

Peptide fractionation and Clean-Up Protocols

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Cation-Exchange Separation SCX and Detergent Removal

Removal of detergents and non polar solutes:

Note the first procedure below was developed for ICAT Cartridge Cation exchange. The second one is used for SCX spin cartridges from Nestgroup. Methods 1 – 4 describe different buffers that can be used. Volatile buffers are more mass spec friendly.

Reagents and Materials

- ICAT Cartridge Cation exchange (Invitrogen)
- Cartridge holder; Outlet tubing kit; Needle-port adapter (Invitrogen)
OR
- PolySULFOETHYL A, Bulk 5µm, 200Å, 1g # PBMSE0502 1G (Nestgroup)
- Empty spin columns, e.g. Centrifuge Columns, 0.8mL # 89868 (Pierce)
OR
- UltraMicroSpin™ (2-100µL elution volume, 5-50µg capacity, # SUM HIL-SCX) (Nestgroup)
- MicroSpin™ Columns (5-200µL elution volume, 10-100µg capacity SEM HIL-SCX) (Nestgroup)
- MacroSpin Column, 50-450µl (100 - 500µg), HILIC or SCX, 96/pk silica based # SMM HIL-SCX (Nestgroup)
- Macro Spin Tubes, 50-150µL elution, PolySULFOETHYL A # PMASCSE1203 (Nestgroup)
- Micro Spin Tubes, 5-25µL elution, PolySULFOETHYL A # PUMSCSE1203 (Nestgroup)
- Monobasic potassium phosphate (KH₂PO₄), HPLC grade # P286-1 (Fisher)
- Potassium chloride (KCl) ACS reagent 1kg # AC42409-0010 (Fisher)
- Acetonitrile (CH₃CN) optima # A996-1 (Fisher)
- Phosphoric acid (H₃PO₄) (85%) HPLC grade # A260-500 (Fisher)
- Sodium azide (NaN₃) # AC19038-0050 (Fisher)

Method 1:

Buffer Load 10 mM KH₂PO₄ pH3 [1.36 g/L], 25% Acetonitrile (CH₃CN) [250 ml/L], H₃PO₄ (85%) to adjust pH
 Buffer Elute 10 mM KH₂PO₄ pH3 [1.36 g/L], 25% Acetonitrile (CH₃CN) [250 ml/L], H₃PO₄ (85%) to adjust pH, 500 mM KCl [37.28 g/L]
 Or for step elutions use different buffers containing 30-500 mM KCl:
 30 mM KCl [2.24 g/L], 40 mM KCl [2.98 g/L], 50 mM KCl [3.73 g/L], 60 mM KCl [4.47 g/L], 70 mM KCl [5.22 g/L], 85 mM KCl [6.34 g/L],
 100 mM KCl [7.46 g/L], 130 mM KCl [9.69 g/L], 160 mM KCl [11.93 g/L], 350 mM KCl [26.09 g/L], 500 mM KCl [37.28 g/L]
 Buffer Clean 10 mM KH₂PO₄ pH3 [1.36 g/L], 25% Acetonitrile (CH₃CN) [250 ml/L], H₃PO₄ (85%) to adjust pH, 1 M KCl [37.28 g/L]
 Buffer Storage 10 mM KH₂PO₄ pH3 [1.36 g/L], 25% Acetonitrile (CH₃CN) [250 ml/L], H₃PO₄ (85%) to adjust pH, 0.1% NaN₃ (sodium azide)

Volatile SCX Buffers

Method 2:

Buffer Load: 250mM Ammoniumacetate (CH₃COO NH₄), 0.5% Formic Acid, pH=5.3
 Buffer Elute: 500mM Ammoniumacetate, 0.5% Formic Acid, pH=5.3 or 4 steps 250mM, 300mM, 400mM, 500mM Ammoniumacetate
 Buffer Clean: 500mM Ammoniumacetate, 0.5% Formic Acid, pH=5.3

Method 3:

Buffer Load: 25% ACN and 0.05% Formic Acid
 Buffer Elute: 25% ACN and 0.05% Formic Acid and 400mM Ammonium bicarbonate (NH₄HCO₃) or 5 Steps: 50mM, 100mM, 150mM, 200mM,
 400mM Ammonium bicarbonate (NH₄HCO₃)
 Buffer Clean: 25% ACN and 0.05% Formic Acid and 400mM Ammonium bicarbonate (NH₄HCO₃)

Method 4: use Ammonium Formate NH₄HCO₂ instead of Ammonium bicarbonate

Procedure ICAT Cartridge Cation exchange

1. Insert into the holder, making sure you can read the writing so the flow is always the same direction.
2. Mount on a stand with a small clamp and prepare an elution tube (1.5mL eppendorf) and a flowthrough tube (double the size of sample, falcon tube).
3. Adjust pH of sample to ~ 3. Using 1- 10% phosphoric acid (when working with acid use glass syringes to minimize contaminants)
4. Inject 2mL of Buffer-Load into the column to condition it. Speed = fast.
5. Inject 1mL of the sample, collect the flowthrough in the FT tube. Speed = slow.
6. Inject 500uL of the Buffer-Load, collect in the FT tube. Speed = fast.
7. Reload the flowthrough (step 5) and wash with 500ul of Buffer-Load
8. Save the FT tube, in case the loading failed.
9. Elute the peptides stepwise through different salt concentrations, 1mL each step. Speed = slow.
 - 70 mM, 100 mM, 130 mM, 160 mM, 500 mM KCl
 - 30 mM, 40 mM, 50 mM, 60 mM, 70 mM, 85 mM, 100 mM, 130 mM, 160 mM, 500 mM KCl
10. To store the column, inject 1mL of Buffer-Clean. Speed = fast.
11. Inject 2mL Buffer-Storage. Speed = fast.
12. Take cartridge out of holder, place back in packaging.
13. Mark the number of times the cartridge was used (do not exceed 10).
14. Clean syringes and holder with Milli-Q water.

