ATLAS ANTIBODIES

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A robust method for the quantification of multiple apolipoproteins in human plasma based on MRM-LC/MS and QPrEST™ internal standards

Application benefits

- Robust and rapid sample preparation with ProteinWorks™ eXpress Direct Digest Kit
- QPrEST™ as internal standard ensures high accuracy and precision
- Short LC-MS method with high multiplexing capacity
- Combining efficient sample prep and QPrEST™ internal standards enables straightforward and robust protein quantification

QPrEST
High Accuracy Targeted Proteomics

Introduction

Apolipoproteins bind to and help solubilize hydrophobic lipids in the blood and play key roles in the structure of lipoprotein particles, as ligands for receptors, and as co-factors for enzymes. As a result, apolipoproteins are associated with a range of diseases, including cardiovascular disease (APOA1 and APOB)[1], Alzheimer's disease (APOE, APOC1 and APOJ)[2], schizophrenia (APOD)[3], and APOM may be involved in atherosclerosis, diabetes, and renal disease[4]. Apolipoproteins have therefore emerged as key risk biomarkers and important research targets.

Enzyme-linked immunosorbent assay (ELISA) is commonly used to quantify apolipoproteins in complex samples. There are, however, disadvantages in using immunoassays for standardized workflows, including limitations in multiplexing, throughput and batch-to-batch variation of the antibodies.

As an alternative to ELISA the combination of liquid chromatography and mass spectrometry (LC-MS) has become a key tool for the absolute quantification of proteins and peptides in biological samples. The complexity of the LC-MS workflow and the need to compensate for method variation, however, requires the use of internal standards to ensure accurate and precise data.

Stable-isotope labeled (SIL) versions of the tryptic peptides are commonly used as internal standards but do not monitor all changes and variations in sample preparation even though they can be added early. The result could be poor accuracy of determination in complex samples. These limitations have led to the development of QPrEST standards (Atlas Antibodies).

QPrEST standards can be used in multiple reaction monitoring (MRM)-based mass spectrometry, which increases sensitivity and robustness, readily supports multiplexing, and enables the detection of proteins for which antibodies are not available[5].

In addition, MRM-based assays that incorporate QPrEST standards enable absolute quantification in complex matrices such as human plasma and cerebrospinal fluid[6] with a broad dynamic range to enable more time-and cost-efficient data generation compared to ELISA.

In this application note, we demonstrate how QPrEST standards in combination with standardized, rapid and efficient protein cleavage using ProteinWorks™ eXpress Direct Digest Kit (Waters Corporation) can be used to accurately quantify apolipoproteins in human plasma.

QPrEST™ - Stable Isotope-Labeled protein standards for protein quantification using MS

QPrEST standards are designed to accurately correct for any variations in protein digestion prior to quantification by MS by reproducing the cleavage of target proteins. To do this, QPrEST standards include amino acid sequences surrounding the digestion site that are known to affect cleavage and generate peptides identical to those of the endogenous protein. QPrEST standards are heavy isotope-labeled MS standards produced recombinantly in a bacterial host. Each standard contains a stretch of 50–150 amino acids identical to the target human protein sequence, including at least two unique tryptic peptides. To generate the tryptic peptides used for quantification, the QPrEST standards are added to the sample prior to digestion, which minimizes quantitative errors caused by variation in digestion efficiency between standard and endogenous protein during the cleavage reaction.

Experimental

Sample preparation

Heavy isotope-labeled QPrEST standards (Atlas Antibodies) targeting apolipoproteins APOA1, APOB, APOD, APOE, APOH and APOM were pooled and spiked into 30 human plasma samples (5 µL). The QPrEST mix was prepared to generate heavy-to-light peptide ratios close to 1 for each of the proteins. Spiked plasma samples were digested with ProteinWorks™ eXpress Direct Digest Kit (Waters Corporation) using the 5-step protocol including reduction and alkylation steps, which took 2 hours. Post-digestion cleanup was performed with ProteinWorks™ µElution SPE Clean-up Kit (Waters Corporation). Sample preparation took less than four hours (two hours digestion).

