ThermoFisher SCIENTIFIC


HPLC columns

# CX-1 pH gradient buffers

## Product manual

thermo scientific

# Contents

# Introduction

## Introduction to the pH gradient buffers

There are two general mechanisms on which proteins are retained and eluted from ion exchange chromatography (IEC) columns. Use of either a salt gradient or a pH gradient results in a high degree of protein fractionation based on protein charge. In salt-gradient-based IEC, the pH of the buffer system is fixed. In addition to choosing the appropriate pH of the starting buffer, its ionic strength is kept low since the affinity of proteins for IEC resins decreases as ionic strength increases. The proteins are then eluted by increasing the ionic strength (salt concentration) of the buffer to increase the competition between the buffer ions and proteins for charged groups on the IEC resin. As a result, the interaction between the IEC resin and proteins is reduced, causing the proteins to elute.

In pH-gradient-based IEC, the pH of the starting buffer is maintained at a constant level to ensure the proteins obtain the opposite charge of the stationary phase and bind to it. The proteins are eluted by changing the buffer pH so the proteins transition to a net zero charge (and ultimately the same charge as the resin) and elute from the column.

Recombinant monoclonal antibodies (mAbs) can be highly heterogeneous due to modifications such as sialylation, deamidation and C-terminal lysine truncation. Salt gradient cation exchange chromatography has been used with some success in characterizing mAbs charge variants. However, additional effort is often required to tailor the salt gradient method for an individual mAbs.

Most mAbs have pI values in the range of 6-10, so theoretically, running a pH gradient from pH 5.6 to pH 10.2 on a cation exchange column can accommodate the majority of the mAb charge variant analyses without further method development. Therefore, the pH gradient method is a platform method for analyses charge variant analysis.

Combined with the most advanced IEC column technology from Thermo Fisher Scientific, the pH gradient method will provide the following benefits:

- Provide a generic, platform method that is applicable to the majority of mAb
- Deliver a highly reproducible, linear pH gradient over the pH range 5.6 to 10.2
- Enable fast analysis with high sample throughput
- Optimize resolution by focusing on narrow pH range
- Facilitate charge variant method development and method transfer
- Predict analyte isoelectric point (pI)

Buffer A and buffer B are each composed of four zwitterionic buffer salts and one electrolyte.

**Table 1. Specifications for pH gradient buffer A**

| Description | |
|---|---|
| pH | 5.6 |
| Form | Liquid |
| Concentrated | 10× |
| Shipping condition | Room temperature |
| Storage condition | 4 – 8 °C |

**Table 2. Specifications for pH gradient buffer B**

| Description | |
|---|---|
| pH | 10.2 |
| Form | Liquid |
| Concentrated | 10× |
| Shipping condition | Room temperature |
| Storage condition | 4 – 8 °C |

# Introduction (continued)

## Recommended cation exchange columns

### ProPac columns

| Description | Dimensions | Particle size | Cat. no |
|---|---|---|---|
| Thermo Scientific™ ProPac™ 3R SCX columns | 2 x 50 mm | 3 µm | 43103-052068 |
| | 2 x 100 mm | 3 µm | 43103-102068 |
| | 4 x 50 mm | 3 µm | 43103-054068 |
| | 4 x 100 mm | 3 µm | 43103-104068 |
| Thermo Scientific™ ProPac™ Elite WCX columns | 2 x 50 mm | 5 µm | 303028 |
| | 4 × 150 mm | 5 µm | 302972 |
| Thermo Scientific™ ProPac™ WCX-10 columns | 4 × 250 mm | 10 µm | 054993 |

### MAbPac SCX-10 columns

| Description | Dimensions | Particle size | Cat. no |
|---|---|---|---|
| Thermo Scientific™ MAbPac™ SCX-10 columns | 4 × 250 mm | 10 µm | 074625 |
| | 4 × 50 mm | 5 µm | 078656 |
| | 4 × 250 mm | 5 µm | 078655 |

**Note**

- If the buffers are frozen during shipping, prior to use be sure to allow the buffers to equilibrate to room temperature and mix well before dilution.
- Set the UV wavelength to 280 nm or above.
- DO NOT dilute buffer A or buffer B more than 10-fold. Over dilution will reduce the overall buffering capacity.
- To achieve optimal chromatographic separation, we recommend using Thermo Scientific ProPac 3R SCX, MAbPac SCX-10, ProPac Elite WCX and ProPac WCX-10 columns.

# Getting started

## Prepare the eluent solutions

Prepare the eluent solutions: dilute the provided 10× buffer A and B ten-fold with deionized water (weight/weight or volume/volume) using Type 1 reagent grade water with a specific resistance of 18.2 megohm-cm or greater filtered through a 0.2 µm filter. With a standard bench top pH meter, measure and record the pH of the 1× buffer A and 1× buffer B. Make sure the pH meter is calibrated before measurement.

## Set up the LC system

Connect the cation-exchange column to the LC system. The system, including all capillaries, should be thoroughly primed before use. The column can be used on a biocompatible iron free LC system that is equipped with a LC pump, a column oven, an injector (or an auto-sampler), and a UV. An online pH and conductivity meter is recommended as a post-detection monitor. Please use 0.005" I.D. tubing for obtaining optimal results. Usage of micro cell (2.5 µL) is highly recommended.

## Condition the column

Slowly ramp up the flow rate to 1 mL/min. If possible, set the flow ramps up and down to 1 mL/min. Equilibrate the column with Eluent A for at least 10 minutes at 1 mL/min or the operational flow rate.

