **4.2 Coupling of antibody to CNBr-activated Sepharose beads**
- **4.3 Pull down (PD)**
- **4.4 Pull down check**
- **4.5 Protein elution**
- **4.6 Reduction, CarboxyAmidoMethylation and trypsin digestion on the beads**
- **4.7 Pull Down checklist**

### 5. In-Gel Digestion procedures

- **5.1 General info**
- **5.2 Recommended procedure for CCB or Oriole protein gel staining:**
  - **5.2.1. Colloidal Coomassie Preparing Staining Solution**
  - **5.2.2. Colloidal Coomassie Gel staining procedure**
- **5.3 In Gel Digestion protocol**
  - **5.3.1. Important remarks**
  - **5.3.2. Procedure**

### 6. Quantitation

#### 6.1 Relative quantitation (Sample compared to Control)

- **6.2 Absolute quantitation**

#### 6.3 Relative quantitation: on column peptide dimethyl labelling

- **6.3.1. Solutions**
- **6.3.2. Method**
- **6.3.3. On-column stable isotope dimethyl labeling**
- **6.3.4. Peptide recovery**

### 7. Phosphopeptide enrichment methods (S and T only)

- **7.1 Phosphopeptides (S, T) enrichment by Titanium Dioxide (TiO2) Chromatography**
- **7.2 Phosphopeptide (S,T) sample preparation by sequential elution from IMAC**
1.2 Abbreviations and some common solutions

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
<th>Concentration/Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>POS / NEG</td>
<td>positive mode / negative mode</td>
<td></td>
</tr>
<tr>
<td>AcN</td>
<td>acetonitril</td>
<td></td>
</tr>
<tr>
<td>ABC or AmBiCar</td>
<td>Ammonium BiCarbonate</td>
<td>NH₂HCO₃ (lab 2) (M=79) 50 mM ABC pH 8.0 = 0.20 g / 50 ml</td>
</tr>
<tr>
<td>AmAc</td>
<td>Ammonium Acetate NH₄CH₃COOH (M = 77) 10 mM = 38.5 mg / 50 ml</td>
<td></td>
</tr>
<tr>
<td>TFE</td>
<td>TriFluoroEthanol (#563 room 2014)</td>
<td></td>
</tr>
<tr>
<td>TFA</td>
<td>TriFluoro-Acetic acid: FUMING = add in fume cupboard only</td>
<td></td>
</tr>
<tr>
<td>TCEP</td>
<td>reductor Tris(CarboxyEthyl)Phosphine (M = 287) 100 mM = 28.7 mg / ml</td>
<td></td>
</tr>
<tr>
<td>- 8 M urea</td>
<td>(always prepare fresh! Do not warm up!) M=60 8 M = 4.80 g / 10 ml</td>
<td></td>
</tr>
<tr>
<td>- DTT</td>
<td>Dithiotreitol (#20) 50 mM ABC M = 154.2 50 mM = 7.7 mg/ml 500 mM = 77 mg/ml</td>
<td></td>
</tr>
<tr>
<td>- Iodoacetamide (#89) FRESH! in 50 mM ABC (IGD) or UT (FASP) M=185 100 mM = 19 mg/ml 500 mM = 92.5 mg/ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Cysteine</td>
<td>(#111, Fluka 30090, &gt;99%) in 50 mM ABC pH 8 M = 121 125 mM = 15 mg/ml 500 mM = 60 mg/ml</td>
<td></td>
</tr>
<tr>
<td>- Trypsin</td>
<td>with 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. Dilute 50° (In Gel Digestion) or 100° (FASP) in ABC before use.</td>
<td></td>
</tr>
<tr>
<td>- Stock Tris (10°):</td>
<td>1 M Tris (M=121) 1 M = 121 g/l 6.0 g / 50 ml</td>
<td></td>
</tr>
<tr>
<td>- ST buffer stock:</td>
<td>4%(w/v) SDS in 100 mM Tris/HCl pH 7.6 1 ml 1M Tris + 0.4 g SDS made up to 10 ml</td>
<td></td>
</tr>
<tr>
<td>- SDT-lysis buffer:</td>
<td>50 mM DTT (M=154) 7.7 mg / 1 ml ST buffer</td>
<td></td>
</tr>
<tr>
<td>- UT:</td>
<td>8 M urea (Sigma, U0631, M=60) in 100 mM Tris/HCl pH 8: 1 ml 1M Tris + 4.8 g urea made up to 10 ml pH will increase to 8.2 due to the addition of urea.</td>
<td></td>
</tr>
<tr>
<td>- 0.5 ml protein LoBind tube:</td>
<td>order# eppe0030108.094</td>
<td></td>
</tr>
<tr>
<td>- 2.0 ml protein LoBind tube:</td>
<td>order# eppe0030108.132</td>
<td></td>
</tr>
<tr>
<td>- Pall 3K or 10K omega filter (20kDa / 50kDa cutoff)</td>
<td>order# Pall OD003C34 = Sigma-Aldrich Z722049-100EA</td>
<td></td>
</tr>
</tbody>
</table>

