Waters™

Application Note

Automated, Kit-Based Sample Preparation Strategy for LC-MS Quantification of Cetuximab in Rat Plasma

Paula Orens, Mary E. Lame, Steven Calciano

Waters Corporation

For research use only. Not for use in diagnostic procedures.

Abstract

This application describes a fully automated kit-based approach using ProteinWorks Auto-eXpress Digest and µ Elution SPE Clean-up Kits, performed on the Hamilton Microlab STAR liquid handler (STAR) for the accurate quantification of the monoclonal antibody, cetuximab.

Benefits

- Automated and standardized approach for high throughput protein quantification; broadly applicable optimized digest kit streamlines method development; high sensitivity cetuximab quantification (100 ng/mL) from plasma.

Introduction

In 2015, the global biologics market was valued at $276.6 billion (USD).[1] Monoclonal antibodies (mAbs) have grown to make up over 30%[2] of that market, with no signs of slowing down. What is it that makes mAbs so popular and successful? MAbs work by attaching to specific antigens found endogenously. This bond blocks communication between cells or elicits an attack from the immune system. With longer circulating half lives, greater target specificity, and higher efficacy, mAbs offer a great advantage over other conventional therapies. This highly targeted approach to treatment is being used as a single agent or combination therapy for oncology, immunological disorders, and infectious diseases.[1]

With the vast increase in large molecule therapeutics, the demand for protein quantification in support of drug research and development has also increased. Traditionally, ligand binding assays (LBAs) have been used to quantify proteins in matrix. However, use of LC-MS/MS has steadily increased due to the many limitations of the LBA approach (e.g., lack of standardization, lot-to-lot reproducibility, issues with specificity, and limited dynamic range). While LC-MS protein quantification offers several advantages, it has presented great challenges for traditional "small molecule" scientists, as these complex molecules require intricate analytical workflows for their quantification. The surrogate peptide, or bottom-up, approach is the most common sample preparation technique employed for LC-MS protein quantification. With many reagents and a multitude of sample processing steps required for this approach, method development can be complex, often requiring highly skilled scientists for their successful execution and transfer. Additionally, to reach the desired low-level detection limits, sample enrichment techniques, such as peptide level solid-phase extraction (SPE) or protein level immunoaffinity, are frequently required. This increases sample preparation complexity and can introduce variability to the method.

An automated and standardized approach to protein bioanalysis, including the necessary reagents and protocols, would enable novice large molecule bioanalysts to successfully and reproducibly quantify proteins with ease, increasing throughput, minimizing human error, and streamlining the process. This application describes a fully automated kit-based approach using ProteinWorks Auto-eXpress Digest and µElution SPE Clean-up Kits, performed on the Hamilton Microlab STAR liquid handler (STAR) for the accurate quantification of the monoclonal antibody, cetuximab (Figure 1). Using this fast and simple sample preparation approach, lower limits of quantification (LLOQ) of 100 ng/mL were easily achieved.

