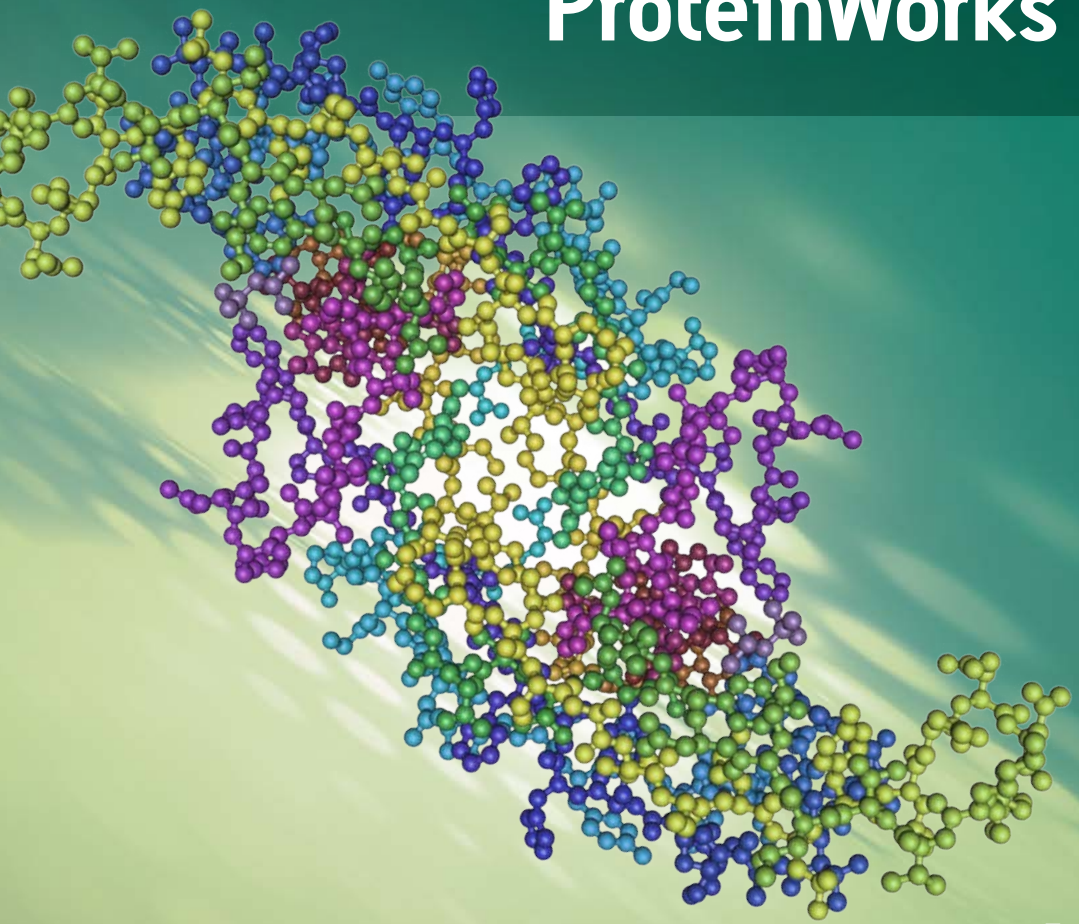
Waters Application Notes

ProteinWorks



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OUR SCIENTISTS

Erin E. Chambers

As a principal scientist, Erin has been working almost exclusively on peptide and protein bioanalysis for the last seven years, while managing small and large molecule bioanalysis and clinical research applications for Waters' consumables business unit. A conversation with her college dean helped put her on the path of scientific study. Ultimately, Erin graduated from Yale University with a degree in chemistry and earned her doctorate from Kings College London. Erin loves the dynamic nature of her job, helping to develop new methods and analytical systems that have a profound influence on illnesses like diabetes and Alzheimer's disease.

Mary E. Lame

Motivated by tough problems, Mary's work at Waters focuses on improving pharmaceutical peptide and protein bioanalysis, testing products, and developing applications. Mary's high school chemistry teacher instilled in her the confidence to pursue chemistry as a career. She went on to receive her undergraduate degree in chemistry from Western Connecticut State University and earned her masters degree at Central Connecticut State University. A risk taker, Mary loves working with the latest, cutting edge technology and developing novel methods for peptide and protein therapeutics.

Sherri Naughton

Passionate about bringing technology to market, Sherri has spent most of her career in business development, strategy and the commercialization of chemistry-focused products, both consumables and instrumentation. Following a B.S. in Chemistry from Union College, and a few years researching therapies for cardiovascular disease, Sherri obtained a masters degree from Rensselaer Polytechnic Institute. Her career at Waters has recently focused on the product management of enabling, chemistry-based solutions for the clinical and life science markets.

Paula Orens

Paula began her career at a contract research organization supporting the high-throughput bioanalysis of small molecules and peptides. Motivated to expand her bioanalytical knowledge of large molecule sample preparation and LC-MS method development, she joined Waters. As a scientist in the consumables business unit, Paula supports new product development and discovery bioanalysis of peptides and proteins. Her favorite part about her role at Waters is the opportunity to work on many different applications using various bioanalytical techniques and LC-MS platforms. Paula earned her bachelor of science degree in chemistry from University of the Sciences.

Hua Yang

Passionate about being involved in every stage of creating a new product: research, development and application, Hua has been involved in cutting-edge research in making advanced materials for analytical separation and sample preparation. In recent years, Hua joined the Waters Technology Advancement Consumable Business Unit as an application scientist to develop applications for Protein Bioanalysis. Before joining Waters, Hua was a post-doctoral fellow in UC San Francisco. Hua also holds a Doctorate degree in Analytical Chemistry from the University of Pittsburgh.

TABLE OF CONTENTS

PROTEINWORKS KITS

Take the Complexity out of Protein Quantification

- A standardized, streamlined approach to protein bioanalysis
- Accurate and precise quantification of monoclonal antibodies and ADCs
- Simplified sample preparation with pre-measured, lot-traceable reagents
- Elimination of method development in Discovery studies

For more information go to www.waters.com/proteinworks

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High Sensitivity Quantification of Infliximab in Rat Plasma Using a Fast, Standardized Kit-Based Approach

Mary Lame, Hua Yang, Sherri Naughton, and Erin Chambers
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Simple, standardized approach to protein quantification; broadly applicable optimized digest kit eliminates method development for discovery studies; samples are ready for LC-MS analysis in 4–6 hours; high sensitivity detection limit of 10 ng/mL for infliximab was achieved.

WATERS SOLUTIONS

ProteinWorks™ eXpress Digest Kit (p/n 176003689)

Intact mAb Check Standard (p/n 186006552)

ACQUITY UPLC® Peptide BEH C_{18}, 300Å 1.7 µm, 2.1 mm x 150 mm, Column (p/n 186003687)

ACQUITY UPLC

Xevo® TQ-S Mass Spectrometer

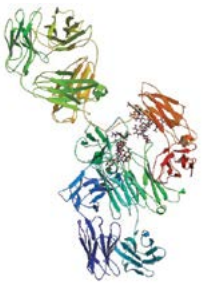
ProteinWorks µElution SPE Clean-up Kit (p/n 186008304)

KEY WORDS

monoclonal antibody, infliximab, Remicade, protein quantification, eXpress Digest

INTRODUCTION

As more drug development efforts focus on large molecules such as antibodies or ADC's, traditional "small molecule" scientists find themselves challenged not only by the complexity and time consuming nature but also the multitude of potential workflows that exist for protein quantification by LC-MS. This is also true for researchers investigating protein biomarkers where the use of ELISA's and other immuno-affinity (IA) methods are commonplace. While IA 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, especially for discovery and early development/pre-clinical studies. LC-MS workflows, however, encompass a multitude of sub-segments, each having many steps. Decisions about specific reagents, as well as the time, temperature, and concentration of the reagents or steps can all affect sensitivity, making it difficult to quickly arrive at a method which produces the desired detection limits. This application note describes the fast, sensitive quantification of infliximab (Figure 1) from rat plasma using the ProteinWorks eXpress Digest Kit and Protocol. Using a single universal sample prep method with pre-weighed, lot-traceable reagents and a set of carefully developed, yet generic set of simple step-wise instructions, an LLOQ of 10 ng/mL infliximab was achieved.



Formula: $C_{6428}H_{9912}N_{1694}O_{1987}S_{46}$
Molecular Weight: ~ 149.1 kD

http://www.drugbank.ca/drugs/DB00065

Figure 1. Infliximab (Remicade) protein structure.

EXPERIMENTAL

Sample description

Infliximab was first immuno-purified from 35 µL rat plasma using a 96-well Protein A agarose-based plate. Samples were then prepared for LC-MS analysis using the ProteinWorks eXpress Digest Kit and Protocol. Finally, signature peptides were cleaned-up using the ProteinWorks µElution SPE Clean-up Kit and Protocol.

Method conditions

LC system:	ACQUITY UPLC
Detection:	Xevo TQ-S Mass Spectrometer, ESI+
Column:	ACQUITY UPLC Peptide BEH C_{18}, 300Å, 1.7 µm, 2.1 mm x 150 mm
Column temp.:	55 °C
Sample temp.:	10 °C
Injection volume:	10 µL
Mobile phase A:	0.1% formic acid in water
Mobile phase B:	0.1% formic acid in acetonitrile

Gradient:

Flow rate (mL/min)	Time (min)	Profile %A	Profile % B	Curve
0.3	0.0	100	0	6
0.3	1.0	100	0	6
0.3	7.0	50	50	6
0.3	8.0	10	90	6

Data management: MassLynx® (v4.1)

MS conditions

Capillary:	3 kv
Cone:	30 V
Source offset:	50 V
Source temp.:	150 °C
Desolvation temp.:	600 °C
Cone gas flow:	150 L/hr
Collision gas flow:	0.15 mL/min
Nebulizer gas flow:	7 Bar

Peptide	MRM Transition	Cone Voltage (V)	Collision Energy (eV)
DILLTQSPAILSVSPGER*	633.10>731.80	31	21
SINSATHYAESVK*	469.6>603.80	40	10
DSTYSLSSTLTLSK	751.88>836.47	31	24
SVSELPIMHQDWLNGK (ISTD)	618.64>834.41	16	12

*Unique Signature Peptide

Table 1. MRM conditions for infliximab peptides and internal standard peptide.

RESULTS AND DISCUSSION

With the infliximab US patent expiration date of 2017 drawing ever closer,[1] the focus on this important drug in CRO's as well as biosimilar research labs has increased. However, typical workflows are incredibly complex, with a multitude of choices and options. This makes the development of high sensitivity methods challenging. In this application note, we have used the ProteinWorks eXpress Digest Kit to simplify and streamline the process. Infliximab samples were affinity purified, digested, and peptides extracted using SPE in under 6 hours total. This enabled data to begin to be acquired the same day, with several 96-well plates being run by the following morning. Multiple unique signature peptides as well as a generic human peptide were simultaneously monitored for use in quantification. The best sensitivity was achieved using the unique peptide SINSATHYAESVK from the heavy chain, while additional unique (DILLTQSPAILSVSPGER, light chain) and generic (DSTYSLSSTLTLSK, light chain) infliximab peptides were monitored for confirmation. A unique peptide (SVSELPIMHQDWLNGK) from a common murine mAb standard (p/n 186006552) was used as the internal standard.

Using the optimized protocol and reagents provided in the kit, only 35 µL of plasma was needed to achieve a detection limit of 10 ng/mL for infliximab (Figure 2). Linearity and accuracy of the standard curves arising from each peptide are summarized in Table 2. The primary, and most sensitive quantitative peptide, SINSATHYAESVK, was linear over 4 orders of magnitude with a mean accuracy of >98% for all points on the curve. The additional two peptides were linear over 3.5 orders of magnitude with average accuracies >99% for all curve points.

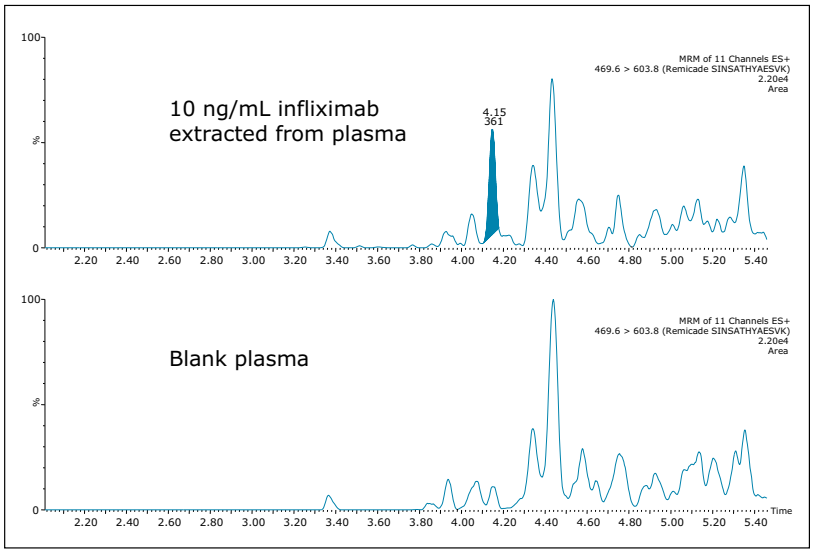


Figure 2. Chromatogram showing 10 ng/mL of infliximab in rat plasma, as compared to blank rat plasma. Infliximab is quantified using the unique peptide SINSATHYAESVK.

