Enhanced biopharmaceutical characterization using the SELECT SERIES™ MRT

INTRODUCTION

Today, an ever-increasing number of approved therapeutics fall into the biologics class. As this number grows so do the requirements of the analytics used to characterize and develop further therapeutics, in particular of liquid chromatography-mass spectrometry. The SELECT SERIES MRT is a first-of-its-kind hybrid quadrupole Multi-Reflecting Time-of-Flight (Q-MRT) mass spectrometer capable of ultra-high mass resolving power of >200,000 FWHM and mass accuracy in the parts-per-billion range. When fitted with an ESI Lockspray ionization source and coupled to Waters state-of-the-art chromatographic hardware the MRT LC-MS system provides a powerful solution to address challenges in the analysis of biologics.

Ions are generated in the universal atmospheric pressure ionisation (API) source and are passed through a second generation StepWave™ XS ion transfer optics which provides improved transmission, especially for more labile compounds.

The quadrupole mass analyser can be operated in both resolving, up to 8 kDa, and non-resolving modes. Ions are subsequently transmitted through a series of RF stacked ring ion guides that lead to a segmented quadrupole collision cell that also provides spatial focusing of the ion beam into the focusing optics prior to the ToF. At the pusher, ions are injected into the MRT analyser via a unique double orthogonal acceleration scheme.

Unlike traditional ToF, the gridless analyser provides three dimensional focusing through multiple intra-ToF lenses allowing 46 reflections within the ToF and a flight path of over 47 meters with minimal losses. Flight times in the MRT analyzer are extremely long, for example *m/z* 1000 has a flight time of approximately 1.37 ms.

An encoded pushing approach is employed to recover lost duty cycle resulting from these long flight times whereby ions are injected into the ToF prior to ions from previous pushes reaching the detector. The relative injection times are varied to encode the data allowing for accurate de-convolution providing mass spectra with high resolution (>200,000 FWHM) and excellent mass accuracy in the parts-per-billion range.

