

High Resolution Glycopeptide Mapping of EPO Using an Agilent AdvanceBio Peptide Mapping Column

Application Note

BioPharma

Abstract

The 2.7 µm Agilent AdvanceBio Peptide Mapping column is specifically designed to improve separations of peptide and peptide mapping applications. The column is based on superficially porous technology and was developed for fast and efficient separation of complex peptide mapping mixtures. This application note demonstrates peptide mapping performance with the AdvanceBio Peptide Mapping column for profiling a tryptic digest of recombinant human erythropoietin (rhEPO) protein as a model digest for glycopeptide profiling. Like many protein therapeutics, rhEPO exhibits a great deal of heterogeneity due to modifications that occur during manufacturing. The AdvanceBio Peptide Mapping column, with an optimized C18 coating technology, provides excellent peptide selectivity and resolution across a broad elution range to enhance separation of the glycopeptide fragments for fast, efficient, and sensitive mass spectrometry analysis.



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Introduction

Peptide mapping using reversed-phase HPLC of proteolytic peptides, combined with electrospray ionization mass spectrometry (ESI/MS), has become the method of choice for establishing the identity and efficacy of biotherapeutic products [1]. Peptide fragment analysis by liquid chromatography/mass spectrometry (LC/MS) is a crucial requirement for the release of these products and plays an important role to complete the protein characterization strategy. For example, the stability of a recombinant protein therapeutic requires long term monitoring throughout the shelflife of the product for important modifications, such as glycosylation. Although glycosylation can be analyzed on the intact protein, glycopeptide mapping is necessary to provide additional information of critical importance, such as sequence information, mass analyses, and identification of the glycosylation sites.

This application note used an rhEPO protein as a model glycoprotein to demonstrate high resolution peptide mapping and glycopeptide analysis by LC/MS using a 2.7 μ m AdvanceBio Peptide Mapping column.

The column was specifically designed to improve separations for peptide mapping applications and provided increased resolution, higher sensitivity, and greater selectivity needed for high efficiency peptide profiling, such as that required for rhEPO mapping and enhanced glycopeptide mining. Furthermore, the 2.7 μ m superficially porous particles enabled the use of a longer column (2.1× 250 mm) at a higher flow rate delivering sub-2 μ m type resolution within HPLC column pressures.

Materials and Methods

Recombinant human EPO was purchased from Creative Biolab, Shirley, NY. Triflouroacetic acid was purchased from Sigma-Adrich Corp., St. Louis, MO, and iso-propanol and acetonitrile were supplied from Honeywell-Burdick & Jackson, Muskegon, MI. High quality sequence-grade trypsin was obtained from the Stratagene division of Agilent Technologies.

Protease digestion was accomplished by adding trypsin protease to a solution including approximately 4.2 mg (2.1 mg/mL, 2 mL) EPO. The ratio of substrate and enzyme was 50:1 (w:w). The mixed solution was incubated at 37 °C for 12 hours. The digestion was quenched by storing the sample at -70 °C. After validation, 3.78 mg (2.1 mg/mL, 1.8 mL) of digested EPO was obtained. An Agilent 1200 Infinity LC System was used, with auto injector (HiP-ALS), binary pump, thermostatted column compartment (TCC) and diode array detector (DAD), coupled to an Agilent 6224 TOF LC/MS. Data was processed using an Agilent MassHunter Qualitative Analysis software and an Agilent MassHunter BioConfirm.

TOF MS parameters

Spectra were recorded in positive ion and in centroid mode.

Gas temperature:	350 °C
Drying gas:	10 L/min
Nebulizer:	45 psi
V _{cap} :	3,500 V
Fragmentor:	170 V
Skimmer:	65 V
Octapole 1 RF:	750 V
MS:	4 Hz
Mass range:	100 to 7,000 <i>m/z</i>
Reference mass:	149.02332 922.009798
Acq. mode:	Extended dynamic range mode (2 GHz)

Chromatographic conditions

Column:	Agilent AdvanceBio Peptide Mapping, 2.1× 250 mm, 2.7 μm (p/n 651750-902)	
Eluent:	A, H ₂ O + 0.1% formic acid (v/v); B, acetonitrile + 0.1% formic acid (v/v)	
Injection volume:	5 μL (2 μg/μL)	
Flow rate:	0.4 mL/min	
Gradient:	Time (min) 0 28 33 34	% B 3 45 60 95
Temperature:	55 °C	

Results and Discussion

Optimized rhEPO HPLC peptide mapping with an AdvanceBio Peptide Mapping column

Systematic method development was performed for mapping the rhEPO digest to determine the best resolved separation under water/ACN conditions. During these evaluations, 2.1 × 150 mm and 2.1 × 250 mm AdvanceBio Peptide Mapping columns were evaluated under various flow, temperature, and gradient profiles. Additionally, since peptide mapping separations work well under conditions that are favorable for ESI/MS, separations were evaluated with trifluoroacetic acid (TFA) and formic acid (FA) ion-pair additives to determine effects on retention and peak shape. The AdvanceBio Peptide Mapping column delivered excellent separation performance and compatibility with both TFA and FA ion-pair additives. The optimized separation displayed in Figure 1 provided the best separation profile for rhEPO mapping. The 2.1 × 250 mm AdvanceBio Peptide Mapping column and optimized chromatographic conditions with formic acid as the ion-pair additive enabled a high resolution separation of the rhEPO tryptic digest, providing very narrow peak widths, increased sensitivity, and unique selectivity across the gradient profile, making this separation highly amendable to ESI/MS analysis.