Protein quantification

Peptide samples were analyzed by targeted LC-MS according to the specified parameters. The average heavy-to-light peptide ratio for each protein in each sample was used to calculate the plasma concentrations of the six target proteins. Three technical replicates were prepared for each plasma sample.

Method conditions

LC-system: Waters ACQUITY UPLC I-Class PLUS System

MS system: Waters Xevo TQ-XS

LC conditions

Column:	Waters ACQUITY PREMIER BEH C18 Column, 1.7 µm, 2.1 x 150 mm
Column temp:	50 ˚C
Sample temp:	10 ˚C
Injection volume:	5 µL
Mobile phases A:	0.1% formic acid in water
Mobile phases B:	0.1% formic acid in acetonitrile

Gradient:

Time	Flow	%A	%B	Curve
Initial	0.5	90	10	Initial
0.2	0.5	90	10	6
5.0	0.5	55	45	6
5.1	0.5	2	98	6
6.5	0.5	2	98	6
6.51	0.5	90	10	6
8.5	0.5	90	10	6

MS conditions

Ionization mode:	ESI+
Capillary voltage:	0.5 kV
Source temp:	150 °C
Desolvation temp:	650 °C
Cone gas flow:	150 L/Hr
Desolvation gas flow:	100 L/Hr
Nebuliser gas flow:	7.0 Bar
MS Software:	Waters MassLynx

Results and Discussion

Method development led to the selection of QPrEST standards comprising peptide sequence pairs for each apolipoprotein analyte (**Figure 1**), and MRM transitions with highest intensity were chosen for quantification (**Table 1**).

Apolipoprotein A

MKAAVLTLAVLFLTGSQARHFWQQDEPPQSPWDRVKDLATVYVDVLKDSGRDYVSQFEGSALGKQLNLKLLNWD
SVTSTFSKLREQLGPVTQEFWDNLEKETEGLRQEMSKDLEEVKAKVQPYLDDFQKKWQEEMELYRQKVEPLRAEL
QEGARQKLHELQEKLSPLGEEMRDRARAHVDALRTHLAPYSDELRQRLAARLEALKENGGARLAEYHAKATEHLSTL
SEKAKPALEDLRQGLLPVLESFKVSFLSALEEYTKKLNTQ

Apolipoprotein B

....SLMIQKAAIQALRKMEPKDKDQEVLLQTFLDDASPGDKRLAAYLMLMRSPSQADINKIVQILPWEQNEQVKNFVAS
HIANILNSEELDIQDLKKLVKEALKESQLPTVMDFRKFSRNYQLYKSVSLPSLDPASAKIEGNLIFDPNNYLPKESMLKT
TLTAFGFASADLIEIGLEGKGFEPTLEALFGKQGFFPDSVNKALYWVNGQVPDGVSKVLVDHFGYTKDDKHEQDMVN
GIMLSVEKLIKDLKSKEVPEARAYLRILGEELG.....

Apolipoprotein D

MVMLLLLLSALAGLFGAAEGQAFHLGKCPNPPVQENFDVNKYLGRWYEIEKIPTTFENGRCIQANYSLMENGKIKVLN
QELRADGTVNQIEGEATPVNLTEPAKLEVKFSWFMPSAPYWILATDYENYALVYSCTCIIQLFHVDFAWILARNPNLPP
ETVDSLKNILTSNNIDVKKMTVT DQVNCPKLS

Apolipoprotein E

...YLRWVQTLSEQVQEELLSSQVTQELRALMDETMKELKAYKSELEEQLTPVAEETRARLSKELQAAQARLGADMED
VCGRLVQYRGEVQAMLGQSTEELRVRLASHLRKLRKRLLRDADDLQKRLAVYQAGAREGAERGLSAIRERLGPLVE
QGRVRAATVGSLAGQPLQERAQAWGERLRARMEEMGSRTRDRLDEVKEQVAEVRAKLEEQAQQIRLQAEAFQAR
LKSWFEPLVEDMQRQWAGLVEKVQAAVGTSAAPVPSDNH

Apolipoprotein H

MISPVLILFSSFLCHVAIAGRTCPKPDDLPFSTVVPLKTFYEPGEEITYSCKPGYVSRGGMRKFICPLTGLWPINTLKCT
PRVCPFAGILENGAVRYTTFEYPNTISFSCNTGFYLNGADSAKCTEEGKWSPELPVCAPIICPPPSIPTFATLRVYKPS
AGNNSLYRDTAVFECLPQHAMFGNDTITCTTHGNWTKLPECREVKCPFPSRPDNGFVNYPAKPTLYYKDKATFGCH
DGYSLDGPEEIECTKLGNWSAMPSCKASCKVPVKKAT...

