The PFG digestion protocol (in solution digests)

Version 9th Jun, 2014. ***Please do not redistribute.***

A key step in almost any proteome analysis is the conversion of protein to (usually tryptic) peptides. This step is so critical because a) it generates analytes that are appropriately charged for MS analysis, and b) are compatible with the nanoscale chromatography systems we use. Failure to achieve complete digestion can lead to missed cleavage (which weakens the strength of database searches) and worse, led to (pre)column clogging.

We should therefore adopt a common digestion protocol that will work for most analyses. The aim is to load onto the column an appropriate amount of material such that a typical protein will be represented at approx 50fmol. In order to calculate this load, it is necessary to make some assumptions. First, that the recovery of material after all digestion/preparation protocols is 100%, secondly, the molecular weight of an 'average' protein for complex protein mixtures, and c) the overall complexity of the mixture.

We can assume that a typical protein has a molecular weight of 40kDa. Thus: 40,000g = 1 mol, 40g = 1mmol, 40mg=1umol, 40µg=1nmol, 40ng=1pmol, 40pg=1fmol. Thus, 2ng=50fmol, the amount loaded on column.

This only works for pure proteins. For a mixture, we need to factor in complexity (C). Let's assume that all proteins are equimolar for now. If the protein is pure, the complexity (C) is 1, such that 2ng loaded should give a good LC-MS/MS trace. If C=10, then if you load 2ng, you'll only get 5fmol of each protein. Therefore:

- If C=10, you need to load a digest equivalent of 20ng
- If C=100, you need to load 200ng
- If C=250, you need to load 500ng
- If C=1000 you need to load 2µg
- For most samples, C>1000, but actually, the dynamic range means that you can assume C=1000

Digestion protocol

This protocol is adopted from the Waters recommendations. The features are the use of a digestion enhancer (Rapigest) that is destroyed after digestion, b) an obligatory reduction and alkylation – this should henceforth be performed as a routine. The digestion is based on large volumes (200uL digest) and large amounts of material (100ug), and can be scaled downwards within reason, say to one quarter of these volumes (a 50uL digest).

Modification history

17th August, 2009: Change method so that the reduction and alkylation and digestion always adds the same volume (10uL) to the vial. Adjusted volumes so that the final volume is 200uL (excluding the 1uL TFA from inactivation).

18th August, 2009: Change method by adjusting the acetic concentration to 10mM for stock trypsin. This will be pH 3.4 which will still be fine for trypsin stability, but the pH of the digest will be more tolerant to the addition of a larger trypsin volume.

9th June, 2014: Minor updates, and typo corrections.

Step	Protocol	Notes
Sample preparation *Volume: 160µL*	Dilute the amount of protein needed, taking into account complexity, into 25mM AmBic. Small Eppendorf tube preferred.	*For example, a cell lysate (C=500) would typically require 100µg of protein. This should be in the smallest volume possibl so that other contaminating or interfering materials are not added in large quantities.*
Detergent treatment *Volume: 170µL* **10min**	Add 10µL of 1% (w/v) RapiGest (~0.05% (w/v) final). Heat at 80°C, 10minutes, vortex briefly at 5min. Spin quickly to return liquid to bottom of tube.	*If you do not intend to use Rapigest, omit this step, and the TFA addition post-digestion. You will start with the sample in a slightly larger volume to compensate for the absence of Rapigest.* *Dissolve only as much as required. 1mg RapiGest dissolved in 100µL 25mM AmBic (added to the RapiGest vial) will be enough for about 9 digestions, pipetted with care.*
Reduction *Volume: 180µL* **10min**	Add 10 µL of a 9.2mg/mL solution of DTT (~3 mM final). Vortex mix. Incubate for 60°C, 10minutes, Cool to RT and quick spin to return liquid to bottom of tube.	*Add approximately 9mg of DTT (assessed by eye –it is the amount on the end of a small spatula) into the appropriate volume of 25mM AmBic to give 9.2mg/mL (60mM). Use within hours.*
Alkylation *Volume: 190µL* **30min**	Add 10 µL of a 33mg/mL solution of iodoacetamide (~9 mM final). Vortex. Incubate at RT, IN THE DARK for 30min	*Add approximately 30mg of IAA (assessed by eye –it is the amount on the end of a small spatula) into the appropriate volume of 25mM AmBic to give 33.3mg/mL (180mM). Keep in dark (wrap foil around vial), and use within hours.*
	THERE IS NO TCA PRECIPITATION STEP HERE	
Digestion *Volume: 200µL* **Overnight**	Add trypsin to 50:1 protein:trypsin ratio. Typically, for 100µg protein, this will be achieved by addition of 10 µL of a 200 µg/mL solution of trypsin in 25mM AmBic. Dissolve 100ug bottle of trypsin in 500uL AmBic Incubate 12-16h (overnight) at 37°C	*Dissolve a 20µg vial of trypsin in 100µL of 25mM AmBic. This will be enough for about 10 (50:1) to 20 (100:1) digestions. If you do not plan to perform this many digestions, share the trypsin, or dissolve in 10mM acetic acid instead (the low pH prevents trypsin autolysing, but it should be stored in the refrigerator if so dissolved).*
Detergent Inactivation *Volume: 201µL* **45min** **15min**	Quickly spin down the digest to bottom of tube. Add trifluoroacetic acid (TFA) to a final concentration of 0.5%(v/v). This is achieved by the addition of ~1µL of TFA. Incubate for between 30 and 45min at 37°C Centrigue to remove all insolubles: 13,000g for 15min. Remove the supernatant faction carefully. If in doubt that all particulates are removed, spin again and remove the supernatant a second time. **This material will be injected directly onto the column.**	*The Rapigest is hydrolysed, and may precipitate. It is safest to assume that insolubles have been generated, and to centrifuge the samples as an **obligatory** step.* *Do not pipette directly from the stock ultrapure TFA, as this will ultimately contaminate the sample. Remove a few tens of microlitres into an Eppendorf tube, and pipette from there. This is also safer, as it reduces the risk of knocking over the TFA bottle.*
MS Analysis	The cleared supernatant fraction is ready for injection without further treatment. Typically, for a 100µg digestion @C=500 (a typical broken cell extract), you would load the equivalent of 500ng protein. This is equivalent to 1µL of the digest, appropriately diluted into starting buffer A. For C=1000, it would be 10µL.	*The amount loaded will depend on the chromatography system and the mass spectrometer. This part can be turned intoinstrument specific guidance.*