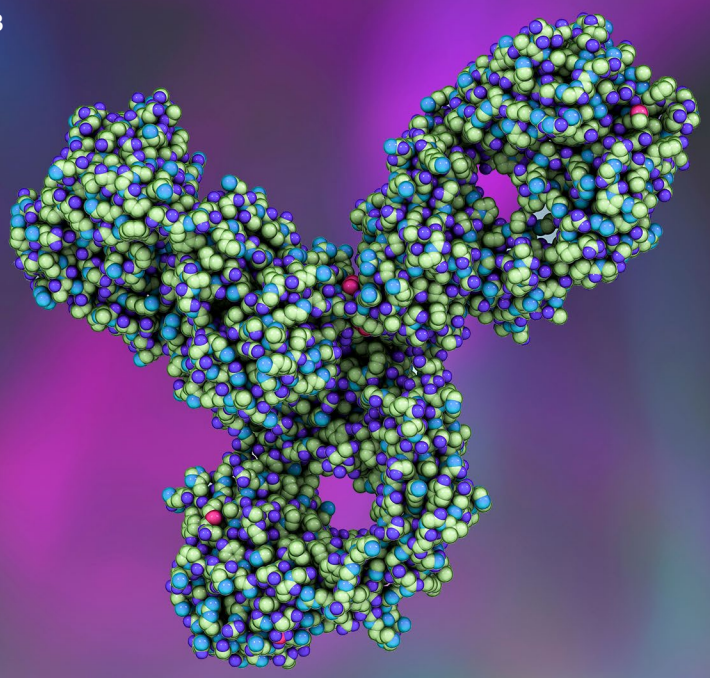
Application note | 002723



Biopharma

# Development of a robust pH gradient method for monoclonal antibody charge variant analysis using cation exchange chromatography and a quality-by-design approach


## Authors

Xuepu Li[1], Xiaoxi Zhang[1], Christof Mitterer[2], Min Du[3], Richard Verseput[4]

[1]Thermo Fisher Scientific, Shanghai, China

[2]Thermo Fisher Scientific, Langerwehe, Germany

[3]Thermo Fisher Scientific, Lexington, Massachusetts, USA

[4]S-Matrix Corporation, Eureka, CA, USA



## Keywords

pH gradient, charge variant analysis, monoclonal antibody, pembrolizumab, cation exchange chromatography, analytical quality by design (AQbD), Vanquish Flex UHPLC, Fusion QbD software platform, S-Matrix Corporation


## Application benefits

- Analytical quality by design (AQbD)-based UHPLC method development workflow for charge variant analysis
- Robust pH gradient method for charge variant analysis of pembrolizumab
- Seamless integration of the S-Matrix™ Fusion QbD™ software platform and the Thermo Scientific™ Chromeleon™ chromatography data system (CDS) to reduce method development time and improve laboratory efficiency

## Goal

Demonstrate the application of an AQbD approach to the development of an efficient and robust UHPLC method for the charge variant analysis of monoclonal antibody pembrolizumab

## Introduction

Charge heterogeneity analysis is a widely used analytical tool for biotherapeutic protein characterization, as the charge variants have a significant impact on the stability and efficacy of the drug. The analysis of charge variants is also a regulatory requirement to ensure that the monoclonal antibodies (mAbs) meet the required levels of quality.[1] Ion exchange chromatography (IEC), particularly cation exchange, is the most widely

used technique for the separation and characterization of charge variants of mAb, which can be performed with either salt or pH gradients.[2] With commercially available pH gradient buffers, such as the Thermo Scientific™ CX-1 pH gradient buffers, the pH gradient method has emerged as a preferred technique to the conventional salt gradient method. These buffer systems simplify the method development procedure by providing generic, fast, and reproducible linear pH gradients for mAbs with different isoelectric points (pI).[3]

In addition to the buffer system, developing a robust and efficient pH gradient method involves the evaluation of other parameters, such as column stationary phase and dimension, gradient slope, flow rate, and column temperature. To meet the FDA's expectations and the proposed ICH guideline for method lifecycle management, a systematic and AQbD-based method development approach is favored. The AQbD approach described in ICH Q14 and USP Chapter <1220> emphasizes quality risk assessment, the investigation of interactions among critical variables, and the definition of a method operable design region (MODR), which can help define a proper control strategy for analytical procedures to control sources of variability and consistently provide credible results with constant quality.[4-5]

Developing an AQbD-based UHPLC method for charge variant analysis is more challenging than for most small molecule methods. The highly resolved separations with sharp peaks common to many small molecule methods are replaced by inadequately resolved acidic and basic peak groups, and there are no generally accepted criteria to evaluate the performance of the separation of charge variants. In this application note, an AQbD approach was carried out using the Fusion QbD software platform combined with the Thermo Scientific™ Vanquish™ Flex UHPLC system and a high-resolution cation-exchange column to develop a pH gradient method for charge variant analysis of pembrolizumab. Fusion QbD software accelerated the whole workflow from method scouting and optimization to robustness evaluation by automatically generating efficient Design of Experiments (DoE)-based studies, best answer predictions, and data visualization. Critical separation metrics including peak-to-valley (p/v) ratio, start p/v and end p/v for specific peaks, retention time difference (RTD), and number of visualized peaks were used to evaluate the mean performance of separation. The final method obtained from Fusion QbD software shows good separation for all charge variants of pembrolizumab, and the method's performance meets or outperforms all performance requirements defined in the analytical target profile (ATP).

## Experimental

### Instrumentation

- Vanquish Flex UHPLC system consisting of:
  - System base (P/N V-S01-A-01)
  - Binary pump F (P/N VF-P10-A)
  - Split sampler FT (P/N VF-A10-A)
  - Column compartment H (P/N VH-C10-A)
  - Variable wavelength detector F (P/N VF-D40-A) with 11 µL standard bio flow cell (P/N 6077.0200)
- Thermo Scientific™ Orion Star™ A211 pH Meter (P/N STARA2110)

### Software

- Chromeleon CDS, version 7.3.1
- Fusion QbD software, version 9.9.2

### Reagents and consumables

- Thermo Scientific™ SureSTART™ 2 mL glass vials (amber) (P/N 6ASV9-2P)
- Thermo Scientific™ SureSTART™ 9 mm vial caps with septum (P/N 6ASC9ST1)
- Deionized water, 18.2 MΩ·cm resistivity or higher
- Thermo Scientific™ CX-1 pH gradient buffer A (P/N 083273)
- Thermo Scientific™ CX-1 pH gradient buffer B (P/N 085348)
- Piperazine, imidazole, Tris base, hydrochloric acid, sodium hydroxide, MOPSO, bicine, CAPSO, CAPS, AR grade, were obtained from Sigma-Aldrich

### Eluents and sample preparation

Two commonly reported pH gradient buffer systems and the CX-1 pH gradient buffer system were used in this experiment, the preparation process is as follows: [6-7]

#### CX-1 pH gradient buffer system (CX-1 buffer):

Eluent A1: 10-fold dilution of CX-1 pH gradient buffer A (pH 5.6) with DI water.

Eluent B1: 10-fold dilution of CX-1 pH gradient buffer B (pH 10.2) with DI water.