Figure 1. Reversed-phase rhEPO peptide map using a 2.1 × 250 mm, 2.7 µm Agilent AdvanceBio Peptide Mapping column (See Chromatographic conditions section).

Rapid rhEPO tryptic digest mapping by ESI-MS

Peptide maps often take 2 hours or longer using a 2.1 × 250 mm column to produce the needed resolution. Additionally, re-equilibration and run-to-run cycle times can add extended time to the completed analysis, significantly affecting laboratory production. While faster maps are desirable, it is critically important that resolution is not compromised. Figure 2 shows the total ion chromatogram (TIC) during a fast LC/MS analysis on a 2.1 × 250 mm column completed in less than 35 minutes. Excellent peak retention, peak shapes, and resolution across the gradient are all indicators of a robust method for LC/MS validation of the peptide mapping analysis.



Figure 2. Total ion chromatogram of an LC/MS analysis on a 2.1 × 250 mm Agilent AdvancedBio Peptide Mapping column accomplished in under 35 minutes.

The faster analysis gained from the AdvancedBio Peptide Mapping column did not compromise the chromatographic map of rhEPO separation. Figure 3 shows the results from the Agilent MassHunter Molecular Feature Extractor (MFE). MFE is an algorithm that finds compounds from complex data and creates averaged MS spectra for each compound. These MFE compound results are then matched back to the rhEPO protein sequence. This 34 minute LC/MS method resulted in 100% sequence coverage of the rhEPO protein.



Figure 3. 100% rhEPO sequence coverage achieved using the 2.1 × 250 mm Agilent AdvanceBio Peptide Mapping column. Data generated using the MFE in Agilent MassHunter qualitative analysis software.

The rhEPO has a predicted molecular mass of 24,000 Dalton and apparent glycosylated molecular mass of 30,400 Dalton, and, thus, serves as an effective glycoprotein model to demonstrate glycopeptide profiling using the AdvanceBio Peptide Maping column. Figure 4 is the extracted peptide glycosylation profile from the total LC/MS analysis shown in Figure 3, while Table 1 shows the mass and glycosylated sequence information. The AdvanceBio Peptide Mapping column enabled the identity of 42 unique glycopeptides and demonstrated the performance advantage and utility of this column for highly complex peptide mapping, where posttranslational modification information, such as glycosylation, is critical to the complete protein characterization.



Figure 4. MFE extracted peptide glycosylation profile using the 2.1 × 250 mm Agilent AdvanceBio Peptide Mapping column. Data generated using Agilent MassHunter Molecular Feature Extractor (MFE) on an Agilent 6224 TOF LC/MS.