Note: Speed slow + ~1 drop / sec, fast = ~ 1 – 1.5ml / min

Procedure for PolyLC Ultra Micro Spin Columns**Reagents and Materials**

- Micro Spin Tubes, 5-100 μ L (10 - 150 μ g) samples (see table below)
- Conditioning buffer (e.g., 10mM potassium phosphate, pH 2.7 w. 20% acetonitrile)
- Releasing buffer (e.g., 10mM potassium phosphate, pH 2.7, 400mM KCl, w. 20% acetonitrile)

| SCX (PolySULFO) | Protein capacity | Elution volume | Part # | Resin |
|------------------|------------------------------------|-----------------|-------------|------------------|
| Micro Spin Tubes | 5-100 μ L (10 – 150 μ g) | 5-25 μ L | PUMSCSE1203 | PolySULFOETHYL A |
| SPE Cartridges | 100 μ L – 1 mL (0.1 – 1 g) | 100-200 μ L | PSPESE1203 | PolySULFOETHYL A |
| Macro Spin Tubes | 50-450 μ L (100 – 500 μ g) | 50-150 μ L | PMASCSE1203 | PolySULFOETHYL A |

Procedure Micro Spin Tubes

- 1) Place the spin column, with the adapter collar in a 2ml micro-centrifuge tube.
- 2) **Conditioning the column:** Pipet 100 μ l of conditioning buffer onto the column and centrifuge it for 4 min at 2000xg @ 8000 rpm with an Eppendorf microcentrifuge. Wash 3 times emptying the tube between each wash. Centrifuge to dryness.
- 3) **Processing the sample:** Place column in a new Eppendorf tube. *This procedure is different than the one included with the columns.* Add acidified sample to the column (up to 100 μ l of protein digest was tested – see notes below). Centrifuge the column for 4 min at 2000xg @ 8000 rpm. Place the flow-through from the column back in the column and centrifuge again. Replace the tube, and wash the column 2-3 times with 100 μ l of conditioning buffer. Centrifuge to dryness making sure the tip of the column is not immersed in liquid at the final step.
- 4) **Releasing the sample:** Use a fresh collecting tube. Add 100 μ l of releasing buffer. Centrifuge for 4 minutes. Collect the flow-through liquid. This is elution 1. Repeat 2 more times, collecting the resulting flow-through liquid for elution 2 and 3. The majority of the peptides will elute in the second elution step. Elution 1 had some peptides, and by elution 3, the amount of peptides was minimal.
- 5) **Notes:** The above protocol will bump off the peptides all in one shot. You may also try successive spins with releasing solvent containing 25-, 50-, 100-, 200-, and 400-mM KCl. The columns can be reused by washing 3 times with 50 μ l of conditioning solvent.
- 6) **Experimentally tested:** Experiments were done with loading 25 μ g (in 25 μ l) and 'overloading' the column with 100 μ g (in 100 μ l) of a BSA digest over brand new columns.
The 25 μ g experiment showed minimal amount of peptide in either the flow-through or the washing step. However, less than 10% peptide was recovered from any single elution step. 10 μ l of elution 1 and 10 μ l of elution 2 were combined, dried in a speed vac and resuspended in 10 μ l of water. This had a yield of 11%.
100 μ g of digest in 100 μ l was loaded in the cartridges and had minimal peptide in the flow-through and wash steps. 24% yield of peptides was found in elution 1, 61% was found in elution 2, 10% in elution #3. Combining elution 1 and 2 in the same manner resulted in a yield of 71%.

Note: The majority of peptides elute in the second elution step. The column has a much higher capacity as the manufacture's protocol suggest, and consequently has severe losses of peptides due to irreversible binding at smaller peptide amounts. The capacity has not been tested past 100 μ g, but at 100 μ g, no peptides were shown to elute from either the flow-through or the washing steps.

Procedure UltraMicroSpin™, MicroSpin™ and MacroSpin™ Columns

The NestGroup (www.nestgrp.com) offers a variety of spin columns and tips with different resins. For detergent removal we recommend to use a SCX (strong cation exchange) resin:

| Column | Protein capacity | Loading volume | Part # | Resin |
|-----------------|------------------|----------------|-----------------|-----------------|
| UltraMicroSpin™ | 5 – 50 µg | 2 – 100 µl | SUM HIL-SCX | SCX (PolySULFO) |
| MicroSpin™ | 10 – 100 µg | 5 – 200 µl | SEM HIL-SCX | SCX (PolySULFO) |
| MacroSpin™ | 50 – 500 µg | 50 – 450 µl | SMM HIL-SCX | SCX (PolySULFO) |
| 96-well | 10 – 100 µg | 10 – 100 µl | SNS HIL-SCX | SCX (PolySULFO) |
| 96-well (-L) | 10 - 300 µg | 10 – 400 µl | SNS HIL-SCX - L | SCX (PolySULFO) |

Procedure UltraMicroSpin™, MicroSpin™ and MacroSpin™ Columns:

- Slide the adapter collar onto the spin column and place it in a 2ml micro centrifuge tube.
- Conditioning the column:
 - Pipette 100 µl (500 µl) of 100% methanol to the column and centrifuge it for 1 min. at ~110x g (@ ~800 rpm Eppendorf microcentrifuge).
 - Flush with at least 2 bed volumes (50 or 100µl or 500 µl, respectively) of 100% water before adding any salt to prevent salt precipitation.
 - Condition the cartridge with a strong buffer for at least one hour prior to its initial use (add 100µl or 500 µl of conditioning buffer, spin for 10 sec. and let stand in the tube) use 0.2 M monosodium phosphate (NaH₂PO₄) + 0.3 M sodium acetate (CH₃COONa) (the pH will be between 3.0 and 6.5).
- Equilibrate the column:
 - Add with 100µl (500 µl) of Buffer-Load (5-15 mM phosphate, pH 3.0) and centrifuge it for 1 min. at about 110x g (@200 rpm with an Eppendorf micro centrifuge).
 - Repeat two more times. Remove the collecting tube and blot dry any moisture on the exterior of the column.
- Processing the sample:
 - Adjust pH of sample to ~ 3 using 1- 10% phosphoric acid (use glass syringes for conc. acids to minimize contaminants)
 - Add 2-200µl (50-450µl) of sample to the column and place it in a new 2ml centrifuge tube
 - Spin the tube 1 min. at 110x g. Peptides and proteins will be retained, while detergents and non polar solutes will elute in the liquid in the collecting tube if some organic solvent is present in the buffer. Retain this Flow Through.
 - Add an additional 50 µl (100 µl) of Buffer-Load and repeat the spin to wash out any trace impurities or un-retained detergent.
 - Repeat once or twice if necessary.
 - Note the first Flow through fraction can be loaded a second time and washed again.
- Releasing the sample:
 - Add 2-50µl (50-250µl) of Buffer Elute to the tube, (containing 5-15 mM phosphate + 0.1 - 0.8M NaCl (or ammonium formate, although elution would be broader under these conditions).
 - Spin as above. Peptides and proteins will be in the liquid in the collection tube.
 - If a sample is especially basic, repeat this step with increasing amounts of salt to elute all of the sample.

