

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

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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 C₁₈,
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.

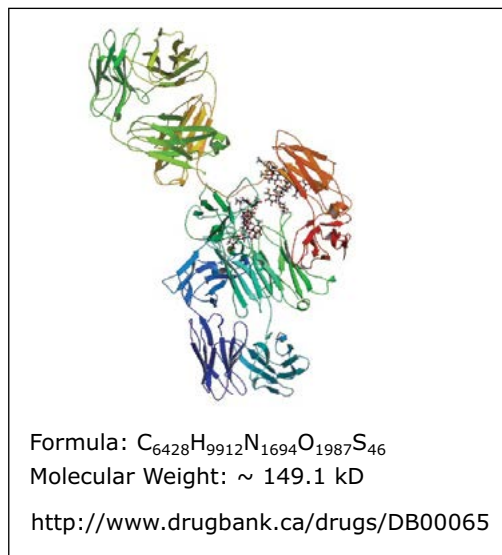


Figure 1. Infliximab (Remicade) protein structure.

EXPERIMENTAL

Sample description

Infliximab was first immuno-purified from 35 μ L 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 μ Elution SPE Clean-up Kit and Protocol.

Method conditions

LC system: ACQUITY UPLC
 Detection: Xevo TQ-S Mass Spectrometer, ESI+
 Column: ACQUITY UPLC Peptide BEH C₁₈, 300Å, 1.7 μ m, 2.1 mm x 150 mm
 Column temp.: 55 °C
 Sample temp.: 10 °C
 Injection volume: 10 μ L
 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		Curve
		%A	%B	
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 (kv): 3
 Cone (V): 30
 Source offset (V): 50
 Source temp. (°C): 150
 Desolvation temp. (°C): 600
 Cone gas flow (L/hr): 150
 Collision gas flow
 (mL/min): 0.15
 Nebulizer gas flow
 (Bar): 7

Peptide	MRM Transition	Cone Voltage (V)	Collision Energy (eV)
DILLTQSPAILSVSPPGER*	633.10>731.80	31	21
SINSATHYAESVK*	469.6>603.80	40	10
DSTYLSSTLTLSK	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 (DILLQSPAILSVSPPGER, light chain) and generic (DSTYLSSTLTLSK, 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 μ L 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.

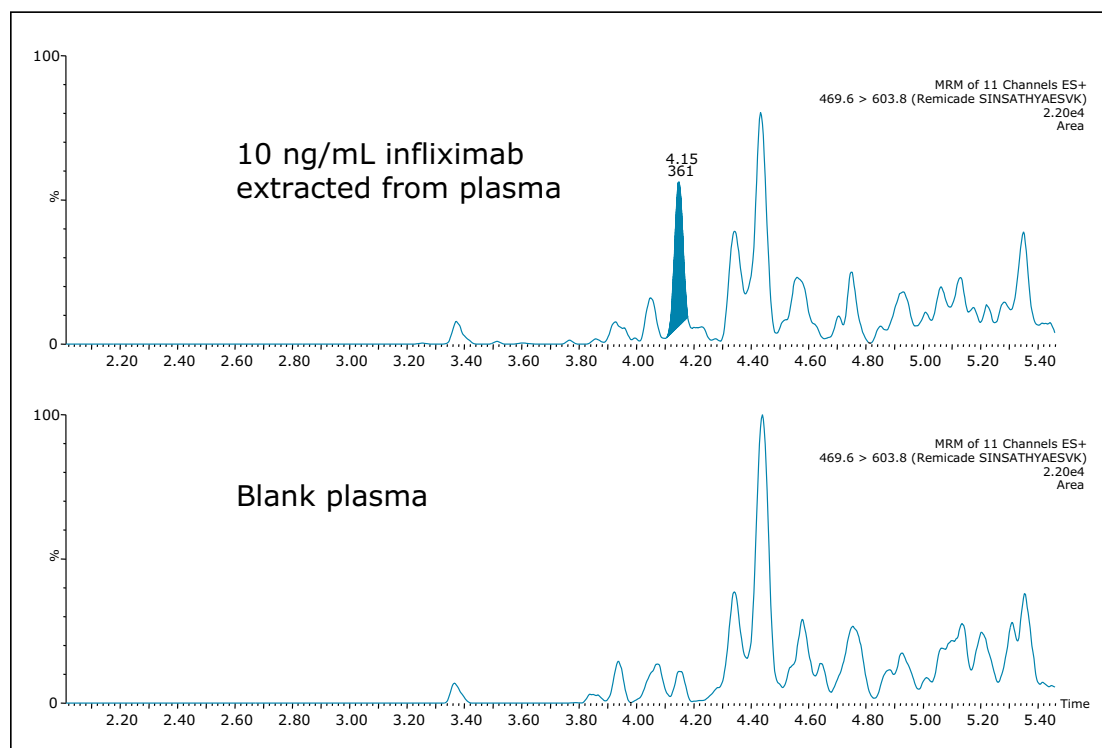


Figure 2. Chromatogram showing 10 ng/mL of infliximab in rat plasma, as compared to blank rat plasma. Infliximab is quantified using the unique peptide SINSATHYAESVK.

