

## High-Resolution Separation of Intact Antibodies Using BioResolve RP mAb Polyphenyl Columns

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### Abstract

This application note provides a high-resolution, reversed-phase based separation method for intact antibodies that take advantage of the purposefully-designed capabilities of the BioResolve RP mAb Polyphenyl Column. Using optimized, silica-based, solid-core particles that are surrounded by a porous layer containing 450Å pores with novel polyphenyl bonded phase, the BioResolve RP mAb Polyphenyl Column increases the efficiency and selectivity of intact antibody separations. This results in improvements in resolution, peak width, peak shape, and carryover compared to current leading porous and non-porous column technologies.

### Benefits

- Improved resolution for reversed-phase separation of intact antibodies using BioResolveRP mAb Polyphenyl Column
  - Reduced on-column carryover and high chromatographic reproducibility for intact antibody separation
  - Robust and specific LC platform method that can be applied to various antibodies
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## Introduction

The market size of therapeutic antibodies has grown significantly since the launch of its first product (Orthoclone OKT3) in 1986.<sup>1</sup> By 2017, a total of 81 antibody related drug products have been approved by FDA/EMA.<sup>2,3</sup> This growth is, in part, due to the specificity that therapeutic antibodies offer as an effective treatment of diseases with controlled risk of adverse effects when compared to traditional drug-based therapies.<sup>4</sup> However, as a biomolecule, antibodies commonly contain high levels of structural heterogeneity, requiring thorough characterization and analyses throughout their product lifecycle to ensure drug safety, efficacy, and reproducibility.<sup>5,6</sup>

Of the various separation techniques employed in the characterization of biomolecules, reversed-phase liquid chromatography (RPLC) is extensively used in antibody development for intact mass analysis, impurity profiling, and stability assessment of drug substance and drug product.<sup>7,8</sup> However, in many of these instances, analysts encounter insufficient resolution and poor column performance because of non-ideal pairing of stationary phase attributes (e.g. morphology, pore size, and surface chemistry) to analyte physicochemical properties (e.g., size). To this end, LC methods that can incorporate column technologies tailored to biomacromolecules, such as monoclonal antibodies (mAbs), that overcome the performance challenges associated with conventional RPLC columns are highly desirable to support the growing market of therapeutic antibodies.

Waters BioResolve RP mAb Polyphenyl Column is specifically designed to address these challenges through its tailored particle morphology and ligand chemistry. The superficially-porous particle and large pore size (450Å, measured by Hg) of the BioResolve RP mAb Polyphenyl Column improve its kinetic properties and performance for biomacromolecules, such as mAbs, while its novel polyphenyl ligand offers favorable desorption behavior and unique selectivity with retentivity comparable to traditional RPLC columns. In addition, the low backpressure introduced by the use of 2.7 µm silica-based solid core particles offers the flexibility to support various LC platforms throughout the products lifecycle without compromise to performance.<sup>9,10</sup>

The objective of this application note is to demonstrate the superior performance of BioResolve RP mAb Polyphenyl Column for intact antibody separations. In this study, six antibodies are selected as surrogates to investigate the range of the column's applicability. A platform method for intact antibody separations will be

developed and used to compare the performance of the BioResolve RP mAb Polyphenyl Column against other leading, commercially-available alternatives designed for this application.

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## Experimental

### Chemical and reagents

NIST mAb (RM 8671) and Waters Intact mAb Mass Check Standard (P/N: 186006552) were used as reference materials in addition to a panel of therapeutic mAbs, including Rituximab, Tocilizumab, Bevacizumab, and Cetuximab. All samples were diluted or dissolved in water to obtain a solution at the concentration of 5 µg/µL. HPLC grade water, acetonitrile, and TFA were purchased from Fisher Scientific and used as received.

