Protein Reduction, Alkylation, Digestion

Contents

Reduction / Alkylation (DTT, IAA and UREA), Trypsin digestion

Reagents and Materials (see Table 1)

- Dithiothreitol DTT; Stock solution: 0.5 - 1 M in H_2O
- Iodoacetamide IAA; Stock solution: 500 mM in H_2O (always prepare fresh, light sensitive)
- Urea
- Ammonium bicarbonate (Fisher, part # A643-500) Stock solution: 500 mM in H_2O
- Tris Base (Fisher, part # BP154-1)
- 200 ng/µl Trypsin in 0.01% acetic acid (modified, sequencing grade)
- All solvents should be HPLC grade, NEVER use pipette tips when transferring acids >2% in concentration!

Reduction / Alkylation (DTT, IAA and UREA)

1. For reduction/alkylation the proteins (concentration up to several mg/ml) should be in reducing buffer containing:
   - 100mM Tris/HCl pH 8.3 OR 100mM Ammonium bicarbonate (AMBIC)
   - 6-8M Urea
2. Add DTT from a 0.5 M stock to a final concentration of 5 mM and incubate for 25-45 min at 56 °C to reduce disulfide bonds. NOTE: Avoid temperatures higher than 60 °C where urea-based carbamylation of lysines and protein N-termini can occur.
3. Allow the protein mixture to cool to room temperature, spin briefly to collect condensation
4. Add iodoacetamide to 14 mM final concentration. Incubate for 30 min at room temperature and in the dark to alkylate cysteines.
5. Quench unreacted iodoacetamide by adding 0.5 M DTT to additional 5 mM and incubating 15 min at room temperature in the dark.

Trypsin digestion:

1. Dilute the protein mixture 1:5 in 25 mM Tris-HCl pH 8.2 or AMBIC, to reduce the concentration of urea to < 2 M
2. Add $CaCl_2$ from a 0.1 M stock to 1 mM.
3. Add trypsin at a minimum concentration of 4–5 ng /µl and 1/100–1/200 enzyme:substrate. Incubate at 37 °C 1-4 hrs or overnight.
4. Allow the digest to cool to room temperature and stop the digestion by acidification with TFA to 0.4% (vol/vol). Verify that the pH is <2.0; otherwise add more acid.
5. Centrifuge at 2,500*g* for 10 min at room temperature and discard the pellet.
6. Clean-up samples with C18 spin columns (e.g., Pierce C18 Spin Columns (89870), Millipore C18 ZipTip (ZTC18S008) or Waters Sep-Pak (WAT054955), Nestgroup spin columns)
7. Dry samples in speed vac or dilute to < 5% ACN prior to MS analysis (typical concentrations 0.5-5 pmol/µl per peptide or 100-1000ng/ul complex protein digest).

Reduction / Alkylation (DTT, IAA and RapiGest or PPS), Trypsin digestion:

Works well for complex mixtures to be analyzed by mass spec.

Reagents and Materials (see Table 1)

- *Rapi*Gest SF Powder (Waters Corporation part # 1860018605 Pack of 1 ml Vials,)
  OR PPS Silent Surfactant (Protein Discovery part #21011)
- Ammonium bicarbonate (AMBIC) (Fisher, part # A643-500) Stock solution: 50 mM in H_2O
- Dithiothreitol DTT (Fisher, part # PI-20291); Stock solution: 500mM in H_2O
- Iodoacetamide IAA (Fisher, part # AC12227-0050); Stock solution: 500 mM in H_2O (always prepare fresh, light sensitive)
- 5 M HCl
- 200 ng/µl Trypsin in 0.01% acetic acid (modified, sequencing grade, Promega, part # V5111, 5 x 20ug)
- All solvents should be HPLC grade, NEVER use pipette tips when transferring acids >2% in concentration!

Reduction / Alkylation (DTT, IAA and RapiGest or PPS)

1. Prepare 0.2% *Rapi*Gest in 50 mM AMBIC pH 7.8 (w/v) (1 mg *Rapi*Gest per 500 µl 50 mM AMBIC pH 7.8).
   *Note: If you use 50-100mM Tris pH 8.5, add twice as much HCl prior to MS analysis.
2. Add 100 µl 0.2% *Rapi*Gest per 100 µl protein mixture (1:1) to get a final concentration of *Rapi*Gest of 0.1 % (w/v). NOTE if protein is in pellet form add 25-50 µl of 0.1% *Rapi*Gest.
3. Vortex the sample.
4. Add DTT to a final concentration of 5mM.
5. Incubate sample at 60° C for 30 minutes.
6. Cool the sample to room temperature and spin the sample for a minute.
7. Add IAA to a final concentration of 15mM.
8. Place sample IN THE DARK at room temperature for 30 minutes.