Peptide	Std. curve range (μ g/mL)	Weighting	Linear fit (r^2)	Mean % accuracy of all points
DILLQSPAILSVSPPGER*	0.05–250	1/X	0.998	100.00
SINSATHYAESVK*	0.01–100	1/X ²	0.995	98.47
DSTYLSSTLTLSK	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
DILLTQSPAILSVPGER*	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
DSTYLSSTLTLK	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.

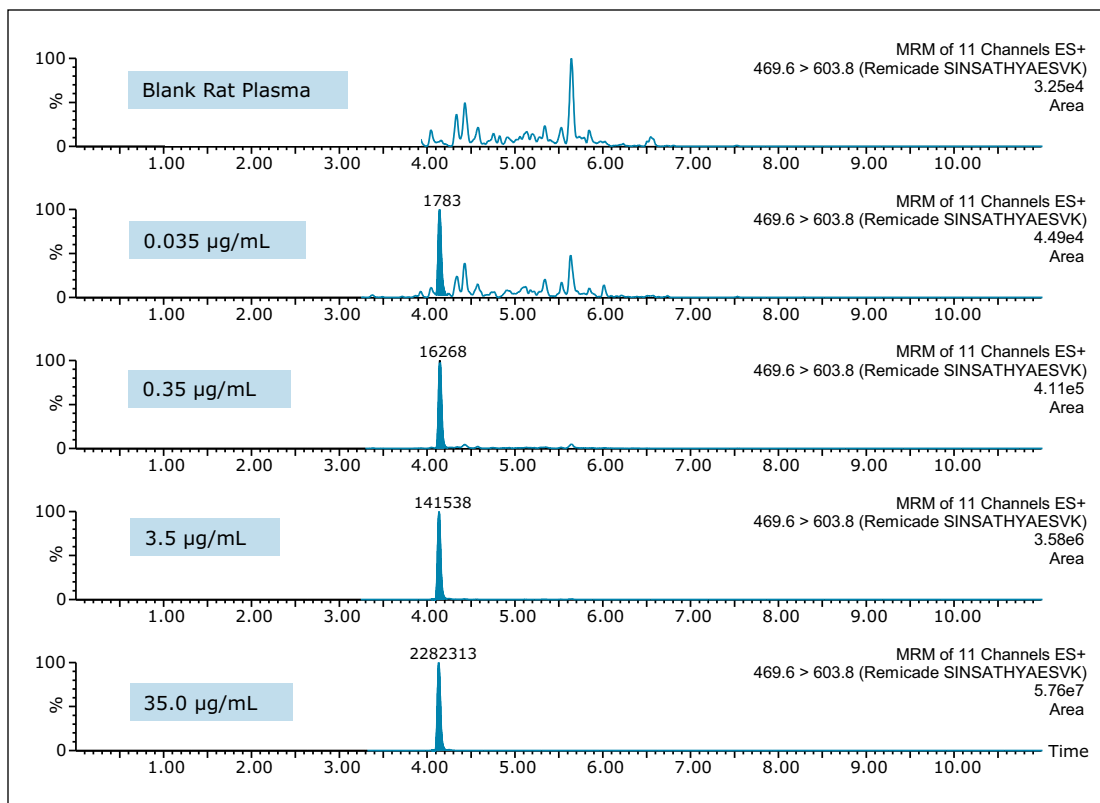


Figure 3. Infliximab QC chromatograms for the SINSATHYAESVK Unique Signature Peptide.

