

## Quantification of the Antibody Drug Conjugate, Trastuzumab Emtansine, and the Monoclonal Antibody, Trastuzumab, in Plasma Using a Generic Kit-Based Approach

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### Abstract

This application note describes the total mAb quantification of the antibody-drug conjugate, ado-trastuzumab emtansine, and the mAb, trastuzumab from rat plasma using the ProteinWorks eXpress Direct Digest Kit and Protocol.

The ProteinWorks eXpress Direct Digest Kit was successfully used for sample preparation prior to LC-MS quantification of trastuzumab and T-DM1 from a typical set of standard curve and QC samples in plasma. The universal, kit-based approach to sample prep allows novice users to achieve high LC-MS sensitivity with a simple step-wise protocol and standardized, premeasured reagents, ensuring both the analytical sensitivity and reproducibility required in discovery studies to make time sensitive and critical project decisions.

### Benefits

Simple, standardized approach for accurate and reproducible quantification of ADC and mAb therapeutics;

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broadly applicable optimized digest kit eliminates method development.

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## Introduction

Monoclonal antibodies (mAbs), as well as antibody-drug conjugates (ADCs) represent a growing class of therapeutics due to their target specificity, lower toxicity and higher potency. With the increasing interest in mAb and ADC therapeutics, the desire for LC-MS bioanalytical quantification in support of drug development is also increasing. Historically, mAbs and ADCs have been quantified using ligand binding assays (LBAs), such as the gold-standard ELISA. While these immuno-based methods are sensitive and simple to execute, poor reagent reproducibility, lack of standardization, cross-reactivity, limited linear dynamic range, and other short-comings have led the drive to convert to LC-MS. In contrast, MS based methodologies offer many advantages over traditional LBAs, such as: multiplexing, broad dynamic range, superior selectivity, and shorter method development times. However, for LC-MS protein quantification challenges still exist. There is no single standardized workflow and the various workflow options can be complex and laborious, making it difficult for the novice bioanalytical scientist to achieve success. Additionally, due to their complex and heterogeneous nature, ADCs often require multiple bioanalytical assays to determine efficacy, toxicity, and PK/PD response during drug development stages. The bottom up approach, using enzymatic digestion of the ADC/mAb, followed by LC-MS/MS analysis is becoming routine for ADC and mAb quantification. Of the many experiments required to characterize and quantify ADC's, total antibody measurements are important. This application note describes the total mAb quantification of the ADC, ado-trastuzumab emtansine, and the mAb, trastuzumab, from rat plasma using the ProteinWorks eXpress Direct Digest Kit and Protocol.

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## Experimental

### Sample description

To prepare standards and quality control samples (QC), trastuzumab or T-DM1 was spiked into rat plasma at various concentrations (0.1–500 µg/mL). An intact murine monoclonal antibody standard (p/n 186006552) was used as a generic internal standard. Plasma samples (35 µL) were then prepared for LC-MS analysis using the

ProteinWorks eXpress Direct Digest Kit and a 5-step digestion protocol which included reduction and alkylation.

## Method conditions

LC system:	ACQUITY UPLC
Detection:	Waters Xevo TQ-S Mass Spectrometer, ESI+
Column:	ACQUITY UPLC Peptide BEH C <sub>18</sub> , 300A 1.7 µm, 2.1 x 150 mm Column
Temp.:	55 °C
Sample temp.:	10 °C
Injection vol.:	10 µL
Mobile phases:	A: 0.1% formic acid in water B: 0.1% formic acid in acetonitrile
Data management:	MassLynx (v4.1)

## Gradient:

Flow rate (mL/min)	Time (min)	%A	%B	Curve
0.3	0.0	100	0	6
0.3	1.0	100	0	6
0.3	16.0	50	50	6

Flow rate (mL/min)	Time (min)	%A	%B	Curve
0.3	16.5	10	90	6
0.3	17.5	10	90	6
0.3	18.0	100	0	6
0.3	20.0	100	0	6

## MS conditions

Capillary (kV):	3
Cone (V):	30
Source offset (V):	50
Source temp. (°C):	150
Desolvation temp. (°C):	600
Cone gas flow: (L/Hr):	150
Desolvation gas flow: (L/Hr):	1000
Collision gas flow (mL/Min):	0.15
Nebuliser gas flow (Bar):	7

