Mapping the Drug Conjugation Sites of an Antibody-Drug Conjugate

Using Automated Sample Preparation and LC/MS Analysis

Application Note

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Introduction

Antibody drug conjugates (ADCs) that combine the specificity of monoclonal antibodies (mAbs) with the potency of cytotoxic molecules have gained significant interest as a promising approach to achieve more selective treatment for cancer. ADCs are more complex and heterogeneous compared to unmodified biotherapeutic antibodies due to a multitude of potential drug conjugation sites and the variation of site occupancy among these potential conjugation sites. To obtain full characterization of the ADC molecule, peptide mapping experiments can be carried out to provide in-depth site-specific information about the ADC conjugation sites. This application note presents an ADC peptide mapping workflow combining automated protein digestion using the Agilent AssayMAP Bravo sample preparation platform, LC/MS analysis using the high resolution accurate mass Agilent 6550 iFunnel Q-TOF system, and Agilent BioConfirm Software. A 98.7 % sequence coverage was achieved for T-DM1. More than 29 lysine sites were identified with drug conjugation.

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Experimental

Reagents and chemicals

Trypsin was from Agilent Technologies (Santa Clara, CA). Eppendorf 96-well PCR plates were from Eppendorf (Hauppauge, NY); all other chemicals were from Sigma-Aldrich (St. Louis, MO).

Automated trypsin digestion

Lyophilized T-DM1 and Herceptin were reconstituted in deionized (DI) water to 20 mg/mL, then aliquoted and stored at –80 °C until used. Antibody digestions were performed on the Agilent AssayMAP Bravo (Santa Clara, CA) using the In-Solution Digestion Protocol: Single-Plate v1.0, which is part of the Protein Sample Prep Workbench software that controls the AssayMAP Bravo. Briefly, T-DM1 and Herceptin were diluted to 1.5 mg/mL; 10 microliters of T-DM1 was dispensed into each well in the first column of a 96-well Eppendorf PCR plate (position A1-H1); 10 µL of Herceptin was dispensed into a well in the second column of the 96-well plate (position A2). Ten microliters of 20 mM dithiothreitol (DTT) dissolved in 50 % 2,2,2-trifluroethanol (TFE), 100 mM ammonium bicarbonate buffer was added to the samples and incubated at 60 °C for one hour on deck using a built-in Peltier heater/cooler. Subsequently, 10 µL of 50 mM iodoacetamide was added to each well and incubated on the deck at room temperature for 30 minutes, protected from the light. Finally, the samples were diluted with 20 µL of 50 mM ammonium bicarbonate buffer, mixed with 30 µL trypsin (10 µg/mL), and incubated at 37 °C on the deck for two hours.

LC/MS analysis

LC/MS analyses were conducted using an Agilent 6550 iFunnel Q-TOF (Santa Clara, CA) equipped with a Dual Agilent Jet Stream ESI source coupled to an Agilent 1290 Infinity UHPLC system (Waldbronn, Germany). Table 1 and Table 2 list the LC/MS parameters used. A total of 1.5 µg of sample was injected for each run.

Data analysis

For ADC conjugation site identification, compounds were extracted by molecular feature extraction (MFE), then matched against the ADC sequence with the following modifications using Agilent MassHunter BioConfirm (version B.07.00):

- Alkylation (iodoacetamide) (C): +57.0215
- Oxidation (M): + 15.9949
- Deamidation (N): +0.9840
- Drug (K): +956.3644

All identified peptides were confirmed by MS/MS spectra.

For the 35 drug conjugated peptides identified, peak areas were integrated from EICs of precursors (M, M+H, M+2H) extracted from MS only data. The % CV were calculated based on peak areas (n = 8).

Table 1. Liquid chromatography parameters.

Parameter	Agilent 1290 Infinity LC System
Column	Agilent ZORBAX Eclipse Plus C18, RRHD, 2.1 × 100 mm, 1.8 µm
Sample thermostat	5 °C
Mobile phase A	0.1 % Formic acid in water
Mobile phase B	0.1 % Formic acid in acetonitrile
Gradient	Time (min) %B 0-16 5-50 16-18 50-90 18-19 90
Post time	3 minutes
Column temperature	55 °C
Flow rate	0.5 mL/min

Table 2. Mass spectrometer parameters.