NOTE from Nestgroup: These spin columns of PolySULFOETHYL Aspartamide™ will retain cationic solutes such as peptides, protein digests, or simple organic amines.

- Since total binding capacity is on the order of 0.05-0.5 mg depending on column size. There will be a considerable Donnan exclusion effect present. To prevent exclusion from the column put sample in 5-15 mM of salt or buffer. Additionally, the gradient will be much more concave than that expected. Thorough equilibration is necessary prior to loading to ensure retention.
- Conditioning and equilibration of cartridges can be done in advance, then stored in the refrigerator until needed.
- Columns can be reused by washing three times with two bed volumes (50µL, 100µL or 500µL, respectively) of 800mM NaCl (aq.) or 80% MeCN, and then washing three times with two bed volumes of the 5mM conditioning solvent.
- Peptides are retained by the positive charge of at least the n-terminal amine and eluted by a combination of total charge, charge distribution and hydrophobicity. If your peptide does not stick to the column, be sure it is in a small amount of buffer, or decrease the concentration of organic in the solvents to 10 or 5%. (Organic solvent concentration is empirically determined).
- Detergents, and non-polar solutes will not be retained if some organic solvent is used in the mobile phase.
- Use of 0.1% TFA or high concentrations of formic acid in the mobile phase is not recommended. The conditioning process is reversed by exposing the column to pure organic solvents.
- Use pH 3 for retention of neutral to slightly acidic peptides. Use of a higher pH may be considered for basic hydrophobic peptides.
- **Sample composition: Important:** The sample and the conditioning solvent should contain comparable amounts of acetonitrile (e.g., 5+%), and salt concentrations should be 5-15 mM. Otherwise, polar solutes such as peptides and proteins might not be retained. Dilute the sample if necessary to decrease the salt concentration.

HPLC Cation Exchange Procedure

Peptide separation procedure using and HPLC equipped with an UV detector

HPLC columns:

- 1) 2.1 mm x 20 cm, 5 µm particles, 300 Å pore size, Polysulfoethyl A strong cation exchange material. Manufacturer's suggested protein max: 1mg protein for good chromatography. (PolyLC Inc.)
- 2) 4.6 mm x 20 cm, 5 µm particles, 300 Å pore size Polysulfoethyl A strong cation exchange material. Manufacturer's suggested protein max: 5mg protein for good chromatography. (PolyLC Inc.)

Buffers:

Buffer A 5 mM K₂HPO₄, 25% CH₃CN, pH 3.0

Buffer B 5 mM K₂HPO₄, 25% CH₃CN, 600 mM KCl, pH 3.0

Gradient:

| Time [min] | %B |
|------------|-----|
| 0 | 0 |
| 30 | 25 |
| 50 | 100 |

Procedure:

- The flow rate is 200 µl/min for the 2.1 mm column and 800 µl/min per minute for the 4.6 mm column.
- We have loaded anywhere from about 120 µg up to about 5 mg of reduced/alkylated and digested total protein on the 2.1 mm column, and up to 30 mg on the 4.6 mm column.
- The samples are usually diluted 1:1 with Buffer A, pH 2.5-3.0. It is important to acidify the samples down to pH 3.0 or below before loading onto the cation exchange because peptides will not be fully charged at higher pH values and may not stick to the column!
- A sample loop with a 2 ml capacity was loaded with sequential 2 ml portions of the total volume of the sample.
- The column and loop are then washed with Buffer A until the baseline returns to approximately zero before loading the next 2 ml (about 15 minutes between each loading).
- The gradient shown is designed to spread out the elution of doubly-charged peptides as much as possible, with these peptides usually eluting starting at about 8-9 minutes into the run until approximately minutes 15-16, after which triply charged peptides begin to elute.
- Phosphopeptides are expected to elute in the earlier fractions, while large multiply charged peptides elute in later fractions.
- Fractions are collected at 1 minute intervals.

Avidin purification

Note this protocol was originally developed for ABI Avidin cartridges to purify ICAT labeled peptides, but it should work fine with other Avidin Agarose products.

Reagents and Materials

- ICAT Cartridge–Avidin # 4326694 (Invitrogen)
- Cartridge holder # 4326688 (Invitrogen); Outlet tubing kit # 4326690 (Invitrogen); Needle-port adapter # 4326689 (Invitrogen)
OR
- Monomeric Avidin Agarose # 20228 (Pierce)
- Empty spin columns, e.g. Centrifuge Columns, 0.8mL # 89868 (Pierce)

| | |
|----------------------------|-------------------------------------------------------------------------------------------------------------------|
| Affinity buffer – Load: | 2 x PBS in H ₂ O pH 7.2 |
| Affinity buffer – Wash 1: | milliQ H ₂ O (optional you can use 1 x PBS in H ₂ O, but less salt is better for mass spec) |
| Affinity buffer – Wash 2: | 50mM Ammonium Bicarbonate pH 8.3, 20% Methanol in H ₂ O |
| Affinity buffer – Elute: | 30% Acetonitrile, 0.4% TFA in H ₂ O |
| Affinity buffer – Storage: | 2 x PBS pH 7.2, 0.1% NaN ₃ in H ₂ O |
| 10 x PBS: | 100mM Sodium phosphate (12g/L), 0.9% NaCl |