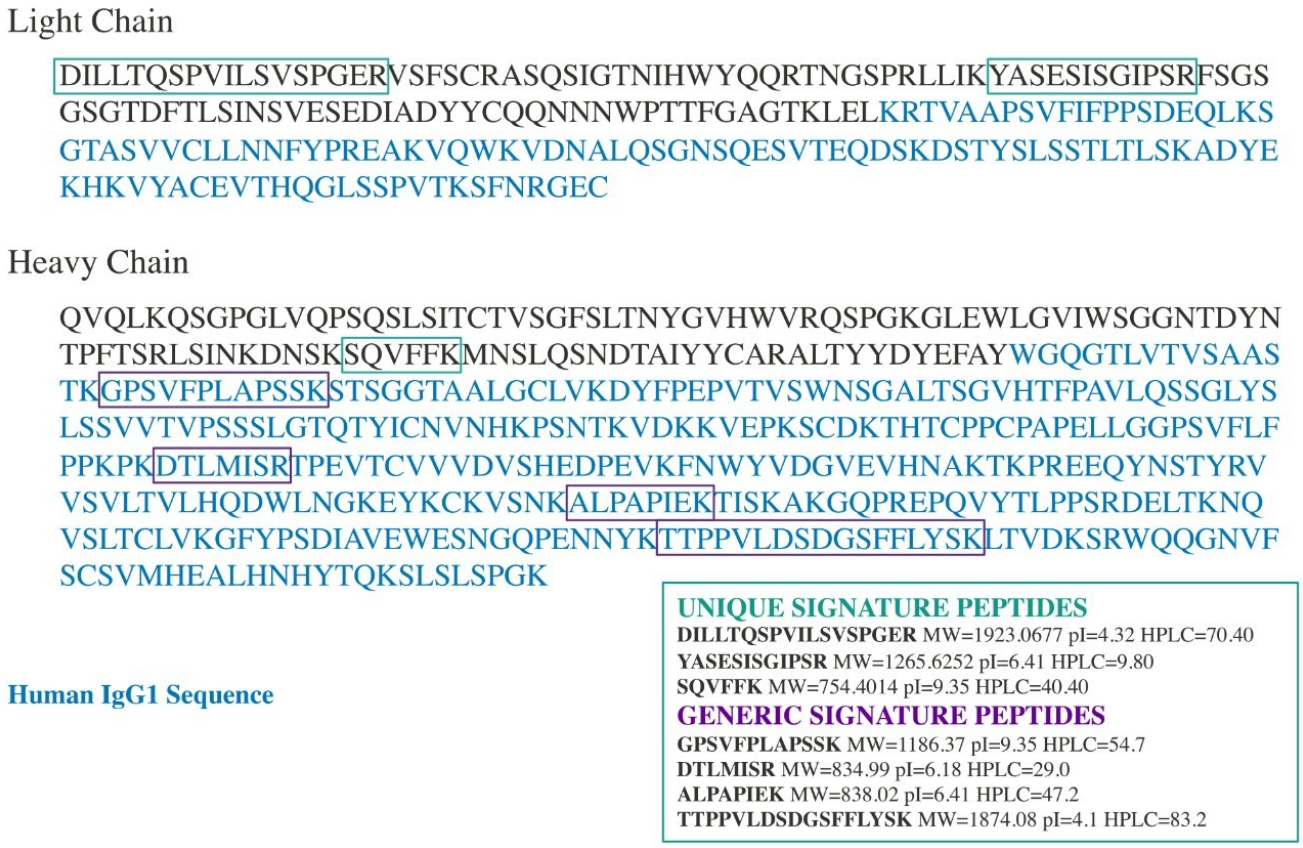


Figure 1. Protein sequence of cetuximab; surrogate peptides used for quantification are boxed in purple and green.

Experimental

Sample description

Rat plasma samples were spiked with cetuximab (0.1–250 µg/mL) and SILu MAB (16 µg/mL) prior to digestion. SILuMAB, a humanized, stable isotopically labeled protein with $^{13}C_6$, $^{15}N_4$-labeled arginine and $^{13}C_6$, $^{15}N_2$-labeled lysine was used as the internal standard (IS) to minimize pre-analytical variability. Since the label occurs where the enzyme, trypsin, cleaves, subsequent labeled tryptic peptides can be used for IS corrections. Calibration curve standards were prepared in duplicate, while QC and blank (non-spiked) plasma samples were prepared in

triplicate. The prepared plasma samples (40 µL) were digested using the ProteinWorks Auto-eXpress High 5 Digest Kit reagents and low volume protocol, which includes the reduction and alkylation of the proteins. Post digestion purification of the subsequent tryptic peptides was performed using the ProteinWorks µElution SPE Clean-up Kit and protocol. Specifically, 100 µL of the digested sample was loaded onto the Oasis MCX µElution plate, processed according to the described protocol, then peptides were eluted using 50 µL of elution solution and diluted with 50 µL of water. Both digestion and SPE sample preparation were carried out on the STAR. The ProteinWorks automation deck layout and protocol includes 2 Hamilton Heater Shakers for incubation and vortexing, a plate sealer, a hold down module for piercing through seals, and a dark box for alkylation. The automated SPE was performed using a CVS Vacuum System (115 V) on the STAR. Digested and purified samples were then subjected to LC-MS/MS analysis.

Method conditions

LC system:	ACQUITY UPLC System
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 vol.:	10 µL
Mobile phases:	A: 0.1% formic acid in water B: 0.1% formic acid in acetonitrile

Gradient

Time (min)	Flow rate (mL/min)	%A	%B	Curve
Initial	0.300	98.0	2.0	6
1.00	0.300	98.0	2.0	6
8.00	0.300	55.0	45.0	6
8.50	0.300	5.0	95.0	6
9.20	0.300	5.0	95.0	6
9.40	0.300	98.0	2.0	6
11.00	0.300	98.0	2.0	6

MS conditions

Capillary:	2.9 kV
Source offset:	35 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
Data management:	MassLynx (v4.1) MS Software
Quantification software:	TargetLynx
Automation platform:	Hamilton Microlab STAR liquid handler

Results and Discussion

Cetuximab was approved by the United States Food and Drug Administration (FDA) in 2004. Since its initial approval, it has been re-approved multiple times for different treatments of colorectal cancer and head and neck cancer.[3] Used as single agent or combination therapy, patients have experienced a better quality of life and better survival outcomes when treated with cetuximab.[3] With US patent expiry in 2016, biosimilar developers are eager to release new products, putting the pressure on small labs and CROs (contract research organizations) to develop methods for its accurate quantification. This has proved challenging for classically small molecule scientists due to the complexity of the LC-MS/MS quantification workflow, with its multiple steps and reagents in need of optimization. In this application, we have used the ProteinWorks Auto-eXpress Digest and µElution SPE Clean-up Kits, fully automated on the Hamilton STAR, to accurately quantify cetuximab in plasma.

A typical set of standards and QCs, spiked with cetuximab and IS, was prepared in rat plasma. For IS peptide selection, SILuMAB tryptic peptides with corresponding, or similar, retention times to cetuximab tryptic peptides provide the best performance. Labeled generic signature peptides digested from SILuMAB should correspond to generic signature peptides digested from cetuximab.

