# Comparison of Biosimilar and Innovator Monoclonal Antibody Rituximab Using the Agilent 1260 Infinity Bio-inert LC System and Agilent OpenLAB Match Compare Software

## Application Note

Biotherapeutics & Biosimilars


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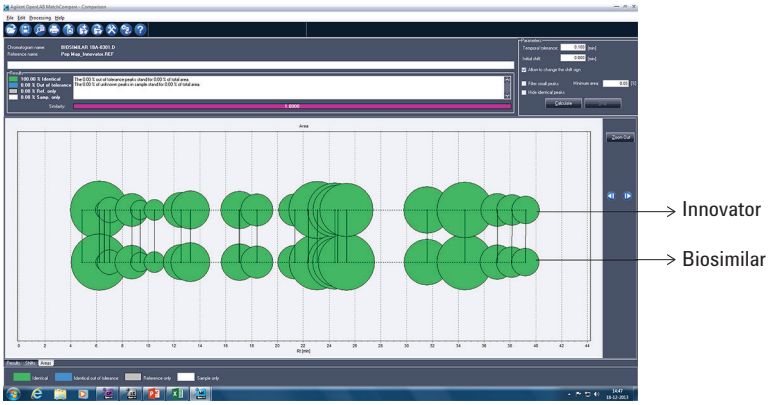
### Abstract

Rituximab is a chimeric mouse/human monoclonal antibody (mAb) consisting of a glycosylated immunoglobulin with human IgG1 constant regions and murine light-chain and heavy-chain variable region sequences. This Application Note shows the workflow solution for the physicochemical and biological characterization of a biosimilar and innovator rituximab using an Agilent 1260 Bio-inert LC system, Bio-columns, and Agilent OpenLAB CDS Match Compare Software tool. Primary and higher-order protein structures were compared using reverse phase high-performance liquid chromatography (RP HPLC), size exclusion chromatography (SEC) under nonreduced and reduced conditions, and peptide mapping with UV detection. The biological activities of the mAb pair were evaluated by a comparative complement dependent cytotoxicity (CDC) assay using a WIL2S cell line. The biosimilar was found to be physico-chemically similar to the innovator rituximab. The CDC bioassay results were also comparable.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## Introduction

Biotherapeutics have enormous potential to improve human health. The number of approved protein and antibody therapeutics continues to grow as this important therapeutic class addresses medical needs. Biosimilar development involves an objective tactic leading to a process that provides a highly similar product. A wide range of comparability programs should be in place to demonstrate similarity to the innovator molecule. In this regard, changes in the manufacturing of innovators have shown comparable products despite shifts in certain quality attributes[1]. The biosimilars industry needs reliable analytical methods to establish the molecular similarity required by regulators. A number of physicochemical and biological methods are required by regulatory authorities for the characterization of monoclonal antibodies[2]. This Application Note demonstrates the suitability of the Agilent 1260 Bio-inert Quaternary LC System and Agilent OpenLAB CDS MatchCompare Software to determine the molecular similarity between the biosimilar and innovator mAb following an LC-UV based approach. Three different analytical approaches were made for comparison. Intact and reduced mAb analysis using reverse phase (RP), SEC, and peptide mapping studies. OpenLab Match Compare Software was used to compare the chromatograms. This tool automates the time-consuming task of comparing chromatograms of samples, making it ideal for assessing the molecular similarity of biomolecules. Subjectivity of the analysis can be addressed by using a software package that provides an objective match of the chromatographic pattern, indicates what the sample really is, and quickly provides a fingerprint analysis of the sample.

