

Peptide Mapping of Innovator and Biosimilar Monoclonal Antibody Using an Agilent 1290 Infinity UHPLC Coupled to an Agilent 6550 iFunnel Q-TOF LC/MS System

Application Note

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Introduction

Monoclonal antibodies (mAbs) are becoming one of the important classes of biomolecules for the treatment of various cancers. Biosimilar mAbs, which are the replica of the licensed innovator product in the market are also gaining lots of attention. According to the FDA definition¹, “*Biosimilars are a type of biological product that are licensed (approved) by FDA because they are highly similar to an already FDA-approved biological product, known as the biological reference product (reference product), and have been shown to have no clinically meaningful differences from the reference product.*” The development of these biosimilars is expanding due to the patent expiry of innovator drugs. Many biopharmaceutical companies are involved in the manufacturing of these biosimilars, more so in developing countries. LC/MS characterization is a powerful and essential tool to show comparability between innovators and biosimilars.

To ensure the quality of biosimilar mAbs, and to show molecular similarity with the innovators, their amino acid sequence confirmation is of crucial importance in biosimilar development. Peptide mapping is one of the vital steps to show similarity in the sequence and modifications between an innovator and biosimilar pair. Peptide mapping involves protease digestion of proteins/mAbs followed by LC/MS analysis. In this Application Note, commercial Rituximab, a chimeric mouse/human monoclonal antibody (used in the treatment of B-cell non-Hodgkin lymphomas, rheumatoid arthritis, and chronic lymphocytic leukemia) from innovator and biosimilar was subjected to trypsin digestion followed by peptide separation and mass determination on LC/Q-TOF MS. The innovator and biosimilar were compared for sequence similarity, oxidation, and deamidation status.



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Experimental

Materials

Rituximab biosimilar and innovator were purchased from a local pharmacy and stored according to manufacturer's instructions. DL-Dithiothreitol (DTT), idoacetamide, Trisbase, and LC/MS grade solvents were purchased from Sigma-Aldrich. High quality sequence grade trypsin was procured from Promega.

Trypsin digestion

Before the digestion of the mAbs with trypsin, the disulfides were reduced and alkylated under denaturation conditions. This pretreatment was done to ensure that the mAB was completely denatured and soluble so that protease can access its substrate efficiently. The mAbs that were in solution were lyophilized, and equal concentrations of both were reconstituted in 8 M urea in 0.25 M Tris buffer, pH 7.6, containing dithiothreitol (DTT). The solutions were then incubated at 37 °C for 30 minutes. Iodoacetamide in 0.25 M Tris buffer, pH 7.6, was added to these solutions, and the sample was incubated at ambient temperature in the dark for 15 minutes. The solutions were diluted with 0.25 M Tris buffer, pH 7.6, before digestion with trypsin.

Trypsin, at a ratio of 20:1 (protein to protease w/w) was added to the above pretreated mAb solutions. The reaction was kept for overnight incubation at 37 °C before LC/MS analysis. The enzymatic activity was quenched by adding 1 µL of 10 % formic acid solution. The samples were either immediately analyzed, or stored at –80 °C until LC/MS/MS analysis.

Instrumentation

LC system

Agilent 1290 Infinity LC System including:

- Agilent 1290 Infinity Binary Pump (G4220A)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C)
- Agilent 1290 Infinity Autosampler (G4226A)

- Agilent 1290 Infinity Thermostat (G1330B)

MS system

Agilent 6550 iFunnel Q-TOF LC/MS System with Agilent JetStream

LC/MS parameters

Parameter	Agilent 1290 Infinity LC System
Column	Agilent AdvanceBio Peptide Mapping, 2.1 × 250 mm, 2.7 µm (p/n 651750-902)
Sample thermostat	5 °C
Mobile phase A	0.1 % formic acid in water
Mobile phase B	90 % acetonitrile in water with 0.1 % formic acid
Gradient (segmented)	At 0 minutes → 3 %B At 40 minutes → 40 %B At 42 minutes → 95 %B At 44 minutes → 95 %B At 44.1 minutes → 3 %B
Stop time	44.1 minutes
Post time	10 minutes
Column temperature	60 °C
Flow rate	0.3 mL/min

