

Application Note

Peptide Mapping of an Antibody-Drug Conjugate Using PeptideWorks Tryptic Protein Digestion Kit

Hua Yang, Stephan M. Koza, Steve Shiner

Waters Corporation

This is an Application Brief and does not contain a detailed Experimental section.

Abstract

To demonstrate the use of PeptideWorks Tryptic Protein Digestion Kits for Antibody-Drug Conjugate (ADC) peptide mapping.

Introduction

Peptide mapping is a powerful tool for protein sequence identification and confirmation, as well as protein modification monitoring, but the procedure is often tedious. It has been shown previously that Waters PeptideWorks Tryptic Protein Digestion Kits (p/n: [176005310](https://www.waters.com/nextgen/global/shop/application-kits/176005310-peptideworks-tryptic-start-up--column-kit.html) <
<https://www.waters.com/nextgen/global/shop/application-kits/176005310-peptideworks-tryptic-start-up--column-kit.html>>) provide an automatable sample preparation procedure and enable reproducible tryptic peptide maps for monoclonal antibodies (mAbs) in less than 2.5 hours.¹ In this Technology Brief, we show that

insightful information can be obtained from ADC peptide mapping using PeptideWorks Tryptic Protein Digestion Kits.

Experimental

Figure 1 shows UV chromatograms of the peptide maps of both trastuzumab (T mAb) and trastuzumab-emtansine (T-DM1, the ADC). The UV detection was done at 252 nm because the small molecule drug absorbs strongly at this wavelength. A number of additional peaks are observed for the T-DM1 sample in the more retained region of the chromatogram that do not appear for T mAb (the blue dashed box and the blown-up figure). These peaks are likely the drug-conjugated peptides because they elute later on the reversed-phase column due to the hydrophobicity of the small molecule drug. Indeed, many of these additional peaks were confirmed to be the drug-conjugated peptides by mass spectrometry data processed by waters_connect software. As the drug is conjugated through lysine residues, trypsin (Rapizyme Trypsin, MS grade, p/n: [186010106 <https://www.waters.com/nextgen/global/shop/standards--reagents/186010106-rapizyme-trypsin-ms-grade-1-pk.html>](https://www.waters.com/nextgen/global/shop/standards--reagents/186010106-rapizyme-trypsin-ms-grade-1-pk.html)) is unable to digest at the drug-conjugated lysine site, leading to missed cleavage between the lysine-containing peptide, and its following peptide. In addition, these peaks elute in pairs (diastereomers) due to two possible stereochemical configurations during conjugation reaction. About a dozen of them are labeled in Figure 1.

