FASP method – Label Free Analysis

Adapted from: Wiśniewski, J.R., 2017. Filter-aided sample preparation: the versatile and efficient method for proteomic analysis. Methods in enzymology, 585, pp.15-27.

Material

- Urea solution (UA): 8 M urea in 0.1 M Tris–HCl, pH 8.5
- Ultracentrifugation filters (10 or 30 kDa), filter on bottom
- Lysis buffer: 50 mM DTT in 100 mM Tris–HCl, pH 7.6
- SDS solution: 20% in water
- Digestion buffer (DB): 0.01 M ammonium bicarbonate, pH 8

Preparation of Lysates from Tissues and Cells

1. Homogenize tissue using a blender, Dounce, or Potter-Elvehjem-type device in about 5- to 10-fold excess (volume) of lysis buffer. To achieve the best results of lysis, it is essential to use enough excess of the lysis buffer over the sample, but preparation of lysates with total protein concentrations below 1 mg/mL should be avoided as too large amounts of the detergent require additional detergent depletion steps in the FASP procedure. Lysis of cultured cells, organelles, or unicellular organisms this homogenization step can be omitted.
2. After addition of SDS to a final concentration of 2% (w/v), sonicate the homogenate sonicated in a Branson type instrument (operating at 20% duty cycle and 3–4 output for 1 min). Note that water bath-type sonicators often are inefficient for this purpose.
3. Place the tubes with the homogenate in a bath with boiling water and incubate for 3–5 min. Cool the sample to room temperature.
4. Clarify the lysate by centrifugation at 10,000 x g for 5 min.

Determination of Total Protein in Lysates

There are several ways the protein concentration in the lysate can be measured. We suggest using either Bradford or BCA assays, which provide fairly accurate readings. Always check that the composition of the buffers and other reagents in the lysate are compatible with the method you selected. ***The ideal concentration for FASP is 2 mg/mL***.

FASP protocol

1. Mix up to 40 µL of protein extract per 200 µL of UA-solution containing, in the ultrafiltration units and centrifuge at 10,000 x g until less than 10 µL of sample remains above the filter. The centrifugation step has to be continued until less than 5% of the initial solution remains above the filter. This usually requires a centrifugation time of 10–15 min. The time needed to achieve this varies between the filter types, lysate properties, and the total protein concentration.
2. Add 200 µL of UA to the ultrafiltration units and repeat the centrifugation.
3. Discard the flow-through from the collection tube.
4. Add 100 µL 50 mM iodoacetamide in UA to the sample and mix at 600 rpm in a thermomixer at room temperature for 1 min.

5. Store filters in the dark for 20 minutes.
6. Centrifuge the ultrafiltration units at 10,000 x g for 10 min.
7. Add 150 µL of UA to the ultrafiltration units and centrifuge at 10,000x g for 15 min. Repeat this step twice. If the amount of sample loaded was > 40 µL, repeat this step one more time.
8. Add 150 µL of Digestion Buffer (DB) to the filtration units and centrifuge at 10,000 x g for 10 min. Repeat this step twice.
9. Add 60 µL of digestion buffer containing a mixture of LysC/Trypsin, with a enzyme to protein ratio of 1:100.
10. Incubate overnight at 37 C.
11. Centrifuge the ultrafiltration units at 10,000 x g until the solution entirely passed the filter membrane (about 5-10 min). The flow-through contains peptides obtained by trypsin digestion.
12. Add 100 µL of DB and centrifuge the ultrafiltration units at 10,000 x g until the solution entirely passed the filter membrane (about 5-10 min). The final volume obtained has all the peptides.
13. Determine concentrations of the LysC and tryptic peptides by A280 measurement in a spectrophotometer or by the fluorometric WF-assay (Wisniewski & Gaugaz, 2015) as described in Section 2.2 using DB buffer instead the urea assay buffer. 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 units (1 mg/mL solution has an A280 of 1.1). Always record a spectrum from 240 to 340 nm. A distinct peak should be observed at 270–280 nm and the extinction at 320 nm should be 0. Measurements of peptide concentrations are reliable when the absorbance value at A280 is above 0.1 in a cuvette with a 10 mm path. Extinction values below 0.1 can be used only for rough protein estimation due to increasing contribution of light scattering. Thus, be aware that measurements carried out using "Nanodrop"-type instruments can be unreliable. Test conducted in the MSF facility using a loading of 50 µg of protein material yielded readings between 0.1 and 0.3 mg/mL at this point. Please note that the blank should be the DB buffer. If needed, a 10 µL aliquot can be dried separately, and resuspended in 1 µL of water to provide a more concentrated sample. In this case, 10 µL of DB buffer need to be prepared in the same way to provide a reliable blank.
14. Dry the peptides and store at -20°C for at least 1 month or -80°C for several months.