Peptide	Std. curve range (µg/mL)	Weighting	Linear fit (r^2)	Mean % accuracy of all points
DILLTQSPAILSVSPGER*	0.05–250	1/X	0.998	100.00
SINSATHYAESVK*	0.01–100	$1/X^2$	0.995	98.47
DSTYSLSSTLTLSK	0.10–500	$1/X^2$	0.997	99.34

*Unique signature peptide.

Table 2. Linear dynamic range and standard curve statistics for signature peptides used to quantify infliximab in rat plasma.

In addition, the accuracy and precision for the QC samples was excellent with %CVs all <6%. This is summarized in Table 3. In fact, the average %CV for QC samples from the SINSATHYAESVK peptide was <3%.

Peptide	QC conc. (µg/mL)	Mean cal. conc. (µg/mL)	Std. dev.	%CV	Mean accuracy
SINSATHYAESVK*	0.035	0.036	0.001	2.78	103.1
	0.350	0.331	0.003	0.80	94.5
	3.500	3.330	0.105	3.15	95.1
	35.000	38.287	1.168	3.05	109.4
	350.000	–	–	–	–
Peptide	**QC conc. (µg/mL)**	**Mean cal. conc. (µg/mL)**	**Std. dev.**	**%CV**	**Mean accuracy**
DILLTQSPAILSVSPGER*	0.035	–	–	–	–
	0.350	0.359	0.015	4.10	102.6
	3.500	3.210	0.026	0.81	91.7
	35.000	37.054	0.581	1.57	105.9
	350.000	327.304	13.672	4.18	93.5
Peptide	**QC conc. (µg/mL)**	**Mean cal. conc. (µg/mL)**	**Std. dev.**	**%CV**	**Mean accuracy**
DSTYSLSSTLTLSK	0.035	–	–	–	–
	0.350	0.333	0.010	2.85	95.3
	3.500	3.271	0.186	5.70	93.5
	35.000	36.256	1.999	5.51	103.6
	350.000	369.975	7.432	2.01	105.7

*Unique signature peptide.

Table 3. Statistics for QC samples from all infliximab peptides used for quantification.

From an assessment of the chromatographic data, it is clear that the quality of the data in terms of peak width and separation from residual endogenous components facilitated both the low level detection and the very high accuracy and precision that were achieved. This can be observed and is highlighted in the QC chromatograms from all signature peptides in Figures 3–5.

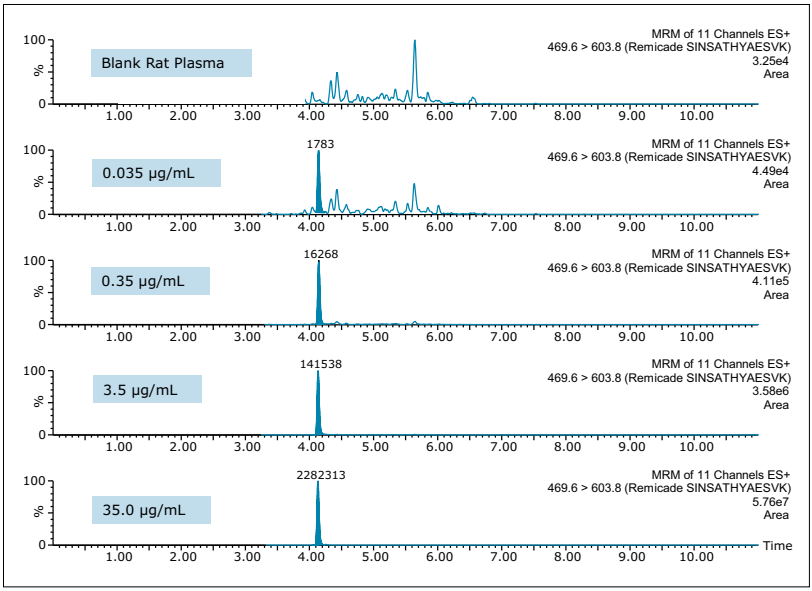


Figure 3. Infliximab QC chromatograms for the SINSATHYAESVK Unique Signature Peptide.

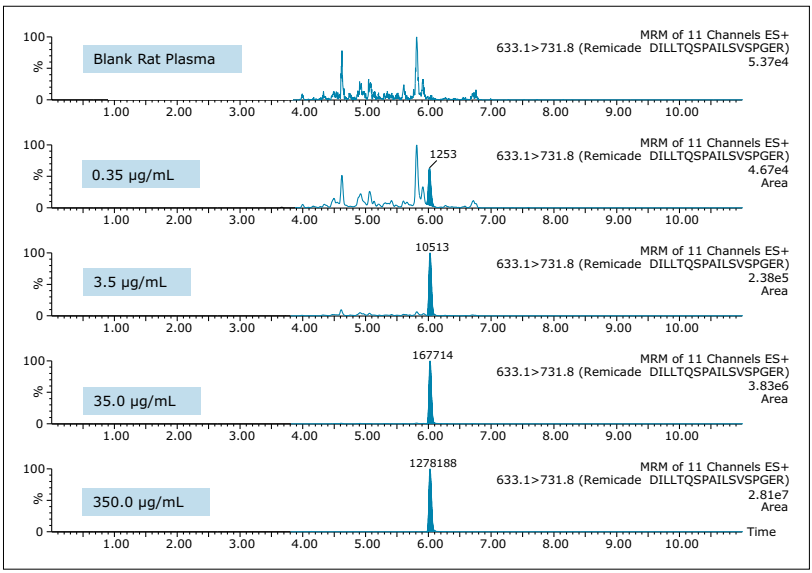


Figure 4. Infliximab QC chromatograms for the DILLTQSPAILSVSPGER Unique Signature Peptide.

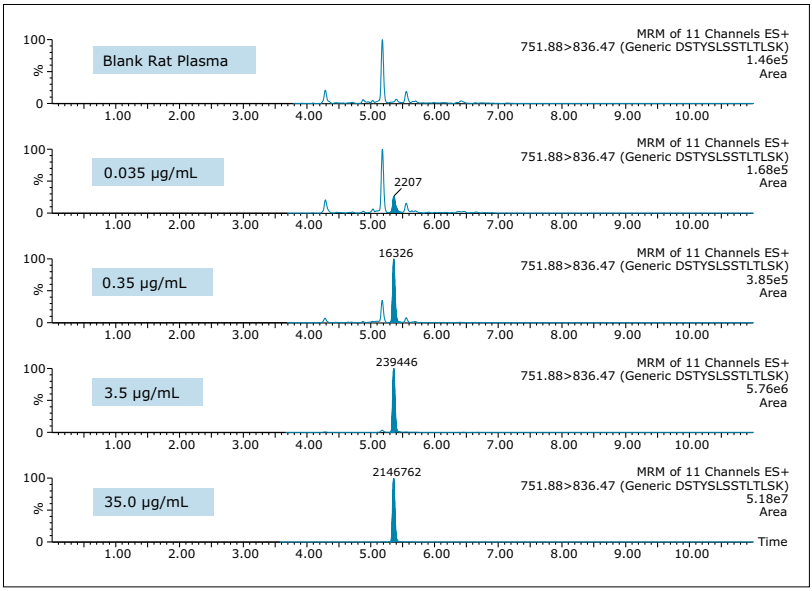


Figure 5. Infliximab QC Chromatograms for the DSTYSLSSTLTLSK Generic Signature Peptide.

CONCLUSIONS

The ProteinWorks eXpress Digest Kit was successfully used to purify infliximab from a typical set of standard curve and QC samples in rat plasma. A limit of quantification of 10 ng/mL was readily achieved, while maintaining excellent linearity and single digit precision. The total sample prep time including an affinity purification step was under 6 hours. The total digest prep time was just over 2 hours. The universal, kit-based approach allows novice users to achieve ultra-low detection limits with a simple step-wise protocol and a set of standardized, pre-measured reagents, ensuring both the sensitivity required and the transferability desired of such methods.

References

1. McKinsey and Company; Data Source: Evaluate Pharma, US Patent Expiration Dates.

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A Generic Kit-Based Approach for Quantifying Monoclonal Antibody Drugs Through Direct Digestion of Discovery Study Samples

Mary Lame, Hua Yang, Sherri Naughton, and Erin Chambers
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

Simple, standardized approach to protein quantification; broadly applicable optimized digest kit eliminates method development; samples are ready for LC-MS analysis in 4–6 hours

WATERS SOLUTIONS

ProteinWorks eXpress Direct Digest Kit (p/n 186003688)

Intact mAb Check Standard (p/n 186006552)

ACQUITY UPLC® Peptide BEH C_{18}, 300Å 1.7 µm, 2.1 mm x 150 mm Column (p/n 186003687)

ACQUITY UPLC

Xevo® TQ-S Mass Spectrometer

ProteinWorks µelution SPE Clean-up Kit (p/n 186008304)

KEY WORDS

Monoclonal antibody, infliximab, Remicade, protein quantification, eXpress Direct Digest, ProteinWorks, adalimumab, Humira, bevacizumab, Avastin, trastuzumab, Herceptin

INTRODUCTION

Over the past 5–10 years, there has been a significant shift towards a greater % of biologics in pharmaceutical pipelines.[1] However, the industry finds itself in the middle of patent expiry for many of the critical monoclonal antibody and other protein-based drugs, with patent expiration dates ranging from ~2012–2020.[2] This has resulted in a focus on protein quantification in bioanalytical labs, innovator pharma and CRO's as well as biomarker research labs. While immunoassay (IA) 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. These LC-MS workflows however, encompass a multitude of sub-segments, each having many steps. Those that are common to most workflows may include affinity purification, denaturation, reduction, alkylation, digestion, and SPE clean-up (each requiring optimization). Such traditional protein quantification protocols often require as much as a day and half for completion. Furthermore, the margin and possibility of error is significant within each individual step. There is a strong need for simpler, more standardized workflows which enable scientists to complete sample preparation and start an analytical run by mid-day. At the same time, ideally using generic, kitted methods, assay sensitivity must be high enough to accurately and precisely quantify low enough levels of the target protein to make critical decisions in discovery. The typical workflow complexity as shown in Figure 1, often leads to errors and poor reproducibility or sensitivity. In this application note, we have used the ProteinWorks™ eXpress Direct Digest Kit to simplify and streamline the workflow process using the same universal protocol and reagents for all monoclonal antibody drugs tested. Infliximab, bevacizumab, trastuzumab, and adalimumab (Figures 2–5) in plasma were directly digested, and peptides extracted using SPE in under 4 hours total time. This enabled data to begin to be acquired the same day, with several 96-well plates being run by the following morning.

EXPERIMENTAL

Sample description

Infliximab, adalimumab, bevacizumab, or trastuzumab were spiked into human plasma. 35 µL plasma samples were then prepared for LC-MS analysis using the ProteinWorks eXpress Direct Digest Kit and Protocol. After digestion, signature peptides were cleaned-up using the ProteinWorks µElution SPE Clean-up Kit and Protocol.

Method conditions

LC System:	ACQUITY UPLC
Detection:	Xevo TQ-S Mass Spectrometer, ESI+
Column:	ACQUITY UPLC Peptide BEH C_{18}, 300Å, 1.7 µm, 2.1 mm x 150 mm
Column temp.:	55 °C
Sample temp.:	10 °C
Injection volume:	10 µL
Mobile phase A:	0.1% formic acid in water
Mobile phase B:	0.1% formic acid in acetonitrile

Gradient:

Flow rate (mL/min)	Time (min)	Profile %A	Profile %B	Curve
0.3	0.0	100	0	6
0.3	1.0	100	0	6
0.3	7.0	50	50	6
0.3	8.0	10	90	6

Capillary:	3 kv
Cone:	30 V
Source offset:	50 V
Source temp.:	150 °C
Desolvation temp.:	600 °C
Cone gas flow:	150 L/hr
Desolvation gas flow:	1000 L/hr
Collision gas flow:	0.15 mL/min
Nebulizer gas flow:	7 Bar

Protein	Peptide	MRM transition	Cone voltage (V)	Collision energy (eV)
Infliximab	SINSATHYAESVK	469.60>603.80	40	10
Bevacizumab	FTFSLDTSK	523.30>797.48	16	14
Adalimumab	APYTFGQGTK	535.30>901.44	40	24
Trastuzumab	FTISADTSK	485.20>721.40	28	20
murine mAb	MNSLQTDDTAK (ISTD)	612.30>978.56	20	20

Table 1. MRM conditions for infliximab, adalimumab, trastuzumab, bevacizumab, and the murine mAb internal standard.

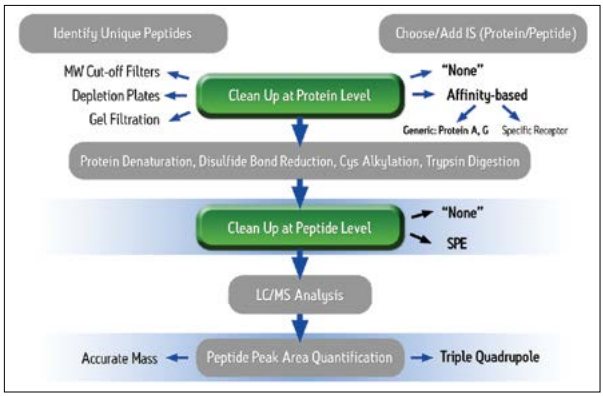


Figure 1. Typical protein bioanalysis workflow.