An example of this unlabeled (cetuximab, A)/labeled (SILuMAB, B) relationship can be seen in Figure 2. Multiple unique and generic human peptides (found in the conserved region of the mAb) were simultaneously monitored for cetuximab quantification. Final MRM conditions for cetuximab and SILuMAB can be found in Table 1. Figure 3 highlights the chromatographic separation of cetuximab tryptic peptides using the ACQUITY Peptide BEH C18,

300Å, 1.7 µm, 2.1 x 150 mm Column. A standard LC gradient of 2% to 45% mobile phase B (MPB) over 7 minutes with the Peptide BEH column provided ample separation of any interfering peaks.

Protein	Peptide	Precursor charge state	MRM transition	Cone (V)	Collision (eV)	Product ion identification
Cetuximab	SQVFFK	$[M+2H]^{2+}$	378.2>540.3	35	13	[1H+]1/y4
	YASESISGIPSR	$[M+2H]^{2+}$	633.8>616.3	35	18	[1H+]1/y6
	DILLTQSPVILSVSPGER	$[M+2H]^{2+}$	962.5>342.2	35	41	[1H+]1/b3
	DTLMISR◊	$[M+2H]^{2+}$	418.2>506.3	35	13	[1H+]1/y4
	ALPAPIEK◊	$[M+2H]^{2+}$	419.8>327.7	35	10	[2H+]2/y6
	GPSVFPLAPSSK◊	$[M+2H]^{2+}$	593.8>418.2	35	25	[1H+]1/y4
	TTPPVLDSDGSFFLYSK◊	$[M+2H]^{2+}$	937.5>234.1	35	39	[1H+]1/y2
SILuMAB	YYAGSVK*	$[M+2H]^{2+}$	398.2>632.4	35	14	[1H+]1/y6
	DTLMISR*	$[M+2H]^{2+}$	423.2>516.3	35	14	[1H+]1/y4
	ALPAPIEK*	$[M+2H]^{2+}$	423.8>662.4	35	14	[1H+]1/y6
	LTVLGQPK*	$[M+2H]^{2+}$	432.3>649.4	35	15	[1H+]1/y6
	GPSVFPLAPSSK*	$[M+2H]^{2+}$	597.8>426.2	35	21	[1H+]1/y4
	TTPPVLDSDGSFFLYSK*	$[M+2H]^{2+}$	941.5>242.2	35	34	[1H+]1/y2

Table 1. MRM conditions for cetuximab and SILuMAB (IS) surrogate peptides.

◊ Indicates generic signature peptides.

**Indicates stable isotopically labeled $^{13}C_6$, $^{15}N_4$-labeled arginine or $^{13}C_6$, $^{15}N_2$-labeled lysine.*

