



**ThermoFisher**  
S C I E N T I F I C

# New chromatographic workflows for charge variant profiling, intact mAb analysis and DAR determination

*Ken Cook, Robert Van Ling*

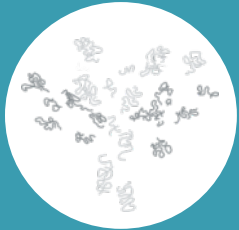
# Workflows to Simplify Biopharmaceutical Analysis

- Thermo Fisher Scientific provides innovative solutions
- Solutions that consist of innovative products
- Such as our biopharmaceutical protein characterisation workflows



WORKFLOW

# 5 Fundamental Workflows



## Peptide mapping

Confirm sequence

Analytical reproducibility  
Peak capacity



## Aggregate analysis

Check monomer vs  
Aggregates

High salt condition,  
biocompatible, low  
dispersion,  
reproducibility



## Charge variant analysis

Check charge  
variation within  
antibody sample

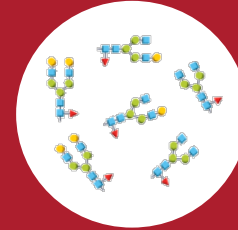
Biocompatible  
selectivity



## Intact protein analysis

Check purity of the  
antibody

Biocompatible,  
gradient and  
thermostating  
capabilities

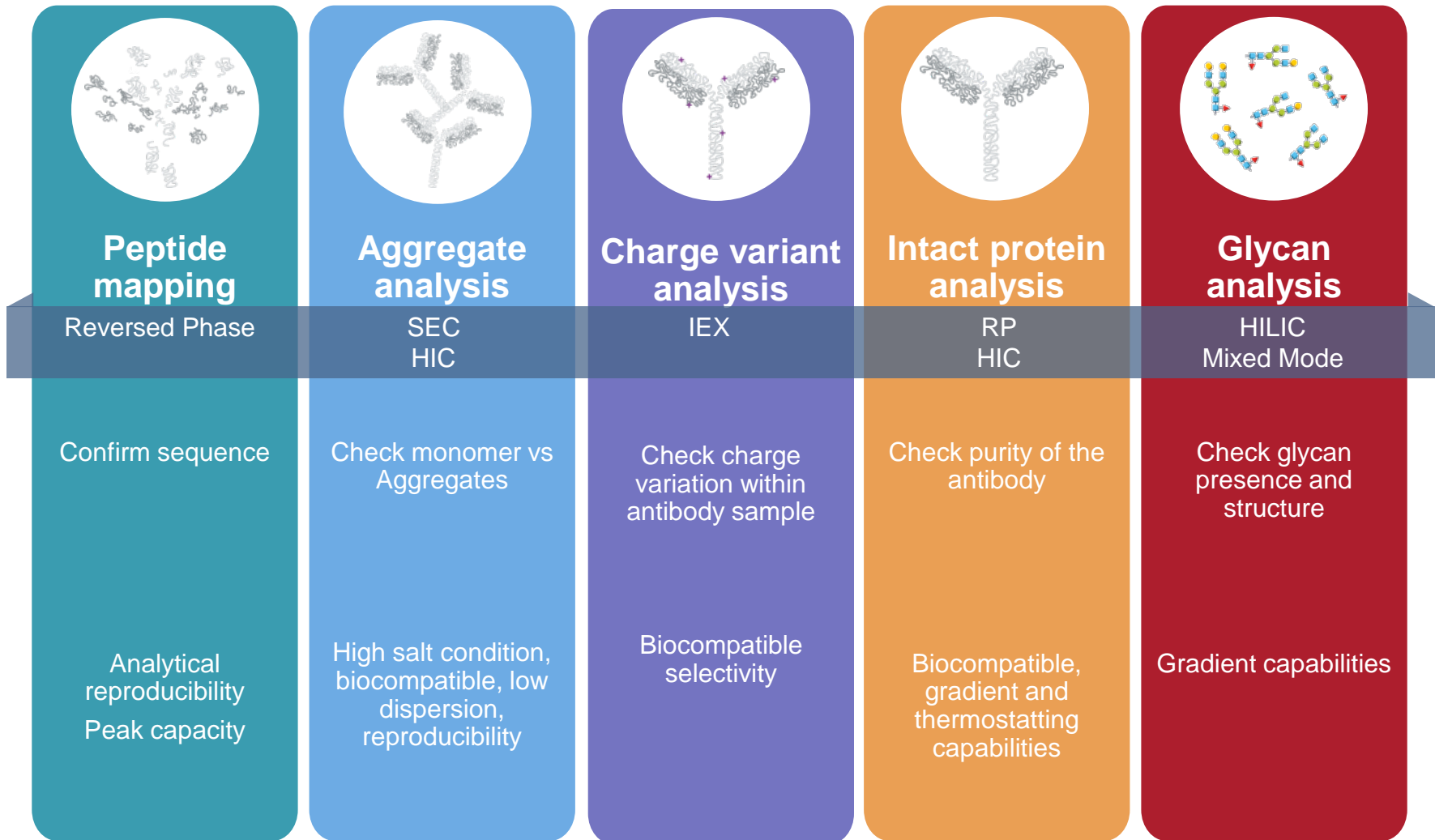


## Glycan analysis

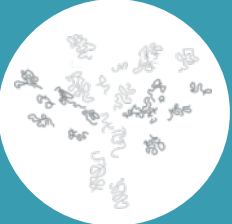
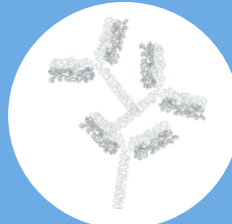
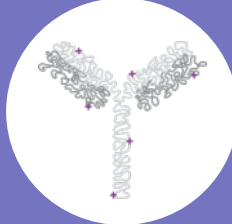
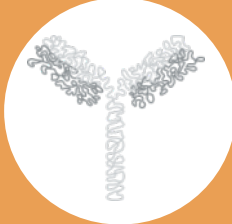
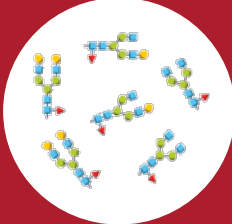
Check glycan  
presence and  
structure