22.796 4272.681 1°G1F/G2F [A147] VYSNFLR 24.569 4396.602 1°G1/G2 [A147] LFRVYSNFLR 22.348 4110.643 1°G0F [NGA2F/G2F A147] LFRVYSNFLR 22.348 4110.643 1°G0F [NGA2F/G2F A147] LFRVYSNFLR 22.348 4110.643 1°G0 [NGA2F/G2F [A147] LFRVYSNFLR 22.348 4110.643 1°G0 [NGA2F/G2F [A147] VKFVAWK 20.752 3688.49 1°G0 [NGA2F/G2F [A147] VKFVAWK 20.396 4039.697 1'3132 2A 06 [A147] VYSNFLR 20.396 4039.697 1'3132 2A 06 [A147] VYSNFLR 20.391 600.6147] VYSNFLR 20.392 21.311 1.40 [A147] VYNFVAWK 20.492 22.404 3027.207 1'31312 2A 36 [A147] VYSNFLR 23.922 2730.183 1'300 0A 06 [A47] VYSNFLR 20.773 3686.512 1'3032 1A 16 or 3122 0A 26 [A147] VYSNFLR 21.72 3737.555 1'3021 2A 16 [A147] LFRVYSNFLR 21.72 4456.615 <	RT	Glyco-Peptide Accurate Mass	Pred glycan modification [seq. location]	Sequence
22.3484110.6431°GUF (NGA2//GE A147)VYSNFLR22.7554218.7551°GU (NGA2//GE [NAA7]UYSNFLR20.7623660.491°GU (NGA2//GE [NAA2] [A47]VYSNFLRG20.7843786.5771°J32 2.406 [A147]VYSNFLRG20.3054038.6971°J32 2.406 [A147]VYSNFLRGKLK20.304053.3161°J312 2.406 [A147]VYSNFLRGKLK20.305303.6971°J312 2.406 [A147]VYSNFLRGKLK21.2022757.3231°J310 A0 G [A47]VYSNFLRGKLK22.4043027.2071°J311 1.406 [A147]VYSNFLRGK20.4022868.5121°J300 A.0 G [A47]VYSNFLRGK20.4123686.5121°J302 1.416 or 3122 0.426 [A147]VYSNFLRGK20.4123685.521°J302 1.416 or 3122 0.426 [A147]VYSNFLRGK21.7133868.5051°J302 1.416 or 3122 0.426 [A147]VYSNFLRGK21.714373.5561°J302 1.416 or 3122 0.426 [A147]VYSNFLRGK21.714373.5561°J302 0.406 (G2F [NA2F] [A24]EAENITTGCAEHCSUNENTVPDTK21.715374.5161°J200 0.406 (G2F [NA2F] [A24]EAENITTGCAEHCSUNENTVPDTK21.712374.5561°J201 0.406 (G47]VYFNAWKR21.712366.5221°J201 0.406 (G47]VYFNAWKR21.713366.5731°J201 0.406 (G47]VYFNAWKR21.714374.5661°J00 0.406 (G0/ [MC42] 1'3000 0.406 (G42A36)EAENITTGCAEHCSUNENTVPDTKNYFAWKR21.715364.5731°J201 0.406 (G41/3000 0.406 (G42A36)EAENITTGCAEHCSUNENTVPDTKNYFAWKR21.7143	22.796	4272.681	1*G1F/G2F [A147]	VYSNFLR
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23.922 2730.163 1'3100 0A.6G A'A' VNFYAWKR 20.642 3668.512 1'3032 1A 16 or 3122 0A 2G [A147] VYSNFLRGK 20.773 3668.505 1'3032 1A 16 or 3122 0A 2G [A147] VYSNFLRGK 22.712 3737.536 1'3022 1A 16 [A147] USNFLRGK 22.712 3737.536 1'302 0A 0G [G2P] (NA2F] [A24] EAFNITGCAEHCSINENITVPDTK 24.12 4456.815 1'2120 0A 0G (G2P) (NGA2F) [A47] VNFYAWKR 15.278 2527.093 1'2100 A 0G (G47] VNFYAWKR 15.283 3147.255 1'2021 0A 1G (A47] VNFYAWKR 17.752 3243.417 1'2021 0A 1G (A47] VNFYAWKR 26.01 3065.322 1'2011 0A 1G [A147] VSNFLR 26.01 3065.322 1'2011 0A 1G [A147] VSNFLR 17.767 8505.473 1'2010 0A 0G (G0) (NGA2)1'3032 0A 2G [A24A36] VERYLLEAKEAENITTGCAEHCSINENITVPDTKVNFYAWK 23.399 7653.184 1'2010 0A 0G (G0) (NGA2)1'3032 0A 2G [A24A36] EAENITTGCAEHCSINENITVPDTKVNFYAWK 15.971 777.212 1'1111 A 0G [A147] VSNFLRGK	22.404	3027.207	1*3111 1A 0G [A47]	VNFYAWK
20.642 5688.512 1'3032 1A 1G or 3122 0A 2G [A147] VYSNFLRGK 20.773 5668.505 1'3032 1A 1G or 3122 0A 2G [A147] VYSNFLRGK 22.712 3'37.536 1'3022 1A 1G [A147] LFRVYSNFLR 19.456 2'46.161 1'3010 0A 0G [A47] VNFYAWKR 24.12 4456.815 1'2120 0A 0G (G2F) [NA2F] [A24] EAENITTGCAEHCSLNENITVPDTK 16.278 2527.093 1'2100 0A 0G (G0F) [NGA2F] [A47] VNFYAWKR 19.283 3147.255 1'2021 0A 1G [A47] VNFYAWKR 26.01 3065.322 1'2010 0A 1G [A47] UFNYSNFLR 26.01 3065.322 1'2011 0A 1G [A147] VYSNFLR 26.01 3065.322 1'2010 0A 1G [A147] VYSNFLR 26.01 3045.706 1'2011 0A 1G 1'G1F/G2F[A24A36] VERYLEAKEAENITTGCAEHCSLNENITVPDTKVNFYAWKR 17.979 7044.846 1'2010 0A 0G (G0) [NGA211'3032 0A 2G [A24A36] EAENITTGCAEHCSLNENITVPDTKVNFYAWKR 23.939 7653.184 1'2000 0A 0G (G0) [NGA21]'3032 0A 2G [A24A36] EAENITTGCAEHCSLNENITVPDTKVNFYAWKR 23.940 777.212 1'1111 1A 0G [A147] YSNFLRGK </td <td>26.057</td> <td>2998.23</td> <td>1*3111 1A 0G [A147]</td> <td>VYSNFLR</td>	26.057	2998.23	1*3111 1A 0G [A147]	VYSNFLR
