A NOVEL PROTEIN DIGESTION WORKFLOW FOR THE ANALYSIS OF SURROGATE PEPTIDES BY LC-MS/MS

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Introduction

Pellet digestion is an extremely effective protocol, yet requires centrifugation and wash steps prior to analysis of the resulting peptides by LC-MS/MS. These steps are time consuming, labor intensive, and difficult to automate procedures. Described here is a novel workflow to streamline the pellet digestion protocol using Control Flow Plate (CFP)[1], Narrow Bore Extraction Plate (NBE)[1], and SMART Digest Kit[2].

We developed a specially designed Flow Control Tube format which eliminates the centrifugation step and allows the protein denaturation and digestion steps to be performed seamlessly in a single tube. If sample matrix requires additional cleanup after protein digestion, NBE (with sorbent) can be used to further purify the samples before LC-MS/MS analysis.

The new workflows provide reproducible quality results with minimal manual steps and are amendable to automation.

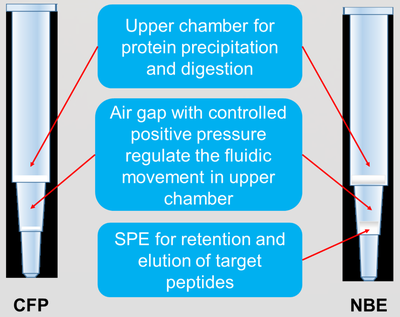
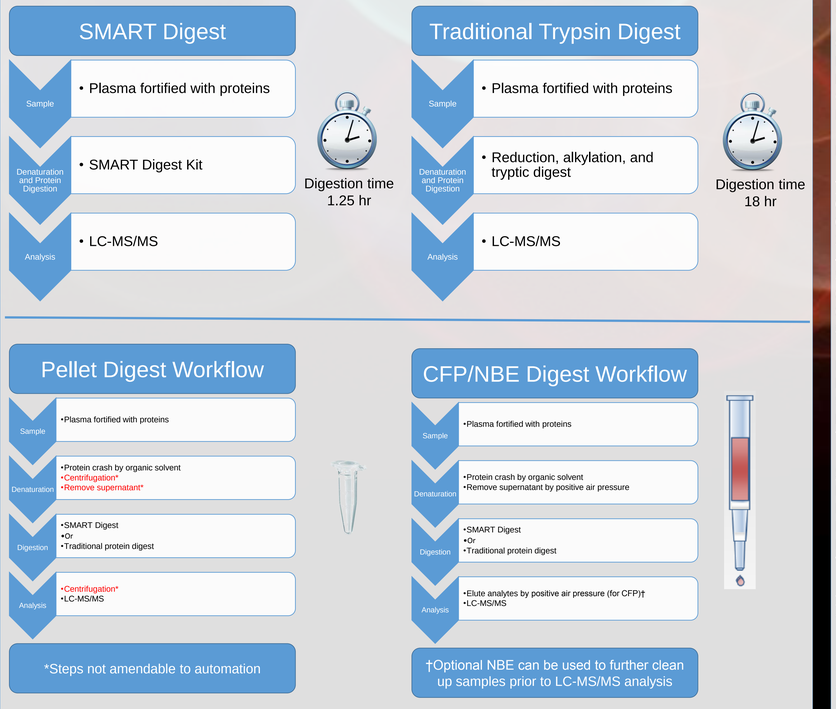


Figure 1. Schematics of CFP and NBE

Method

The CFP can accommodate up to 96 reactions in a plate format. Each tube has a unique design which introduces an air gap between two semi-permeable proprietary filters. Proteins can be retained on the top filter (above the air gap) by organic solvent precipitation or immunoaffinity[i] capture techniques.

Human IgG obtained from Sigma-Aldrich were used as test articles to validate the utility of the CFP and NBE. 20 µL of rat plasma spiked with test article was added into the CFP / NBE followed by protein precipitation with 200 µL ACN. The CFP / NEB was briefly vortexed and soluble components were removed by applying positive air pressure to the CFP / NBE. Proteins retained on the top filter were digested by adding SMART Digest buffer[2] and enzyme[2] and was incubated at 70 °C with shaking at 700 rpm for 75 min. The reaction was quenched by adding 18 µL of 5% formic acid then followed by addition of 20 µL of internal standards. The peptides formed could be either eluted directly into autosampler vials or further purified by integrated solid phase extraction technique (optional) – see 'NBE (post digest workflow)', prior to LC-MS/MS analysis. Samples can be processed in single tube or in 96-well plate format.