### LC conditions - Developed method

|                |                                                                                                                                                                                                                                                                                           |
|----------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| System:        | ACQUITY UPLC H-Class Bio                                                                                                                                                                                                                                                                  |
| Detectors:     | ACQUITY UPLC TUV 5 mm flow cell, $\lambda = 280 \text{ nm}$                                                                                                                                                                                                                               |
| Columns:       | BioResolve RP mAb Polyphenyl, 450Å, 2.7 µm<br>(*Average pore diameter is measured by Hg porosimetry), 2.1 mm x 50 mm, w/ Intact mAb and mAb Subunit Stds<br><br>ACQUITY UPLC Protein BEH C <sub>4</sub> Column, 300Å, 1.7 µm, 2.1 mm X 50 mm<br><br>Polymeric, 1500Å, 4.0 µm, 2.1 x 50 mm |
| Column temp.:  | 80 °C                                                                                                                                                                                                                                                                                     |
| Sample vial:   | LCMS Certified Clear Glass 12 x 32 mm Screw Neck Total Recovery Vial                                                                                                                                                                                                                      |
| Mobile phases: | Water and acetonitrile                                                                                                                                                                                                                                                                    |

|                   |          |
|-------------------|----------|
| MP additive:      | 0.1% TFA |
| Mass load:        | 2.5 µg   |
| Injection volume: | 0.5 µL   |

## Gradient

| Time (min) | Flow rate (mL/min) | %A | %B |
|------------|--------------------|----|----|
| Initial    | 0.5                | 75 | 25 |
| 10         | 0.5                | 55 | 45 |
| 11         | 0.5                | 20 | 80 |
| 11.5       | 0.5                | 20 | 80 |
| 11.51      | 0.5                | 75 | 25 |
| 15         | 0.5                | 75 | 25 |

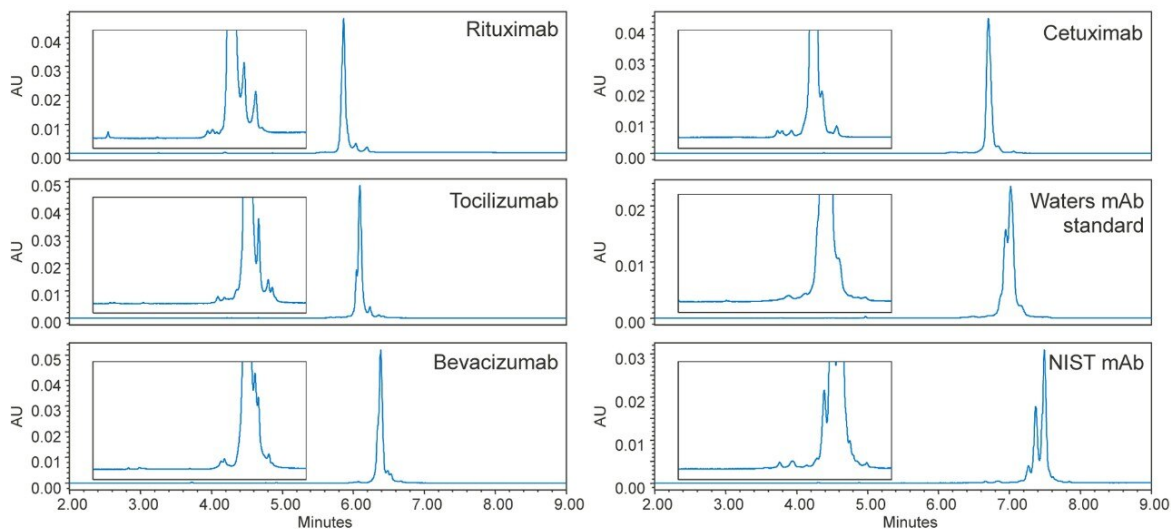
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## Results and Discussion

A high level of heterogeneity is commonly present in antibody-based products due to various post-translational modifications and process/product related impurities.<sup>5</sup> In addition, the large size of mAbs (typically 150 kDa) elevates the complexity of characterization due to restricted diffusion imposed by the small pore size of conventional RPLC stationary phases. To characterize this heterogeneity, an RPLC method for intact antibody separation using the BioResolve RP mAb Polyphenyl Column was developed and optimized for peak-to-peak resolution at an acceptable analysis time. To explore the applicability of this method, a broad array of antibodies were selected for analysis, comprised of Rituximab, Tocilizumab, Bevacizumab, Cetuximab, Waters Intact mAb Mass Check Standard, and NIST mAb RM 8671. As shown in Figure 1, using a 10 minute gradient from