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 (BioRad is not recommended). 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. Wash your hands under running tap water before you start and as often as possible in between handlings. Discharge yourself and your materials (especially the Eppendorf tubes) by using a discharge wire. 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. Do not wear clothes of wool. Try not to lean over the samples too much. Never ever use glass vials for proteins. You will lose a lot protein. Use new (or only used for proteomics) throw away (polypropylene) plastics. Do not use hand creams. Do not use any glassware that has been cleaned with detergent (e.g. in a washing machine). Cheap (non-Eppendorf) micro tubes may contain polymers, mold release agents, plasticizers, etc. Do not use pipet tips that have been sterilized by heating them. The heat may release plasticer compounds. Do not use siliconized Eppendorf tubes but:

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

From:
1.4 Recommended procedures

<table>
<thead>
<tr>
<th>Method</th>
<th>Protein Identification</th>
<th>Unlabeled relative Quantiation</th>
<th>Silac labeled Relative Quantiation</th>
<th>Dimethyl labeled Relative Quantiation</th>
<th>Absolute Quantiation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silac</td>
<td></td>
<td></td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FASP</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>In Gel Digestion</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>On column Dimethyl labeling</td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Ovalbumin Standard addition</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peptide (labeled) Standard addition</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peptide fractionation</td>
<td>+ (always possible)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Measurement</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Raw Data handling: MaxQuant (Protein identification and relative quantitation)

MQ data handling: Perseus (extra filtering of MQ result, statistics and intensity or ratio based clustering of proteins)

Bioinformatics:
First pathway analysis can be done with eg: Reactome ([www.reactome.org](http://www.reactome.org)), PathVisio ([http://www.pathvisio.org](http://www.pathvisio.org)), KEGG ([http://www.genome.jp/kegg/tool/map_pathway2.html](http://www.genome.jp/kegg/tool/map_pathway2.html)), Biocyc ([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](http://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:
FASP alternative “in Gel Digestion” (IGD):
Dimethyl labeling:

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

<table>
<thead>
<tr>
<th>Method</th>
<th>Protein load</th>
<th>Peptide identifications</th>
<th>Protein identifications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spin filter</td>
<td>50 µg</td>
<td>5,369</td>
<td>642</td>
</tr>
<tr>
<td>Short SDS-PAGE</td>
<td>4,176</td>
<td>593</td>
<td></td>
</tr>
<tr>
<td>TFE</td>
<td>4,663</td>
<td>593</td>
<td></td>
</tr>
<tr>
<td>Spin filter</td>
<td>86</td>
<td>46</td>
<td></td>
</tr>
<tr>
<td>Short SDS-PAGE</td>
<td>298</td>
<td>106</td>
<td></td>
</tr>
<tr>
<td>TFE</td>
<td>626</td>
<td>150</td>
<td></td>
</tr>
</tbody>
</table>

1.5 Sample losses, methods comparison.

Taken from:
1.6 Peptide concentrations in MS samples
Maldi-tof samples: The peptide concentrations are not critical since samples (that have to be salt free!, see below) will be diluted in matrix solution using the tip-in method, 1:1, 10^*, 100^*, and 1000^*. Samples will be measured as such and the optimal dilution will be found.

LC-MS-MSMS samples:
Hand in minimally 50 ul acidic sample (pH 2 – 4) with a concentration of approximately 10 – 500 ng/ul.

Info FASP: the yield is approximately 50% of the starting amount.
Info InGelDigestion: the yield of an “In gel digestion” sample is approximately 20% of the starting amount. So, for a:
- 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.

Peptide concentrations may be increased by using the Eppendorf concentrator and/or C18 uColumns (see 1.8 below). Do NOT use these techniques on proteins.

1.7 Protein sample solution for total MW determination by ESI-tof or Maldi-tof
Maldi: Prepare >= 2 ul protein (>= 10 uM, for MW = 100.000 ⊙ >= 1 mg/mL) preferentially in 1% TFA + 20% AcNi or 50 mM ABC (NEG) for direct Maldi-tof or ESI-tof measurement. Alternative: 10 – 25 mM AmAc or another volatile buffer. Maldi samples will be diluted with matrix before measurement. ESI samples will be diluted in 10 mM NH4Ac pH 5.

The sample solution should not contain charged nonvolatile ions like SDS, TRIS and/or phosphate or at least less than 10 mM charged ions. Proteins can be transferred into the appropriate solution by treatment with a Microcon (3, 5 or 10 kDa) filter. Also, see 3.2. Filter aided sample preparation (FASP).