LC-MS/MS Conditions

Column: Waters Xbridge C8, 2.1x50 mm, 5 µm
Mobile phase A: 0.1% formic acid in water
Mobile phase B: 0.1% formic acid in methanol
Column temperature: 40 °C
Injection volume: 10 µL

LC gradient flow rate: 500 µL/mL

Time (min)	A (%)	B (%)
0.0	90	10
0.5	90	10
2.0	75	25
4.0	75	25
5.5	40	60
6.5	40	60
6.6	90	10
8.0	90	10

Mass spectrometer: Sciex API 5500 Qtrap
Ionization: Turbo IonSpray
Scan type: Positive Ion SRM
IonSpray voltage: 5500 V
Turbo heater temperature: 450 °C

Surrogate peptides	SRM
DTLMISR	418.22/506.28
DTLMISR	418.22/619.36
FNWYVDGVEVHNAK	839.40/968.48
FNWYVDGVEVHNAK	839.40/1067.55
VVSVLTVLHQDWLNGK	603.70/805.50

† NBE (post digest workflow)

- Sample transfer: apply positive air pressure to transfer sample to lower chamber containing SCX sorbent
- Wash: 150 µL of 1% formic acid
- Elution: 2 x 150 µL of 0.6% NH_3 in ACN-MeOH-H_2O (4:4:2)
- Evaporation: blow dry solvent from eluate using N_2 at room temperature for 30 min
- Reconstitution: dissolve the eluate in 150 µL of MeOH-H_2O (2:8)

Result

Protein denaturation and digestion in CFP offer comparable results to traditional pellet protein digestion method (>= 90% surrogate peptides recovered) while significantly simplifying the method. Linearity of surrogate peptides for the quantitation of Human IgG in rat plasma are from 5 µg/mL to 500 µg/mL (r = 0.999) – see figure 2. The accuracy of LLOQ is between 90% and 110% - see table 1. The accuracy and precision of low, mid, and high QCs are between 85% and 115% and ≤15% CV respectively – see table 1. Choice of denaturation solvent, digestion time, solid phase extraction sorbent material (optional), wash solvent, and elution solvent were optimized for this study. Non-specific binding of the surrogate peptides to the CFP / NBE device were not observed.

PEPTIDE	DESCRIPTION	LLOQ	LQC	MQC	HQC
DTLM_1	• Nominal Conc (ug/mL)	5	15	80	350
	• N	2	5	5	5
	• % CV	n/a	7.8	6.9	2.8
	• % Nominal	95.4	107.6	99.6	108.0
DTLM_2	• Nominal Conc (ug/mL)	5	15	80	350
	• N	2	5	5	5
	• % CV	n/a	7.3	7.2	1.1
	• % Nominal	93.6	104.1	100.5	107.1
FNWY_1	• Nominal Conc (ug/mL)	5	15	80	350
	• N	2	5	5	5
	• % CV	n/a	4.5	4.0	3.0
	• % Nominal	92.1	107.1	92.6	101.8
FNWY_2	• Nominal Conc (ug/mL)	5	15	80	350
	• N	2	5	5	5
	• % CV	n/a	4.1	6.5	4.2
	• % Nominal	95.5	99.9	95.5	103.6
VVSV	• Nominal Conc (ug/mL)	5	15	80	350
	• N	2	5	5	5
	• % CV	n/a	3.2	12.2	4.3
	• % Nominal	100.8	99.9	89.4	105.1

Table 1. Accuracy and precision of Hu IgG LLOQ and QC samples using CFP workflow

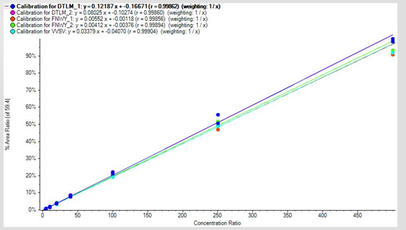


Figure 2. Hu IgG standard curves using CFP workflow

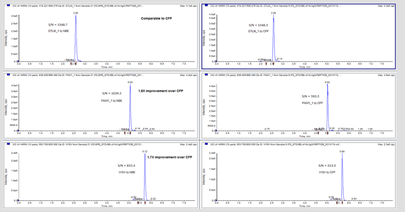


Figure 3. Comparison of NBE vs CFP

Conclusion

- Results indicate that the CFP and NBE workflows are good alternative to traditional protein digestion protocols, increasing both the throughput and robustness.
- Protein precipitation by organic solvent can be performed on the top filter (above the air gap) in CFP or NBE without loss of fluid through gravity filtration.
- CFP and NBE workflows do not require centrifugation steps or manual removal of supernatant as compared with pellet digestion method.
- The CFP and NBE experimental layout could be easily adapted to robotic automation platform.
- NBE demonstrated improvement in analyte S/N ratio over CFP – see figure 3 (the results are peptide dependent and could be further improved by optimization of sorbent material, washing and elution parameters).
- Combined with SMART Digest kit, the overall sample preparation protocol is simplified, the sample digestion time is reduced from 18 hr to 1.25 hr, and resulting in enhancement of assay success rate.

Reference

i) C F-Metzier, B Baker, R Buerger, J O'Grady, K Meyer, R C King, A Fast Immunoaffinity Capture Workflow for Bioanalysis of Proteins: Affinity and Rapid Digestion in One Well, AAPS National Biotech Conference.