### Tris base/piperazine/imidazole buffer system (Tris base buffer):

A stock buffer with 116 mM piperazine, 15 mM imidazole, and 24 mM Tris base in DI water was prepared and stored at room temperature.

Eluent A2: 10-fold dilution of the stock buffer with DI water, then adjusted the pH to 6.0 with hydrochloric acid.

Eluent B2: 10-fold dilution of the stock buffer with DI water, then adjusted the pH to 9.5 with hydrochloric acid.

### MOPSO/bicine/CAPSO/CAPS buffer system (MOPSO buffer):

Eluent A3: 1.0 L eluent A contains 7.1 mM MOPSO, 5.3 mM bicine, 14.9 mM CAPSO, 0.7 mM CAPS, and 1.0 L DI water; the pH was adjusted to 6.5 using sodium hydroxide.

Eluent B3: 1.0 L eluent B contains 14.6 mM MOPSO, 4.9 mM bicine, 1.4 mM CAPSO, 7.1 mM CAPS, and 1.0 L DI water; the pH was adjusted to 10.3 using sodium hydroxide.

### Sample:

Commercially available pembrolizumab was diluted to 1.0 mg/mL using DI water.

## Results and discussion

### Quality-by-design workflow

The AQbD-based method development workflow shown in Figure 1 describes the science and risk-based approach for developing and maintaining analytical procedures. It starts with ATP identification, followed by a risk assessment and method development/validation, and ends with control strategy definition. In the method development phase, multi-variate experiments need to be conducted to explore ranges and interactions between identified parameters using the DoE approach. This leads to more robust analytical procedures, a better understanding of the impact of analytical procedure parameters, more flexibility for lifecycle management such as wider operating ranges, and associated reporting categories for changes. The blue box in Figure 1 shows how to use the Fusion QbD software combined with Chromeleon CDS to conduct method development/optimization and create the design space. Fusion QbD is a platform that can be seamlessly integrated with Chromeleon CDS to automate AQbD-based method development and validation. Fusion QbD automatically generates the most efficient experiment design to characterize the independent and interactive effects of the study parameters at each development stage.

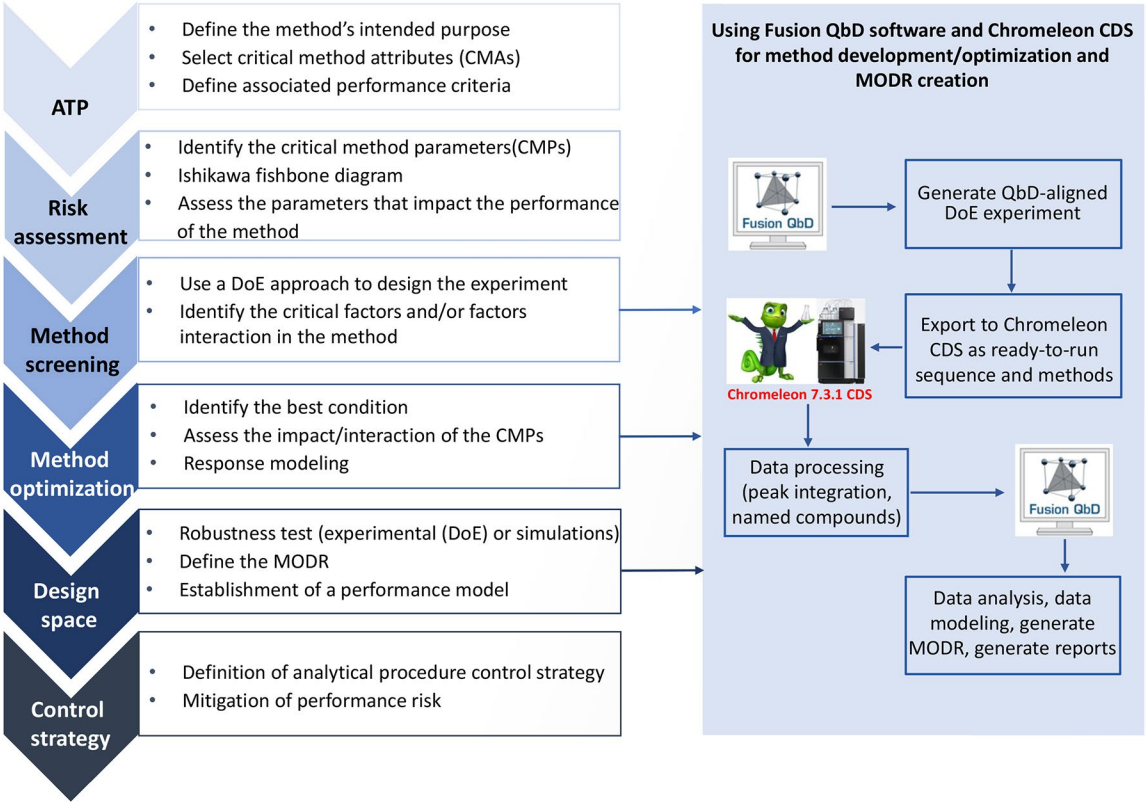



**Figure 1. AQbD-based UHPLC method development workflow.** The left flow chart depicts the main steps of the AQbD process: ATP definition, risk assessment, method screening, method optimization, design space, and establishment of the method control strategy. The right blue box shows how to use the Fusion QbD software combined with Chromeleon CDS to conduct method development/optimization and create the design space.

Then the Fusion QbD software export operation automatically transfers these designs to Chromeleon CDS as ready-to-run methods and sequences. Chromeleon CDS is used for data acquisition and chromatogram processing. After peak integration in Chromeleon CDS, the data is imported into Fusion QbD software to perform data analysis, mathematical modeling, and MODR generation.

## ATP identification

An ATP consists of a description of the intended purpose, appropriate details on the product attributes to be measured, and relevant performance characteristics with associated criteria. For the charge variant analysis, as peaks cannot be baseline separated, the p/v ratio, start p/v and end p/v for specific peaks, and RTD (also called retention delta) results were used to evaluate the method's mean performance (described in Figure 2), and the % RSD results for the main peak area and retention time results were used to evaluate method robustness. The complete ATP was therefore defined as follows:

- Maximize the separation of the main peak (MP) from its adjacent acidic peak (AAP) and adjacent basic peak (ABP).

  Goals:

  - Maximize the end p/v of AAP, with a lower performance limit of 1.2.
  - Maximize the start p/v of ABP, with a lower performance limit of 1.2.
  - Maximize the RTD of the MP from the AAP, with a lower performance limit of 0.5 min.
  - Maximize the RTD of the ABP from the MP, with a lower performance limit of 0.5 min.

- Aim to detect all the potential charge variants in pembrolizumab. According to previous research, at least 9 peaks (4 acidic peaks, 1 main peak, 4 basic peaks) should be detected.[8]

  Goal:

  - Maximize the number of peaks, with a lower bound of 9.

- Maximize the separation of different charge variants.

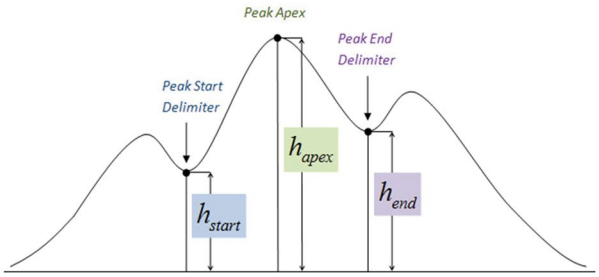
  Goal:

  - Maximize the number of peaks with p/v > 1.20, with a lower performance limit of 4.

