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Application Note

Quick and Robust Sample Preparation for Tryptic Peptide Mapping With the PeptideWorks™ Kit Using Simple, Automatable Workflows

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Abstract

Here, we present an automatable sample preparation protocol for peptide mapping using the PeptideWorks Tryptic Protein Digestion Kit. The kit provides a reproducible tryptic protein digest developed for regulated and research peptide mapping applications.

Benefits

- Comprehensive kit and protocol for preparation of tryptic protein digests intended for quality control (QC), bioprocess, analytical development, and research environments

- Automatable preparation of 24 tryptic peptide mapping samples in under 2.5 hours
- Reproducible preparation of tryptic peptide mapping samples without sacrificing digestion completion or generating high levels of method-induced peptide modifications
- 93% reduction in missed cleavages and 55% reduction in non-specific cleavages compared to a leading immobilized trypsin digest kit

Introduction

Peptide mapping is used in QC, bioprocess, analytical development, and research environments to deliver comprehensive information about the primary structure of biotherapeutic proteins.[1] Generally, the peptide map of a test article is compared to that of a reference material; sample-to-sample reproducibility is imperative for detection of real changes between the test article and reference material. Reliable day-to-day sample preparation is also a critical factor in generating an effective peptide mapping method. Sample preparation for peptide mapping is complex. Prior to analysis, protein samples are treated in alkaline buffer at high temperatures and enzymatically digested to generate peptides. These conditions can result in method-induced peptide modifications, over- or under-digestion of the protein, and autolysis of the proteolytic enzyme, each of which complicate data analysis and interpretation.

Waters™ PeptideWorks Tryptic Protein Digestion Kits deliver fast and reliable sample preparation for routine peptide mapping of therapeutic proteins. The sample preparation kit is centered around RapiZyme™ Trypsin, Waters' homogeneously methylated, recombinant porcine trypsin. RapiZyme Trypsin enables speed, digestion fidelity, and low levels of trypsin-derived peptides through its autolysis resistance, purity, and high activity.[2,3,4] RapiZyme Trypsin can be used at high concentrations to achieve faster digestions without requiring high temperatures or sacrificing digestion completeness. The PeptideWorks workflow is shown in Figure

1. Reagents and reaction conditions used in the PeptideWorks kit were optimized to enable fast and complete digestion of proteins by RapiZyme Trypsin.

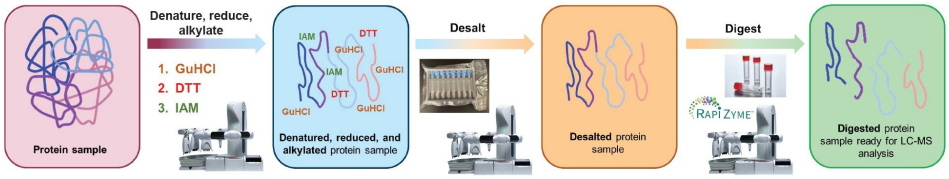


Figure 1. Workflow for the preparation of tryptic protein digests using the PeptideWorks Tryptic Protein Digestion Kit. The protocol can be performed manually or with automation on the Andrew+™ Pipetting Robot.

Experimental

Reagent Preparation

The NIST Monoclonal Antibody (NISTmAb) Reference Material® was obtained from NIST (p/n: 8671), Tris HCl (1 M, pH 7.5) was obtained from ThermoFisher Scientific (p/n: 15567027), and formic acid was obtained from Fisher (p/n: A117-50). All other reagents and required devices are included in the PeptideWorks Tryptic Protein Digestion Kit (p/n: 176005311) and were prepared following the protocol outlined in the PeptideWorks Tryptic Protein Digestion Kit Care and Use Manual 720007980 < https://www.waters.com/webassets/cms/support/docs/720007980en.pdf> .

Sample Preparation

NISTmAb tryptic digest samples were prepared both manually and with automation using the procedure outlined below. Automated sample preparation was performed on the Andrew+ Pipetting Robot equipped with the Extraction+ Connected Device. NISTmAb samples (10 mg/mL) were denatured and reduced in a solution containing 5 M guanidine hydrochloride (GuHCl) and 5 mM dithiothreitol (DTT) for

30 minutes at room temperature. Iodoacetamide (IAM) was then added to a final concentration of 10 mM and the samples were incubated for 30 minutes at room temperature in the dark. Samples were desalted with Sep-Pak™ SEC Desalting Cartridges and buffer exchanged with digestion buffer (10 mM $CaCl_2$ and 100 mM tris HCl, pH 7.5). The concentration of desalted samples was measured with a UV plate reader and normalized to 0.1 mg/mL using the digestion buffer as a diluent. RapiZyme Trypsin was added to each sample at a 1:5 enzyme:protein ratio and digestion proceeded for 30 minutes at 37 ºC. Finally, the reaction was quenched with 1% formic acid to a final concentration of 0.1%.