## Experimental

All chromatographic analyses were performed on an Agilent 1260 Infinity Bio-inert Quaternary LC system consisting of the following modules:

- Agilent 1260 Infinity Bio-inert Quaternary LC Pump (G5611A)
- Agilent 1260 Infinity Bio-inert High Performance Autosampler (G5667A)
- Agilent 1200 Infinity Series Thermostat (G1330B)
- Agilent 1260 Infinity Thermostatted Column Compartment containing bio-inert click-in heating elements (G1316C, option 19)
- Agilent 1260 Infinity DAD VL with a 10-mm bio-inert standard flow cell (G1315D, option 28)
- Agilent 1260 Infinity Bio-inert Analytical-scale Fraction Collector (G5664A)

### Software

- Agilent OpenLAB CDS, ChemStation Edition for LC & LC MS Systems, Rev. C.01.02 [14]
- Agilent OpenLAB CDS Match Compare, A.01.01. 1

### Reagents, samples, and materials

Rituximab biosimilar and innovator were purchased from a local pharmacy and stored, according to the manufacturer's instructions. Sodium phosphate dibasic dihydrate, sodium phosphate monobasic dihydrate, sodium chloride, hydrochloric acid (HCl), and sodium hydroxide were purchased from Sigma-Aldrich. All the chemicals and solvents were HPLC grade, and highly purified water was from a Milli Q water purification system (Millipore Elix 10 model, USA). Acetonitrile was of gradient grade and purchased from Lab-Scan (Bangkok, Thailand).

### Reduction and alkylation of intact mAbs

Biosimilar and innovator mAbs were diluted to 2 mg/mL using 100 mM Tris HCl and 4 M guanidine HCl, pH 8.0. An aliquot of 10 µL of 0.5 M DTT stock was added to obtain a final concentration of 5 mM. The mixture was held at 37 °C for 30 minutes. The reaction mixture was cooled briefly to room temperature. An aliquot of 26 µL of 0.5 M iodoacetamide stock was added for a final concentration of 13 mM, and allowed to stand for 45 minutes. Once removed, the solution was quenched with 20 µL of DTT for a final concentration of 10 mM.

### Peptide mapping

Tryptic digestion of mAbs were carried out as described in Application Note 5991-1694EN[3].

### Biological assay for biosimilar and innovator mAb

The biological activity of the biosimilar and innovator mAb was assessed using comparative dependent cytotoxicity (CDC) assay using a WIL2S cell line. The data were analyzed using GraphPad Prism software.

### Chromatographic parameters

Table 1. Chromatographic parameters used.

| Parameter | HPLC (intact and reduced) | SEC HPLC | RP HPLC (peptide mapping) |
|---|---|---|---|
| Column oven | 50 °C | 25 °C | 50 °C |
| Acquisition rate | 20 Hz | 20 Hz | 20 Hz |
| Data acquisition | 214 and 280 nm | 214 and 280 nm | 214 and 280 nm |
| Injection volume | 5 µL (Needle with wash, flush port active for 7 seconds) | 5 µL (Needle with wash, flush port active for 7 seconds) | 2.1 µL (Needle with wash, flush port active for 7 seconds) |
| Sample thermostat | 5 °C | 5 °C | 5 °C |
| Mobile phase A<br>Mobile phase B | Water + 0.1 % TFA<br>Acetonitrile + 0.09 % TFA | 150 mM Sodium phosphate buffer, pH 7.0 + 150 mM NaCl | Water + 0.1 % TFA<br>Acetonitrile + 0.09 % TFA |
| Column | Agilent Poroshell 120 SB-C18, 4.6 × 150 mm, 2.7 µm | Agilent Bio SEC-3, 300Å, 7.8 × 300 mm, 3 µm | Agilent AdvanceBio Peptide mapping, 4.6 × 150 mm, 2.7 µm |
| Gradient | 5–95 % B in 15 minutes | Isocratic 30 minutes | At 0 minutes, 2 % B<br>At 15 minutes, 20 % B<br>At 50 minutes, 40 % B<br>At 55 minutes, 60 % B<br>At 60 minutes, 90 % B<br>At 61 minutes, 2 % B |
| Post run time | 5 minutes | 10 minutes | 10 minutes |
| Flow rate | 1.0 mL/min | 0.8 mL/min | 1.0 mL/min |

## Results and Discussion

### RP HPLC analysis - intact mAb analysis

The biosimilar mAb was characterized using the innovator as the reference standard. The optimized RP HPLC separation of intact biosimilar and innovator rituximab on the Poroshell 120 SB-C18 column displayed excellent separation, demonstrating homogenous profiles of mAbs within a total run time of 15 minutes (Figure 1).

Normally, one would expect the 120Å pore size to be too small for the intact and reduced mAb work. But we did not find any issues even when the smaller pore size column was used for such separations.