Procedure

1. Activating the Avidin Affinity column
 - Insert the Avidin affinity cartridge (in either direction, but reuse always in the same direction) into the column holder.
 - Inject 1ml of the Affinity buffer–Elute (this step is critical to the performance of the Avidin affinity column)
 - Inject 2ml of the Affinity buffer–Load
2. Loading Sample on the Avidin Affinity column
 - Neutralize the cation exchange fraction by adding **500µl** Affinity buffer–**Load**, check for **pH 7 – 7.5**
 - Slowly inject (~drop/second) the **neutralized fraction** onto the column and collect the flow-through
 - Optional: reload the flow-through
3. Removing Non-labeled material
 - Inject **500µl** Affinity buffer–**Load** and continue to collect the flow-through
 - Inject **1ml** of Affinity buffer–**Wash 1 or MilliQ H₂O**, sending the output to waste. (This step reduces the salt concentration)
 - To remove non-specifically bound peptides and lower salt concentration, inject **1ml** Affinity buffer–**Wash 2**, sending the output to waste.
4. Eluting ICAT labeled Material
 - To elute the peptides, slowly inject (~1 drop/second) **800µl** Affinity buffer–**Elute**
 - Collect the eluate into a **1ml glass vial** (clear, with caps, Waters # WAT025054, Lot#71)
 - **dry** eluate in Speed Vac and keep at **–20°C**
5. Cleaning and storing the Avidin Affinity column
 - Inject **1ml** of the Affinity buffer–**Elute** to clean the cartridge
 - If you have additional cation exchange fractions, repeat steps 1. - 5., starting with 1.2.
 - Neutralize the column by injecting **2ml** Affinity buffer–**Load**
 - To store the cartridge:
 - **Overnight**, inject **2ml** of the Affinity buffer–**Load**
 - **More than one day**, inject **2ml** Affinity buffer–**Storage**

Open the bayonet mount of the column holder and remove the Avidin cartridge.
Cap the ends of the cartridge with the two end caps before storing at 2-8°C.

IMAC (immobilized metal affinity chromatography)

Reference: The SCX/IMAC enrichment approach for global phosphorylation analysis by mass spectrometry. Villén J, Gygi SP. Nat Protoc. 2008;3(10):1630-8.

Note peptides were separated via SCX and desalted, see ref for complete details

There are many protocols and kits available ... it's worth doing some extra research.

IMAC buffers

Binding buffer: 40% ACN (vol/vol), 25 mM FA in H₂O

Elution buffer A: 50 mM K₂HPO₄, adjust to pH 10 with NH₄OH

Elution buffer B: 500 mM K₂HPO₄, pH 7.

IMAC phosphopeptide enrichment

1. Prepare 100 µl of IMAC beads by washing them with 1 ml of IMAC binding buffer, turning over the vial a few times to resuspend all beads and spinning to remove the liquid. Repeat three times and prepare 50% slurry in the same buffer.
 2. Prepare twelve 250-µl Eppendorf tubes and place 10 µl of IMAC beads slurry into each. Cutting the end of the tip facilitates pipetting of beads.
 3. Dissolve each peptide fraction obtained from SCX in 120 µl of IMAC-binding buffer and transfer to the IMAC beads
 4. Incubate peptides on beads for 60 min, with vigorous shaking, at room temperature.
 5. During this time, prepare StageTips²⁶ by packing two disks of Empore 3M C18 material onto 250-µl pipette tips.
 6. Wash and equilibrate StageTips by passing through sequentially 20 µl of MeOH, 20 µl of 50% ACN 0.5% HAcO and twice through 20 µl of 1% FA. For convenience and increased throughput, one can use a centrifuge by holding the StageTips within a 2-ml Eppendorf tube with the top part of a 500-µl Eppendorf body as an adaptor, limiting spinning speed to 2,000g and time to the minimum to get the liquid passed through
 7. From this step, one can keep IMAC enrichment and phosphopeptide desalting separated (option A), or for convenience, experienced users might consider combining them into a single procedure (Option B). Both protocols are provided below
 - A. **IMAC enrichment followed by phosphopeptide desalting**
 - i. Remove liquid from the tubes (Step 31) and transfer to a tube labeled 'IMAC flowthrough'. These peptide mixtures can be analyzed in the mass spectrometer if one is interested in nonphosphorylated peptides.
 - ii. Wash the resin with 120 µl of IMAC binding buffer. Repeat this step twice.
 - iii. Elute phosphopeptides by incubating for 5 min with 40 µl of IMAC elution buffer A (50 mM K₂HPO₄/NH₄OH, pH 10.0). Repeat this step twice. Combine eluates from the same sample into the same tube, containing 40 µl of 10% FA to neutralize pH.
 - iv. Dry samples by vacuum centrifugation at room temperature.
 - v. Resuspend the samples from Step A(iv) in 20 µl of 1% FA and load them into the StageTips prepared on Steps 32 and 33.
 - vi. Wash to desalt with 20 µl of 1% FA.
 - B. **Combined IMAC enrichment and phosphopeptide desalting**
 - i. After the 60-min incubation in Step 31, transfer IMAC beads to the top of the StageTips and spin down. The beads will get retained on the StageTip and the solution will pass through. As the buffer contains 40% ACN, nonphosphorylated peptides, which are not retained in the IMAC resin, will not be retained by the C18 material. These peptide mixtures can be collected and analyzed as well in the mass spectrometer.
 - ii. Wash with 50 µl of IMAC binding buffer. Repeat this step once.
 - iii. Wash with 40 µl of 1% FA. This step allows equilibrating the StageTip C18.
 - iv. Wash with 70 µl of 500 mM K₂HPO₄, pH 7. Repeat this step twice. At this point, phosphopeptides are eluted from IMAC resin and retained on the C18.
 - v. Wash with 40 µl of 1% FA to remove phosphate salts.
 8. Elute phosphopeptides from StageTips into vials for MS analysis with 40 µl of 50% ACN 0.5% HAcO.
 9. Dry down the samples from Steps A(i) and B(i) (nonphosphorylated peptides) and Step 8 (phosphopeptides) by vacuum centrifugation. **Pause Point At this point, samples can be stored at -20 °C for several weeks.**
 10. Points from here up to are related to LC-MS/MS analysis Resuspend samples in 10 µl of 5% ACN, 4% FA. For nonphosphopeptide samples, prepare a 1/100 dilution.
- Inject 1 µl of each sample onto the LC-MS/MS system. Analyze each sample in duplicate.