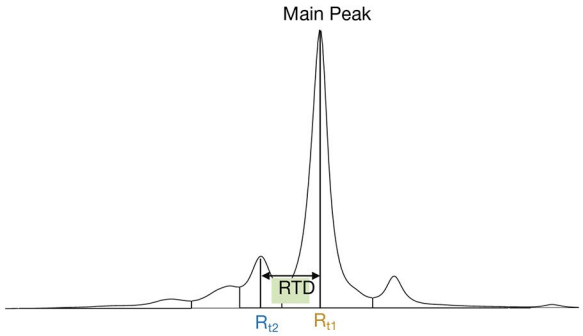
- The method should be robust and reproducible.

  Goal:

  - Minimize the peak area and peak retention time % RSD results for the MP, with an upper performance limit of 5.0%.




Start p/v = $Ratio_{start} = h_{apex}/h_{start}$
End p/v = $Ratio_{end} = h_{apex}/h_{end}$
Peak-to-valley ratio: The variable always reports the minimum ratio of the two calculated ratios.




$RTD_{main\ peak} = R_{t1}-R_{t2}$
For a given peak RTD (also called retention delta) is the difference in the retention time between the peak and its preceding peak.

**Figure 2. Peak-to-valley ratio and RTD are used to evaluate the performance of the separation.** The p/v ratios are calculated based on the United States Pharmacopeia guidelines.[9]

## Risk assessment

After the ATP definition, a risk assessment should be conducted to determine the criticality of the method attributes and key method parameters. A fishbone diagram in Figure 3 displays all method attributes and parameters that affect the charge variant analysis method performance characteristics defined in the ATP. The diagram is constructed based on sound knowledge and empirical experience. However, not all these parameters need to be assessed. For example, some parameters can be easily controlled by setting them as constant, such as column stationary phase, pump type, and solvent type, while others such as column reproducibility cannot be controlled by the analyst in the lab. The selection of parameters for the study is therefore based on the identification of those significant parameters for which optimum settings and robust operating ranges need to be determined. In this case, the parameters were scored based on their criticality and likelihood, some of the results are shown in Table 1. After taking the score and detectability into consideration, the buffer system, gradient, column dimensions, column temperature, flow rate, injection volume, and sample stability were selected as CMPs.

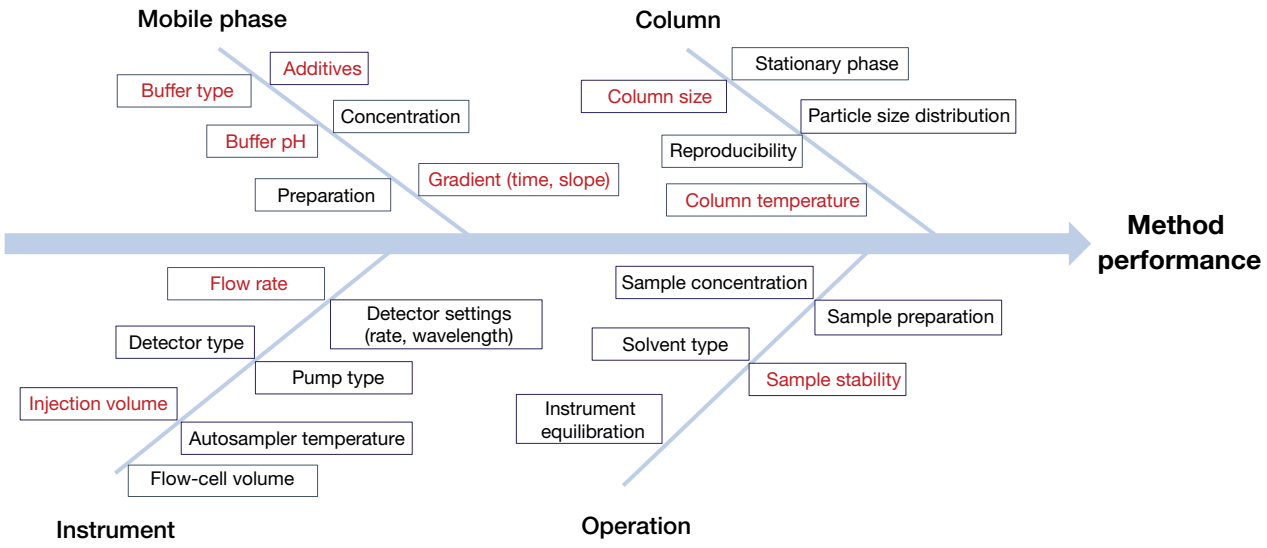



**Figure 3. The fishbone diagram shows the method attributes and parameters that affect the method performance; attributes in red were selected for this study.**

**Table 1. Risk scoring results based on fishbone diagram, scientific judgment, and risk assessment**

| Factors | Likelihood | Criticality | Scoring |
|---|---|---|---|
| Buffer type | Low (1) | High (3) | 3 |
| Buffer pH | High (3) | High (3) | 9 |
| Gradient (time, slope) | Low (1) | High (3) | 3 |
| Column size and stationary phase | Low (1) | High (3) | 3 |
| Column temperature | Medium (2) | High (3) | 6 |
| Column reproducibility | Low (1) | High (3) | 3 |
| Flow rate | Low (1) | High (3) | 3 |
| Injection volume | Medium (2) | Medium (2) | 4 |
| Detector setting | Low (1) | Low (1) | 1 |
| Sample preparation | Low (1) | High (3) | 3 |
| Sample stability | Medium (2) | High (3) | 6 |

**Table 2. UHPLC parameters used for method scouting**

| Study factor | Types/Range |
|---|---|
| **Columns** | Thermo Scientific™ ProPac™ 3R SCX columns:<br>1. 50 mm × 2 mm, 3 µm (P/N 43103-052068)<br>2. 100 mm × 4 mm, 3 µm (P/N 43103-104068) |
| **Eluents** | 1. CX-1 pH gradient buffer system<br>2. Tris base/piperazine/imidazole buffer system<br>3. MOPSO/bicine/CAPSO/CAPS buffer system |
| **Gradient slope** | 0–100% B, tG= 10–20 min |
| **Constants** | **Level settings** |
| **Column temp.** | 35.0 °C |
| **Flow rate** | 0.2 mL/min for column 1, 0.4 mL/min for column 2 |
| **Inj. volume** | 10 µL |
| **Autosampler temp.** | 4.0 °C |
| **Detector** | 280 nm |

## Method screening

The goal of the method screening is to perform a rapid experiment to screen CMPs that are likely to have the largest impact on the performance. Therefore, SCX columns with different dimensions, buffer types, and gradient times were investigated in this phase; other constant parameters are listed in Table 2. The ATP goal at this phase was defined as maximizing both the number of peaks and the separation of AAP/ABP with the main peak. These metrics were used to identify the best combination of column dimensions, buffer type, and initial and end %B in terms of acceptable separation of pembrolizumab charge variants. These results would then be promoted to method optimization.