## Verify the performance of the system, the buffer, and the column

Check the performance of the column using a protein standard such as bovine ribonuclease A, and compare the result with the one in the Figure 1. After the column is fully equilibrated, multiple injections should be made until the reproducible retention is obtained.

**Note**

Due to various reasons, such as difference of LC systems, oven temperature control, etc., you may observe slightly different retention times from those in the Figure 1.

## Real sample analysis

Once the column performance is satisfactorily confirmed in step "verify the performance of the system, the buffer, and the column", the column is ready for real sample analysis.

**Note**

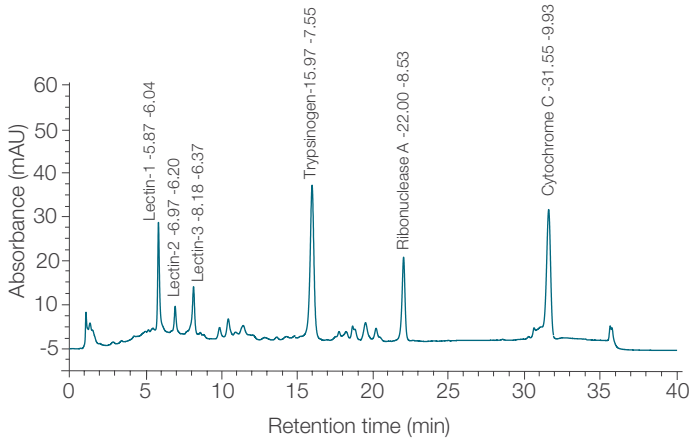
It is recommended that the column performance test be performed periodically to monitor the condition of the column.

# Applications using pH gradient

## Separation of four protein standards

Four proteins with a range of pI values from 6 to 10 are effectively separated on a MAbPac SCX-10, 10 µm, 4 × 250 mm column. These proteins are lectin (including three isoforms, lectin-1, lectin-2, and lectin-3), trypsinogen, ribonuclease A, and cytomchrome C. The chromatogram is shown in Figure 10. The pH value measured in this experiment as a function of time is plotted in Figure 2. The pH gradient is essentially linear from pH 5.6 to pH 10.2 over a 30 minute period. The correlation coefficient value $R^2$ is 0.9996.

An analysis is performed to show that there is a correlation between the elution pH for the peaks and the corresponding pI values of the protein components. Figure 3 is a graph comparing the measured pH values for six protein component peaks in Figure 10 as a function of the corresponding pI values. The measured pH values for the six protein component peaks exhibits a strong linear correlation to the literature based pI values. Thus, after a calibration procedure, this example supports the fact that a linear regression coupled with the gradient method described here can be used to estimate the pI of a protein component based on the peak retention time and measured pH.




| MAbPac SCX-10, 10 µm, 4 × 250 mm | |
|---|---|
| **Cat. no** | 074625 |
| **Mobile phase** | Buffer A: 1x, CX-1 buffer A pH 5.6<br>Buffer B: 1x, CX-1 buffer B pH 10.2 |
| **Flow rate** | 1 mL/min |
| **Inj. volume** | 10 µL |
| **Temperature** | 30 °C |
| **Detection** | 280 nm |
| **Sample** | lentil lectin, bovine trypsinogen, bovine ribonuclease A, equine heart cytochrome C |
| **Gradient** | 0-1 min, 0% B; 1-31 min, 0-100% B; 31-34 min, 100% B; 34-40 min, 0% B |

**Figure 1. Chromatogram of four proteins separated on a 30-min linear pH gradient on a MAbPac SCX-10, 10 µm, 4 × 250 mm column.** Protein name, retention time, and corresponding pH values are labeled for each protein peak.

# Applications using pH gradient (cotinued)

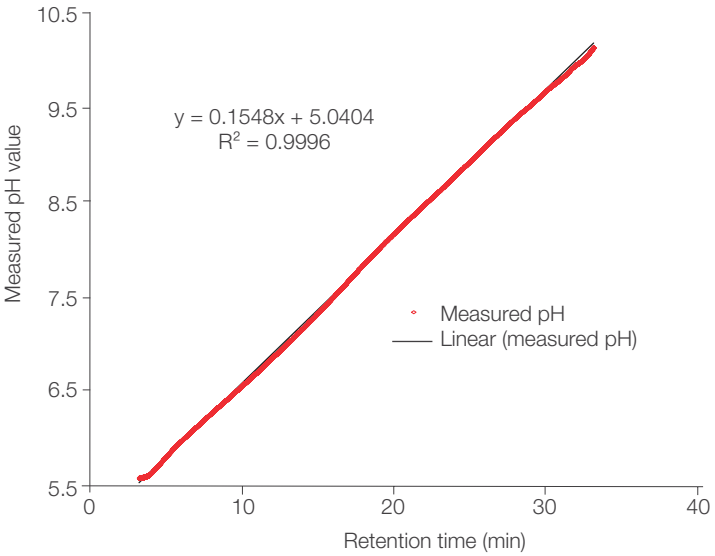



**Figure 2. A graph showing measured pH values as a function of time.** The measured pH values are exported from the same experiment shown in Figure 1.