Parameter	Agilent 6550 Q-TOF LC/MS System
Ion mode	Positive ion mode
Source	Agilent Dual JetStream
Drying gas temperature	250 °C
Drying gas flow	12 L/min
Sheath gas temperature	250 °C
Sheath gas flow	10 L/min
Nebulizer	25 psi
Capillary voltage	3,500 V
Nozzle	0 V
MS range	<i>m/z</i> 300–1,700
MS/MS range	<i>m/z</i> 50–1,700
MS scan rate (spectra/second)	6
MS/MS scan rate (spectra/second)	8
Ramped collision energy	Charge state slope offset 2 3.1 1 3 and >3 3.6 –4.8 1 3.5 6
Data analysis	The data obtained from LC/MS were analyzed using Agilent MassHunter Qualitative Analysis Software B.07 and Agilent MassHunter BioConfirm Software B.07

Results and Discussion

Peptide mapping is a regularly employed technique for evaluating the quality of antibodies in the pharmaceutical industry. Peptide mapping is regarded as the fingerprint of the protein under analysis. Therefore, it is an excellent technique to compare the similarity between innovator and its biosimilar version. Figure 1 shows the Total Ion Chromatogram (TIC) of trypsin-digested mAb obtained using

LC/MS for innovator and the biosimilar with AdvancedBio peptide mapping column. Inspection of the chromatograms reveals peptide peaks with no undigested protein product. They look similar in peak appearance across the chromatogram except for few minor differences.

Peptides masses were obtained from the LC/MS run using the Molecular Feature Extraction feature (MFE) of Bioconfirm. The masses of peptides obtained were

matched with the theoretical digest. The results of MS only gives 95 % sequence coverage of heavy chain, and 100 % for the light chain for trypsin digestion for both antibodies. The great separating power of the AdvancedBio peptide mapping column maximizes resolution and efficiency. This, in combination with the high analytical sensitivity and high accuracy MS, enables a good comparison of the innovator and biosimilar pair.

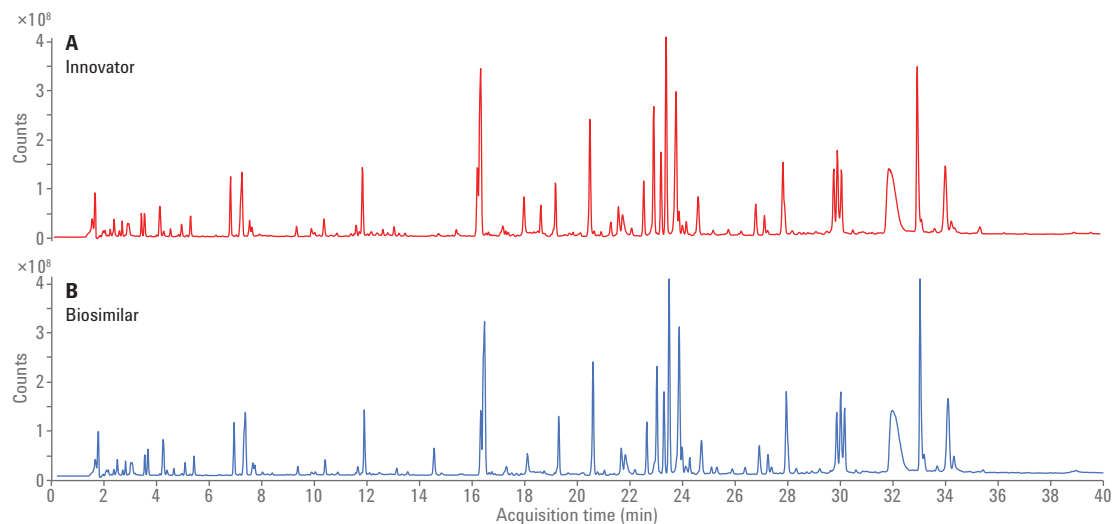


Figure 1. Total ion chromatogram (TIC) of peptide digest from Innovator (A) and Biosimilar (B).