FASP (Filter-aided sample preparation) on whole cell lysates

References:

1. Sample preparation and digestion for proteomic analyses using spin filters. Manza LL, Stamer SL, Ham AJ, Codreanu SG, Liebler DC. *Proteomics*. 2005 May ; 5(7): 1742-5
2. Universal sample preparation method for proteome analysis. Wisniewski JR, Zougman A, Nagaraj N, Mann M. *Nat Methods*. 2009 May ; 6(5): 359-62
3. Proteome, phosphoproteome, and N-glycoproteome are quantitatively preserved in formalin-fixed paraffin-embedded tissue and analyzable by high-resolution mass spectrometry. Ostasiewicz P, Zielinska DF, Mann M, Wisniewski JR. *J Proteome Res*. 2010 Jul 2; 9(7): 3688-700

Use the FASP Protein Digestion Kit whenever you're concerned that SDS, reducing agents, salts, or other contaminants might interfere with trypsin digest efficiency. The FASP procedure depletes contaminants such as salts, detergents, and reducing agents from the sample prior to alkylation and digestion.

The following protocols describes a method for generation of tryptic peptides from crude lysates for LC-MS analysis. The method allows analysis of detergent lysed cells and tissues. Therefore it is particularly suitable for studying entire proteomes and fractions containing biological membranes. The key features of the method are:

1. Protein digests free from nucleic acids and other cell components
2. Can be applied to samples containing high concentrations of detergents
3. There are no precipitation and the concentration of sample is kept high
4. In a single filter device 0.2-200 µg of total protein can be processed
5. The yield and purity of peptides can be monitored by UV-spectrometry allowing QC of the digest.

Materials and Reagents

- FASP™ Protein Digestion Kit (Protein discovery 44250) OR
- FASP-FFPE Protein Digestion (Protein discovery 44255) Kit for paraffin embedded tissue
- OR all of the following
- Lysis buffer: 4%(w/v) SDS, 100mM Tris/HCl pH 7.6, 0.1M DTT (prepare fresh)
- Urea 8.5: 8 M urea (Sigma, U5128) in 0.1 M Tris/HCl pH 8.5 (prepare fresh)
- Urea 8.0: 8 M urea (Sigma, U5128) in 0.1 M Tris/HCl pH 8.0 (prepare fresh)
- IAA solution: 50 mM iodoacetamide in Urea 8.5. Prepare 0.1 ml per 1 sample (prepare fresh)
- Endoproteinase Lys-C from Wako Bioproducts (Richmond, VA) Stock 5 µg/µl or (Princeton Separations EN-130) optional
- Trypsin, Stock 0.4 µg/µl
- 0.5 M NaCl in water
- Ambic: 50 mM NH₄HCO₃ in water. Prepare 0.25 ml per 1 sample
- Amicon Ultra-0.5, Ultracel-30 Membrane, 30 kDa (Millipore # UFC503008), or Amicon Ultra-0.5, Ultracel-10 Membrane, 10 kDa (Millipore # UFC501008), or Nanosep® Centrifugal Devices with Omega™ Membrane – Volumes < 1 mL 10k MWCO, (PALL Cooperation OD010C33)
- Benchtop centrifuge capable of 14,000 x g (centrifugation speed and time should be in accordance with the spin filter manufacturers protocol)
- Incubator set at 37 °C

Cell Lysate preparation:

Lyse cells or homogenized tissues (homogenized with a blender in the Lysis buffer) in Lysis buffer using 1:10 sample to buffer ratio at 95°C for 3-5 min, e.g. 50 µl cell pellet (one 15cm dish) or 25 mg of tissue in 150 ul Lysis buffer (50 µl HeLa pellet or 25 mg brain or liver contains roughly 2 mg protein). The DNA has to be sheared by sonication to reduce the viscosity of the sample. Before starting sample processing in the filter unit the lysate has to be clarified by centrifugation at 16,000 x g for 5 min.

FASP Procedure for Trypsin digest

1. Mix up to 30µl of a protein extract with 200µl of Urea 8.5 in the filter unit and centrifuge at 14,000 x g for 15-40 min.
2. Add 200µl of Urea 8.5 to the filter unit and centrifuge at 14,000 x g for 15-40 min.
3. Discard the flow-through from the collection tube.
4. Note if your lysis step did not include a reduction step using DTT you should add 100µl of 10 mM DTT in Urea 8.5, vortex for 1 min and incubate at 50°C for 15 min and centrifuge at 14,000 x g for 15-40 min. Discard the flow-through from the collection tube.
5. Add 100 µl IAA solution and vortex for 1 min and incubate without mixing for 20 min in the dark.
6. Centrifuge at 14,000 x g for 15-40 min.
7. Add 100 µL of Urea Sample Solution to the Spin Filter and 6. centrifuge at 14,000 x g for 15 min. Repeat this step twice

8. Discard the flow-through from the collection tube
9. Add 100 µl of Ambic to the filter unit and centrifuge at 14,000 x g for 10-30 min. Repeat this step twice.
10. Add 75 µl Ambic with trypsin (enzyme to protein ratio 1:100) and vortex for 1 min. Wrap the tops of the tubes with Parafilm to minimize the effects from evaporation.
11. Incubate the Spin Filter in an incubator at 37 °C for 4 – 18 h
12. Transfer the filter units to new collection tubes.
13. Centrifuge the filter units at 14,000 x g for 10 min.
14. Add 40 µl Ambic and centrifuge the filter units at 14,000 x g for 10 min, repeat twice.
15. Add 50 µL 0.5 M Sodium Chloride Solution and centrifuge the Spin Filter at 14,000 x g for 10 min (this helps release any peptides bound to the membrane)
16. Filtrate contains digested proteins. Acidify the filtrate with TFA or FA to the desired pH and desalt