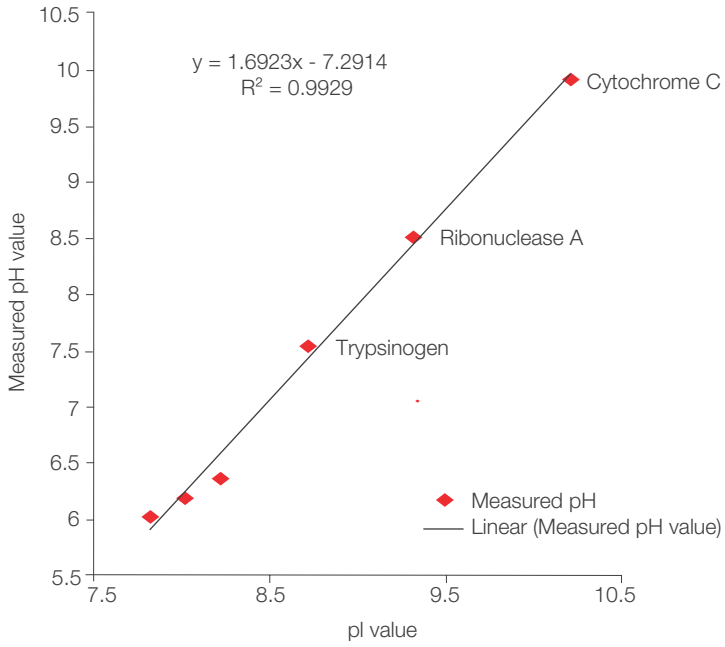



**Figure 3. A graph plotting the measured pH values for six protein component peaks as a function of the corresponding pI value.** The measured pH values of all six components were exported from the same experiment shown in Figure 1.

# Applications using pH gradient (cotinued)

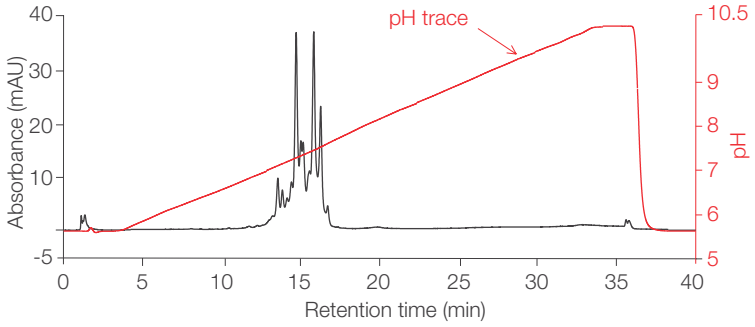
## Separation of mAb charge variants

Most mAbs have pI values in the range of 6 to 10. The pH gradient separation method can serve as a platform for charge variant separation. Using a full range of pH gradient from pH 5.6 to pH 10.2, the pH elution range of mAb is established in the initial run with a pH gradient slope of 0.153 pH unit/min (Figure 4).

Further optimization of separation can simply be achieved by running a shallower pH gradient over a narrower pH range. Figure 5 shows the separation profile from pH 5.6 to pH 7.9 with pH gradient slope at 0.076 pH unit/min. Figure 6 shows the separation profile from pH 6.75 to pH 7.9 with pH gradient slope at 0.038 pH unit/min.

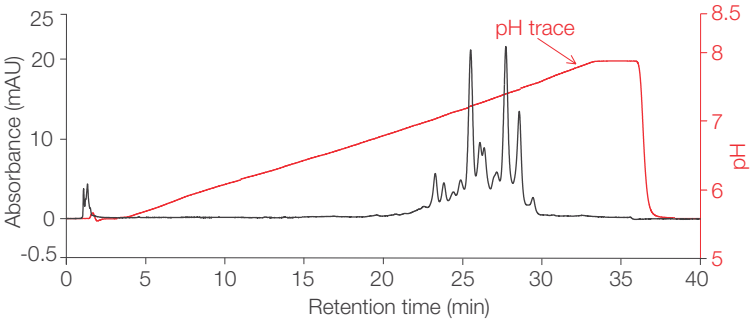
The pH traces in Figure 4, 5, and 6 demonstrates that the pH gradient maintain linear when the slope is reduced to ½ or ¼ of the initial run. And therefore, the chromatographic profile of the variants is predictable when running a shallower pH gradient.

Pump methods for chromatogram shown in Figure 5 and 6 can be automatically generated by writing a post-acquisition script using the mAb variant pH elution range information collected in the initial run (Figure 4). This example illustrates the advantages of using pH gradient separation platform, which is to simplify and automate the method development for mAb charge variant separation.




| MAbPac SCX-10, 5 µm, 4 × 250 mm | |
|---|---|
| **Cat. no** | 078655 |
| **Mobile phase** | A: 1× buffer A, pH 5.6<br>B: 1× buffer B, pH 10.2 |
| **Flow rate** | 1 mL/min |
| **Inj. volume** | 10 µL |
| **Temperature** | 30 °C |
| **Detection** | 280 nm |
| **Sample** | MAb |
| **Gradient** | 0-1 min, 0% B; 1-31 min, 0-100% B;<br>31-34 min, 100% B; 34-40 min, 0% B |

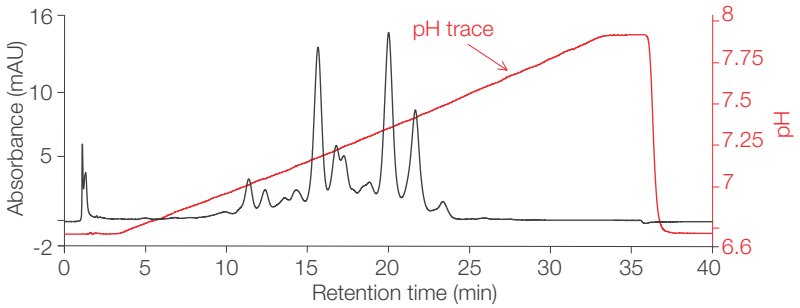
**Figure 4. MAb charge variants separation with a pH gradient slope of 0.153 pH unit/min.** The separation is carried out using a full pH gradient from pH 5.6 to pH 10.2 in 30 min. The UV profile is labeled in black and the pH trace is labeled in red.