RESULTS AND DISCUSSION

In a pre-clinical setting, there is an emphasis on simple, broadly applicable, generic protocols as method development time and expertise are at a premium. Multiple signature peptides were used to quantify 4 different monoclonal antibody drugs in human plasma using direct digestion and the ProteinWorks eXpress Direct Digest Kit. For each protein, sensitivity, linearity, accuracy and precision data all met typical method validation requirements using the same broadly applicable ProteinWorks Kit. Through a direct digest of a 35 µL plasma sample, quantification limits ranged from 250 ng/mL–2.5 µg/mL for the 4 monoclonal antibody-based drugs. Standard curves were linear over 3.5–4 orders of magnitude with the average accuracies for standard curve points typically within 95–105%. Summary statistics from standard curves for infliximab, adalimumab, trastuzumab, and bevacizumab are shown in Table 2 below.

Protein	Peptide	Std. curve range (µg/mL)	Weighting	Linear fit (r^2)	Mean % accuracy of all points
Infliximab	SINSATHYAESVK	0.25–250	1/X	0.996	101.74
Bevacizumab	FTFSLDTSK	0.50–500	1/X	0.999	100.00
Adalimumab	APYTFGQGTK	2.50–500	$1/X^2$	0.997	99.99
Trastuzumab	FTISADTSK	2.50–500	$1/X^2$	0.997	100.01

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

Remicade Light chain [2]:

DILLTQSPAILSVSPGERVSFSCRASQFVGSSIHWYQQRTNGSPRLLIKYASESMSGIPSRFSGSGSGTDFTLSINTVESEDIADYYCQQSH
SWPFTFGSGTNLEVKTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKAD
YEKHKVYACEVTHQGLSSPVTKSFNRGEC

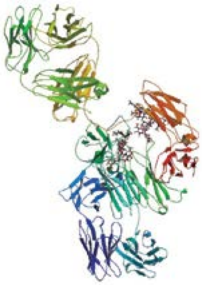
Remicade Heavy chain [2]:

EVKLEESGGGLVQPGGSMKLSCVASGFIFSNHWMNWVRQSPEKGLEWVAEIRSKSINSATHYAESVKGRFTISRDDSKSAVYLQMNSLRTED
TGVYYCSRNYYGSTYDYGQGTTLTVSXASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSL
SSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDP
EVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQV
SLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

Conserved region: blue
variable regions: red
CDR regions: green

Unique signature
Generic signature

Van Dongen et al. 61st ASMS, MP525 Minneapolis, Minnesota, USA 9-13 June 2013.



Formula: $C_{6428}H_{9912}N_{1694}O_{1987}S_{46}$
Molecular Weight: ~ 149.1 kD

http://www.drugbank.ca/drugs/DB00065

Figure 2. Structures of the monoclonal antibody-based drug infliximab (Remicade).

Conserved region Surrogate Peptides

Anti-HER2 Light chain DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPS
RFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKRTVAAPSVFIFPP
SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

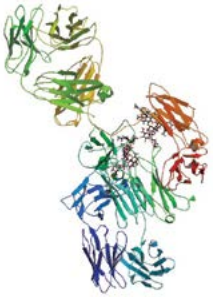
Anti-HER2 Heavy chain EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYTRY
ADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGTLVTVSS
ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSS
GLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPPKSCDKTHTCPPCPAPELLG
GPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY
NSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRD
ELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

Unique Signature
Generic Signature

Formula: $C_{6470}H_{10012}N_{1726}O_{2013}S_{42}$
Molecular Weight: ~ 145.5 kDa (claims are 148 package insert)

http://www.drugbank.ca/drugs/DB00072

Figure 3. Structures of the monoclonal antibody-based drug trastuzumab (Herceptin).



Bevacizumab light chain
DIQMTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQKPGKAPKVLIYFTSSLHSGVPSRFSGSGSGTDFTLTIS
SLQPEDFATYYCQQYSTVPWTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDN
ALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
Bevacizumab heavy chain
EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVRQAPGKGLEWVGWINTYTGEPTYAADFKRRFTFSLDTSK
STAYLQMNSLRAEDTAVYYCAKYPHYYGSSHWYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVK
DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKVEPKSCDKTH
TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTY
RVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPS
REEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHE
ALHNHYTQKSLSLSPGK

Unique Signature
Generic Signature

Drug Bank

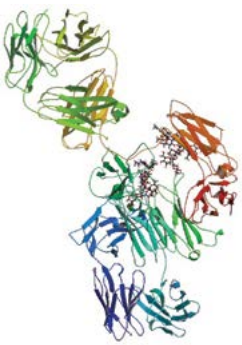
Formula:
$C_{6538}H_{10034}N_{1716}O_{2033}S_{44}$
Molecular Weight: ~ 149.0 kD

http://www.drugbank.ca/drugs/DB00065

Figure 4. Structures of the monoclonal antibody-based drug bevacizumab (Avastin).

Light chain: DIQMTQSPSSLSASVGDRVTITCRASQGIRNYLAWYQQKPGKAPKLLIYAASTLQSGVPS
RFSGSGSGTDFTLTISSLQPEDVATYYCQRYNRAPYTFGQGTKVEIKRTVAAPSVFIFPP
SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSK
ADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

Heavy chain: EVQLVESGGGLVQPGRSLRLSCAASGFTFDDYAMHWVRQAPGKGLEWVSAITWNSGHIDY
ADSVEGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCAKVSYLSTASSLDYWGQGTLVTVS
SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQS
SGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC DKTHTCPPCPAPELLG
GPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY
NSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRD
ELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSR
WQQGNVFSCSVMHEALHNHYTQKSLSLSPGK



Unique Signature
Generic Signature

$C_{6428}H_{9912}N_{1694}O_{1987}S_{46}$
Protein average weight 144190.3000

Drug Bank
http://www.drugbank.ca/drugs/DB00051

Figure 5. Structures of the monoclonal antibody-based drug adalimumab (Humira).

At the same time, QC statistics (summarized in Table 3 below) also easily met regulatory guidelines,[3] with average precision values well under 15%, and averaging in the single digits.

For a typical discovery study, detection limits ~1 µg/mL are common for monoclonal antibody type drugs. Using the ProteinWorks eXpress Direct Digest kit, these limits are easily obtained for all 4 drugs evaluated. Low QC chromatograms are shown in Figure 6 which demonstrate that adequate sensitivity is achieved with a single universal protocol and the kit.

In this study, the single universal digest protocol and SPE method designed for tryptic peptides eliminated the need for discovery-stage method development. The fact that the kit was able to accurately and precisely quantify 4 monoclonal antibody drugs in plasma without the need for optimization demonstrates its broad applicability and utility in an environment where time is critical and experience with protein bioanalysis may be limited. Furthermore, the application of a kit with lot-traceable, pre-measured reagents ensures that methods may be seamlessly transferred across sites and labs, or analysts.

Protein	Peptide	QC conc. (µg/mL)	Mean cal. conc. (µg/mL)	Std. dev.	%CV	Mean accuracy
Infliximab	SINSATHYAESVK	0.350	0.333	0.010	3.10	95.0
		3.500	3.816	0.098	2.56	109.0
		35.000	36.075	0.576	1.60	103.1
		350.000	359.301	19.892	5.54	102.6
		QC conc. (µg/mL)	**Mean cal. conc. (µg/mL)**	**Std. dev.**	**%CV**	**Mean accuracy**
Bevacizumab	FTFSLDTSK	0.350	0.356	0.004	1.08	101.7
		3.500	3.393	0.196	5.78	96.9
		35.000	38.461	1.282	3.33	109.9
		350.000	369.788	28.066	7.59	105.6
		QC conc. (µg/mL)	**Mean cal. conc. (µg/mL)**	**Std. dev.**	**%CV**	**Mean accuracy**
Adalimumab	APYTFGQGTK	0.350	–	–	–	–
		3.500	3.978	0.570	14.34	113.7
		35.000	36.567	1.023	2.80	104.5
		350.000	380.963	18.143	4.76	108.8
		QC conc. (µg/mL)	**Mean cal. conc. (µg/mL)**	**Std. dev.**	**%CV**	**Mean accuracy**
Trastuzumab	FTISADTSK	0.350	–	–	–	–
		3.500	3.663	0.067	1.82	104.7
		35.000	39.182	2.389	6.10	112.0
		350.000	374.080	14.010	3.75	106.9

Table 3. Statistics for QC samples of infliximab, adalimumab, trastuzumab, and bevacizumab in plasma digested and extracted using the ProteinWorks eXpress Direct Digest Kit.

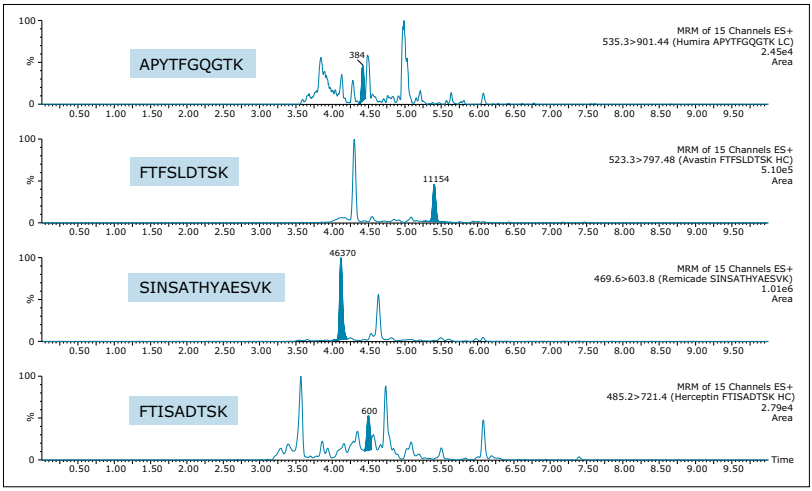


Figure 6. Low QC chromatograms (3.5 µg/mL) for adalimumab, bevacizumab, infliximab, and trastuzumab.

CONCLUSIONS

The ProteinWorks eXpress Direct Digest Kit was successfully used to purify and simultaneously quantify infliximab, adalimumab, bevacizumab, and trastuzumab from a typical set of standard curve and QC samples in human plasma. Quantification limits of 250 ng/mL to 2.5 µg/mL for each antibody were readily achieved, while maintaining excellent linearity and precision. The total sample prep time including digestion and SPE was just over 3 hours. The standardized, kit-based approach enables inexperienced users to immediately obtain meaningful data in discovery studies in order to make time sensitive and critical project decisions.

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Single Digit Reproducibility Using ProteinWorks eXpress Digest Kits

Mary Lame, Paula Orens, Sherri Naughton, and Erin Chambers
Waters Corporation, Milford, MA, USA

GOAL

To demonstrate the high reproducibility of ProteinWorks™ eXpress Digest Kits resulting from a standardized protocol and pre-weighed, lot-traceable reagents.

BACKGROUND

In any bioanalytical assay, one of the greatest sources of variability arises from the sample preparation. This is especially true for protein quantification workflows which often contain many segments-each with multiple steps capable of introducing variability. This can be of particular concern when assays are transferred from sponsor-to-CRO or from lab-to-lab within the same company, or across sites for example. Furthermore, given the multitude of possible options within a typical workflow, method development time and the expertise required are significant. The difficulty in implementing these assays is further aggravated by the complexity of the troubleshooting required when analytical goals are not met. At the same time, an assay is expected to meet acceptable accuracy and precision guidelines and provide reliable, reproducible results to make critical research and discovery-stage decisions. Thus, there is a strong need for simpler, more standardized workflows. These would ideally employ generic, kitted methods that provide a "recipe" and the reagents necessary to streamline the workflow, reduce variability and allow for implementation by less experienced scientists.

Protein quantification workflows are not only time consuming and complex, but due to their elaborate, multi-segment nature, the margin for error and the potential variability across sites, analysts, and even day-to-day can be very high. Poor reproducibility in protein quantification analytical data and a general lack of expertise strongly support the requirement for a standardized, kit-based approach.

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THE SOLUTION

ProteinWorks eXpress Digest Kits are flexible, broadly applicable, sample preparation kits containing pre-measured, lot traceable reagents optimized for the accurate, precise, reproducible and robust LC-MS quantification of proteins via the surrogate peptide approach.

Reproducibility within an assay (intra-kit) and between assays (inter-kit) was evaluated with two (2) analysts, on different days, using a total of five (5) unique lots of kits, and six (6) technical replicates per kit. Both the ProteinWorks eXpress Direct Digest Kit (p/n 176003688), for direct digestion of whole plasma, and ProteinWorks eXpress Digest Kit (p/n 176003689), for digestion of affinity-purified plasma, were employed. Using the included generic protocol, several signature peptides (a combination of both generic and unique), resulting from the digestion of the monoclonal antibody drug bevacizumab (Avastin) in plasma, were evaluated and analyzed by LC-MS. The coefficient of variation (CV), also known as relative standard deviation (RSD), was used to evaluate reproducibility, as high CV values are indicative of poor reproducibility and precision. Raw area counts for multiple tryptic peptides from the aforementioned protein were used to make the assessment.

For the two types of kits, both intra-kit and inter-kit % CV's on average were ≤10. Figures 1A and 2A summarize intra and inter-kit reproducibility data from direct digestion of whole plasma using ProteinWorks eXpress Direct Digest Kits. Figures 1B and 2B summarize intra and inter-kit reproducibility data from affinity purified plasma using ProteinWorks eXpress Digest Kits. Additionally, using two (2) analysts, on two (2) separate days, the calculated mAb concentrations of the multiple tryptic peptides, when compared, were within 10% of each other, with % CV's across the six (6) technical replicates ≤15. Data from direct plasma digests are shown in Figure 3A, while data from affinity purified plasma digests are shown in Figure 3B.