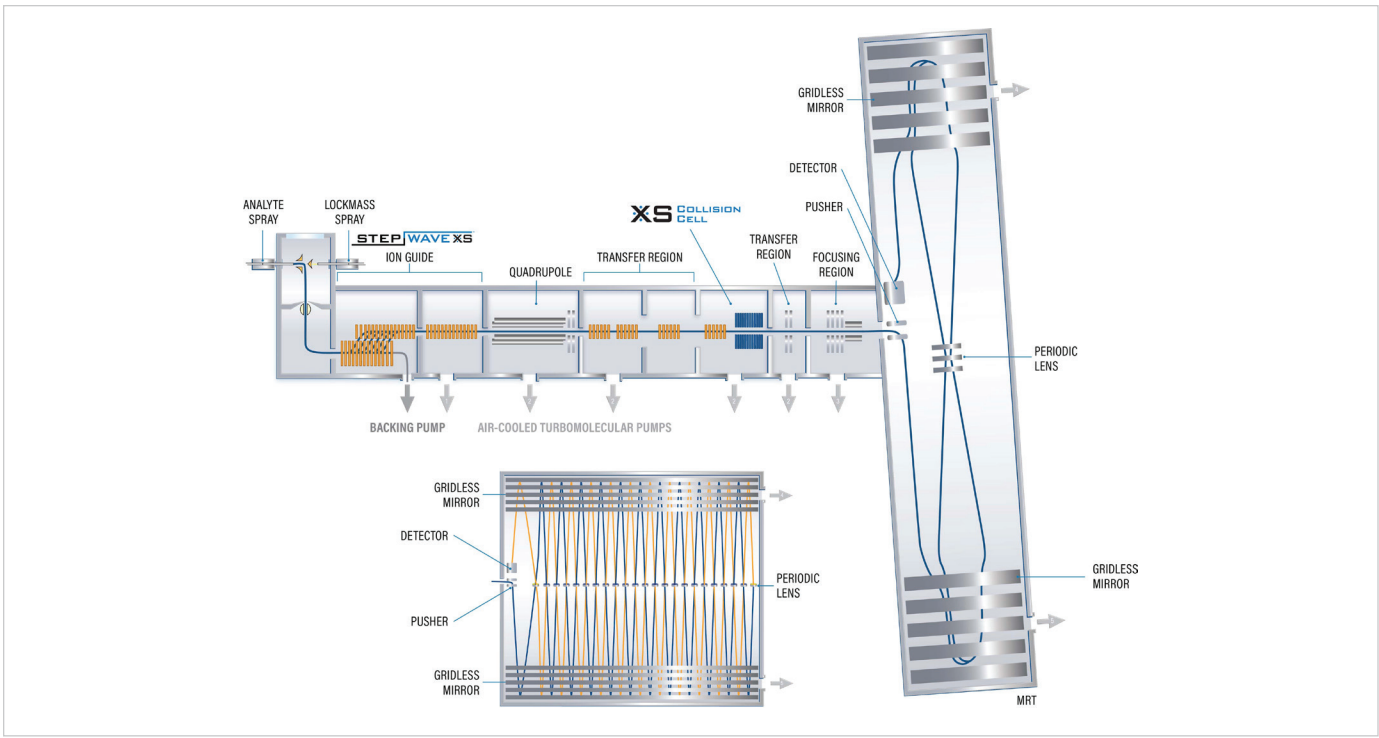

Figure 1. SELECT SERIES MRT instrument schematic.

EXPERIMENTAL CONDITIONS

Sample	Waters mAb Tryptic Digestion Standard (P/N 186009126) / Waters Humanized mAb Mass Check Standard (P/N 186009125)

LC Conditions	
LC System	Waters ACQUITY™ UPLC I-Class
Column	ACQUITY PREMIER Peptide CSH 2.1 × 100 mm / ACQUITY Protein C_4 2.1 × 50 mm
Column temperature	45 °C / 80 °C
Injection volume	1 – 5 µL
Mobile phase A	Water + 0.1 % formic acid (v/v)
Mobile phase B	Acetonitrile + 0.1 % formic acid (v/v)

MS Conditions	
Acquisition:	MS^E / MS
Capillary voltage	2 kV
Nebulising gas	7.5 psi
Desolvation gas	800L/hr
Source temperature	120 °C
Desolvation temperature	500 °C
Cone / source offset	30 V (peptide mapping) / 70 V (subunit analysis) / 120 V (intact mAb)
Mass range	50 – 2400 *m/z* (peptide and subunit) / 50 – 9600 *m/z* (intact mAb)
Acquisition rate	0.5 s/scan (peptide mapping) / 1 s/scan (subunit and intact)
Acquisition / Processing Software	MassLynx™ v4.2 SCN1024 / UNIFI v1.9

EXPERIMENTAL CONDITIONS

UNIFI processing parameters	
Peptide mapping	
Mass tolerance	1 ppm (precursor) / 2 ppm (product)
Digest	Trypsin
Missed cleavages	2
Modifications	Carbamidomethyl C/G0F/G1F/G2F/G0/Deamidation/lysine C-term/Pyroglu N-term
Lockmass	Leucine enkephalin - 556.276575 *m/z*
Subunit	
Workflow	RT window
Algorithm	BayesSpray, detailed isotope model
Modifications	Carbamidomethyl C/G0F/G1F/G2F/G0/Deamidation/lysine C-term/Pyroglu N-term
Lockmass	Leucine enkephalin - 556.276575 *m/z*

LC GRADIENTS

Peptide Mapping

Tim (min)	Flow rate (ml/min)	% A	% B
0.00	0.2	99	1
1.00	0.2	99	1
50.0	0.2	65	35
56.0	0.2	15	85
60.0	0.2	15	85
65.0	0.2	99	1
79.0	0.2	99	1

Subunit

Tim (min)	Flow rate (ml/min)	% A	% B
0.00	0.5	95	5
0.50	0.5	95	5
0.51	0.4	80	20
7.61	0.4	65	35
8.00	0.4	50	50
10.01	0.4	10	90
11.00	0.4	10	90
11.01	0.5	95	5
14.0	0.5	95	5

Intact

Tim (min)	Flow rate (ml/min)	% A	% B
0.00	0.4	95	5
0.50	0.4	95	5
0.51	0.2	95	5
3.50	0.2	5	95
3.51	0.4	5	95
4.00	0.4	5	95
4.25	0.4	95	5
4.50	0.4	5	95
4.75	0.4	95	5
5.50	0.4	95	5

RESULTS

PEPTIDE MAPPING

The SELECT SERIES MRT is easily integrated into biopharmaceutical discovery/characterization workflows such as peptide mapping and intact mass measurement. For peptide mapping the excellent mass accuracy of the MRT analyzer for both precursor and product ions provides higher confidence in peptide identifications. Figure 2 shows a UNIFI data summary for a peptide map of the NISTmAb reference standard with 98 % sequence coverage using a 1 ppm mass tolerance. The RMS mass error for the top 100 detections was observed to be an excellent 485 ppb. The RMS mass error for the top 1000 product ions was 0.5 mDa.