| MAbPac SCX-10, 10 µm, 4 × 250 mm | |
|---|---|
| **Cat. no** | 078655 |
| **Mobile phase** | Buffer A: 1x, CX-1 buffer A pH 5.6<br>Buffer B: 1x, CX-1 buffer B pH 10.2 |
| **Flow rate** | 1 mL/min |
| **Inj. volume** | 10 µL |
| **Temperature** | 30 °C |
| **Detection** | 280 nm |
| **Sample** | MAb |
| **Gradient** | 0-1 min, 25% B; 1-31 min, 25-50% B;<br>31-34 min, 50% B; 34-40 min, 25% B |

**Figure 5. MAb charge variants separation with a pH gradient slope of 0.076 pH unit/min.** The separation is carried out using a pH gradient from pH 5.6 to pH 7.9 in 30 min. The UV profile is labeled in black and the pH trace is labeled in red.

# Applications using pH gradient (cotinued)

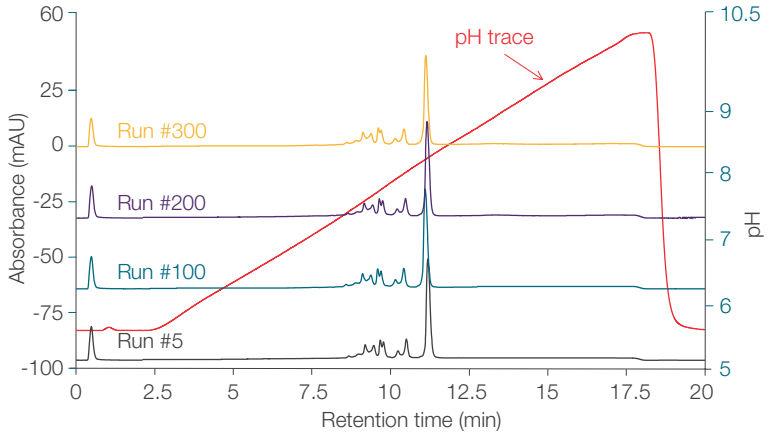



| MAbPac SCX-10, 10 µm, 4 × 250 mm | |
|---|---|
| **Cat. no** | 078655 |
| **Mobile phase** | Buffer A: 1x, CX-1 buffer A pH 5.6<br>Buffer B: 1x, CX-1 buffer B pH 10.2 |
| **Flow rate** | 1 mL/min |
| **Inj. volume** | 10 µL |
| **Temperature** | 30 °C |
| **Detection** | 280 nm |
| **Sample** | MAb |
| **Gradient** | 0-1 min, 25% B; 1-31 min, 25-50% B;<br>31-34 min, 50% B; 34-40 min, 25% B |

**Figure 6. MAb charge variants separation with a pH gradient slope of 0.038 pH unit/min.** The separation is carried out using a pH gradient from pH 6.75 to pH 7.9 in 30 min. The UV profile is labeled in black and the pH trace is labeled in red.

## Ruggedness of pH gradient

In addition, the pH gradient method is fast and rugged. Figure 7 shows a pH gradient run on a MAbPac SCX-10, 5 µm, 4 × 50 mm column. Gradient time is 15 min and the total run time is 20 min. The ribonuclease A peak retention time RSD is less than 0.8% over 300 runs.




| MAbPac SCX-10, 5 µm, 4 × 50 mm | |
|---|---|
| **Cat. no** | 078656 |
| **Mobile phase** | A: 1×, CX-1 buffer A pH 5.6<br>B: 1×, CX-1 buffer B pH 10.2 |
| **Flow rate** | 1 mL/min |
| **Inj. volume** | 10 µL |
| **Temperature** | 30 °C |
| **Detection** | 280 nm |
| **Sample** | Bovine ribonuclease A |
| **Gradient** | 0-1 min, 0% B; 1-16 min, 0-100% B;<br>16-17 min, 100% B; 17-20 min, 0% B |

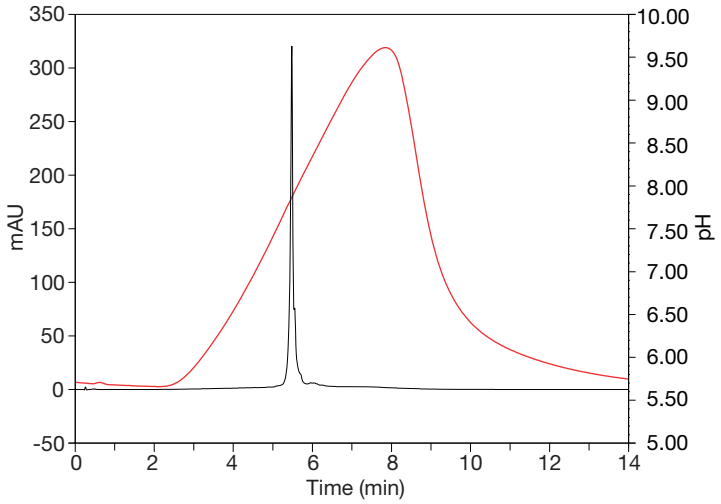
**Figgure 7. Ruggedness testing of pH gradient on a MAbPac SCX-10, 5 µm, 4 × 50 mm column.**

# Applications using pH gradient (cotinued)

## Method development for pH gradient analysis of monoclonal antibodies using a 3 µm monodisperse particle strong cation exchange chromatography column

Monoclonal antibodies (mAbs) are a preferred class of protein therapeutics used for the treatment of various diseases because of their ability to target specific tissues for drug delivery or the modulation of cellular activities. Cellular production and downstream manufacturing processes commonly introduce heterogeneity to the mAb structure by way of post-translational or chemical modifications that can have potential effects on product efficacy, safety, and stability. As such, thorough characterization of mAbs is required to fulfill regulatory requirements to bring new therapeutics to market.