LC Conditions

LC system:	ACQUITY™ UPLC I-Class PLUS
Sample plate:	Eppendorf twin.tec® PCR Plate 96-well, skirted, green (p/n: 951020443)
Column:	ACQUITY Premier Peptide CSH™ C_{18} Column 1.7 µm, 2.1 x 150 mm (p/n: 186009489)
Column temp.:	65 °C
Sample temp.:	6 °C
Injection volume:	10 µL
Mobile phase A:	0.1% Formic Acid in H_2O
Mobile phase B:	0.1% Formic Acid in

	Acetonitrile

Gradient Table

Time (min)	Flow (mL/min)	%A	%B	Curve
Initial	0.25	99	1	Initial
5	0.25	99	1	6
65	0.25	60	40	6
68	0.25	30	70	6
70	0.25	30	70	6
71	0.25	99	1	6
85	0.25	99	1	6

ACQUITY RDa Detector Settings

Mass range:	50–2000 *m/z*
Mode:	Full scan with fragmentation
Ionization mode:	ESI+
Sample rate:	2 Hz
Cone voltage:	20 V
Fragmentation cone voltage:	60–120 V
Desolvation temperature:	350 °C
Capillary voltage:	1.20 kV
LockMass:	waters_connect™

LockMass solution

Data Management

Chromatography software: waters_connect

Results and Discussion

The PeptideWorks Workflow

The PeptideWorks workflow was optimized to enable fast and complete digestion of proteins by RapiZyme Trypsin. The workflow can be performed manually or with automation on the Andrew+ Pipetting Robot; both manual and automated workflows follow the steps detailed in Figure 2.

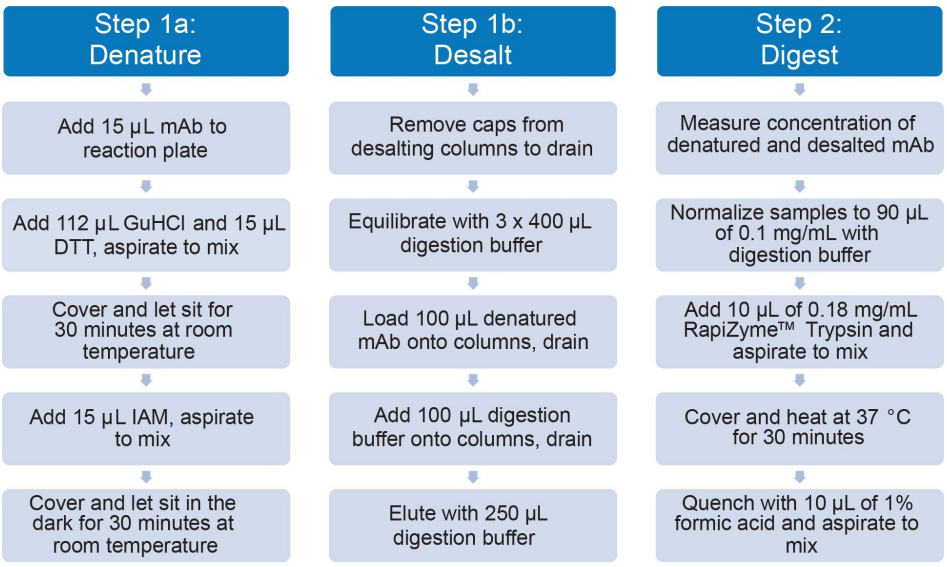
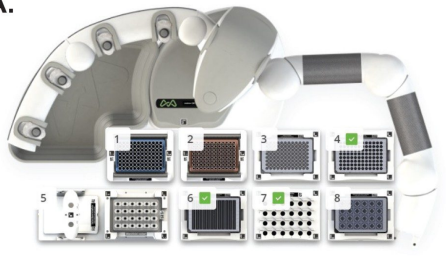


Figure 2. Flow diagram outlining the PeptideWorks sample preparation workflow. Both manual and automated sample preparation follow the depicted workflow.

The automated PeptideWorks workflow is split into two protocols: protocol A executes denaturation, reduction, and alkylation and protocol B executes concentration normalization and digestion. The automated workflow can accommodate up to 24-samples. The deck layouts for a full 24-sample workflow are shown in Figure 3. While concentration normalization is recommended to ensure accurate enzyme:protein ratios during digestion, an automated workflow without concentration normalization was also developed. The total execution time for 24-samples is 2 h 40 m with concentration normalization and 2 h 30 m without concentration normalization.