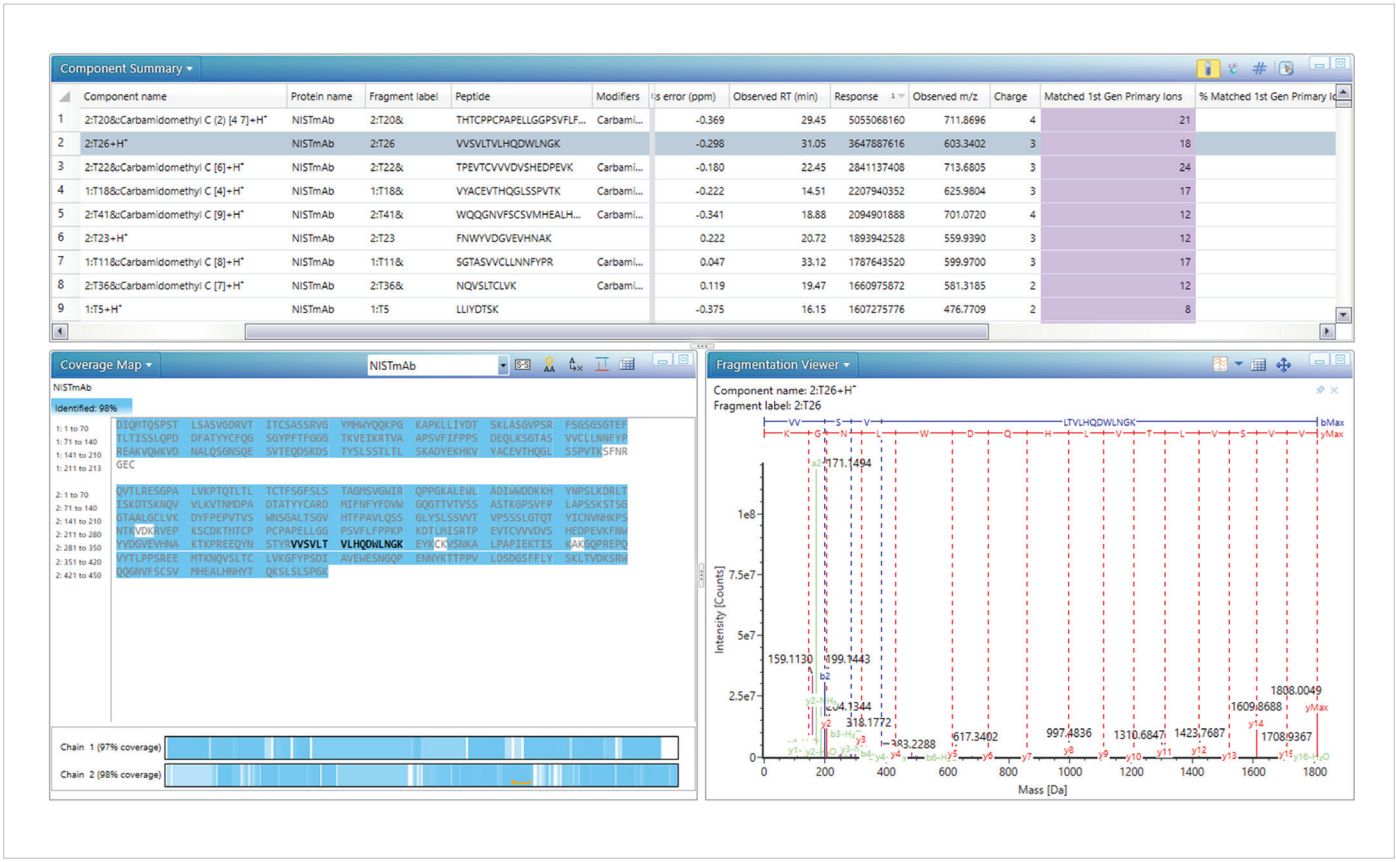

Figure 2. High sequence coverage of biotherapeutics using narrow mass tolerances. 98% coverage was obtained in this case for the NISTmAb tryptic digest using a 1 ppm mass filter.