### Determining gradient starting conditions

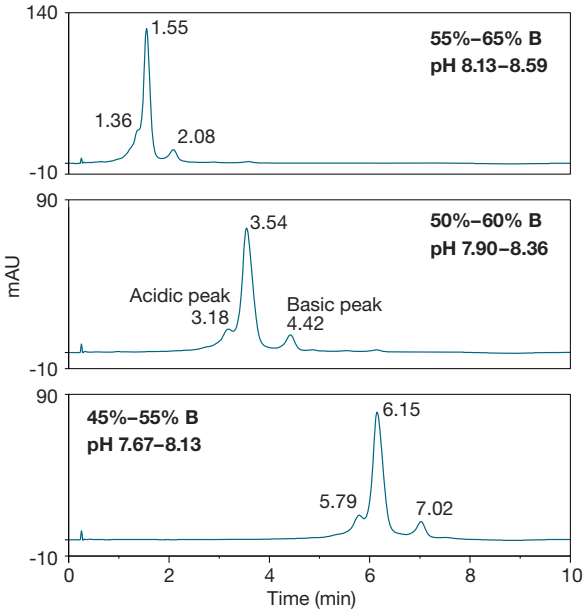



| ProPac 3R SCX, 3 µm, 2 × 50 mm | | | |
|---|---|---|---|
| **Cat. no** | 43103-052068 | | |
| **Mobile phase** | Buffer A: 1x, CX-1 buffer A pH 5.6<br>Buffer B: 1x, CX-1 buffer B pH 10.2 | | |
| **Flow rate** | 0.3 mL/min | | |
| **Inj. volume** | 1 µL | | |
| **Temperature** | 30 °C | | |
| **Detection** | 280 nm | | |
| **Sample** | NISTmAb 10 mg/mL | | |
| **Gradient** | **Time (min)** | **%A** | **%B** |
| | -0.2 | 100 | 0 |
| | 0.0 | 100 | 0 |
| | 5.0 | 0 | 100 |
| | 6.0 | 0 | 100 |
| | 6.1 | 100 | 0 |
| | 7.0 | 100 | 0 |
| | 7.1 | 100 | 0 |
| | 14.0 | 100 | 0 |

**Figure 8. pH gradient analysis of NISTmAb and associated variants on a 2 × 50 mm ProPac 3R SCX column using a gradient over the total buffer pH range, pH 5.6–10.2.** The red trace indicates mobile phase pH (right axis) as measured by the PCM-3000 pH and conductivity monitor.

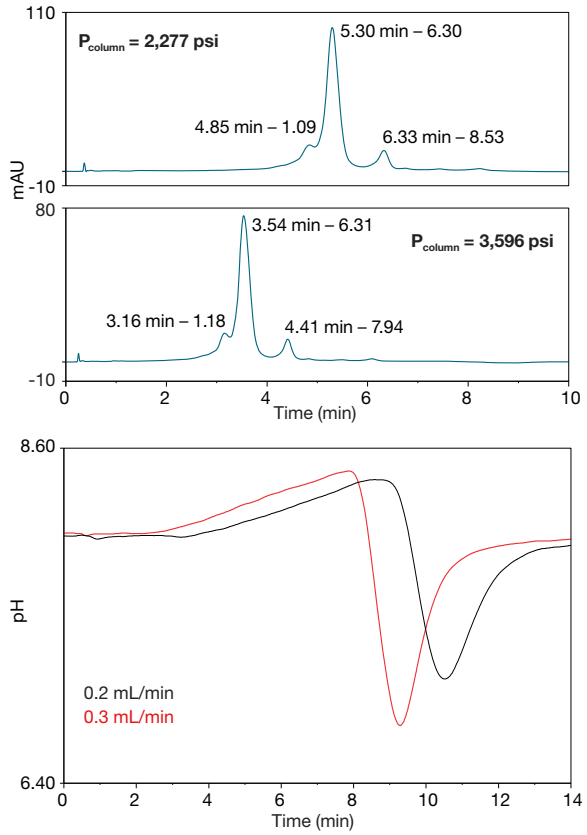
# Applications using pH gradient (cotinued)

## Flow rate effect on pH gradient separation




| ProPac 3R SCX, 3 µm, 2 × 50 mm | |
|---|---|
| Cat. no | 43103-052068 |
| Mobile phase | Buffer A: 1x, CX-1 buffer A pH 5.6<br>Buffer B: 1x, CX-1 buffer B pH 10.2 |
| Flow rate | 0.3 mL/min |
| Inj. volume | 1 µL |
| Temperature | 30 °C |
| Detection | 280 nm |
| Sample | NISTmAb 10 mg/mL |
| Gradient | See chromatogram for change in %B over 5 minutes |