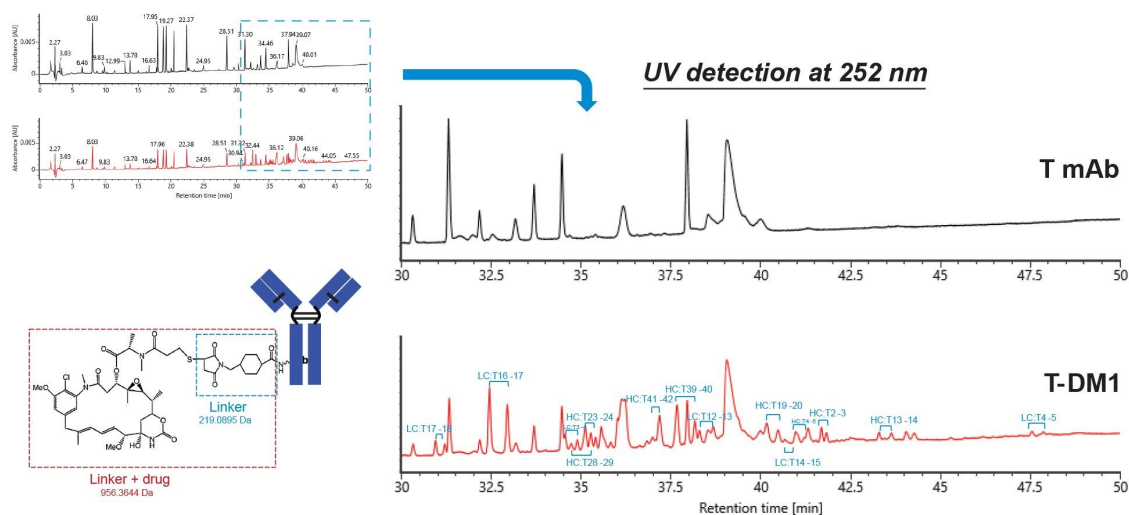


Figure 1. UV (252 nm) chromatograms of approximately 10 μ g of trypsin-digested trastuzumab and trastuzumab-emtansine (T-DM1, the ADC) peptide separation on an XSelect Premier Peptide CSH C₁₈, 130 Å, 2.5 μ m, 2.1 x 150 mm column (p/n: 186009906). Mobile phase A: 0.1% formic acid in H₂O, Mobile phase B: 0.08% formic acid in acetonitrile. Gradient: 0.5–50% B in 60 mins. Flow rate: 0.2 mL/min. Column temperature: 60 °C. Xevo G2 XS mass spectrometer and waters_connect software were used for peak confirmation. MS conditions: m/z 50–2000, ESI⁺, Cone voltage: 30V, source temperature: 100 °C, desolvation temperature: 350 °C, capillary voltage: 1.2kV, high collision energy ramp: 30–50 V. The digestion was carried out manually following the PeptideWorks Tryptic Protein Digestion Kit Care and Use Manual.² The digestion procedure can be automated using Andrew+ Pipetting Robot.¹ The kit contains a novel chemically optimized recombinant trypsin, RapiZyme Trypsin.³ Some diastereomer peptide pairs, resulted from two possible stereochemical configurations during conjugation reaction, are confirmed on T-DM1 sample using MS data. The structure and the molecular weight of the linker and the small molecule drug is shown on the lower left of the figure.

As an example, Figure 2a shows mass spectrometry data of a drug-conjugated peptide-light chain T16–17 (ADYEKHK) peptide. Based on the molecular weight of the peptide and the drug, m/z of 616.27 (+3 charge) was extracted from the BPI chromatograms. As predicted, two diastereomer peaks appeared in the extracted ion chromatogram (XIC) for the T-DM1 sample while no peaks with meaningful information appeared for the T mAb sample. Additionally, the combined spectra of the diastereomer peaks both showed m/z of 616.27 (+3 charge)

and 923.90 (+2 charge) as the highest intensity, indicating that the major component of the peaks is the drug-conjugated light chain T16-17 peptide. The diastereomers also appear on the UV chromatogram for the T-DM1 sample but not for the T mAb sample, shown on Figure 2b.

