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Filter Aided Sample Preparation (FASP) with trypsin digestion

Wiśniewski, J. R., Zougman, A., Nagaraj, N., Mann, M. Universal sample preparation method for proteome analysis. *Nat. Methods* **2009**, *6* (5), 359–62.

With FASP you can use detergent based lysis buffers for preparation of samples for LC-MS/MS. There is an approximate 30-50% loss of protein with this procedure. In a single filter device (Microcon YM-30, Millipore #MRCF0R030) **0.2-200 µg** (*max 300 µg*) protein can be processed.

Golden tip: *Gently pipette up and down (2x, without touching the filter) when adding new solution into the filter with the protein sample, or mix the samples for 1 minute at 650 rpm at RT on a thermoshaker . It is very important to filter homogeneous solutions.*

IMPORTANT! *The centrifugation speed must not exceed 12 000 x g when using FASP filters and they should always be capped during centrifugation. Do not allow the membrane in the filter devices to dry out once wet. If you are not using the device immediately after rinsing, leave fluid on the membrane until the device is used.*

DO NOT CENTRIFUGE PAST MINIMUM VOLUME OF APPROX. 50µL

FASP Protocol, Day 1.

1. **Test the filters** by **adding 0.4 ml of Urea solution** and centrifuge at 11 000 x g for 5 min. Approx. 1/4 of the volume should pass through "Good" filters. Discard filters with higher flow-through. Keep centrifuging the "Good" filters for about 15 min. This first step will also clean the filters (traces of glycerine) before sample addition.
2. Mix **30 µl sample** *(max 50 µl)* with **200 µl Urea solution** *(max 350 µl)* to reduce the SDS concentration to 0.5 % (w/v) that is compatible with the filters. *Tip: If you have larger sample volume than 30-50 µl, dilute with Urea to 0.5% SDS and concentrate in the filter using max 400 µl for each centrifugation.* **Centrifuge at 11 000 x g** at RT for 15-20 min. The same centrifugation settings are used throughout the protocol, unless otherwise stated.

   NOTE: centrifugation time varies from sample to sample and must be optimized. Make sure that the filters are not spun dry (filters should be covered with solution all times, minimum 50µl). Discard the FT anytime the collector is getting full.

3. Add **200 µl of Urea solution** to the filter unit, mix the solution well (*e.g. 1 minute at 400-650 rpm at RT on a thermoshaker*), and centrifuge for 15-20 minutes, repeat *(total of 2 washes)*

0.5M Tris-HCl stock, pH 8.5

*Dissolve **30.29 g Tris** (Sigma Aldrich, #252859) in 400 ml deionized water dH_2O. Adjust pH to 8.5 with conc. HCl. Bring final volume to 0.5 liter with dH_2O*

Urea solution: 8M Urea/0.1M Tris-HCl, pH 8.5

***480 mg Urea** (Sigma-Aldrich, # U1250) and 430 µl dH2O and 200 µl 0.5M Tris-HCl pH 8.5*

FASP Protocol, Day 1 continued…

4. If your lysis step did not include reduction with DTT, add **100 µl 10 mM DTT in Urea solution** *(Dilute 10µl 1M DTT stock to 1ml with Urea solution)*. Mix the sample solution (*as above*), and incubate the tubes for 1h at RT. Centrifuge for 10-20 min.
5. Add **100 µl 50 mM IAA solution**, mix sample solution, and incubate in the dark for 20 min. Centrifuge for 10-20 min.
6. Add **200 µl Urea solution**, mix sample solution, and centrifuge for 10-20 min. Repeat twice *(total of 3 washes)*, and discard FT.

NOTE: SDS concentration needs to be below 0.0005% for protein amounts below 10-15 µg

7. Add **100 µl of 50mM Ambic** to the filter unit, mix sample solution, and centrifuge for 10 min *(check how long it takes, it goes faster with Ambic)*. Repeat twice. (*total of 3 washes, DO NOT discard FT*)
8. Add a **50-100µl Trypsin** solution with trypsin to protein ratio 1:50, to the spin filters *(50-75µl for global proteome, and 100µl for phosphoproteome)*. Mix sample solution.

NOTE: Wrap the tubes in parafilm to minimize evaporation during incubation and do not discard the liquid in the collecting tubes.

Incubate the samples in a heating closet at 37 °C for 16 h.

1M Dithiotreitol (DTT) stock solution

154 mg DTT *(Sigma Aldrich, #D-9163) in 1ml water (may be aliquoted, and kept in freezer for 6 months), or purchase the 1M DTT solution from Sigma Aldrich # 43816.*

50 mM iodoacetamide (IAA) in Urea solution

9.25 mg IAA *(Sigma Aldrich, #I-6125) to 1ml Urea solution (make fresh, keep dark).*

50 mM ammoniumbicarbonate (Ambic) solution

3.95 mg Ambic in 1 ml dH_2O. (Sigma Aldrich, #09830)

Trypsin solution (50mM Ambic as w/1mM $CaCl_2$) is made by adding 10µl 0.1M $CaCl_2$ dissolved in dH2O to 1 ml 50mM Ambic solution

Note: for phosphoproteomics, do not add the $CaCl_2$

FASP Protocol, Day 2.

9. Transfer the filter to **new collecting tubes**. Centrifuge for 10-12 min.
10. Add **40-50 µl 50 mM Ambic** and centrifuge for 10-12 min. Repeat twice.
11. Add **50 µl 0.5 M NaCl** and centrifuge for 10-12 min. *(this releases all peptides bound to the filter)*.
12. Transfer the peptide solution to low binding tubes, and measure protein/peptide concentration with Nanodrop.

NOTE: concentration should be 0.1 µg/µl before acidification for phospho analysis).

13. Acidify with **10% TFA** to reach final concentration of 0.5-1% TFA. Check that solution is acidic.
14. Add **100 µl 0.1 %TFA** to increase volume *unless your volume is >300µl*. Clean-up samples using Oasis HLB µElution Plate from Waters *(2mg sorbent, loading approx. 40* µg *peptides per well)*. Freeze the samples, and dry in the FreezeVac.

0.5M NaCl solution

29.2 mg NaCl in1ml dH_2O (Sigma Aldrich, #71380)

10% and 0.1% TFA solution