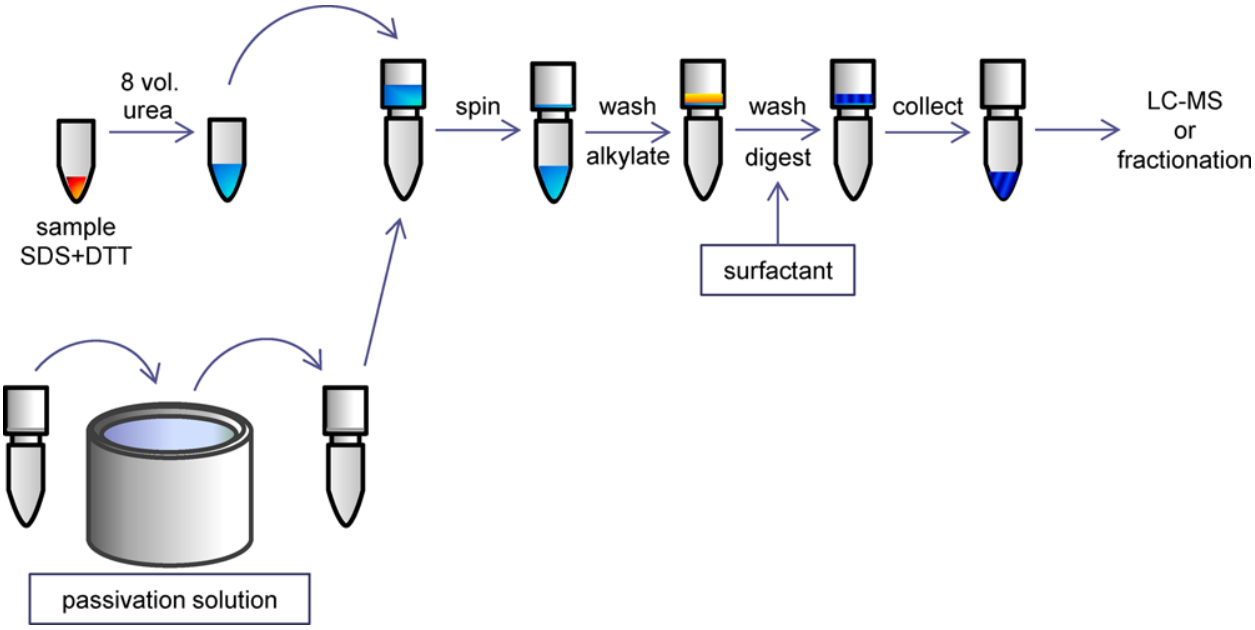
OHSU Proteomics Shared Resources

eFASP Digestion Protocol

Adapted from below reference

Ref: "Enhanced FASP (eFASP) to Increase Proteome Coverage and Samplerecovery for Quantitative proteomic Experiments", *J. Proteome Res. 2014, 13, 1885−1895*.



Reagents

Tween 20: SigmaP7949-100ml

Deoxycholic acid: Sigma, Catlg# D4297-5G, MW 392.57

Triethyl Ammonium Bicarbonate (TEAB) buffer, 1M: Thermo Scientific, Catlg # 90114

Ethyl acetate: AR (ACS) Macron Fine Chemicals, Ctlg# 4992-04

Formic acid: Optima LC/MS, Fisher scientific, Catlg# A117-50, 50ml, FW 46.02

TCEP HCl: Thermo Scientific, Ctlg#20490, MW 286.65

Alpha-Iodoacetamide (IAA): Calbiochem, Catlg# 407710, MW 185

NaOH: ACS, Fisher Scientific, Ctlg# S318-500

Urea: Ultrapure Urea, Invitrogen, Ctlg# 15505-050, MW 60.06

Sodium DodecylSulfate (SDS): ReagentPlus grade, >98.5%(GC), Sigma-Aldrich, Ctlg# L4509-10G, MW 288.38

HPLC grade Water: Fisher Scientific, Optima LC/MS, Ctlg# W6-1

Methanol: Fisher Scientific, Ctlg# A452-4

Trifluoroacetic acid (TFA): 100g, Pierce, Catlg# 28903

Trypsin: Promega Sequencing grade, Modified porcine trypsin, Ctlg# V5111, 5x20ug

Solutions

Passivation Solution: 5% (v/v) TWEEN-20. Dissolve 5ml of Tween-20 in 95ml of HPLC water.

20% SDS + 1%DCA: Dissolve 1g of SDS + 50mg DCA in 4ml of HPLC water. Make up to 5ml with HPLC water.

