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# Rapid Protein Sequencing Using TripleTOF™ 5600 System with MRMPilot™ and ProteinPilot™ Software

Yan Ke, Nicole Roenker, Elise Snide and Yong-Xi Li

Medpace Bioanalytical Laboratories, 5365 Medpace Way, Cincinnati, OH 45227

## Overview

This study demonstrates a systematic approach for identification and confirmation of extensive sequence information over the whole length of a protein in a single series of experiments using TripleTOF™ 5600 System with MRMPilot™ and ProteinPilot™ Software..

In this method, herceptin was denatured with guanidine hydrochloride in Tris-HCL buffer, and then treated with dithiothreitol (DTT) to break disulfide linkages between cysteine residues for complete reduction. To prevent the disulfide bonds from re-forming, iodoacetamide (IAA) was used to modify the reactive cysteine-SH groups, forming s-carboxylmethylated cysteines. After alkylation, the proteases such as Lys-C or trypsin were used for digesting the protein into a population of peptides that were able to be identified by the mass spectrometer. MRMPilot™ and ProteinPilot™ Software can help to select optimized MRM transitions of peptides based on the protease digestion of the protein.

Following the denature, reduction, alkylation and digestion, the protein sequencing and sequence confirmation were performed by TripleTOF™ 5600 System.

This method has been successfully applied to clinical pharmacokinetic studies.

## Introduction

The development of bioanalytical techniques for rapid and accurate identification of proteins is important in biopharmaceutical industry. Peptide mapping by LC-MS/MS is one of the most powerful qualitative assays to confirm the primary sequence of proteins or antibodies and to detect subtle changes in the primary structure of biologics. TripleTOF™ 5600 System has become a valuable tool for characterization of large biomolecules due to its reliability, speed, resolution and sensitivity. MRMPilot™ and ProteinPilot™ Software can help to predict potential MRM of peptides based on the protease digestion of the protein.

In this study, herceptin was used for analysis. Herceptin, also named Trastuzumab, a high-affinity humanized monoclonal antibody that recognizes HER-2, is a novel targeted-therapy for breast cancer overexpressing HER-2 receptor.

## Methods

**Sample Preparation:**

Herceptin was denatured with guanidine hydrochloride in Tris-HCL buffer, and was reduced and alkylated by DTT and IAA. After alkylation, the proteases such as Lys-C or trypsin were used for digesting the protein into a population of peptides that were able to be identified by the mass spectrometer. Following the denature, reduction, alkylation and digestion, The digested product was transferred to LC vials for the analysis by TripleTOF™ 5600 System.

**Liquid Chromatography:**

Pump: Shimadzu LC-30A
Autosampler: Shimadzu SIL-30AC
System Controller: Shimadzu CBM-20A
Analytical Column: HyperClone BDS C18 column, 2.0 x 150 mm, 5 µm, 130A
Gradient: The analyte was eluted using a gradient of mobile phase A (0.1% TFA in water) and mobile phase B (ACN:H2O:TFA (90:9.92:0.08) from 10% to 80% mobile phase B in 20 min.
Injection Volume: 10 µL

**Mass Spectrometry:**

MS System: AB/Sciex TripleTOF™ 5600
Condition: LC/(+)ESI-MS/MS, (High Resolution MRM)

## Structure

Herceptin

## Results and Discussion

| Sequence | Precursor Mass (Da) | Fragment Mass (Da) | RT (min) | Fragment Type | Mean Height |
|---|---|---|---|---|---|
| RTVAAPSVFIFPPSDEQLK | 701.38 | 913.46 | 15.9 | 3+ / y8 | 112980 |
| SFNRGEC[CAM] | 435.18 | 635.26 | 3.4 | 2+ / y5 | 97420 |
| DSTYSLSSTLTLSK | 751.88 | 1036.59 | 14.3 | 2+ / y10 | 50380 |
| SGTASVVC[CAM]LLNNFYPREAK | 709.36 | 1138.56 | 15.9 | 3+ / y9 | 36100 |
| ADYEK | 313.14 | 439.22 | 1.7 | 2+ / y3 | 28260 |
| VQWK | 280.66 | 333.19 | 6.6 | 2+ / y2 | 15840 |
| VDNALQSGNSQESVTEQDSK | 712.66 | 893.42 | 6.7 | 3+ / y8 | 11320 |
| VYAC[CAM]EVTHQGLSSPVTK | 938.47 | 1154.62 | 10.3 | 2+ / y11 | 6140 |
| DIQMTQSPSSLSASVGDRVTITC[CAM]RASQDVNTAVAWYQQK | 860.62 | 1094.56 | 15.8 | 5+ / y9 | 100 |

