APPLICATION NOTE 000382

# H-SRM isolation: improved selectivity and sensitivity for quantitation of protein biotherapeutics in plasma

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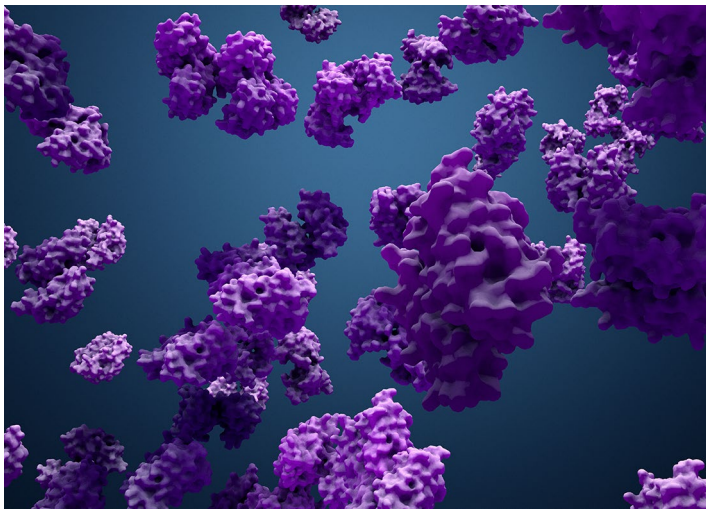

## Goal

The performance of high-resolution selective-reaction monitoring (H-SRM) over conventional unit-resolution SRM in the quantification of four biotherapeutics in biological matrix was evaluated. Here we show that the H-SRM feature on the Thermo Scientific™ TSQ Altis™ Plus mass spectrometer improves selective isolation of the target precursors, resulting in significantly reduced background chemical noise, which leads to increased signal-to-noise ratio (S/N) and improved limit of quantitation (LOQ).

## Introduction

Therapeutic proteins, especially monoclonal antibodies (mAbs), represent an important class of new drugs. Quantitative analysis of these drugs is critical for their discovery, development, and evaluation. Although liquid chromatography (LC)-selected reaction monitoring (SRM)-MS offers considerable potentials for selective analysis of mAbs in plasma and tissues,[1, 2] insufficient sensitivity due to high matrix interferences in digested plasma/tissue samples remains a prominent challenge. Consequently, an approach to selectively reduce the interference to improve quantitative sensitivity is urgently needed. The TSQ Altis Plus triple quadrupole mass spectrometer provides high-resolution-Q1 SRM, which utilizes a narrower isolation window at Q1 (0.2 Da FWHM) to decrease co-isolated interfering compounds and improve sensitivity (Figure 1). Here we evaluated the S/N and LOQ improvement afforded by H-SRM using targeted quantification of mAbs via their signature peptides, directly from a total plasma digest as the model system.

## Experimental

Monoclonal antibodies 4B9 and 28H1 were kindly provided by Roche, and AB095 was kindly provided by AbbVie. Evolocumab was purchased from Amgen. Stable-isotope-labeled peptide internal standards with K[$^{15}$N, $^{13}$C] or R[$^{15}$N, $^{13}$C] at the C-terminus for each signature peptide were obtained from Synpeptide (Shanghai, China). To prepare calibration curves, standard proteins (with purity accurately determined by amino acid analysis) were spiked at variable levels of concentration in pooled mouse plasma and were proteolytically digested following a surfactant aided precipitation/on-pellet digestion protocol[3] for analysis. A sensitive, high-throughput, and robust LC system described previously was employed.[4] The Thermo Scientific™ UltiMate™ 3000 HPLC system contains a SRD-3400 degasser, NCS-3500RS CAP pumps, a high-flow binary gradient pump, and a WPS-3000RS autosampler. A gradient separation (12 min run time) was conducted on a Thermo Scientific™ Acclaim™ C18 column (150 mm x 0.5 mm, 2.2 µm, 120 Å) at a flow rate of 25 µL/min for all targets. The TSQ Altis Plus MS instrument settings are listed in Table 1 and SRM transition parameters of four mAbs are listed in Table 2. To allow a fair comparison, H-SRM and unit-resolution SRM (Q1 isolation at 0.2 and 0.7 Th, respectively) were performed in adjacent scan events of the *same LC-MS run* with otherwise the same scan parameters; to separately extract the data, the precursor *m/z* was set to differ by 0.001 Da for the two approaches. Appropriate dwell times were set in order to get 10–15 data points per peak for both Q1 resolution settings. Data was processed using Skyline software.