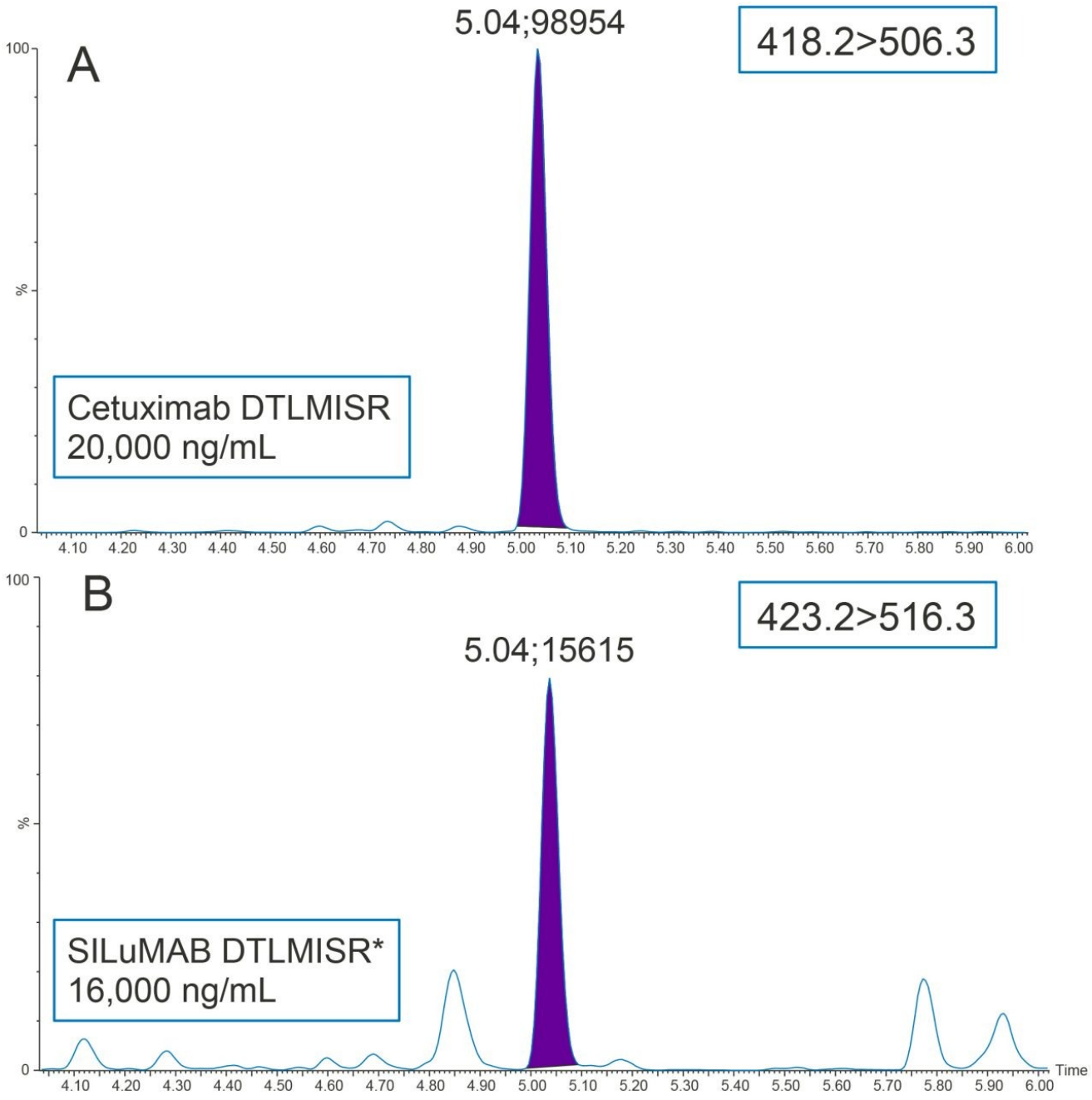


Figure 2. Representative chromatogram for the DTL signature peptide of cetuximab (20 µg/mL, Panel A) used for quantification compared to labeled DTL peptide of SILuMAB (16 