Apolipoprotein M

MFHQIWAALLYFYGIILNSIYQCPEHSQLTTLGVDGKEFPEVHLGQWYFIAGAAPTKEELATFDPVDNIVFNMAAGSAP
MQLHLRATIRMKDGLCVPRKWIYHLTEGSTDLRTEGRPDMKTELFSSSCPGGIMLNETGQGYQRFLLYNRSPHPPEK
CVEEFKSLTSCLDSKAFLLTPRNQEACELSNN

Figure 1 *QPrEST™ APOA1, APOB, APOD, APOE, APOH and APOM sequences. The protein sequence (in light grey), corresponding QPrEST sequence (in black) and the tryptic peptides (blue and orange) used for quantification.*

QPrEST standards for the apolipoproteins were added to human plasma samples at levels that approximated the expected levels of the target apolipoproteins to maximize the accuracy of quantification. The spiked samples were then digested using ProteinWorks eXpress Direct Digest Kit (Waters Corporation) with a 5-step protocol including reduction and alkylation steps. Representative chromatograms for the peptides used to quantify the apolipoproteins are shown in **Figure 2**.

Protein	QPrEST standard	Peptide	MRM Transition	Cone voltage (V)	Collision energy (eV)
APOA1	QPrEST22529	VQPYLDDFQK (1) EQLGPVTQEFWDNLEK (2)	626.8>1025.5 645.0>804.4	35 35	22 14
APOB	QPrEST22605	IEGNLIFDPNNYLPK (3) SVSLPSLDPASAK (4)	874.0>1107.6 636.4>885.5	35 35	31 22
APOD	QPrEST22652	IPTTFENGR (5) WYEIEK (6)	517.8>461.2 434.2>681.4	36 35	18 15
APOE	QPrEST22530	AKLEEQAQQIR (7) LQAEAFQAR (8)	657.4>1114.6 517.3>792.4	35 35	28 18
APOH	QPrEST22549	FICPLTGLWPINTLK (9) VCPFAGILENGAVR (10)	887.0>685.4 751.9>1243.7	35 35	35 39
APOM	QPrEST22218	AFLLTPR (11) FLLYNR (12)	409.3>599.4 413.2>565.3	35 35	14 14

Table 1. *Tryptic peptides with MS conditions included in the final MRM method that took only eight minutes to run.*