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25–45% acetonitrile with 0.1% TFA, multiple impurities were resolved from the main mAb peaks in the form of shoulder peaks (insets) for many of the mAb samples. This is highly-desirable information on product related impurities likely corresponding to oxidation among other chemical and physical modifications. In addition, it is apparent that peaks are symmetrical and quite narrow for each sample, suggesting that efficient mass transfer is indeed afforded by the optimized symmetrical, 2.7  $\mu\text{m}$  particle morphology and 450Å pore size of the BioResolve RP mAb Polyphenyl stationary phase.



*Figure 1. Separation of intact antibodies using a BioResolve RP mAb Column. High-resolution separation of sample heterogeneity is demonstrated in the zoomed views of the chromatograms. Gradient: 25–45% acetonitrile in 10 min with 0.1% TFA. Flow rate: 0.5 mL/min.*

To compare the performance of the BioResolve RP mAb Polyphenyl Column with other RPLC stationary phases, this optimized method was used as the basis for comparison between the BioResolve RP mAb Polyphenyl Column against that of two other leading commercially-available columns, one based on C4 bonded, fully-porous particles and another based on a polymeric divinylbenzene particles. Taking Rituximab as a detailed case study, chromatograms obtained with the three different columns were overlaid and compared (Figure 2A). Full width at half maximum (w50%) of the main peak was calculated to be 3.40 s for the separation using the BioResolve RP mAb Polyphenyl Column, 4.84 s for the polymeric column, and 4.00 s for the C<sub>4</sub> column. Moreover, as shown in Figure 2B, the use of BioResolve RP mAb Polyphenyl Column resulted in improved resolution of shoulder peaks and low abundance impurities. To verify the results were not analyte specific, the column evaluations were extended to a broader set of mAbs. As shown in Figure 3, the superior performance of the BioResolve RP mAb Polyphenyl Column, in terms of resolution and peak shape, was clearly demonstrated

as exemplified with the resolved shoulder peaks found in the chromatograms for Tocilizumab, Bevacizumab, and the Waters Intact mAb Mass Check Standard.

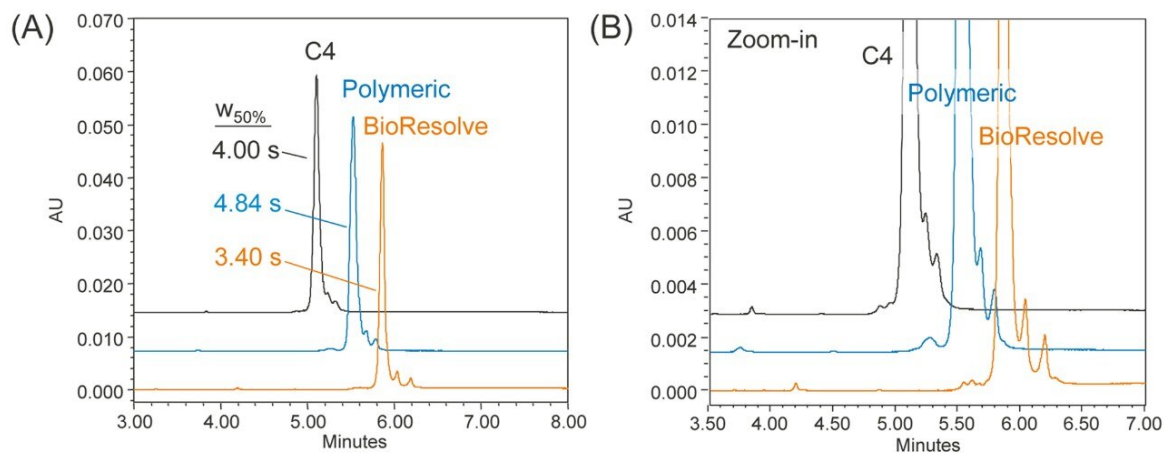


Figure 2. Comparison of Rituximab chromatograms obtained using three different columns. A) Peak widths at half height ( $w_{50\%}$ ) have been labeled for each peak. B) Zoomed view chromatograms. The BioResolve RP mAb Column provided the highest resolution and narrowest peak width as compared to leading, commercially available  $C_4$  and polymeric columns. Gradient: 25–45% acetonitrile in 10 min with 0.1% TFA. Flow rate: 0.5 mL/min.