To probe the differences between the peptide maps of innovator and biosimilar, a mirror plot of TIC as shown in Figure 2A was generated using the Compare Files feature of Agilent MassHunter Comparative Analysis software. The mirror plots show certain differences between the peptide maps. As an example in Figure 2A, a peak around 14.4 minutes is prominently found in the biosimilar, and not in the innovator sample. This peak corresponded to a C-terminal sequence (SLSLSPGK). The abundance of the C-terminal can be attributed to additional charge variants

(lysine addition and basic variants) seen in the biosimilar mAb, which was reported earlier². The extracted compound chromatogram (EIC) for the peptide (SLSLSPGK) is also shown in Figure 2B. A similar peak around 18.7 minutes (Figure 2C) corresponds to the des-lysine peptide, which is enriched in the innovator.

Peptide maps were also used to quantify the extent of oxidation and deamidation, as these are the two most commonly occurring modifications seen during storage, formulation, and sample handling.

Oxidation

The commonly used DLTMSR peptide sequence from the mAb was used to access the degree of oxidation in both the innovator and biosimilar mAbs³. The peptide masses obtained from the MFE analysis from MS-only data were matched with the theoretical digest with a preferred modification of oxidation included for the theoretical peptide digest. The theoretical digestion list of peptides for the trypsin-digested oxidized mAb was generated using the BioConfirm sequence editor (define and match sequences). The relative percentage oxidation in the samples was calculated using Equation 1.

$$\text{Relative \% oxidation} = \frac{\Sigma \text{ Height of Met - oxi peptide ions}}{(\Sigma \text{ Height of Met - oxi peptide ions}) + (\Sigma \text{ Height of Met - non-oxi peptide ions})} \times 100$$

Equation 1.