Gradient capabilities

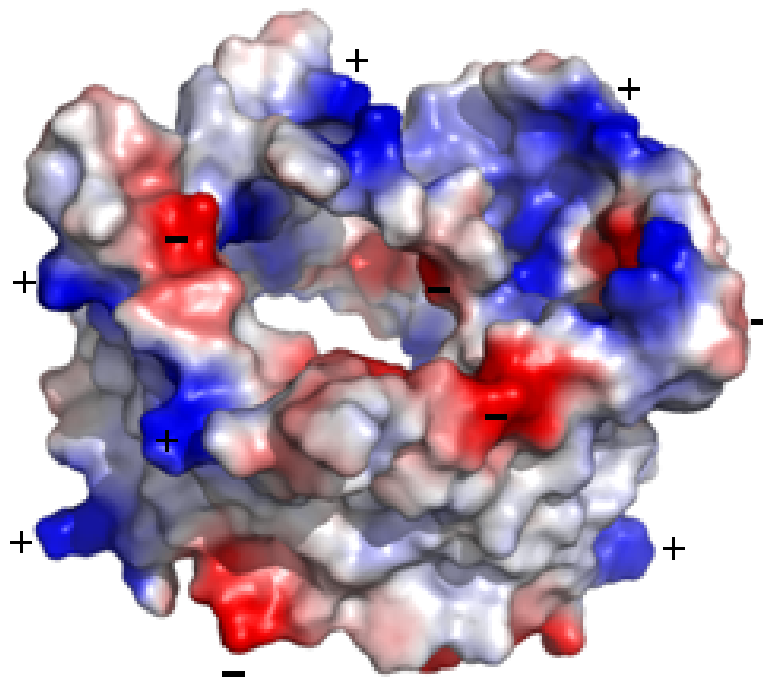
# 5 Fundamental Workflows



# 5 Fundamental Workflows

				
<b>Peptide mapping</b>	<b>Aggregate analysis</b>	<b>Charge variant analysis</b>	<b>Intact protein analysis</b>	<b>Glycan analysis</b>
Reversed Phase	SEC HIC	IEX	RP HIC	HILIC Mixed Mode
Confirm sequence	Check monomer vs Aggregates	Check charge variation within antibody sample	Check purity of the antibody	Check glycan presence and structure
UV and UV-MS	UV (UV-MS)	UV	UV and UV-MS	FLD, FLD-MS, CAD
Analytical reproducibility Peak capacity	High salt condition, biocompatible, low dispersion, reproducibility	Biocompatible selectivity	Biocompatible, gradient and thermostating capabilities	Gradient capabilities

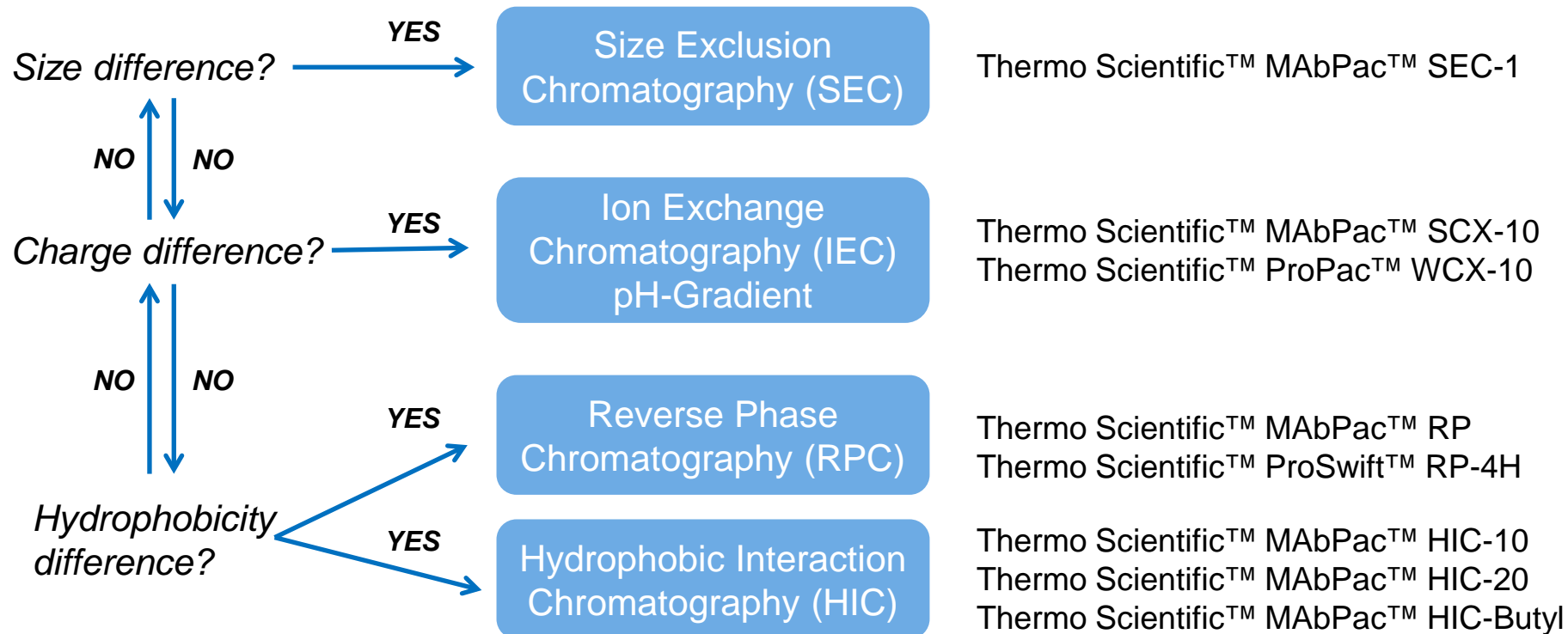
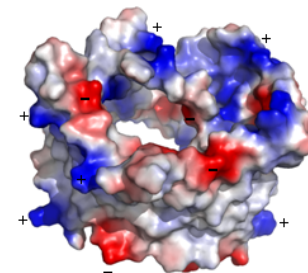
- Protein properties:
  - Size
  - Charge
  - Hydrophobicity
  - Affinity or Recognition