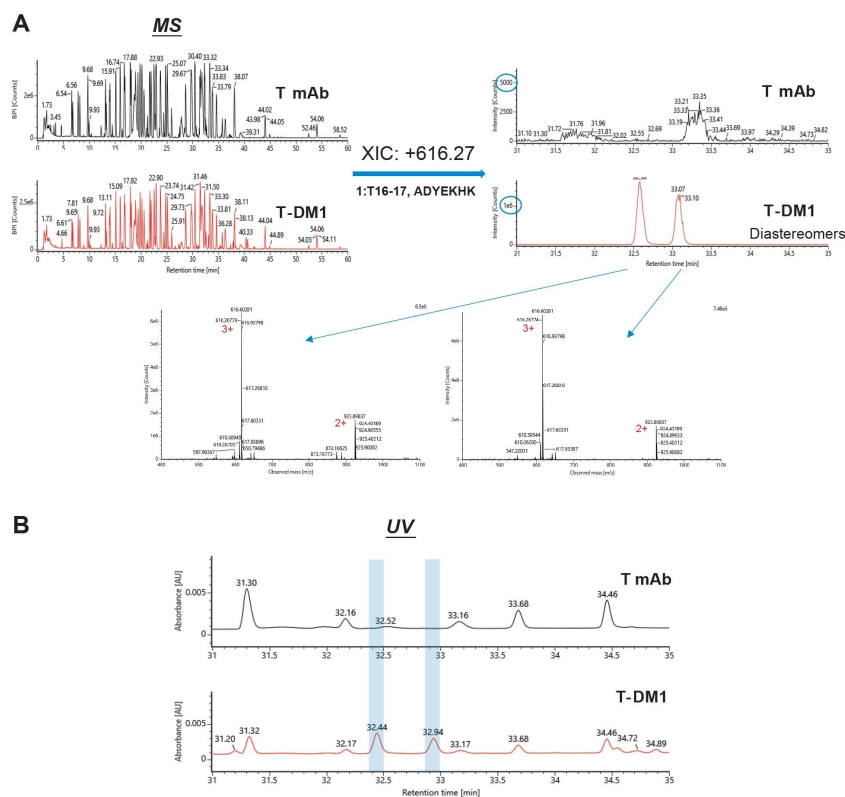


Figure 2. a. Base peak intensity (BPI) chromatograms of T mAb and T-DM1 peptide mapping are shown in the top left. Extracted ion chromatograms (XIC) are obtained by extracting a m/z of 616.27. Two peaks (diastereomers) appear for the T-DM1 sample with retention time of 32.57 min and 33.07 min. Combined spectra showed +3 charge (m/z 616.27) and +2 charge (m/z 923.90) species, consistent with the molecular mass of light chain T16-17 (ADYEKHK) peptide with conjugated drug. b. The diastereomers also appear on UV chromatograms for the T-DM1 sample while no major peaks appear for the T mAb sample.

Figure 3 shows MS/MS spectra of the drug-conjugated light chain T16-17 fragment ions. As shown in the

Fragmentation View of waters-connect software, y1-ion, y2-ion, b2-ion, and b3-ion are confirmed, strongly suggesting that the drug-conjugation site is the 5th amino acid (lysine) in the peptide sequence. Signature fragment ions specific to drug-conjugated peptide are also present (m/z in orange box), and the reputed structures are shown on top of the spectra.^{4,5}

