

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

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### Abstract

This application note describes the fast, sensitive quantification of infliximab from rat plasma using the ProteinWorks eXpress Digest Kit and Protocol.

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.

### 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.

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## 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:  $\sim 149.1$  kD

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

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*Figure 1. Infiximab (Remicade) protein structure.*

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## Experimental

### Sample description

Infliximab was first immuno-purified from 35  $\mu$ 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  $\mu$ 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 C <sub>18</sub> , 300Å, 1.7 $\mu$ m, 2.1 mm x 150 mm
Column temp.:	55 °C
Sample temp.:	10 °C
Injection volume:	10 $\mu$ L
Mobile phase A:	0.1% formic acid in water
Mobile phase B:	0.1% formic acid in acetonitrile
Data management:	MassLynx (v4.1)

### Gradient

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Flow rate (mL/min)	Time (min)	Profile %A	Profile % B	Curve
0.3	0.0	100.0	0.0	6.0
0.3	1.0	100.0	0.0	6.0
0.3	7.0	50.0	50.0	6.0
0.3	8.0	10.0	90.0	6.0

## MS conditions

Capillary (kv):	3.0
Cone (V):	30.0
Source offset (V):	50.0
Source temp. (°C):	150.0
Desolvation temp. (°C):	600.0
Cone gas flow (L/hr):	150.0
Collision gas flow (mL/min):	0.15
Nebulizer gas flow (Bar):	7.0

Peptide	MRM Transition	Cone Voltage (V)	Collision Energy (eV)
DILLTQSPAILSVPGER*	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,<sup>1</sup> 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 (DILLTQSPAILSVPGER, 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 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.