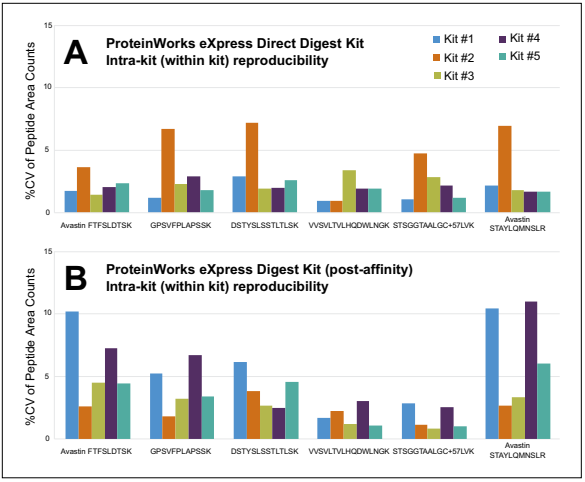

Figure 1. Intra-kit (N=5) % CV's of raw area counts for unique and generic signature peptides from bevacizumab; Panel A: ProteinWorks eXpress Direct Digest Kit (direct digestion of whole plasma) and Panel B: ProteinWorks eXpress Digest Kit (from affinity purified plasma).

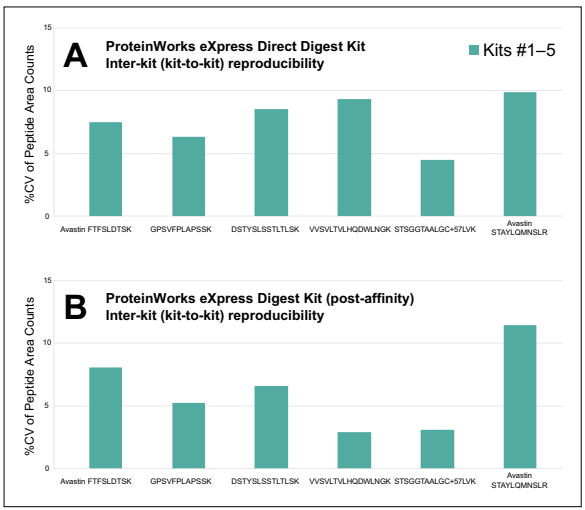

Figure 2. Inter-kit (N=5) % CV's of raw area counts for unique and generic signature peptides from bevacizumab; Panel A: ProteinWorks eXpress Direct Digest Kit (direct digestion of whole plasma) and Panel B: ProteinWorks eXpress Digest Kit (from affinity purified plasma).

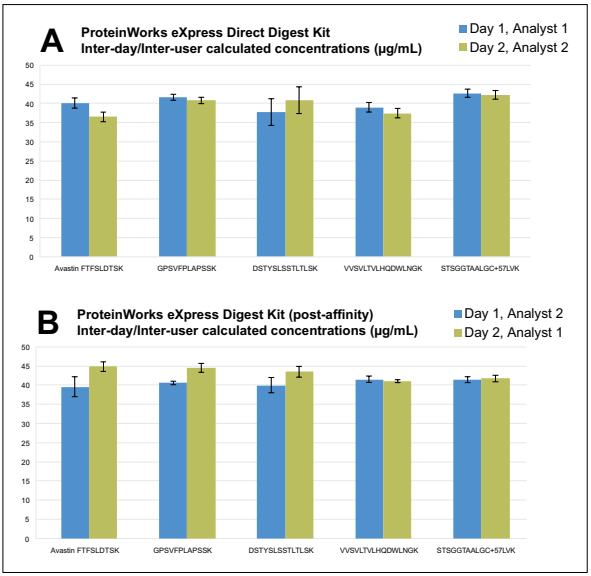

Figure 3. Inter-day/Inter-user calculated concentrations, ProteinWorks eXpress Direct Digest Kit (Panel A) and ProteinWorks eXpress Digest Kit (Panel B), for unique and generic signature peptides from a 40 µg/mL bevacizumab plasma sample.

SUMMARY

This work demonstrates that single digit reproducibility, accuracy and precision is achievable using this kit-based approach to protein quantification. These data suggest that a high degree of standardization can be achieved across analysts and sites implementing the kit strategy outlined herein.

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Simple, Standardized, and Sensitive Quantification of Bevacizumab (Avastin) Using ProteinWorks eXpress Digest Kits

Mary Lame, Hua Yang, Sherri Naughton, and Erin Chambers
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Simple, standardized approach to protein quantification; broadly applicable optimized digest kit eliminates method development for discovery studies; samples are ready for LC-MS analysis in 4–6 hours; high sensitivity detection limit of 100 ng/mL for bevacizumab was achieved

WATERS SOLUTIONS

ProteinWorks™ eXpress Digest Kit (p/n 176003689)

Intact mAb Check Standard (p/n 186006552)

ACQUITY UPLC® Peptide BEH C_{18}, 300Å, 1.7 µm, Column (p/n 186003687)

ACQUITY UPLC System

Xevo® TQ-S Mass Spectrometer

ProteinWorks µElution SPE Clean-up Kit (p/n 186008304)

KEY WORDS

monoclonal antibody, bevacizumab, Avastin, protein quantification, eXpress Digest

INTRODUCTION

During the period of 2013-2020, many of the world's top selling antibody-based drugs come off patent,[1] including bevacizumab, an almost $6 billion dollar drug, expiring in 2020. In addition, as of 2013, there were 338 new monoclonal antibody drugs in development.[2] This represents the largest class of biologics in the pipelines. As patents for bevacizumab and other important antibody therapies expire and additional antibody drugs are developed, the need for streamlined LC-MS protein bioanalysis methods and approaches continues to grow. This is particularly true as bioanalysis studies using LC-MS were historically dominated by small molecule therapies with far simpler and more straightforward sample prep and analysis methods. Bevacizumab (Avastin, Genentech/Roche) is a tumor angiogenesis inhibitor, which selectively binds to and neutralizes the biologic activity of human vascular endothelial growth factor (VEGF) and is an adjunct IV therapy for colorectal, lung, cervical and kidney cancer, amongst others.[3,4] Typical dosing is in the 5–15 mg/kg range every 2–3 weeks, depending on indication. It was reported that doses >1 mg/kg produced serum levels of bevacizumab ≥10 µg/ml for at least 14 days.[5] This information suggests that a quantification method with a detection limit of ≥100 ng/mL and an upper limit of quantification (ULOQ) of several hundred µg/mL would be more than sufficient. While this would be a trivial exercise for a small molecule bioanalyst, the lack of expertise in biological molecule handling and the techniques associated with protein quantification via the surrogate peptide approach make it challenging for those same individuals to readily obtain high quality bioanalytical data in support of antibody drug programs. A generic, yet standardized approach to protein bioanalysis using LC-MS which is broadly applicable to large molecule drug development would enable novice scientists to successfully support discovery studies. In addition, one such universal proven methodology could facilitate transfer of methods between sites and ensure reproducibility of results. This application note describes the fast, sensitive quantification of bevacizumab (Figure 1) from rat plasma using the ProteinWorks eXpress Digest Kit and Protocol. A single universal sample prep method using pre-weighed, lot-traceable reagents and a carefully developed, yet generic set of simple step-wise instructions produced an LLOQ of 100 ng/mL bevacizumab from 35 µL of rat plasma.

EXPERIMENTAL

Sample description

Bevacizumab was first immuno-purified from 35 µL rat plasma using a 96-well Protein A agarose-based plate. Samples were then prepared for LC-MS analysis using the ProteinWorks eXpress Digest Kit and protocol. Finally, signature peptides were cleaned-up using the ProteinWorks µElution SPE Clean-up Kit and Protocol.

Method conditions

LC system:	ACQUITY UPLC
Detection:	Xevo TQ-S Mass Spectrometer, ESI+
Column:	ACQUITY UPLC Peptide BEH C_{18}, 300Å, 1.7 µm, 2.1 mm x 150 mm
Column temp.:	55 °C
Sample temp.:	10 °C
Injection volume:	10 µL
Mobile phase A:	0.1% formic acid in water
Mobile phase B:	0.1% formic acid in acetonitrile

Gradient:

Flow rate (mL/min)	Time (min)	Profile %A	Profile %B	Curve
0.3	0.0	100	0	6
0.3	1.0	100	0	6
0.3	7.0	50	50	6
0.3	8.0	10	90	6

Data management: MassLynx® (v4.1)

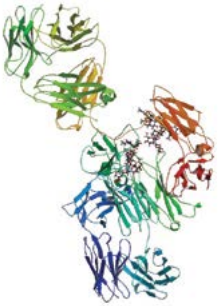
MS conditions

Capillary:	3 kv
Cone:	30 V
Source offset:	50 V
Source temp.:	150 °C
Desolvation temp.:	600 °C
Cone gas flow:	150 L/hr
Desolvation gas flow:	1000 L/hr
Collision gas flow:	0.15 mL/min
Nebuliser gas flow:	7 Bar

Peptide	MRM transition	Cone voltage (V)	Collision energy (eV)
FTFSLDTSK*	523.30>797.48	16	14
STAYLQMNSLR*	642.30>748.45	36	20
DSTYSLSSTLTLSK	751.88>836.47	31	24
STSGGTAALGC[+57.0]LVK	661.34>576.32	31	25
NTQPIMDTDGSYFVYSK (ISTD)	983.95>397.21	32	26

*Unique Signature Peptide

Table 1. MRM conditions for bevacizumab peptides and the internal standard peptide.



Bevacizumab light chain
DIQMTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQKPGKAPKVLIYFTSSLHSGVPSRFSGSGSGTDFTLTIS
SLQPEDFATYYCQQYSTVPWTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDN
ALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

Bevacizumab heavy chain
EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVRQAPGKGLEWVGWINTYTGEPTYAADFKRRFTFSLDTSK
STAYLQMNSLRAEDTAVYYCAKYPHYYGSSHWYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTALGCLVK
DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKVEPKSCDKTH
TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTY
RVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPS

REEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHE
ALHNHYTQKSLSLSPGK

Unique Signature

Generic Signature

Drug Bank

Formula: $C_{6538}H_{10034}N_{1716}O_{2033}S_{44}$
Molecular Weight: ~ 149.0 kD

http://www.drugbank.ca/drugs/DB00065

Figure 1. Bevacizumab (Avastin) Protein Structure.

RESULTS AND DISCUSSION

With the bevacizumab US patent expiration date of 2020 drawing ever closer, the focus on this important drug in CRO's as well as biosimilar research labs has increased. However, typical workflows are incredibly complex, with a multitude of choices and options. This makes the development of high sensitivity LC-MS methods for this and other monoclonal antibody-based drugs particularly challenging. In this application note, we have used the ProteinWorks eXpress Digest Kit to simplify and streamline the process. Bevacizumab samples were affinity purified, digested, and peptides extracted using SPE in under 6 hours total. This enabled same day data acquisition, with several 96-well plates being run by the following morning. Multiple unique signature peptides as well as a generic human peptide were simultaneously monitored for use in quantification. The best sensitivity and specificity were achieved using the unique peptide FTFSLDTSK from the variable region of the heavy chain, while additional generic (STSGGTAALGC+57LVK, heavy chain) and specific (STAYLQMNSLR, heavy chain) bevacizumab peptides were monitored for confirmation. A signature peptide (NTQPIMDTDGSYFVYSK) from a common murine mAb standard (p/n 186006552) was used as the internal standard.

Using the optimized protocol and reagents provided in the kit, only 35 µL of plasma was needed to achieve a quantification limit of 100 ng/mL for bevacizumab (Figure 2). Linearity and accuracy of the standard curves arising from each peptide are summarized in Table 2. The primary, and most sensitive and specific quantitative peptide, FTFSLDTSK, was linear over 3.5 orders of magnitude with a mean accuracy of 100% for all points on the curve. The additional three peptides were linear over 3–3.5 orders of magnitude with average accuracies >99% for all curve points.

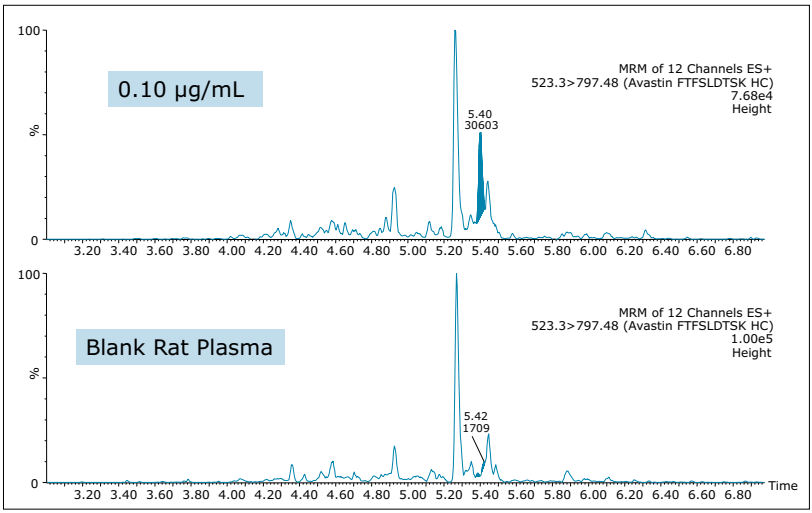


Figure 2. Chromatogram showing 100 ng/mL of bevacizumab in rat plasma, as compared to blank rat plasma. The unique peptide FTFSLDTSK is shown.