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## Results and Discussion

Trastuzumab is a humanized anti-HER2 monoclonal antibody that was approved by the FDA in 1998. With EU patent expiry in July 2014, and impending US patent expiry in 2019, the focus on this drug, as well as next and new generation drugs, such as ADCs, has steadily increased. Ado-trastuzumab emtansine (T-DM1) is an FDA approved ADC, marketed under the brand name Kadcyla, and used as treatment for patients with advanced breast cancer<sup>1-3</sup> ADCs, like T-DM1, are composed of cytotoxic small molecule drug (payload) covalently bound to an antibody by a linker. Due to their complex and heterogeneous nature, ADCs require multiple bioanalytical assays to quantify both conjugated and unconjugated forms of the ADC, total mAb, cytotoxic payload, and various other catabolites/metabolites. LC-MS quantification of ADCs and mAbs typically employs enzymatic digestion (most commonly trypsin), followed by quantification of one or multiple representative tryptic peptides using multiple reaction monitoring (MRM).

Using the ProteinWorks eXpress Direct Digest Kit and protocol, a direct digest of plasma (35  $\mu$ L) containing either T-DM1 or trastuzumab was performed. LC-MS/MS quantification of signature peptides was performed using a Xevo TQ-S triple quadrupole MS. Chromatographic separation was achieved using an ACQUITY UPLC System with an ACQUITY UPLC Peptide BEH C<sub>18</sub>, 300A, 1.7  $\mu$ m, 2.1 x 150 mm Column. Three signature tryptic peptides were used for quantification: IYPTNGYTR, FTISADTSK, and GPSVFPLAPSSK. MS conditions are summarized in Table 1.

Protein	Peptide	MRM transition	Cone voltage (V)	Collision Energy (eV)
T-DM1/Trastuzumab	IYPTNGYTR	542.77>249.16	36	16
		542.77>808.40	12	16
Trastuzumab	FTISADTSK	485.20>721.40	28	22
		485.20>608.30	28	22
Trastuzumab	DTYIHWVR	543.30>597.30	28	24
		545.30>710.40	28	28
Trastuzumab	GPSVFPLAPSSK*	593.83>699.40	31	21
T-DM1 miscleavage with small molecule drug attached	FTISADTSKNTAYLQMNSLR	1073.17>547.20	35	38
		1073.17>485.22	35	38
	GPSVFPLAPSSKSTSGGTAALGCLVK*	1149.23>547.20	35	38
Murine mAb (IS)	SVSELPIMHQDWLNGK*	618.64>834.41	16	12
	VNSAAFPAPIEK	622.30>654.44	28	16

Table 1. MRM conditions for trastuzumab and trastuzumab emtansine (T-DM1).

\*Generic IgG peptide

From an analytical perspective, tryptic digestion and choice of signature peptide poses a challenge for quantification of T-DM1, since it is a lysine-conjugated ADC. Trypsin cleaves peptides on the C-terminal side of lysine amino acid residues and if a lysine residue is occupied with the cytotoxic drug, cleavage will not occur ("miscleavage"). Thus, if one were to choose a lysine containing peptide to quantify T-DM1, there is potential for miscleavage on the lysine residue when it is conjugated with the small molecule drug. Because the signature peptide IYPTNGYTR lacks a lysine residue, one can confidently and accurately use it to quantify both T-DM1 and trastuzumab. For this same reason one would need to be cautious of using the two lysine containing peptides, FTISADTSK and GPSVFPLAPSSK for accurate quantification of T-DM1. Both of these peptides have some degree of small molecule drug occupancy and thus, due to potential miscleavage of the lysine residue may result in lower calculated concentrations than a non-lysine containing peptide.

For this application, sensitivity, linearity, accuracy and precision data met typical method validation requirements.

<sup>4</sup> Standard curves were linear over 3.5 orders of magnitude with the average accuracies of 100% for the standard curve points. For the IYPTNGYTR, FTISADTSK, and GPSVFPLAPSSK tryptic peptides, quantification limits between 0.5–1.0 µg/mL were achieved. Summary statistics from standard curves for trastuzumab are shown in Table 2. In addition, the accuracy and precision for trastuzumab and T-DM1 QC samples, quantified using the trastuzumab standard curve, were excellent with % CVs <8. This is summarized in Table 3.

Peptide	Std. curve range ( $\mu\text{g/mL}$ )	Weighting	Linear fit ( $r^2$ )	Mean % accuracy
IYPTNGYTR	0.5–500	$1/x^2$	0.995	100.01
FTISADTSK	1.0–500	$1/x$	0.999	100.01
GPSVFPLAPSSK*	0.5–500	$1/x^2$	0.990	100.00

Table 2. Linear dynamic range, weighting and average accuracy for standard curves for Trastuzumab plasma digested and extracted using the ProteinWorks eXpress Direct Digest Kit.