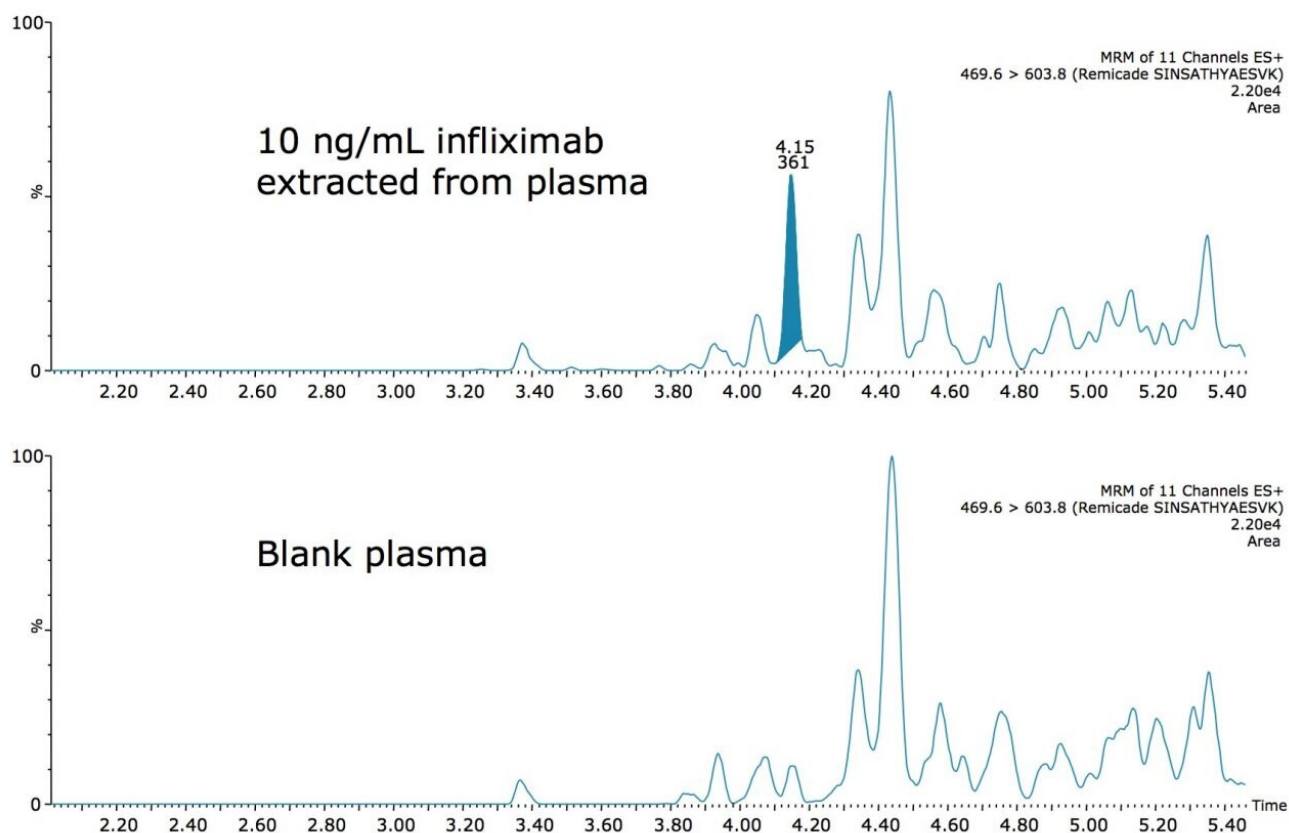


Figure 2. Chromatogram showing 10 ng/mL of infliximab in rat plasma, as compared to blank rat plasma. Infliximab is quantified using the unique peptide SINSATHYAESVK.

Peptide	Std. curve range ( $\mu\text{g/mL}$ )	Weighting	Linear fit ( $r^2$ )	Mean % accuracy of all points
DILLTQSPAILSVPGER*	0.05–250	1/X	0.998	100.00
SINSATHYAESVK*	0.01–100	1/X <sup>2</sup>	0.995	98.47
DSTYSLSSTLTLSK	0.10–500	1/X <sup>2</sup>	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 ( $\mu\text{g/mL}$ )	Mean cal. conc ( $\mu\text{g/mL}$ )	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 ( $\mu\text{g/mL}$ )	Mean cal. conc ( $\mu\text{g/mL}$ )	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 ( $\mu\text{g/mL}$ )	Mean cal. conc ( $\mu\text{g/mL}$ )	Std. dev.	%CV	Mean accuracy
DSTYLSSTLTLSK	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.