Peptide	Std. curve range (µg/mL)	Weighting	Linear fit (r^2)	Mean % accuracy of all points
FTFSLDTSK*	0.05–250	1/X	0.998	100.00
STAYQMNSLR*	0.50–500	$1/X^2$	0.997	100.02
DSTYSLSSTLTLSK	0.25–500	$1/X^2$	0.996	100.00
STSGGTAALGC+57LVK	0.05–250	$1/X^2$	0.996	100.60

*Unique signature peptide

Table 2. Linear dynamic range and standard curve statistics for signature peptides used to quantify bevacizumab in rat plasma.

In addition, the accuracy and precision for the QC samples was excellent with %CVs all ≤7%. This is summarized in Table 3. In fact, the average %CV for QC samples from the FTFSLDTSK peptide was 4%. Similarly, the average %CV for QC samples from the STAYLQMNSLR peptide was only 3%. Furthermore, the precision of the low QC across all peptides was 3%. Mean accuracies for all peptides hovered close to 100%.

Peptide	QC conc. (µg/mL)	Mean cal. conc. (µg/mL)	Std. dev.	%CV	Mean accuracy
FTFSLDTSK*	0.035	–	–	–	–
	0.350	0.342	0.013	3.82	97.7
	3.500	3.553	0.126	3.56	101.5
	35.000	32.386	0.611	1.89	92.5
	350.000	290.135	21.024	7.25	82.9
Peptide	**QC conc. (µg/mL)**	**Mean cal. conc. (µg/mL)**	**Std. dev.**	**%CV**	**Mean accuracy**
STAYLQMNSLR*	0.035	–	–	–	–
	0.350	0.345	0.004	1.12	98.6
	3.500	3.819	0.110	2.89	108.3
	35.000	35.065	1.262	3.60	100.2
	350.000	335.347	15.208	4.54	95.8
Peptide	**QC conc. (µg/mL)**	**Mean cal. conc. (µg/mL)**	**Std. dev.**	**%CV**	**Mean accuracy**
DSTYSLSSTLTLSK	0.035	–	–	–	–
	0.350	0.351	0.016	4.61	100.3
	3.500	3.397	0.045	1.32	97.1
	35.000	32.082	0.469	1.46	91.7
	350.000	320.836	9.141	2.85	91.7
Peptide	**QC conc. (µg/mL)**	**Mean cal. conc. (µg/mL)**	**Std. dev.**	**%CV**	**Mean accuracy**
STSGGTALGC+57LVK	0.035	–	–	–	–
	0.350	0.366	0.012	3.36	104.4
	3.500	3.509	0.063	1.79	100.2
	35.000	33.015	1.406	4.26	94.3
	350.000	305.140	3.259	1.07	87.2

Table 3. Statistics for QC samples from all bevacizumab peptides used for quantification.

We consistently achieved single digit accuracy and precision during bevacizumab analysis through a combination of three primary factors: high fidelity chromatographic data, an optimized, well controlled protocol, and the use of a set of standardized, pre-measured reagents. These three critical components were realized through application of the ProteinWorks eXpress Digest Kit, which facilitated low level detection, separation from residual endogenous interferences, and the very high accuracy and precision. This performance is highlighted in the QC chromatograms from representative signature peptides in Figures 3 and 4.

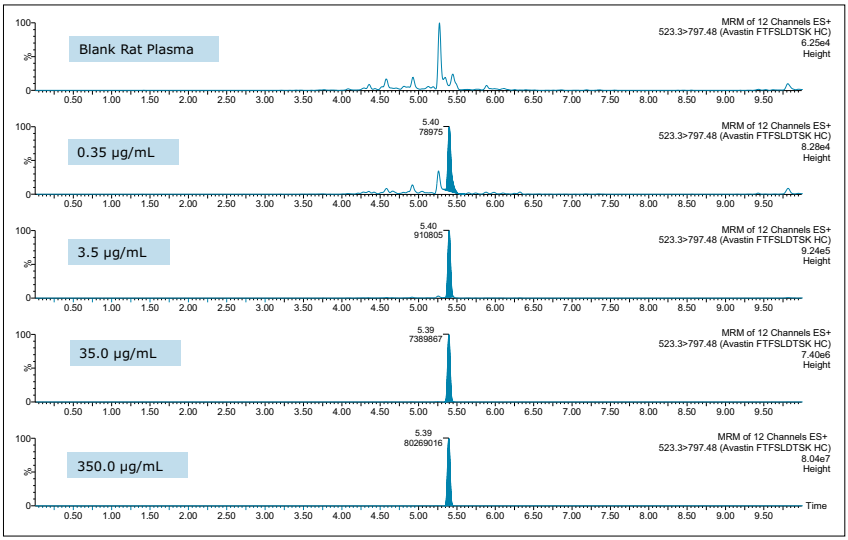


Figure 3. Bevacizumab QC Chromatograms for the FTFSLDTSK Unique Signature Peptide.

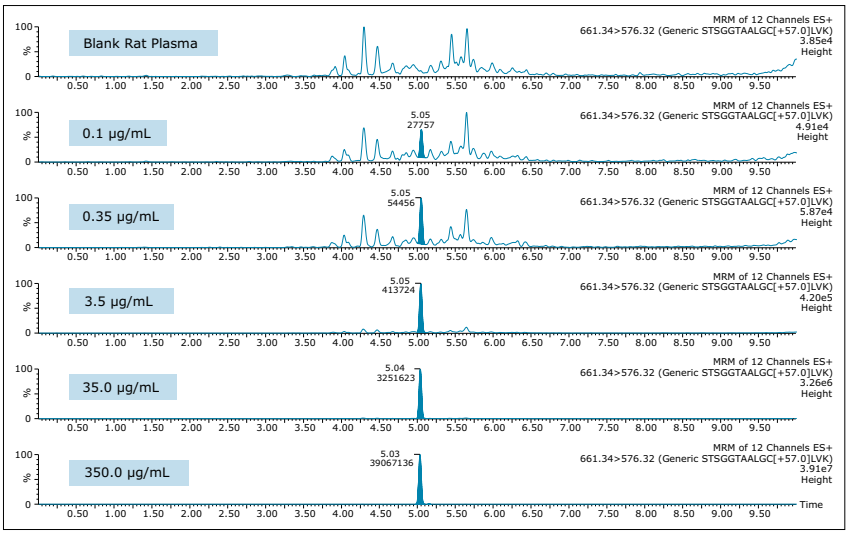


Figure 4. Bevacizumab QC Chromatograms for the STSGGTAALGC+57LVK Generic Signature Peptide.

CONCLUSIONS

The ProteinWorks eXpress Digest Kit was successfully used to purify bevacizumab from a typical set of standard curve and QC samples in human plasma. A limit of quantification of 100 ng/mL was readily achieved from only 35 µL rat plasma, while maintaining excellent linearity and single digit precision. The total sample prep time including an affinity purification step was under 6 hours. The total digest prep time was just over 2 hours. The universal, kit-based approach allows novice users to achieve low detection limits with a simple step-wise protocol and a set of standardized, pre-measured reagents, ensuring both the sensitivity required and the transferability desired of such methods.

In addition, the kit is optimized and flexible enough to enable simultaneous, sensitive quantification of both unique and generic signature peptides from monoclonal antibodies. This is important as confirmatory data from multiple peptides is critical in supporting the confident use of LC-MS for protein bioanalysis workflows.

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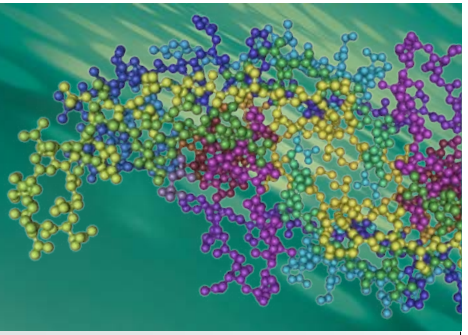
A Universal, Optimized SPE Protocol for Clean-up of Tryptic Peptides in Protein Bioanalysis

Mary Lame, Hua Yang, Sherri Naughton, and Erin Chambers



GOAL

To demonstrate the performance of an optimized method for tryptic peptide clean-up in protein bioanalysis.

BACKGROUND

During a typical protein bioanalytical workflow, it is common to either directly digest an unpurified plasma/serum sample or to perform affinity purification followed by digestion. An affinity step may be included to isolate a class of proteins (for example, the use of Protein A/G which isolates an IgG fraction) or a specific target from the plasma/serum sample. Depending on the degree of protein-level clean-up, which ranges from none (direct plasma digestion) to significant (specific affinity capture of a target) the concentration of resultant peptides can be extremely high. In small molecule SPE the majority of proteins (undigested, of course) pass through the extraction device on the load step. In contrast, in a protein bioanalysis workflow, 10's of thousands of digested peptides from as high as 50–60 mg/mL starting protein concentration, are all now capable of binding to the SPE sorbent bed. This creates a significant capacity challenge. A possible solution that might occur to a "small molecule" scientist would be to use a larger sorbent bed size, for example 10, 30 or even 60 mg sorbent beds. However, these larger bed sizes require larger volumes to elute, which must then be dried down and reconstituted. The same practices, when applied in large molecule quantification, could result

As of 2013, there were 338 new monoclonal antibody drugs in development,[1] representing the largest class of biologics in drug development pipelines. Recently, LC-MS has been gaining ground as the technique of choice over ligand binding assays (LBAs) for the support of biologic drug development programs. An important component of these LC-MS workflows is the post-digest purification of tryptic peptides. While SPE is commonly used in small molecule sample preparation, the same methods and guidelines do not apply to peptide extraction. Knowledge of peptide handling concerns as well as the ability to efficiently manipulate wash and elution conditions for tryptic peptides have become critical capabilities in bioanalytical labs. Historically, these labs have been dominated by work on small molecule therapies with far simpler and more straightforward sample prep and analysis methods. The expertise in peptide and protein bioanalysis, if it exists, tends to be a rare and valuable skill, held by only a few individuals.

in significant peptide/protein losses due to adsorption during evaporation. Therefore, it is critical to avoid the dry-down step commonly associated with small molecule SPE methods.

In addition to the requirement for clear capacity guidelines for loading protein digests, a method tailored to targeted elution of tryptic peptides is also needed. A broadly applicable protocol which effectively removes excess digest reagents, digestion buffer, phospholipids, and other plasma/serum components could not only increase sensitivity by reducing matrix effects, but would also improve system robustness.

THE SOLUTION

A total of 17 signature peptides (a combination of both generic and unique) were used in the development and verification of an SPE protocol, as well as an optimal device format, which specifically targets tryptic peptide clean-up during protein bioanalysis experiments. The result of this research is the ProteinWorks™ µElution SPE Clean-up Kit (p/n 186008304). Key attributes of the kit include: the ability to concentrate digests without evaporation, orthogonal retention mechanism for maximum specificity, and a protocol designed expressly for efficient purification of tryptic peptides. Oasis® MCX was chosen as the ideal sorbent due to its strong ion exchange binding to the positively charged termini produced through cleavage at the basic residues arginine and lysine during trypsin digestion. In addition, very polar peptides are more efficiently trapped by ion exchange on the Oasis MCX plate than they would be on a traditional reversed-phase only sorbent. The µElution 96-well device format is characterized by elution in as little as 25 µL, which enables significant concentration of extracts without evaporation. The entire 96-well plate can be processed in under 20 minutes using either vacuum or positive pressure, and is amenable to automation.

Performance of the SPE Kit was evaluated using a broad range of chemically diverse representative peptides. Key peptide characteristics are summarized in Table 1.

Through extensive testing and optimization, recovery was maximized for all peptides and sample loading guidelines were created. The comprehensive guidelines, which are related to starting protein content, address both direct plasma/serum digest samples and extracts resulting from affinity purification of the target protein or class of proteins. Recovery of the various peptides using the optimized protocol in the SPE Kit is summarized in Figure 1. The average peptide recovery for the antibody drugs is 104%. Peptides from the generic murine mAb IS average 84% recovery.

Table 1. Relevant characteristics of signature peptides tested during the development and verification of the ProteinWorks µElution SPE Clean-up Kit

Protein Name	Peptide	MW	pI	# of Residues	HPLC Index
Herceptin	FTISADTSK	969	6.2	9	26.5
Humira	APYTFGQGTK	1069	9.1	10	39.3
Herceptin	DTYIHWVR	1089	7.4	8	31.1
Generic Human IgG	GPSVFPLAPSSK	1186	9.4	12	54.7
Murine	MNSLQTDDTAK	1223	4.1	11	21.5
Murine	VNSAAFPAPIEK	1243	6.4	12	47.4
Avastin	STAYLQMNSLR	1283	9.2	11	48.4
Avastin	FTFSLDTSK	1285	7.3	11	40.5
Generic Human IgG	STSGGTAALGC[+57.0]LVK	1322	9.4	14	50.8
Humira	NSLYLQMNSLR	1339	9.2	11	54.6
Remicade	SINSATHYAESVK	1407	7.4	13	7.1
Humira	NYLAWYQQKPGK	1496	9.7	12	52.2
Generic Human IgG	DSTYSLSSTLTLSK	1503	6.2	14	47.8
Generic Human IgG	VVSVLTVLHQDWLNGK	1808	7.4	16	77.7
Murine	SVSELPIMHQDWLNGK	1854	5.5	16	52.5
Remicade	DILLTQSPAILSVSPGER	1869	4.3	18	74.2
Murine	NTQPIMDTDGSYFVYSK	1966	4.1	17	35.1

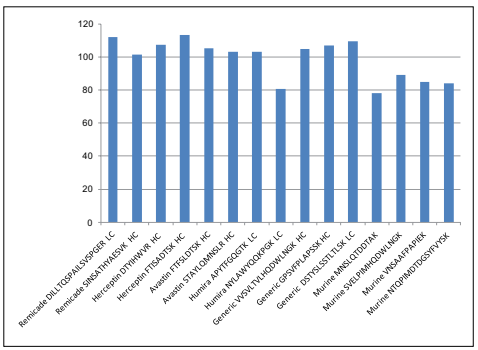


Figure 1. MCX µElution SPE recovery of unique and generic signature peptides from infliximab, bevacizumab, adalimumab, trastuzumab, and a generic murine mAb internal standard.