µg/mL, Panel B), digested and extracted from rat plasma.

** Indicates stable isotopically labeled $^{13}C_6$, $^{15}N_4$-labeled arginine.*

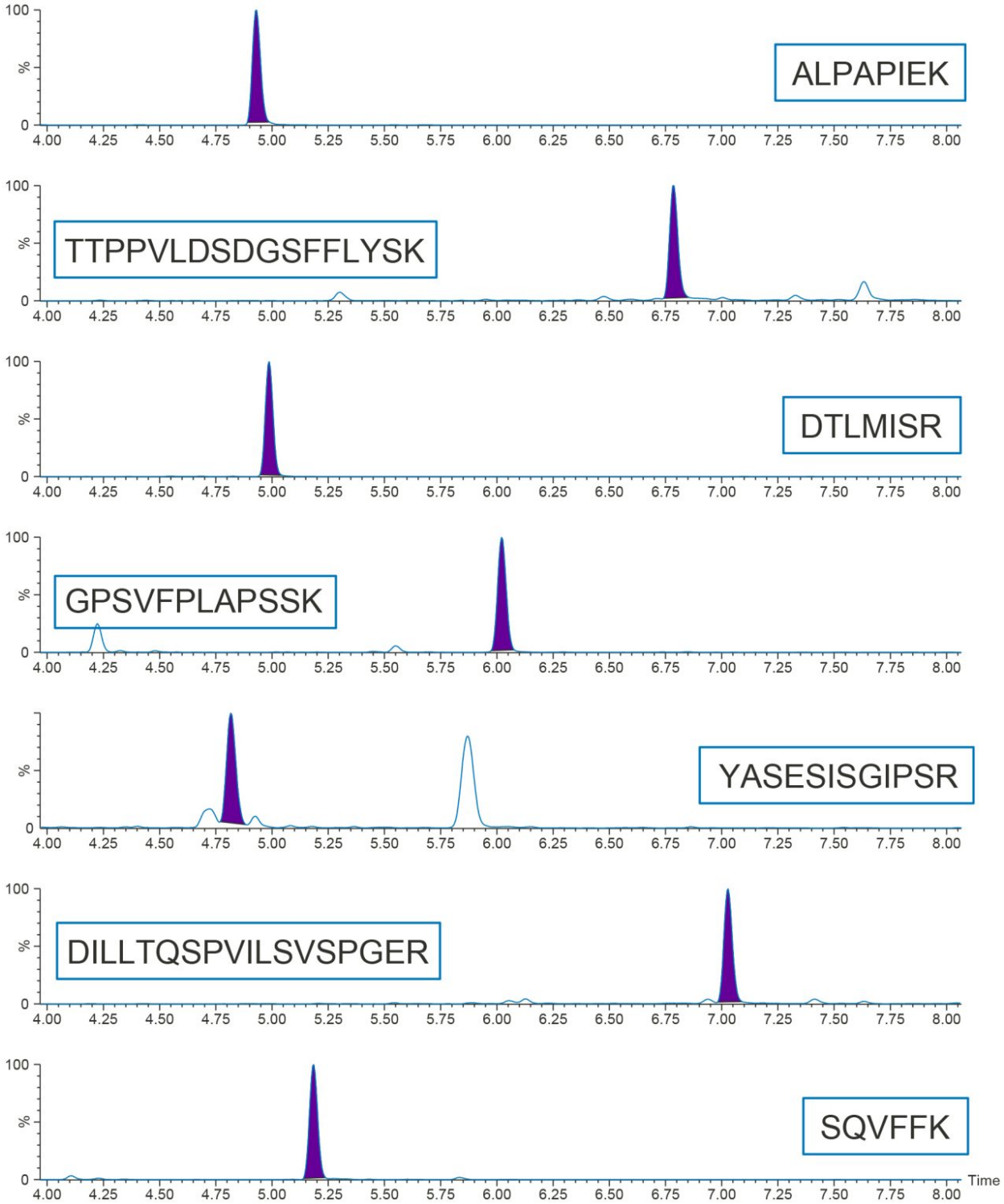
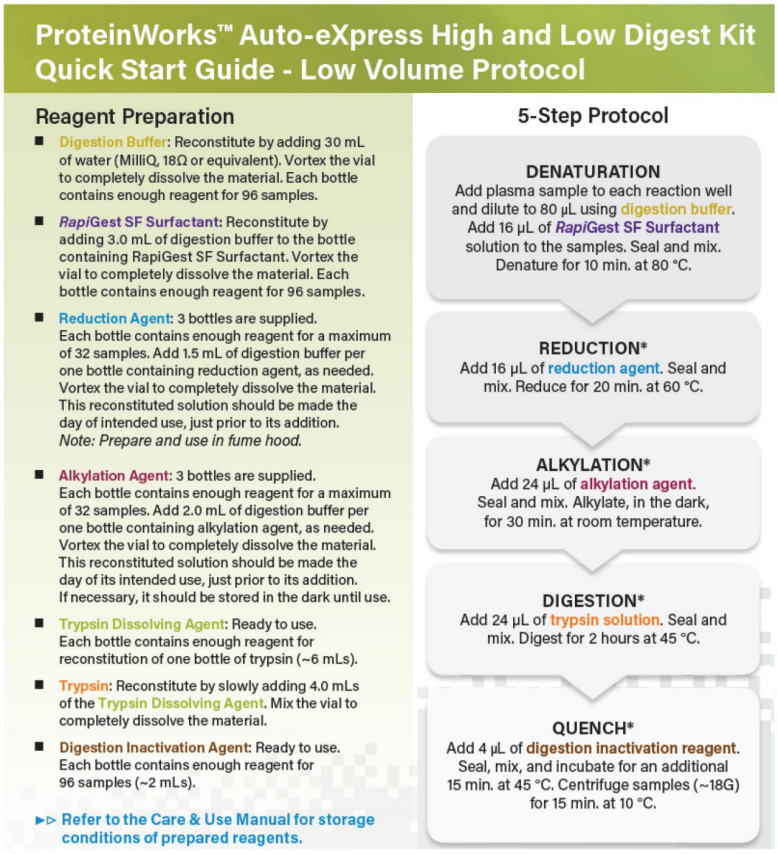