Parameter	Agilent 6550 Q-TOF LC/MS System
Ion mode	Positive ion mode
Source	Agilent Dual Jet Stream
Drying gas temperature	150 °C
Drying gas flow	13 L/min
Sheath gas temperature	250 °C
Sheath gas flow	12 L/min
Nebulizer	35 psi
Capillary voltage	3,500 V
Nozzle	250 V
Fragmentor voltage	250 V
Oct RF Vpp	750 V
MS acquisition mode	Extended dynamic range, 2 GHz; Auto MS/MS mode for drug conjugation site identification; MS only mode for reproducibility assessment
MS range	300–2,000 *m/z*
MS/MS range	100–2,000 *m/z*

Results and Discussion

To identify drug conjugated sites of T-DM1, the ADC and its corresponding mAb, Herceptin, were reduced, alkylated, and digested using the AssayMAP Bravo's automated sample preparation. They were then analyzed with an Agilent 6550 Q-TOF coupled to an Agilent 1290 Infinity LC. Raw data were processed with Agilent BioConfirm Software for identification of drug conjugated peptides (Figure 1).

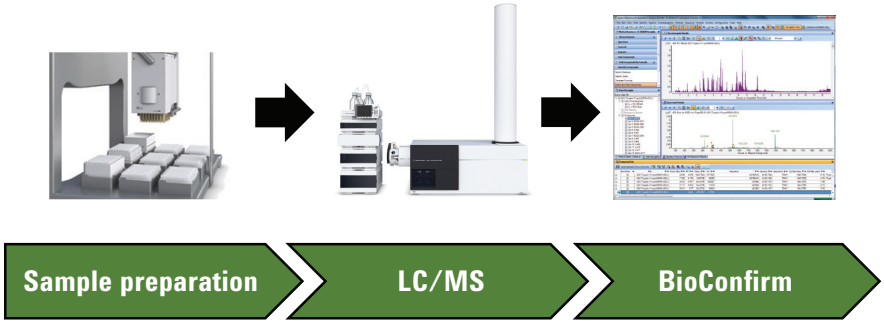

Figure 1. ADC peptide mapping workflow using the Agilent AssayMAP Bravo and Agilent 1290 Infinity LC/MS system.

Similar profiles were observed between the base peak chromatograms (BPCs) of T-DM1 and Herceptin, with additional peaks observed in the latter half of the chromatogram of T-DM1 (Figure 2A). Since DM1 is known to produce a strong fragment ion at 547.22, the EIC of this ion was extracted from the MS/MS spectra. The EIC at 547.22 showed numerous peaks in the latter half of the chromatogram, between 8 minutes and 15 minutes, suggesting the elution of drug conjugated peptides at these retention times (Figure 2B). As expected, no peak with appreciable signal intensity was observed in the EIC at 547.22 from Herceptin.