Peptide mapping studies are conducted to determine the critical quality attributes (CQA) of molecules in biopharmaceutical development. CQAs include post-translational modifications and degradation products such as deamidations. It can often be difficult to identify and quantify deamidations due to the +0.98 Da mass shift leading to an overlap of the isotope distributions between the modified and the native peptide. The native peptide is often in such large excess that the deamidated form can be obscured in the tail of its the chromatographic peak. The resolving power of the SELECT SERIES MRT addresses this challenge enabling the native and deamidated peptide isotope distributions to be resolved, allowing accurate identification and quantification. Figure 3A shows the chromatogram of the tryptic peptide T36 (FNWYVDGVEVHNAK) from the heavy chain of NISTmAb in the above peptide map. The two deamidations (Figure 3A peaks 2 and 3) are present in the chromatographic peak tail of the native peptide.

(1). The mass spectra of the 3+ charge state (Figure 3B) are shown for each peak demonstrating the resolution of the deamidated forms from the native peptide.

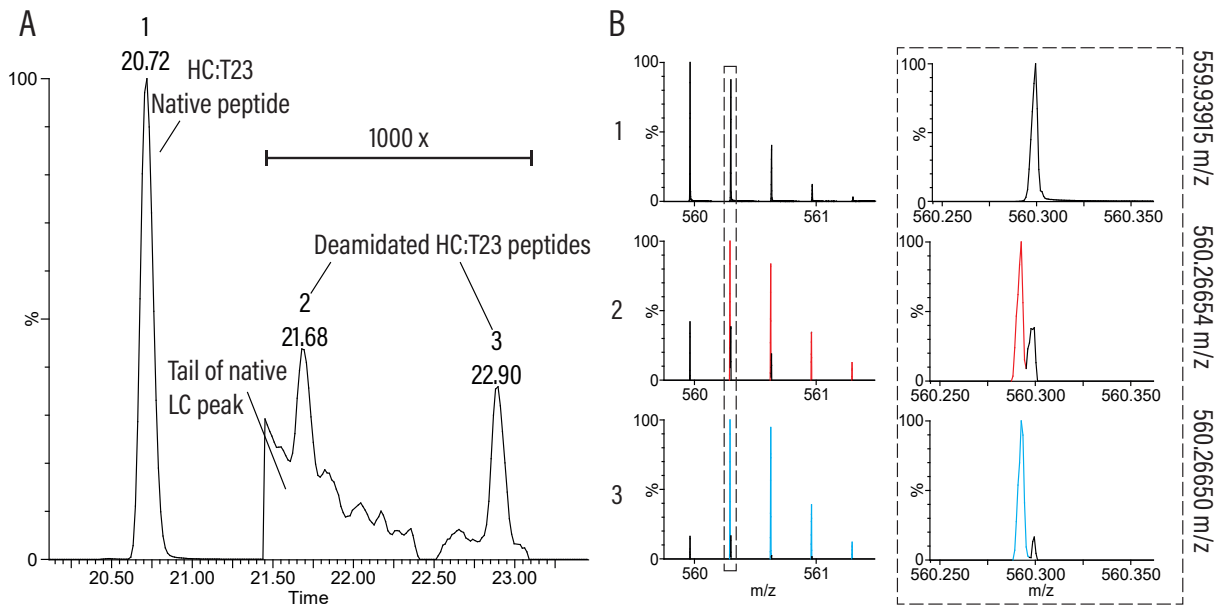

Figure 3. Resolving deamidations for confident quantification. A) The deamidated forms of peptide HC:T23 elute in the tail of the native peptide peak. Without the ultra-high resolution of the SELECT SERIES MRT as shown in (B) identification and quantification of the deamidations.