Figure 3. Representative chromatograms for cetuximab tryptic peptides, 100 µg/mL digested in rat plasma.

Using the ProteinWorks Auto-eXpress Digest and µElution SPE Clean-up Kits and protocols (Figure 4, Panels A and B, respectively) automated on the Hamilton STAR (Figure 5) with only 40 µL of plasma, sub µg/mL LLOQs and high accuracies for cetuximab were achieved. Figure 6 compares the performance of blank rat plasma against the LLOQ at 100 ng/mL for the cetuximab tryptic peptide, DILL. Calibration curves for each of the tryptic peptides were linear, r^2>0.99, with mean accuracies averaging 99%, well within regulatory guidlines.5 Standard curves were linear over 3 orders of magnitude for the various cetuximab signature peptides. A summary of standard curve performance is shown in Table 2. Additionally, QC performance was excellent with average CVs ≤15% across all peptides. This performance is summarized in Table 3. QC chromatographic performance for the SQVFFK cetuximab tryptic peptide is highlighted in Figure 7.

A



B

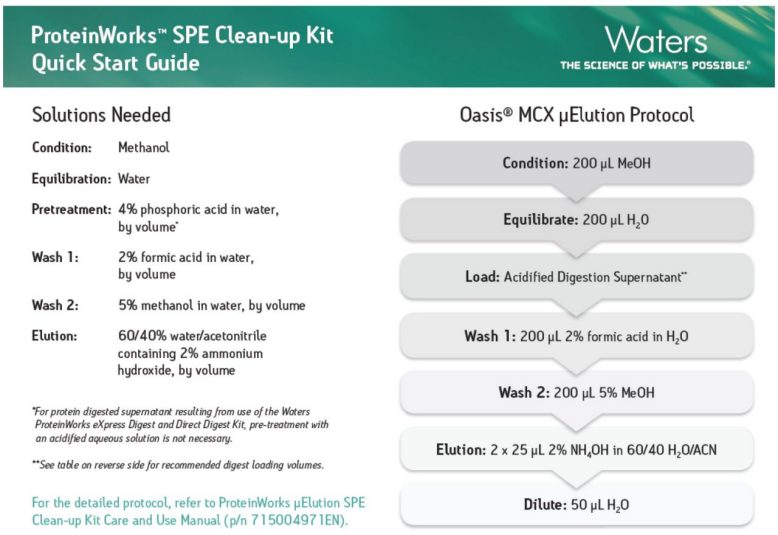
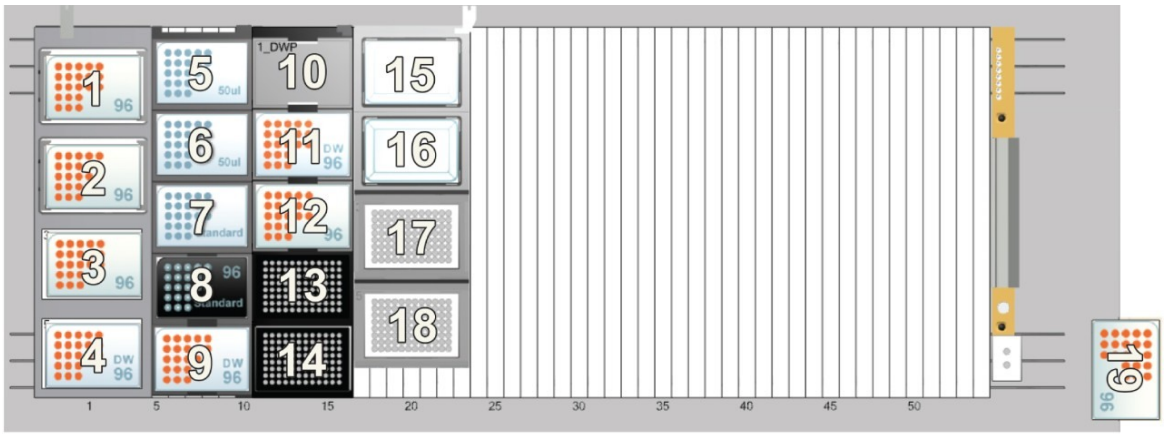


Figure 4. ProteinWorks Auto-eXpress High 5 Digestion Low Volume (Panel A) and ProteinWorks µElution SPE

Clean-up (Panel B) protocols.



1. Hamilton heater shaker (1) @ 80 °C, RT
2. Hamilton heater shaker (2) @ 60 °C, 45 °C
3. Alkylation location (dark box cover)
4. Dark box park
5. 50 µL NTR tips
6. 50 µL NTR tips
7. 300 µL NTR tps
8. Piercing tip location
9. ProteinWorks reagent reservoir
10. Deep well plate location
11. Sample plate (Oasis 96-well plate)
12. Processing plate (350 µL PCR plate)
13. Down holder park
14. Piercing location
15. SPE waste collection park
16. SPE wash solvents
17. Vacuum manifold park
18. Vacuum manifold
19. Agilent PlateLoc plate sealer

Figure 5. Hamilton STAR deck layout for protein digestion andsubsequent peptide purification with ProteinWorks Auto-eXpress Digest and µElution SPE Clean-up Kits.