OpenLAB CDS Match Compare Software is an add-on software tool that automates the time-consuming task of comparing quality control chromatograms of samples. The software provides the ability to objectively compare unknown samples to a known standard. The software quickly identifies similar chromatographic peaks between two chromatograms and compares the areas against predetermined ranges. OpenLab CDS Match Compare Software provides the ability to streamline quality control processes for higher throughput in a variety of GC and LC applications. OpenLab CDS Match Compare can:

- Integrate seamlessly with OpenLAB Match Compare Software
- Identify and match peaks between two complex softwares
- Save time and monitor product content uniformity by peak area comparison

Matching peaks between a reference and a sample chromatogram, even when retention times have shifted over several minutes, is easy with OpenLAB CDS Match Compare Software. Matched peaks between a reference and the sample are graphically linked for easy identification. Missing peaks or impurities are represented by different colors both on-screen, and in the report. Automatic peak matching with OpenLAB CDS Match Compare Software eliminates errors and speeds data review[4].

OpenLAB CDS Match Compare Software was used to compare the innovator chromatogram (reference) with the biosimilar chromatogram (sample) to find peak differences. Matched peaks or impurities are represented by different colors both on-screen and in the report. Automatic peak matching with OpenLAB CDS Match Compare Software eliminates errors and speeds data review. The OpenLAB CDS Match Compare Software analysis of intact biosimilar and innovator indicated that the samples were similar (Figure 2).

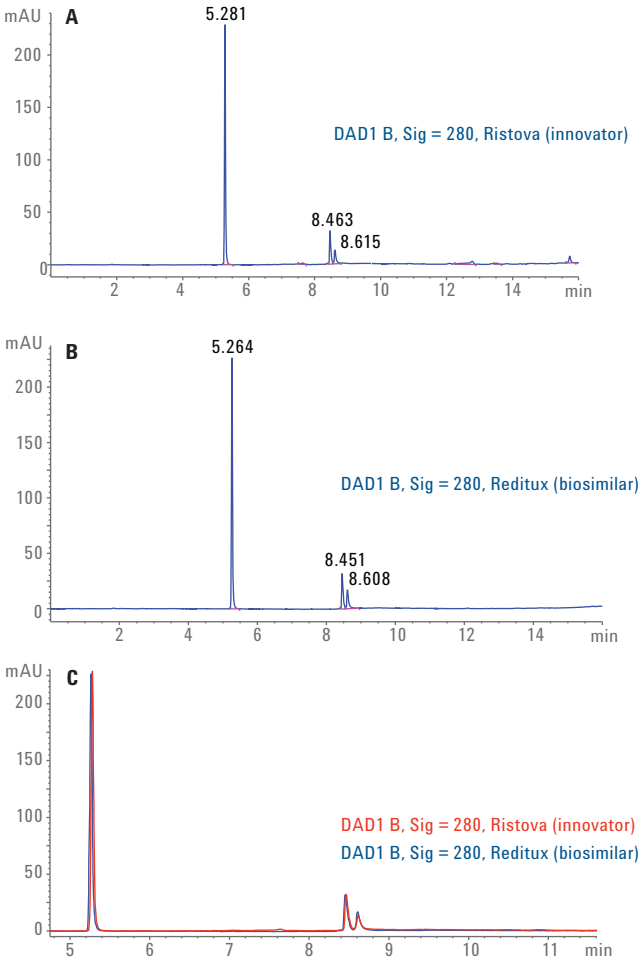


Figure 1. RP HPLC profile of intact innovator (A) and biosimilar (B) mAb on an Agilent Poroshell 120 SB C18, 4.6 × 150 mm, 2.7 µm column, and their overlays (C).