Trypsin digestion

1. Add Trypsin for a final concentration of 1:100 enzyme:protein.
2. Incubate for 1 hour with shaking at 37° C. Note incubation times may vary 2-4 hrs should completely digest your protein, if unsure check on a gel.
3. Samples can be stored at -20° C if needed.
4. Prior to mass spectrometry analyses, add 5 M HCl to a final concentration of 200mM.
5. Incubate at 37° C for 45 minutes while shaking.
6. Spin sample at 14,000 rpm, 4°C for 10 minutes.
7. A cloudy pellet should appear. Separate your supernatant form the pellet into a fresh Eppendorf tube.
8. Spin again if needed to make sure you have completely removed the cloudy material.
9. Also you can add 5% acetonitrile or Buffer A (5% acetonitrile, 95% water, 0.1% formic acid) to reduce the risk of clogging the column.

Trypsin Activity in various buffers:

Trypsin solution [a]	Trypsin activity in % [b]
No additive	100
0.1% RapiGest	100
0.5% RapiGest	87
0.1% SDS	20
0.5% SDS	1
0.1% RapiGest 0.1% SDS	58
50% Methanol	31
50% Acetonitrile	92
1 M Urea	97
2 M Urea	83
0.5 M Guanidine HCl	21
1 M Guanidine HCl	8

[a] 0.5 µg of trypsin was added to 1 ml of 50 mM ammonium bicarbonate, pH7.9 containing 0.2 mM of BAEE(N-a-benzoyl-L-arginine ethyl ester).

[b] Measured as delta BAEE absorbance at 253 nm (slope within 5 minutes)

Source: RapiGest Application Note: http://www.waters.com/webassets/cms/library/docs/720003102en.pdf

Cell Lysis and Tryptic Digest of Mammalian Cells

Reagents and Materials (see Table 1)

- PBS
- Lysis buffer (10 mM NaPO4, pH 7.0; 0.5% SDS)
- Urea powdered
- TBP, 200 mM stock
- Ammonium bicarbonate50 mM NH4HCO3, pH 7.8
- 1M solution of CaCl2
- Trypsin
- 3 mL C18 SPE column

Procedure

1. Sample size: 300.0 µL of cells suspended in 1xPBS.
2. Wash cells 3 times with 2.0 mL of ice-cold PBS. Pellet the cells after each wash by centrifugation at 10 000 rpm for 2 min.
3. Add 1.5 mL of Lysis Buffer (10 mM $NaPO_4$, pH 7.0; 0.5% SDS) to the cells and gently agitate to resuspend cells. Do not vortex.
4. Sonicate cells for 10 min in cold sonication bath containing water and a layer of ice.
5. Perform BCA Protein Assay to check protein concentration.
6. Add powdered form of Urea to a final concentration of 8 M (484.6 mg/mL solution).
7. Add TBP to a final concentration of 5 mM.
8. Incubate the sample at 37°C for 1 hour.
9. Dilute the sample 8-fold with 50 mM NH_4HCO_3, pH 7.8 to reduce the Urea concentration to 1M.
10. Add sufficient amount of a 1M solution of $CaCl_2$ to obtain a sample concentration of 1 mM $CaCl_2$.
11. Digest sample for 5 hours with Trypsin at 37°C at a concentration of 1 unit trypsin/50 units protein.
12. After trypsin incubation sample can be stored at -80 ^oC.
13. Clean up sample , e.g. using a SepPak column
14. Concentrate sample in Speed-Vac. And perform BCA protein assay on sample.
15. Store at –80°C until needed for analysis.

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

See Methods/ProteinDigestion/Enzymatic Digestion Protocols/FASP

Lys-C Trypsin Digestion

1. Dissolve proteins in 8 M urea, 100 mM Tris pH 8.5 (60-100 µl).
2. Add 0.3 µl 1 M TCEP (5 mM final conc.). Incubate at RT for 20 min.
3. Add 1.2 µl 500 mM iodoacetamide (10 mM final conc.) (make fresh daily, 0.046 g/500 µl H_2O).
4. Incubate at RT for 15 min in the dark.
5. Lys-C Digest
    1. Add in Lys-C (0.1 µg/µl), 1/100th total amount of protein
    2. Incubate in the dark for 4 h at 37 ºC.
6. Trypsin Digest (optional)
    1. Dilute sample by a factor of 4 (i.e. 180 µl) with 100 mM Tris pH 8.5 (final conc. = 2 M, vol=240 µl)
    2. Add 100 mM $CaCl_2$ to a final conc. of 1 mM (i.e. 2.4 µl)
    3. Add in Trypsin (0.5 µg/µl) at 1:20 to 1:100 ratio.
    4. Incubate at 37 ºC in the dark from several hours to O/N.
7. Add 13.5 µl 90% formic acid (5% final conc.).
8. Spin at top speed for 10-20 min, transfer the sup to a new tube, and freeze at –80 ºC.
9. Clean-up samples with C18 spin columns (e.g., Pierce C18 Spin Columns (89870), Millipore C18 ZipTip (ZTC18S008) or Waters Sep-Pak (WAT054955))
10. Dry samples in speed vac.