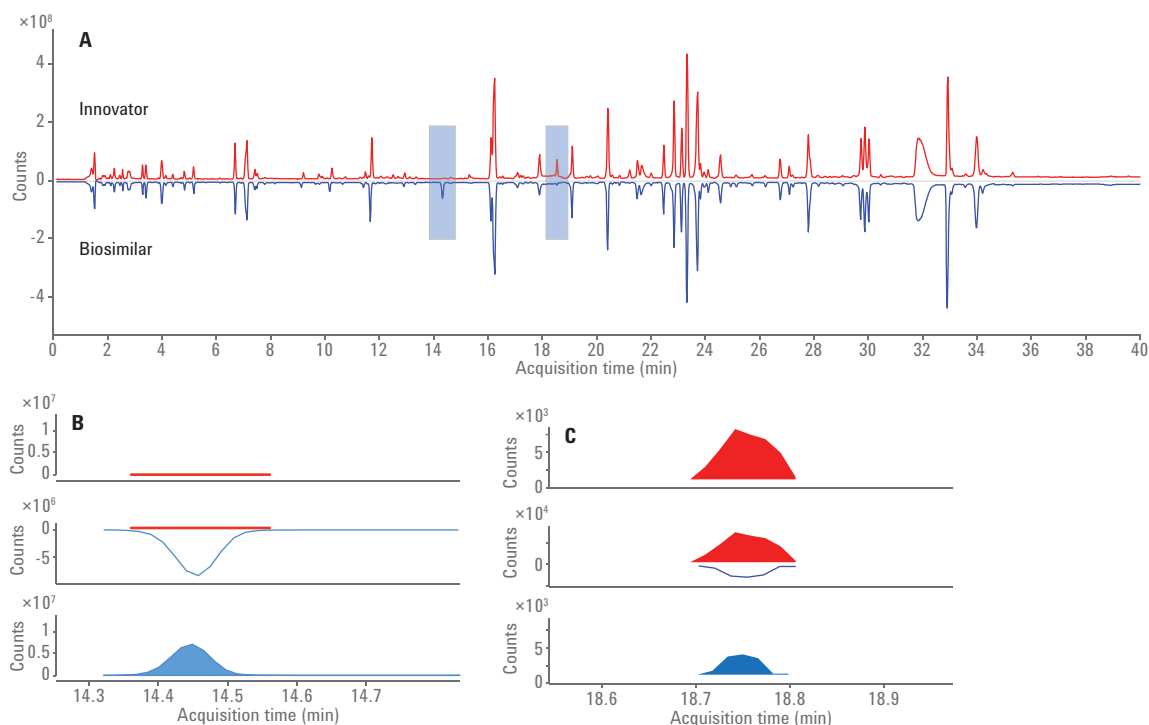


Figure 2. Mirror plot of TIC between innovator (red trace) and biosimilar (blue trace) (A). The region at around 14.4 minutes is highlighted to show the difference of SLSLSPGK peptide. The EIC of SLSLSPGK peptide, shows that this peptide is enriched in biosimilar (B). Similarly, the peak around 18.7 minutes corresponds to SLSLSPG peptide (lysine truncated). The EIC of SLSLSPG peptide is shown (C), which is enriched in innovator.

From the bar graph (Figure 3A), it can be seen that the biosimilar has relatively more oxidation species compared to the innovator. MS/MS data further confirmed the site of modification on the DLTMISR

peptide in both mAbs. Figure 3B shows representative MS/MS spectra for DLTMISR peptide in both modified and unmodified peptide derived from the biosimilar sample. Comparison of the

y ions from the MS/MS spectrum in Figure 3C table for modified y_4 and y_5 ions shows an increase of ~16 Da over those ions in unmodified peptide, suggesting that oxidation is occurring on the Met position.