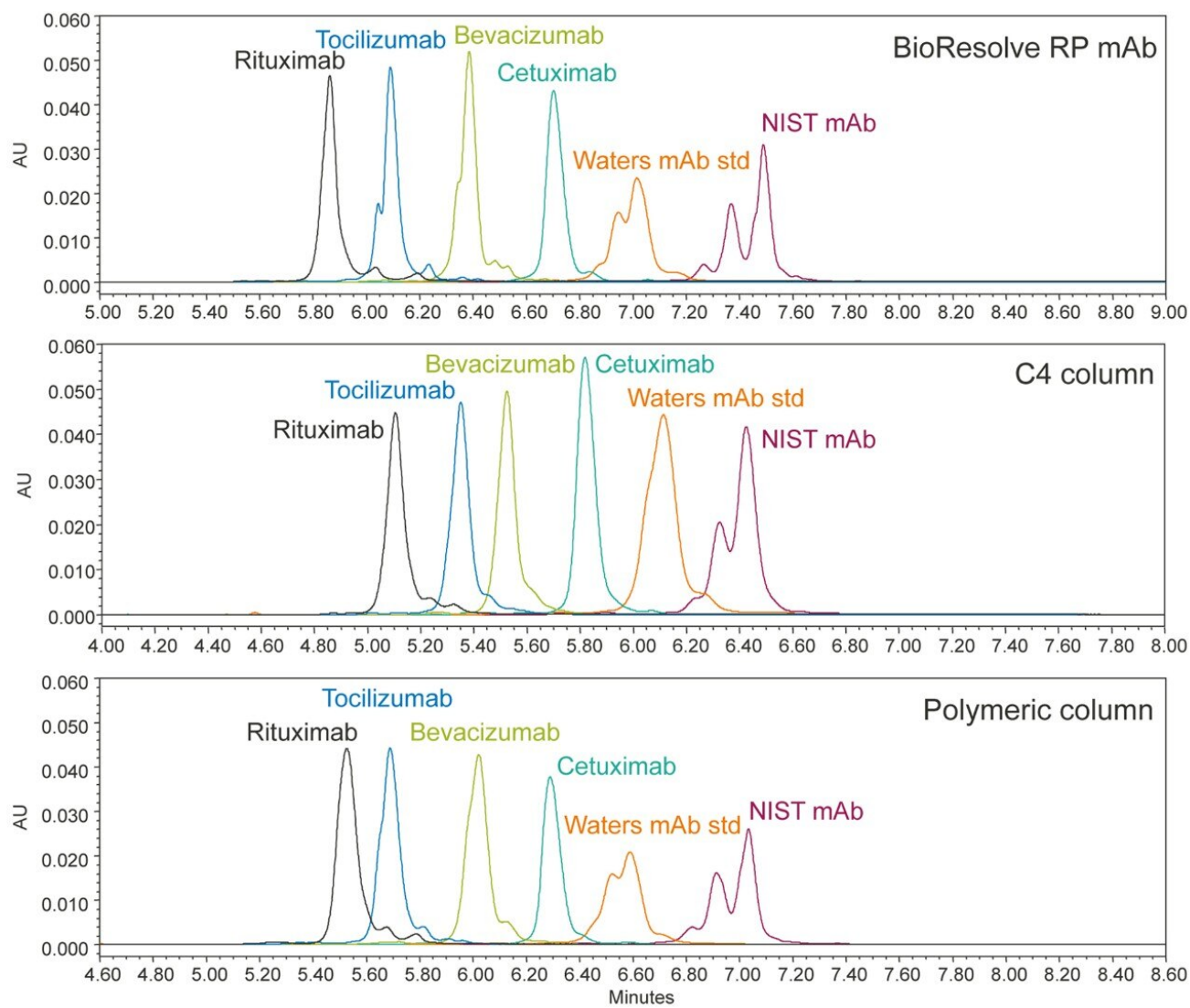
