

# A Novel pH Gradient Separation Platform for Monoclonal Antibody (MAb) Charge Variant Analysis

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## Key Words

CX-1 pH Gradient Buffer Kit, MAbPac SCX-10, UltiMate 3000 BioRS, MAb charge variant analysis

## Abstract

MAb charge variants and protein standards with various pI values have been successfully separated using cation exchange chromatography with a linear pH gradient. This linear pH gradient is generated by running a linear pump gradient from 100% buffer A (at pH 5.6) to 100% buffer B (at pH 10.2). Ruggedness testing of this pH gradient on Thermo Scientific™ MAbPac™ SCX-10 columns shows that retention time RSD is less than 0.8% over 300 runs.

## Introduction

There is an extensive and increasing development pipeline for monoclonal antibody (MAb) therapeutics, which, combined with advances in automation in upstream processes such as cell culture and purification process development is driving a requirement for innovative analytical tools in order to deliver greater productivity. Recombinant MAbs can be highly heterogeneous due to modifications such as sialylation, deamidation and C-terminal lysine truncation. During development and production of these products, it is essential to detect, characterize, and quantify impurities as well as structural variants and modifications, and to monitor product stability. This is key to demonstrating their safety and efficacy as biotherapeutics and is required by the U.S. FDA and other regulatory agencies.

Traditionally, cation exchange chromatography using salt gradients has been successfully employed to characterize MAb charge variants. However, additional effort is often required to tailor the salt gradient method for individual MAbs. In the fast-paced drug development environment, a generic platform approach which saves time in method development and facilitates method transfer for a wide range of MAb charge variants, while still using an LC system, is desirable [1].

In this study, we present a novel pH gradient method for cation exchange chromatography. The linear pH gradient was achieved by employing a multi-component buffer system containing multiple zwitterionic buffer species with pI values ranging from 6 to 10. Eluent A was titrated



to pH 5.6 and eluent B was titrated to pH 10.2. In this pH range, each buffer species was either neutral or negatively charged. Therefore, they were not retained by the cation exchange stationary phase and served as good buffers for the mobile phase and the stationary phase.

The linear gradient is run from 100% buffer A (at pH 5.6) to 100% buffer B (at pH 10.2). Using an online pH meter, it is confirmed that a linear pH gradient was achieved in the range of pH 6 to 10. Furthermore, there is a strong correlation between the measured pH values of model proteins and their pI values. Since the majority of MAbs have their pI values in the range of pH 6 to 10, this pH gradient based separation method can serve as a more generic platform method for MAb charge variant analysis. Once the approximate pH elution range of the target MAb has been established in the initial run, further

optimization of the separation can simply be achieved by running a shallower pH gradient over a narrower pH range. Ruggedness testing of this pH gradient on a MAbPac SCX-10, 5  $\mu$ m column has shown that peak retention time RSD is less than 0.8% over 300 runs. Extensive testing of this pH gradient method using various cation exchange columns for charge variant separation of MABs and other protein samples has demonstrated its capability for fast and high resolution separations.

## Experimental Details

### Consumables Part Number

All standard proteins were purchased from Sigma. The monoclonal antibody was obtained from a local biotech company. Proteins and MAb were dissolved in deionized water.

Thermo Scientific™ CX-1 pH Gradient Buffer Kit 083274  
 This kit includes 125 mL of 10X buffer A concentrate (pH 5.6) and 125 mL of 10X buffer B concentrate (pH 10.2).

### Columns Part Number

MAbPac SCX-10, 10  $\mu$ m, 4  $\times$  250 mm 074625

MAbPac SCX-10, 5  $\mu$ m, 4  $\times$  50 mm 078656

### Liquid Chromatography

HPLC experiments were carried out using a Thermo Scientific™ Dionex™ UltiMate™ 3000 BioRS System equipped with:

SRD-3600 Membrane Degasser

DGP-3600RS Biocompatible Dual-Gradient Rapid Separation Pump

TCC-3000SD Thermostated Column Compartment with two biocompatible 10-port valves

WPS-3000TBRS Biocompatible Rapid Separation Thermostated Autosampler

VWD-3400RS UV Detector equipped with a micro flow cell

PCM-3000 pH and Conductivity Monitor

### Solutions

Eluents A and B each were prepared by diluting the corresponding 10X buffer concentrates 10 fold using deionized water.