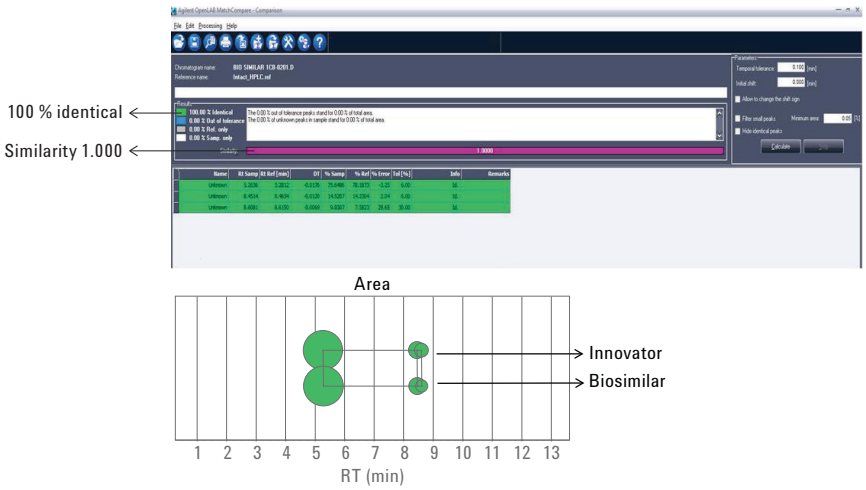


Figure 2. The intact RP HPLC data files of biosimilar and innovator compared. The bottom is the biosimilar and the top is the innovator. The size of the circles indicates the intensity of the peaks, while the color indicates how closely the match is for each peak.

## RP HPLC analysis - reduced mAb analysis

When we studied the more detailed structure of the mAb, chemical methods were used to separate free antibody light and heavy chains. Poroshell 120 SB-C18 columns are very effective in providing high-velocity, high resolution separations of antibody fragments. The profiles in Figure 3 show a rapid reversed phase analysis optimized for separation of antibody fragments with peaks eluting in less than 8 minutes.

The OpenLAB CDS Match Compare Software analysis of reduced mAbs indicates that their RT and areas are highly similar. However, in this case, the temporal tolerance criterion cannot be satisfied, but the peaks are matched. This kind of match is called out of temporal tolerance (Figure 4).

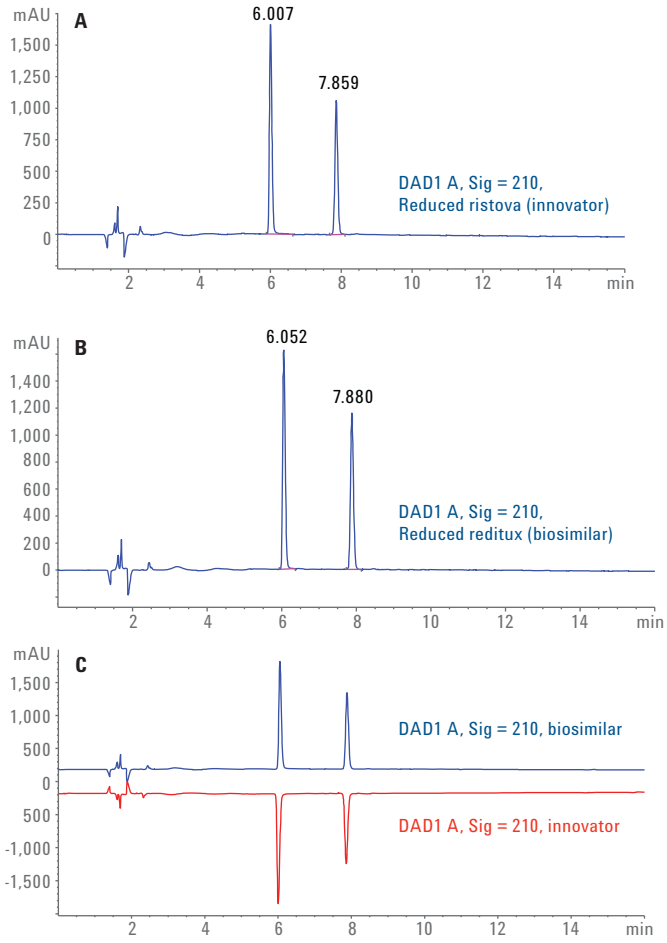


Figure 3. RP HPLC profiles of innovator (A) and biosimilar (B) mAb separated on an Agilent Poroshell 120 SB C18, 4.6 × 150 mm, 2.7 µm column after treatment with DTT to break disulfide bonds. Mirror plot image overlays (C).