The screening results presented in Figure 4 show that using the CX-1 buffer generates the maximum number of peaks and the best separation between the peaks immediately adjacent to the main peak, and there is no obvious improvement in the resolution by increasing the length of the column. As the pH range of different buffer systems is slightly different, to further verify the capability of the CX-1 buffer system, pembrolizumab was injected with a fixed *pH unit slope* (0.2 ΔpH/min) using these three buffer systems. As shown in Figure 5, the CX-1 buffer provides a better separation for pembrolizumab and its charge variants. Additionally, the linear pH gradient of the CX-1 buffer makes it easy to determine the initial and end %B in the optimization phase, and risks caused by buffer preparation also can be easily controlled by using the CX-1 buffer.[3] Based on the screening results, the short (50 mm) column and CX-1 buffer with an endpoint of 25% B were selected for the following optimization experiment.

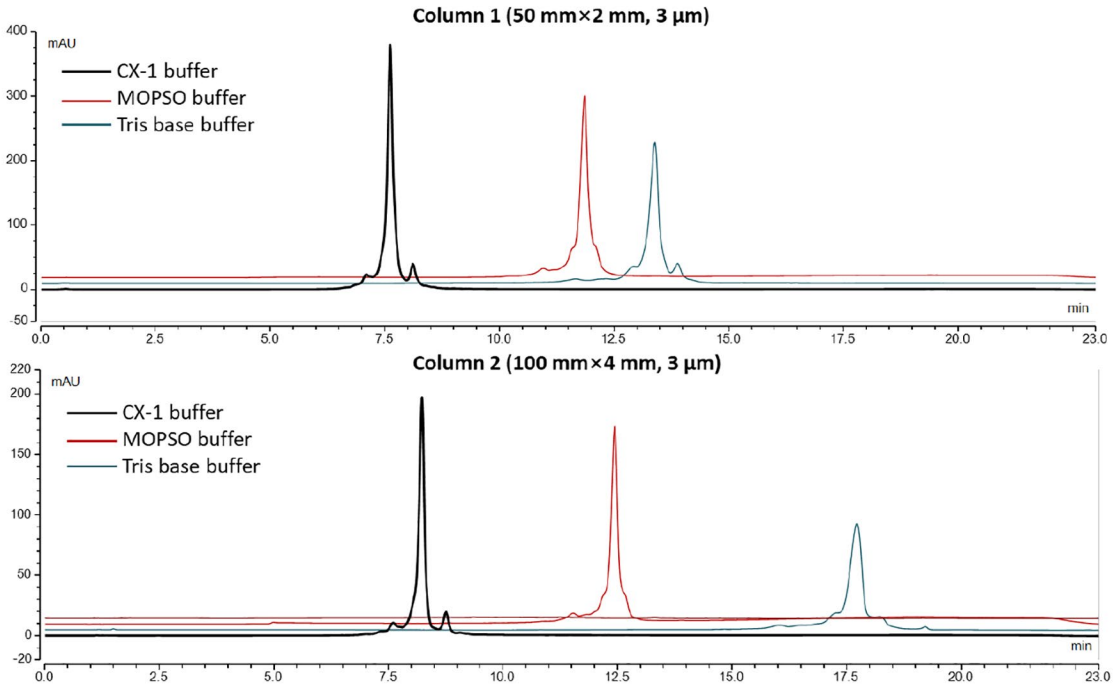



Figure 4. Screening results using different buffer systems and ProPac 3R SCX columns; the gradient time is 15 minutes.

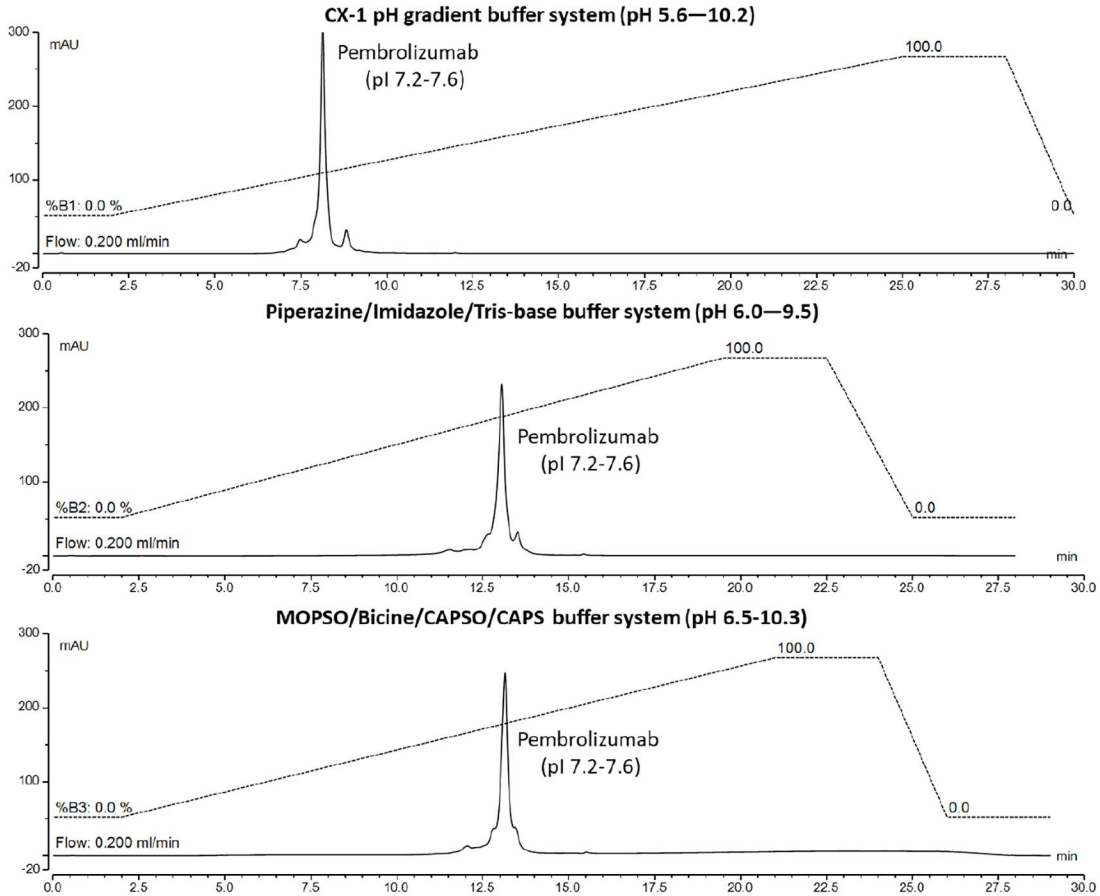



Figure 5. Chromatograms of pembrolizumab using different buffer systems on column 1 with a 0.2 ΔpH/min gradient

## Method optimization

In the optimization phase, gradient slope, flow rate, and column temperature were studied to achieve a better separation for the charge variants. The study ranges of these variables are shown in Table 3, with an injection volume of 10.0 µL and a detector wavelength of 280 nm. Note that gradient slope was studied in detail in this experiment by including both initial %B and gradient time. The gradient time was extended to 30 minutes in the optimization experiment, as it was found in the screening phase that longer gradient times can provide better resolution overall. In this optimization, a 30-run statistical experimental design was generated, which required about 28 hours of instrument time (including the conditioning runs and system suitability test). Compared with traditional manual method development, this procedure is fully automated and unattended. The time spent on method development was reduced from several weeks to several days, which reduced the cost and improved the efficiency in the lab.