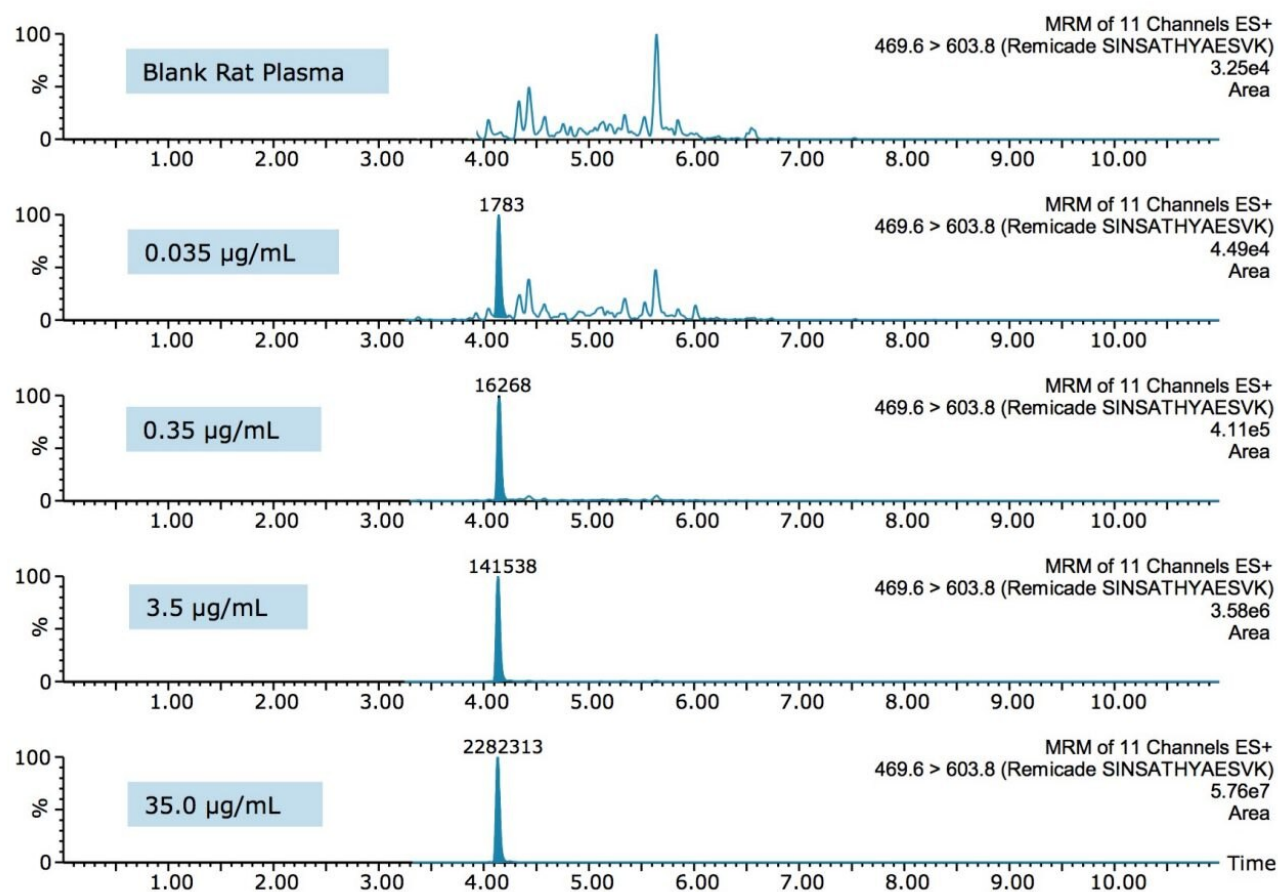


Figure 3. Infliximab QC chromatograms for the SINSATHYAESVK Unique Signature Peptide.

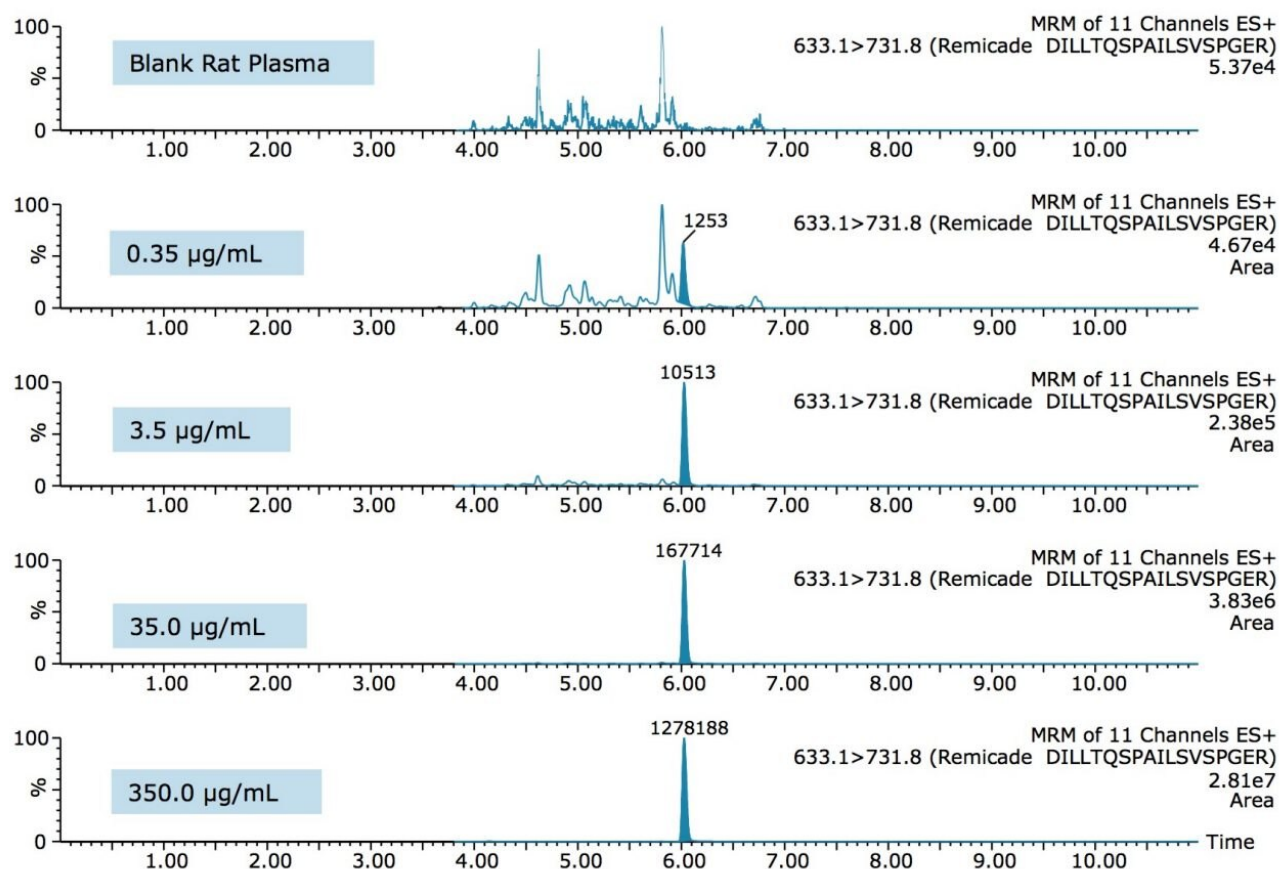


Figure 4. Infliximab QC chromatograms for the DILLTQSPAILSVSPGER Unique Signature Peptide.