### Gradients

The linear pH gradient was generated by running from 100% eluent A (pH 5.6) to 100% eluent B (pH 10.2). For pH gradient analysis carried out on the MAbPac SCX-10, 10  $\mu$ m, 4  $\times$  250 mm, cation exchange columns, the gradient method in Table 1 was used unless stated otherwise. For pH gradient analysis carried out on the MAbPac SCX-10, 5  $\mu$ m, 4  $\times$  50 mm columns, the gradient method in Table 2 was used unless stated otherwise. Both methods cover the pH range from pH 5.6 to pH 10.2.

Time (Minutes)	Flow rate (mL/min)	%A	%B
0-1	1	100	0
1-31	1	100-0	0-100
31-34	1	0	100
34-40	1	100	0

Table 1: 30 minute linear gradient method used with the MAbPac SCX-10, 10  $\mu$ m, 4  $\times$  250 mm, cation exchange column. Total run time is 40 min. The linear pH range covers from pH 5.6 to pH 10.2.

Time (Minutes)	Flow rate (mL/min)	%A	%B
0-1	1	100	0
1-16	1	100-0	0-100
16-17	1	0	100
17-20	1	100	0

Table 2: 15 minute linear gradient method used with the MAbPac SCX-10, 5  $\mu$ m, 4  $\times$  50 mm, cation exchange column. Total run time is 20 min. The linear pH range covers from pH 5.6 to pH 10.2.

### Data Processing and Software

Thermo Scientific™ Dionex™ Chromeleon™ 6.8 Chromatography Data System

## Results

### pH gradient linearity

Using the gradient method shown in Table 1, six proteins with a range of pI values from 6 to 10 were effectively separated on a MAbPac SCX-10, 10  $\mu$ m, 4  $\times$  250 mm column. These proteins were lectin (including three isoforms, lectin-1, lectin-2, and lectin-3), trypsinogen, ribonuclease A, and cytochrome C. The chromatogram was shown in Figure 1. The pH value measured in this experiment as a function of time was plotted in Figure 2. The pH gradient was found to be linear from pH 5.6 to pH 10.2 over a 30 minute gradient, with a correlation coefficient value  $R^2$  of 0.9996.

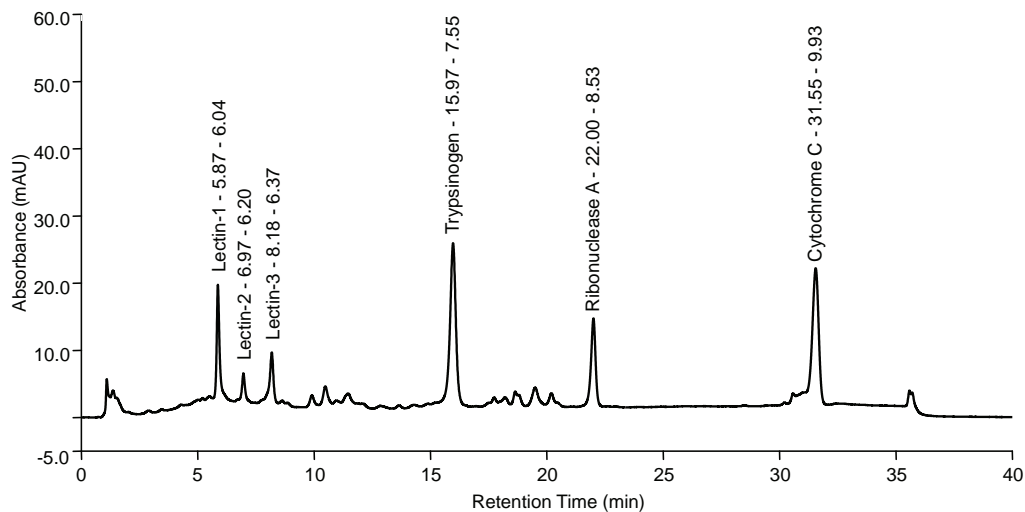


Figure 1: Chromatogram of six proteins separated on a 30 min linear pH gradient on a MAbPac SCX-10, 10  $\mu$ m, 4  $\times$  250 mm column. Protein name, retention time, and corresponding pH values are labeled for each protein peak.