\*Generic IgG peptide

mAb/ADC	Peptide	QC conc. (µg/mL)	Mean cal. conc. (µg/mL)	Std. dev.	%CV	Mean % accuracy
Trastuzumab	IYPTNGYTR	0.65	0.64	0.03	4.58	99.77
		3.5	3.25	0.19	5.96	92.90
		6.5	6.83	0.16	2.29	105.13
		35	36.41	0.42	1.16	104.03
		65	63.31	2.18	3.44	97.40
		350	345.64	18.66	5.40	98.73
T-DM1	IYPTNGYTR	0.65	0.65	0.05	6.94	100.50
		3.5	3.36	0.24	7.10	95.87
		6.5	7.1	0.05	0.66	109.20
		35	34.51	1.09	3.17	98.57
		65	59.74	3.72	6.22	91.90
		350	324.72	17.06	5.25	92.80
mAb/ADC	Peptide	QC conc. (µg/mL)	Mean cal. conc. (µg/mL)	Std. dev.	%CV	Mean % accuracy
Trastuzumab	FTISADTSK	3.5	3.47	0.17	4.89	99.20
		6.5	6.70	0.11	1.69	103.07
		35	38.30	0.28	0.73	109.47
		65	64.12	1.68	2.63	98.67
		350	357.47	9.65	2.70	102.13
T-DM1	FTISADTSK	3.5	3.03	0.06	1.87	86.60
		6.5	6.91	0.30	4.37	106.30
		35	33.35	0.65	1.94	95.27
		65	58.70	2.93	4.99	90.30
		350	322.02	7.51	2.33	92.00
mAb/ADC	Peptide	QC conc. (µg/mL)	Mean cal. conc. (µg/mL)	Std. dev.	%CV	Mean % accuracy
Trastuzumab	GPSVFPLAPSSK	0.65	0.66	0.05	7.97	100.87
		3.5	3.04	0.07	2.29	86.90
		6.5	6.27	0.09	1.41	96.50
		35	35.5	1.62	4.55	101.43
		65	71.38	3.04	4.26	109.83
		350	379.79	21.64	5.70	108.50
T-DM1	GPSVFPLAPSSK	0.65	0.67	0.02	2.84	103.30
		3.5	3.1	0.06	1.80	88.47
		6.5	6.19	0.31	4.98	95.15
		35	33.55	1.44	4.29	95.87
		65	63.08	4.04	6.40	97.03
		350	336.36	15.35	4.56	96.10

Table 3. Statistics for Trastuzumab and T-DM1 QC samples from the peptides, IYPTNGYTR,



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*FTISADTSK, and GPSVFPLAPSSK, used for quantification.*

QC chromatographic performance and demonstration of sensitive quantification for all three signature peptides is highlighted in Figures 1–3, Panels A (trastuzumab) and B (T-DM1), respectively.