The mathematical models in Fusion QbD software were automatically built and used to predict the "Best Overall Answer." Table 4 shows the best answer predicted by Fusion QbD software from the optimization study. The chromatogram using the best conditions is shown in Figure 6. It shows that the achieved experimental results for all mean performance metrics are in excellent agreement with the predicted results for all ATP performance requirements.

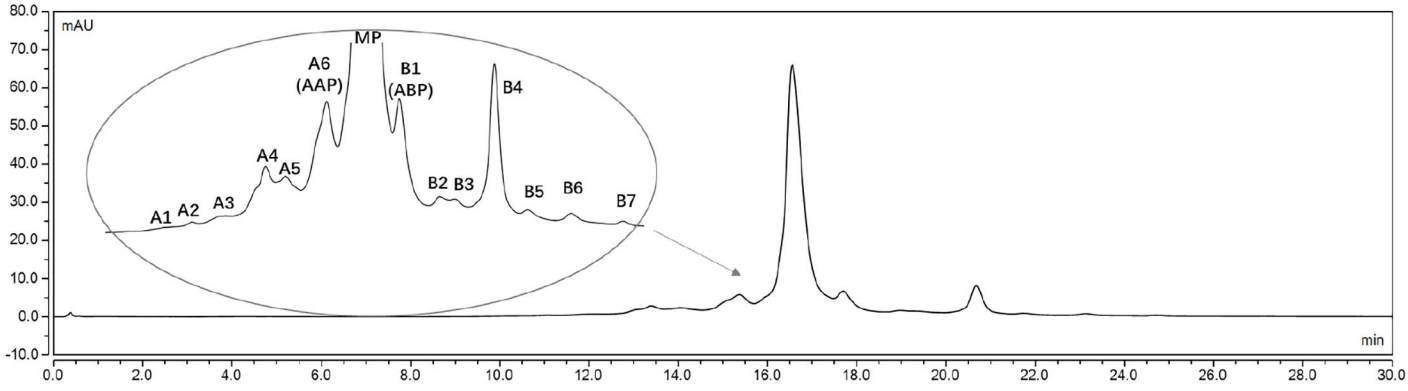
The effects of the various study parameters on method mean performance can be evaluated by generating an initial MODR, or analytical design space in Fusion QbD software. Figure 7 presents a 3 × 3 trellis graph series that displays the variable effects and the initial MODR. In these graphs, each critical performance characteristic is assigned a color, and the graph region shaded with that color identifies method conditions that fail to meet the specified performance requirements for that characteristic.

**Table 3. DOE platform for UHPLC method optimization**

| Variable | DOE range |
|---|---|
| Column temperature | 25.0 °C to 45.0 °C |
| Flow rate | 0.15–0.30 mL/min |
| Gradient time | 10.0–30.0 min |
| Gradient slope (%B) | Start point = (0.0%–10.0%)<br>End point = 25.0% |

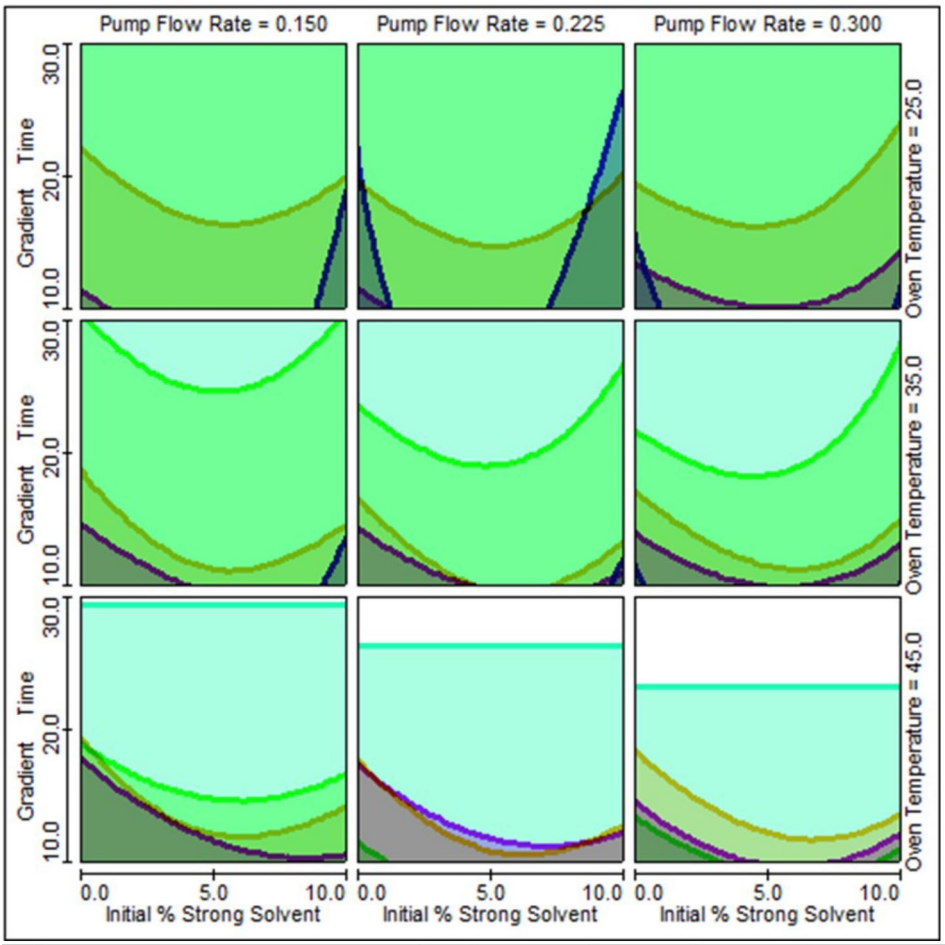
**Table 4. Best UHPLC conditions predicted by Fusion QbD software**

| Name | Level setting |
|---|---|
| Column temperature | 45.0 °C |
| Pump flow rate | 0.30 mL/min |
| Gradient time | 30 min |
| Initial B% | 5.0% |




| Name | Goal | Predicted results | Achieved values | Residual |
|---|---|---|---|---|
| No. of peaks | Maximize, lower bound: 9 | 13.7 | 14 | 0.3 |
| No. of peaks ≥1.20 peak-to-valley ratio | Maximize, lower bound: 7 | 7.5 | 8 | 0.5 |
| ABP-start p/v | Maximize, ≥1.2 | 1.276 | 1.240 | -0.036 |
| AAP-end p/v | Maximize, ≥1.2 | 1.535 | 1.570 | 0.035 |
| ABP-Retention delta | Maximize, ≥0.5 min | 1.049 | 1.100 | 0.051 |
| MP-Retention delta | Maximize, ≥0.5 min | 1.064 | 1.198 | 0.134 |

**Figure 6. Chromatogram of pembrolizumab obtained using the best conditions predicted by Fusion QbD software demonstrates that all previously defined optimization goals have been met or exceeded after method optimization.** The inserted graph is a zoom-in to show the acidic and basic variants peaks. A1-A6 represent the acidic peaks and B1-B7 represent the basic peaks.




| | Name | Units | Goal | Lower Bound | Upper Bound | Color |
|---|---|---|---|---|---|---|
| ☑ | No. of Peaks | * | Maximize | 9.0 | | Red |
| ☑ | No. of Peaks >= 1.20 - Peak to Valley Ratio | | Maximize | 4.0 | | Blue |
| ☑ | AAP - End p/v | | Maximize | 1.200 | | Green |
| ☑ | ABP - Retention delta | | Maximize | 0.500 | | Orange |
| ☑ | ABP - Start p/v | | Maximize | 1.200 | | Teal |
| ☑ | MP - Retention delta | | Maximize | 0.500 | | Purple |

**Figure 7. Trellis graphs visually display the interaction of the CMPs to the method performance.** The graph region shaded with a color identifies method conditions that fail to meet the specified performance requirements for a given characteristic, while the unshaded region is the MODR.