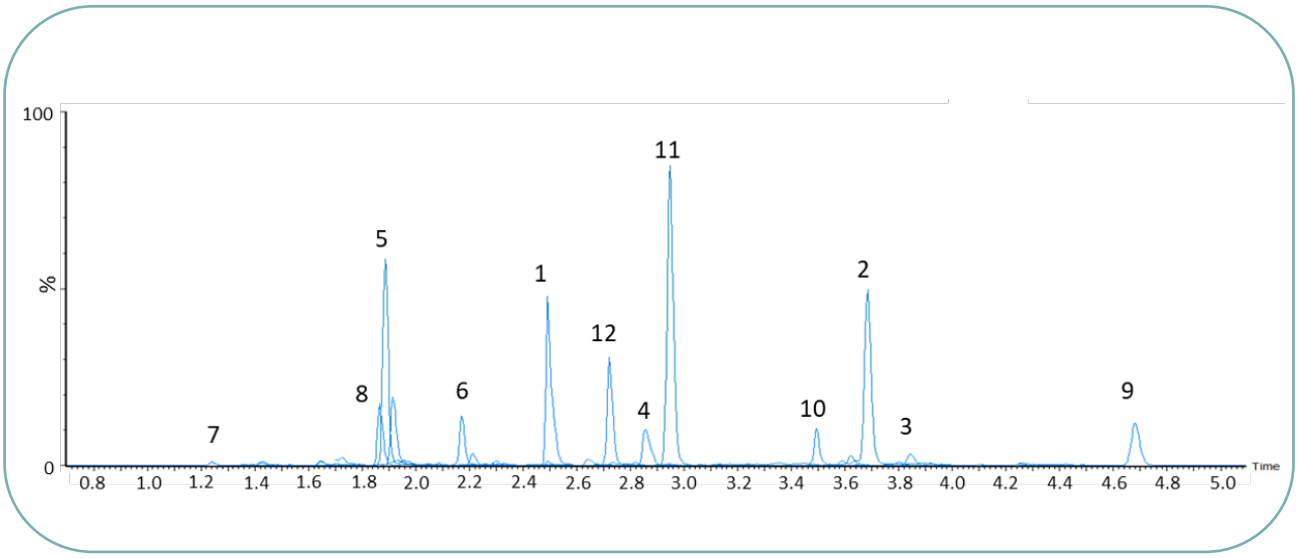

Figure 2. *Representative chromatograms for the peptides used to quantify the apolipoproteins. Peptides were separated using an ACQUITY PREMIER BEH C18, 1.7 µm, 2.1 x 150 mm column. The corresponding peptide of each peak is listed in Table 1.*

Each apolipoprotein was quantified using two QPrEST peptides, which were cleaved together with the native, full-length protein. The technical replicates showed a high level of reproducibility within replicates and peptide standard pairs, indicating efficient digestion using the ProteinWorks™ eXpress Direct Digest Kit and reliable quantification using the QPrEST standards (**Figure 3**).

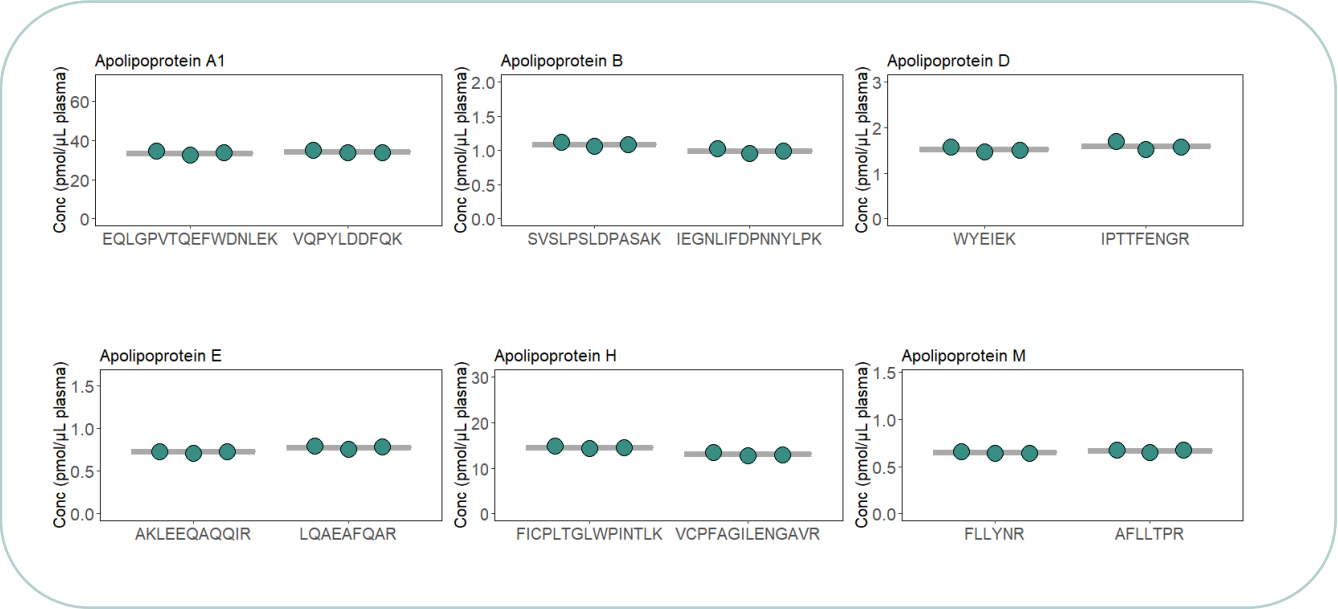

Figure 3. *The measured protein concentration in triplicate for each peptide in a representative human plasma sample.*