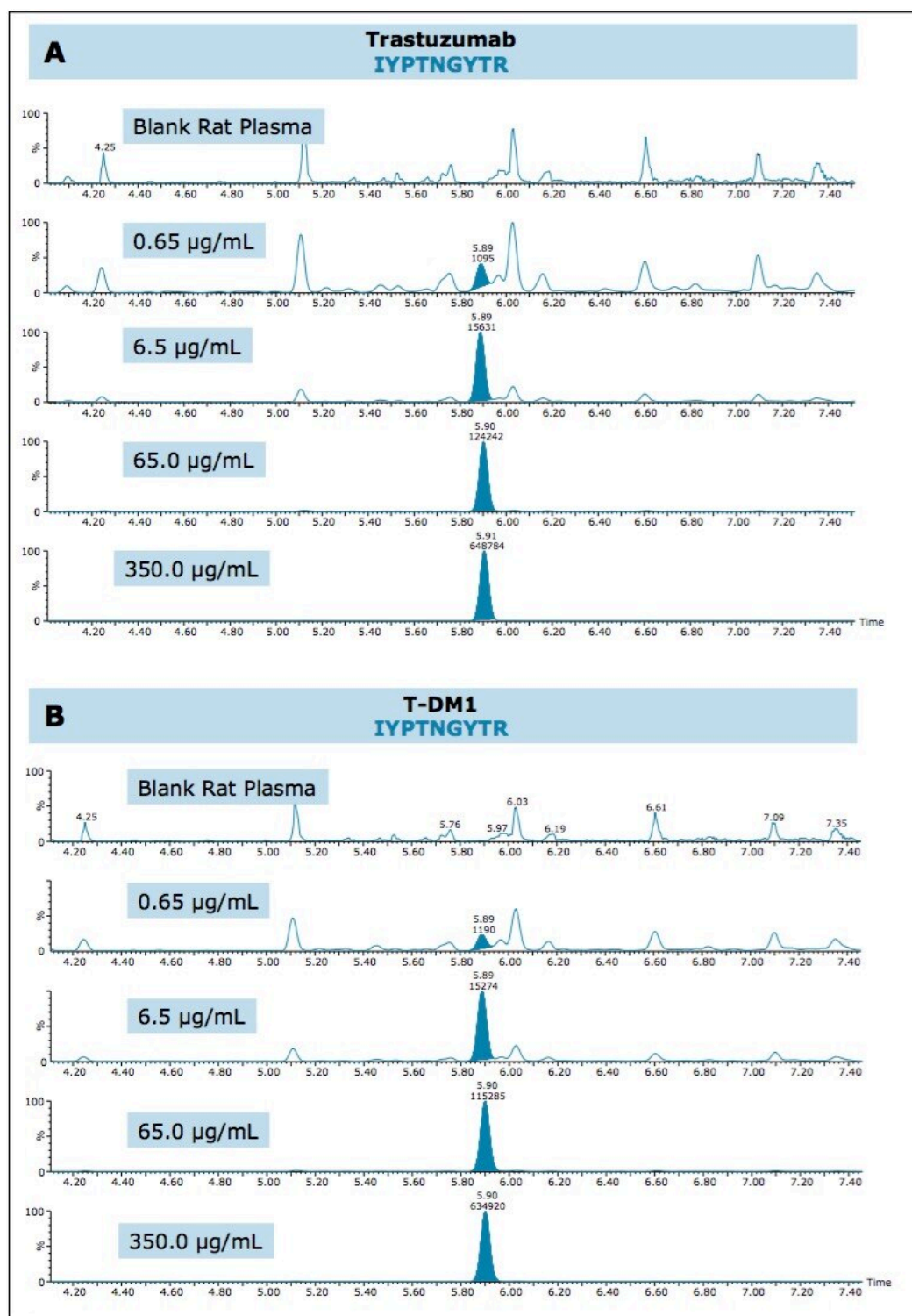


Figure 1. QC chromatograms of Trastuzumab (A) and T-DM1 (B) for the IYPTNGYTR unique signature