The remaining unshaded region in the graph is the mean performance MODR—the region containing the methods that simultaneously meet or exceed all mean performance requirements specified for resolution and number of peaks. Within the MODR, parameters can be varied independently or simultaneously without compromising any of the mean performance requirements. The results show that MP-ABP is the critical peak pair; in most conditions, the start p/v of ABP determines the area of the MODR. To separate the ABP from the main peak with a p/v ≥1.2, the column temperature must be higher than 35.0 °C. The effect of column temperature on the separation between MP and ABP is further demonstrated in Figure 8, which shows that when column temperature is increased, the start p/v of ABP is also increased. However, it should be noted that this method is used to separate proteins. Therefore, to maintain the non-denaturing condition and the native conformations of the proteins, the column temperature should be controlled in an appropriate range.

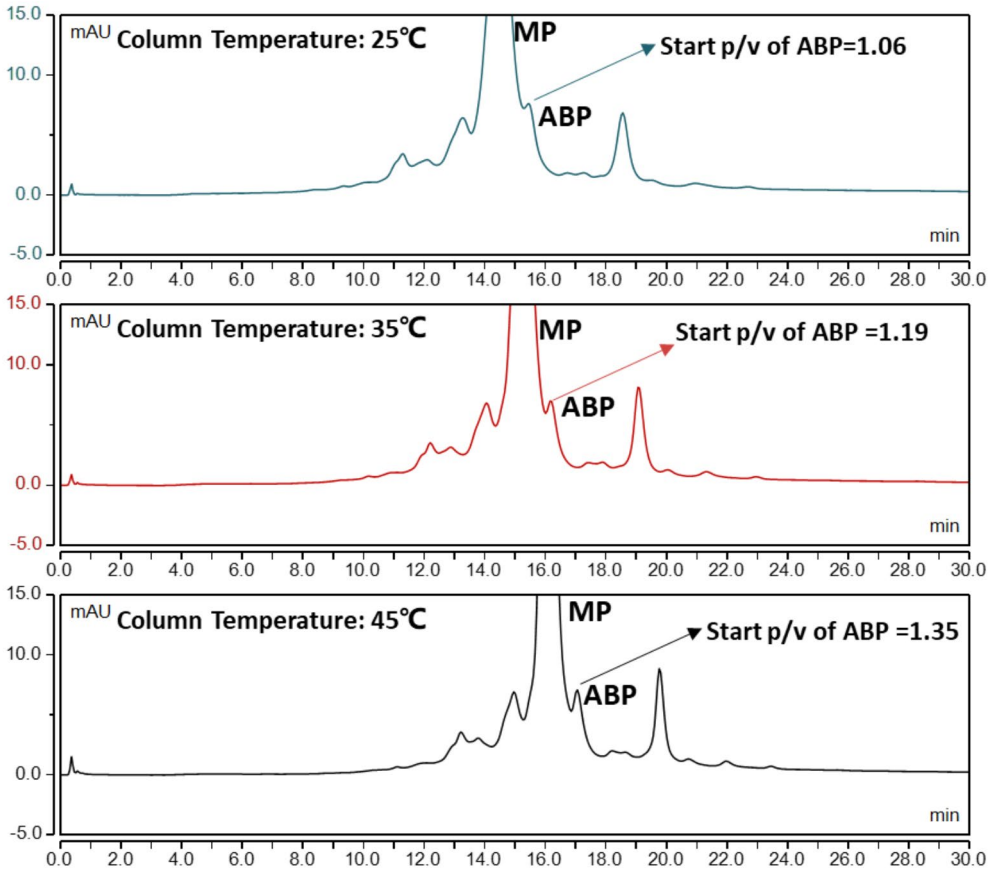



**Figure 8. The effect of column temperature on the separation between the MP and ABP.** Other UHPLC conditions were the same with the best conditions predicted from Fusion QbD software.

## Robustness simulation

The MODR in Figure 7 reflects the predicted mean performance of a given method for all included critical method performance characteristics. To establish a final robust MODR, the robustness simulation in Fusion QbD software was added to reflect changes in method performance caused by variations in the critical parameters that the method will experience in the lab over time. Figure 9 shows the variable settings in Fusion QbD software. First, the maximum expected variation in each included critical parameter of the method should be defined. Second, Fusion QbD software creates the MODR that combines the robustness and average performance of the method. It should be noted that variation in gradient time represents slope variation, which is in turn a variation in mobile phase composition. This is why, as seen in Figure 9, Fusion QbD software has automatically converted the gradient time and initial B% study factors into a single mobile phase composition parameter for the robustness simulation setup. The commonly used maximum expected variations are ±3σ values, which is normally about ±2.0% mobile phase composition variation at given points along the gradient.

In this case, to reflect the possible variations across instruments and personnel on method transfer and normal use in the lab over time, the variation of flow rate is extended to 0.03 mL/min, which is about 10% of the optimal flow rate, and the variation of the column temperature is enlarged to 3.0 °C, which is about 7% of the optimal column temperature.