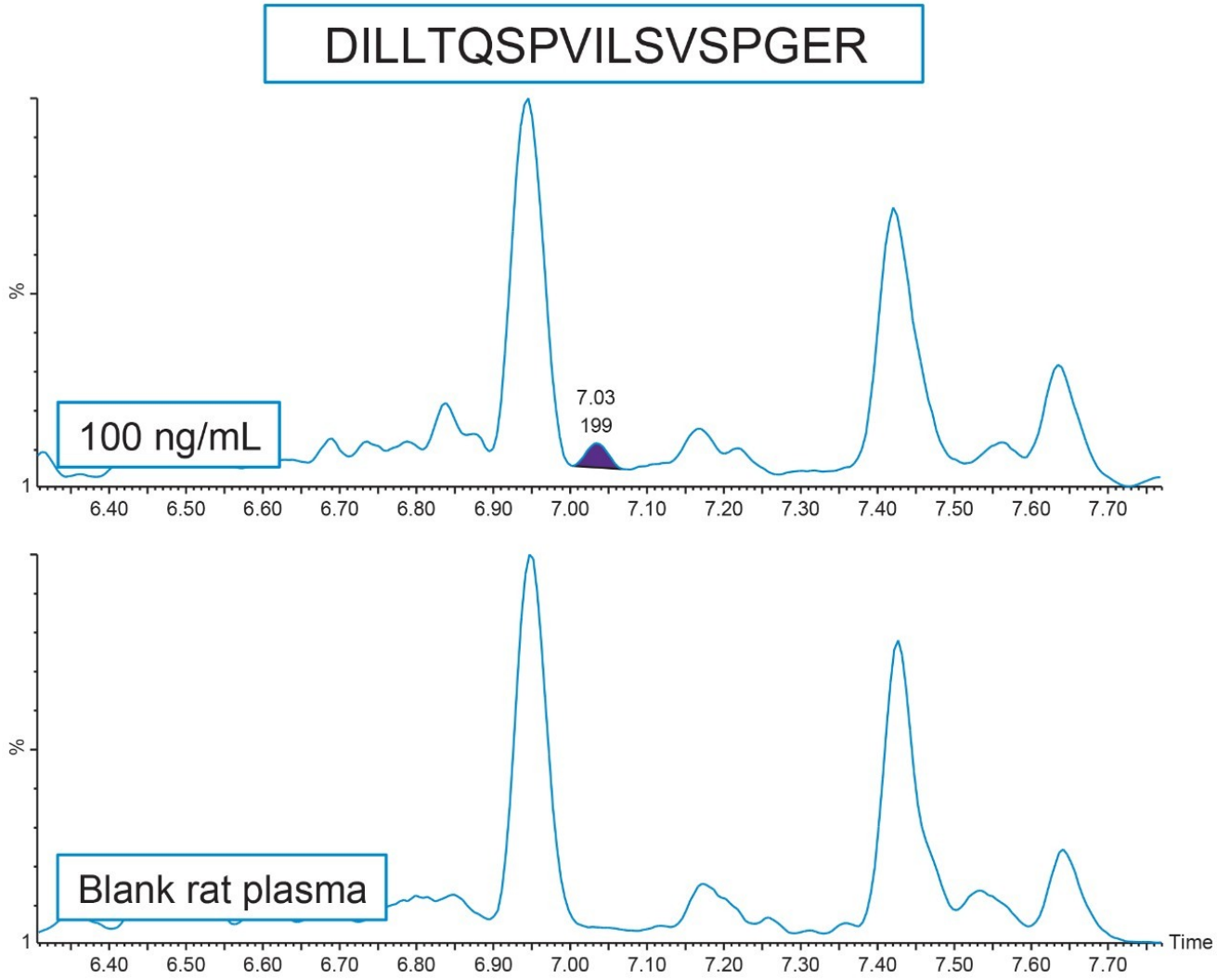


Figure 6. Chromatogram showing 100 ng/mL of cetuximab in rat plasma, compared to blank rat plasma. Cetuximab is quantified using the unique peptide DILLTQSPVILSVSPGER.

Peptide	Std. Curve Range (µg/mL)	r^2	Weighting	Mean % Accuracy
SQVFFK	0.10–250	0.9989	1/x	99.62
YASESISGIPSR	0.50–250	0.9957	1/x	98.90
DILLTQSPVILSVSPGER	0.10–250	0.9979	1/x	100.04
DTLMISR	0.10–250	0.9982	1/x	100.56
ALPAPIEK	0.10–250	0.9951	$1/x^2$	99.56
GPSVFPLAPSSK	0.10–250	0.9942	$1/x^2$	99.91
TTPPVLDSDGSFFLYSK	0.50–250	0.9953	1/x	99.90

Table 2. Linear dynamic range and standard curve statistics for cetuximab tryptic peptides used for quantification. Plasma samples were digested using ProteinWorks Auto-eXpress High 5 Digest and µElution SPE Clean-up Kits and protocols, performed on the Hamilton STAR.

Peptide	QC conc. (µg/mL)	Mean Calc. QC conc. (µg/mL)	Mean %CVs	Mean % accuracy (n=3)	Replicates
SQVFFK	0.25	0.23	7.06	92.35	2 of 3
	4.0	4.14	3.27	103.47	3 of 3
	40.0	40.13	4.37	100.30	3 of 3
	200.0	192.96	6.07	96.47	3 of 3
YASESISGIPSR	4.0	3.75	0.82	93.80	3 of 3
	40.0	40.78	1.34	101.97	3 of 3
	200.0	184.99	4.90	92.50	3 of 3
DILLTQSPVILSVSPGER	0.25	0.27	2.17	105.43	3 of 3
	4.0	4.01	10.58	100.35	2 of 3
	40.0	40.08	2.40	100.23	3 of 3
	200.0	186.77	4.15	93.37	3 of 3
DTLMISR	0.25	0.22	0.64	88.40	2 of 3
	4.0	4.15	2.96	103.67	3 of 3
	40.0	42.34	2.79	105.83	3 of 3
	200.0	189.57	1.78	94.77	3 of 3
ALPAPIEK	0.25	0.24	6.96	97.70	3 of 3
	4.0	4.31	2.78	107.70	3 of 3
	40.0	39.43	1.27	98.57	3 of 3
	200.0	180.35	2.80	90.20	3 of 3
GPSVFPLAPSSK	0.25	0.25	5.90	100.67	3 of 3
	4.0	4.32	4.74	107.93	3 of 3
	40.0	38.57	2.18	96.43	3 of 3
	200.0	187.51	0.97	93.77	3 of 3
TTPPVLDSDGSFFLYSK	4.0	4.14	3.27	103.47	3 of 3
	40.0	40.13	4.37	100.30	3 of 3
	200.0	192.96	6.07	96.47	3 of 3

Table 3. Statistics for QC samples of cetuximab digested in rat plasma, extracted using ProteinWorks Auto-eXpress High 5 Digest and µElution SPE Clean-up Kits and protocols, performed on the Hamilton STAR.