1.8 General sample cleanup procedures with uColumns.
Peptide solutions can be concentrated and salt as well as beads (from IP/chromatography/SP3) can be removed using uColumns (= 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)
1. Prepare your own μcolumn by:
Use the “cookie cutter” method to cut a small (~1mm) 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 1 ml syringe, or with the vacuum manifold (connected to a vacuum pump) in lab1. Whatever method you use, do not let the uColumns run dry.
2. Wash the uColumn ones more with 100 ul MeOH.
3. Equilibrate the uColumn 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 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 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 °C for 2 hours or more 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 ul. Sonicate (water bath sonicator) or vigorously mix (thermomixer) 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 11, 14, 17, 21 and 40% AcNi with 20 mM NH4Formate], to fractionate peaks according to their high pH affinities for the C18 column.

1.9 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 H2O2 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.

2. Protein determination (BCA).

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

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 NH₄HCO₃.

The exact concentration of Ovalbumin or BSA can be directly measured when in 50 mM NH₄HCO₃ at 280 nm:

\[
\begin{align*}
C_{\text{Ovalbumin}} &= E_{280} \times 1.30 \text{ (mg/ml)} \\
C_{\text{BSA}} &= E_{280} \times 1.50 \text{ (mg/ml)}
\end{align*}
\]

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 cuvet as described in the table below. Seal tightly with parafilm.

Measure \(E_{562}\) after mixing and incubation at 21 ºC for 60 min or at 37 ºC for 30 min.

Example measurement:

Ovalbumin dissolved at 1 mg/ml a absorbance at 280 nm was measured: \(E_{280} = 0.618\)

Therefore the real concentration was: \(0.618 \times 1.30 = 0.803 \text{ mg/ml}\)

<table>
<thead>
<tr>
<th>Volume BCA ws (ul)</th>
<th>Volume Ovalbumin Std (ul)</th>
<th>Volume sample (ul)</th>
<th>Volume H2O (ul)</th>
<th>Calc. amount Ovalbumin (ug)</th>
<th>Measured (E_{562}) (1)</th>
<th>Measured (E_{562}) (2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>950</td>
<td>0</td>
<td>-</td>
<td>50</td>
<td>0</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>950</td>
<td>10</td>
<td>-</td>
<td>40</td>
<td>8.03</td>
<td>0.186</td>
<td>0.186</td>
</tr>
<tr>
<td>950</td>
<td>20</td>
<td>-</td>
<td>30</td>
<td>16.1</td>
<td>0.346</td>
<td>0.351</td>
</tr>
<tr>
<td>950</td>
<td>30</td>
<td>-</td>
<td>20</td>
<td>24.1</td>
<td>0.510</td>
<td>0.509</td>
</tr>
<tr>
<td>950</td>
<td>40</td>
<td>-</td>
<td>10</td>
<td>32.1</td>
<td>0.653</td>
<td>0.655</td>
</tr>
<tr>
<td>950</td>
<td>50</td>
<td>-</td>
<td>0</td>
<td>40.2</td>
<td>0.746</td>
<td>0.730</td>
</tr>
<tr>
<td>950</td>
<td>-</td>
<td>20</td>
<td>30</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**BCA protein determination**

Linear between 0 – 30 ug:

\[
\text{ug protein} = \frac{[E_{562} - 0.0135]}{0.0203}
\]

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) which 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. Protein Digestion methods.

3.1 Trypsin digestion tips.
Just as for a direct protein measurement, the starting sample solution should not contain charged nonvolatile ions like SDS, TRIS and/or phosphate or at least less than 10 mM charged ions for Maldi MS. 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), an SDS gel-electrophoresis step (see section 5), C18 uColumn cleanup (see section 1.8) or iST/SP3 cleanup step. Trypsin cleaves C terminal of R and K (sometimes of N, slight chymotrypsin activity is sometimes possible).

Sometimes the cysteine reduction/alkylation procedure decreases the peak intensities measured, probably due to protein precipitation. An easy procedure without cysteine reduction/CarboxyAmidoMethylation:
Make 100 ul 1 uM (or 1 mg/ml) protein in 50 mM ABC pH 8. Unfold protein by heating when necessary. Add 2 ul Trypsin sequencing grade (0.5 ug/ul 1 mM HCl, bovine). Incubate while shaking at 37 °C for 4 hours.

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

3.2.1. Solutions and Reagents
- **ABC**: 0.05M NH₄HCO₃ in water (M=79) 0.20 g / 50 ml
- **Stock Tris (10*)**: 1 M Tris (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 1 ml 1M Tris + 0.4 g SDS made up to 10 ml
- **SDT-lysis buffer**: 0.1M DTT (M=154) 15.4 mg / 1 ml ST buffer
- **UT**: 8 M urea (Sigma, U0631, M=60) in 100 mM Tris/HCl pH 8: 1 ml 1M Tris + 4.8 g urea made up to 10 ml
- **IAA solution**: 0.05 M Iodoacetamide in UT (pH 8!, not higher, not lower!) (M=185) 9 mg / ml UT
- **Trypsin** Stock 0.5 μg/μl Dilute 100* in ABC.