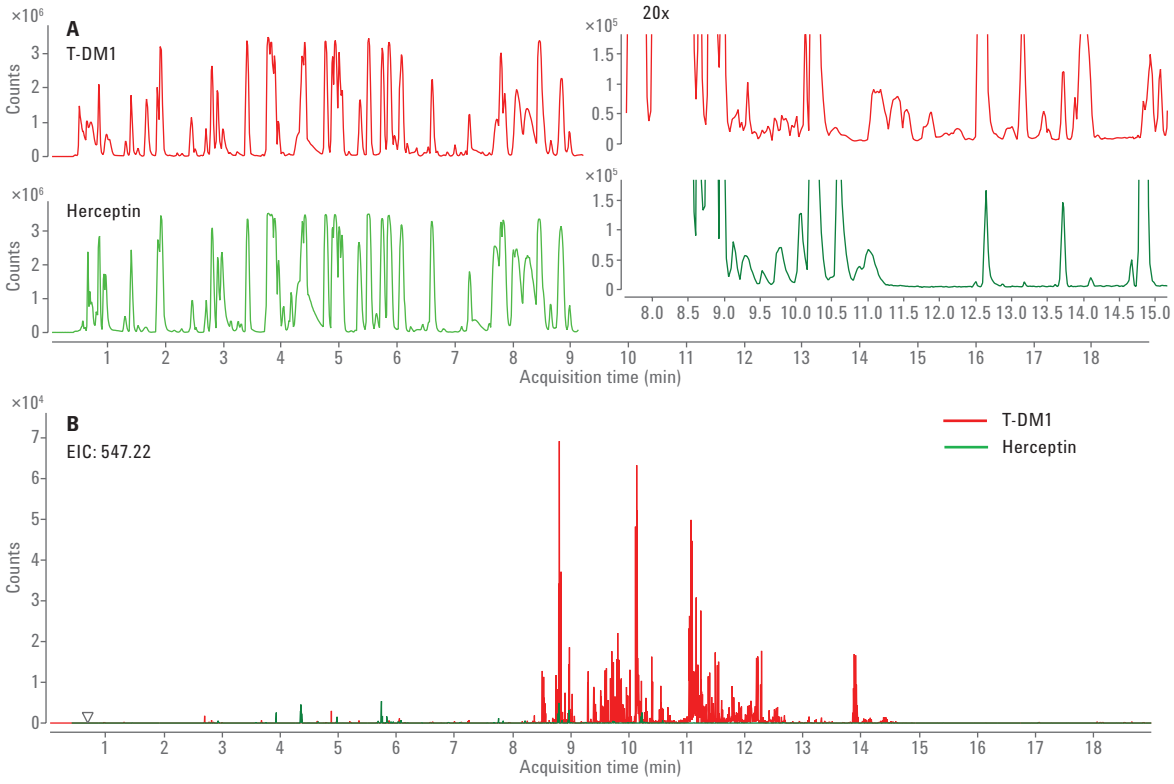

Figure 2. Base peak chromatograms (A) and extracted ion chromatograms of 547.22 from MS/MS spectra (B) for digested T-DM1 and Herceptin.

Figure 3 shows the precursor EICs and MS/MS spectra of two representative drug conjugated peptides identified. The drug conjugated peptides formed diastereomers, and eluted in pairs (Figures 3A and 3C). In addition to the expected y and b ions, the drug fragment ions 140.07, 453.19, 485.22, and 547.22 were commonly present in the MS/MS spectra of the drug conjugated peptides. Y ions or precursor ions losing a mass of 546.21 (loss of 547.22 and gaining of a hydrogen) were also frequently observed (Figures 3B and 3D). A 98.7 % sequence coverage was achieved for T-DM1. All 44 lysine sites were covered by at least one unique peptide, with 29 confirmed drug conjugated sites, five potential drug conjugation sites (unique drug conjugated peptides were identified based on both MS and MS/MS, but the exact

