In-solution trypsin digestion

In-solution digest for crude protein mixtures dissolved in SDS/DTT buffer, done in Ultrafiltration devices. Based on the improved 'FASP II' protocol in Wisniewsky et al, Analytical Biochemistry 410 (2011) 307-309.

Materials:

1. Vivacon 30 kDa (Sartorius)

Devices with oblique membranes (like Millipore Ultra) work as well but give slightly lower yield. 10 kDa does also work, but centrifugation times are three times longer.

2. Bench-top eppendorf centrifuge at room temperature (Urea will precipitate in the cold).
3. 200 µl and 1000 µl pipets and tips
4. Iodoacetamide,
5. trypsin MS-grade.

Sample buffer:

Ideally samples should be denatured in some neutral SDS/DTT buffer, and less than ~200 µg. Then dilute by adding 3x sample volume of UA buffer (see end of the document for composition).

Problems arise with other detergents but SDS (NP40, Triton, TWEEN-20 do not pass the membrane hence concentrates up) and large volumes. Refer to section 'Samples which cannot be directly diluted with UA' further below about options in these cases.

Procedure:

The 30 kDa centrifugation devices should be spun 14000 g at room temperature. After centrifugation 80 - 95 % of the initial volume should be in the flow-through, adjust centrifugation times accordingly. Do not process more than 200 µg in one device, otherwise the concentrate becomes viscous which decreases the flow rate. Eventually proteins will precipitate and block the membrane.

- wash column with 200 µl of UA buffer, three times
- add the sample to column, spin. Bring the supernatant volume down to 30 µl.
- add 200 µl sample in UA, spin (three times)
- add 200 µl 10 mM DTT in UA, shake 1 minute 600 rpm
- keep 45 minutes at room temperature, spin 15 minutes
- add 100 µl 50 mM Iodo-Acetamide in UA, shake 1 minute 600 rpm
- keep 30 minutes at room temperature in the dark, spin 15 minutes
- add 200 µl UA, spin (three times)
- add 100 µl (for >50 µg protein use 200 µl) 40 mM NH_4HCO_3, spin (two times).

Be sure to narrow down > 90 % in this 40 mM NH_4HCO_3 wash step, prolong centrifugation time if necessary. Too much remaining Urea inactivates the trypsin and might modify peptides by decomposition products.

- provide a new tube to capture the flow-through
- add 40 µl trypsin solution in 40 mM NH_4HCO_3 to achieve a Enzyme:Protein ratio 1:100 or more.
- incubate over night at 37°C in a wet chamber and close the lid of the centrifugation device to

prevent drying.

- spin 15 minutes

- add 40 µl 40 mM NH_4HCO_3, shake 1 minute 600 rpm, spin 15 minutes.

The peptides are now in the flow-through.

- add 3 µl 25 % TFA to the flow-through, transfer to LoBind eppi. Discard filtration device.

- dry in SpeedVac

- dissolve in 20-50 µl 0.1 % TFA and desalt

To confirm digestion apply the concentrate in the fitration device onto a SDS gel. Mix 15 µl concentrate + 5 µl 4x SDS sample buffer, heat 5 minute 95 °C, load and run the gel. You should see the trypsin at ~ 24 kDa and otherwise nothing if slight background smear. New users are advised for this check.

Buffers/Solutions:

UA buffer (8M Urea 0.1M TRIS pH 8.5)

Prepare UA freshly. You need ~ 1.6 ml/sample. Store at room temperature. Good for one day.

	10 ml	**20 ml**	**30 ml**
Urea [g]	4.8	9.6	14.4
1 M TRIS pH 8.5 [µl]	1000	2000	3000
Filtered deionized water [ml]	5.7	11.4	17.1

Dissolve by vigorous shaking. Heat the solution in a 23°C water bath (higher temperatures will decompose the Urea). Filtrate 0.2 µm.

10 mM DTT in UA

Prepare fresh. Mix 10 µl stock 1 M DTT + 990 µl UA.

50 mM Iodo-Acetamide in UA

Prepare fresh.

50 mM NaH_4CO_3

Mix 1000 µl stock 2 M NH_4HCO_3 + 39 ml filtered deionized water.

150 ng/µl trypsin

Dissolve 25 µg trypsin (Roche proteomics grade) in 166 µl 40 mM NH_4HCO_3

Preparation of samples which cannot be directly diluted with UA

Samples which contain other detergents than SDS

Please follow the TCA-precipitation procedure (see 'TCA-precipitation.doc') until the -20°C acetone addition. Repeat the acetone wash. Remove the acetone and add 0.2 µl 10 % SDS + 10 µl 1M TRIS pH 8.5 + 20 µl water, sonicate for 5 minutes. Add 500 µl UA and concentrate down to ~ 30 µl. Continue with FASP protocol.