**Note**: SDT (the DTT part), Urea and IAA solutions must be prepared freshly and used within a day.

3.2.2. Sample processing
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 3 mg of DTT (100 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 protein sample in SDT (50 ug protein) and incubate for 1 min while mildly shaking at room temperature.
6. Pipet 10 ug alkylated protein sample (= 100 to maximally 500 ul) into a Pall 3K omega filter (10-20kDa cutoff, OD003C34) and centrifuge at 13 kRPM for 30 min (100 ul) or more. 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 5 seconds) and incubate gently shaking at room temperature overnight or at 37 °C for 4 hours or at 45 °C for 2 hours.
11. Centrifuge the filter units 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 uColumn for desalting (see section 1.8 General Sample cleanup procedures) and/or Dimethyl labeling (see section 6.1 Relative quantitation: on column peptide dimethyl labeling).

3.2.3. When you have a limited amount of protein then you can use a minimal volume (50 ul) of ST buffer to do the sonication. Also, you can concentrate the peptide solution using the Eppendorf concentrator or the C18 uColumn concentrating procedure (1.8 General Sample cleanup procedures). Alternatively, you can try the TriFluoroEthanol method described below in part 3.4 or one of the newer iST or SP3 methods.
In general, FASP will give about 50% peptide recovery. This can be 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)
3.3 Normal ‘In solution’ trypsin digestion

3.3.1. Dissolve 1-10 ug protein in 100 ul 50 mM ABC (pH 8) (10 ug BSA = 0.15 nmol = 5.3 nmol Cys)

3.3.2. Add 10 ul 100 mM DTT† dissolved into 50 mM ABC (pH 8.5 with NH3):
   Incubate at 60 °C for 1 hour (max 2 hours) (1.0 umol)

3.3.3. Check the pH. Make pH 8.5 with 10* diluted NH3. (Usually not necessary!)

3.3.4. Add 15 ul 100 mM Iodoacetamide* dissolved into 50 mM ABC (pH 8.5 with NH3):
   Incubate at 20 °C in the dark for 1 hour exactly. (1.5 umol)

3.3.5. Add 15 ul 125 mM cysteine dissolved into 50 mM ABC to remove the excess Iodoacetamide (1.9 umol) and, for procedure 3.4 (below) 360 ul ABC to decrease the TFE or MeOH concentration to 10%.

3.3.6. 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. Longer reaction times may increase the amount of chymotryptic cleavages.

3.3.7. Add 2.5 ul 10* diluted TFA to decrease the pH to 2. Measure the pH by putting 0.1 ul on a piece of pH paper. Add more 10* diluted TFA when necessary.

3.3.8. Perform the sample cleanup with uColumns as described in: 1.8 General sample cleanup procedures with uColumns. 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 CarboxyAmidoMethylation (to prevent auto-oxidation) = +57 Da per cysteine.


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 3.2. Filter aided sample preparation), pull-down (see chapter 4. Pull down procedure for Mass Spectrometry), by running an SDS gel (see chapter 5 In-Gel Digestion procedures) or by a specific detergent removal method e.g. commercial available SDS removal spin columns mentioned in 3.1.

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


(iST) sample prep method:
   Max 5 ug protein (= 25 ug washed yeast cells) for a C18 StageTip prepared with a 14 gauge needle
   Lysis in: 10 ul 6M GuHCl (or 8M urea), 10mM TCEP, 40mM CAA (chloroacetamide), 100mM Tris pH8.5 (1-5 ug protein/ul lysis buffer): boil (5') and sonicate (waterbath 1min).
   Dilute 5* with: 40 ul 10% (v/v) ACN, 25mM Tris pH 8.5 with Trypsin (100*dil): inc rT ON. Add 2 ul 10% TFA to acidify and elute through.
   Wash: 2 * 100 ul 1 ml/l HCOOH.
   Elute: 50 ul 50% AcNi/ 50% 1ml/l HCOOH.

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

1) To washed cells:
   + 20 ul of Lysis buffer (1% SDS, 1× cOmplete Protease Inhibitor Cocktail-EDTA (Roche), 5 mM EDTA, 5 mM EGTA, 10 mM NaOH, and 10 mM DTT, in 10 mM HEPES buffer at pH 8.5.
   + 20 ul SP3 beads (rinsed with water before use, 2 ul of 10 ug/ul beads mix)
   + 20 ul of neat trifluoroethanol
   → sonicate for 15 min 10 cycles (30 s on, 30 s off) on the setting 'high' without cooling.
   + 0.75 ul of 0.1% formic acid to neutralize. Samples were then heated at 95°C for 5 min and placed on ice before proceeding with reduction and alkylation steps.