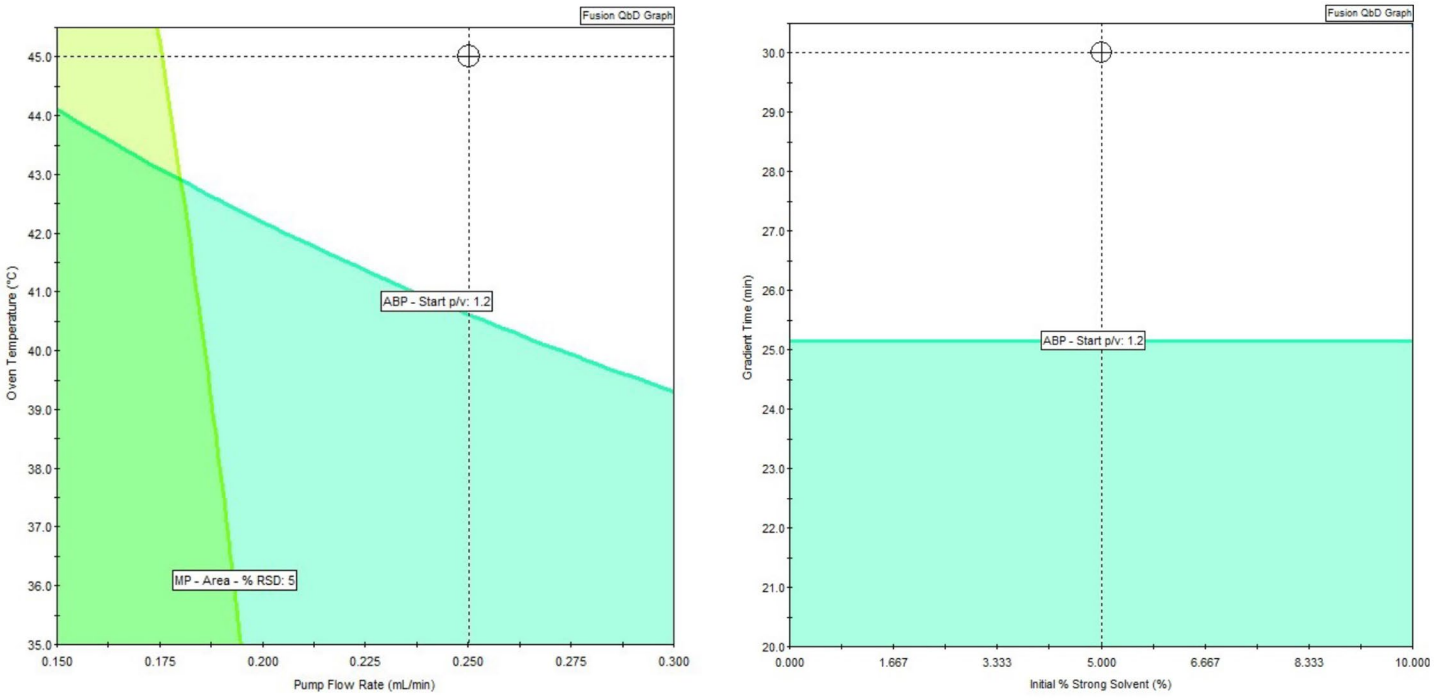
The main peak area and retention time %RSD results were selected to characterize the method's robustness. The graph presented in Figure 10 illustrates the MODR of mean performance and robustness. The left graph indicates that adding the two response goals reduced the area of MODR. The %RSD value for the peak area of the main peak is >5.0% when the flow rate is lower than 0.2 mL/min (with initial B% = 5.0% and gradient time = 30.0 min). The right graph in Figure 10 shows that the area associated with a gradient time of 25.5 min to 30.0 min and initial %B from 0% to 10% can simultaneously meet the mean performance goal and robustness goals. This area indicates that the target method has excellent robustness performance at the target setpoint conditions of pump flow rate and column temperature.

Variable Settings

| Enabled | Experiment Variable | Units | Maximum Expected Variation (±3σ Value) |
|---|---|---|---|
| ☑ | Pump Flow Rate | mL/min | 0.030 |
| ☑ | Oven Temperature | °C | 3.0 |
| ☑ | Mobile Phase Composition (MPC)* | % | 2.0 |

* - MPC variation is composition (blend) variation due to pump precision limits. A commonly used ±3σ value = ±2.0%.
The value you enter will be applied to all Gradient Slope factors (e.g., Time, Slope, and Ramp Steps) in the experiment design.

**Figure 9. Variable settings in Robustness simulator.** Fusion QbD software has automatically transformed the gradient time and initial %B study factors into a single mobile phase composition factor for robustness simulation.




| | Name | Units | Goal | Lower Bound | Upper Bound | Color |
|---|---|---|---|---|---|---|
| ☑ | No. of Peaks | * | Maximize | 9.0 | | Red |
| ☑ | No. of Peaks >= 1.20 - Peak to Valley Ratio | | Maximize | 4.0 | | Blue |
| ☑ | AAP - End p/v | | Maximize | 1.200 | | Green |
| ☑ | ABP - Retention delta | | Maximize | 0.500 | | Orange |
| ☑ | ABP - Start p/v | | Maximize | 1.200 | | Teal |
| ☑ | MP - Retention delta | | Maximize | 0.500 | | Purple |
| ☑ | MP - Area - % RSD | * | Minimize | | 5.000 | Lime |
| ☑ | MP - Retention Time - % RSD | * | Minimize | | 5.000 | Sky |

**Figure 10. Graph illustrates the MODR of mean performance and robustness.** The non-graphed variables for the left graph: initial B% = 5.0% and gradient time = 30.0 min; for the right graph: flow rate = 0.30 mL/min and column temperature = 45 °C.

## Point Predictions feature

The Point Predictions feature in Fusion QbD software was used to generate predictions for the five verification run points in the MODR pictured in Figure 11. Fusion QbD software generates all the critical method performance characteristics for these five conditions. The five conditions were then validated on a Vanquish Flex UHPLC system. Figure 11 shows the chromatograms obtained from running these five conditions. The performance of the AAP, MP, and ABP were predicted in Fusion QbD software. The right graph in Figure 11 shows that the predicted retention time of these three peaks is in excellent agreement with the experiment results.

The p/v performance (for AAP and ABP) and number of peaks from the predicated and experimental results are compared in Table 5. All the experimental results were consistent with the predicted results, which shows the high degree of accuracy associated with the point predictions in Fusion QbD software.

## Control strategy

Using Fusion QbD software, it is easy to evaluate the effect of each CMP on the method's performance characteristics. To obtain consistent performance and reliable data in the lab, the control strategy presented in Table 6 was therefore proposed based on the risk assessment and the experimental studies from Fusion QbD software. According to their degree of influence on p/v (resolution) and robustness, different parameters were labeled as red, yellow, and green. The control strategy, study type, and values/types are also listed in this table, and the MODR and ranges of the parameters are used for the following control strategy.

The best condition predicted from Fusion QbD software is the upper limit of the flow rate, gradient time, and column temperature in the MODR. To get a better control strategy, the point (0.25 mL/min, 28.0 min, and 43 °C) in the center of the unshaded regions within the MODR was chosen as the final method (Figure 10). This point was further verified and the results met or outperformed all goals defined in the ATP. Injection volume was investigated separately after the DoE study and showed that the appropriate injection volume was 5.0 to 20.0 µL. In this range, the change in the injection volume will not affect the performance of the method. The sample stability was studied by injecting the 4.0 °C stored sample on the 1, 3, 5, and 7 days three times, and then comparing the retention time and peak area with the freshly prepared sample. The sample is considered stable if the average %RSD of the peak area and retention time is less than 5.0%. The stability results show that the sample is stable after 1 week of storage at 4.0 °C.