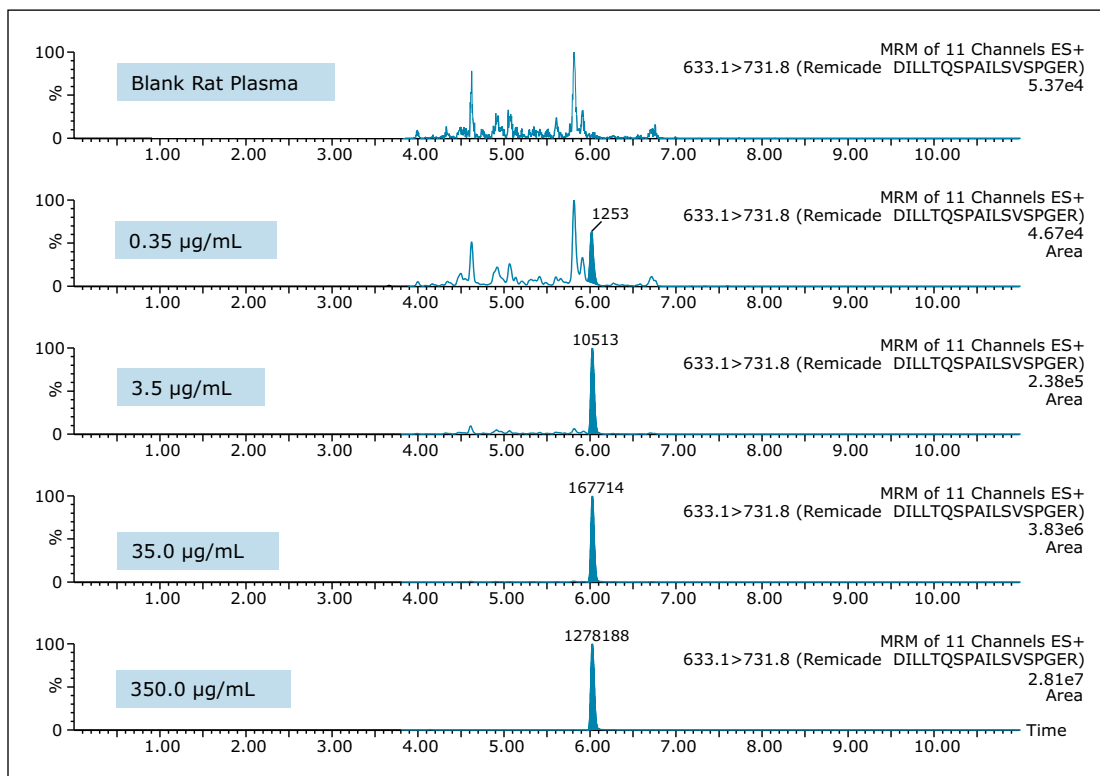


Figure 4. Infliximab QC chromatograms for the DILLTQSPAILSVSPPGER Unique Signature Peptide.

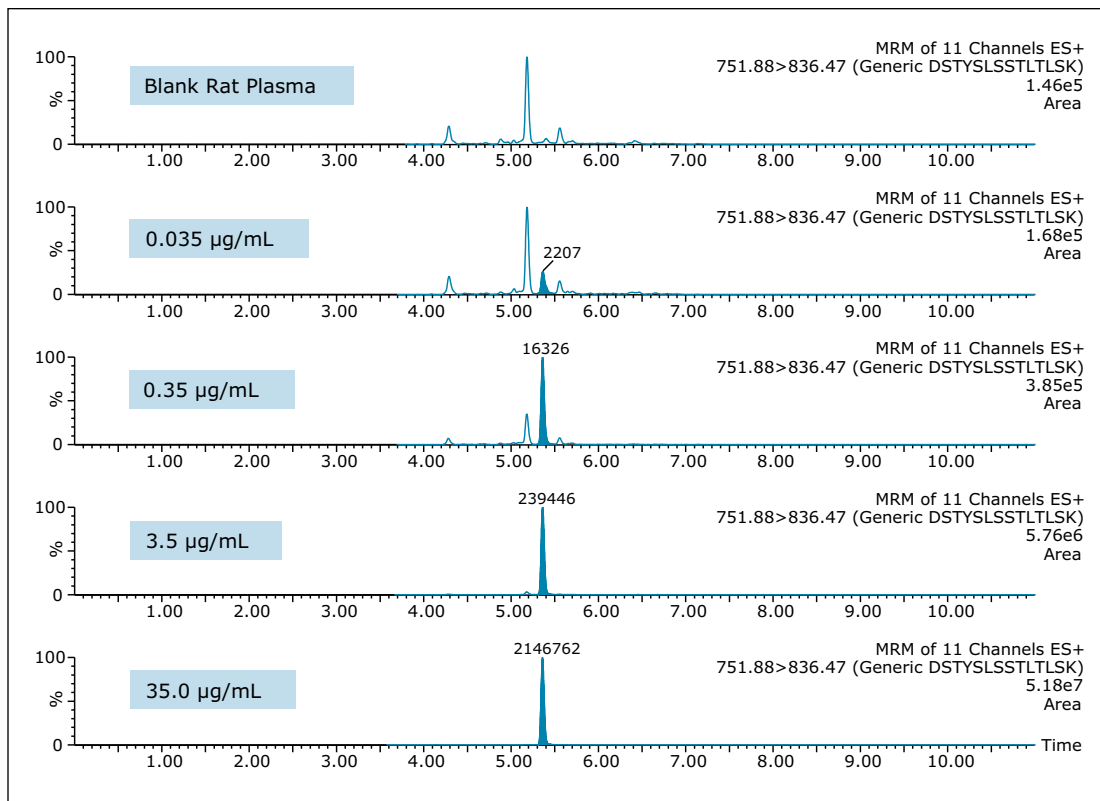


Figure 5. Infliximab QC Chromatograms for the DSTYLSSTLTLSK Generic Signature Peptide.

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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