2) Reduction: + 2 μL of 200mM DTT (Bio-Rad) per 40 μL of lysate. 45°C for 30 min.
   Alkylation: + 4 μL of 400mM iodoacetamide (IAA) per 40 μL of lysate. 24°C in the dark for 30 minutes.
   Quench: + 4 μL of 200 mM DTT per 40 μL of lysate (SB: better use cysteine).

3) Acidify with formic acid to pH 2 (SB: better 10% TFA) + 80 ul 100% AcNi to 50% AcNi. 8 min inc r T --> magnet 2 min. Remove sup.
   + 200 ul 70% EtOH. 30 s inc., remove sup.
   Repeat 70% EtOH wash 1*.
   + 180 ul 100% AcNi, inc., remove sup.

4) + 5 μL of 50mM HEPES pH 8 that contained ~250 ng of Trypsin/LysC enzyme mix (Promega) → incubated for 14 hours at 37°C.
   + 195 ul 100% AcNi (final concentration >95%), incubated for 8 minutes, followed by 2 minutes on a magnetic rack. Remove sup.
   + 180 μL of 100% acetonitrile. Remove sup.Elute peptides off the beads in 10 μL of 2% DMSO with pipette mixing for 5 minutes. Can beads be removed completely to be injection ready? Samples were acidified with formic acid prior to MS-analysis.

Preferentially use commercially available (magnetic) beads e.g. from Miltenyi, Invitrogen, Chromotek or Pierce.

Alternatively, you may use CNBr-activated Sepharose 4B to prepare your own antibody linked affinity beads described by the procedure below.

In case of CNBr activated Sepharose, the active group on the beads (cross-linked agarose support) will react with primary amines of proteins. Therefore, the sample solution may not contain amines (e.g., Tris or glycine) apart from the ones you want to couple as they will quench the reaction. Remove amines before coupling by dialysis or desalting.

Example (data Rumyana Karlova):
Sample: GFP-BRI1 from A. thaliana pulled down with purified GFP antibodies (Willy vd Berg) linked to Micro-link beads. → SDS gel electrophoresis stained with colloidal Coomassie → in gel digestion → LCMS by nLC – LCQ classic (so, we can do much better now).
4.1 Pull down general tips:
Mix non-magnetic beads gently by hand or with an end-over-end mixer. Do not use a magnetic stirrer to do this! Centrifuge the non-magnetic beads after each step at low speed and maximally for 1 minute (1000*g, 1 min max.). When you do not use covalently linked antibody-beads commercially bought, than measure the protein content of the antibody solution before and after coupling to determine the coupling efficiency.
Measure the binding capacity of the coupled antibody-agarose beads with GFP or YFP or CFP only.

4.2 Coupling of antibody to CNBr-activated Sepharose beads
(Taken from GE Healthcare 17-0439-01).
See: D:\Sjef's Documents\TEKSTEN\Downloads\CNBr activated Sepharose instructions for use.pdf.

Wash solution:  1 mM HCl.
Coupling buffer:  0.1 M NaHCO3 + 0.5 M NaCl pH 8.3.  Avoid NH2 containing buffers.
Store buffer:   coupling buffer + 0.05% NaN3  or  20% ethanol / coupling buffer.

4.2.1 Prepare beads. Weigh out 100 mg lyophilized powder and suspend it in 1 ml 1 mM HCl. Gently shake for 5 min. Wash 3* with 1 mM HCl. Remove the supernatant. This will yield 0.35 ml medium.

4.2.2 Couple antibody. Reserve a portion of the anti-tag-antibodies solution for measuring the starting protein content to determine the coupling efficiency.

4.2.3 Add 1 ml of the antibody (1 – 3.5 mg/ml in coupling buffer) and rotate the mixture end-over-end for 1 h at room temperature or overnight at 4 °C. Save the supernatant to measure the coupling efficiency by measuring the protein content with the Pierce BCA kit (see section 2. Protein determination (BCA)).

4.2.4 Wash away excess ligand with 2* 1 ml coupling buffer.

4.2.5 Block any remaining active groups. Add 1 ml 0.1 M Tris-HCl buffer, pH 8.0 or 1 M ethanolamine, pH 8.0. Mix. Let it stand for 2 hours.

4.2.6 Wash the medium with 1 ml of 0.1 M acetic acid/sodium acetate, pH 4.0 containing 0.5 M NaCl followed by a wash with 0.1 M Tris-HCl, pH 8 containing 0.5 M NaCl.

4.2.7 Repeat wash step 6 with both buffers for 2 times.

4.2.8 Add 300 ul of store buffer. This procedure gives a 50% slurry. Shake before use.

29. Measure the coupling efficiency by measuring the depleted supernatant obtained AFTER antibody coupling or measure the binding capacity of the coupled antibody-agarose beads with purified tag only.

