# HIGH SELECTIVITY MRM (HS-MRM) ASSAY FOR QUANTIFICATION OF HOST CELL PROTEINS IN BIOTHERAPEUTICS

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## OVERVIEW

An HS-MRM assay was developed for quantification of low abundance peptide standards spiked in a monoclonal antibody (mAb) digest to demonstrate that it has the required sensitivity and dynamic range (at least 3 orders of magnitude) for a typical HCP monitoring assay.

## INTRODUCTION

- Analysis of low-levels (1-100 ppm) protein impurities (e.g. host cell proteins–HCPs) in protein biotherapeutics is a challenging assay requiring high sensitivity and a wide dynamic range.
- Mass spectrometry based quantification assays for proteins typically involve protein digestion followed by selective reaction monitoring/multiple reaction monitoring (SRM/MRM) quantification of peptides using a low-resolution (Rs~1,000) tandem quadrupole mass spectrometer. One of the limitations of this approach is the interference phenomenon observed when the peptide of interest has the "same" precursor and fragment mass (in terms of m/z values) as other co-eluting peptides present in the sample (within 1 Da window). To avoid this phenomenon we propose an alternative mass spectrometric approach, a high selectivity (HS) MRM assay which combines ion mobility separation of peptide precursors with high-resolution (Rs~40,000) MS-detection of peptide fragments.
- We explored the capabilities of this approach for quantification of low abundance peptide standards spiked in a monoclonal antibody (mAb) digest and demonstrate that it has the sensitivity and dynamic range (at least 3 orders of magnitude) required for HCP analysis. All six peptide standards were detected at concentrations as low as 0.1 nM (1 femtomole loaded on a 2.1 mm ID chromatographic column) in the presence of a high-abundance peptide background (2 µg of a mAb digest loaded on-column). When considering the MW of spiked-in peptides, the LOQ of this assay is at the 1-2 ppm level. Relative standard deviations (RSD) of peak areas (n=4 replicates) were less than 15% across the entire concentration range investigated (0.1-100 nM or 1-1,000 ppm) in this study.

## METHODS

### Sample Preparation

A licensed mAb biosimilar (Inflectra, 10 mg/mL) was denatured with 0.04% RapiGest surfactant (60°C, 15 min), reduced with 20 mM DTT (60°C, 1h), alkylated with 10 mM IAM (RT, 30 min) and digested with a mixture of Lys-C and porcine trypsin (Promega) overnight. A MassPREP digest standard containing six rabbit phosphorylase (PHO) peptides (Waters P/N 186006011) was spiked post-digestion in the mAb digest at the following concentrations: 0.1, 1. 10, 100 and 1000 nM, while keeping the amount of mAb digest constant. Two MassPREP digest standards (1 µM and 100 nM) were prepared in 0.1% FA (formic acid), in the absence of the Inflectra digest background.

### LC Conditions

An ACQUITY™ UPLC® H-class system equipped with a CSH (charged-surface hybrid) C18 column (2.1 x 150 mm, 1.7 µm particles) was used to separate the mAb digest at a flow rate of 200 µL/min and a column temperature of 60 °C. Gradient elution was performed from 1% to 40% mobile phase B in 30 min and the total LC runtime was 50 min. The mobile phase composition was: Solvent A: 0.1% FA in DI water and Solvent B: 0.1% FA in acetonitrile.

### MS conditions

Data-independent acquisition following precursor level ion mobility separations (HDMS$^E$) were performed on a VION IMS QTof mass spectrometer, over the m/z range of 100-2000 with an acquisition time of 0.5 sec. A single HS-MRM acquisition method designed to monitor all six PHO peptides was then created following the Tof-MRM optimization experiments (see Table II for detailed parameters).

### Informatics

UNIFI Scientific Information System 1.8.1 was used for data acquisition and processing.

### Workflow (5 steps)

1. HDMS$^E$ data acquisition
2. Tof-MRM collision energy (CE) optimization
3. Setup the final HS-MRM method
4. Setup a processing method in UNIFI
5. Process the HS-MRM data in UNIFI


*VION IMS QTof MS system*

## RESULTS

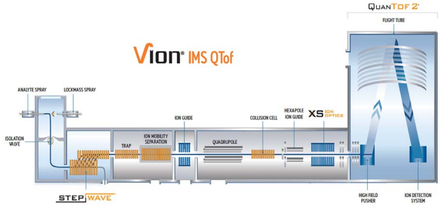


Figure 1. Diagram of the VION IMS QTof mass spectrometer.
In the high selectivity MRM acquisition mode (HS-MRM) the precursors of the peptide that is being quantified are separated from other co-eluting (interfering) peptide precursors in the ion mobility cell, isolated by the quadrupole and fragmented with a fixed collision energy in the collision cell. The signal produced by the peptide fragment ions is enhanced by adjusting the pusher frequency and peptide quantification is performed using the high MS resolution (>40,000) signals produced by the most intense fragment ion of each peptide.