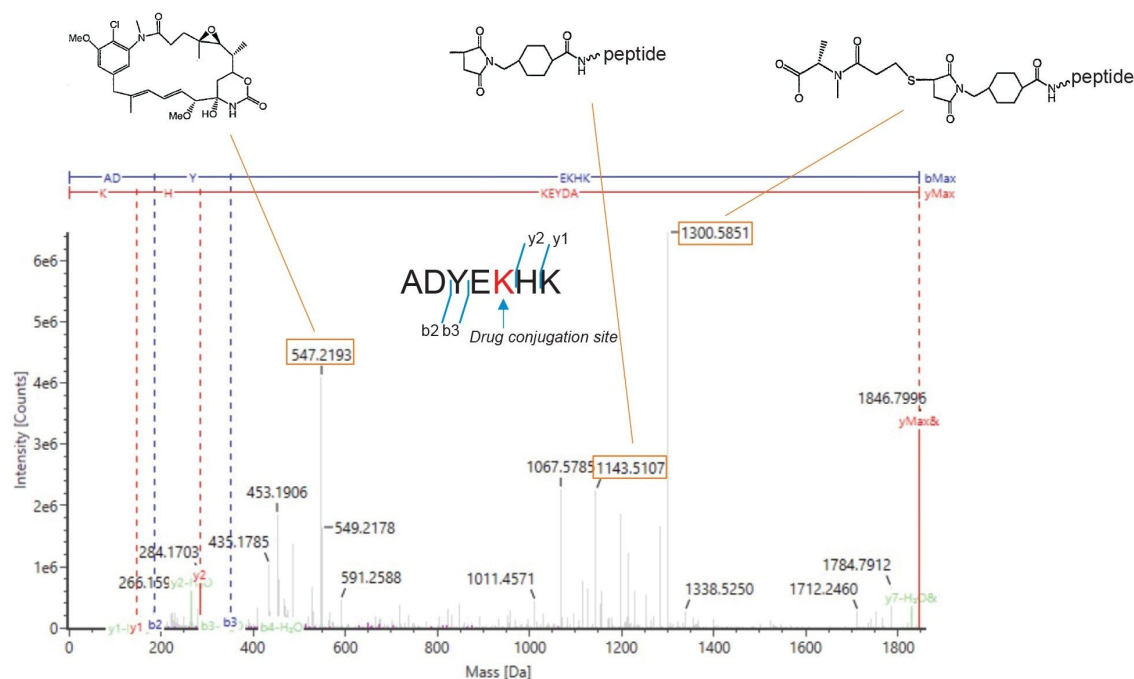


Figure 3. MS/MS spectra of the drug-conjugated light chain T16-17 fragment ions. y1-ion, y2-ion, b2-ion, and b3-ion are confirmed, strongly suggesting that the drug-conjugation site is the 5th amino acid (lysine) in the peptide sequence. A few signature fragment ions specific to drug-conjugated peptide are present (m/z in orange box), and the structures are shown on top of the spectra.

Table 1 shows a list of drug-conjugated peptides and their sequences. Most of the lysines are conjugated, consistent with previous results.⁴

	Tryptic peptide	Sequence	Observed RT (min)	
Light chain	1:T3-4	ASQDVNTAVAWYQQKPGKAPK	35.03	34.70
	1:T4-5	APKLLIYSASFLYSGVPSR	48.16	47.94
	1:T7	SGDFTLTISLQPEDFATYYCQQHYTTPTFGQGTK	28.65	28.66
	1:T8-9	VEIKR	37.33	37.78
	1:T12-13	EAKVQWK	38.83	38.44
	1:T13-14	VQWKVDNALQSGNSQESVTEQDSK	39.84	40.11
	1:T14-15	VDNALQSGNSQESVTEQDSKDYSLSTLTLSK	40.82	41.19
	1:T15-16	DSTYLSSTLTLSKADYEK	43.73	44.02
	1:T16-17	ADYEKHK	33.08	32.58
	1:T17-18	HKVYACEVTHQGLSSPVTK	31.08	31.34
	1:T18-19	VYACEVTHQGLSSPVTKSFNR	36.39	36.15
	2:T2-3	LSCAASGFNIKDTYIHWVR	41.84	41.99
	2:T4-5	QAPGKGLEWVAR	41.45	41.12
	2:T7-8	YADSVKGR	37.31	37.78
Heavy chain	2:T9-10	FTISADTSKNTAYLQMNSLR	41.36	41.70
	2:T12-13	WGDDGFYAMDYWGQGLTVTVSSASTKGPSVFPLAPSSK	48.21	47.92
	2:T13-14	GPSVFPLAPSSKSTSGGTAALGCLVK	43.79	43.45
	2:T13	GPSVFPLAPSSK	42.64	43.02
	2:T15-16	DYFPEPVTWSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDK	42.49	42.29
	2:T15	DYFPEPVTWSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTK	44.38	44.18
	2:T16-17	VDKK	36.18	36.77
	2:T19-20	SCDKTHTCPPCPAPELLGGPSVFLFPPKPK	40.63	40.32
	2:T20-21	THTCPPCPAPELLGGPSVFLFPPKPKDTLMISR	42.87	42.71
	2:T20	THTCPPCPAPELLGGPSVFLFPPKPK	44.85	45.07
	2:T23-24	FNWYVDGVEVHNAKTKPR	35.27	35.55
	2:T24-25	TKPREEQYNSTYR	34.46	30.18
	2:T24	TKPR	35.24	35.80
	2:T26-27	VVSVLTVLHQDWLNGKEYK	43.61	43.84
	2:T27-28	EYKCK	35.69	36.24
	2:T28-29	CKVSNK	34.87	35.41
	2:T29-30	VSNKALPAPIEK	39.54	39.20
	2:T30-31	ALPAPIEKTISK	41.50	41.19
	2:T32-33	AKGQPR	35.20	35.77
	2:T35-36	EEMTKNQVSLTCLVK	41.48	41.84
	2:T37-38	GFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSK	46.34	46.63
	2:T39-40	LTVDKSR	37.83	38.31
	2:T41-42	WQQGNVFSCSVMHEALHNHYTQKSLSLSPGK	37.22	37.06