A.



Position	Domino
1, 2	Tip Insertion Domino
3	Microplate Domino
4	Storage Plate Domino
5	Extraction+
6	Deepwell Microplate Domino
7	Microtube Domino
8	Deepwell Microplate Domino

B.

Position	Domino
1	Tip Insertion Domino
2, 3	Microplate Domino
4	Deepwell Microplate Domino
5	Microtube Domino
6	96-PCR Plate Peltier+

Figure 3. Deck layouts for the 24-sample automated PeptideWorks workflow. Protocol A executes denaturation, reduction, and alkylation and protocol B executes concentration normalization and digestion.

Manual vs Automated Sample Preparation

The PeptideWorks Tryptic Protein Digestion Kit was used to digest NISTmAb manually and with automation on the Andrew+ Pipetting Robot. NISTmAb digests were analyzed via UPLC-MS; chromatograms of NISTmAb digests prepared using the manual and automated PeptideWorks workflow are shown in Figure 4. Both manual and automated sample preparation yield comparable chromatographic results with high sequence coverage (>88% expected peptides). Additionally, the relative abundances of modified peptides are consistent between manual and automated sample preparation workflows, as discussed below.

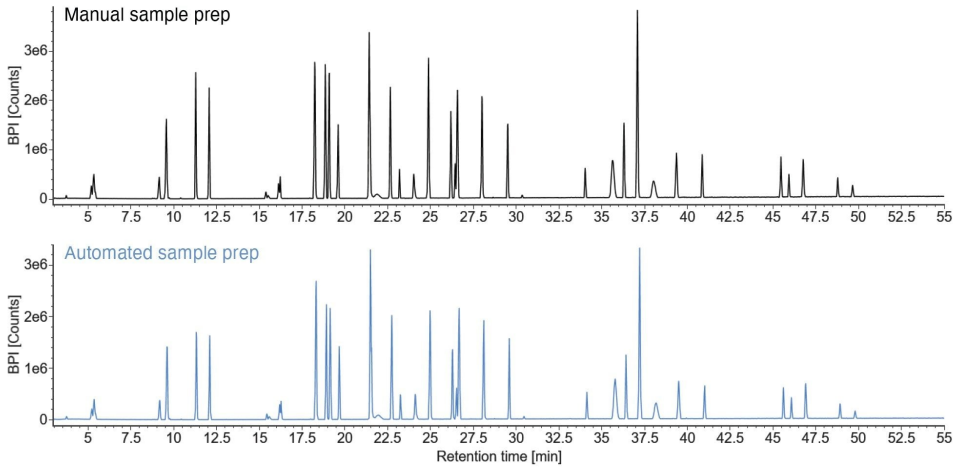


Figure 4. BPI chromatograms of NISTmAb digests prepared using the manual and automated PeptideWorks workflows, showing equivalent results.

The chromatography data were processed in waters_connect using the Peptide Map workflow. Relative levels of missed and non-specific cleavages were calculated using published methods.[5] Figure 5 displays the relative missed and non-specific cleavage results for NISTmAb digests prepared using manual and automated PeptideWorks workflows. Both PeptideWorks workflows deliver NISTmAb digests with less than 5% missed and non-specific cleavages, indicating high digestion efficiency without over-digestion of the protein. Missed and non-specific cleavage results for NISTmAb digests prepared using a leading immobilized trypsin digest kit are based on published results and shown in red in Figure 5.[6] PeptideWorks yields a 93% reduction in missed cleavages and 55% reduction in non-specific cleavages compared to the immobilized trypsin digest kit. In total, PeptideWorks delivers a far more complete and specific digestion, increasing reliability, and enabling faster data processing.