20.7333668.5051.3032 1A 1G or 3122 0A 2G [A147]VYSNFLRGK22.712373.7581.3022 1A 1G [A147]LRVYSNFLR19.456276.611.3010 0A 0G [A47]VNFYAWKR24.124456.8151.2120 0A 0G (G2F) [NA2F] [A24]EAENITTGCAEHCSLNENITVPDTK19.2833147.2551.2120 0A 0G (G0F) (NGA2F) [A47]VNFYAWKR17.7522243.4171.2022 1A 1G [A47]VNFYAWKR20.013065.3221.2101 1A 0G [A147]UFRVYSNFLR20.212655.0731.2011 1A 0G [A147]VSNFLR17.723045.7061.2011 0A 1G 1A16 I*G2F [A24A36]VLERYLEAKEAENITTGCAEHCSLNENITVPDTKVNFYAWK17.733505.4731.2011 0A 1G 1A16 I*G2F [A24A36]VLERYLEAKEAENITTGCAEHCSLNENITVPDTKVNFYAWK17.743505.4731.2011 0A 1G 1A10 [A147]VSNFLR23.3907653.1841.2000 0A 0G (G0) (NGA2) 1*3032 0A 2G [A24A36]EAENITTGCAEHCSLNENITVPDTKVNFYAWK24.013509.501.1111 0AG [A147]VSNFLRGK24.01277.2121.1111 1A 0G [A147]VSNFLRGK24.01265.1971.1110 0A 0G [A147]VSNFLRGK25.1971.1110 0A 0G [A147]VSNFLRGK25.1983063.761.1011 0A 1G [A147]VSNFLRGK25.1971.1110 0A 0G [A147]VSNFLRGK25.198365.3761.1011 0A 1G [A147]25.1971.1011 0A 1G [A147]VSNFLRGK25.198363.761.1011 0A 1G [A147]25.198363.761.1011 0A 1G [A147]25.1991.1011 0A 1G [A147]VSNFLRGK	23.922	2730.163	1*3100 0A 0G [A47]	VNFYAWKR
22.712 3737.536 1'3022 IA 1G [A147] LFRVYSNFLR 19.456 2746.161 1'3010 0A 0G [A47] VNFYAWKR 24.12 4456.815 1'2120 0A 0G (G2F) [NA2F] [A24] EAENITTGCAEHCSLNENITVPDTK 16.278 2527.093 1'2100 0A 0G (G0F) (NGA2F) [A47] VNFYAWKR 19.283 3147.255 1'2022 IA 1G [A47] VNFYAWK 17.752 3243.417 1'2021 0A 1G [A47] VNFYAWK 26.01 3065.322 1'2011 A 0G [A147] LFRVYSNFLR 20.296 2665.073 1'2010 A 1G [A147] VSNFLR 17.77 8505.473 1'2010 A 0G (G0) (NGA2) I'3032 0A 2G [A24A36] VLERYLLEAKEAENITTGCAEHCSLNENITVPDTKVNFYAWK 17.979 7044.846 1'2010 A 0 G (G0) (NGA2) I'3032 0A 2G [A24A36] EAENITTGCAEHCSLNENITVPDTKVNFYAWK 23.399 7653.184 1'2000 0A 0G (G0) (NGA2) I'3032 0A 2G [A24A36] EAENITTGCAEHCSLNENITVPDTKVNFYAWK 16.827 2777.212 1'1111 1 A 0G [A147] VSNFLR 24.019 6396.629 1'2000 0A 0G (G0) (NGA2) I'3030 0A 0G [A24A36] EAENITTGCAEHCSLNENITVPDTKVNFYAWK 16.827 2777.212 1'1110 1 0A	20.642	3668.512	1*3032 1A 1G or 3122 0A 2G [A147]	VYSNFLRGK
19.456 2746.161 1'3010 0.0 G(A47) VNFYAWKR 24.12 4456.815 1'2120 0.0 G(GEF) (NA2F) [A24] EAENITTGCAEHCSLNENITVPDTK 16.278 2527.093 1'2100 0.0 G(GF) (NGA2F) [A47] VNFYAWKR 19.283 3147.255 1'2021 0.1 G [A47] VNFYAWKR 17.752 3243.417 1'2021 0.1 G [A47] LFRVYSNFLR 20.01 3065.322 1'2011 0.0 G [A147] LFRVYSNFLR 20.296 2665.073 1'2011 0.1 G [A147] VSNFLR 20.297 8505.473 1'2011 0.0 G [G1]' 1302 0.1 G [A24A36] VLERYLLEAKEAENITTGCAEHCSLNENITVPDTK 17.767 8505.473 1'2011 0.0 G [G1]' 1303 0.0 G [A24A36] EAENITTGCAEHCSLNENITVPDTKVNFYAWK 23.399 7653.184 1'2010 0.0 G [G0] (NGA2)1'3030 0.0 G [A24A36] EAENITTGCAEHCSLNENITVPDTKVNFYAWKR 24.019 6396.629 1'2000 0.0 G (G0) (NGA2)1'3030 0.0 G [A24A36] EAENITTGCAEHCSLNENITVPDTKVNFYAWKR 24.019 6396.629 1'2010 0.0 G [A147] VSNFLRGK 24.019 6396.629 1'1011 0.0 G [A147] VSNFLRGK 24.110 2300.096 1'1101 0.0 G [A147]	20.773	3668.505	1*3032 1A 1G or 3122 0A 2G [A147]	VYSNFLRGK
24.12 4456.815 1'2120 0A 0G (GDF) (NA2F) [A24] EAENITTGCAEHCSLNENITVPDTK 16.278 2527.093 1'2100 0A 0G (GDF) (NGA2F) [A47] VNFYAWKR 19.283 3147.255 1'2021 AA 1G [A47] VNFYAWK 17.752 3243.417 1'2021 0A 1G [A47] LFRVYSNFLR 26.01 3065.322 1'2011 0A 1G [A147] LFRVYSNFLR 20.296 2665.073 1'2011 0A 1G [A147] VYSNFLR 18.735 9045.706 1'2011 0A 1G 1'3022 1A 1G [A2436] VLERYLLEAKEAENITTGCAEHCSLNENITVPDTK 17.767 8505.473 1'2011 0A 1G 1'3022 1A 1G [A2436] VLERYLLEAKEAENITTGCAEHCSLNENITVPDTKVNFYAWK 17.979 7044.846 1'2010 0A 0G (G01) (NGA2)1'3030 0A 0G [A24A36] EAENITTGCAEHCSLNENITVPDTKVNFYAWK 23.399 7653.184 1'2000 0A 0G (G01) (NGA2)1'3030 0A 0G [A24A36] EAENITGCAEHCSLNENITVPDTKVNFYAWK 16.927 2777.212 1'1111 1A 0G [A147] VYSNFLR 23.401 2565.197 1'1010 0A 0G [A147] VYSNFLR 23.412 2665.037 1'1010 0A 0G [A147] VYSNFLR 23.413 2300.996 1'1110 0A 0G [A147] VYSNFLR 23.414 2565.197 1'1010	22.712	3737.536	1*3022 1A 1G [A147]	LFRVYSNFLR