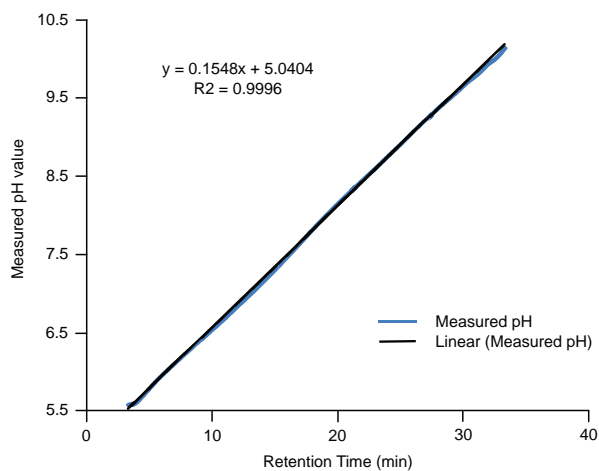


Figure 2: Graph showing measured pH values as a function of time. The measured pH values were exported from the same experiment shown in Figure 1.

The correlation between the elution pH for the peaks and the corresponding pI values of the protein components was assessed. This is shown in Figure 3, which compares the measured pH values for the six protein component peaks shown in Figure 1, as a function of their corresponding pI values. The measured pH values for the six protein component peaks exhibited a strong linear correlation to the literature based pI values. Thus, after a calibration procedure, this example supports the fact that linear regression coupled with the pH gradient method described here can be used to estimate the pI of a protein component based on the peak retention time and measured pH.

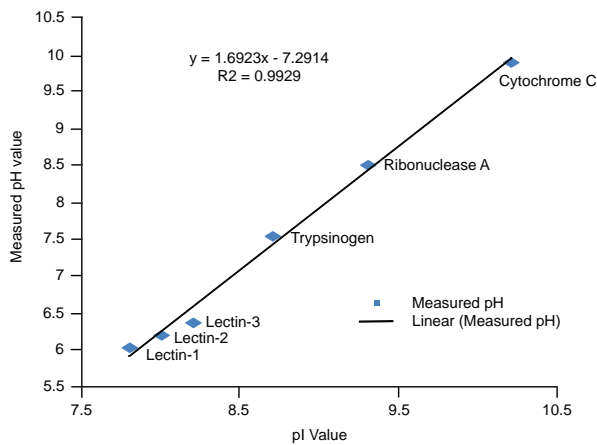


Figure 3: Graph plotting the measured pH values for six protein component peaks as a function of the corresponding pI value. The measured pH values of all six components were exported from the same experiment shown in Figure 1.

#### Comparison between pH gradient and salt gradient

Figure 4 shows the separation of a MAb on a MAbPac SCX-10, 10  $\mu$ m, 4  $\times$  250 mm column. Using a shallow salt gradient (from 84 mM NaCl to 132 mM NaCl in 30 min), MAb variant peaks were not very sharp, although they were somewhat separated. In order to further optimize the salt method it may be necessary to experiment with changing parameters such as buffer salt and pH. Instead of using a salt gradient, a pH gradient approach was used to separate the charge variants. In the initial run (Figure 5a) the pH elution range from pH 5.6 to pH 10.2 with a gradient slope of 0.153 pH unit/min was chosen.

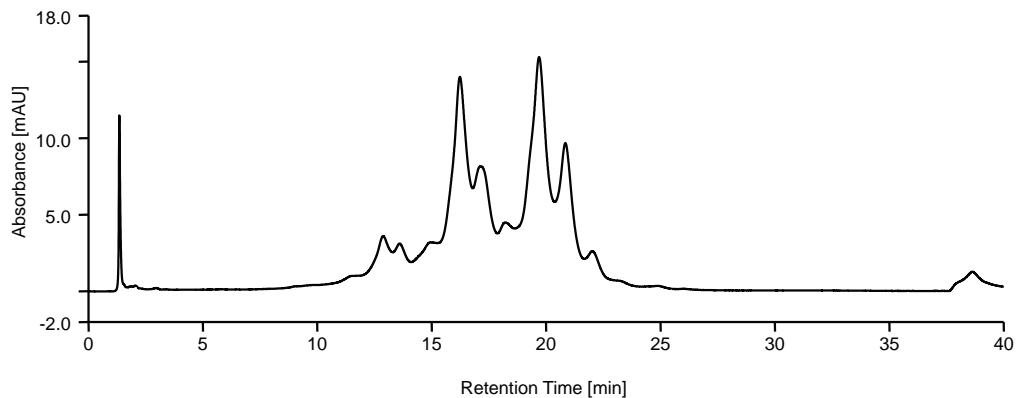


Figure 4: An example of MAb charge variant separation using a salt gradient. The separation was carried out on a MAbPac SCX-10, 10  $\mu$ m, 4  $\times$  250 mm column. Eluent A contained 20 mM MES and 60 mM NaCl (pH 5.6) and eluent B contained 20 mM MES and 300 mM NaCl (pH 5.6). Flow rate was at 0.76 mL/min. A shallow salt gradient was run from 10% B to 30% B from 2 to 32 min, followed by a 2 min wash at 30% B and 1 min wash at 100% B. The column was pre-equilibrated for 15 min at 10% B prior to the gradient. The total run time was 55 min.