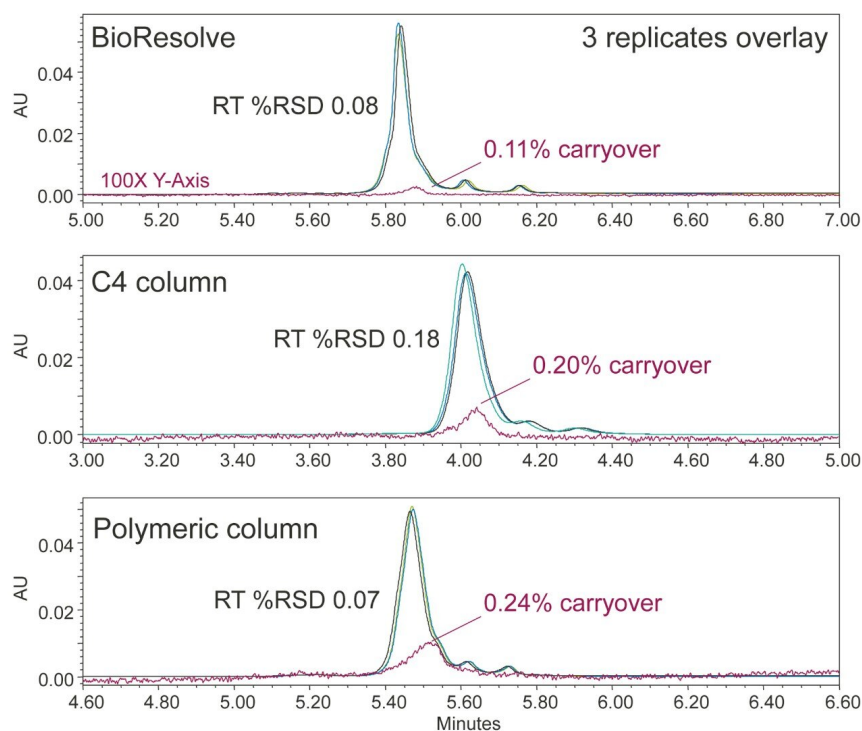