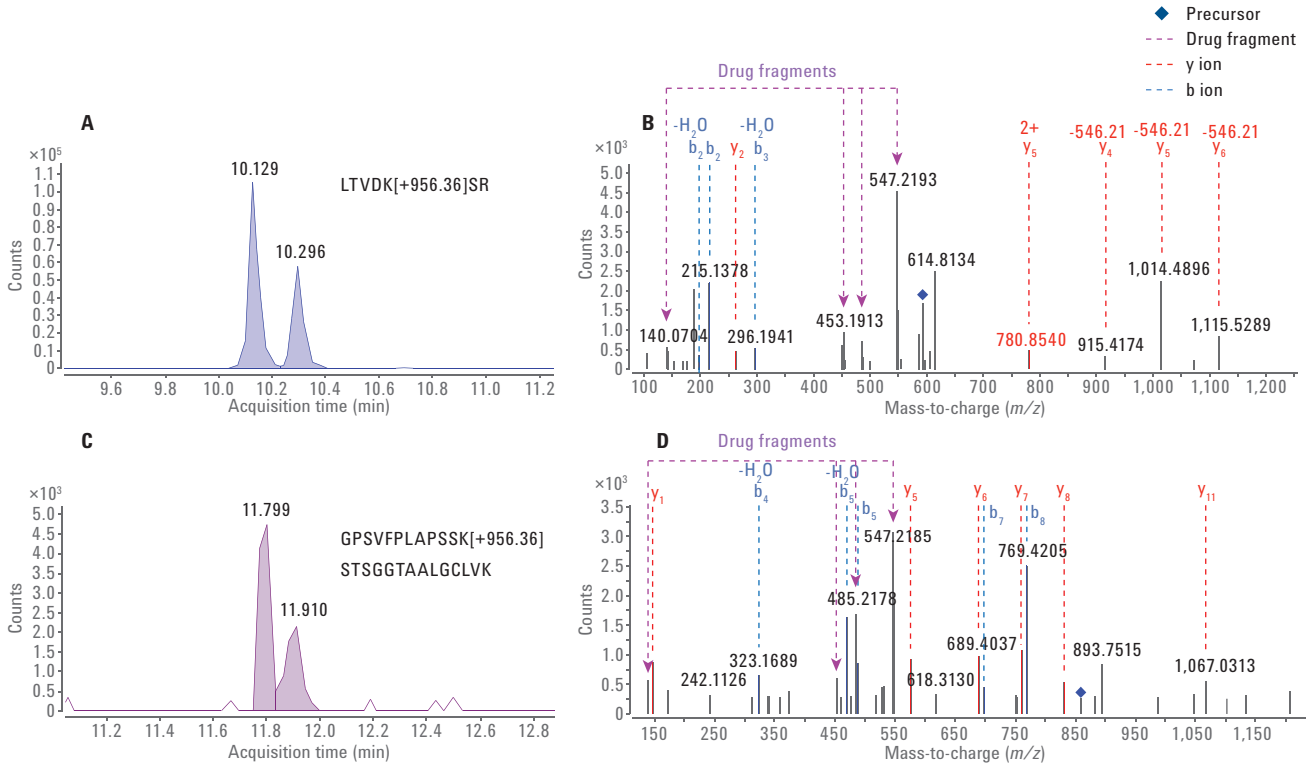

Figure 3. Representative precursor EICs and MS/MS spectra of drug conjugated peptides. A) EIC of the precursor ion of drug conjugated peptide LTDKSR. B) MS/MS spectrum of drug conjugated peptide LTDKSR. C) EIC of the precursor ion of drug conjugated peptide GPSVFPLAPSSKSTSGGTAALGCLVK. D) MS/MS spectrum of drug conjugated peptide GPSVFPLAPSSKSTSGGTAALGCLVK.

Light chain

DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPSRFSGS
RSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASV
VCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEV
THQGLSSPVTKSFNRGEC

Heavy chain

EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYTRYADSV
KGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGTLVTVSSASTKGPS
VFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVP
SSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMIS
RTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGK
EYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWES
NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG

Identified sequence
K Confirmed drug conjugation site
K Potential drug conjugation site
K Unconjugated site identified
29 (Light-9; Heavy-20)
5 (Heavy-5)
10 (Light-4; Heavy-6)

Figure 4. Confirmed drug conjugation sites, potential drug conjugation sites and unconjugated site identified.

conjugation site could not be located among the multiple lysine sites on the peptide), and 10 unconjugated sites (no evidence of conjugation found) (Figure 4).

Table 3 shows a list of 35 unique drug conjugated peptides, with the confirmed drug conjugation sites, potential drug conjugation sites, and sites for which there is no evidence of conjugation labeled in green, orange, and blue, respectively.

Table 3. Unique drug conjugated peptides identified. Confirmed drug conjugation sites are labeled in green. The potential drug conjugation sites are labeled in orange where the site of conjugation cannot be determined based on MS/MS spectra. Lysine residues that are not conjugated are labeled in blue.