It is recommended that the total protein load in the digested sample on the Oasis MCX µElution plate not exceed 1.25 mg. In addition, for best performance, the minimum SPE loading volume range is 15–25 µL. The sample loading guidelines, summarized in Table 2, were designed to produce the highest sensitivity by identifying the maximum digest volume which should be loaded.

Table 2. Recommended maximum digest loading volumes for the ProteinWorks µElution SPE Clean-up Kit

Oasis MCX 96-well µElution Plate Recommended Maximum Digest Loading Volumes		
	SPE Loading Volume (µL)	
Starting Plasma/Serum Volume (µL)*	Direct Digest**	Post-Generic Affinity Digestion†
15	110-200	Total Digest Supernatant
25	70-140	Total Digest Supernatant
35	50-100	Total Digest Supernatant
50	35-70	170-200
70	25-50	120-200

** Starting volume of plasma added for protein digestion or affinity purification using the ProteinWorks eXpress Digest Kit and Protocols, with a final digestion volume of 200 µL.*

***Based on a total protein content of 75 mg/mL in whole plasma/serum.*

†Based on a total protein content 15 mg/mL post-generic affinity purified plasma (Protein A/G) and assuming all Affinity captured sample is used for digestion.

Plasma was spiked with infliximab, trastuzumab, adalimumab, bevacizumab, and a common murine mAb IS and then digested. The resultant tryptic peptides were isolated from digest reagents (which are washed off during loading and wash steps), phospholipids (which remain stuck to the SPE plate after peptide elution), and concentrated using the ProteinWorks SPE Clean-up Kit. Chromatographic purity of the signature peptides extracted from the plasma digest sample is demonstrated in Figure 2.

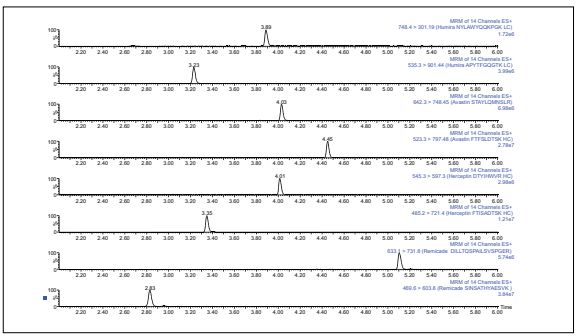


Figure 2. LC-MS chromatograms for representative unique signature peptides from a plasma digest containing adalimumab, bevacizumab, trastuzumab, and infliximab.

SUMMARY

The ProteinWorks µElution SPE Clean-up Kit was successfully used to isolate peptides resulting from plasma digests of bevacizumab, trastuzumab, adalimumab, and infliximab as well as a common murine mAb internal standard. The simple 96-well µElution format enabled clean-up and concentration of digest samples in <20 minutes, without the need for evaporation and reconstitution, preserving low levels of precious tryptic peptides. Furthermore, the kit takes advantage of the orthogonal nature of mixed-mode SPE (binding tryptic peptides by ion-exchange) to provide the degree of specificity required for low-level protein quantification studies. Finally, the specific sample loading guidelines and an optimized kit-based approach allow novice users to quickly, easily, and effectively clean-up protein digests. This strategy generates data with the best possible reproducibility and highest peptide recovery for challenging protein bioanalysis studies.

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Antibody Quantification Using ProteinWorks eXpress Digest Kits and Multiple Plasma Volumes

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GOAL

To demonstrate the broad applicability across a range of sample volumes, and the inherent flexibility of the ProteinWorks™ eXpress Direct Digest Kits for the quantification of monoclonal antibody (mAb) drugs by LC-MS/MS.

BACKGROUND

Over the past decade, MS platform technologies have steadily increased in sensitivity and ease of use. At the same time, this increase in sensitivity has facilitated the use of smaller sample volumes. As such, microsampling techniques have become common place. These techniques allow for collection from pre-clinical species such as rodent, and minimize animal use. Additionally, as biological drug development increases, LC-MS assays to quantify both therapeutic and endogenous peptides and proteins are growing. The desire to use small sample volumes presents an even greater challenge for protein and peptide quantification analyses due to the complexity of the workflow and their inherent lower MS sensitivity, relative to small molecules. While some studies are dominated by the need to use sample volumes <50 µL, others require more sample to increase sensitivity. Therefore, there is a need for a simple, kit-based approach that accommodates a range of plasma volumes and is easily implemented by scientists unfamiliar with the workflows.

Recently there has been a trend towards LC-MS for the bioanalytical quantification of peptide and protein therapeutics due to the many benefits it affords (multiplexing, improved specificity, broader dynamic range and fast method development time). However, protein quantification challenges still exist with regard to assay sensitivity, small sample volume requirements, and laborious and often complex workflows. This is especially in contrast to ligand binding assays. Thus, there is a strong need for simpler and more broadly applicable standardized workflows, ideal for low sample volume assays (<50 µL), for the quantification of proteins.

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THE SOLUTION

LC-MS for quantification of therapeutic and endogenous proteins is steadily gaining in its use, and unlike small molecule LC-MS analysis, it presents many new and unique challenges. Typically one needs to perform an enzymatic digestion (most commonly trypsin) in plasma, followed by quantification of one or multiple representative tryptic peptides using multiple reaction monitoring (MRM). There is no single standard work flow for this task, and it is often traditional "small molecule" scientists faced with learning and implementing these unfamiliar workflows, which can be complex and laborious. Required sample volumes will vary depending on assay or sensitivity requirements. This can make it difficult to know where to start, particularly if one considers the diversity of proteins, as they can vary greatly in their size, structure, and amino acid sequence. For this reason, we have developed a fully flexible yet generic, kit-based sample preparation strategy using the ProteinWorks eXpress Direct Digest Kit (p/n 176003688) for the simultaneous quantification of the monoclonal antibody drugs: infliximab, adalimumab, bevacizumab, and trastuzumab with plasma volumes ≤70 µL. In short, the mAb's were spiked into plasma at concentrations between 5–50 µg/mL. Sample aliquots of 15, 35, and 70 µL of the mAb spiked plasma were then directly digested with the ProteinWorks eXpress Direct Digest Kit using the generic protocol provided. For all plasma sample volumes, and using the ProteinWorks kits for digestion, standard curves for all peptides, from all mAb drugs, were linear with R^2 ≥0.99, using 1/x weighting. Mean % accuracies of the standard curve points were >99% (Table 1). Standard curves arising from a range of starting plasma volumes are shown in Figure 1, Panels A–C. The representative infliximab tryptic peptide (DILLTQSPAILSVSPGER) is shown as an example. The chromatographic performance is highlighted in Figure 2, panels A–D. Multiple calibration levels from representative signature peptides of infliximab, bevacizumab, trastuzumab, and adalimumab are shown.

Table 1. Linear dynamic range and standard curve statistics for the various plasma digestion volumes, for infliximab, adalimumab, trastuzumab, and bevacizumab using the ProteinWorks eXpress Direct Digest Kit.

Protein	Peptide	Linear fit (r^2) with 1/x weighting			Mean % accuracy		
Standard curve range 5.0–50.0 (µg/mL)		15 µL plasma	35 µL plasma	70 µL plasma	15 µL plasma	35 µL plasma	70 µL plasma
Infliximab	SINSATHYAESVK	0.999	0.999	0.997	100.00	99.99	99.99
	DILLTQSPAILSVSPGER	0.999	0.998	0.994	99.99	100.00	100.01
Trastuzumab	FTISADTSK	0.997	0.993	0.998	100.00	100.01	99.99
	DTYIHWVR	0.995	0.995	0.996	100.00	100.02	100.02
	IYPTNGYTR	0.998	0.996	0.991	99.98	99.98	98.79
Bevacizumab	STAYLQMNSLR	0.999	0.998	0.995	100.00	100.01	99.99
	FTFSLDTSK	0.999	0.999	0.993	100.02	100.00	100.00
Adalimumab	APYTFGQGTK	0.994	0.997	0.995	99.99	99.99	99.99
	NYLAWYQQKPGK	0.997	0.998	0.999	99.99	100.02	100.01

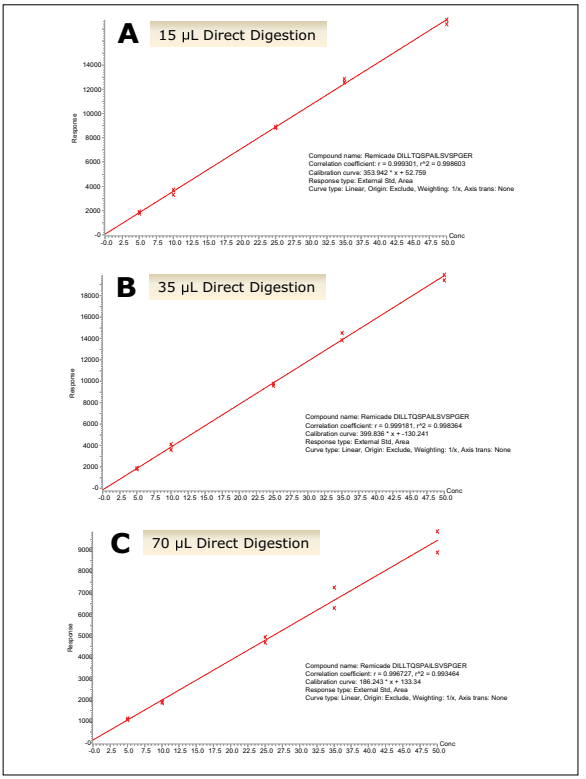

Figure 1. Representative calibration curves arising from digestion of 15, 35, and 70 µL of plasma. The infliximab signature peptide DILLTQSPAILSVSPGER is shown as an example.

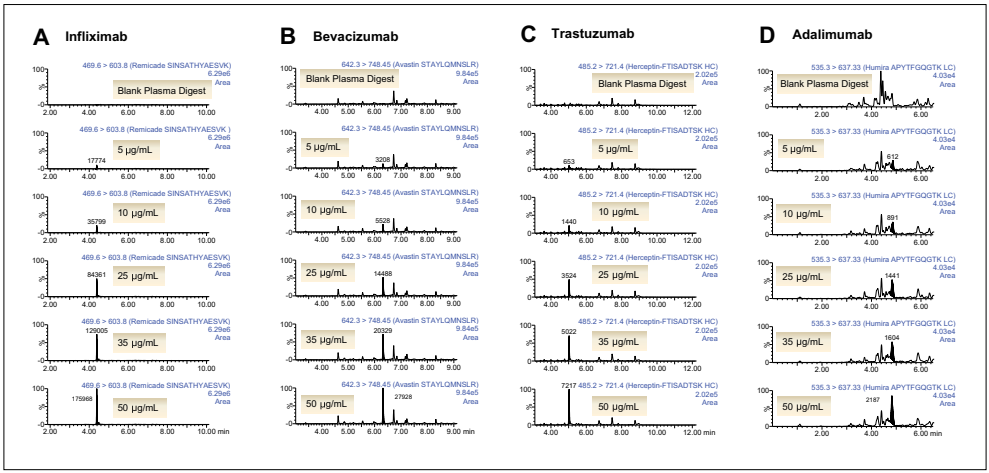

Figure 2. Chromatograms from calibration levels (5–50 µg/mL) of infliximab, bevacizumab, trastuzumab, and adalimumab (15 µL starting plasma) are shown in panels A–D, respectively.

SUMMARY

In this study, a flexible 'kit-based' approach, using a single protocol which accommodates a range of sample volumes (15–70 µL) eliminated the need for method development in discovery studies, and facilitated the accurate quantification of 4 monoclonal antibody drugs.

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LC-MS Quantification of Trastuzumab Using Pellet Digestion and ProteinWorks eXpress Direct Digest Kits

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Protein quantification workflows are often complex and laborious, and extensive sample clean-up to achieve specificity and sensitivity for accurate quantification from complex biological matrices is required. While affinity purification at the protein level can significantly increase the ultimate sensitivity of the assay, the cost and time necessary are not realistic for discovery studies. Identification and implementation of a simpler pre-fractionation step, such as protein precipitation, can improve sensitivity, while a standardized, kit-based approach minimizes method development. Both provide a common generic option for inexperienced analysts to achieve sensitive, accurate and robust quantification of proteins.

GOAL

To demonstrate the compatibility of ProteinWorks™ Kits with pellet digestion and to show improved sensitivity/selectivity for the monoclonal antibody (mAb) trastuzumab (Herceptin) by combining pellet digestion and ProteinWorks eXpress Direct Digest Kits.