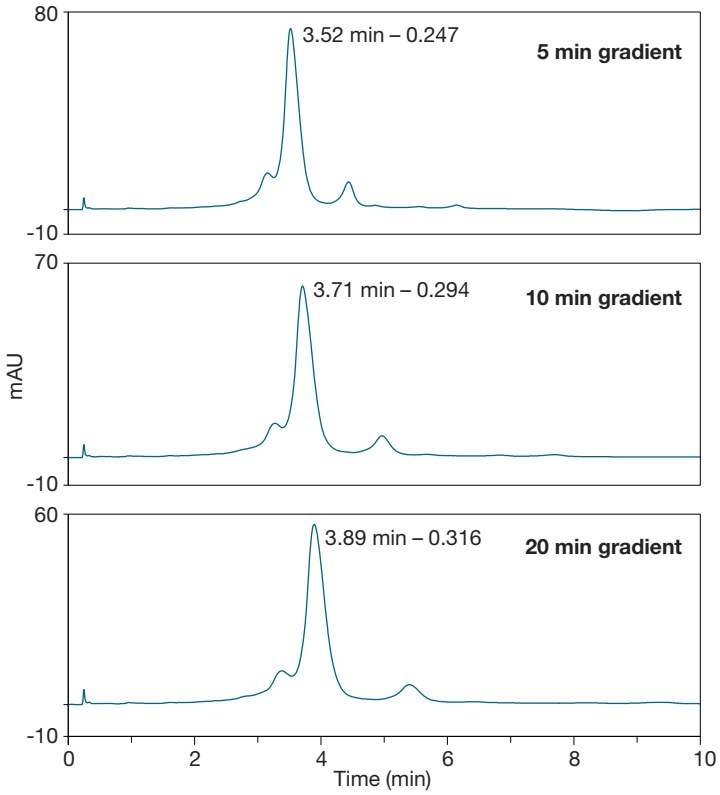
Figure 9. Effect of gradient starting conditions on NISTmAb and associated variants separation




| ProPac 3R SCX, 3 µm, 2 × 50 mm | |
|---|---|
| Cat. no | 43103-052068 |
| Mobile phase | Buffer A: 1x, CX-1 buffer A pH 5.6<br>Buffer B: 1x, CX-1 buffer B pH 10.2 |
| Flow rate | Black – 0.2 mL/min<br>Red – 0.3 mL/min |
| Inj. volume | 1 µL |
| Temperature | 30 °C |
| Detection | 280 nm |
| Sample | NISTmAb 10 mg/mL |
| Gradient | 50–60% B over 5 minutes |

Figure 10. Effect of flow rate on the separation of NISTmAb and associated charge variants (top) and associated pH traces (bottom)

# Applications using pH gradient (cotinued)




| ProPac 3R SCX, 3 µm, 2 × 50 mm | |
|---|---|
| Cat. no | 43103-052068 |
| Mobile phase | Buffer A: 1x, CX-1 buffer A pH 5.6<br>Buffer B: 1x, CX-1 buffer B pH 10.2 |
| Flow rate | 0.3 mL/min |
| Inj. volume | 1 µL |
| Temperature | 30 °C |
| Detection | 280 nm |
| Sample | NISTmAb 10 mg/mL |
| Gradient | 50–60% B over 5 minutes |

Figure 11. Effect of gradient time on the separation of NISTmAb and associated variants

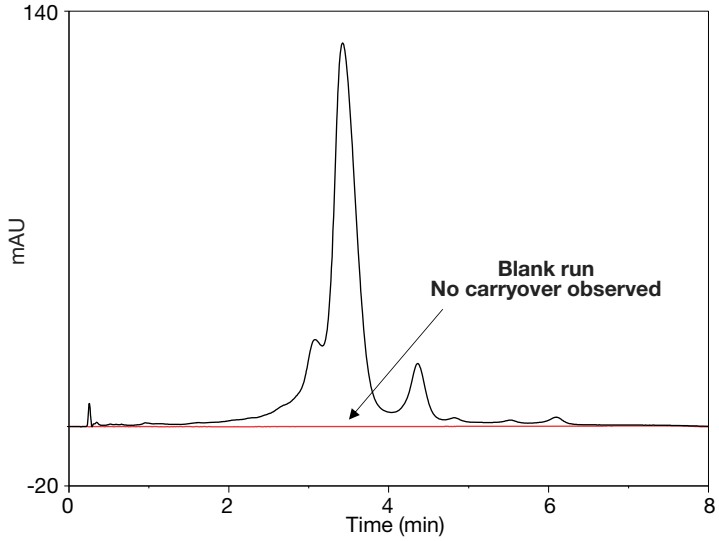



Figure 12. Overlaid chromatograms showing a 20 µg injection and elution of NISTmAb and the following blank run to measure carryover using the 0.3 mL/min pH gradient method