4.3 Pull down (PD)
Pull Down Buffer: 50mM Tris-HCl + 150 mM NaCl + protease inhibitors mix cocktail (Roche, 1 pill per 50 ml buffer), final pH=7.5
Extraction buffer for Arabidopsis total protein extraction: PD Buffer + 1% Triton X100 final pH=7.5.

4.3.1 Grind 10 day’s old seedlings in liquid nitrogen. Mix the sample with 2 volumes of Extraction buffer. Keep 30 min. on ice for solubilisation. Centrifuge 10 min at maximum speed. Take the supernatant. Dilute with buffer (without Triton) to adjust the Triton concentration of the plant extract to 0.2% before doing the Pull Down.

4.3.2 Wash the GFP-antibodies linked beads 3 times with buffer (without triton) to get rid of sodium azide. Spin 1000rpm 1min.

4.3.3 Pre-clear the plant protein extract with beads containing antibody with blocked active sites by adding 25 ul of a 50% slurry in buffer, 0.5 h at 4 °C with rotating. When these pre-clear beads are unavailable, then skip this step. Centrifuge 2min at 1000 rpm. Pellet = pre-clear beads. As a blank, treat the pre-clear beads further as the washed sample beads described under C) reduction, CAM Trypsin digestion. Proteins observed on the pre-clear beads have been a-specifically bound by the (blocked) anti-body or the beads.

4.3.4 Transfer the supernatant to a 0.5 ml low binding ep.

4.3.5 Incubate 1-10 mg of total protein (200 ul 10 mg/ml = 2 mg) with 25ul 50% of GFP-antibodies linked beads for maximally 1 hour at 4 °C with (end-over-end) mixing.

4.3.6 Proceed with the next steps swiftly. Transfer the magnetic beads to the magnet or spin down the non-magnetic beads at 1000 rpm for 1min. Elute or Remove the supernatant. Check the supernatant on blot.

4.3.7 Wash swiftly 2 times with 400 ul extraction buffer with 0.1% triton within 2* 5 min. Remove the supernatant.

4.4 Pull down check
Use 10% of the beads to check binding of your bait protein by SDS gel-electrophoresis → Blot on NitroCellulose membrane (generally gives a more clear background then PVDF (RK) → detect on the membrane with a specific antibody detection. Also, stain de SDS gel with a suitable protein stain to detect all proteins present. Continue from here only when you see a clear band on the blot and not too many other proteins on the SDS gel. If this is not the case, then contact your supervisor.
4.5 Protein elution

It is possible now to go to section 4.6 "Reduction, CarboxyAmidoMethylation and Trypsin digestion on the beads" from here but this will result in very high MS peaks from peptides from highly abundant (a-specifically bound) proteins. This will obstruct measurement of the more interesting peptides. It is better to elute the protein complexes from the beads by:

Bait-GFP → GFP-antibodies-agarose: + 50 ul 0.1 M Triethylamine pH 11.8/0.1% Triton X-100 (Sullivan 2008)
or: + 50 ul 0.1 M glycine pH 2.5 (advised for Chromotek GFP-trap)
or: + 50 ul buffer + 6 M urea (FRESH!)
Bait-FLAG → M2-agarose: + 50 ul 50mM NH₄HCO₃ pH=8 + 400 uM FLAG peptide
Bait-SulfoNHS-SS-Biotin → Streptavidin-Sepharose: + 50 ul 50mM NH₄HCO₃ pH=8 + 5% 2-Mercaptoethanol.

Alternative (a-specific): elution with ST buffer or gel Sample Loading buffer:
+ 20 or 40 ul ST or non-reducing SDS gel loading buffer, boil 5', centrifuge.

→ FASP with 10 ul supernatant (see section 3.2. Filter aided sample preparation (FASP)) → LCMS
or → SDS gel-electrophorese (all of the supernatant using 1.5 mm SDS gel) → in Gel digestion on protein bands (see chapter 5 In-Gel Digestion procedures) → General sample cleanup procedures with uColumns (section 1.8) → LCMS

4.6 Reduction, CarboxyAmidoMethylation and trypsin digestion on the beads

4.6.1 TritonX100 removal for direct “On Beads Digestion”: Wash swiftly 2 times with 400 ul 50mM ABC pH=8 within 10 min. Change Eppendorf tube for non-magnetic beads at least once during a washing step. For magnetic beads: put a clean low binding Eppendorf tube under the holder and remove the holder from the magnet. Wash the beads out of the holder into 2 Eppendorf tubes with 2 * 45 ul ABC. The second ep should not contain too many beads. From this point, interactors will be eluting from the beads so do not remove supernatants anymore. Continue with ep 1 that should have 95% of the beads:

4.6.2 To the beads that should now be in 45 ul of clean ABC: add 5 ul 100 mM DTT* (Dithiotreitol) in 50 mM ABC pH 8 (15.4 mg/ml) and incubate at 60 ºC for 1 hour. (500 nmol)
4.6.3 Add 6 ul 100 mM Iodoacetamide* in 50 mM ABC pH 8 (18.5 mg/ml) and incubate at room temperature in the dark for 1 hour . (600 nmol)
4.6.4 Add 7 ul 100 mM cysteine to destroy excess IAcAm and stop the alkylation. (700 nmol)
4.6.5 Add 1ul Trypsin sequencing grade (0.5 ug/ul 1 mM HCl). Incubate over night at 20 ºC or at 37 ºC for 4 hours while shaking. Longer reaction times may increase the amount of chymotrypsinic cleavages.
4.6.6 Option: partly digest with Trypsin in 2M urea for 1 to 2 h. Remove beads by centrifugation and continue digesting.
4.6.7 Carefully add 10% TFA to make the pH approximately pH 3 (= about +1.2 ul 10% TFA). Check the pH by putting 0.1 ul on a piece of pH paper. Perform the “General sample cleanup procedures with uColumns” procedure (section 1.8) → Store the samples in the freezer.

4.7 Pull Down checklist

When you want to have pull-down samples measured by LCMS, you will be asked to do the Pull Down check and deliver the following information:

1. What was the antibody coupling efficiency for the beads you used?
The protein concentration decreased from … mg/ml to … mg/ml per … mg of dry beads.

2. What is the mass of the tagged protein including the tag: … kDa.

3. How much of the sample was used to do the pull down check by SDS-page and Western blot: … V/V %

4. Does the Western blot obtained for the “pull down check” clearly show the tagged protein: Yes / No

5. Does the SDS gel show the presence of a lot of unwanted proteins? Yes / No

6. Please supply clear electronic pictures of both the SDS gel and of the blot.

Should I do a sample fractionation for my pull-down sample?

<table>
<thead>
<tr>
<th>Info obtained from a Colloidal Coomassie stained gel</th>
<th>Fractionate?</th>
</tr>
</thead>
<tbody>
<tr>
<td>I clearly see my tagged protein and its interactors.</td>
<td>No</td>
</tr>
<tr>
<td>The protein amounts are large enough to be measured.</td>
<td></td>
</tr>
<tr>
<td>I only see some faint protein spots</td>
<td>No</td>
</tr>
<tr>
<td>Minimize sample handlings and do an on-beads digestion.</td>
<td></td>
</tr>
<tr>
<td>I see plenty of protein but not my tagged protein and/or its interactors</td>
<td>Yes</td>
</tr>
<tr>
<td>Fractionate into 3 to 5 fractions. This can be done on the protein level (gel, preferred method) or peptide level (High pH RP).</td>
<td></td>
</tr>
</tbody>
</table>
5. In-Gel Digestion procedures

5.1 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 (BioRad gels are not recommended).

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!

5.2 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.

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.

5.2.1 Colloidal Coomassie Preparing Staining Solution
Shake the Stainer B solution before using it. Prepare the solutions fresh 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..

<table>
<thead>
<tr>
<th>Solution*</th>
<th>ml per gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deionized Water</td>
<td>13.75</td>
</tr>
<tr>
<td>Methanol</td>
<td>5</td>
</tr>
<tr>
<td>Stainer B</td>
<td>1.25</td>
</tr>
<tr>
<td>Stainer A</td>
<td>5</td>
</tr>
</tbody>
</table>

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

5.2.2 Colloidal Coomassie Gel staining procedure
1. Shake gel in staining solution for 1 - 3 hours (maximally 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.

3. For long-term storage (over 3 days), keep the gel in a 10% Methanol solution at 4°C.
5.3 In Gel Digestion protocol

5.3.1 Important 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….

Tip: Use narrow Bio-Rad 20 ul pipet tips to remove supernatant from gel slices in between the steps when necessary.

5.3.2 Procedure
1. Sample preparation.
   a. Make a concentrated protein sample in a suitable buffer e.g. by sonicating in a 1.5 ml low binding Eppendorf tube.
      E.g. 50 mg washed cells in 450 ul SDT-lysis buffer pH 8 ➔ ca 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. Incubate at 95°C (heating block) for 10 min. Cool down to room temperature. Centrifuge at 13 kRPM for 10 min. Use 200 ul clear supernatant to continue.
   c. Add 1/4 of the present volume of Sample loading buffer (e.g. Pierce 39000 or [50% glycerol/50% water + 1 g/l bromophenol blue]) (+ 50 ul) ➔ circa 4 ug protein/ul from 20 mg cells or
      8 ng BSA/ul from 2 ug BSA
   d. Apply 2 μg (one purified protein) to 60 μg (protein mix) or more protein per sample well.
      Each sample well holds maximally 50μL (but then you have to pipet very carefully, 40 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 6 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) 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 at least 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 (8 – 24) and cut them into small pieces of ca. 1 mm². 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.