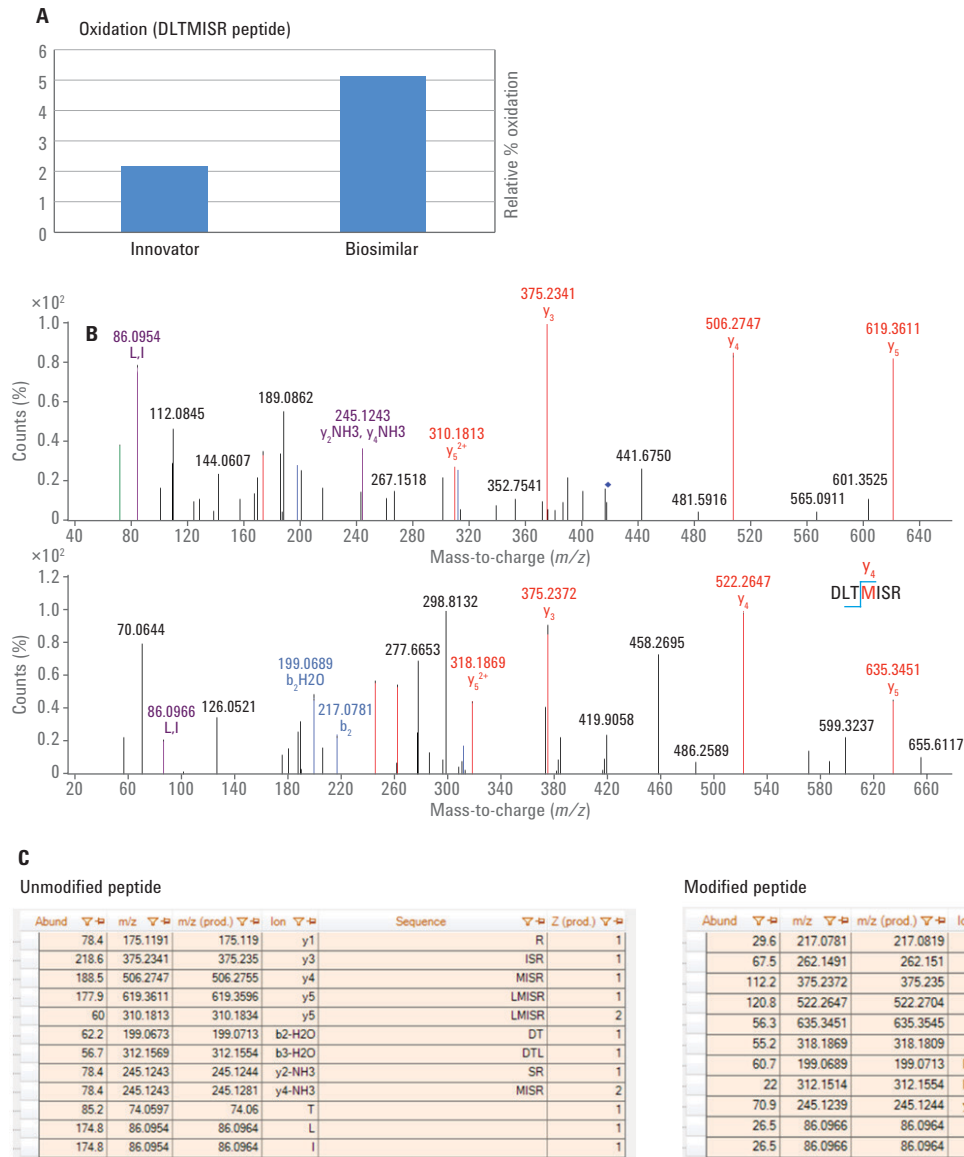


Figure 3. Bar graph between innovator and biosimilar for percentage of oxidation species in the sample (A). MS/MS spectra between unmodified and modified DLTMISR peptide (B). Table showing the fragment ions for unmodified and modified DLTMISR peptide (C).

Deamidation

Deamidation is an important PTM to study, as this modification can cause structural and functional changes to the mAb. Deamidation is a nonenzymatic chemical reaction in which asparagine residues are converted to isoaspartyl and aspartyl residues. It can be identified

through a LC/MS/MS peptide mapping experiment.

The GFYPSDIAVEWESNGQPENNYK peptide sequence (375–396), from the mAb heavy chain was used for accessing the degree of deamidation in the innovator and biosimilar mAb⁴. The relative percentage deamidation

of the amino acid was calculated using Equation 2. From the bar graph (Figure 4A), it can be seen that both mAbs have similar deamidation species, at about 8 %. From the MS/MS experiments, heavy chain peptide (375–396), which contains Asn 388, was identified to have undergone the deamidation in both mAbs.

$$\text{Relative \% deamidation} = \frac{\sum \text{Height of Asp - dem peptide ions}}{(\sum \text{Height of Asp - dem peptide ions}) + (\sum \text{Height of Asp - non-dem peptide ions})} \times 100$$

Equation 2.