| Peptide ID | Peptide Sequence | Charge states | | | |
|---|---|---|---|---|---|
| | | + 1 | + 2 | + 3 | + 4 |
| Pep 1 | VLYPNDNFFEGK | 1442.6951 | **721.8512** | 481.5699 | 361.4292 |
| Pep 2 | TCAYTNHTVLPEALER | 1874.9065 | 937.9569 | **625.6404** | 469.4821 |
| Pep 3 | IGEEYISDLDQLRK | 1678.8646 | 839.9360 | **560.2931** | 420.4716 |
| Pep 4 | LLSYVDDEAFIR | 1440.7369 | **720.8721** | 480.9172 | 360.9397 |
| Pep 5 | LITAIGDVVNHDPVVGDR | 1890.0080 | 945.5076 | **630.6742** | 473.2574 |
| Pep 6 | VFADYEEYVK | 1262.5939 | **631.8006** | 421.5362 | 316.4039 |

Table I. PHO peptide standards contained in the Mass PREP digest mixture (P/N 186006011) spiked in the Inflectra digest. The most abundant precursors from each peptide are highlighted in bold.

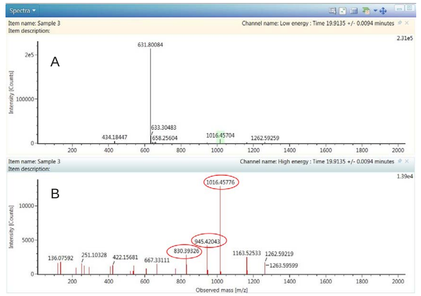


Figure 2. Example of HDMS$^E$ data: (A) Low-energy spectrum showing the Pep 6 precursor ion; (B) high-energy fragmentation spectrum of the same peptide, displaying the top three most abundant fragments ions (circled) selected for Tof-MRM collision energy (CE) optimization.

| Peptide ID | Peptide Sequence | Retention time (min) | Peptide precursor | | Most abundant fragment ions/charge | | | Optimum |
|---|---|---|---|---|---|---|---|---|
| | | | m/z & charge | Drift time (ms) | I | II | III | CE (V) |
| Pep 2 | TCAYTNHTVLPEALER | 16.0 | 625.6404 (+3) | 6.2 | **714.3781 (+1)** | 807.4177 (+2) | 827.4621 (+1) | 24 |
| Pep 4 | LLSYVDDEAFIR | 21.1 | 720.8721 (+2) | 7.5 | 865.4050 (+1) | 964.4734 (+1) | **1214.5688 (+1)** | 22 |
| Pep 5 | LITAIGDVVNHDPVVGDR | 19.7 | 630.6742 (+3) | 6.3 | 642.3570 (+1) | 689.8391 (+2) | **832.4236 (+2)** | 20 |
| Pep 6 | VFADYEEYVK | 17.7 | 631.8006 (+2) | 7.0 | 830.3931 (+1) | 945.4200 (+2) | **1016.4571 (+1)** | 24 |

Table II. Results of the Tof-MRM CE optimization experiment: the three most abundant fragments of each PHO peptide quantified in this study are indicated along with the corresponding optimized collision energy.

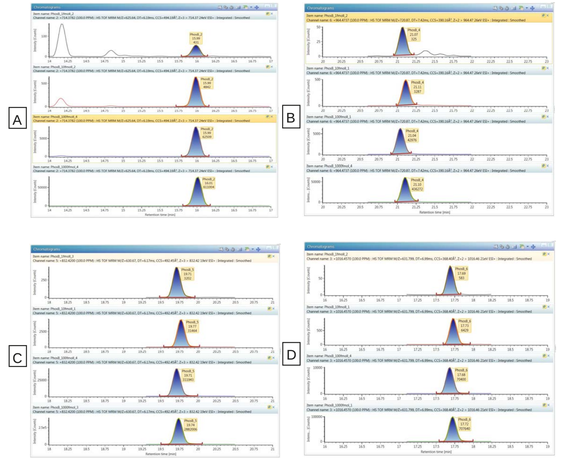


Figure 3. HS-MRM chromatograms recorded for 4 PHO peptides at 4 different concentrations spanning 3 orders of magnitude (0.1, 1, 10 and 100 nM); (A) Pep 2 chromatograms; (B) Pep 4 chromatograms; (C) Pep 5 chromatograms; (D) Pep 6 chromatograms.