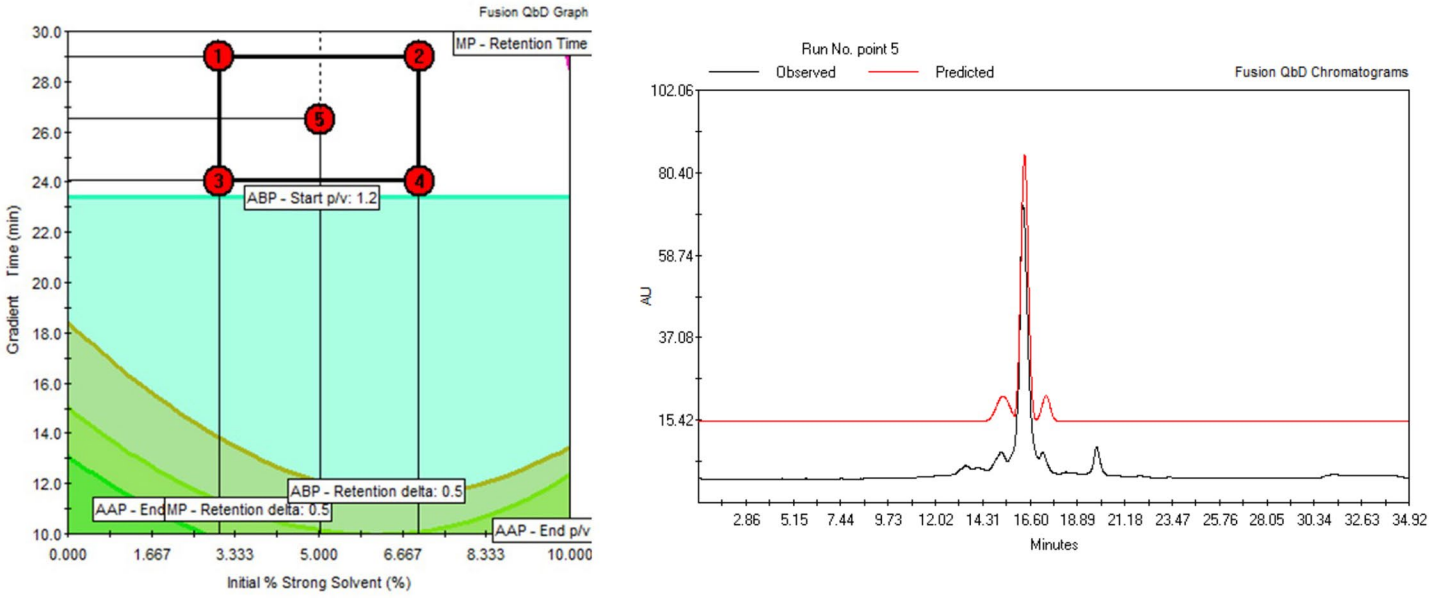



**Figure 11. Five verification run points in the MODR were used for point prediction.** The comparison of predicted and experimental chromatograms for point five in the MODR is shown on the right.

**Table 5. The comparison of the predicted results and experimental results for 5 points in MODR**

| Run ID | Pump flow rate (mL/min) | Gradient time (min) | Initial solvent B% (%) | Column temperature (°C) | End p/v of AAP | | Start p/v of ABP | | Peak no. | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Predicated | Experimental | Predicated | Experimental | Predicated | Experimental |
| Point 1 | 0.3 | 29.0 | 3.0 | 45.0 | 1.522 | 1.50 | 1.264 | 1.30 | 13.8 | 14 |
| Point 2 | 0.3 | 29.0 | 7.0 | 45.0 | 1.501 | 1.56 | 1.264 | 1.32 | 14.0 | 14 |
| Point 3 | 0.3 | 24.0 | 3.0 | 45.0 | 1.445 | 1.42 | 1.207 | 1.26 | 14.1 | 14 |
| Point 4 | 0.3 | 24.0 | 7.0 | 45.0 | 1.434 | 1.49 | 1.207 | 1.28 | 14.3 | 14 |
| Point 5 | 0.3 | 26.5 | 5.0 | 45.0 | 1.484 | 1.53 | 1.236 | 1.32 | 14.1 | 14 |

Table 6. Risk assessment and control strategies for charge variant analysis of pembrolizumab

| CMAs | | Risk assessment | | Value/Types | Study type | Control strategy |
|---|---|---|---|---|---|---|
| | | Resolution | Robustness | | | |
| **Buffer system** | pH and salts | High | Medium | CX-1 pH gradient buffer | DOE | Fixed, not changeable until proven equivalent |
| **Column** | Stationary phase | High | High | ProPac 3R SCX | DOE | Fixed, not changeable until proven equivalent |
| | Size | Low | Medium | 50 mm × 2 mm, 3 µm | DOE | |
| | Temperature | High | Low | 43.0 °C | DOE | MODR |
| **Gradient** | Time | High | Low | 28.0 min | DOE | MODR |
| | Initial B% | Medium | Low | 5.0% eluent B | DOE | MODR |
| **Instrument** | Configuration | Medium | High | Vanquish Flex UHPLC | / | Fixed |
| | Flow rate | High | Medium | 0.25 mL/min | DOE | MODR |
| | Injection volume | Low | Low | 10 µL | Range Study | Range |
| **Sample preparation** | Solvent | Low | Medium | Water | / | Fixed |
| | Sample stability | Medium | Medium | 4.0 °C, 1 week | Range | Range |

## Conclusion

In this application note, we demonstrated the AQbD-based pH gradient method development approach for charge variant analysis of pembrolizumab using Fusion QbD software, a Vanquish Flex UHPLC, a CX-1 pH gradient buffer, and a ProPac 3R SCX column. The predicted "Best Overall Answer" by Fusion QbD software is in excellent agreement with the experimental results, which showed a good separation for pembrolizumab and all charge variants. The method was further proven to be robust within the variations of ±2.0% to 10.0% of the variable setpoints using the robustness simulator in Fusion QbD software. Finally, a control strategy was defined based on the risk assessment and the experimental studies from Fusion QbD software to ensure consistent performance and reliable data in the laboratory.

## References


1. ICH Q6B: Specifications: Test Procedures and Acceptance Criteria for Biotechnological/ Biological Products, International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use: Geneva, Switzerland, 1999.
2. Baek, J.; Schwahn, A.B.; Lin, S.; et al. New insights into the chromatography mechanisms of ion-exchange charge variant analysis: dispelling myths and providing guidance for robust method optimization. *Analytical Chemistry,* **2020,** *92*(19), 13411–13419.
3. Lin, S.; Baek, J.; Rao, S.; et al. Thermo Scientific Application Note 20784: A novel pH gradient separation platform for monoclonal antibody (mAb) charge variant analysis, **2013.** https://assets.thermofisher.com/TFS-Assets/CMD/Application-Notes/an-20784-gradient-separation-platform-mab-an20784-en.pdf
4. ICH Q14: Analytical Procedure Development Q14 (Draft version). International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use: Geneva, Switzerland, 2022. https://database.ich.org/sites/default/files/ICH_Q14_Guideline_2023_1116.pdf
5. McDowall, R. Understanding the lifecycle approach for analytical procedures. *LCGC North America,* **2020,** *38*(4).
6. Zhang, L.; Patapoff, T.; Farnan, D.; et al. Improving pH gradient cation-exchange chromatography of monoclonal antibodies by controlling ionic strength. *Journal of Chromatography A,* **2013,** *1272,* 56–64.
7. Lingg, N.; Tan, E.; Hintersteiner, B.; et al. Highly linear pH gradients for analyzing monoclonal antibody charge heterogeneity in the alkaline range. *Journal of Chromatography A,* **2013,** *1319,* 65–71.
8. Zhang, X.; Chen ,T.; Li, V.; et al. Cutting-edge mass spectrometry strategy based on imaged capillary isoelectric focusing (icIEF) technology for characterizing charge heterogeneity of monoclonal antibody. *Analytical Biochemistry,* **2023,** *660,* 114961.
9. USP, USP General Chapter <621>, "Chromatography". https://doi.org/10.31003/USPNF_M99380_06_01 (accessed 2023-05-01).


Learn more at **thermofisher.com/methodlifecyclemanagement**