# Applications using pH gradient (cotinued)

## Sample loading and carryover analysis

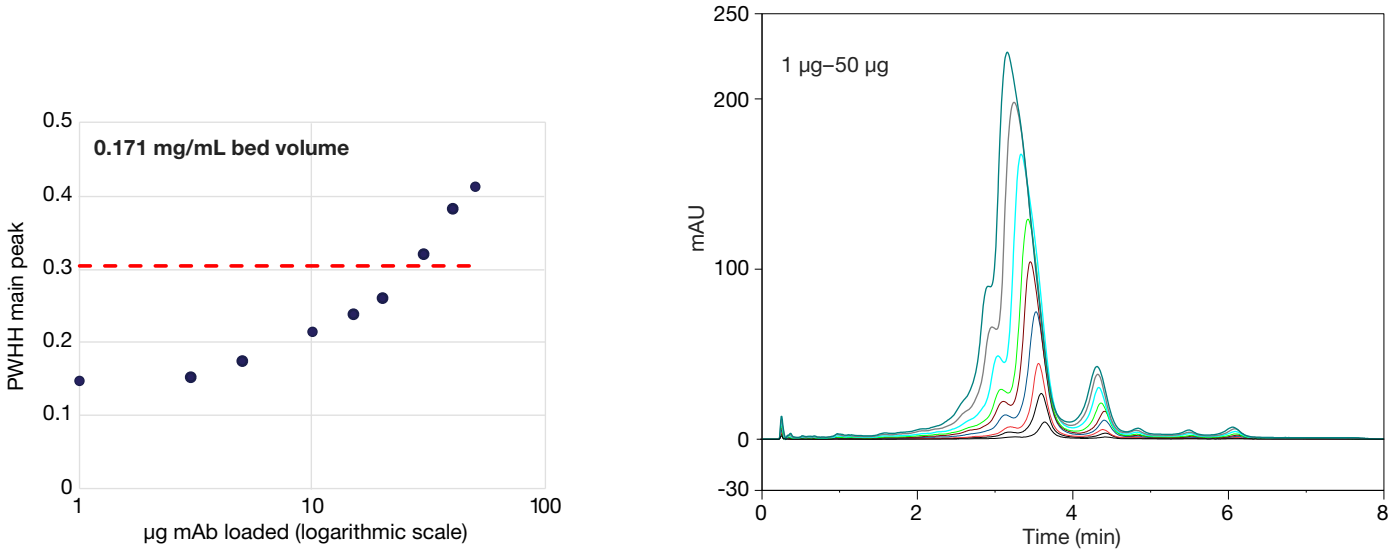
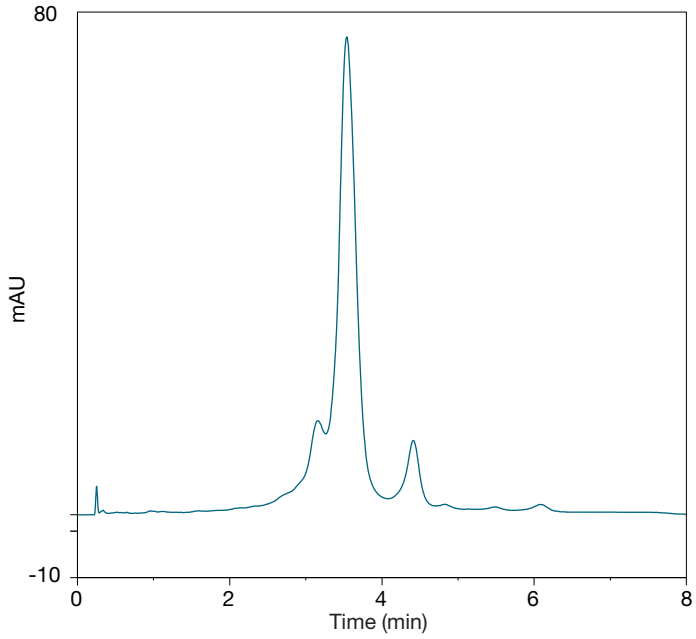



**Figure 13. Chromatogram overlays showing the dynamic loading analysis of NISTmAb using the 0.3 mL/min pH gradient method.** The plot shows the corresponding PWHH of the main mAb peak against the masses of mAb loaded in the column.

## Final pH gradient method




| ProPac 3R SCX, 3 µm, 2 × 50 mm | | | |
|---|---|---|---|
| **Cat. no** | 43103-052068 | | |
| **Mobile phase** | Buffer A: 1x, CX-1 buffer A pH 5.6<br>Buffer B: 1x, CX-1 buffer B pH 10.2 | | |
| **Flow rate** | 0.3 mL/min | | |
| **Inj. volume** | 1 µL | | |
| **Temperature** | 30 °C | | |
| **Detection** | 280 nm | | |
| **Sample** | NISTmAb 10 mg/mL | | |
| **Gradient** | **Time (min)** | **%A** | **%B** |
| | -0.2 | 50 | 50 |
| | 0.0 | 50 | 50 |
| | 5.0 | 40 | 60 |
| | 6.0 | 40 | 60 |
| | 6.1 | 100 | 0 |
| | 7.0 | 100 | 0 |
| | 7.1 | 50 | 50 |
| | 14.0 | 50 | 50 |