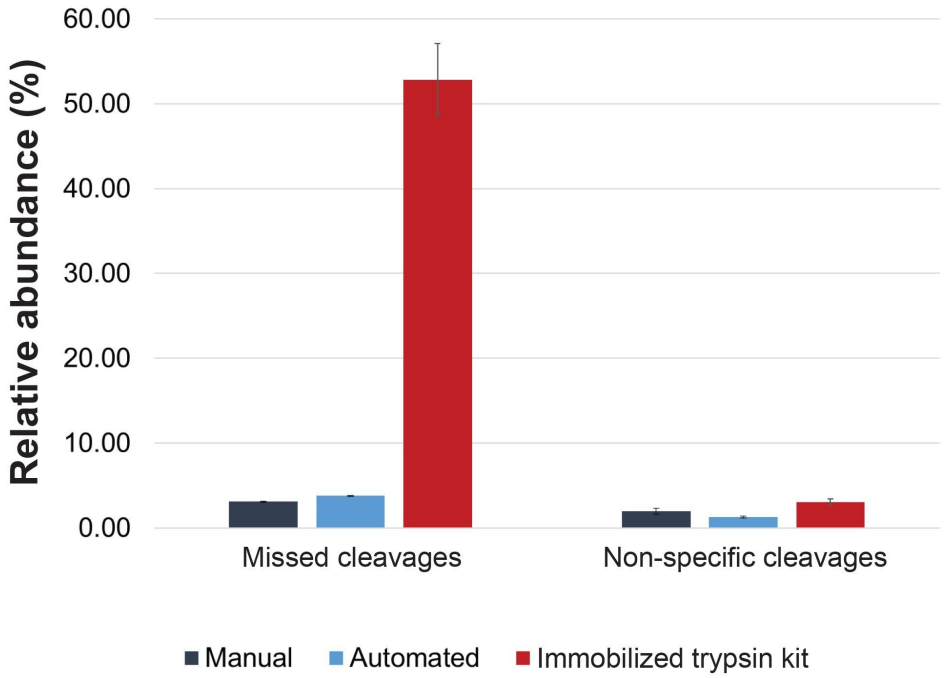
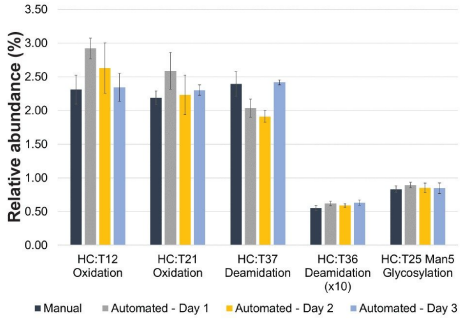


Figure 5. Relative abundance of missed and non-specific cleavages for NISTmAb digests prepared using the manual and automated PeptideWorks workflows and a leading immobilized trypsin kit. Error bars represent one standard deviation. PeptideWorks delivers a more complete and specific digestion.

Peptide Modification Characterization and Reproducibility

The relative abundance of select peptide modifications for three batches of NISTmAb digests prepared across three days with the automated PeptideWorks workflow are shown in Figure 6; results for NISTmAb digests prepared with the manual workflow are also shown to demonstrate consistency between manual and automated sample preparation. The values reported in Figure 6 are well within range of published NISTmAb tryptic digest data.[6–9] The consistency of the modification values reported here with those reported in the literature highlight the efficacy of PeptideWorks. PeptideWorks enables fast preparation of tryptic peptide mapping samples without sacrificing digestion completion or inducing high levels of method-induced deamidation or oxidation.

Variability for unmodified peptide abundance and modified peptide relative abundance, expressed as relative standard deviation (RSD), are detailed in the table in Figure 6. The day-to-day variability of the unmodified peptide abundance was less than 15% for each peptide, demonstrating consistent peptide recovery and therefore consistent quantification limits when using PeptideWorks for sample preparation. Day-to-day variability of the modified peptide relative abundance was less than 10% for each peptide modification.



Peptide (modification)	% RSD	
	Unmodified peptide abundance	Modified peptide relative abundance
HC:T12 (Ox.)	11.80	8.06
HC:T21 (Ox.)	6.02	6.79
HC:T37 (Deam.)	8.09	8.82
HC:T36 (Deam.)	4.62	3.01
HC:T25 (Man5)	6.47[a]	2.12

[a] The combined abundance of the G0F, G1F, and G2F glycoforms of HC:T25 were used in place of the unmodified peptide abundance.

Figure 6. Left: Bar chart representing the relative abundance of select peptide modifications for three batches of NISTmAb digests prepared using the automated PeptideWorks workflow. Results for NISTmAb digests prepared with the manual workflow are also shown to demonstrate consistency between the manual and automated sample preparation. Error bars represent one standard deviation. Right: Table outlining the %RSD of the unmodified peptide abundance and modified peptide relative abundance to demonstrate consistent peptide recovery and therefore consistent quantification limits when using PeptideWorks for sample preparation.

Conclusion

PeptideWorks Tryptic Protein Digestion Kit is a comprehensive kit for the preparation of tryptic peptide mapping samples suitable for QC, bioprocess,

analytical development, and research environments. The PeptideWorks protocol can be performed manually or with automation, yielding 24 digested samples in under 2.5 hours. As demonstrated with NISTmAb, PeptideWorks enables reproducible preparation of tryptic peptide mapping samples without sacrificing digestion completion or inducing high levels of peptide modifications.

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