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.

   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 uColumn cleanup procedure with a C18 uColumn as described in section 1.8 “General sample cleanup procedures with uColumns”. 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 uColumn as well.

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.
6. Quantitation

6.1 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 (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 starts from a factor 2.

**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%).

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 (CH₂O, CD₂O or ¹³CD₂O) and cyanoborohydride (NaBH₃CN or NaBD₃CN) and is described in detail 2 pages further. 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%).
6.2 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 (NMR quantitated) 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 6-30 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.
6.3 Relative quantitation: on column peptide dimethyl labelling

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

\[
R-\text{NH}_2 + 2. \text{H}_2\text{CO}/\text{D}_2\text{CO} + \text{NaBH}_3\text{CN} \rightarrow R-\text{N}[\text{CHD}_2]_2 + \text{CO}_2 + \text{H}_3\text{O}^+ \\
\Delta M = + C_2H_4 = + 28.0313 \\
+ C_2D_4 = + 32.0564 \\
+ ^{13}C_2D_6 = + 36.0757 \\
d\Delta M = 4 / 8 \text{ Da per group}
\]

6.3.1 Solutions

<table>
<thead>
<tr>
<th>Solution</th>
<th>Concentration</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>500 mM NaH₂PO₄.1 H₂O (M= 138)</td>
<td>6.9 g/l</td>
<td>3.45 g / 50 ml</td>
</tr>
<tr>
<td>500 mM Na₂HPO₄ (M= 142)</td>
<td>7.1 g/l</td>
<td>3.55 g / 50 ml</td>
</tr>
<tr>
<td>35% or 20% (vol/vol) formaldehyde in water (CH₂O, CD₂O or 13CD₂O)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.6 M cyanoborohydride in water (NaBH₃CN or NaBD₃CN): (M= 64.8)</td>
<td>3.9 mg / 100 ul</td>
<td></td>
</tr>
</tbody>
</table>

6.3.2 Method

Prepare per sample/label:

10 ul 500 mM NaH₂PO₄.1 H₂O + 35 ul 500 mM Na₂HPO₄ + 425 ul H₂O (50 mM pH 7.5)
+ 2.9 ul 35% or 5 ul of 20% (vol/vol) formaldehyde in water (CH₂O, CD₂O or 13CD₂O).
+ 25 ul of 0.6 M cyanoborohydride in water (NaBH₃CN or NaBD₃CN) 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 °C and not stored longer than 24 h to ensure labeling efficiency.

Light: CH₂O + NaBH₃CN
Intermediate: CD₂O + NaBH₃CN
Heavy: C₁₃D₂O + NaBD₃CN

6.3.3 On-column stable isotope dimethyl labeling (TIMING: 1 h):
(i) Make C₁₈⁺ Stage tips by: Use the “cookie cutter” method to put a small (~1mm) 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 three C₁₈⁺ Stage tip columns with 200 ul of methanol.
(iii) Condition the C₁₈⁺ Stage tip columns 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 columns 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.

6.3.4 Peptide recovery
Manually elute and collect the labeled samples in 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 °C for 2 hours or more 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) or vigorously mix (thermomixer) 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.
7. 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)  
Electrospray ionization will mainly give 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.  

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

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

Prepare ca 5 mm uColumns with 10 ul TiO2 beads slurry (50% beads in 100% MeOH) packed in a 200 ul tip fitted with a C8 filter. (Optimal: use 0.5 mg TiO2 per 100 ug of peptide when you know the amount of peptide)

Wash column with 100 ul MeOH.
Wash column with 100 ul ultrapure AcNi (e.g. HPLC gradient grade) and equilibrate with 100 ul 50 mg/ml 25DHB (Fluka, or D33 in Willems lab) in 80% acetonitrile, 1% TFA. The column may turn orange in this step.

Dissolve dried samples in:  
100 ul loading buffer (50 mg/ml 25DHB (slightly yellow powder, Fluka) in 80% acetonitrile, 1% TFA).
Load samples onto the TiO2 microColumn.
Wash with 100 ul of loading buffer (to remove non-phospho peptides).
Wash with 100 ul of wash buffer (80% acetonitrile, 1% TFA without DHB) to remove all 25DHB.
Elute with 50 ul of ammonium water pH 11.3 (10 ul of 25% ammonia solution in 490 ml of MilliQ water) into a clean low binding Eppendorf tube *(. Elute with 50 ul of 80% acetonitrile/ 1% TFA to elute all phosphopeptides bound to the C8 disk as well into the same Eppendorf tube. Check the pH of the mixed eluates to be around pH 3.

Dry samples in a rotating concentrator at 45 °C.
7.2 Phosphopeptide (S,T) sample preparation by sequential elution from IMAC.

Uses the Pierce Fe-NTA Phosphopeptide Enrichment Kit: PX0088300


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.