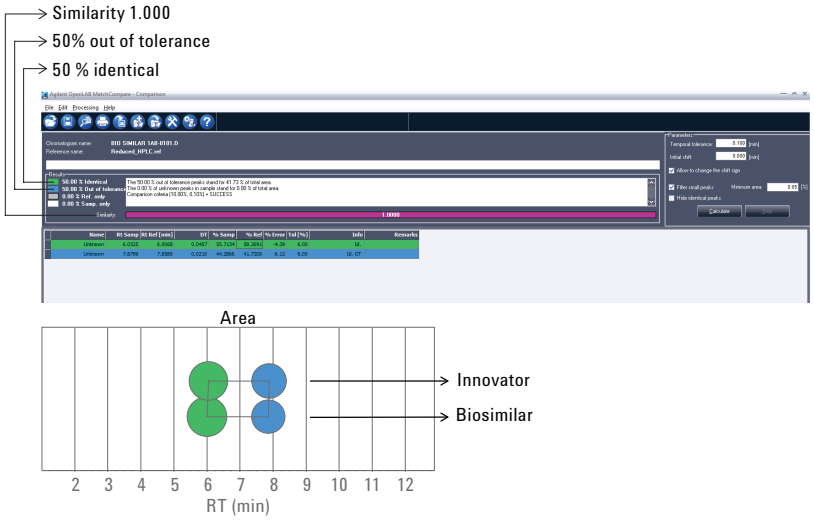


Figure 4. Agilent OpenLAB MatchCompare Software analysis of innovator and biosimilar mAb after reduction with DTT.

## SEC HPLC analysis - intact mAb analysis

SEC is a critical tool for monitoring monomers, dimers, aggregates and potential degradants. Figure 5 demonstrates the excellent separation of an intact mAb pair in 30 minutes, exhibiting a sharp and symmetrical peak with no nonspecific interactions. SEC, under nonreducing conditions, separates monomer from aggregates or degradation products due to optimum exclusion limit. The RT of the biosimilar and the innovator were comparable, and the purity by area percent was 99.52 % for the innovator and > 99.25 % for the biosimilar respectively.

The OpenLAB CDS Match Compare Software analysis of intact biosimilar and innovator after SEC indicated that the samples were similar (Figure 6).

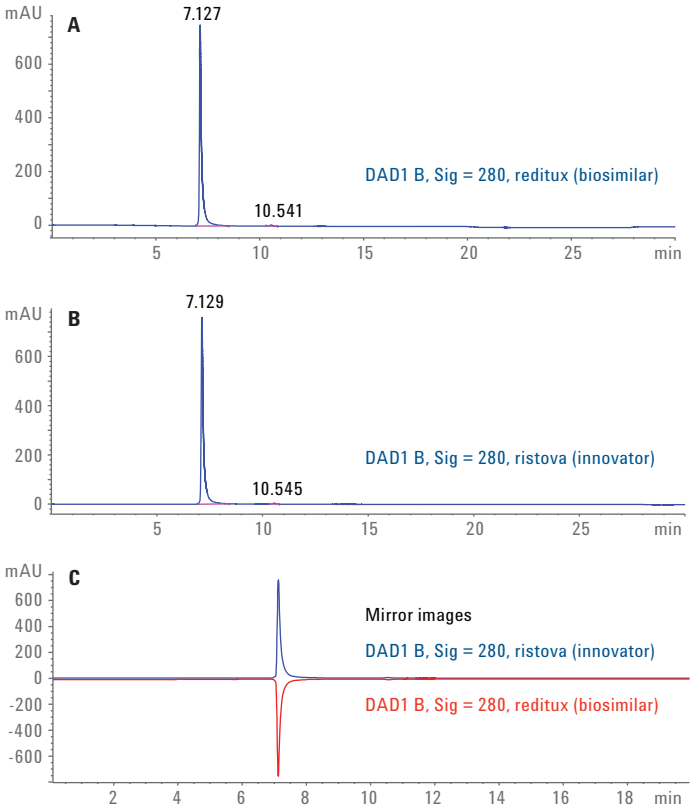


Figure 5. SEC profile of intact biosimilar (A) and innovator (B) mAb on an Agilent Bio SEC-3, 300Å, 7.8 × 300 mm, 3 µm column. Mirror plot image overlays (C).