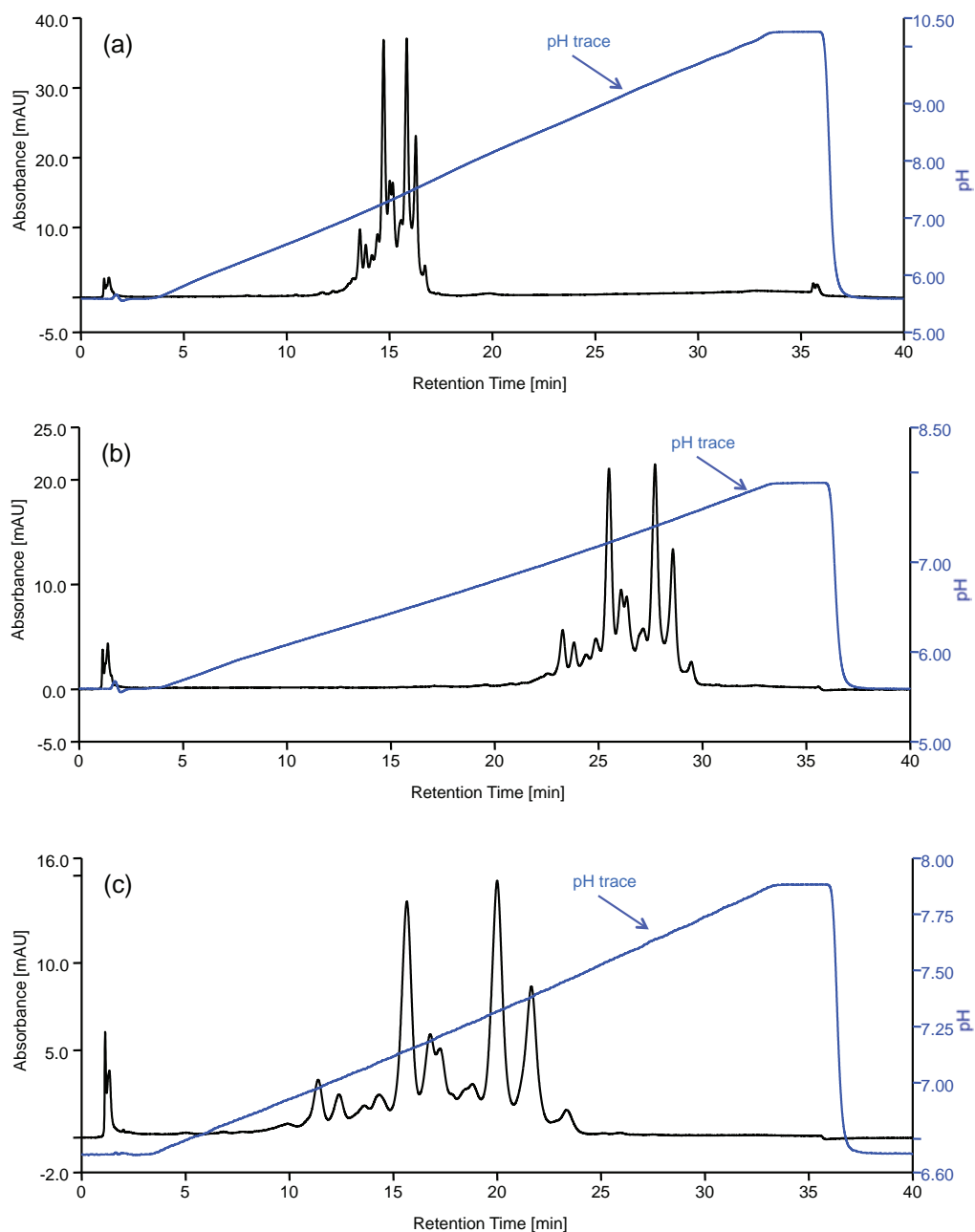


Figure 5: An example of MAb charge variant separation using a linear pH gradient. The separation was carried out on a MAbPac SCX-10, 10  $\mu$ m, 4  $\times$  250 mm column.