FASP Procedure for Lys-C and Trypsin digestion

1. Mix up to 30µl of a protein extract with 200µl of Urea 8.5 in the filter unit and centrifuge at 14,000 x g for 15-40 min.
2. Add 200µl of Urea 8.5 to the filter unit and centrifuge at 14,000 x g for 15-40 min.
3. Discard the flow-through from the collection tube.
4. Note if your lysis step did not include a reduction step using DTT you should add 100µl of 10 mM DTT in Urea 8.5, vortex for 1 min and incubate at 50°C for 15 min and centrifuge at 14,000 x g for 15-40 min. Discard the flow-through from the collection tube.
5. Add 100 µl IAA solution and mix at 600 rpm in a thermo-mixer for 1 min and incubate without mixing for 20 min in the dark.
6. Centrifuge the filter units at 14,000 x g for 10-30 min.
7. Add 100 µl of Urea 8.0 to the filter unit and centrifuge at 14,000 x g for 15-40 min. Repeat this step twice.
8. Add 40 µl of Urea 8.0 with Lys-C (enzyme to protein ration 1:50) and mix at 600 rpm in thermo-mixer for 1 min.
9. Incubate the filter units in incubator at 37 °C overnight.
10. Transfer the filter units to new collection tubes.
11. Add 120 µl Ambic with trypsin (enzyme to protein ration 1:100) and mix at 600 rpm in thermo-mixer for 1 min.
12. Incubate the units in a incubator at 37°C for 4 -18 h.
13. Transfer the filter units to new collection tubes.
14. Centrifuge the filter units at 14,000 x g for 10-40 min.
15. Add 50 µl Ambic (or 0.5 M NaCl) and centrifuge the filter units at 14,000 x g for 10-40 min.
16. Acidify with CF₃COOH and desalt the filtrate.

Yield determination

Concentration of the peptides can be estimated by UV spectrometer assuming that 0.1% solution of vertebrate proteins has at 280 nm an extinction of 1.1 absorbance units (1mg/ml solution has 1.1 au). Always record a spectrum from 240-340 nm. It should have a distinct peak with a maximum at 270-280 nm and the extinction at 320 nm has to be 0.

Notes:

1. Quarz cuvettes are required for correct absorption spectra.
2. Extinction values below 0.1 allow only rough protein estimation.

MWCO Selection for Protein Applications, Biomolecule MW refers to the MW to be retained on top of the filter:

| MWCO | Membrane Nominal Pore Size | Biomolecule Size | Biomolecule Molecular Weight |
|--------|----------------------------|------------------|------------------------------|
| 1K | | | 3K - 10K |
| 3K | | | 10K - 20K |
| 5K | | | 15K - 30K |
| 10K | | | 30K - 90K |
| 30K | | | 90K - 180K |
| 50K | 5 nm | 15 - 30 nm | 150K - 300K |
| 100K | 10 nm | 30 - 90 nm | 300K - 900K |
| 300K | 35 nm | 90 - 200 nm | 900K - 1,800K |
| 1,000K | 100 nm | 300 - 600 nm | >3,000K |

FASP (Filter Aided Sample Preparation)

Use the FASP Protein Digestion Kit whenever you're concerned that SDS, reducing agents, salts, or other contaminants might interfere with Trypsin digest efficiency. The FASP procedure depletes contaminants such as salts, detergents, and reducing agents from the sample prior to alkylation and digestion.

Materials

- Ultrafree-MC low-binding regenerated cellulose spin filters with a 5000 molecular weight cutoff (MWCO) UFC30HV25 from Millipore (Billerica, MA, USA) or any of the filters mentioned above.
 - Methanol (LC-MS grade)
 - Water (LC-MS grade)
 - 100 mM ammonium bicarbonate
 - 10 mM DTT
 - IAA
 - Trypsin
1. Spin filters are rinsed with methanol and then sequential 300 μ L washes of methanol and water are centrifuged through the filters at 4500xg at 20°C.
 2. The protein sample (approximately 20 μ g in 10 μ L 100 mM ammonium bicarbonate) is diluted with 50 μ L 100 mM ammonium bicarbonate and added to the upper chamber of the spin filter.
 3. The samples then are centrifuged at 4500xg to pass the solution through the filter.
 4. The proteins on the filter then are washed with 300 μ L each water and 100 mM ammonium bicarbonate followed each time by centrifugation at 4500xg and the filtrates are discarded.
 5. The protein is resuspended in 100 μ L 100 mM ammonium bicarbonate containing 10 mM DTT and the filters are capped and incubated in a water bath at 50°C for 15 min.
 6. Iodoacetamide is added to a final concentration of 20 mM for 15 min to convert thiols to carboxamidomethyl derivatives.
 7. Centrifuge at 14,000 x g for 15-40 min.
 8. Add 100 μ L of Urea Sample Solution to the Spin Filter and 6. centrifuge at 14,000 x g for 15 min. Repeat this step twice
 9. Discard the flow-through from the collection tube
 10. Add 100 μ L of Ambic to the filter unit and centrifuge at 14,000 x g for 10-30 min. Repeat this step twice.
 11. Transfer the filter units to new collection tubes
 12. Add 75 μ L modified porcine sequencing-grade trypsin (Promega, Madison, WI, USA) then is added in a 1:50 protein:Trypsin ratio and the samples are incubated at 37°C for 18–24 h.
 13. Tryptic peptides then are collected by centrifugation through the filter at 4500xg and the filtrate is acidified with 0.5 μ L of concentrated formic acid.

Peptide desalting (C18 resin)

Peptides must be desalted before LC-MS analysis to remove salts and urea from the digestion buffer. The NestGroup (www.nestgrp.com) offers a variety of spin columns and tips with different resins, C4, C8 or C18 resins will retain non-polar solutes such as peptides, proteins, and detergents. Salts, and polar solutes like DNA will not be retained. This permits the removal of SDS from samples prior to mass spectrometry. Use of 1.0% TFA will increase the binding of peptides and proteins.