Figure 3. Overlay of separation of six different antibodies using three different columns. Across all six samples, the BioResolve RP mAb Column provides the highest resolution as compared to leading, commercially available C<sub>4</sub> and polymeric columns. Gradient: 25–45% acetonitrile in 10 min with 0.1% TFA. Flow rate: 0.5 mL/min.

As an extension to this study, repeatability, carryover, and batch-to-batch reproducibility were evaluated. In Figure 4, overlays of three consecutive injections of Rituximab showed comparable reproducibility for the BioResolve RP mAb Polyphenyl, C<sub>4</sub>, and polymeric column. In contrast, carryover measurements suggested a disparity in performance. Carryover was tested by running a blank injection after the three consecutive Rituximab separations and calculating the residual peak area. Calculated results showed the BioResolve RP mAb Polyphenyl Column to have the lowest carryover (0.11%) when compared with the C<sub>4</sub> column (0.20%) and

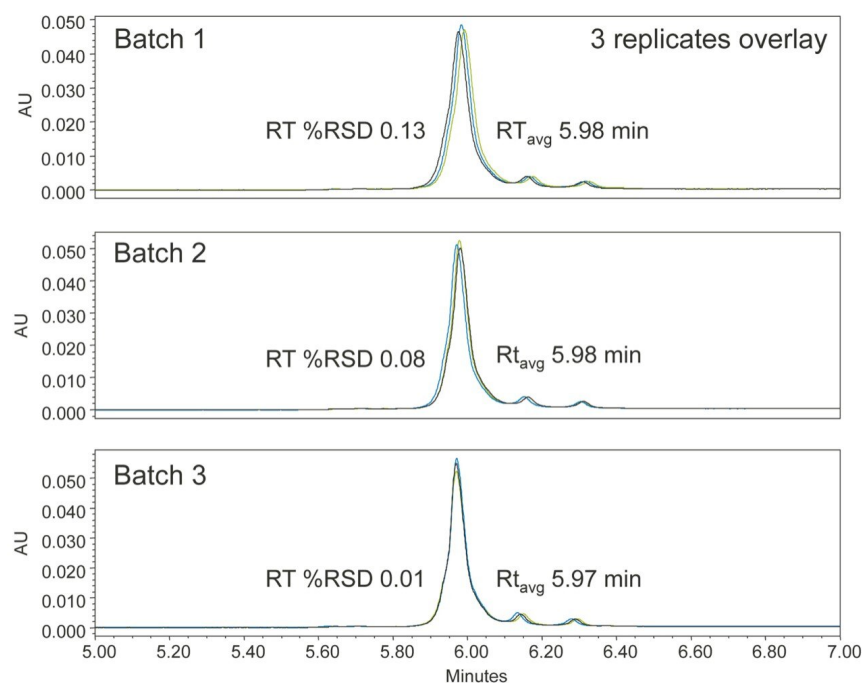
polymeric column (0.24%).

To evaluate the batch-to-batch reproducibility, three BioResolve RP mAb Polyphenyl Columns were selected from different batches and used for the separation of Rituximab. As shown in Figure 5, the %RSD of average retention time for the three columns was calculated to be 0.11, indicating a high batch-to-batch reproducibility of the BioResolve RP mAb Polyphenyl Column. Collectively, this study demonstrates that the BioResolve RP mAb Polyphenyl Column can be used to develop efficient and robust methods for the intact antibody heterogeneity assessment for improved confidence in drug product safety and efficacy assessment.



*Figure 4. A comparison of the carryover and injection-to-injection (n=3) reproducibility for a separation of Rituximab. Red traces shows a subsequent blank injection with the y-axis zoomed-in 100-fold. The BioResolve RP mAb Column provides the lowest carryover with comparable reproducibility.*





*Figure 5. Evaluation of batch-to-batch reproducibility of the BioResolve RP mAb Column for separation of Rituximab. Three BioResolve RP mAb Columns from different batches were used for comparison and all showed high injection-to-injection reproducibility. The %RSD of average retention time for the three columns was calculated to be 0.11, suggesting a high batch-to-batch reproducibility of the BioResolve RP mAb Column.*

## Conclusion

This work provides a high-resolution, reversed-phase based separation method for intact antibodies that takes advantage of the purposefully-designed capabilities of the BioResolve RP mAb Polyphenyl Column. Using optimized, silica-based, solid core particles that are surrounded by a porous layer containing 450Å pores with novel polyphenyl bonded phase, the BioResolve RP mAb Column increases the efficiency and selectivity of intact antibody separations. This results in improvements in resolution, peak width, peak shape, and carryover compared to current leading porous and non-porous column technologies. The data shown supported the ability of the BioResolve RP mAb Polyphenyl Column to deliver improved intact antibody separations by

producing 20% narrower peak widths and showing 50% less carryover compared to conventional RP columns, making it an ideal separation media for antibody characterization and analysis.

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ACQUITY UPLC TUV Detector <<https://www.waters.com/514228>>

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## Available for purchase online:

BioResolve RP mAb Polyphenyl Column, 450Å, 2.7 µm, 2.1 mm x 50 mm, 1/pkg w/ Intact mAb and mAb Subunit Stds <<https://www.waters.com/waters/partDetail.htm?partNumber=176004156>>

ACQUITY UPLC Protein BEH C4 Column, 300Å, 1.7 µm, 2.1 mm X 50 mm <  
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Intact mAb Mass Check Standard <  
<https://www.waters.com/waters/partDetail.htm?partNumber=186006552>>

LCMS Certified Clear Glass 12 x 32mm Screw Neck Total Recovery Vial <  
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