SUBUNIT (MIDDLE-UP) ANALYSIS

The ultra-high resolution of the MRT has benefits for protein analysis in that fully-resolved isotope distributions can be obtained, aiding in deconvolution to monoisotopic mass. After performing digestion with the IdeS protease and subsequent reduction and alkylation (Figure 4A) LC-MS was performed in MRT mode. Three chromatographic peaks consistent with each of the three subunits (Fc, Fd, and LC) were observed (Figure 4B). The mass spectrum for each peak was inspected and revealed fully-resolved isotope distributions (Figure 4C and D). Deconvolution to monoisotopic mass using the BayesSpray algorithm in the UNIFI app yielded mass errors of 0.8 and 0.7 ppm for the G0F and G1F glycoforms of the Fc subunit, respectively, 0.8 ppm for the Fd subunit and 2.0 ppm for the LC subunit, which is excellent for these proteins.

Table 1. Mass errors after BayesSpray deconvolution of the subunit (middle-up) analysis.

Subunit	Observed Mass $[M + H]^+$ (Da)	Observed Mass $[M + H]^+$ (Da)	Mas error (ppm)
Fc (G0F)	25449.53625	25449.55707	-0.8
Fc (G1F)	25611.59271	25611.60989	-0.7
Fd	26072.94347	26072.96461	-0.8
Light chain (LC)	23399.37180	23998.41933	-2.0

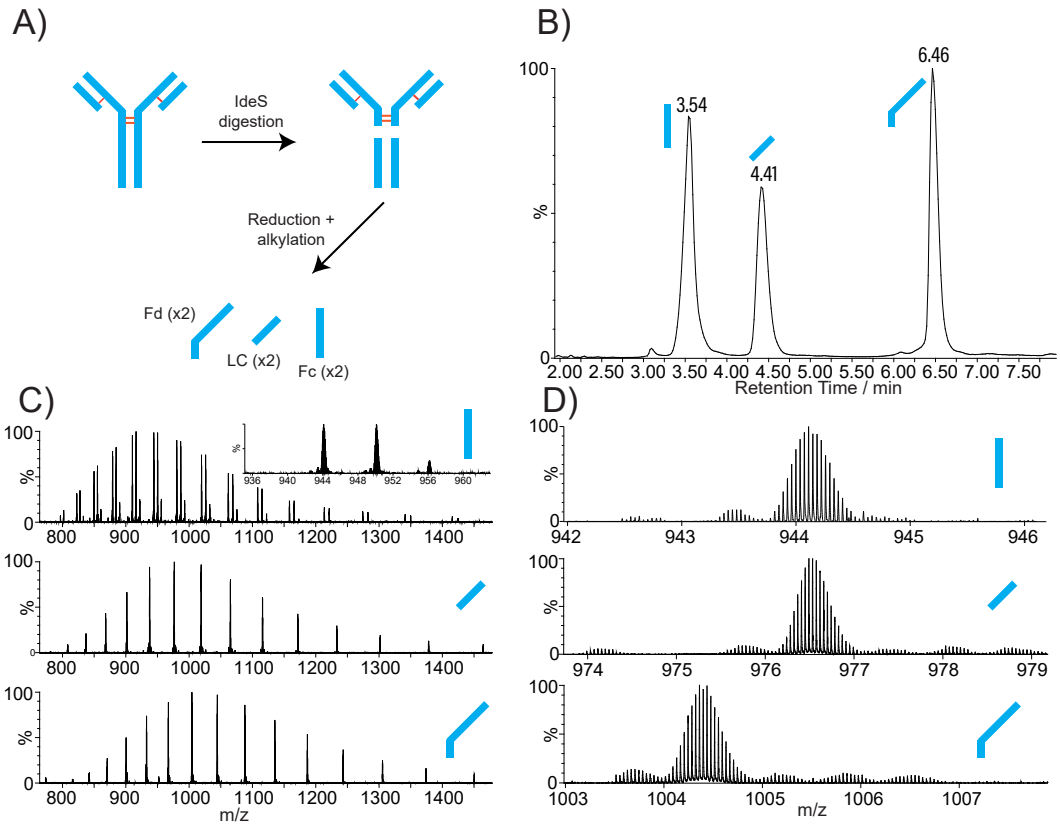

Figure 4. Subunit (middle-up) analysis on the SLECT SERIES MRT. A) The subunits were generated by IdeS digestion followed by reduction and alkylation. B) Three chromatographic peaks were observed, corresponding to the Fc (3.54 min), LC (4.41 min) and Fd (6.46 min) subunits. C) Mass spectra of each of the LC peaks D) Zooms of individual isotope distributions demonstrating the resolving power of the system.