# Protein and MAb Separation by LC

- Protein properties:

- Size
- Charge
- Hydrophobicity
- Affinity or Recognition

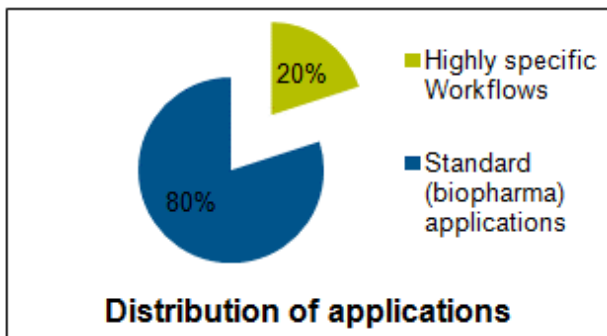


# LC Systems for Bio-Therapeutic Protein Analysis

Thermo Scientific™ Vanquish™ UHPLC &  
Thermo Scientific™ Vanquish™ Flex UHPLC systems



Thermo Scientific™ UltiMate™ 3000 BioRS system



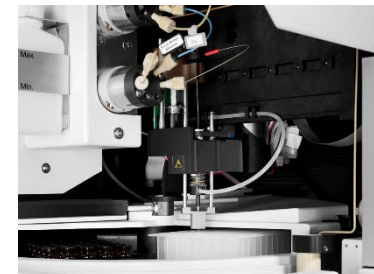


# LC Systems for Bio-Therapeutic Protein Analysis

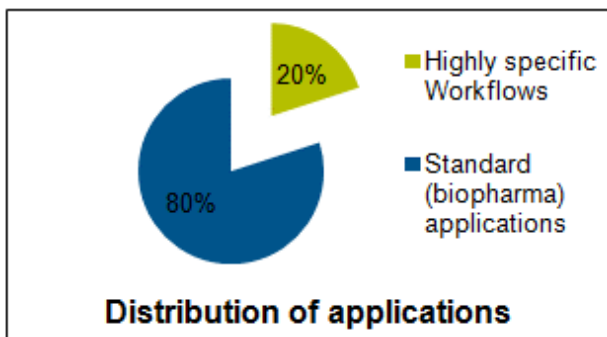
Thermo Scientific™ Vanquish™ UHPLC &  
Thermo Scientific™ Vanquish™ Flex UHPLC systems



Thermo Scientific™ UltiMate™ 3000 BioRS system



High Resolution,  
Cooled Fractionation



pH and Conductivity

# Charged Variant Analysis Workflow



Protein Variants



pH gradient ion exchange UHPLC

## 1. Buffers

- Thermo Scientific™ CX-1 pH Gradient buffers, 10X concentrated

## 2. Chemistries

- MAbPac SCX-10 columns

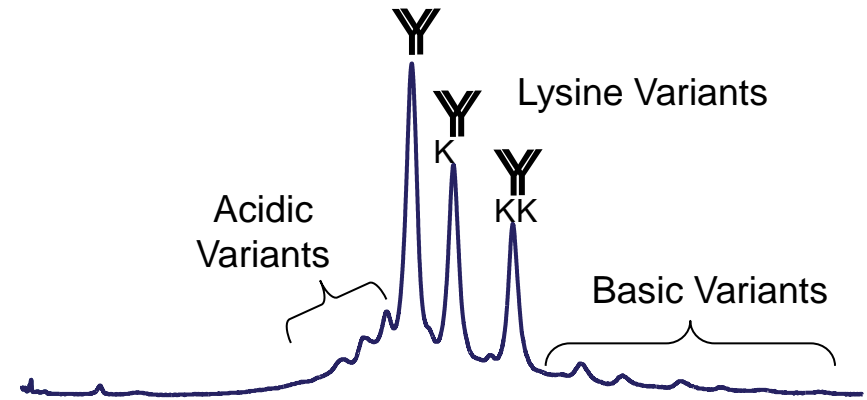
## 3. Separations & Detection

- Vanquish Flex UHPLC or UltiMate 3000 BioRS UHPLC system
- Automated desalting on polymeric Thermo Scientific™ MSPac™ DS-10 de-salter cartridge