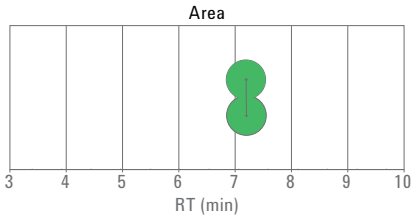


Figure 6. Agilent OpenLAB MatchCompare Software analysis of innovator and biosimilar mAb after size exclusion chromatography.

## SEC HPLC analysis – reduced mAb analysis

For reduced SEC, rituximab samples were reduced using guanidine hydrochloride, and the free sulfydryl groups were alkylated with iodoacetamide to prevent disulfide shuffling. The elution profiles of reduced rituximab (Figure 7) indicate that the heavy chain (HC) and light chain (LC) were eluted later than that of the intact sample, and that their elution times were comparable. The identities of HC and LC were established based on the molecular elution order. The OpenLAB CDS Match Compare Software analysis of the reduced mAb pair (Figure 8) was found to be comparable with the SEC results, confirming that biosimilar and innovator rituximab have the same purity.

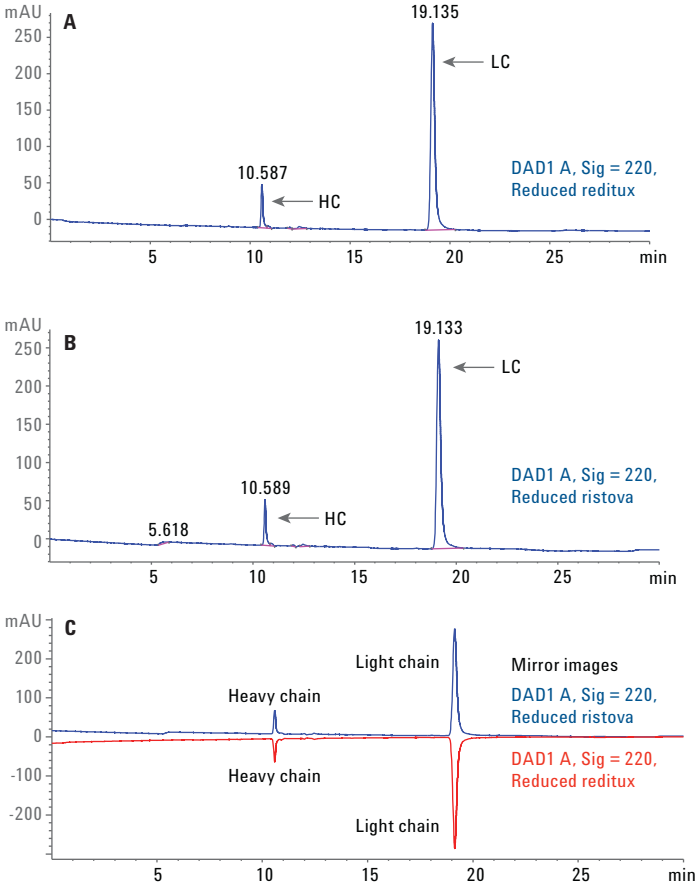


Figure 7. SEC profile of reduced biosimilar (A) and innovator (B) mAb on an Agilent Bio SEC-3, 300Å, 7.8 × 300 mm, 3 µm column. Mirror plot image overlays (C).