| Conc (nM) | Amount on-column (fmoles) | Pep 2 Peak Areas | | | | | |
|---|---|---|---|---|---|---|---|
| | | Rep01 | Rep02 | Rep03 | Rep04 | Mean | RSD (%) |
| 0.1 | 1 | 490 | 439 | 462 | *431* | 456 | 5.8 |
| 1 | 10 | 5121 | 4842 | 5198 | *4482* | 4911 | 6.6 |
| 10 | 100 | 63853 | 64279 | 66111 | *62509* | 64188 | 2.3 |
| 100 | 1000 | 612392 | 605553 | 613229 | *611004* | 610545 | 0.6 |

| Conc (nM) | Amount on-column (fmoles) | Pep 4 Peak Areas | | | | | |
|---|---|---|---|---|---|---|---|
| | | Rep01 | Rep02 | Rep03 | Rep04 | Mean | RSD (%) |
| 0.1 | 1 | 275 | 359 | *325* | 288 | 312 | 12.2 |
| 1 | 10 | 3559 | 3694 | *3287* | 3754 | 3574 | 5.8 |
| 10 | 100 | 45259 | 45775 | *42976* | 45548 | 44890 | 2.9 |
| 100 | 1000 | 459374 | 467927 | *436272* | 458994 | 455642 | 3.0 |

| Conc (nM) | Amount on-column (fmoles) | Pep 5 Peak Areas | | | | | |
|---|---|---|---|---|---|---|---|
| | | Rep01 | Rep02 | Rep03 | Rep04 | Mean | RSD (%) |
| 0.1 | 1 | 3194 | 3243 | *3202* | 3257 | 3224 | 1.0 |
| 1 | 10 | 31464 | 31150 | *31464* | 31433 | 31378 | 0.5 |
| 10 | 100 | 313638 | 320712 | *311943* | 311943 | 314559 | 1.3 |
| 100 | 1000 | 2845736 | 2840031 | *2882006* | 2864052 | 2857956 | 0.7 |

| Conc (nM) | Amount on-column (fmoles) | Pep 6 Peak Areas | | | | | |
|---|---|---|---|---|---|---|---|
| | | Rep01 | Rep02 | Rep03 | Rep04 | Mean | RSD (%) |
| 0.1 | 1 | 490 | *583* | 440 | 440 | 488 | 13.8 |
| 1 | 10 | 6429 | *6429* | 6848 | 6623 | 6582 | 3.0 |
| 10 | 100 | 71295 | *70400* | 71563 | 70400 | 70915 | 0.9 |
| 100 | 1000 | 707640 | *707640* | 694461 | 729490 | 709808 | 2.0 |

Table III. Peak areas of 4 PHO peptides recorded for each LC/MS injection (16 LC/MS runs, 4 concentrations tested, 4 replicate injections for each concentration). The relative standard deviation was better than 15% for all peptides over the entire concentration range investigated. Peak areas highlighted in *italics* correspond to the HS-MRM chromatograms displayed in Figure 3.

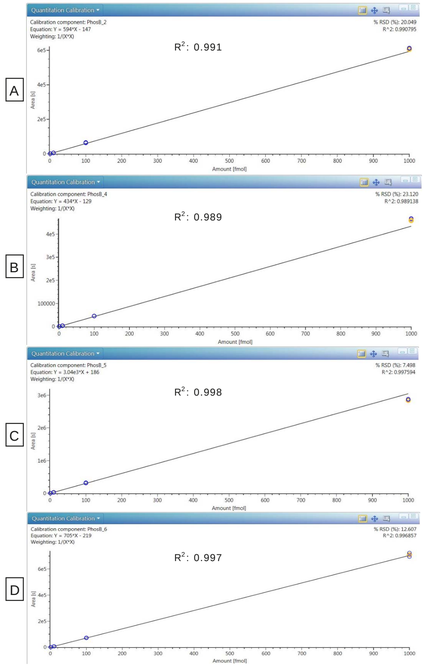


Figure 4. Calibration curves generated in UNIFI for 4 PHO peptides across 4 different concentrations (0.1, 1, 10 and 100 nM). (A) Pep 2 calibration; (B) Pep 4 calibration; (C) Pep 5 calibration; (D) Pep 6 calibration.

## CONCLUSIONS

- All six peptide standards were detected at concentrations as low as 0.1 nM (1 femtomole loaded on a 2.1 mm ID chromatographic column) in the presence of a high-abundance peptide background (2 µg of a mAb digest loaded on-column.
- When considering the MW of spiked-in peptides, the LOQ of this assay is at the 1-2 ppm level.
- Relative standard deviations (RSD) of peak areas (n=4 replicates) were less than 15% across the entire concentration range investigated (1-1000 nM).
- The HS-MRM assay has great potential for becoming a fast, high-throughput monitoring assay for multiple HCPs across multiple batches of biopharmaceuticals.
- Vion IMS QTof can be used for biopharmaceutical characterization as well as quantification of low-level protein impurities.

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