8M Urea, 100mM TEAB buffer: 4.805g Urea + 1ml of 1M TEAB, pH 8 in 10ml of HPLC water.

500mM TCEP: Dissolve 35.8mg of TCEP in 0.25ml of 100mM TEAB. Check pH.

500mM IAA: Dissolve 46.25mg of IAA in 0.5ml of 8M Urea, 100mM TEAB, pH 8

1M NaOH: Dissolve 2g in 50ml of HPLC grade water.

Lysis Buffer w/o TCEP: 4% SDS, 0.2% DCA (deoxycholic acid), 100mM TEAB (pH 8.0). Dilute 2ml of 20% SDS, 1%DCA + 1ml of 1M TEAB (pH8) to 10ml with HPLC water.

Alkylation Buffer: 8M Urea, 50mM iodoacetamide, 100mM TEAB, pH8. 3.6ml of 8M urea, 100mM TEAB + 400ul of 500mM IAA to get 4ml.

Exchange Buffer (minus TCEP): 8M Urea, 0.2% DCA, 100mM TEAB, pH8. Weigh out 60mg DCA into a 15ml polypropylene tube. Add 3ml of 1M TEAB and vortex. Add 225ul of 1M NaOH to bring pH to 8 and allow to dissolve. To a 50ml centrifuge tube add 14.41g Urea and 25ml of HPLC water to dissolve. Add the 3ml DCA/TEAB solution. Bring up to 30ml final volume with HPLC water. Need 1.2ml/sample.

Digestion Buffer: 0.2% DCA, 50mM TEAB, pH 8. Weigh out 40mg DCA, add 1ml 1M TEAB, 1ml HPLC water and 200ul 1M NaOH to dissolve. Add to 50ml polypropylene centrifuge tube and bring up to 20ml with HPLC water (prep. 7/14/15 and stored at rt). Need 0.8ml/sample.

Trypsin Stock: 200ng/ul in 1mM HCl. Dissolve 20ug lyophilized trypsin in 100ul of 1mM HCl. Store left over stock in 10ul aliquots in 0.5ml lobind tubes at -80C.

100mM TEAB: Dilute 100ul of 1M TEAB to 1ml with HPLC water

50mM TEAB: Dilute 250ul of 1M TEAB to 5ml with HPLC water

50% Methanol: Dilute 2.5ml of methanol to 5ml with HPLC water

Peptide Recovery Buffer: 50mM TEAB, pH8. Dilute 50ul of 1M TEAB to 1ml with HPLC water

LC/MS Peptide Dissolution Buffer: 5%FA/3%ACN in HPLC water. Take 0.5ml of Formic acid, 0.3ml of Acetonitrile and make up to 10ml w HPLC water

Note: DCA/TEAB solutions should be prepared immediately before use because DCA settles over time.

Equipment

Amicon Ultra centrifugal filters, 0.5ml, 30K membrane, 30000 MWCO, Ctlg# UFC503063, 100/Pk. $500. $5/filter.

Eppendorf Centrifuge 5430R in MRB 524

Eppendorf Thermomixer C initially set to 90C

Eppendorf Protein LoBind tubes, 0.5ml, Ctlg# 022431064, 2ml, Ctlg# 022431102

Fisher Scientific Digital Vortex Mixer

Methods

Surface Passivation

1. Incubate the filter unit and collection tube overnight in Passivation Solution on a shaker. For small batches, this can be done in a 50 mL Falcon tube.

2. Remove components and quickly rinse with a squeeze bottle containing MS gradeH2O or rinse w Millipore water. Do at least 10 rinses.

3. Transfer components to a clean container with 250 to 500 mL of MS-grade water. Place on a shaker at low speed for 30 minutes. Repeat this step once or twice with fresh water. Rinse at least five times with MS grade H2O. Air dry at room temp. Store in a plastic bag.

4. Reserve the passivated collection tube for peptide collection.

Sample lysis

1. Add Lysis Buffer w/o TCEP to cell pellet and mix at 600 rpm in a thermo-mixer for 10 minutes at 90°C. Protein concentration should be high enough such that 25 µl of lysate will provide the desired amount of protein for processing (max 100 µg protein).
2. Sonicate lysate in three 10-second intervals and centrifuge at 14,000 g for 10 minutes. Repeat this step once.
3. Sonicate the supernate and pellet, and cool to 37°C.
4. Determine protein concentration using Pierce BCA Assay Kit

Sample processing

Note: Sample volumes higher than 25ul can be processed with this protocol, Please adjust the volumes accordingly to get required concentration in total volume. Also increase the number of exchange buffer spins to completely remove SDS from the samples.