## 4. Characterization

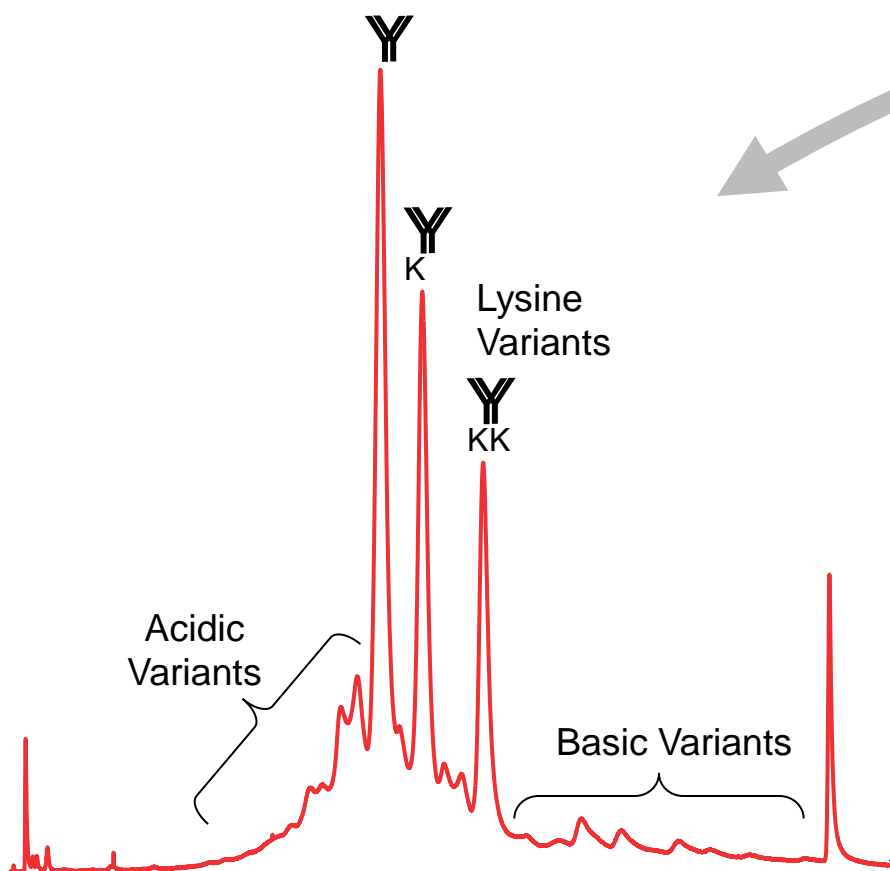
- Thermo Scientific™ Exactive™ Plus or Thermo Scientific™ Q Exactive™ Plus Mass Spectrometer
- Thermo Scientific™ BioPharma Finder™ Software
- Full scan method, intact mass deconvolution