16.278 2527.093 1*2100 0A 0G (GP) (NGA2F) [A47] VNFYAWKR 19.283 3147.255 1*2022 1A 1G [A47] VNFYAWK 17.752 3243.417 1*2021 0A 1G [A47] LFRVYSNFLR 26.01 3065.322 1*2011 1A 0G [A147] LFRVYSNFLR 20.296 2665.073 1*2011 0A 1G [A147] VYSNFLR 18.735 9045.706 1*2011 0A 1G [A147] VYSNFLR 17.767 8505.473 1*2010 0A 0G (G0) (NGA2)1*3030 0A 0G [A24A36] EAENITTGCAEHCSLNENITVPDTKVNFYAWK 17.767 8505.473 1*2010 0A 0G (G0) (NGA2)1*3030 0A 0G [A24A36] EAENITTGCAEHCSLNENITVPDTKVNFYAWK 23.399 7653.184 1*2000 0A 0G (G0) (NGA2)1*3030 0A 0G [A24A36] EAENITTGCAEHCSLNENITVPDTKVNFYAWKR 24.019 6396.629 1*2000 0A 0G (G0) (NGA2)1*3030 0A 0G [A24A36] EAENITTGCAEHCSLNENITVPDTKVNFYAWKR 16.927 2777.212 1*1111 0 AG [A147] VYSNFLR 23.401 2565.197 1*1100 0A 0G [A147] VYSNFLR 23.412 24.62 1*101 0 A 1G [A147] VYSNFLR 23.855 3063.376 1*101 0 A 1G [A147] VYSNFLRGK	19.456	2746.161	1*3010 0A 0G [A47]	VNFYAWKR
19.283 3147.255 1'2022 1A 1G [A47] VNFYAWK 17.752 3243.417 1'2021 0A 1G [A47] LFRVYSNFLR 26.01 3065.322 1'2011 1A 0G [A147] LFRVYSNFLR 20.296 2665.073 1'2011 0A 1G [A147] VYSNFLR 18.735 9045.706 1'2011 0A 1G 1'G2F[A24A36] VLERYLLEAKEAENITTGCAEHCSLNENITVPDTK 17.767 8505.473 1'2010 0A 0G (G1)''3030 0A 0G [A24A36] EAENITTGCAEHCSLNENITVPDTKVNFYAWK 23.399 7653.184 1'2000 0A 0G (G0) (NGA2)''3032 0A 2G [A24A36] EAENITTGCAEHCSLNENITVPDTKVNFYAWK 24.019 6396.629 1'2000 0A 0G (G0) (NGA2)''3030 0A 0G [A24A36] EAENITTGCAEHCSLNENITVPDTKVNFYAWK 16.927 2777.212 1'1111 1A 0G [A147] VYSNFLRGK 17.586 2462 1'1010 0A 0G [A147] VYSNFLRGKLK 17.586 2462 1'1011 0A 1G [A147] VYSNFLRGK 15.701 7365.966 1'1011 0A 1G 1'2022 2A 0G [A24A36] EAENITTGCAEHCSLNENITVPDTKVNFYAWK 19.961 2340.001 1'1011 0A 1G [A147] VYSNFLRGK 17.586 266.197 1'1011 0A 1G [A147] VYSNFLRG	24.12	4456.815	1*2120 0A 0G (G2F) (NA2F) [A24]	EAENITTGCAEHCSLNENITVPDTK
17.7523243.41712021 0.A 1 G (A47)LFRVSNFLR26.013065.32212011 A.O G (A17)LFRVSNFLR20.2022665.07312011 0.A 1 G (A17)VSNFLR18.7359045.0612011 0.A 1 G (A16, 164, 264, 264, 264, 264, 264, 264, 264, 2	16.278	2527.093	1*2100 0A 0G (G0F) (NGA2F) [A47]	VNFYAWKR
26.113065.3221/2011 A 0 G [A147]LFRVSNFLR20.266265.0731/2011 0 A 1 G [A147]VYSNFLR18.7369045.7061/2011 0 A 1 G 1 G 1 G 2 G A 2 A 3 GVLERYLEAKEAENITGCAEHCSLNENITVPDTKVNFYAWK17.7678505.4731/2011 0 A 1 G 1 3 302 2 I A 1 G [A24A36]VLEAKEAENITGCAEHCSLNENITVPDTKVNFYAWK17.9797044.861/2010 0 A 0 G (G) (NGA2)1'303 0 A 0 G [A24A36]EAENITGCAEHCSLNENITVPDTKVNFYAWK23.9801/30.08 0 G (G) (NGA2)1'303 0 A 0 G [A24A36]EAENITGCAEHCSLNENITVPDTKVNFYAWK24.019636.6291/2000 A 0 G (G) (NGA2)1'300 0 A 0 G [A24A36]EAENITGCAEHCSLNENITVPDTKVNFYAWK16.927877.2121/111 1 A 0 G [A147]VYSNFLRGK17.840200.9961/110 0 A 0 G [A147]VYSNFLRGK23.441265.1971/110 0 A 0 G [A147]VYSNFLRGK17.5563063.3761/101 0 A 1 G [A147]LFRVSNFLRGK15.710365.9661/101 0 A 1 G [A147]KFNYSNFLRGK15.711305.9661/101 0 A 0 G [A47]VYFAWKR19.812240.011/100 A 0 G [A47]VYFAWKR19.813304.0011/100 A 0 G [A47]VYFAWKR20.825340.0061/100 A 0 G [A47]VYFAWKR20.8361/100 A 0 G [A47]VYFAWKR20.845340.0061/100	19.283	3147.255	1*2022 1A 1G [A47]	VNFYAWK
20.2962665.0731'2011 0A 1 G [A147]VYSNFLR18.7359045.7061'2011 0A 1 G 1'G1F/G2F[A24A36]VLERYLLEAKEAENITTGCAEHCSLNENITVPDTK17.7678505.4731'2011 0A 1 G 1'3022 1A 1 G [A24A36]YLLEAKEAENITTGCAEHCSLNENITVPDTKVNFYAWK17.9797044.8461'2010 0A 0G (G1)1'3030 0A 0G [A24A36]EAENITTGCAEHCSLNENITVPDTKVNFYAWK23.3997653.1841'2000 0A 0G (G0) (NGA2)1'3032 0A 2G [A24A36]EAENITTGCAEHCSLNENITVPDTKVNFYAWKR24.0196396.6291'2000 0A 0G (G0) (NGA2)1'3000 0A 0G [A24A36]EAENITTGCAEHCSLNENITVPDTKVNFYAWKR16.9272777.2121'1111 1A 0G [A147]VYSNFLR23.4012565.1971'1100 0A 0G [A147]VYSNFLRGKLK23.8953063.3761'1011 0A 1G [A147]VYSNFLRGK15.7017365.9661'1011 0A 1G [A147]LFRVYSNFLRGK19.9612340.0011'1010 0A 0G [A47]VNFYAWKR20.2852340.0061'101 0A 0G [A47]VNFYAWKR17.868750.1051'101 0A 0G [1'3132 2A 0G [A24A36]EAENITTGCAEHCSLNENITVPDTKVNFYAWK	17.752	3243.417	1*2021 0A 1G [A47]	LFRVYSNFLR