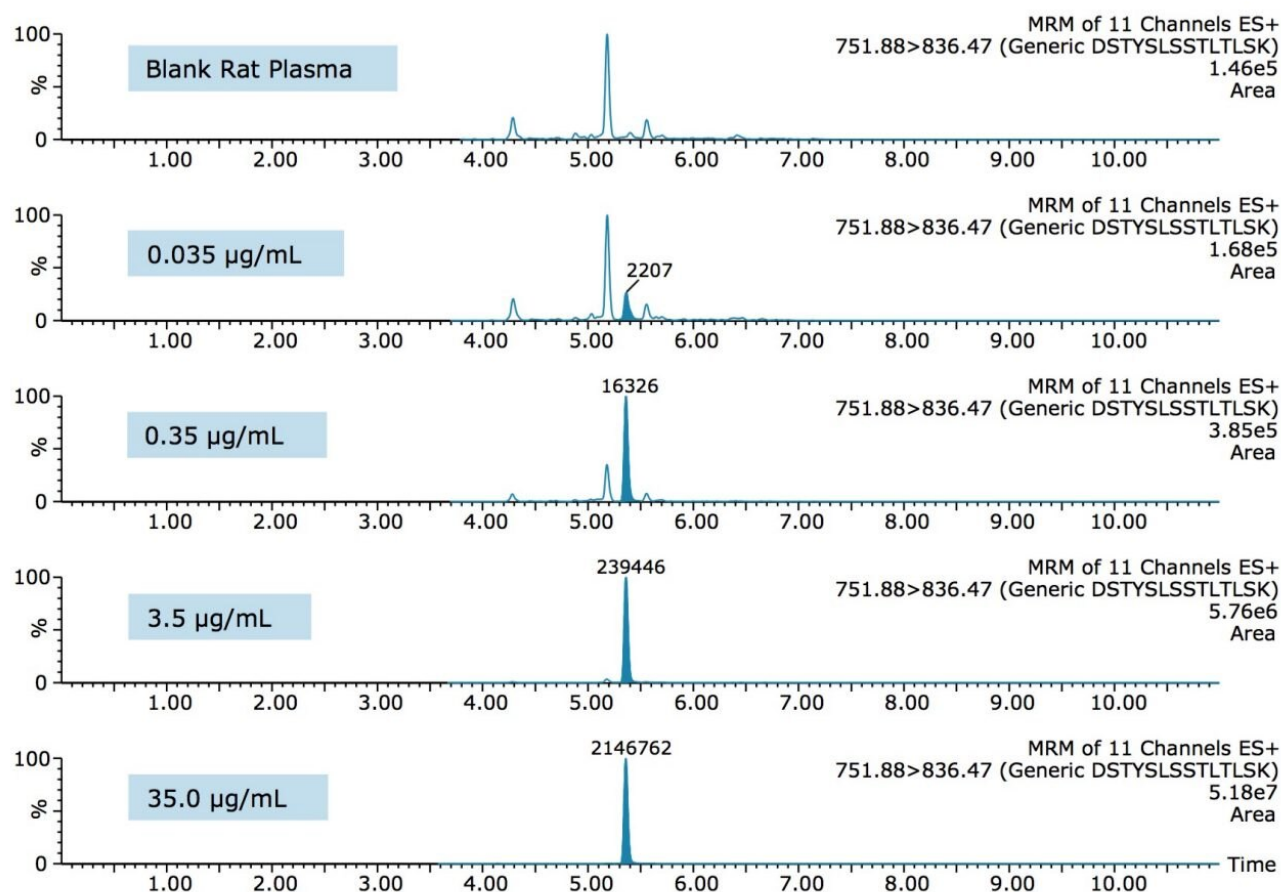


Figure 5. Infliximab QC Chromatograms for the DSTYLSSTLTLSK Generic Signature Peptide.

## Conclusion

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.

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## References

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

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## Featured Products

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

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

### Available for purchase online:

ProteinWorks eXpress Digest Kit <<https://www.waters.com/waters/partDetail.htm?partNumber=176003689>>

Intact mAb Mass Check Standard <  
<https://www.waters.com/waters/partDetail.htm?partNumber=186006552>>

ACQUITY UPLC Peptide BEH C18, 300Å, 1.7 µm, 2.1 mm X 150 mm Column <  
<https://www.waters.com/waters/partDetail.htm?partNumber=186003687>>

ProteinWorks uElution SPE Clean-up Kit <  
<https://www.waters.com/waters/partDetail.htm?partNumber=186008304>>

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