# Next-generation CEX Column – MAbPac SCX-10



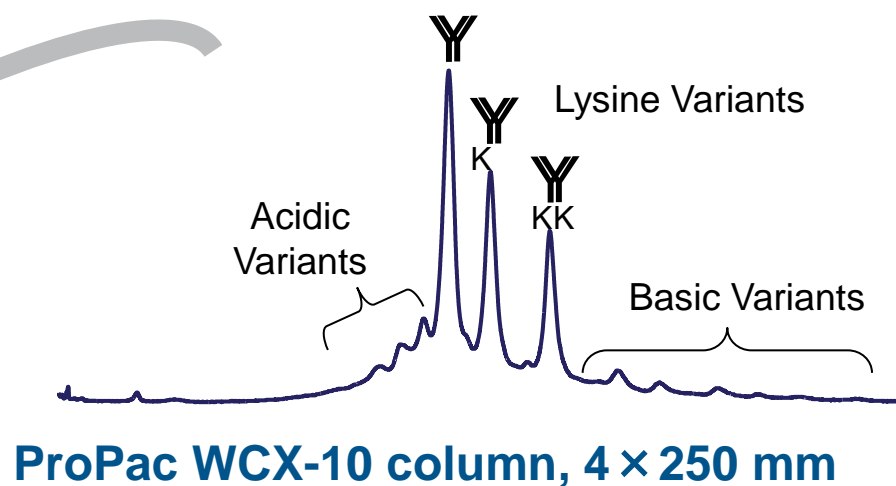
**ProPac WCX-10 column, 4 × 250 mm**

# Next-generation CEX Column – MAbPac SCX-10



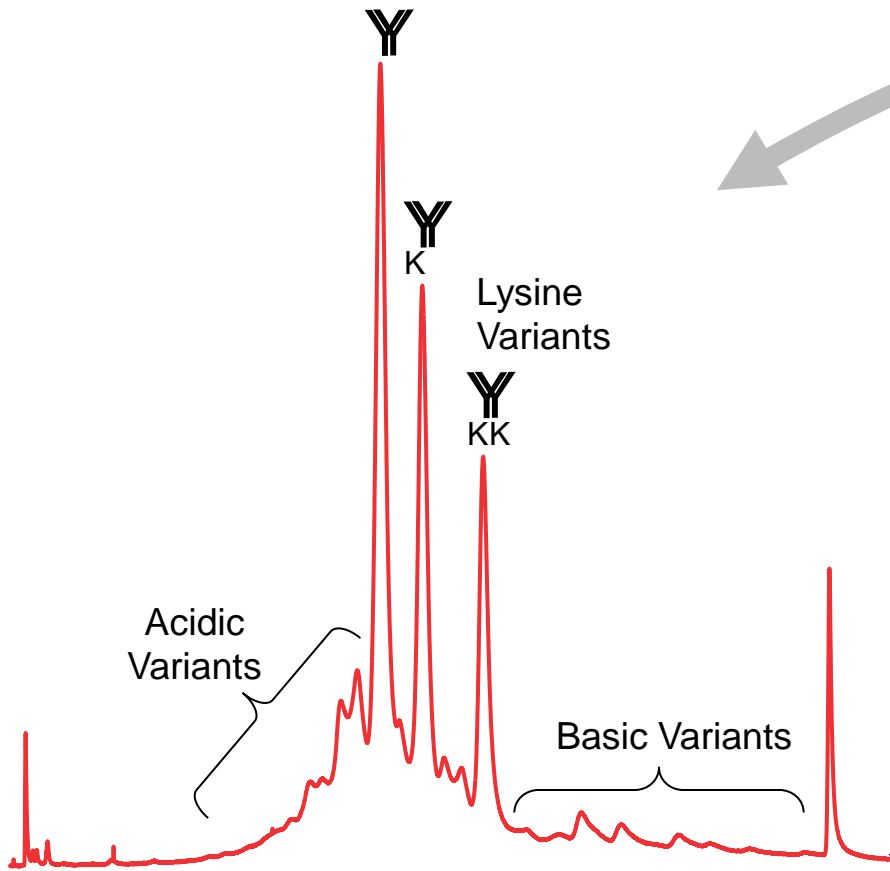
**MAbPac SCX-10 column, 4 × 250 mm**

60 min. total analysis time



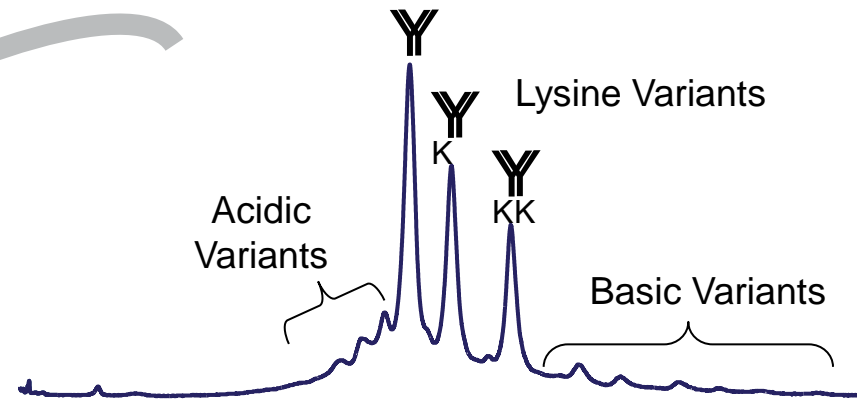
**ProPac WCX-10 column, 4 × 250 mm**

# Next-generation CEX Column – MAbPac SCX-10

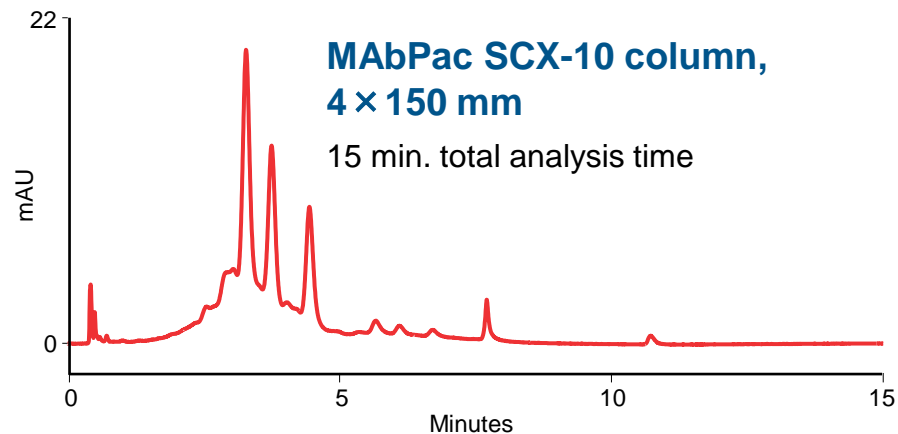


**MAbPac SCX-10 column, 4 × 250 mm**

60 min. total analysis time



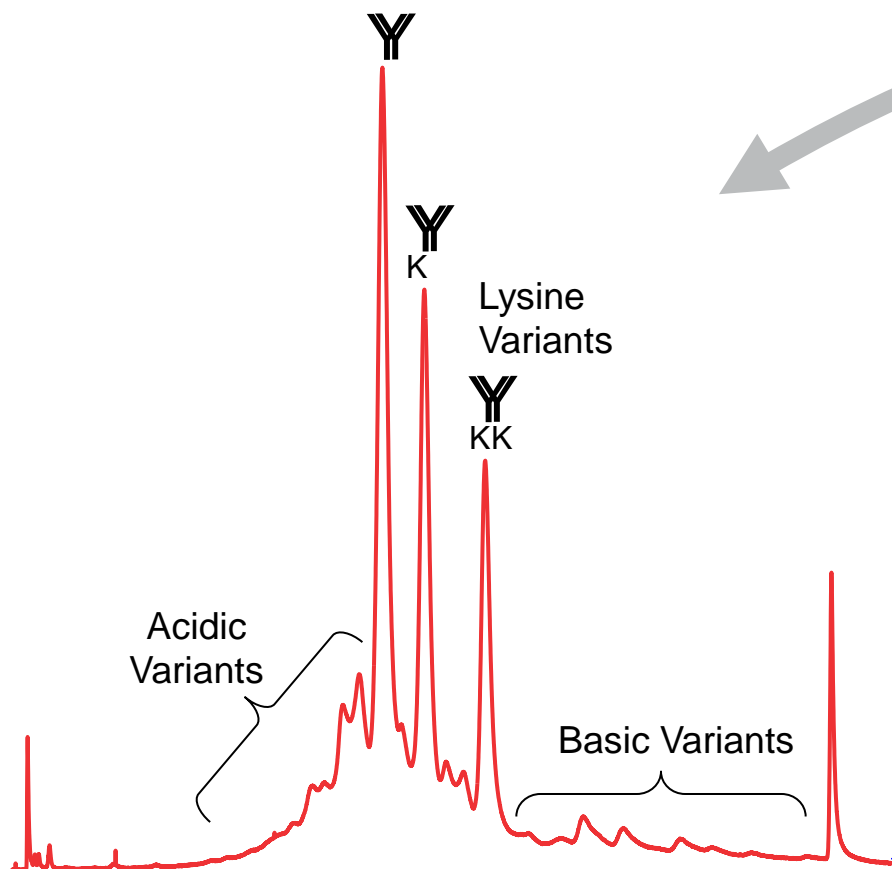
**ProPac WCX-10 column, 4 × 250 mm**



**MAbPac SCX-10 column,  
4 × 150 mm**

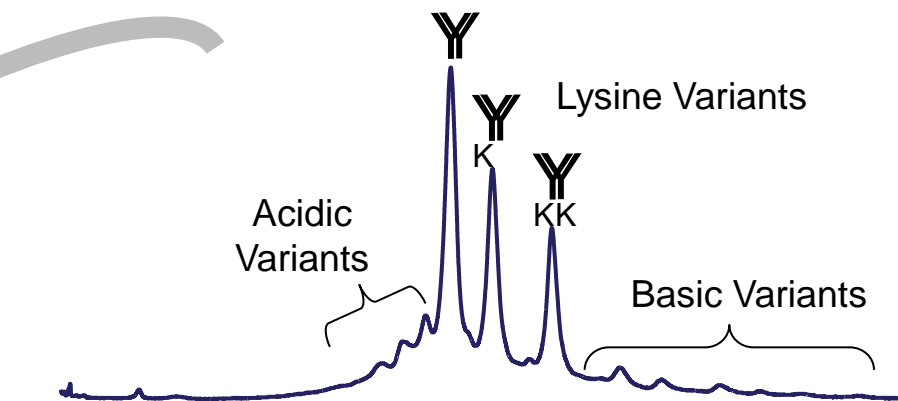
15 min. total analysis time