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*peptide.*

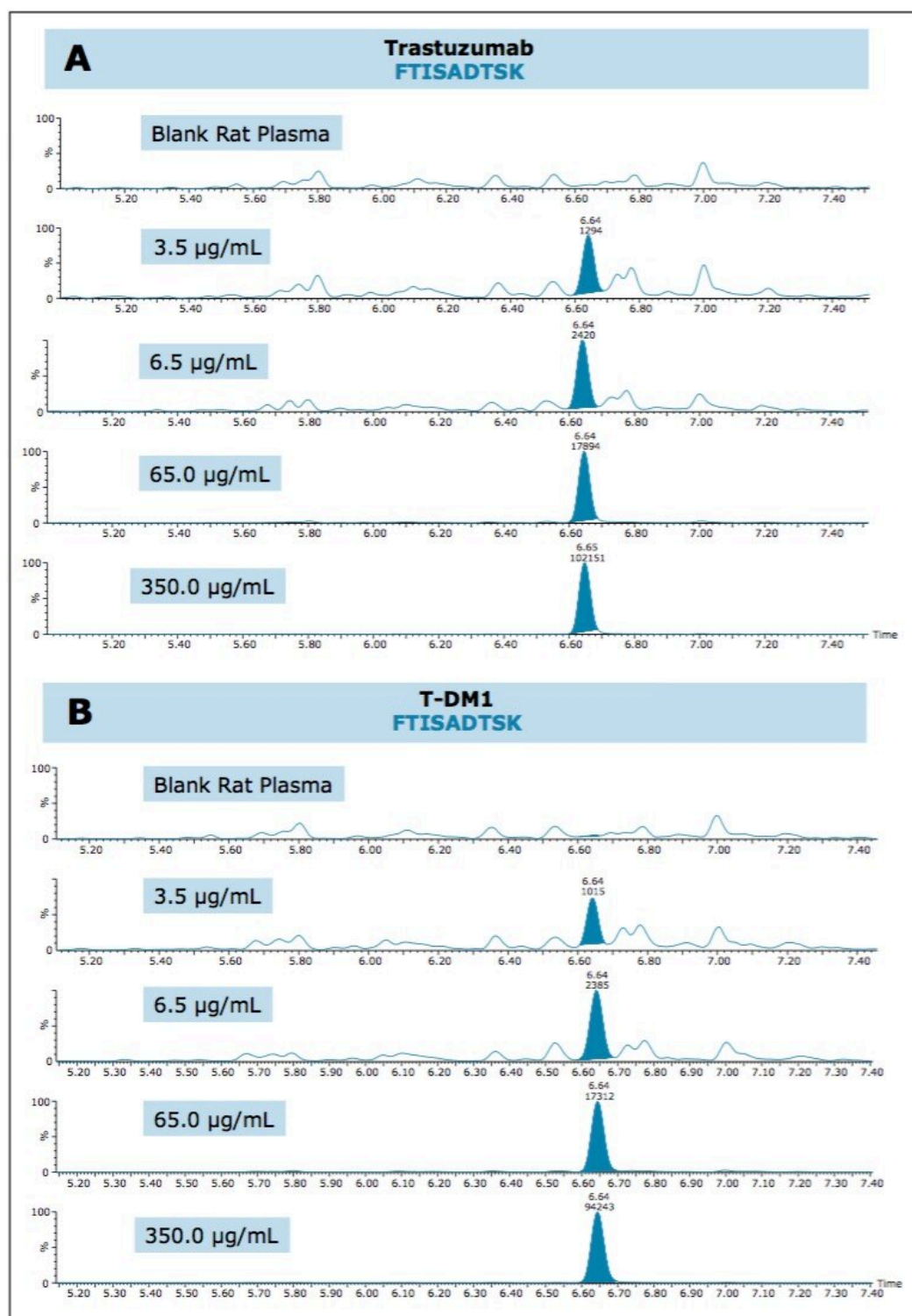


Figure 2. QC chromatograms of Trastuzumab (A) and T-DM1 (B) for the FTISADTSK unique signature