18.735 9045.706 1*2011 0A 1G 1*G1F/G2F[A24A36] VLERYLLEAKEAENITTGCAEHCSLNENITVPDTK 17.767 8505.473 1*2011 0A 1G 1*3022 1A 1G [A24A36] YLLEAKEAENITTGCAEHCSLNENITVPDTKVNFYAWKR 17.779 7044.846 1*2010 0A 0G (G)1'3030 0A 0G [A24A36] EAENITTGCAEHCSLNENITVPDTKVNFYAWKR 23.399 7653.184 1*2000 0A 0G (G0) (NGA2)1*3032 0A 2G [A24A36] EAENITTGCAEHCSLNENITVPDTKVNFYAWKR 24.019 6396.629 1*2000 0A 0G (G0) (NGA2)1*3000 0A 0G [A24A36] EAENITTGCAEHCSLNENITVPDTKVNFYAWKR 16.927 2777.212 1*111 1A 0G [A147] VYSNFLRGK 23.401 2300.996 1*1110 0A 0G [A147] VYSNFLRGK 23.402 265.197 1*110 0A 0G [A147] VYSNFLRGK 23.885 3063.376 1*1011 0A 1G [A147] VYSNFLRGK 15.701 7365.966 1*1011 0A 1G [A147] LFRVYSNFLRGK 19.961 2340.001 1*1010 0A 0G [A47] VNFYAWKR 20.285 2340.006 1*1010 0A 0G [A47] VNFYAWKR 20.285 2340.006 1*1010 0A 0G [A47] VNFYAWKR 20.285 2340.006 1*1010 0A 0G [A47]	26.01	3065.322	1*2011 1A 0G [A147]	LFRVYSNFLR
17.7678505.4731*2011 0A 1G 1*3022 1A 1G [A24A36]YLLEAKEAENITTGCAEHCSLNENITVPDTKVNFYAWK17.9797044.8461*2010 0A 0G (G1)1*3030 0A 0G [A24A36]EAENITTGCAEHCSLNENITVPDTKVNFYAWK23.3997653.1841*2000 0A 0G (G0) (NGA2)1*3032 0A 2G [A24A36]EAENITTGCAEHCSLNENITVPDTKVNFYAWKR24.0196396.6291*2000 0A 0G (G0) (NGA2)1*3000 0A 0G [A24A36]EAENITTGCAEHCSLNENITVPDTKVNFYAWKR16.9272777.2121*1111 1A 0G [A147]VYSNFLRGK18.4332300.9961*1100 0A 0G [A147]VYSNFLRGKLK23.4012565.1971*1101 0A 1G [A147]VYSNFLRGKLK17.58624621*1011 0A 1G [A147]VYSNFLRGK23.8853063.3761*101 0A 1G [A147]LFRVYSNFLRGK15.7017365.9661*1011 0A 1G 1*2022 2A 0G [A24A36]EAENITTGCAEHCSLNENITVPDTKVNFYAWK19.9612340.0011*100 0A 0G [A47]VNFYAWKR20.2852340.0061*101 0A 0G [A47]VNFYAWKR17.5867570.1051*101 0A 0G 1*3132 2A 0G [A24A36]EAENITTGCAEHCSLNENITVPDTKVNFYAWK	20.296	2665.073	1*2011 0A 1G [A147]	VYSNFLR
17.9797044.8461*2010 0A 0G (G1)1*3030 0A 0G [A24A36]EAENITTGCAEHCSLNENITVPDTKVNFYAWKR23.3997653.1841*2000 0A 0G (G0) (NGA2)1*3032 0A 2G [A24A36]EAENITTGCAEHCSLNENITVPDTKVNFYAWKR24.0196396.6291*2000 0A 0G (G0) (NGA2)1*3000 0A 0G [A24A36]EAENITTGCAEHCSLNENITVPDTKVNFYAWKR16.9272777.2121*1111 A 0G [A147]VYSNFLRGK18.4332300.9961*1110 0A 0G [A147]VYSNFLRGK23.4012565.1971*1100 0A 0G [A147]VYSNFLRGKLK17.58624621*1011 0A 1G [A147]VYSNFLRGK23.8853063.3761*1011 0A 1G [A147]LFRVYSNFLRGK15.7017365.9661*1011 0A 1G 1*2022 2A 0G [A24A36]EAENITTGCAEHCSLNENITVPDTKVNFYAWK19.9612340.0011*1010 0A 0G [A47]VNFYAWKR20.2852340.0061*1010 0A 0G [A47]VNFYAWKR17.8687570.1051*1010 0A 0G 1*3132 2A 0G [A24A36]EAENITTGCAEHCSLNENITVPDTKVNFYAWK	18.735	9045.706	1*2011 0A 1G 1*G1F/G2F[A24A36]	VLERYLLEAKEAENITTGCAEHCSLNENITVPDTK
23.3997653.1841*2000 0A 0G (G0) (NGA2)1*3032 0A 2G [A24A36]EAENITTGCAEHCSLNENITVPDTKVNFYAWKR24.0196396.6291*2000 0A 0G (G0) (NGA2)1*3000 0A 0G [A24A36]EAENITTGCAEHCSLNENITVPDTKVNFYAWKR16.9272777.2121*1111 1A 0G [A147]VYSNFLRGK18.4332300.9961*1110 0A 0G [A147]VYSNFLR23.4012565.1971*1100 0A 0G [A147]VYSNFLRGKLK17.58624621*1011 0A 1G [A147]VYSNFLR23.8853063.3761*1011 0A 1G [A147]LFRVYSNFLRGK15.7017365.9661*1011 0A 1G 1*2022 2A 0G [A24A36]EAENITTGCAEHCSLNENITVPDTKVNFYAWK19.9612340.0011*1010 0A 0G [A47]VNFYAWKR20.2852340.0061*101 0A 0G [A47]VNFYAWKR17.8687570.1051*101 0A 0G 1*3132 2A 0G [A24A36]EAENITTGCAEHCSLNENITVPDTKVNFYAWK	17.767	8505.473	1*2011 0A 1G 1*3022 1A 1G [A24A36]	YLLEAKEAENITTGCAEHCSLNENITVPDTKVNFYAWK