BACKGROUND

Due to the similarity of therapeutic proteins with each other and other plasma/serum proteins, there are often significant matrix and isobaric interferences which can severely limit selectivity and sensitivity of a quantitative LC-MS assay for antibody and other protein-based drugs. One of the major culprits in this respect is serum albumin. Present at 35–50 mg/mL, albumin not only interferes significantly and causes ion suppression chromatographically, but its presence can also reduce digestion efficiency, and at the very least, increases the amount of enzyme required, adding significant cost to the assay. For this reason, additional clean-up strategies (e.g., protein precipitation, immuno-capture with Protein A/G or specific capture reagents, solid phase extraction, and molecular weight cut-off filters) are often employed to reduce sample complexity and to impart additional specificity and sensitivity. Of these techniques, protein precipitation (PPT) with organic solvents prior to protein digestion is the most attractive. PPT results in an aqueous/organic layer containing small molecules, phospholipids,

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salts, and some proteins. The pellet typically contains larger precipitated proteins. Depending on the choice of organic and the ratio of organic to plasma, it is possible to precipitate proteins such as antibodies, while maintaining solubility of the majority of albumins in the liquid layer so that they may be removed. This has the advantage of being a simple, inexpensive, and fast way to enrich antibodies and other large proteins while removing the majority of interfering albumins, detergents, small proteins, phospholipids and other endogenous components of biological matrices. Ultimately, incorporating this option into a generic, yet standardized LC-MS approach for protein bioanalysis enables novice scientists to more successfully support discovery studies.

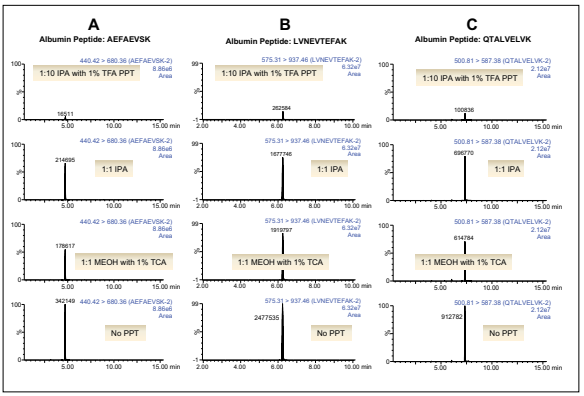

Figure 1. Chromatograms showing albumin depletion (three representative peptides) following PPT pre-treatment and digestion using the ProteinWorks eXpress Direct Digest Kit. Results of the three PPT pre-treatments, compared to no PPT, and are shown in panels A–C, respectively.

THE SOLUTION

Preliminary experiments were conducted using a series of PPT conditions such as varying the starting plasma volume, ratio of organic to plasma, nature of organic, and time and rate of centrifugation. To measure performance, raw area counts from tryptic peptides of both human serum albumin (HSA) and trastuzumab were monitored. Conditions which produced the greatest removal in albumin (as represented by a reduction in albumin peptide area counts) while maintaining or improving trastuzumab sensitivity/recovery (as represented by increased trastuzumab peptide area counts) were progressed for further study.

Following PPT of plasma samples (15 µL), pellets were re-suspended with buffer and subsequently digested using the ProteinWorks eXpress Direct Digest kit and protocol. Using a targeted UPLC-MS/MS method for the representative tryptic peptides (mAb and albumin), digested plasma samples with and without the PPT pre-treatment were analyzed. Of the various plasma PPT pre-treatments, three PPT conditions (1:1 isopropanol, 1:1 methanol containing 1% trichloroacetic acid, and 1:10 isopropanol containing 1% trifluoroacetic acid) were chosen based on sensitivity increases in the mAb tryptic peptides (area count) and reduction of several human

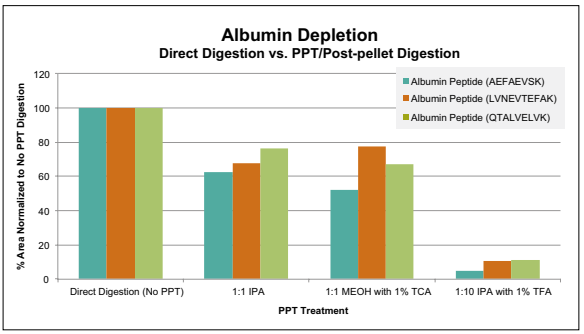

Figure 2. Bar Graph demonstrating reduction of representative albumin peptides (AEFAEVSK, LVNEVTEFAK, and QTALVELVK), resulting from preparation using ProteinWorks eXpress Direct Digest Kits with and without PPT pre-treatment.

serum albumin peptides (area count) when compared to non-PPT treated plasma samples. The reduction of 3 particular albumin peptides (AEFAEVSK, LVNEVTEFAK, and QTALVELVK) with the various PPT treatments is demonstrated in Figure 1, panels A–C. While all 3 treatments provided some degree of albumin removal, PPT with isopropanol (IPA) with 1% trifluoroacetic acid (TFA), at a ratio of 1:10, provided the most effective removal of the albumin peptides (>90%), as shown in Figure 2. Area counts for all 3 unique tryptic peptides from trastuzumab increased significantly using any of the top three PPT conditions (Figure 3). In fact, for the three best PPT pre-treatments, peptide area counts increased 2–8x on average, as is demonstrated in Figure 4.

Standard curves were then prepared in plasma. 15 µL aliquots were subjected to the different PPT conditions, followed by the generic ProteinWorks eXpress Direct Digest Kit and Protocol. Three unique trastuzumab peptides were assessed for linearity and accuracy as an initial gauge of performance. For all PPT treatments, and using the ProteinWorks kits for digestion, standard curves for all peptides were linear with R^2 ≥0.99, using 1/x weighting. Mean % accuracies of the standard curve points were >99% (Table 1). Representative calibration curves for the three trastuzumab tryptic peptides (DTYIHWVR, IYPTNGYTR, and FTISADTSK) prepared using PPT pre-treatment (1:10 IPA containing 1% TFA) of the plasma samples and pellet digestion with ProteinWorks eXpress Direct Digest Kits, are illustrated in Figure 5, panels A–C.

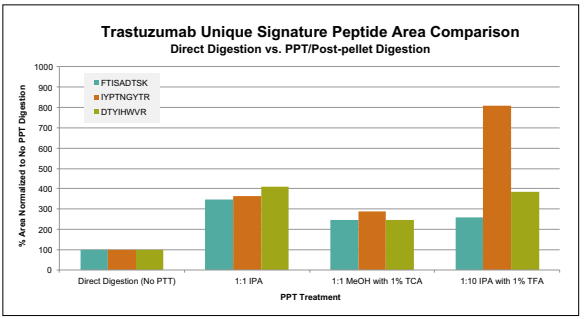

Figure 3. Bar Graph demonstrating improved area counts for 3 unique tryptic peptides of trastuzumab, resulting from preparation using ProteinWorks eXpress Direct Digest Kits with and without PPT pre-treatment.

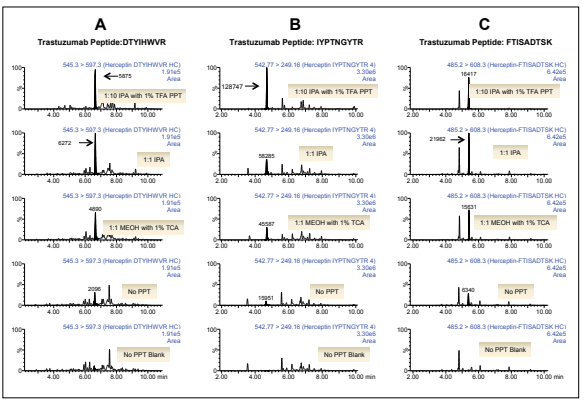

Figure 4. Chromatograms demonstrating improved chromatographic performance and intensity of 3 unique tryptic peptides of trastuzumab, resulting from preparation using ProteinWorks eXpress Direct Digest Kits with and without PPT pre-treatment.

Protein	Peptide	Linear fit (r^2) with 1/x weighting			Mean % accuracy		
Standard curve range 5.0-50.0 (µg/mL)		15 µL plasma IPA with 1% TFA (1:10)	15 µL plasma IPA (1:1)	15 µL plasma MeOH with 1% TCA (1:1)	15 µL plasma IPA with 1% TFA (1:10)	15 µL plasma IPA (1:1)	15 µL plasma MeOH with 1% TCA (1:1)
Trastuzumab	FTISADTSK	0.996	0.997	0.992	99.98	99.99	98.64
	DTYIHWVR	0.994	0.995	0.993	100.00	100.01	98.73
	IYPTNGYTR	0.999	0.997	0.995	100.00	100.01	99.31

Table 1. Linear dynamic range and standard curve statistics for signature peptides used to quantify trastuzumab in plasma following a PPT and pellet digestion with ProteinWorks eXpress Direct Digest Kits.

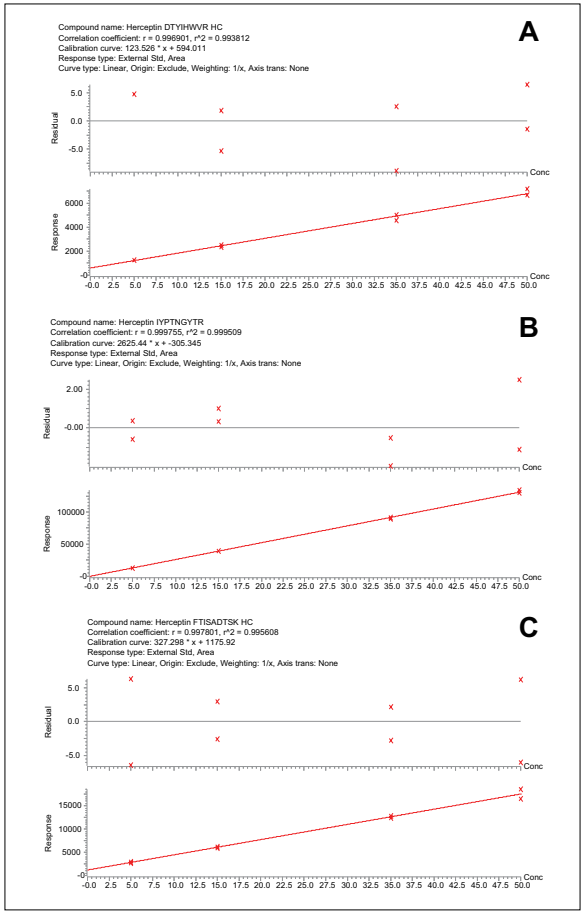

Figure 5. Representative calibration curves for the three tryptic peptides (DTYIHWVR, IYPTNGYTR, and FTISADTSK) of trastuzumab in plasma following PPT pre-treatment (1:10 isopropanol containing 1% trifluoroacetic acid) and pellet digestion with ProteinWorks eXpress Direct Digest Kits, are shown in panels A-C, respectively.

SUMMARY

We describe here a robust and reproducible pellet digestion and LC-MS/MS methodology to quantify the mAb trastuzumab. A simple PPT clean-up and post-pellet digestion using the ProteinWorks eXpress Direct Digest Kit yields accurate, precise and robust LC-MS quantification of trastuzumab via the surrogate peptide approach. Area counts for trastuzumab peptides increased 2–8x using the above strategy, significantly improving sensitivity and specificity, whilst achieving accurate and reproducible quantitative results.

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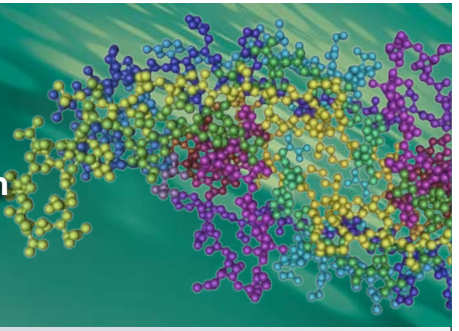
An Intact Murine Monoclonal Antibody for Use as a Generic Internal Standard and Workflow Check Standard in Protein Bioanalysis Studies

Authors: Mary Lame, Hua Yang, Sherri Naughton, and Erin Chambers



GOAL

To demonstrate the value of an intact murine monoclonal antibody standard as both a generic internal standard and as a workflow check/system health standard in protein bioanalysis.

BACKGROUND

For those small molecule bioanalytical scientists now faced with performing bioanalysis studies on proteins, the task may be a difficult one. One of the first challenges encountered is the choice of internal standard (IS). While small molecule workflows have become commonplace and are simple and straightforward, protein quantification involves many unfamiliar processes such as digestion and affinity purification. In order to monitor the efficiency of these steps, an appropriate internal standard must be used. Options such as labeled and/or extended tag surrogate/signature peptides are not effective in compensating for or identifying changes in digestion efficiency or upfront, protein-level pull-down. The optimal IS for protein quantification via the surrogate peptide approach would naturally be a protein. While labeled antibody IS's exist such as SILu™Mab, they can be prohibitively expensive.

In addition, protein bioanalysis workflows not only contain many segments and multiple steps within each segment, but a significant fraction of the workflow is comprised of techniques which are new to typical small molecule

Protein quantification workflows are complex and replete with challenges, not the least of which are choice of internal standard and the ability to demonstrate analyst capability. While ligand binding assays (LBAs) have long been the gold standard for large molecule quantification, LC-MS has risen to the top as a technique of choice due to its improved specificity, improved accuracy and precision, and elimination of long development timelines for often poorly standardized biological reagents. In spite of its obvious advantages, LC-MS for protein bioanalysis is relatively new and is often carried out by scientists with traditional small molecule training. For this reason, we have developed a murine monoclonal antibody for use as both a generic internal standard for discovery studies involving antibody-based drugs as well as for use in evaluating staff proficiency with these new workflows.

bioanalysts. It is the unfamiliar nature of the steps, coupled with the preparation of numerous specialty (some biological) reagents that creates opportunities for error, imprecision, and poor reproducibility. A well characterized workflow test standard with an accompanying "kit" and protocol recipe would be an ideal way to not only learn and validate the process, but also to demonstrate competence.