No.	Chain	Seq Loc	Sequence	Modifications	Mass	Diff (Bio, ppm)	MS/MS Count
1	Light	25-45	ASQDVNTAVAWYQQKPGKAPK	1*Drug(+956.36444)A41	3,242.5245	-4.82	1
2	Light	43-61	APKLLIYSASFLYSGVPSR	1*Drug(+956.36444)A44	3,024.4894	-3.55	1
3	Light	104-108	VEIKR	1*Drug(+956.36444)A106	1,599.7626	-2.24	3
4	Light	109-142	TVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPR	1*Drug(+956.36444); 1*Alkylation (iodoacetamide) (+57.021464)A125A133	4,680.2396	-4.69	2
5	Light	143-149	EAKVQWK	1*Drug(+956.36444)A144	1,843.8448	-3.31	1
6	Light	150-183	VDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSK	1*Drug(+956.36444)A168	4,575.0418	-5.4	1
7	Light	184-190	ADYEKHK	1*Drug(+956.36444)A187	1,845.7912	-1.44	2
8	Light	189-207	HKVYACEVTHQGLSSPVTK	1*Alkylation (iodoacetamide) (+57.021464); 1*Drug(+956.36444)A193A189	3,096.4260	-3.87	1
9	Light	191-211	VYACEVTHQGLSSPVTKSFNR	1*Alkylation (iodoacetamide) (+57.021464); 1*Drug(+956.36444)A193A206	3,335.5169	-3.51	1
10	Heavy	20-38	LSCAASGFNIKDTYIHWVR	1*Alkylation (iodoacetamide) (+57.021464); 1*Drug(+956.36444)B22B30	3,193.4581	-3.59	1
11	Heavy	39-50	QAPGKGLEWVAR	1*Drug(+956.36444)B43	2,267.0663	-3.37	1
12	Heavy	60-67	YADSVKGR	1*Drug(+956.36444)B65	1,850.814	-3.43	2
13	Heavy	99-136	WGGDGFYAMDYWGQGTLVTVSSASTKGPSVFP LAPSSK	1*Drug(+956.36444)B124	4,907.1999	-2.29	1
14	Heavy	125-136	GPSVFPLAPSSK	1*Drug(+956.36444)B136	2,142.0028	-0.46	3
15	Heavy	125-150	GPSVFPLAPSSKSTSGGTAALGCLVK	1*Alkylation (iodoacetamide) (+57.021464); 1*Drug(+956.36444)B147B136	3,444.6523	-3.41	1
16	Heavy	151-213	DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYS LSSVVTVPSSSLGTQTYICNVNHKPSNTK	1*Alkylation (iodoacetamide) (+57.021464); 1*Drug(+956.36444)B203B208	7,668.634	-4.9	6
17	Heavy	214-217	VDKK	1*Drug(+956.36444)B216	1,444.6554	-3.4	1
18	Heavy	214-221	VDKKVEPK	1*Drug(+956.36444) (B216orB217orB221)	1,897.9142	-2.56	1
19	Heavy	217-221	KVEPK	1*Drug(+956.36444)B217	1,555.7243	-2.83	1
20	Heavy	222-251	SCDKTHTCPPCPAPELLGGPSVFLFPPKPK	3*Alkylation (iodoacetamide) (+57.021464); 1*Drug(+956.36444) B232B229B223B225	4,289.9795	-4.61	3
21	Heavy	226-251	THTCPPCPAPELLGGPSVFLFPPKPK	2*Alkylatio (iodoacetamide) (+57.021464); 1*Drug(+956.36444)B232B229B249	3,799.799	-4.19	2

No.	Chain	Seq Loc	Sequence	Modifications	Mass	Diff (Bio, ppm)	MS/MS Count
22	Heavy	226-258	THTCPPCPAPELLGGPSVFLFPPKPKDTLMISR	2*Alkylation (iodoacetamide) (+57.021464); 1*Drug(+956.36444) B232B229(B249orB251)	4,616.2141	-3.68	2
23	Heavy	259-291	TPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAK	1*Alkylation (iodoacetamide) (+57.021464); 1*Drug(+956.36444)B264B291	4,753.1541	-3.07	3
24	Heavy	278-295	FNWYVDGVEVHNAKTKPR	1*Drug(+956.36444)(B291orB293)	3,115.441	-4.72	1
25	Heavy	292-295	TKPR	1*Drug(+956.36444)B293	1,456.6701	-0.95	1
26	Heavy	305-323	VVSVLTVLHQDWLNGKEYK	1*Drug(+956.36444)B314B320	3,183.5605	-1.27	2
27	Heavy	305-325	VVSVLTVLHQDWLNGKEYKCK	1*Alkylation (iodoacetamide) (+57.021464); 1*Drug(+956.36444) B324(B320orB323orB325)	3,471.6801	-2.89	3
28	Heavy	321-325	EYKCK	1*Alkylation (iodoacetamide) (+57.021464); 1*Drug(+956.36444)B324B323	1,682.6981	-2.05	1
29	Heavy	324-329	CKVSNK	1*Alkylation (iodoacetamide) (+57.021464); 1*Drug(+956.36444)B324B325	1,690.7356	-1.99	1
30	Heavy	326-337	VSNKALPAPIEK	1*Drug(+956.36444)B329	2,222.0921	-2.98	1
31	Heavy	330-341	ALPAPIEKTISK	1*Drug(+956.36444)B337	2,223.111	-3.67	3
32	Heavy	342-347	AKGQPR	1*Drug(+956.36444)B343	1,611.7385	-1.53	1
33	Heavy	348-373	EPQVYTLPPSREEMTKNQVSLTCLVK	1*Alkylation (iodoacetamide) (+57.021464); 1*Drug(+956.36444) B370(B363orB373)	4,002.899	0.75	1
34	Heavy	374-412	GFYPSDIAVEWESNGQPENNYKTTPPVLDSDGS FFLYSK	1*Drug(+956.36444)B395	5,354.3867	-1.08	1
35	Heavy	413-419	LTVDKSR	1*Drug(+956.36444)B417	1,773.8267	-1.98	1