Materials and reagents:

For desalting peptides we recommend to use a C18 resin, the size of the columns should be selected based on the amount of starting protein, considering their loading capacities:

| Column | Protein capacity | Loading vol. | Void vol. | Part # | Resin |
|-------------------|------------------|--------------|-----------|-------------|-----------------|
| UltraMicroSpin | 10 – 50 µg | 2 – 100 µl | 25 µl | SUM SS18V | Silica C18 300A |
| MicroSpin | 20 – 100 µg | 5 – 200 µl | 50 µl | SEM SS18V | Silica C18 300A |
| MacroSpin | 100 – 500 µg | 50 – 450 µl | 250 µl | SMM SS18V | Silica C18 300A |
| UltraMicro Tip | 3 – 30 µg | 5 – 100 µl | 50 µl | STU SS18V | Silica C18 300A |
| 96-Well MiniSpin | 10 – 150 µg | 10 – 100 µl | 75 µl | SNS SS18V | Silica C18 300A |
| 96-Well MACROSpin | 20 - 300 µg | 20 – 200 µl | 225 µl | SNS SS18V-L | Silica C18 300A |

Solvent A (loading): 80% ACN, 0.1% TFA in water (or 99.9% ACN, 0.1% TFA)
 Solvent B (equilibration): 5% ACN, 0.1% TFA in water (optional use 0-5% ACN)
 Solvent C (elution): 80% ACN, 0.1% FA in water (or up to 25mM formic acid)

UltraMicroSpin and MicroSpin columns Or MacroSpin™ columns:

1. Dilute sample to 5% ACN, 0.1% TFA in water, and ensure pH is acidic. If not, add small aliquots of 0.5% TFA and check with pH paper until it is.
2. Slide the adapter collar onto the spin column and place it in a 2ml micro centrifuge tube.
3. Condition the column using Solvent A (80% ACN / 0.1% TFA). Add 100 µl or 500µl and centrifuge 2 min at 2000 rpm, repeat twice (manufacturers instruction: 110x g (@ ~800 rpm with an Eppendorf micro centrifuge)
4. Empty collecting tube as needed throughout equilibration and washing processes
5. Equilibrate column by adding 50 or 100µl or 500µl Solvent B, respectively, centrifuge 2 min at 2000 rpm, repeat twice.
6. Remove the collecting tube and blot dry any moisture on the exterior of the column and place it in a new 2ml centrifuge tube
7. Add up to max volume/protein of protein digest (sample) per column. Centrifuge 2 min at 2000 rpm. Collect flowthrough and pass protein digest through column once more
8. Wash column with 25 or 50µl or 250µl of solvent B to wash out any traces of salts, repeat twice
9. Place column in a new 2ml centrifuge tube and collect clean desalted sample by adding 2-50µl or 50-250µl of solvent C, optional repeat and combine eluates (Peptides will be in the liquid in the collection tube. If a sample is especially non-polar, it may be necessary to repeat this step to elute all of the sample.)
10. Speed Vac to near dryness (not less than 10uL). Do not dry completely!
11. Resuspend in 5% ACN / 0.1% Formic Acid (to a peptide concentration of 1-10 µg/µl) and store at -80 °C
12. Prior to LC-MS analysis spin sample and transfer supernatant to an autosampler vial.

NOTES:

- Columns can be reused by washing three times with two bed volumes (50µL, 100µL or 500µL, respectively) of 100% ACN, MeOH or *n*-PrOH containing 25 mM formic acid (aq.) and then washing three times with two bed volumes of loading or equilibration buffer.
- **Sample composition Important:** The sample and the equilibration buffer should contain comparable amounts of acetonitrile (e.g., 0 - 5%). Otherwise, polar solutes such as peptides and proteins might not be retained. Including 1.0% TFA increases binding capacity for peptide capture. Decrease the organic solvent concentration of the sample if yields are low.

96-Well Spin and 96-Well MACROSpin RPC Plates*Conditioning the column:*

1. Tap the column gently to ensure that the dry column material is settled at the bottom of the columns and condition as above. Foil is for sealing purposes only. All 96 wells do not need to be opened at the same time. Remove foil from as many rows as desired for your application. Foil should be cut with a razor or other sharp blade.
2. Place the 96-Well Spin Column into a collection plate and pipette 200 μ L of organic solvent (400 μ L for the MACROSpin Plates) into all opened wells and centrifuge the plate for 1 minute in the collection plate at 110x g to wet the RPC phase then repeat with equilibration buffer (5% ACN, 0.1% TFA in water) to equilibrate.

Processing the sample:

3. You can reuse the emptied collection plate for sample loading. Blot dry any liquid on the exterior of the column. Add your 50-100 μ L sample (50-400 μ L for the MACROSpin Plates) to the top of a well. Be careful to ensure that the sample is placed in the center of the well. Having 0.1% TFA in the sample can facilitate binding, but it isn't necessary.

Releasing the sample:

4. Place the column in a new collection plate when the appropriate elution solvent is added (i.e. higher concentrations of ACN or organic solvent). Spin the plate for 1 minute at 110x g. After centrifugation, the purified sample will be in the collecting tube and will be ready for further use. If a sample is especially non-polar, it may be necessary to repeat this step to elute all of the sample.

SepPak tC18 solid-phase extraction cartridges from Waters

(adapted from Villen et. al. Nat. Protocols 2008, vol. 3, no. 10, p1630)

| Description | Vendor | Part# |
|-------------------------------------------------------------|--------|-----------|
| Sep-Pak® Vac tC18 cartridge 1cc/50mg 37-55 μ m 100/box | Waters | WAT054960 |
| Sep-Pak® Vac tC18 cartridge 1cc/100mg 37-55 μ m 100/box | Waters | WAT036820 |
| Sep-Pak® Vac tC18 cartridge 3cc/500mg 37-55 μ m 50/box | Waters | WAT036815 |

SepPak solvents

Binding buffer: 0.1% TFA (vol/vol) in H₂O

Elution buffer: 50% ACN (vol/vol), 0.5% HAcO (vol/vol) in H₂O.