Table 1: Proposed and Found peptide by MRMPilot on AB/Sciex TripleTOF™ 5600 from Lys-C Digested Herceptin Light Chain

| Sequence | Precursor Mass (Da) | Fragment Mass (Da) | RT (min) | Fragment Type | Mean Height |
|---|---|---|---|---|---|
| GPSVFPLAPSSK | 593.83 | 699.40 | 14.7 | 2+ / y7 | 435453 |
| ALPAPIEK | 419.76 | 486.29 | 9.7 | 2+ / y4 | 228840 |
| NQVSLTC[CAM]LVK | 581.32 | 820.46 | 14.4 | 2+ / y7 | 186780 |
| STSGGTAALGC[CAM]LVK | 661.34 | 760.44 | 13.0 | 2+ / y7 | 95900 |
| FNWYVDGVEVHNAK | 839.40 | 968.48 | 15.2 | 2+ / y9 | 48720 |
| LTVDK | 288.17 | 462.26 | 4.9 | 2+ / y4 | 28560 |
| TTPPVLDSDGSFFLYSK | 937.46 | 948.48 | 15.9 | 2+ / y8 | 22840 |
| DTYIHWVRQAPGK | 785.91 | 1078.59 | 12.3 | 2+ / y9 | 17320 |
| DTLMISRTPEVTC[CAM]VVVDVSHEDPEVK | 985.82 | 1154.53 | 15.8 | 3+ / y10 | 14420 |
| PSNTK | 273.65 | 449.24 | 1.3 | 2+ / y4 | 13620 |
| VEPK | 472.28 | 244.17 | 1.7 | 1+ / y2 | 11320 |
| GRFTISADTSK | 591.81 | 721.37 | 8.8 | 2+ / y7 | 6600 |
| SLSLSPGK | 394.73 | 588.34 | 8.7 | 2+ / y5 | 5100 |
| GFYPSDIAVEWESNGQPENNYK | 848.72 | 949.44 | 15.8 | 3+ / y8 | 4720 |
| SC[CAM]DEK | 255.10 | 292.14 | 1.3 | 2+ / y2 | 4300 |
| GLEWVARIYPTNGYTRYADSVK | 853.77 | 1159.57 | 15.9 | 3+ / y10 | 2920 |
| THTC[CAM]PPPC[CAM]PAPELLGGPSVFLFPPK | 873.77 | 1031.59 | 16.0 | 3+ / y9 | 680 |
| SRWQQGNVFSC[CAM]SVMHEALHNHYTQK | 761.86 | 927.44 | 15.3 | 4+ / y7 | 560 |
| NTAVLQMNSLRAEDTAVYYC[CAM]SRWGGDGFYAMDYWGQGTLVTVSSASTK | 1079.09 | 1150.63 | 16.0 | 5+ / y12 | 120 |
| EVQLVESGGGLVQPGGSLRLSC[CAM]AASGFNIK | 758.40 | 1167.58 | 15.8 | 4+ / y11 | 260 |
| GQPREPQVYTLPPSREELTK | 771.07 | 945.50 | No Peak | 3+ / y8 | No Peak |
| PREQVYNSTYRVVSVLTVLHQDWLNGK | 808.67 | 997.49 | No Peak | 4+ / y8 | No Peak |
| GQPREPQVYTLPPSREEMTK | 791.73 | 1074.52 | 11.3 | 3+ / y9 | 31820 |
| VEPPK | 285.17 | 341.22 | No Peak | 2+ / y3 | No Peak |

Table 2: Proposed and Found peptide by MRMPilot on AB/Sciex TripleTOF™ 5600 from Lys-C Digested Herceptin Heavy Chain