**Table 1. TSQ Altis Plus MS instrument settings**

| Parameter | Settings |
|---|---|
| Ion source type | Heated ESI |
| Positive ion spray voltage | 3,500 V |
| Sheath gas | 8 Arb |
| Aux gas | 6 Arb |
| Sweep gas | 0 Arb |
| Ion transfer tube temp. | 325 °C |
| Vaporizer temp. | 50 °C |
| Resolution | 0.2 Th Q1 for H-SRM<br>0.7 Th Q1 for unit-resolution SRM<br>0.7 Th for Q3 for both approaches |

**Table 2. SRM transition parameters for quantification of the four mAbs**

| mAb | Signature peptide | Transition | Collision energy | RF lens |
|---|---|---|---|---|
| 4B9 | LLINVGSR | 436.272/319.172 | 14 | 90 |
| 28H1 | LLIIGASTR | 472.300/491.257 | 14 | 64 |
| AB095 | GPSVFPLAPSSK | 593.827/699.404 | 20 | 90 |
| Evolocumab | GPSVFPLAPCSR | 644.330/800.408 | 22 | 95 |

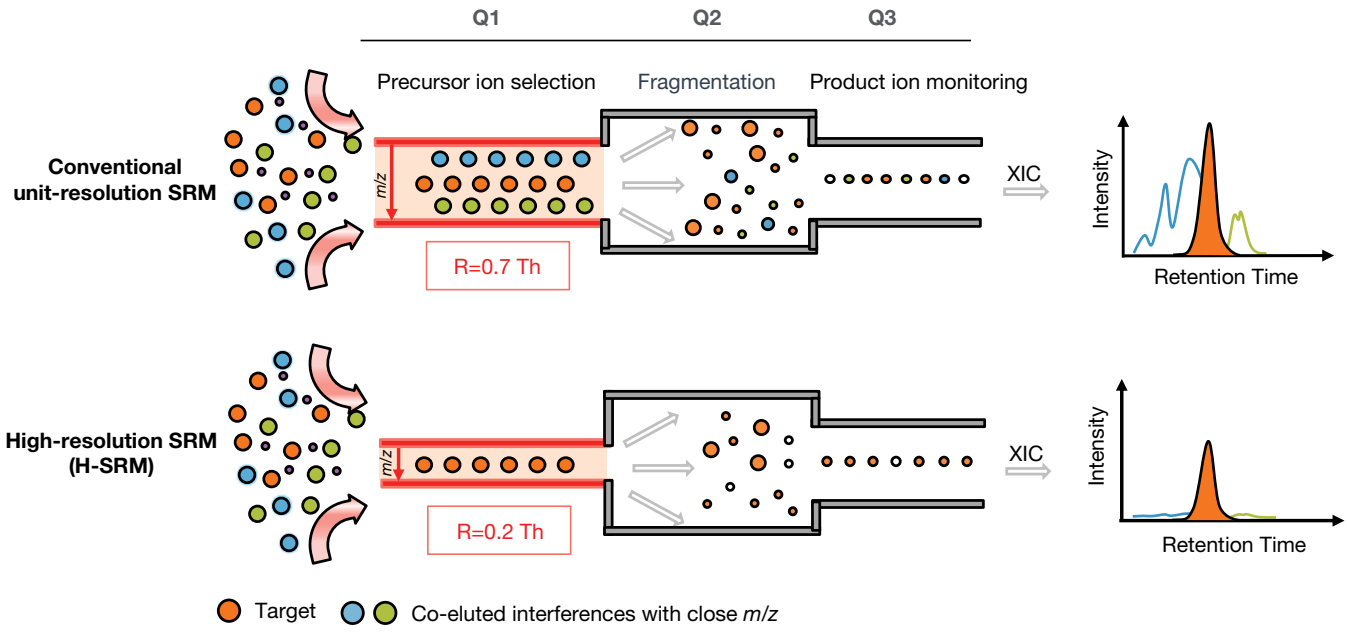


**Figure 1. A scheme comparing conventional unit-resolution SRM and H-SRM.** Conventional SRM uses unit-resolution at Q1 (R=0.7 Th), while H-SRM uses a narrower *m/z* window (R=0.2 Th) for precursor isolation on Q1. The H-SRM decreases the chemical noise from interferences in the matrix with close *m/z* to the analyte, improving selectivity to enable greater S/N for quantification in highly complicated matrices. There will be a 50–70% decrease in the signal response than when the unit-resolution SRM is used, but the chemical noise will be decreased to a much higher extent, resulting in considerable net gains of S/N.