24.0196396.6291*2000 0A 0G (G0) (NGA2)1*3000 0A 0G [A24A36]EAENITTGCAEHCSLNENITVPDTKVNFYAWK16.9272777.2121*1111 1A 0G [A147]VYSNFLRGK18.4332300.9961*1110 0A 0G [A147]VYSNFLR23.4012565.1971*1100 0A 0G [A147]VYSNFLRGKLK17.58624621*1011 0A 1G [A147]VYSNFLR23.8853063.3761*1011 0A 1G [A147]LFRVYSNFLRGK15.7017365.9661*1011 0A 1G 1*2022 2A 0G [A24A36]EAENITTGCAEHCSLNENITVPDTKVNFYAWK19.9612340.0011*1010 0A 0G [A47]VNFYAWKR20.2852340.0061*1010 0A 0G [A47]VNFYAWKR17.8687570.1051*1010 0A 0G 1*3132 2A 0G [A24A36]EAENITTGCAEHCSLNENITVPDTKVNFYAWK	17.979	7044.846	1*2010 0A 0G (G1)1*3030 0A 0G [A24A36]	EAENITTGCAEHCSLNENITVPDTKVNFYAWK
16.9272777.2121*1111 A OG [A147]VYSNFLRGK18.4332300.9961*1110 0A OG [A147]VYSNFLR23.4012565.1971*1100 0A OG [A147]VYSNFLRGKLK17.58624621*1011 0A 1G [A147]VYSNFLR23.8553063.3761*1011 0A 1G [A147]LFRVYSNFLRGK15.7017365.9661*1011 0A 1G 1*2022 2A 0G [A24A36]EAENITTGCAEHCSLNENITVPDTKVNFYAWK19.9612340.0011*1010 0A 0G [A47]VNFYAWKR20.2852340.0061*1010 0A 0G [A47]KNFYAWKR17.8687570.1051*1010 0A 0G 1*3132 2A 0G [A24A36]EAENITTGCAEHCSLNENITVPDTKVNFYAWK	23.399	7653.184	1*2000 0A 0G (G0) (NGA2)1*3032 0A 2G [A24A36]	EAENITTGCAEHCSLNENITVPDTKVNFYAWKR
18.4332300.9961*1110 0A 0G [A147]VYSNFLR23.4012565.1971*1100 0A 0G [A147]VYSNFLRGKLK17.58624621*1011 0A 1G [A147]VYSNFLR23.8853063.3761*1011 0A 1G [A147]LFRVYSNFLRGK15.7017365.9661*1011 0A 1G 1*2022 2A 0G [A24A36]EAENITTGCAEHCSLNENITVPDTKVNFYAWK19.9612340.0011*1010 0A 0G [A47]VNFYAWKR20.2852340.0061*1010 0A 0G [A47]VNFYAWKR17.8687570.1051*1010 0A 0G 1*3132 2A 0G [A24A36]EAENITTGCAEHCSLNENITVPDTKVNFYAWK	24.019	6396.629	1*2000 0A 0G (G0) (NGA2)1*3000 0A 0G [A24A36]	EAENITTGCAEHCSLNENITVPDTKVNFYAWK
23.4012565.1971*1100 0A 0G [A147]VYSNFLRGKLK17.58624621*1011 0A 1G [A147]VYSNFLR23.8853063.3761*1011 0A 1G [A147]LFRVYSNFLRGK15.7017365.9661*1011 0A 1G 1*2022 2A 0G [A24A36]EAENITTGCAEHCSLNENITVPDTKVNFYAWK19.9612340.0011*1010 0A 0G [A47]VNFYAWKR20.2852340.0061*1010 0A 0G [A47]VNFYAWKR17.8687570.1051*1010 0A 0G 1*3132 2A 0G [A24A36]EAENITTGCAEHCSLNENITVPDTKVNFYAWK	16.927	2777.212	1*1111 1A 0G [A147]	VYSNFLRGK
17.58624621*1011 0A 1G [A147]VYSNFLR23.8853063.3761*1011 0A 1G [A147]LFRVYSNFLRGK15.7017365.9661*1011 0A 1G 1*2022 2A 0G [A24A36]EAENITTGCAEHCSLNENITVPDTKVNFYAWK19.9612340.0011*1010 0A 0G [A47]VNFYAWKR20.2852340.0061*1010 0A 0G [A47]VNFYAWKR17.8687570.1051*1010 0A 0G 1*3132 2A 0G [A24A36]EAENITTGCAEHCSLNENITVPDTKVNFYAWK	18.433	2300.996	1*1110 0A 0G [A147]	VYSNFLR
23.885 3063.376 1*1011 0A 1G [A147] LFRVYSNFLRGK 15.701 7365.966 1*1011 0A 1G 1*2022 2A 0G [A24A36] EAENITTGCAEHCSLNENITVPDTKVNFYAWK 19.961 2340.001 1*1010 0A 0G [A47] VNFYAWKR 20.285 2340.006 1*1010 0A 0G [A47] VNFYAWKR 17.868 7570.105 1*1010 0A 0G 1*3132 2A 0G [A24A36] EAENITTGCAEHCSLNENITVPDTKVNFYAWK	23.401	2565.197	1*1100 0A 0G [A147]	VYSNFLRGKLK
15.701 7365.966 1*1011 0A 1G 1*2022 2A 0G [A24A36] EAENITTGCAEHCSLNENITVPDTKVNFYAWK 19.961 2340.001 1*1010 0A 0G [A47] VNFYAWKR 20.285 2340.006 1*1010 0A 0G [A47] VNFYAWKR 17.868 7570.105 1*1010 0A 0G 1*3132 2A 0G [A24A36] EAENITTGCAEHCSLNENITVPDTKVNFYAWK	17.586	2462	1*1011 0A 1G [A147]	VYSNFLR
19.961 2340.001 1*1010 0A 0G [A47] VNFYAWKR 20.285 2340.006 1*1010 0A 0G [A47] VNFYAWKR 17.868 7570.105 1*1010 0A 0G 1*3132 2A 0G [A24A36] EAENITTGCAEHCSLNENITVPDTKVNFYAWK	23.885	3063.376	1*1011 0A 1G [A147]	LFRVYSNFLRGK
20.285 2340.006 1*1010 0A 0G [A47] VNFYAWKR 17.868 7570.105 1*1010 0A 0G 1*3132 2A 0G [A24A36] EAENITTGCAEHCSLNENITVPDTKVNFYAWK	15.701	7365.966	1*1011 0A 1G 1*2022 2A 0G [A24A36]	EAENITTGCAEHCSLNENITVPDTKVNFYAWK
17.868 7570.105 1*1010 0A 0G 1*3132 2A 0G [A24A36] EAENITTGCAEHCSLNENITVPDTKVNFYAWK	19.961	2340.001	1*1010 0A 0G [A47]	VNFYAWKR
	20.285	2340.006	1*1010 0A 0G [A47]	VNFYAWKR
24.685 7518.254 1*1010 0A 0G 1*2040 0A 0G [A24A36] YLLEAKEAENITTGCAEHCSLNENITVPDTKVNFYAWK	17.868	7570.105	1*1010 0A 0G 1*3132 2A 0G [A24A36]	EAENITTGCAEHCSLNENITVPDTKVNFYAWK
	24.685	7518.254	1*1010 0A 0G 1*2040 0A 0G [A24A36]	YLLEAKEAENITTGCAEHCSLNENITVPDTKVNFYAWK