# Next-generation CEX Column – MAbPac SCX-10

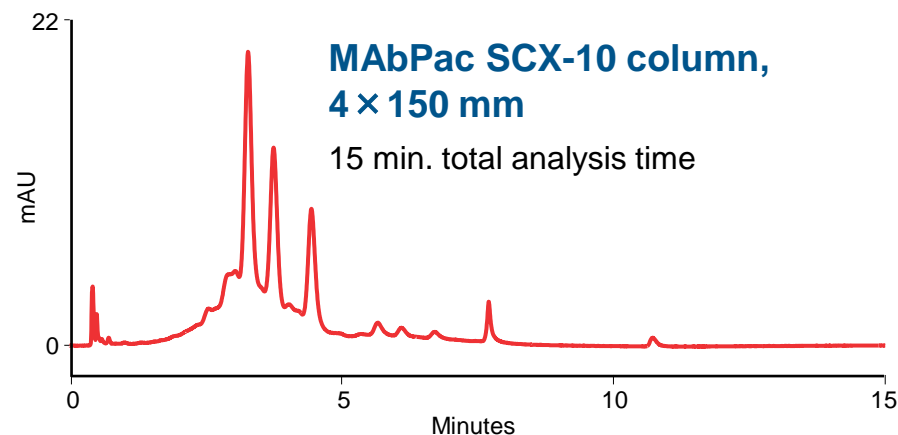


**MAbPac SCX-10 column, 4 x 250 mm**

60 min. total analysis time



**ProPac WCX-10 column, 4 x 250 mm**



**MAbPac SCX-10 column,  
4 x 150 mm**

15 min. total analysis time

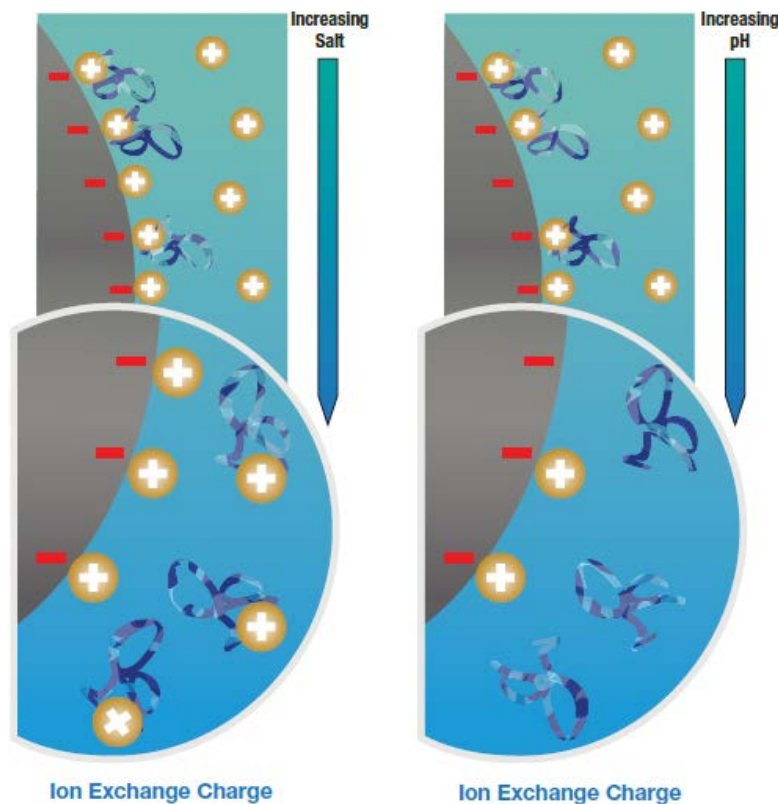
*Improve resolution or sample throughput through column chemistry*

# Charge Variant Analysis by CEX

## Ion Exchange Elution - Cation Chromatography

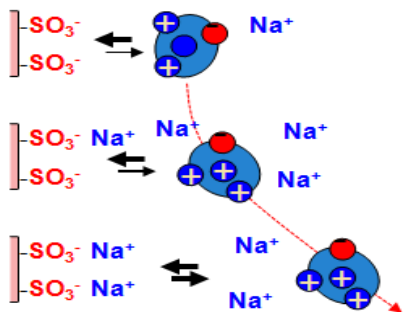
Salt Gradient

pH Gradient



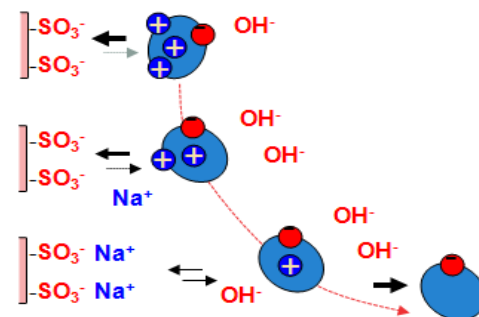
### Salt gradient elution

- Based on ionic strength
- Competition / displacement for interaction with functional groups of IEX matrix
- Multiple interactions with IEX matrix