## Results and discussion

### Comparison of the background in blank matrix at two Q1 resolutions

As shown in Figure 2, substantially higher interference peaks at the retention time of each analyte were observed at unit-resolution SRM that compromised the ability to selectively detect the target analyte. In comparison, H-SRM reduced most of the interference peaks and achieved a much lower baseline..

### Comparison of the analyte S/N in LOQ samples at two Q1 resolutions

H-SRM substantially decreased chemical noise for all the signature peptides examined here, resulting in considerable net gains of S/N. In the four analytes shown in Figure 3, H-SRM yielded a 3- to 21-fold S/N improvement compared with unit-resolution SRM. For example, in the case of 28H1, the target peak was undetectable at a concentration of 50 ng/mL with unit-resolution SRM because of the high and irregular interference peaks. In comparison, the target peak was selectively detected when analyzed with H-SRM to achieve an increased S/N=21.

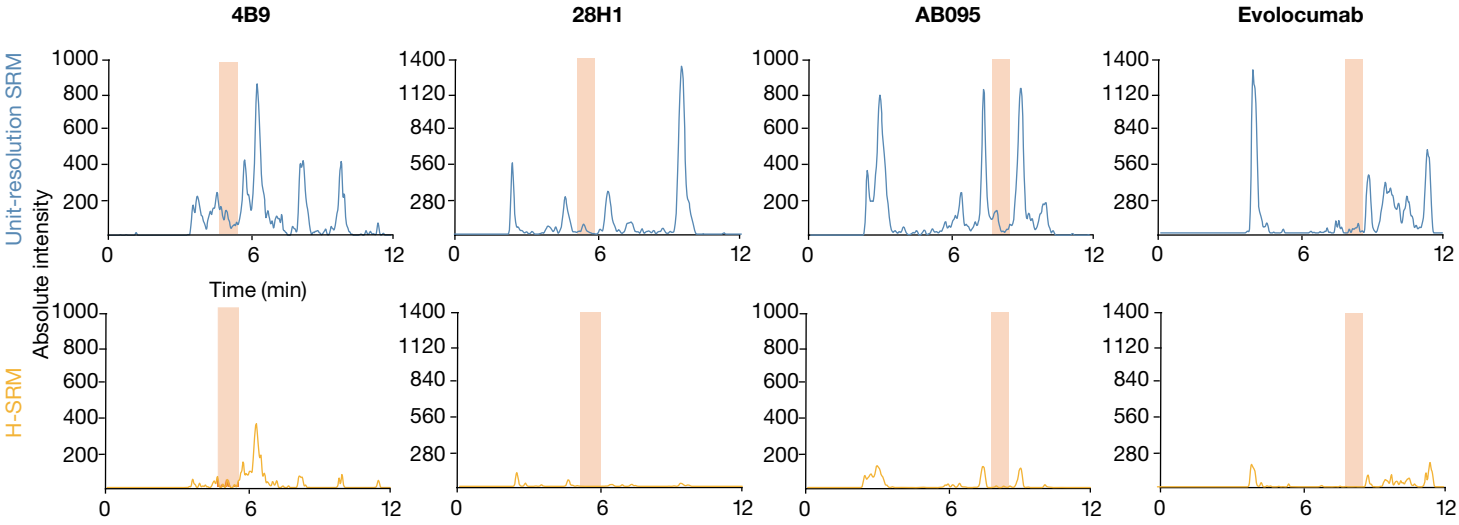


**Figure 2. Comparison of the background in blank matrix at two Q1 resolutions.** H-SRM reduced most interference peaks and achieved a much lower baseline. The retention time window of targets is highlighted.