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*peptide.*

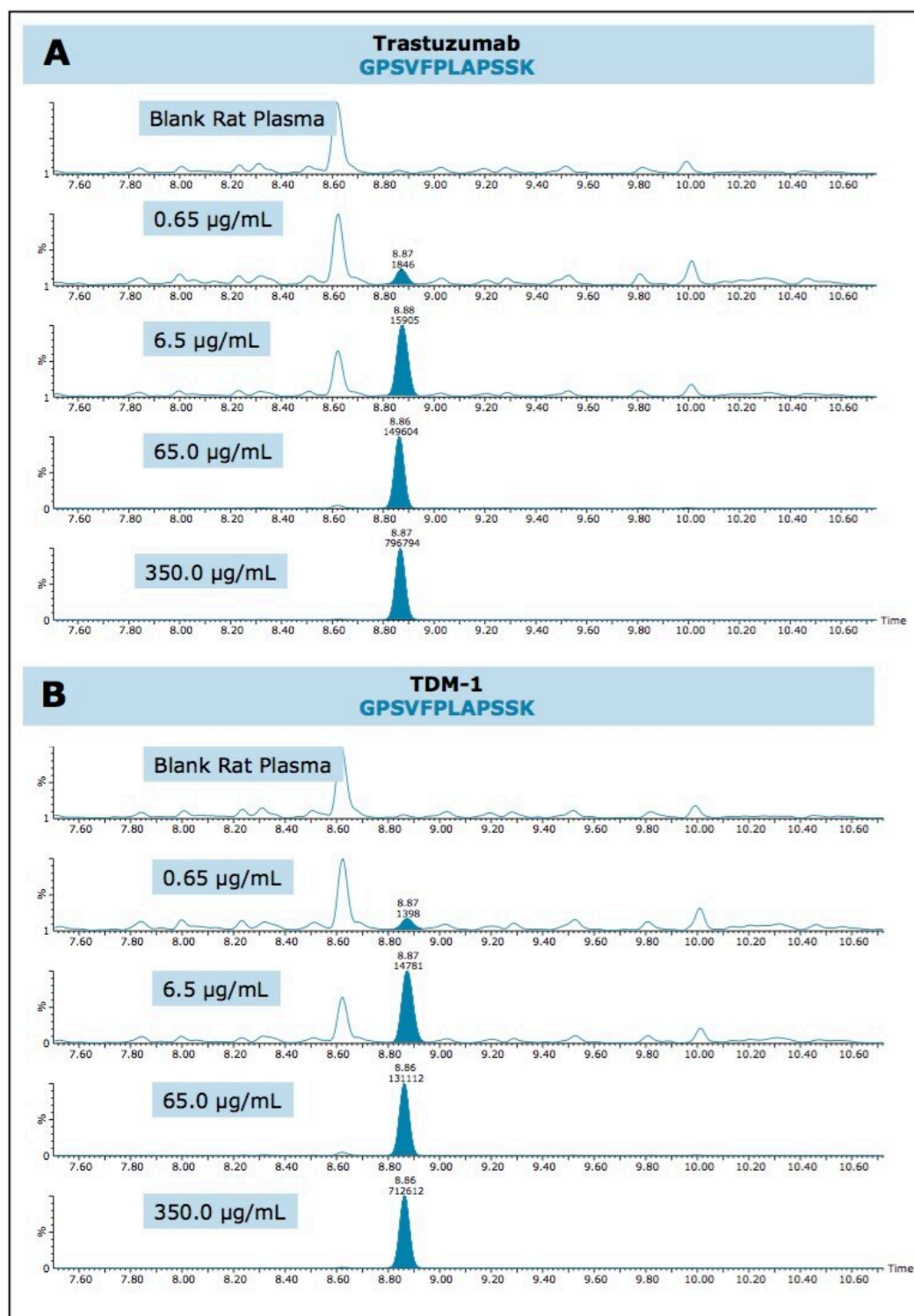


Figure 3. QC chromatograms of Trastuzumab (A) and T-DM1 (B) for the GPSVFPLAPSSK generic IgG

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*signature peptide.*

Due to the hydrophobic nature of the cytotoxic drug molecule attached to the antibody and differences in stereo chemical configurations, conjugated TDM-1 peptides generally will elute later in a chromatographic run as diastereomeric pairs. Additionally, TDM-1 peptides, by collision induced disassociation (CID), produce a common fragment (547.2  $m/z$ ). This fragment corresponds to part of the drug molecule broken down by the CID process.

In this application, we were successfully able to detect two conjugated, “miscleavage” peptides of TDM-1 (FTISADTSKNTAYLQMNSLR and GPSVFPLAPSSKSTSGGTAALGCLVK). These conjugate peptides contained a common fragment 547.2  $m/z$  from the conjugated payload and eluted later in the chromatographic run, as pairs (isomers from the conjugation). Figure 4, panels A and B illustrate the presence of these conjugated peptides in TDM-1 plasma samples (350  $\mu\text{g/mL}$ ), as compared to Trastuzumab (350  $\mu\text{g/mL}$ ), and blank rat plasma. Presence of the FTISADTSKNTAYLQMNSLR conjugated peptide was confirmed by multiple MRM transitions, and is shown in Figure 5. Additionally, both of these conjugated TDM-1 peptides increased with increasing concentration of TDM1. This is highlighted in Figure 6, panels A and B.