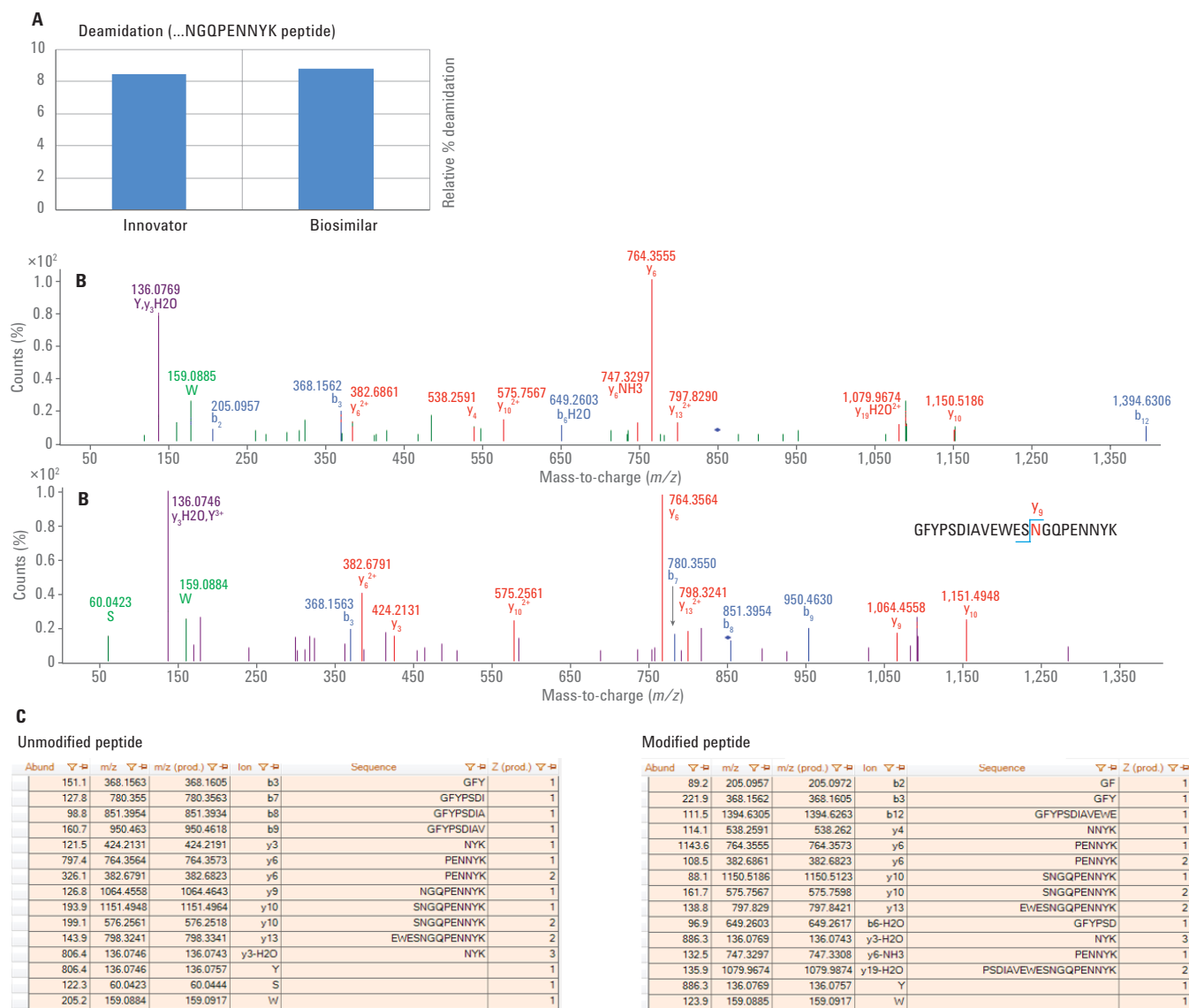


Figure 4. Bar graph between innovator and biosimilar for percentage of deamidation species in the samples (A). MS/MS spectra of unmodified and modified GFYPSDIAVEWESNGQPENNYK peptide (B). MS/MS fragment ions for unmodified and modified peptides (C). Peptide GFYPSDIAVEWESNGQPENNYK peptide (C).

Figure 4B shows a representative MS/MS spectrum for the GFYPSDIAVEWESNGQPENNYK peptide in both modified and unmodified peptide forms. If we compare the γ ions from the MS/MS spectrum in Figure 4C for modified ions from γ_9 onwards, we can clearly see an increase of ~ 0.98 Da over those ions in unmodified peptide suggesting that deamidation is occurring on the Asn 388 position.

Conclusions

This Application Note demonstrates peptide mapping of innovator and biosimilar monoclonal antibodies using an Agilent 1290 Infinity LC System coupled to an Agilent 6550 iFunnel Q-TOF LC/MS System.

- Peptide mapping was used to compare and find similarities and differences between innovator and biosimilar mAbs.
- Oxidation and deamidation levels between the innovator and biosimilar mAbs were assessed by comparing specific peptides.
- Agilent MassHunter BioConfirm software and Agilent MassHunter Comparative Analysis software provided automated data extraction, sequence matching, PTM identification, and sequence coverage calculation.

References

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2. *Agilent Technologies*, publication number 5991-5557EN.
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4. Chelius, D.; Rehder, D. S.; Bondarenko, P. V. Identification and characterization of deamidation sites in the conserved regions of human immunoglobulin gamma antibodies. *Anal Chem.* **2005**, *77*, 6004-6011.

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Published in the USA, November 2, 2017
5991-6522EN



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