1. The size of the cartridge should be selected on the basis of the amount of starting protein, considering their capacities are about 5% (wt/wt) of the packing material's weight. For example, for 20 mg of a protein digest, a SepPak with 500 mg of tC18 beads is recommended. In this protocol, we assume the use of 500 mg of SepPak (500-mg bulk material, 3 or 6 ml, 800- μ l bed volume). Volumes should be adapted accordingly for different sizes. A vacuum manifold can be used to increase solvent flow rates through the cartridge, or alternatively, air pressure is recommended for high-capacity SepPaks, as it provides more uniform peptide loading and elution.
2. Wash and condition the cartridge using 9 ml of ACN followed by 3 ml of 50% ACN and 0.5% HAcO.
3. Equilibrate with 9 ml of 0.1% TFA.
4. Load sample in 0.4% TFA.
5. Wash/desalt with 9 ml of 0.1% TFA.
6. Wash (to remove TFA) with 900 μ l of 0.5% HAcO.
7. Elute with 5 ml of 50% ACN 0.5% HAcO and collect eluate in a 15-ml conical tube.
8. Freeze the eluate with liquid N₂ and lyophilize. The result here should be a white (sometimes yellowish) fluffy powder. **At this point, samples can be stored at -20 °C for several weeks.**

Table 1: Materials and Reagents

Note those are just suggestions, there are other vendors, quantities or formats for most consumables, shopping around may save you some money.

| Description | Vendor | Part # |
|-----------------------------------------------------------------------------|----------------------|-----------------|
| 0.1% Formic Acid in Acetonitrile, Optima LC/MS, Solvent Blends | Fisher | LS120-4 |
| 0.1% Formic Acid in Water, Optima LC/MS, Solvent Blends | Fisher | LS118-4 |
| Acetic Acid, Optima LC/MS, AA | Fisher | A113-50 |
| Acetonitrile (Optima LC/MS), ACN | Fisher | A955-4 |
| Ammonium bicarbonate | Fisher | A643-500 |
| Calcium Chloride, CaCl ₂ | Fisher | BP510-100 |
| DTT Dithiothreitol | Fisher | PI-20291 |
| Formic Acid, Optima LC/MS Grade, FA | Fisher | A117-50 |
| IAA Iodoacetamide | Fisher | AC12227-0050 |
| Methanol (Optima LC/MS), MeOH | Fisher | A456-4 |
| Monobasic potassium phosphate (KH ₂ PO ₄) | Fisher | P286-1 |
| PBS 10x (Phosphate Buffered Saline) | Fisher | BP665-1 |
| Phosphoric acid (H ₃ PO ₄) (85%) HPLC grade | Fisher | A260-500 |
| Potassium chloride (KCl) ACS reagent 1kg | Fisher | AC42409-0010 |
| Sodium azide (NaN ₃) | Fisher | AC19038-0050 |
| TCEP (tris(2-carboxyethyl)phosphine) | Fisher | PRP5481 |
| Trifluoroacetic Acid, Biochemical Grade, TFA | Fisher | AC29381-0250 |
| Tris Base | Fisher | BP154-1 |
| Urea | Fisher | AC14075-0010 |
| Water (Optima LC/MS) | Fisher | W6-4 |
| Cartridge holder | Invitrogen | 4326688 |
| ICAT Cartridge Cation exchange | Invitrogen | 4326695 |
| ICAT Cartridge-Avidin | Invitrogen | 4326694 |
| Needle-port adapter | Invitrogen | 4326689 |
| Outlet tubing kit | Invitrogen | 4326690 |
| Amicon Ultra-0.5, Ultracel-10 Membrane, 10 kDa | Millipore | UFC501008 |
| Amicon Ultra-0.5, Ultracel-30 Membrane, 30 kDa | Millipore | UFC503008 |
| 96-well | NestGroup | SNS HIL-SCX |
| 96-well (-L) | NestGroup | SNS HIL-SCX - L |
| 96-Well MACROSpin 300Å C18, 20-200µl (20 - 300µg), ea. Silica Based | NestGroup | SNS SS18V-L |
| 96-Well MiniSpin 300Å C18, 10-100µl (10 - 150µg), ea. Silica Based | NestGroup | SNS SS18V |
| Macro Spin Tubes, 50-150µL elution, PolySULFOETHYL A | NestGroup | PMASCSE1203 |
| MacroSpin Column, 50-450µl (100 - 500µg), HILIC or SCX, 96/pk silica based | NestGroup | SMM HIL-SCX |
| MacroSpin Columns, 50-450µl (100 - 500µg), 96/pk; Silica C18 300Å | NestGroup | SMM SS18V |
| Micro Spin Tubes, 5-25µL elution, PolySULFOETHYL A | NestGroup | PUMSCSE1203 |
| MicroSpin Column, 5-200µl (20-100µg), HILIC or SCX, 96/pk silica based | NestGroup | SEM HIL-SCX |
| MicroSpin Columns, 5-200µl (20-100µg), 96/pk; Silica C18 300Å | NestGroup | SEM SS18V |
| PolySULFOETHYL A, Bulk 5µm, 200Å, 1g | NestGroup | PBMSE0502 |
| SPE Cartridges, 100-200µL elution, PolySULFOETHYL A | NestGroup | PSPSE1203 |
| UltraMicro TIP Columns, 5-100µl (3-30µg), 96/pk; Silica C18 300Å | NestGroup | STU SS18V |
| UltraMicroSpin Column, 2-100µl (10-50µg), HILIC or SCX, 96/pk silica based | NestGroup | SUM HIL-SCX |
| UltraMicroSpin Columns, 2-100µl (10-50µg), 96/pk; Silica C18 300Å | NestGroup | SUM SS18V |
| Nanosep® Centrifugal Devices with Omega™ Membrane – Volumes < 1 mL 10k MWCO | PALL Cooperation | OD010C33 |
| Empty spin columns, e.g. Centrifuge Columns, 0.8mL | Pierce | 89868 |
| Monomeric Avidin Agarose | Pierce | 20228 |
| Lysine-C 3 x 5 µg vials | PrincetonSeparations | EN-130 |
| Trypsin modified, sequencing grade, 5 x 20ug | Promega | V5111 |
| FASP-FFPE Protein Digestion | Protein discovery | 44255 |
| FASP™ Protein Digestion Kit | Protein discovery | 44250 |
| PPS Silent Surfactant | Protein Discovery | 21011 |
| Endoproteinase Lys-C from Lysobacter enzymogenes, 5ug | Sigma | P3428-1VL |
| Sodium dodecyl sulfate, SDS | Sigma | L4509-10G |
| | Fisher | BP166-500 |