Table 1. A list of drug-conjugated peptides and their sequences, identified by waters-connect software.

It is worth noting that the coverage for T mAb and T-DM1 peptide map is 92% and 95%, respectively, with the predicted trypsin cleavage sites. When the drug conjugation site is taken into consideration, the coverage increases to 98% for T-DM1. This shows that PeptideWorks Tryptic Protein Digestion Kit can be a useful tool for mAb and ADC peptide mapping work.

Conclusion

PeptideWorks Tryptic Protein Digestion Kits can be used for ADC peptide mapping with automatable sample preparation procedure under 2.5 hours. Compared to the peptide map of the mAb without drug conjugation, additional peaks are observed on the ADC sample in the more retained region of the chromatogram. Using MS data, some diastereomer peptide pairs, resulted from two possible stereochemical configurations during conjugation reaction, are confirmed on the ADC sample.

References

1. Hanna C.M., Danaceau J.P., Koza S.M., Shiner S., Trudeau M. Quick and Robust Sample Preparation for Tryptic Peptide Mapping With the PeptideWorks™ Kit Using Simple, Automatable Workflows. Waters Application Note. [720008019](#). July, 2023.
2. PeptideWorks Tryptic Protein Digestion Kit Care and Use Manual. [720007980](#) <<https://help.waters.com/help/global/support/library-details.html?documentid=720007980>> . October, 2024.
3. RapiZyme Trypsin Care and Use Manual. [720007851](#) <<https://help.waters.com/help/global/support/library-details.html?documentid=720007851>> . February, 2023.
4. Chen L., Wang L., Shion H., Yu C., Yu Y.Q., Zhu L., Li M., Chen W., and Gao K. In-depth structural characterization of Kadcyla® (ado-trastuzumab emtansine) and its biosimilar candidate. *MABS*, VOL. 8, NO. 7, 1210–1223, October, 2016.
5. Luo Q., Chung H. H., Borths C., Janson M., Wen J., Joubert M. K., and Wypych J. Structural Characterization of a Monoclonal Antibody–Maytansinoid Immunoconjugate. *Anal. Chem.* 88, 695–702, January, 2016.

Featured Products

[Xevo G3 QToF](https://www.waters.com/nextgen/global/products/mass-spectrometry/mass-spectrometry-systems/xevo-g3-qtof.html) <<https://www.waters.com/nextgen/global/products/mass-spectrometry/mass-spectrometry-systems/xevo-g3-qtof.html>>

[PeptideWorks Tryptic Protein Digestion Kits](https://www.waters.com/nextgen/global/products/application-kits/peptideworks-tryptic-protein-digestion-kits.html) <<https://www.waters.com/nextgen/global/products/application-kits/peptideworks-tryptic-protein-digestion-kits.html>>

waters_connect Informatics <https://www.waters.com/nextgen/global/products/informatics-and-software/waters_connect.html>

720008664, January 2025



© 2025 Waters Corporation. All Rights Reserved.

[Terms of Use](#) [Privacy Policy](#) [Trademarks](#) [Careers](#) [Legal and Privacy Notices](#) [Cookies](#)
[Cookie Preferences](#)