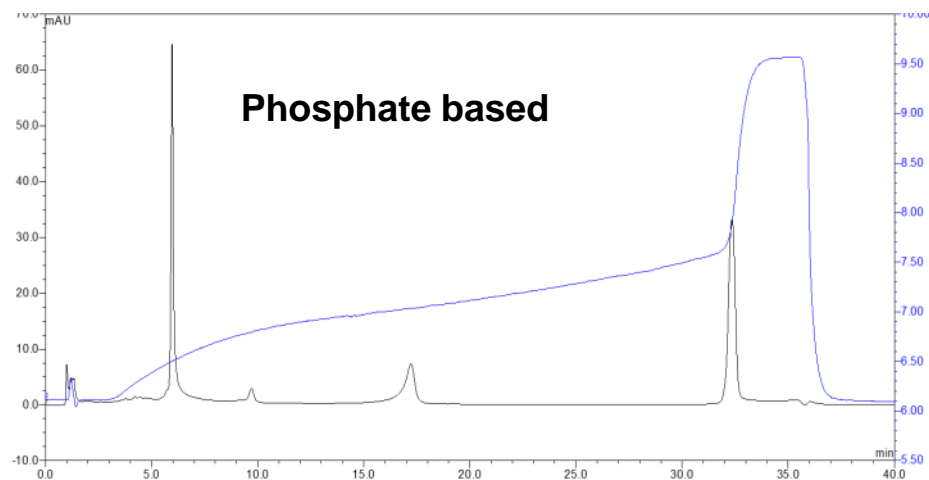
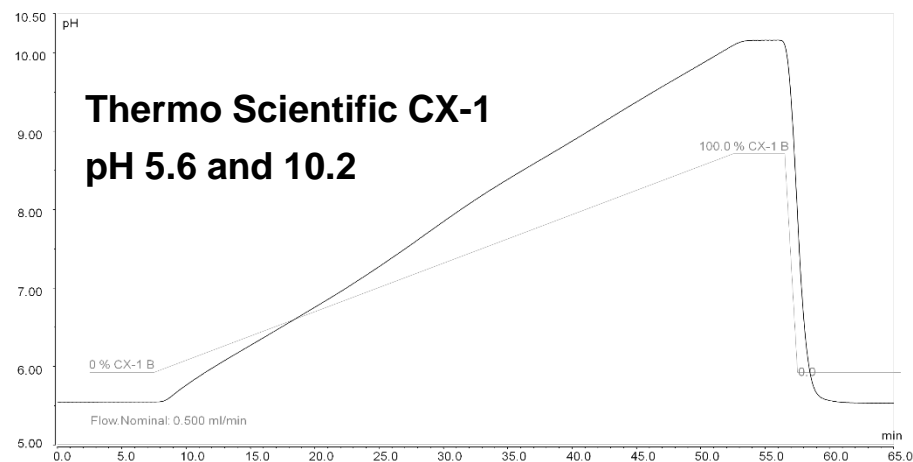
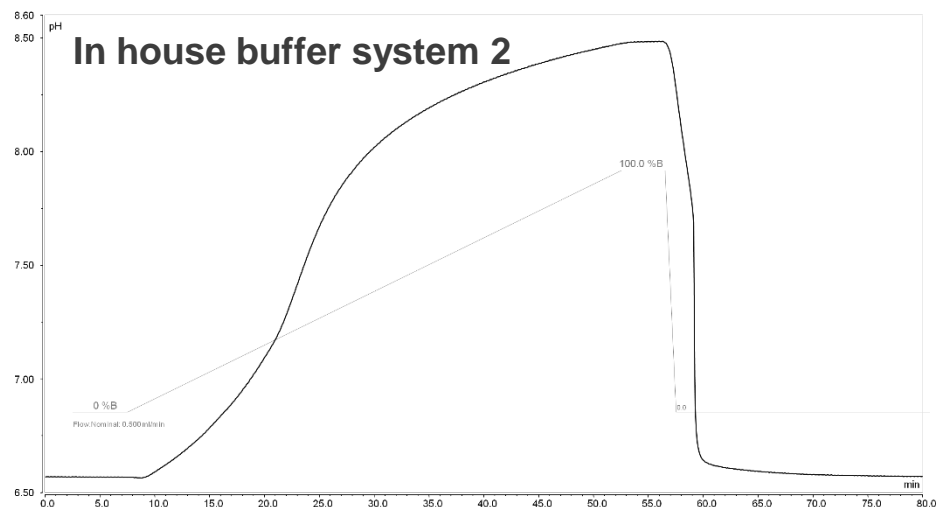
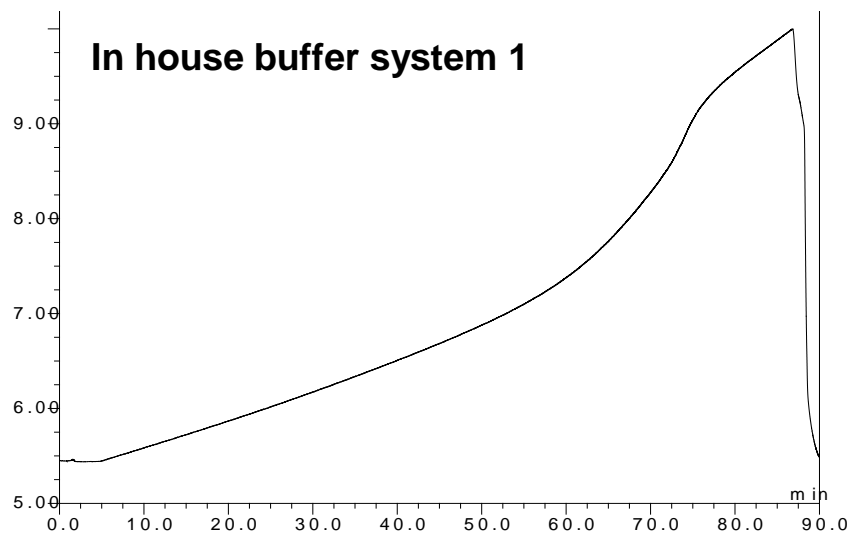