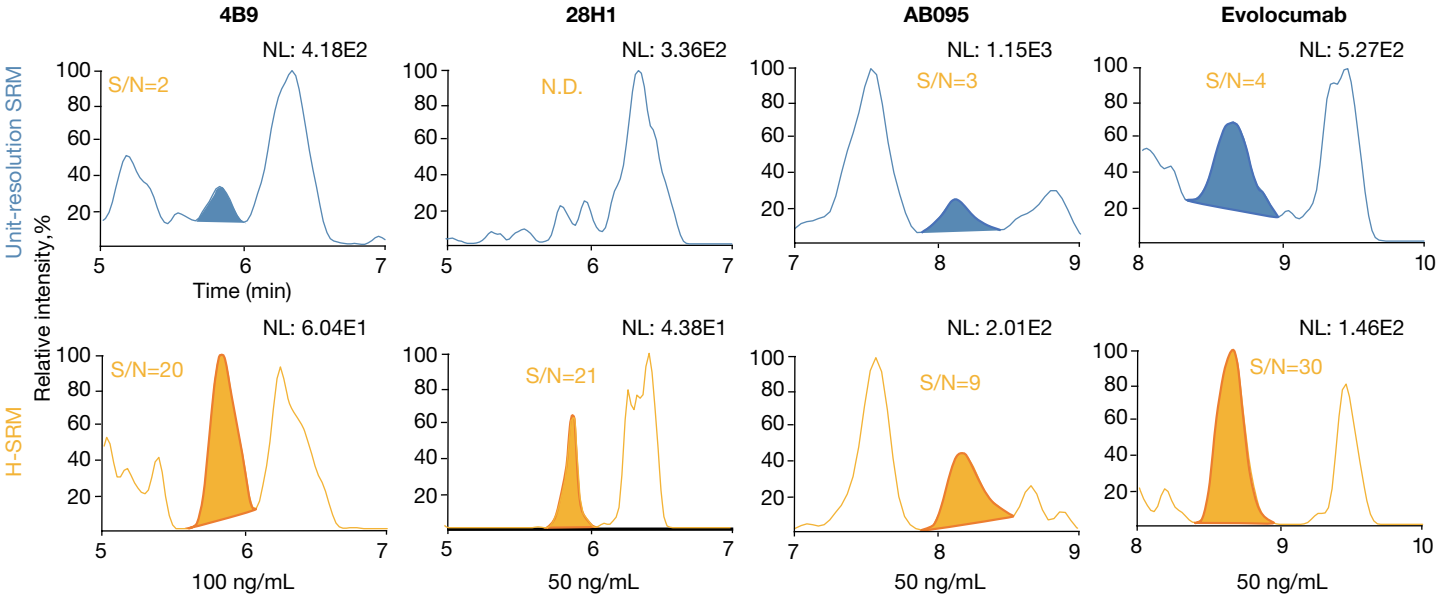


**Figure 3. S/N comparison at two Q1 resolutions.** H-SRM provided 3- to 21- fold of S/N improvement compared with unit-resolution SRM.

### Comparison of calibration and validation results for the model proteins at two Q1 resolutions

As shown in Figure 4, H-SRM provided a 3- to 6-fold LOQ improvement over unit-resolution SRM for the four model proteins. Calibration and validation results using H-SRM are listed in Table 3. Overall, because of interference, the calibration curve by unit-resolution SRM was not linear at the lower levels (50 to 200 ng/mL). On the contrary, good linearity was achieved using H-SRM in the same concentration range for each analyte. In the case of 4B9, accuracy of quality control (QC) samples at a concentration of 100 ng/mL was 241.1% with unit-resolution SRM, while it was 117.7% when analyzed with H-SRM. For 28H1, coefficient of variation (%CV) of QC samples at a concentration of 50 ng/mL was 51.0% with unit-resolution SRM and 7.8% when analyzed with H-SRM. Therefore, H-SRM both improved quantification accuracy and minimized variations at lower concentration levels, which markedly improved quantitative sensitivity as well as the data quality.