The method was then used to determine the concentration of the apolipoproteins in plasma samples from 30 individuals in triplicate (**Figure 4**). The concentrations of the investigated proteins spanned roughly two orders of magnitude with only small variation between samples for each protein analyte, as expected from healthy individuals. Within each sample, the technical variation between three separate digested samples was low, with a coefficient of variation generally <5 % (**Table 2**). This by far exceeds the variation criteria for an LC-MS assay, where a coefficient of variation below 20% is usually considered acceptable[7]. The high precision of this data indicates that the QPrEST LC-MS/MS workflow is resistant to variations both in sample preparation and MS analysis.

Protein	Lowest conc (pmol/µL) N=30	Highest conc (pmol/µL) N=30	Mean CV (%) N=30
APOA1	25.0	47.0	4.2
APOB	0.62	1.97	2.4
APOD	1.38	3.91	2.8
APOE	0.41	1.12	1.9
APOH	9.62	18.2	2.4
APOM	0.39	1.01	1.7

Table 2. *Concentration intervals (pmol/µL plasma) and average coefficient of variation (CV) between three technical replicates across 30 human plasma samples.*

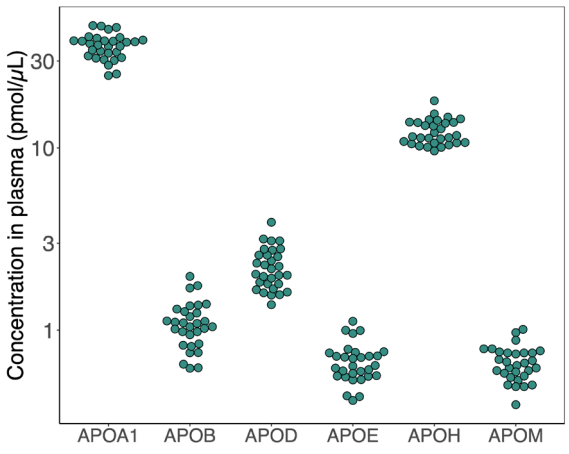

Figure 4. *Concentrations of apolipoproteins in 30 human plasma samples determined using QPrEST internal standards. Each circle represents one patient sample and shows averaged data from two peptides measured in three replicates. Concentrations are shown in log scale to clearly visualize the dynamic range between the apolipoproteins.*

The performance of the QPrEST LC-MS/MS method was also compared with ELISA (**Figure 5**). ELISA data for four of the target apolipoproteins was generated with MabTech ELISAPRO kits according to the manufacturer's instructions, using the same plasma samples. There was good correlation between the results from ELISA and QPrEST LC-MS/MS. The differences in absolute values between the two methods could be due to differences in the internal or external standards that rely on an accurate concentration measurement of the standard*. In addition, epitope availability and/or batch-to-batch variation of antibodies can affect the amount of protein measured in antibody-based assays.