INTACT mAb ANALYSIS

The SELECT SERIES MRT has an alternative flight path geometry, the 'Diamond' mode of operation, that is capable of an extended mass range of 20,000 *m/z*, suitable for the analysis of intact denatured, native mAbs and more. Figure 5 shows spectra for NISTmAb acquired under denaturing RPLC and native infusion conditions. Excellent glycoform resolution is obtained in both cases demonstrating how the global glycosylation profile can be obtained on biotherapeutic molecules.

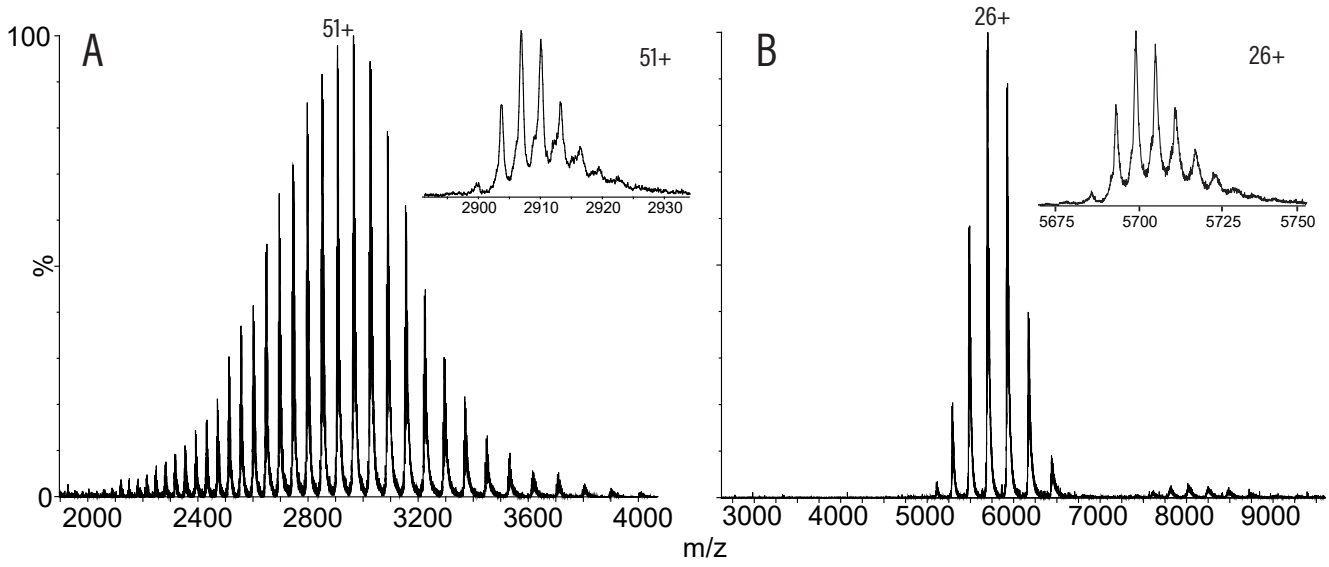

Figure 5. Intact and native mAbs on the SELECT SERIES MRT. A) Intact denatured NISTmAb from RPLC. B) Native NISTmAb acquired by static nanospray infusion using borosilicate glass capillaries. High quality spectra are obtained in both cases using the 'Diamond' mode of operation.

SUMMARY

The excellent mass accuracy afforded by the SELECT SERIES MRT system enables high sequence coverage with a stringent 1 ppm mass tolerance allowing higher confidence in peptide identifications. The ultra-high resolving power of the MRT analyzer provides a means to separate the overlapping isotope distributions of a native peptide and its deamidations for more facile identification and quantification of these important modifications. Furthermore, for more global mAb analysis such as IdeS-digested subunits, the MRT provides fully resolved isotope distributions enabling deconvolution to monoisotopic mass with higher confidence. Finally, for intact mAb analysis under either denatured or native conditions, an alternative acquisition mode capable of a mass range of 20,000 *m/z* was employed, yielding high quality data for both these approaches. Overall, the state-of-the-art SELECT SERIES MRT offers a powerful solution to tackle challenges in biopharmaceutical analysis.

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Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
waters.com