THE SOLUTION

We have developed and verified an intact murine (mouse) antibody (p/n 186006552) for use as both a generic internal standard and as a tool, in conjunction with ProteinWorks™ eXpress Digest Kits, to both learn and then test aptitude in protein bioanalysis workflows. This standard can also be used on a periodic basis to confirm system readiness/health for these same workflows. The murine antibody is an ideal internal standard for antibody quantification via the surrogate peptide approach as it is added to the sample prior to any type of sample preparation and, subsequently, efficiently and effectively compensates for changes and or sample spillage throughout every step of these complex multi-stage workflows. This includes its ability to adjust for efficiency, recovery, or other changes in the affinity purification (if used) of the protein drug from plasma. It is also ideally suited to compensate for changes in digestion efficiency or volume as it is essentially an analog of any antibody-based drug, thus making it a suitable generic IS for discovery studies. For use as an IS, we recommend a final concentration in plasma of 20–50 µg/mL.

In order to broaden its utility, we have identified several signature peptides from this murine mAb. Depending on the signature peptide used, one can obtain specificity in human, rat, or other species. For example, while the SVSELPIMHQDWLNGK and VNSAAFPAPIEK peptides from the heavy chain are specific in rat, human and mouse plasma, the MNSLQTDDTAK peptide is specific in human and

Figure 1. Sequence and signature peptide options for the intact murine antibody standard.

mouse, but not rat plasma. The final peptide, NTQPIMDTDGSYFVYSK , is also specific in human and mouse plasma, but not necessarily in rat. MRM and chromatographic test conditions are provided in order to confirm and monitor the workflow performance. The sequence and available signature peptides from the intact murine mAb IS/check standard are shown in Figure 1. When the murine mAb is used with and according to the directions of the ProteinWorks eXpress Digest Kits, its signature peptides elute in the same gradient window as both unique and generic signature peptides from humanized monoclonal antibody-type drugs. For example, Figure 2 demonstrates the elution profile of the murine peptides as compared to multiple representative unique and generic signature peptides from mAb therapeutics such as trastuzumab, infliximab, adalimumab, and bevacizumab, following the recommended chromatographic conditions from the ProteinWorks eXpress Digest Kit. Furthermore, as an internal standard, its performance enabled single digit accuracy and precision and a quantification limit of 10 ng/mL for infliximab in Waters Application Note 720005535EN.

SUMMARY

An intact murine (mouse) antibody has been developed and is available for use in protein bioanalysis workflows as either a generic IS or as a workflow test/system health standard, in combination with the specified MRM and LC conditions. In conjunction with a universal, proven protocol and the pre-measured, lot-traceable reagents in ProteinWorks eXpress Digest Kits, one has a recipe which simplifies protein bioanalysis and enables novice users to quickly generate high quality data in discovery studies. In addition, this generic commercially available antibody can be used to evaluate analyst proficiency and to monitor LC-MS system performance.

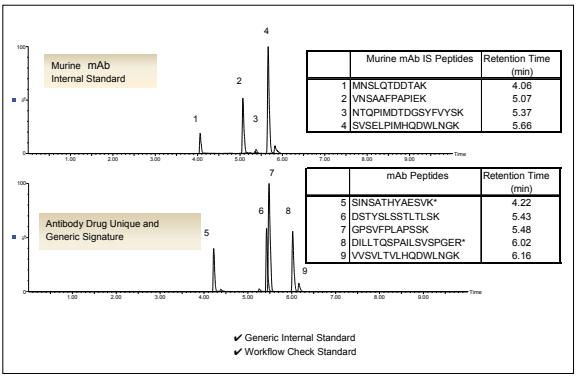


Figure 2. Chromatographic elution profile for signature peptides from the intact murine mAb check standard (top pane) as compared to signature peptides from various mAb therapeutics (bottom pane).

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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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APPLICATION BENEFITS

Simple, standardized approach for accurate and reproducible quantification of ADC and mAb therapeutics; broadly applicable optimized digest kit eliminates method development.

WATERS SOLUTIONS

ProteinWorks eXpress Direct Digest Kit (p/n 186003688)

Intact mAb Check Standard (p/n 186006552)

ACQUITY UPLC® Peptide BEH C_{18}, 300Å 1.7 µm, 2.1 mm x 150 mm Column (p/n 186003687)

ACQUITY UPLC

Xevo® TQ-S Mass Spectrometer

KEY WORDS

antibody drug conjugate, monoclonal antibody, ado-trastuzumab emtansine, T-DM1, trastuzumab, protein quantification, eXpress Direct Digest, ProteinWorks

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.

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_{18}, 300Å 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

Gradient:

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

Data management: MassLynx (v4.1)

MS conditions

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

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.1-3 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 µL) 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_{18}, 300A, 1.7 µm, 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.[4] 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 (µg/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, 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.

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.

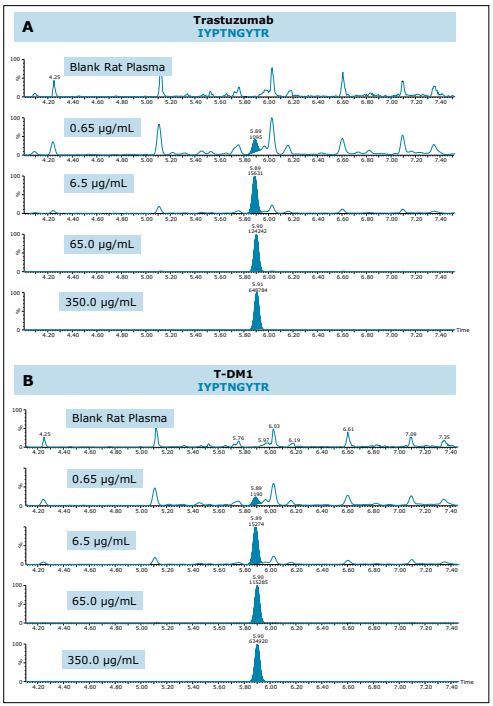

Figure 1. QC chromatograms of Trastuzumab (A) and T-DM1 (B) for the IYPTNGYTR unique signature peptide.

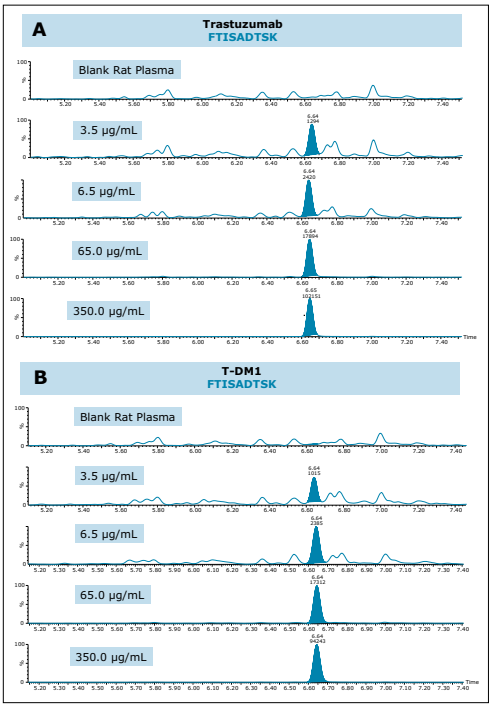

Figure 2. QC chromatograms of Trastuzumab (A) and T-DM1 (B) for the FTISADTSK unique signature peptide.

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 µg/mL), as compared to Trastuzumab (350 µg/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 T-DM1. This is highlighted in Figure 6, panels A and B.

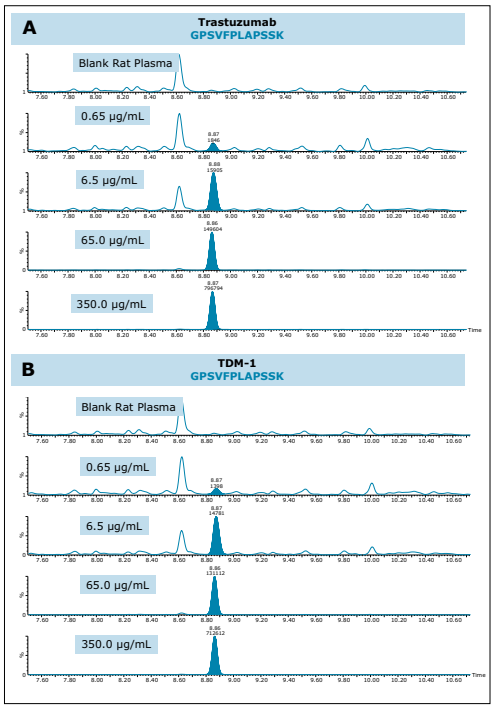

Figure 3. QC chromatograms of Trastuzumab (A) and T-DM1 (B) for the GPSVFPLAPSSK generic IgG signature peptide.

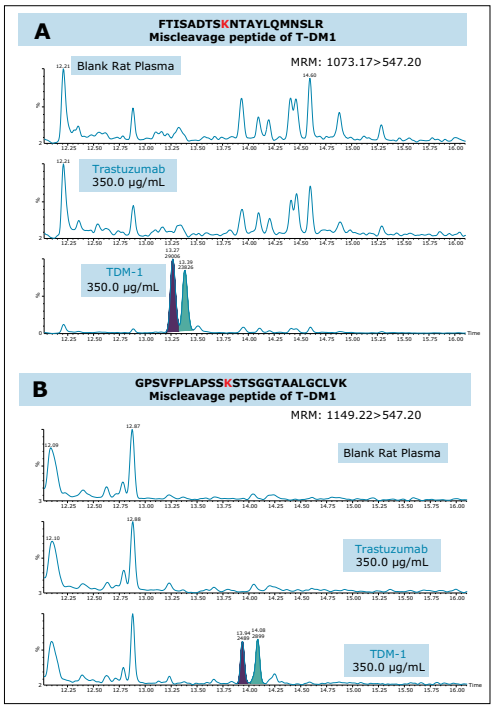

Figure 4. Chromatograms demonstrating the presence of the miscleavage peptides in T-DM1 (350 µg/mL), as compared to Trastuzumab (350 µg/mL), and blank rat plasma when digested and extracted using the ProteinWorks eXpress Direct Digest kit; Panel A: FTISADTSKNTAYLQMNSLR and Panel B: GPSVFPLAPSSKSTSGGTAALGCLVK.

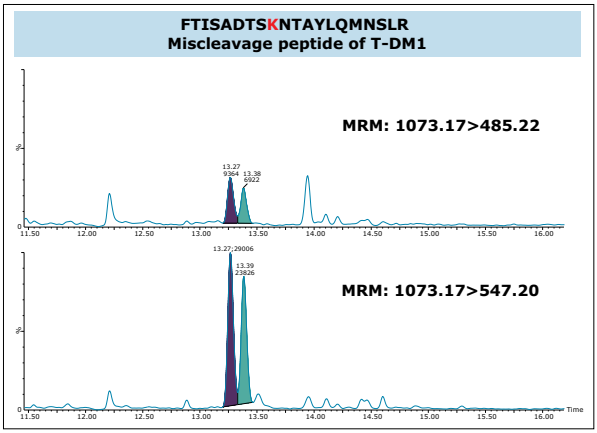

Figure 5. Chromatograms demonstrating the presence of the FTISADTSKNTAYLQMNSLR conjugated peptide confirmed by multiple MRM transitions (1073.17>547.20 m/z and 1073.17>485.22 m/z).

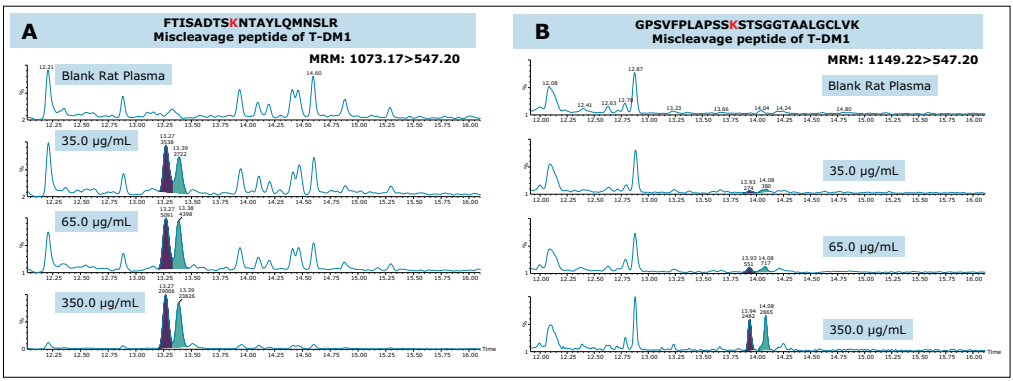

Figure 6. Chromatograms demonstrating increase of the conjugated peptides of T-DM1 with increasing T-DM1 concentration in plasma, when digested and extracted using the ProteinWorks eXpress Direct Digest Kit; Panel A: FTISADTSKNTAYLQMNSLR and Panel B: GPSVFPLAPSSKSTSGGTAALGCLVK.

CONCLUSIONS

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, pre-measured reagents, ensuring both the sensitivity and reproducibility required in discovery studies to make time sensitive and critical project decisions.

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