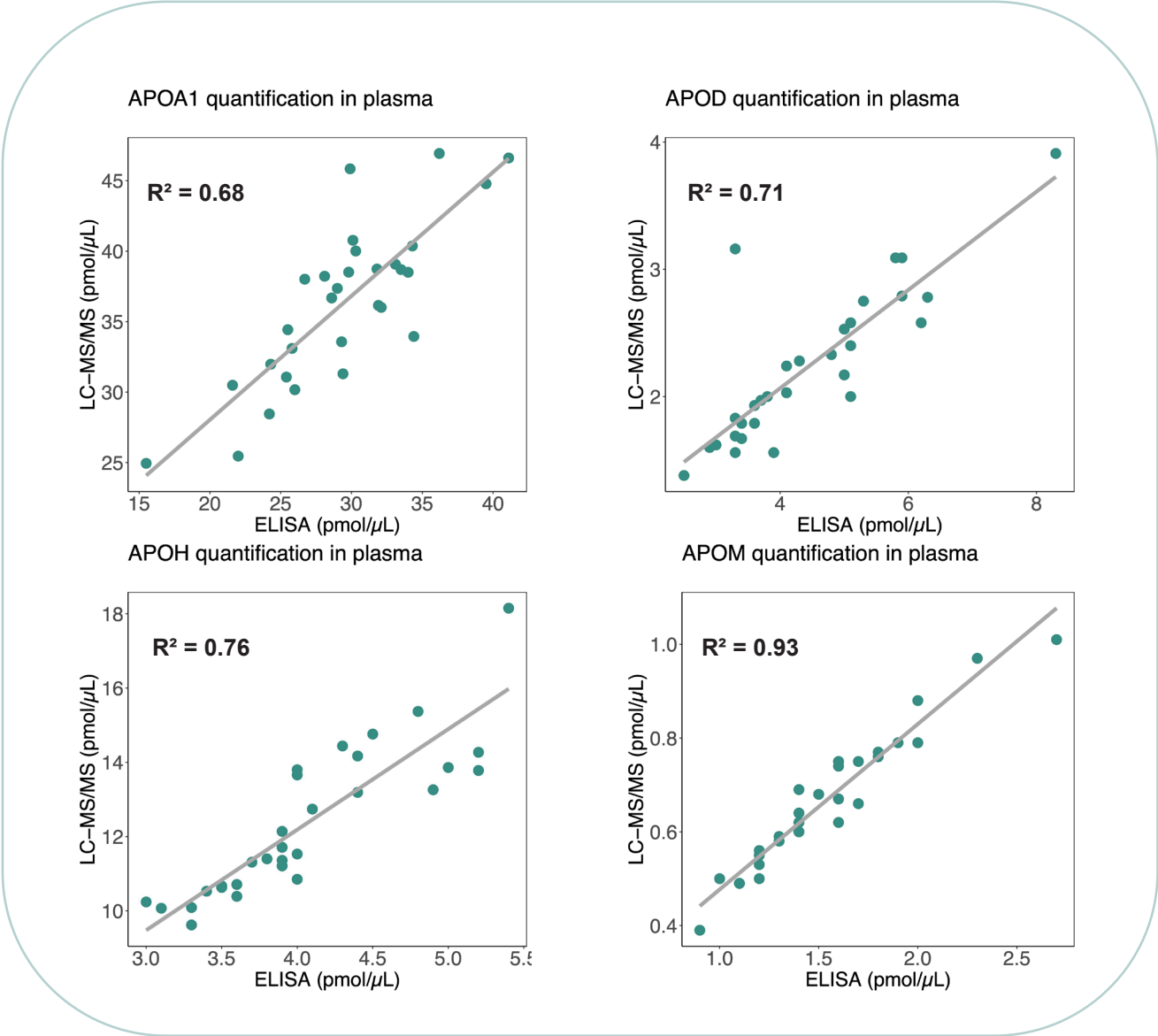

Figure 5. *Correlation of plasma protein concentrations determined by the QPrEST LC-MS/MS method and ELISA for APOA1, APOD, APOH and APOM. LC-MS/MS data is shown on the x-axis and ELISA data is shown on the y-axis. Correlation coefficients (R^2) are presented for each protein.*

***QPrEST™** concentration is determined using an LC-MS/MS setup with an amino acid analyzed protein as reference*

Conclusions

These results show that efficient, standardized protein cleavage using ProteinWorks eXpress Direct Digest Kit and QPrEST internal standards can be combined in a multiplex method to quickly generate accurate and precise data on the levels of several apolipoproteins in human plasma samples. Sample preparation using the ProteinWorks eXpress Direct Digest Kit with 5-step protocol took three hours and the MRM method enabled the quantification of six proteins in eight minutes.

This robust, standardized approach enables the rapid quantification of proteins in complex mixtures even when antibodies are not available. The high correlation between LC-MS/MRM and ELISA data demonstrates that these methods can be used for cross-validation and gives confidence in the LC-MS/MRM results.

References

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Atlas Antibodies AB in collaboration with Waters Sverige AB.

Atlas Antibodies AB
Voltavägen 13A
SE-168 69 Bromma, Sweden
atlasantibodies.com

Phone +46(0)8 54 59 58 50
contact@atlasantibodies.com
order@atlasantibodies.com
support@atlasantibodies.com

052 - 210320