**Figure 14. Zoomed in chromatogram of NISTmAb analysis using 5-minute gradient at 0.3 mL/min flow rate**

# Applications using pH gradient (cotinued)

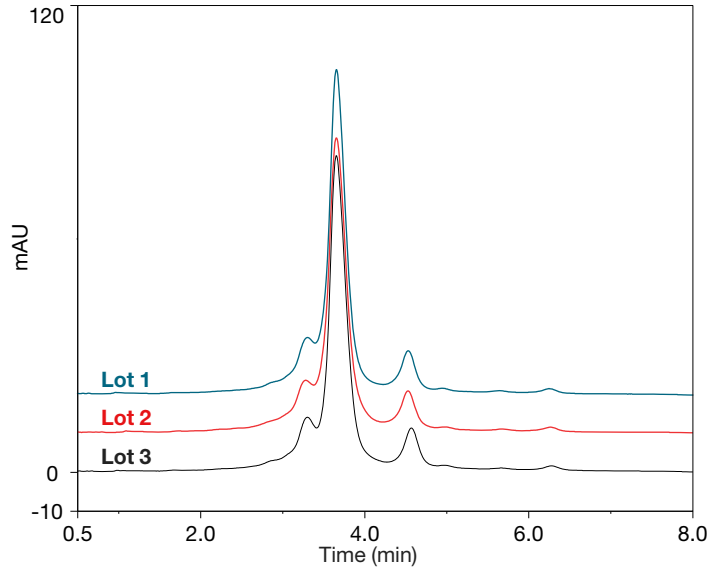
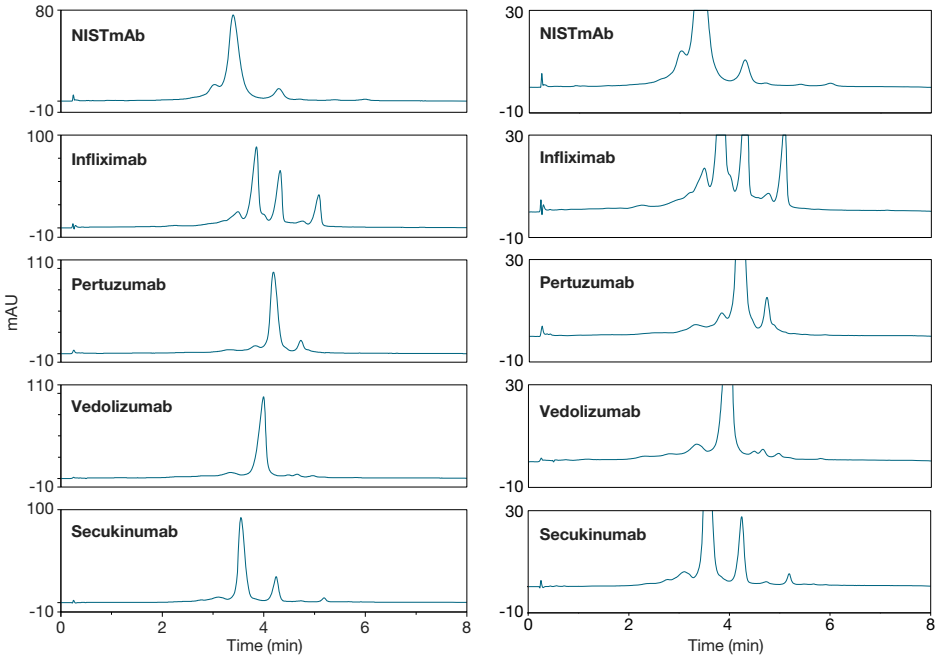



**Figure 15. Zoomed-in view of chromatograms of three different lots using optimized method with a 5-minute gradient at 0.3 mL/min flow rate.** Retention time of main mAb peak is normalized to aid comparison of variant separation.




| ProPac 3R SCX, 3 µm, 2 × 50 mm | | |
|---|---|---|
| Cat. no | 43103-052068 | |
| Mobile phase | Buffer A: 1x, CX-1 buffer A pH 5.6<br>Buffer B: 1x, CX-1 buffer B pH 10.2 | |
| Flow rate | 0.3 mL/min | |
| Temperature | 30 °C | |
| Gradient | Time (min) | %B |
| | -0.2 | Initial |
| | 0.0 | Initial |
| | 5.0 | Final |
| | 6.0 | Final |
| | 6.1 | 0 |
| | 7.0 | 0 |
| | 7.1 | Initial |
| | 14.0 | Initial |

**Figure 16. Analysis of mAbs using pH gradient detailed in Table 3.** Left chromatograms show the full signal strength, and the right chromatograms show the zoomed-in detailed view of the mAb variants.

**Table 3. Gradient parameters for analysis of mAbs using pH gradient**

| mAb | Initial %B | Final %B | Concentration | Inj. vol. (µL) |
|---|---|---|---|---|
| NISTmAb | 50 | 60 | 10 mg/mL | 1.0 |
| Infliximab | 24 | 44 | 5 mg/mL | 3.0 |
| Pertuzumab | 36 | 51 | 5 mg/mL | 1.5 |
| Vedolizumab | 23 | 38 | 5 mg/mL | 1.5 |
| Secukinumab | 27 | 47 | 5 mg/mL | 1.5 |

# Frequently asked questions

**What molecules are suitable to be analyzed on a cation exchange column using CX-1 pH gradient buffer kit?**

Proteins, such as monoclonal antibodies (mAbs), with pI value in the range of 6-10.

**What components are included in Buffer A and B?**

Buffer A and buffer B each contains four zwitterionic buffer salts and one electrolyte. Buffer A and buffer B each comes in a pre-made concentrated solution (10×) in a 125 mL, 250 mL, 500 mL or 1000 mL bottle.

**Would Thermo Fisher Scientific verify the correct pH value before shipping these buffers in a kit to control for any accidental $CO_2$ intake?**

Buffer A solution is titrated to pH 5.6 and buffer B solution is titrated to pH 10.2 (error range ± 0.1). The ten-fold dilution hardly changes the pH values. Buffer bottles are purged with inert gas and the package is vacuum sealed. A stability test has been conducted comparing the pH of the freshly manufactured buffers and two and a half year old buffers. There were less than 1% change in the pH for both buffer A and buffer B.

**Besides MAbPac SCX-10 and ProPac 3R SCX, do ProPac Elite WCX and ProPac WCX-10 columns work well with the pH buffer kit?**

Yes. However, because the column stationary phase is a weak-cation exchanger, there is a slight pH gradient delay compared to MAbPac SCX-10 or ProPac 3R SCX.

**What UV wavelength should be used while running pH gradient?**

It is recommended to use a UV wavelength of 280 nm or above. At lower wavelengths, there will be higher background interference.

# Ordering information

### Thermo Scientific CX-1 pH gradient buffers

| Description | Size | pH | Cat. no. |
|---|---|---|---|
| pH gradient buffer A | 125 mL | pH 5.6 | 083273 |
| | 250 mL | | 085346 |
| | 500 mL | | 302779 |
| | 1000 mL | | 303274 |
| pH gradient buffer B | 125 mL | pH 10.2 | 083275 |
| | 250 mL | | 085348 |
| | 500 mL | | 302780 |
| | 1000 mL | | 303275 |

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