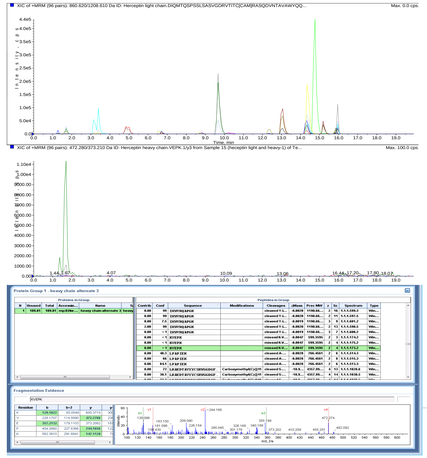

Figure 1. Representative Chromatogram of Lys-C Digested Herceptin (Verify Sequence "VEPK" for single proline)

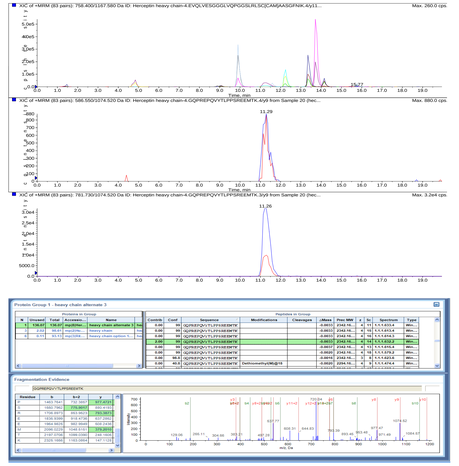

Figure 2. Representative Chromatogram of Lys-C Digested Herceptin (Verify Sequence "SREEMTK", no evidence for DELTK)

**Herceptin light chain**

DIQMTQSPSS LSASVGDRVT ITCRASQDVN TAVAWYQQKP GKAPKLLIYS ASFLYSGVPS
RFSGSRSGTD FTLTISSLQP EDFATYYCQQ HYTTPPTFGQ GTKVEIKRTV AAPSVFIFPP
SDEQLKSGTA SVVCLLNNFY PREAKVQWKV DNALQSGNSQ ESVTEQDSKD STYSLSSTLT
LSKADYEKHK VYACEVTHQG LSSPVTKSFN RGEC

**Herceptin heavy chain-4**

EVQLVESGGG LVQPGGSLRL SCAASGFNIK DTYIHWVRQA PGKGLEWVAR IYPTNGYTRY
ADSVKGRFTI SADTSKNTAY LQMNSLRAED TAVYYCSRWG GDGFYAMDYW GQGTLVTVSS
ASTKGPSVFP LAPSSKSTSG GTAALGCLVK DYFPEPVTVS WNSGALTSGV HTFPAVLQSS
GLYSLSSVVT VPSSSLGTQT YICNVNHKPS NTKVDKKVEP KSCDKTHTCP PCPAPELLGG
PSVFLFPPKP KDTLMISRTP EVTCVVVDVS HEDPEVKFNW YVDGVEVHNA KTKPREEQYN
STYRVVSVLT VLHQDWLNGK EYKCKVSNKA LPAPIEKTIS KAKGQPREPQ VYTLPPSREE
MTKNQVSLTC LVKGFYPSDI AVEWESNGQP ENNYKTTPPV LDSDGSFFLY SKLTVDKSRW
QQGNVFSCSV MHEALHNHYT QKSLSLSPGK

Figure 3, Results from determination by MRMPilot on AB/Sciex TripleTOF™ 5600 system for Herceptin.
Note: Red sequence was observed in digest.
Yellow sequence was not observed.
Black sequence was not estimated by MRMPilot

• Confirmation of the protein sequence was accomplished by TripleTOF™ 5600 System analysis combined with MRMPilot™ and ProteinPilot™ data processing and sequence similarity database searching tools.

•After complete denature, reduction and alkylation, the protein was digested by the enzyme into a collection of peptides which were then separated by LC and analyzed by TripleTOF™ 5600 System based on the MRM transitions optimized automatically by MRMPilot™ and ProteinPilot™ Software. Extensive sequence coverage was achieved.

• The protein herceptin has a light chain and a heavy chain, where the heavy chain has some sequence variants with only one or two amino acid difference. The peptide mapping results not only identified and confirmed the extensive sequence information over the whole length of the protein herceptin including both light chain and heavy chain, but also demonstrated the correct version.

## Conclusions

This method demonstrates the advantage of fast chromatography combined with high resolution, high mass accuracy mass spectrometry to confirm the sequence identity of complex proteins. The method is a simple, rapid, accurate and useful LC-HRMS-based approach for protein sequence mapping analysis of protein therapeutics.