1) Aliquot the # ul into 1.5ml Lobind tubes to get 25ng-100ug sample.

2) Add 2.5ul of 500mM TCEP to the sample. Add additional lysis buffer (4% SDS, 0.2% DCA (deoxycholic acid), 100mM TEAB (pH 8.0) w/o TCEP) to bring volume to 25ul. Concn. of TCEP in 25ul is 50mM.

3) Vortex, quickly spin down, and then heat for 10min at 90C. Use incubator in lab w shaker set at 700rpm.

4) Vortex and quickly spin samples down to collect from sides.

5) **Testing eFASP filter:** Add 500ul of exchange buffer (8M Urea, 0.2% deoxycholic acid (DCA), 100mM TEAB) to each filter and centrifuge at 14,000 rpm for 5min. About 1/3 of the buffer should pass through the filter. Discard the filter that shows complete filtration. Keep centrifuging the working filter to fully condition the unit for a further 15min leaving just a layer of buffer on top of the filter.

6) Add 200ul exchange buffer (8M Urea, 0.2% deoxycholic acid (DCA), 100mM TEAB) to each sample. Dispense the diluted lysate into the passivated 30kDa filter unit with non-passivated collection tube. Use collection tubes provided w 30kDa filters only. Will lose sample if use other 2ml tubes. (Filter unit passivated overnight with 5% Tween, and then washed extensively with water. See Surface passivation protocol).

7) Centrifuge at 14,000xg for 10minutes. Save filtrate and store at -80C or discard.

8) Add 200ul exchange buffer and centrifuge at 14000xg for 10minutes. Discard filtrate. Repeat step two more times.

9) Add 100ul alkylation buffer (8M Urea, 50mM iodoacetamide, 100mM TEAB, pH8) and incubate w mixing in thermomixer at 37degC for 1 hour. Wrap tops of the spin columns w parafilm to minimize evaporation (use HYBAID incubator in MRB Rm524).

10) Centrifuge at 14000xg for 10minutes. Discard filtrate.

11) Add 200ul exchange buffer (8M Urea, 0.2% deoxycholic acid (DCA), 100mM TEAB) and centrifuge at 14000xg for 10minutes. Discard filtrate.

12) Add 200ul digestion buffer (0.2% DCA, 50mM TEAB, pH 8) and centrifuge at 14000xg for 10minutes. Discard filtrate. Repeat this step two more times.

13) Transfer filter to a passivated 2ml collection tube.

14) Add 100ul Digestion buffer (0.2% DCA, 50mM TEAB, pH 8) to each filter.

15) Add 7ul of 200ng/ul trypsin stock (1400ng) to each sample (final 1:36 (wt:wt) trypsin:protein, 14ng/ul final trypsin concentration). Trypsin in 50mMTEAB. Discard left over trypsin. Wrap top of each sample tube w parafilm before incubating at 37C to prevent evaporation. **Note:** Can go up to 1:25 (wt:wt) tryspin:protein if needed.

16) Mix at low speed (500rpm) in HYBAID incubator in MRB Rm524 at 37degC overnight (in MRB524). Minimum 12h.

17) Next day, centrifuge samples at 14,000xg for 10minutes.

18) Add 50ul of Peptide recovery buffer (50mM TEAB, pH 8) to each tube and centrifuge at 14,000xg for 10minutes. Repeat this step one more time.

Phase Transfer

The following was done to remove DCA from samples.

1) Add 200ul ethyl acetate to the collection tube that has the peptide-containing filtrate sample and transfer to a 2ml Eppendorf Lobind tube. Add 2.5ul TFA (pure) and vortex. Detergent will precipitate. White thread-like precipitate may be visible for large quantities of peptides.

2) Add 400ul ethyl acetate and vortex vigorously for 1minute. Centrifuge at 16,000 x g for 10minutes.

3) Carefully remove and discard as much of the upper organic layer as much as possible without disturbing boundary layer.

4) Repeat steps 2 and 3 twice.

5) Open the tubes and leave in fume hood for 30min so that ethyl acetate can evaporate. Store at 4C if not drying.

6) Dry down the samples in a speedvac. It takes about 2h.

7) Add 100ul of 50% methanol and dry down in speedvac. Add another 100ul of 50% methanol, vortex and spin down. If see small white undissolved pellet incubate samples at 37C for 20min w shaking.

8) Dry left over samples in speedvac and store at -80C till TMT labeling or LC/MS/MS analysis.