Peptide desalting

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.

C18 cartridges from NestGroup (www.nestgrp.com)

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 300Å
MicroSpin	20 – 100 µg	5 – 200 µl	50 µl	SEM SS18V	Silica C18 300Å
MacroSpin	100 – 500 µg	50 – 450 µl	250 µl	SMM SS18V	Silica C18 300Å
UltraMicro Tip	3 – 30 µg	5 – 100 µl	50 µl	STU SS18V	Silica C18 300Å
96-Well MiniSpin	10 – 150 µg	10 – 100 µl	75 µl	SNS SS18V	Silica C18 300Å
96-Well MACROSpin	20 - 300 µg	20 – 200 µl	225 µl	SNS SS18V-L	Silica C18 300Å

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)

Procedure UltraMicroSpin and MicroSpin 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.

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 37-55µm 100/box	Waters	WAT054960
Sep-Pak® Vac tC18 cartridge 1cc/100mg 37-55µm 100/box	Waters	WAT036820
Sep-Pak® Vac tC18 cartridge 3cc/500mg 37-55µm 50/box	Waters	WAT036815

SepPak solvents

Binding buffer: 0.1% TFA (vol/vol) in H_2O

Elution buffer: 50% ACN (vol/vol), 0.5% HAcO (vol/vol) in H_2O.

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-µl 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% AA.
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 µl of 0.5% AA.
7. Elute with 5 ml of 50% ACN 0.5% AA and collect eluate in a 15-ml conical tube.
8. Freeze the eluate with liquid N_2 and lyophilize. The result here should be a white (sometimes yellowish) fluffy powder.
9. At this point, samples can be stored at -20 °C for several weeks.
10. Dry in speed vac or dilute to < 5%ACN (typical concentrations 0.5-5 pmol/µl per peptide or 100-1000ng/ul complex protein digest).

ZipTip® Protocol

(EMD Millipore ZTC18S008 pk of 8, ZTC18S096 pk of 96, ZTC18S960 pk of 960)

Use ZipTip®C18 pipette tip protocol for peptide purification and sample concentration.

These conditions may not be optimal for all proteins/peptides.

1. Prepare ZipTip® tips by washing with 10µl of 100% ACN, then washing 2–3 times with 10µl of 0.1% TFA.
2. Reconstitute the samples with 10µl of 0.1% TFA.
3. Draw the sample into ZipTip® tips by pipetting fully into and out of tips 4–5 times. Expel liquid.
4. Wash ZipTip® tips 2–3 times with 10µl of 0.1% TFA to remove contaminants.
5. Elute peptides in 2.5µl of 70% ACN/0.1% TFA (or use 0.1% formic acid in elution buffer) and dry them in speed vac or dilute to <5%ACN prior to mass spec analysis (typical concentrations 0.5-5 pmol/µl per peptide or 100-1000ng/ul complex protein digest).

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), MetOH	Fisher	A456-4
PBS (Phosphate Buffered Saline)	Fisher	BP665-1
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
MacroSpin Columns Silica C18 Vydac, loading volume 50-450µl, protein capacity 100 - 500µg	Nestgroup	SMM SS18V.25
MicroSpin Columns Silica C18 Vydac, loading volume 5-200µl, protein capacity 20-100µg	Nestgroup	SEM SS18V.25
UltraMicro TIP Columns Silica C18 Vydac, loading volume 5-100µl, protein capacity 3-30µg	Nestgroup	STU SS18V.25
Lysine-C 3 x 5 µg vials	PrincetonSeparations	EN-130
Trypsin modified, sequencing grade, 5 x 20ug	Promega	V5111
PPS Silent Surfactant	Protein Discovery	21011
Endoproteinase Lys-C from Lysobacter enzymogenes, 5ug	Sigma	P3428-1VL
Sodium dodecyl sulfate, SDS	Sigma Fisher	L4509-10G BP166-500
TBP Tributylphosphine 200 mM Stock Solution, 10 x 0.5 ml flame sealed ampules	Sigma	T 7567
*Rapi*Gest SF Powder 1 ml Vial	Waters	1860018605
Sep-Pak® Vac tC18 cartridge 1cc/100mg 37-55µm 100/box	Waters	WAT036820
Sep-Pak® Vac tC18 cartridge 1cc/50mg 37-55µm 100/box	Waters	WAT054960
Sep-Pak® Vac tC18 cartridge 3cc/500mg 37-55µm 50/box	Waters	WAT036815
HCl		
$NaPO_4$		