To assess the reproducibility of the workflow, the EICs of the precursor mass for the 35 identified drug conjugated peptides acquired in MS only mode were extracted and integrated. Figure 5A shows the overlaid EICs of two representative drug conjugated peptides for eight replicate runs. Figure 5B shows the integrated peak areas of the eight replicates from four representative peptides with different signal intensity. For all 35 unique drug conjugated peptides, 22 had a CV between 5 % and 10 % (average 7.79 %), nine had a CV% between 10 % and 20 % (average 12.29 %), and four had a CV% higher than 20 %, which correlated with low signal intensity (Figure 5C).

Conclusion

An integrated peptide mapping workflow was developed for in-depth characterization of antibody drug conjugates. Robust and reproducible automated in-solution trypsin digestion was demonstrated using the Agilent AssayMAP Bravo system for T-DM1. Excellent mass accuracy and sensitivity were achieved using an Agilent 6550 iFunnel Q-TOF LC/MS system to ensure comprehensive sequence coverage and accurate peptide identification. Automated data extraction, sequence matching, and drug conjugation site identification were provided using Agilent MassHunter BioConfirm Software.

For T-DM1, 98.7 % sequence coverage was achieved. The peptides identified covered all 44 lysine sites. There were 29 out of 44 sites confirmed to be drug conjugation sites. Additional peptides were identified covering another five sites, but the exact site modified cannot be unambiguously determined. Ten lysine sites have no evidence of drug conjugation.

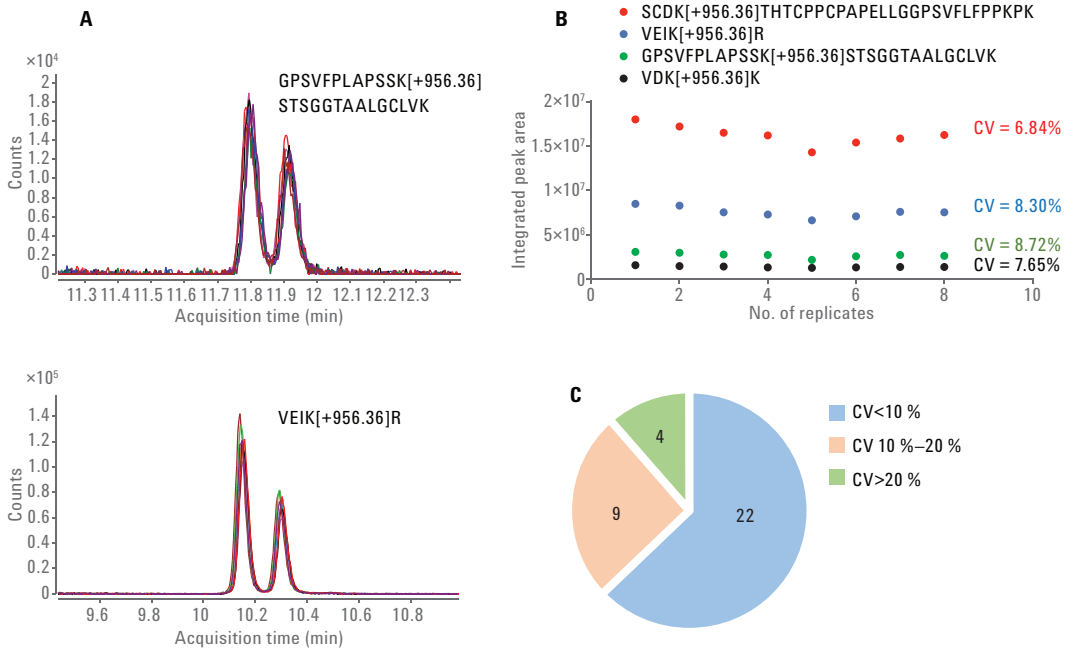

Figure 5. Reproducibility of the peptide mapping workflow. A) Overlay of eight precursor EICs for two identified drug conjugated peptides. B) Scatter plot of integrated EIC peak area for four representative drug conjugated peptides. C) Pie chart of the number of peptides with %CV less than 10 %, between 10 % and 20 %, and over 20 %.

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