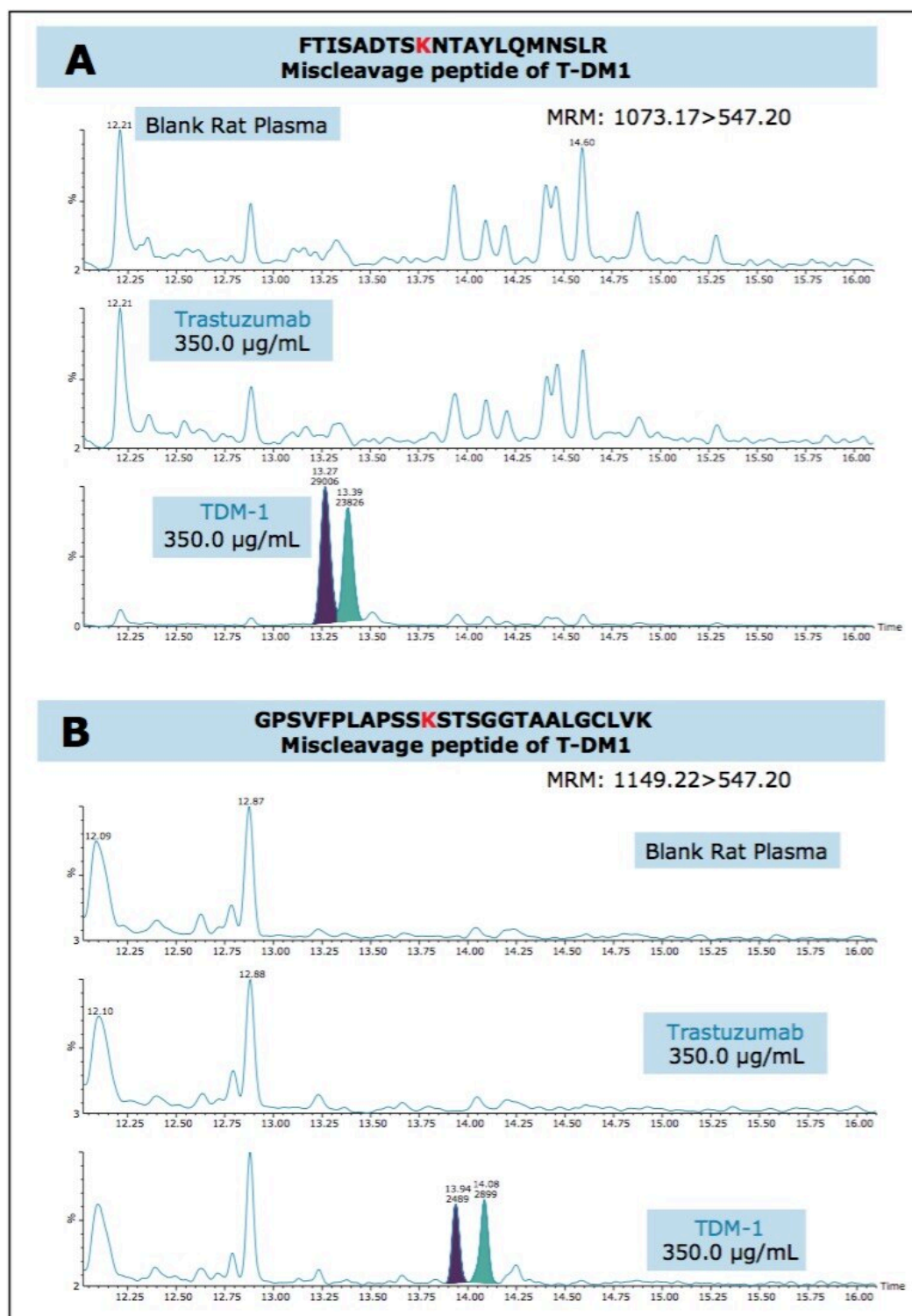


Figure 4. Chromatograms demonstrating the presence of the miscleavage peptides in T-DM1 (350 µ



g/mL), as compared to Trastuzumab (350 µg/mL), and blank rat plasma when digested and extracted using the ProteinWorks eXpress Direct Digest kit; Panel A: FTISADTSKNTAYLQMNSLR and Panel B: GPSVFPLAPSSKSTSGGTAALGCLVK.