Table 1. Predicted N-linked glycan modifications and sequence for recombinant human EPO.

RT	Glyco-Peptide Accurate Mass	Pred glycan modification [seq. location]	Sequence
19.896	1992.853	1*1000 0A 0G [A147]	VYSNFLR
22.134	7293.135	1*1000 0A 0G 1*3031 0A 1G [A24A36]	VLERYLLEAKEAENITTGCAEHCSLNENITVPDTK
29.128	7842.206	1*0100 0A 0G 1*G0F (NGA2F)/G1F [A24A36]	EAENITTGCAEHCSLNENITVPDTKVNFYAWKR
22.303	6912.043	1*0100 0A 0G1*3011 0A 1G [A24A36]	VLERYLLEAKEAENITTGCAEHCSLNENITVPDTK
17.671	6095.53	1*0100 0A 0G1*2010 0A 0G (G1) [A24A36]	EAENITTGCAEHCSLNENITVPDTKVNFYAWK

Table 1. Predicted N-linked glycan modifications and sequence for recombinant human EPO (continued).

Conclusions

The Agilent 2.7 μ m AdvanceBio Peptide Mapping column demonstrated excellent utility for fast and efficient LC/MS peptide mapping using a recombinant human erythropoietin protein digest. A 2.1 × 250 mm column dimension, and chromatographic conditions optimized for mass spectrometry analysis, provided high resolution separation of the tryptic rhEPO digest across the entire gradient profile demonstrating unique selectivity and retention features critical to generating a well-defined and resolved peptide map. The AdvanceBio Peptide Mapping column enabled 100% rhEPO sequence coverage by ESI/MS and provided 42 glycopeptide matches, making this column an excellent choice for highly complex peptide mapping applications.

Reference

1. L. J. Campbell, J. Y. Le Blanc. *Bioanalysis*, 3, 645 (2011).

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