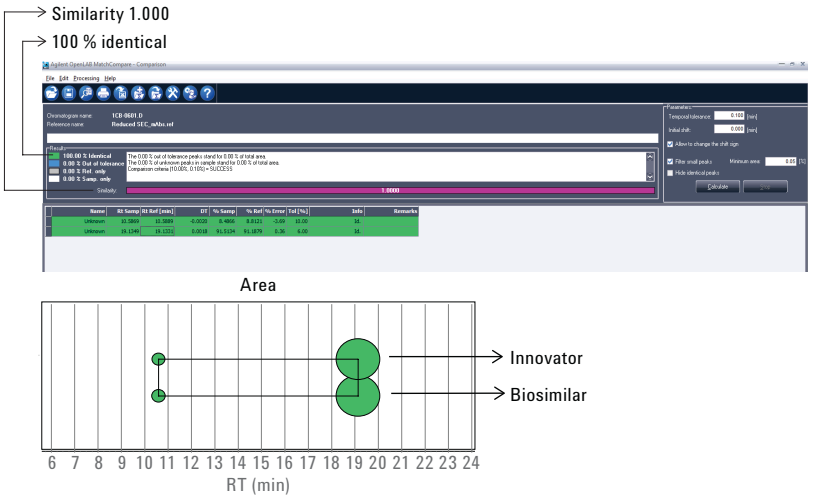


Figure 8. Agilent OpenLAB CDS Match Compare Software analysis of innovator and biosimilar mAb after reduced size exclusion chromatography.

## Peptide mapping Analysis

Peptide mapping is an identity test that confirms the primary structure of recombinant proteins and demonstrates process consistency. The peptide mapping analysis of tryptic digested biosimilar and innovator rituximab on an Agilent AdvanceBio Peptide mapping, 4.6 × 150 mm, 2.7 µm column, resulted in indistinguishable chromatograms (Figure 9).

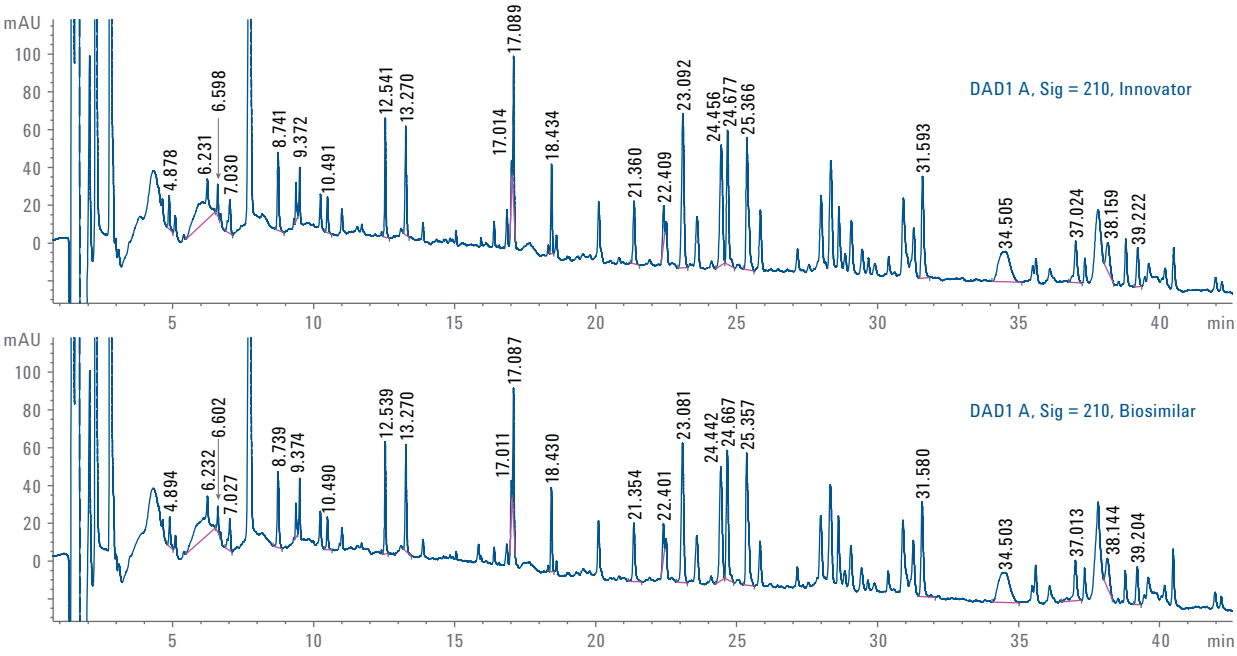


Figure 9. Peptide maps of innovator and biosimilar rituximab separated on an Agilent AdvanceBio Peptide mapping, 4.6 × 150 mm, 2.7 µm column. Peaks selected for comparison using Agilent OpenLAB CDS Match Compare Software are annotated.