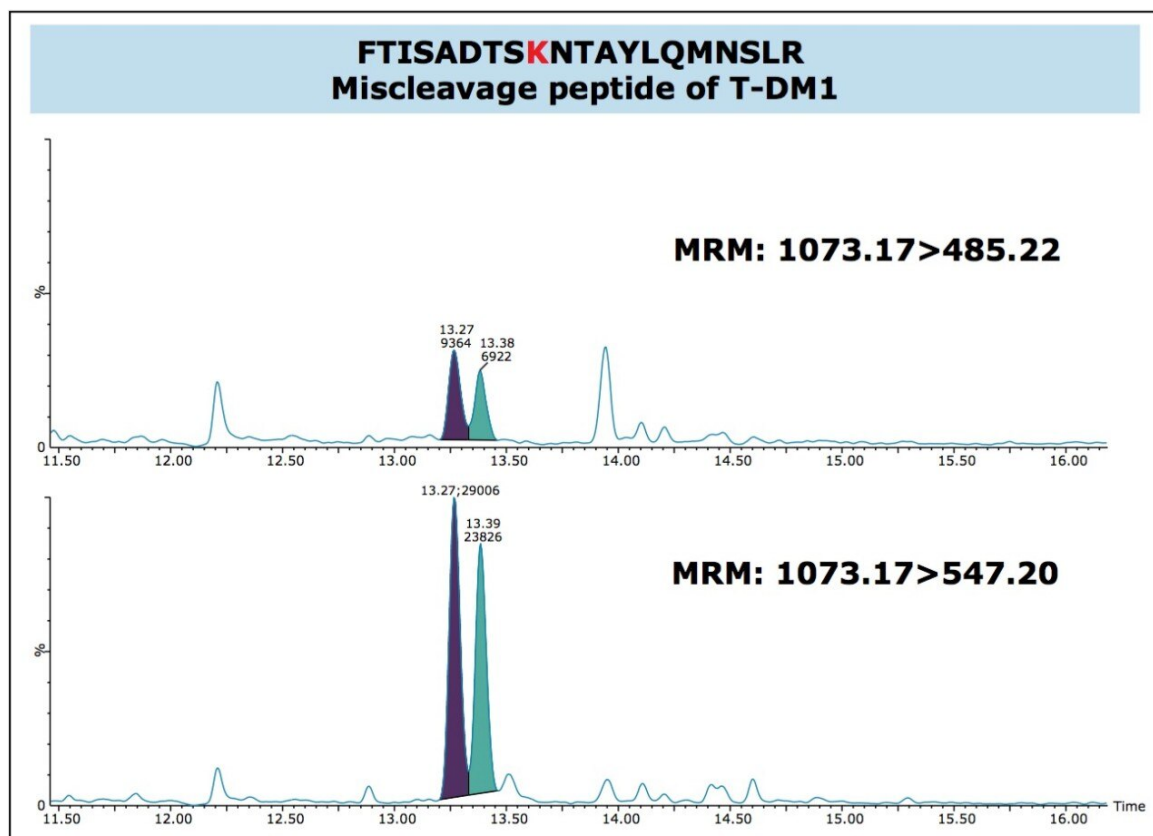


Figure 5. Chromatograms demonstrating the presence of the FTISADTSKNTAYLQMNSLR conjugated peptide confirmed by multiple MRM transitions (1073.17 > 547.20 m/z and 1073.17 > 485.22 m/z).

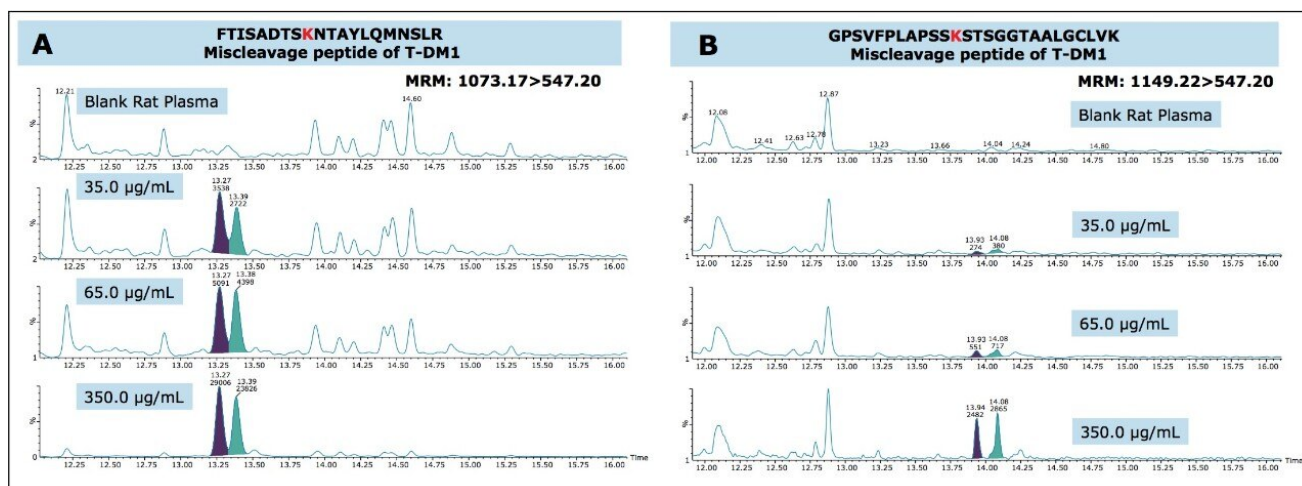


Figure 6. Chromatograms demonstrating increase of the conjugated peptides of T-DM1 with increasing T-DM1 concentration in plasma, when digested and extracted using the ProteinWorks eXpress Direct Digest Kit; Panel A: FTISADTSKNTAYLQMNSLR and Panel B: GPSVFPLAPSSKSTSGGTAALGCLVK.

## Conclusion

The ProteinWorks eXpress Direct Digest Kit was successfully used to quantify trastuzumab and the ADC, T-DM1, from a typical set of standard curve and QC samples in plasma. Through direct digestion of 35 µL of plasma, quantification limits of 0.5–1.0 µg/mL were achieved, while maintaining excellent linearity, precision and accuracy. The universal, kit-based approach allows novice users to achieve high sensitivity with a simple step-wise protocol and standardized, premeasured reagents, ensuring both the sensitivity and reproducibility required in discovery studies to make time sensitive and critical project decisions.

## References

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