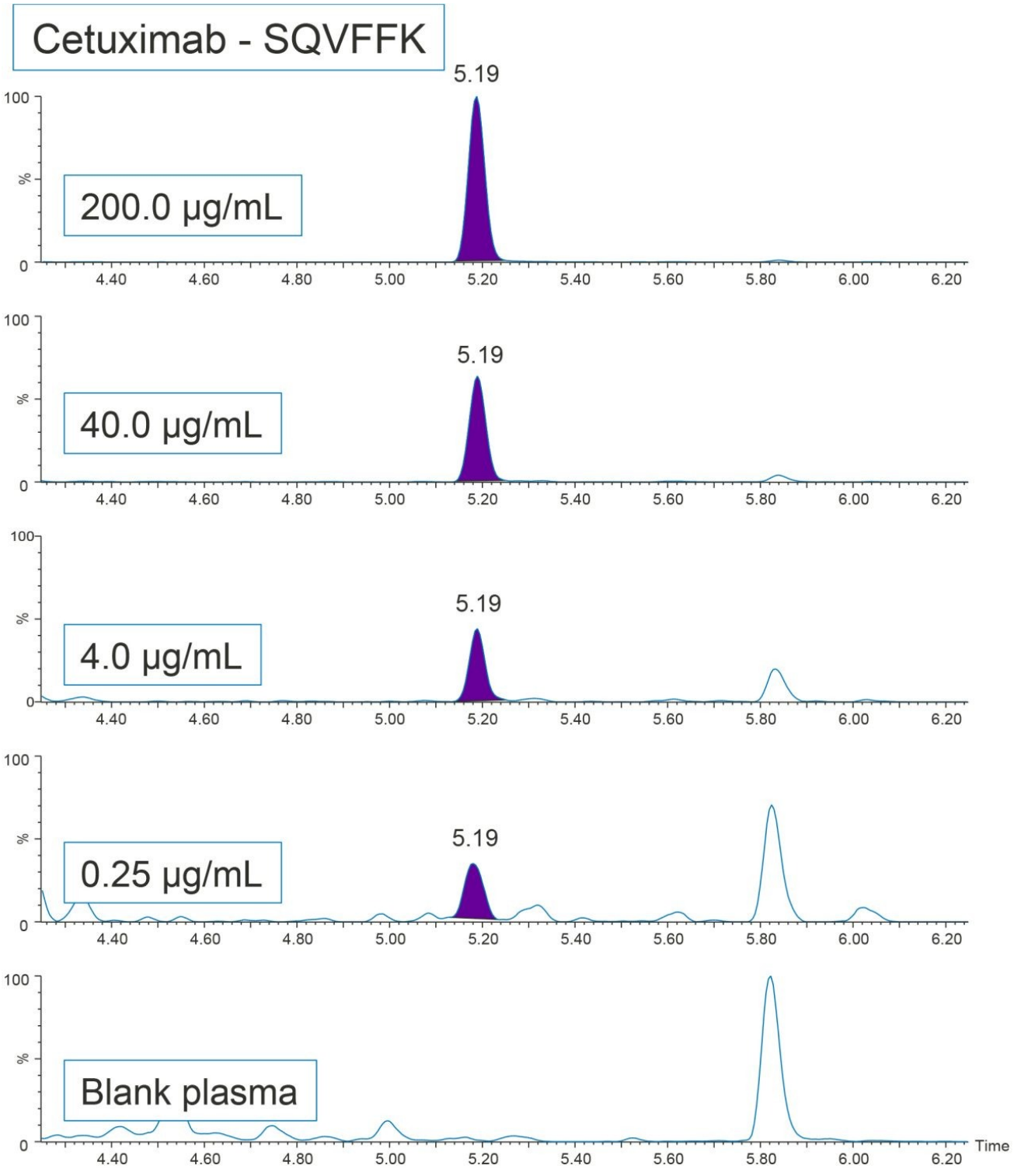


Figure 7. Cetuximab QC chromatograms for the SQVFFK unique signature peptide.

Conclusion

The ProteinWorks Auto-eXpress Digest and µElution SPE Clean-up Kits, fully automated on the Hamilton STAR, were successfully used to quantify cetuximab from rat plasma. This fully automated, kit-based, sample preparation solution facilitates fast digestion and sample enrichment in 6 hours, ensuring sensitive, accurate, and reproducible cetuximab quantification down to 100 ng/mL for any bioanalyst.

References

1. Grand View Research (2017 Mar) Biologics market size worth $399.5 billion by 2025 | growth rate: 3.9%. Retrieved from: http://www.grandviewresearch.com/press-release/global-biologics-market.
2. Grand View Research (2016 Nov) Monoclonal antibodies (mAbs) market size worth $138.6 billion by 2024. Retrieved from: http://www.grandviewresearch.com/press-release/global-monoclonal-antibodies-market.
3. Marnett Research Laboratory, Department of Biochemistry (Accessed 2017 Nov) Cetuximab. Retrieved from: https://medschool.vanderbilt.edu/marnett-lab/cetuximab.
4. FDA Guidance for Industry for Bioanalytical Method Validation, CDER.

Featured Products

ACQUITY UPLC System <https://www.waters.com/514207>

Xevo TQ-S <https://www.waters.com/10160596>

MassLynx Mass Spectrometry Software <https://www.waters.com/513164>

720006209EN, February 2018