The acquired LC data were processed by OpenLab Match Compare Software for the randomly selected peaks across the whole chromatogram to find the reference peaks in the biosimilar chromatogram. Figure 10 shows the data files of the biosimilar and innovator compared using OpenLab Match Compare Software, indicating a striking similarity between the mAbs under study.

## Biological assay for biosimilar and innovator mAb

To assess Fc-effector functions, CDC bioassays for the biosimilar and innovator rituximab were compared. The results of the bioassays indicate that, when compared to the innovator, the biosimilar showed a relative potency of 111 % (Figure 11).

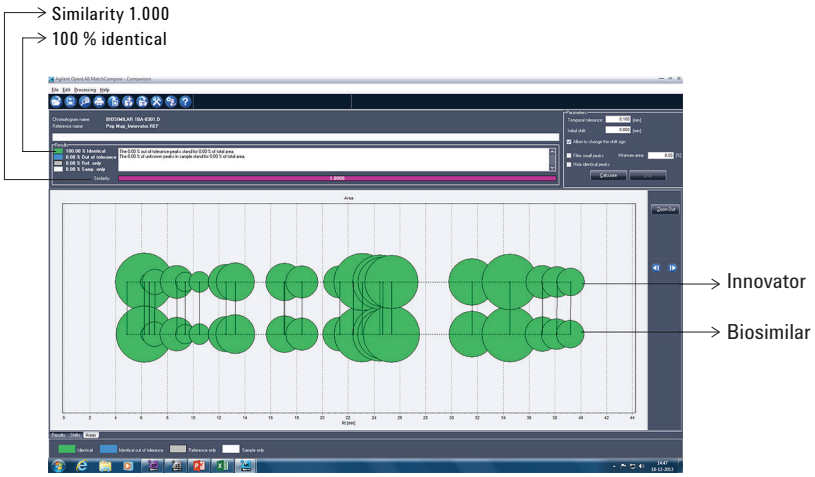


Figure 10. Comparision of peptide maps of innovator and biosimilar mAb using Agilent OpenLab CDS Match Compare Software.

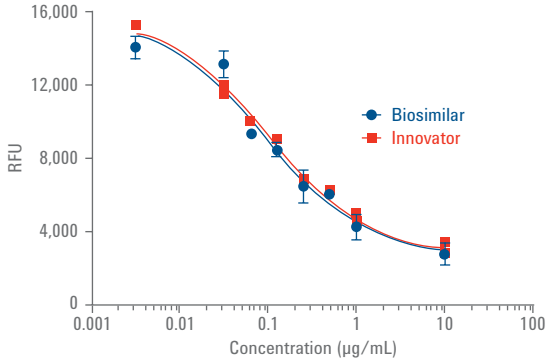


Figure 11. Comparative dependent cytotoxicity (CDC) assay of Biosimilar and innovator using WIL2S cell line.

## Conclusion

Biosimilars are a version of branded biologics introduced after the patents of an innovator product have expired. Analytical studies have to demonstrate that the biosimilar is highly similar to the reference product. Thus, accurate and precise bioanalytical data is critical in establishing comparability between biosimilar and innovator. This Application Note has demonstrated an LC-UV based approach to define the molecular similarity between biosimilar and innovator rituximab. We first used the Agilent 1260 Infinity Bio-inert Quarternary LC system and Bio-columns to develop a simple and high-resolution separation of the mAb pair using RP HPLC, SEC, and peptide mapping techniques. Agilent OpenLAB CDS Match Compare Software was used to compare peaks between the innovator and biosimilar chromatogram to identify chromatographic peak matches. Automatic peak matching eliminates visual errors and speeds data review. Such simple and reproducible methods, coupled with powerful data analysis, make this solution particularly suitable for the comparability analysis analysis of monoclonal antibody for the biopharmaceutical industry.

## References


1. Jan Visser; *et al.* Physicochemical and Functional Comparability Between the Proposed Biosimilar Rituximab GP2013 and Originator Rituximab. *BioDrugs* **2013**, *27*, pp 495–507.
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4. OpenLAB CDS Match Compare for Chromatographic Peak Matching and Comparison, *Agilent Technologies Data Sheet*, publication number 5991-2553EN.



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