(a) Separation by pH gradient, 0% B (pH 5.6) to 100% B (pH 10.2), gradient method was shown in Table 1

(b) Separation by pH gradient, 0% B (pH 5.6) to 50% B (pH 7.9)

(c) Separation by pH gradient, 25% B (pH 6.75) to 50% B (pH 7.9)

Further optimization of separation can simply be achieved by running a shallower pH gradient over a narrower pH range. Figure 5b shows the separation profile from pH 5.6 to pH 7.9 with pH gradient slope at 0.076 pH unit/min. Figure 5c shows the separation profile from pH 6.75 to pH 7.9 with pH gradient slope at 0.038 pH unit/min. The pH traces in Figure 5a, 5b, and 5c demonstrate that the pH gradient maintains linearity when the slope was reduced to  $\frac{1}{2}$  or  $\frac{1}{4}$  of the initial run. The chromatographic profile and therefore the elution order of the variants remained predictable when running a shallower pH gradient. Pump methods for the chromatograms shown in Figure 5b and 5c can be automatically generated by writing a post-acquisition script using the MAb variant pH elution range information collected in the initial run (Figure 5a). This elution predictability demonstrates one of the major advantages of using a pH gradient separation platform, which is to simplify and automate the method development for MAb charge variant separation.

## Ruggedness

In addition, the ruggedness of a fast pH gradient method was assessed using a protein standard. Figure 6 shows the elution of ribonuclease A using pH gradient run on a MAbPac SCX-10, 5  $\mu$ m, 4  $\times$  50 mm column. The gradient time was 15 min with a total run time of 20 min. The retention time RSD of the ribonuclease A peak was less than 0.8% over 300 runs. This demonstrates the high level of reproducibility with which the pH gradient can be applied to charge variant separations.

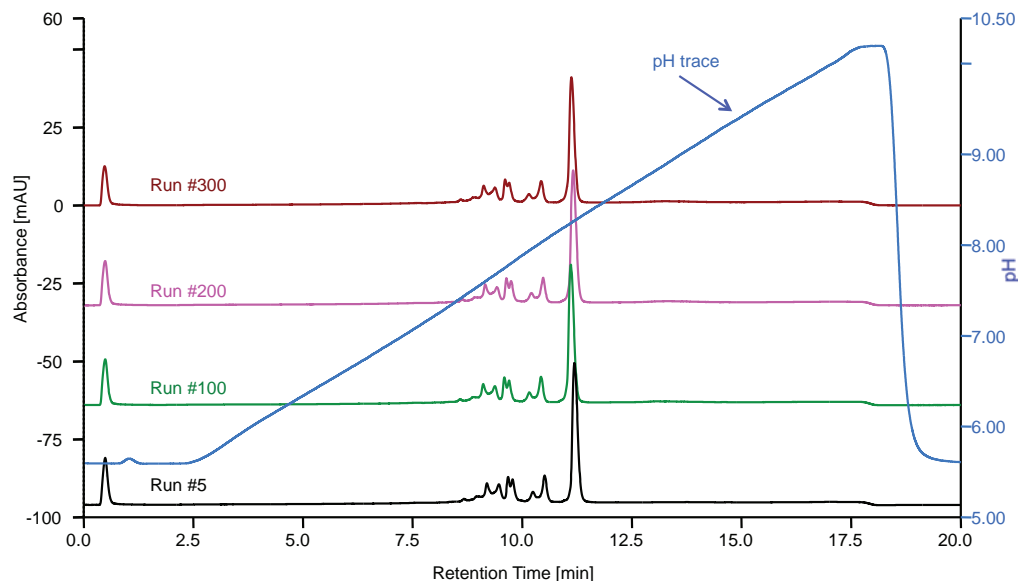


Figure 6: Ruggedness testing of pH gradient on a MAbPac SCX-10, 5  $\mu$ m, 4  $\times$  50 mm column. Gradient method was shown in table 2. The sample was ribonuclease A.

## Conclusion

- A linear pH gradient from pH 5.6 to pH 10.2 can be generated reproducibly using a multi-component zwitterionic buffer system and a MAbPac SCX-10 column.
- The pH gradient can be easily optimized, thereby simplifying charge variant separation method development.
- The pH gradient separation platform enables high resolution, fast and rugged MAb charge variant analysis.

## Reference

[1] Farnan, D. and Moreno, T., Multiproduct high-resolution monoclonal antibody charge variant separations by pH gradient ion-exchange chromatography. *Anal. Chem.* 81 (2009) 8846–57.

[thermoscientific.com/pHgradientbuffer](http://thermoscientific.com/pHgradientbuffer)

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