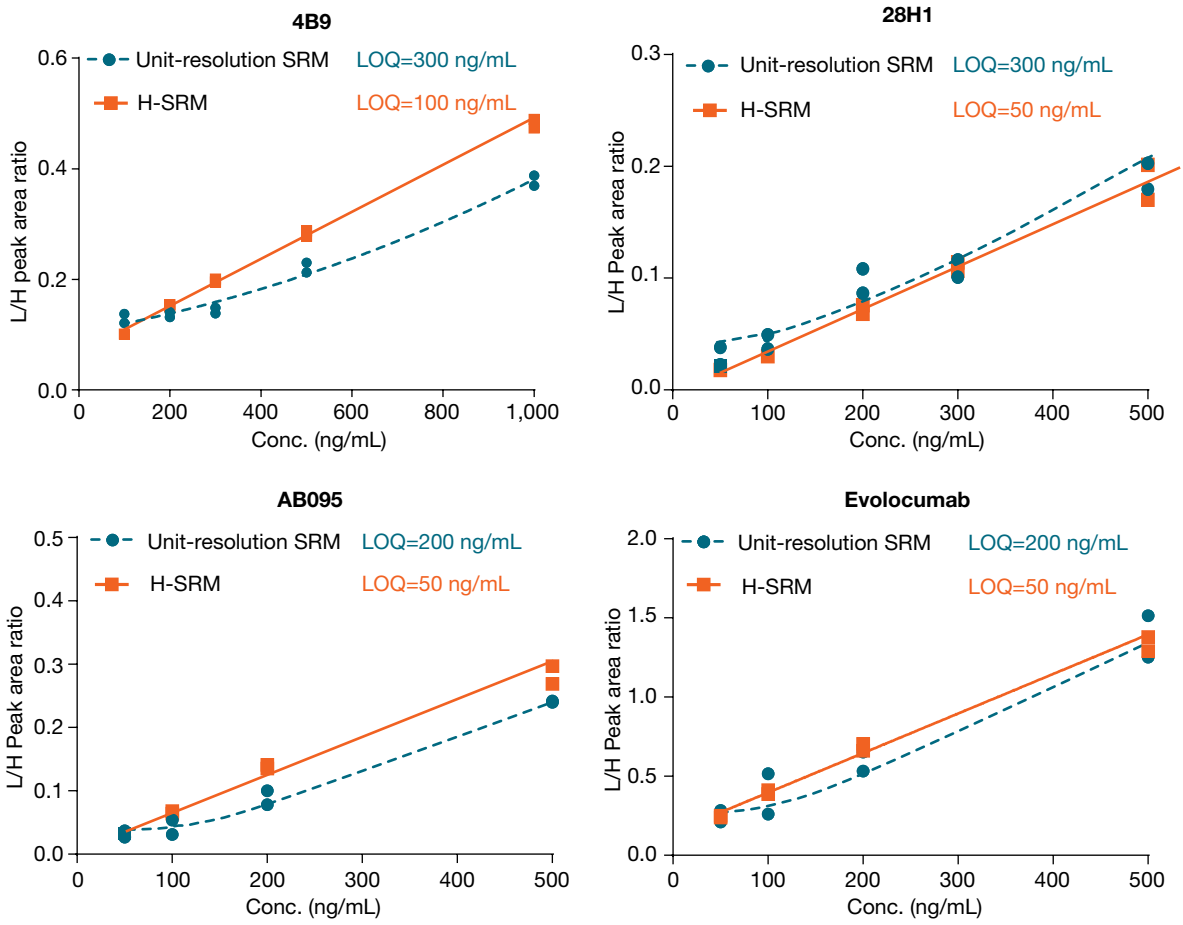


**Figure 4. Calibration curve comparison (lower concentration levels zoom-in).** H-SRM both improved quantification accuracy and minimized variations at lower concentration levels, which markedly improved quantitative sensitivity and data quality.

**Table 3. Calibration and validation results for quantification of the four mAbs using H-SRM**

| mAbs | Calibration range (ng/mL) | $R^2$ 1/× weighted) | LLOQ (ng/mL) | Accuracy%/ CV% | LQC (ng/mL) | Accuracy%/ CV% |
|---|---|---|---|---|---|---|
| 4B9 | 100–25,000 | 0.9945 | 100 | 117.7/5.7 | 300 | 99.6/3.6 |
| 28H1 | 50–25,000 | 0.9947 | 50 | 93.4/7.8 | 100 | 109.5/8.6 |
| AB095 | 50–25,000 | 0.9918 | 50 | 92.8/17.1 | 100 | 112.2/4.4 |
| Evolocumab | 50–25,000 | 0.9906 | 50 | 112.2/4.9 | 100 | 104.8/6.4 |

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## Conclusion

Compared with traditional unit-resolution SRM, H-SRM performed on the TSQ Altis Plus mass spectrometer substantially decreased chemical noise and interfering peaks. It resulted in a 3- to 21- fold S/N improvement and 3- to 6- fold LOQ improvement in the quantification of four mAbs in plasma. Owing to the improved selectivity, H-SRM may also significantly reduce the demand on the LC separation, which is helpful in achieving shorter analytical cycles to improve the throughput of LC-MS quantitative protein analysis.


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