### pH gradient elution

- Based on pI of protein
- Loss of retention with progressive pH gradient, depending on pI
- “Single” binding event, trapping at pH < pI (for CEX)



# Comparison of pH gradient buffer systems





# Thermo Scientific CX-1 pH Gradient Buffers



- Dilute buffers 10-fold with DI water
- A linear pH gradient (pH 5.6 - 10.2) is generated by running a linear pump gradient from 100% Buffer A to 100% Buffer B
- Generic, fast & high-resolution!

	Buffer A	Buffer B
pH	5.6	10.2
Form	Liquid	Liquid
Concentrate	10X	10X
Shipping condition	Room Temp	Room Temp
Storage condition	4 ~ 8 °C	4 ~ 8 °C

# Thermo Scientific CX-1 pH Gradient Buffers

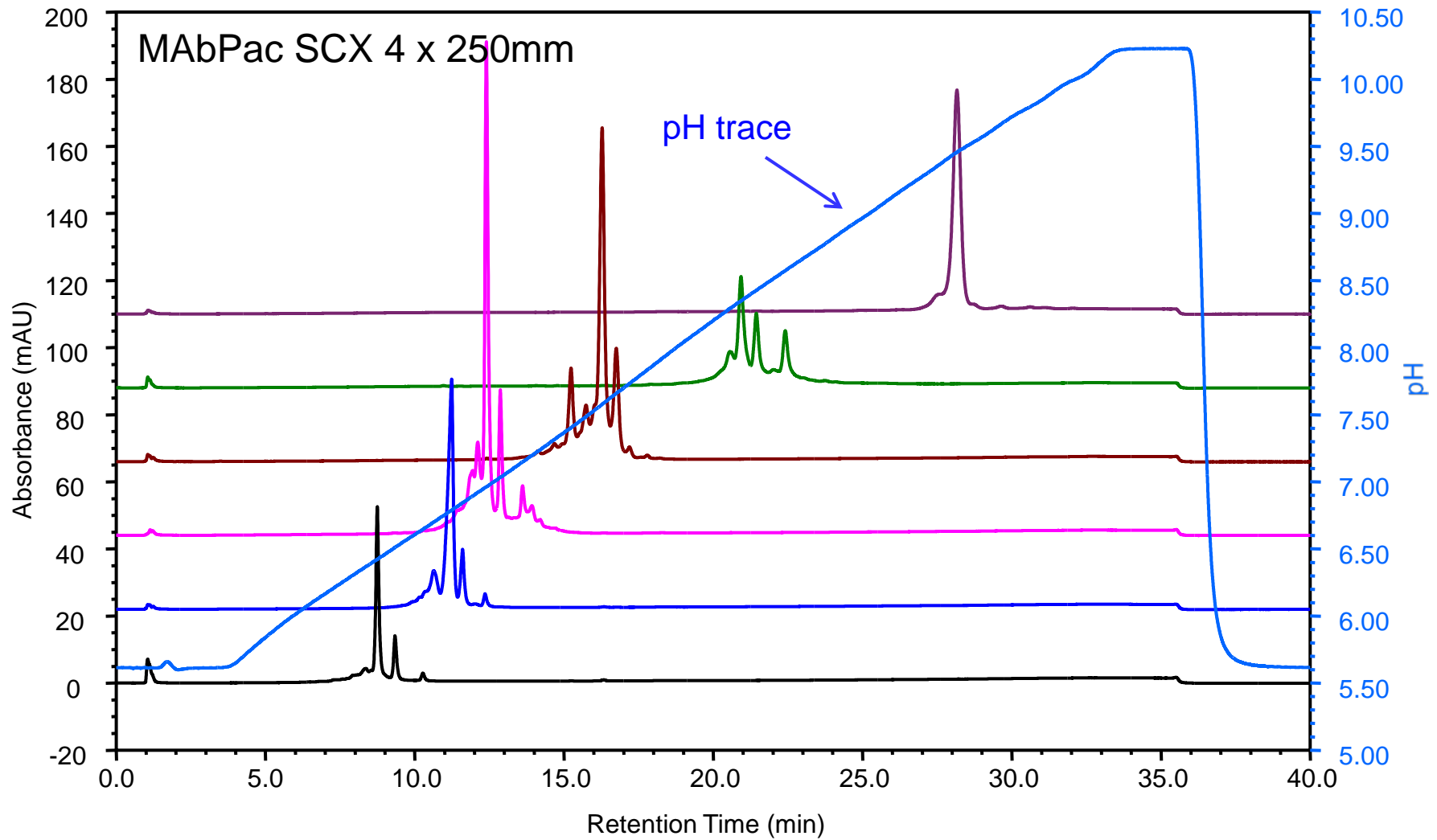


- Dilute buffers 10-fold with DI water
- A linear pH gradient (pH 5.6 - 10.2) is generated by running a linear pump gradient from 100% Buffer A to 100% Buffer B
- Generic, fast & high-resolution!

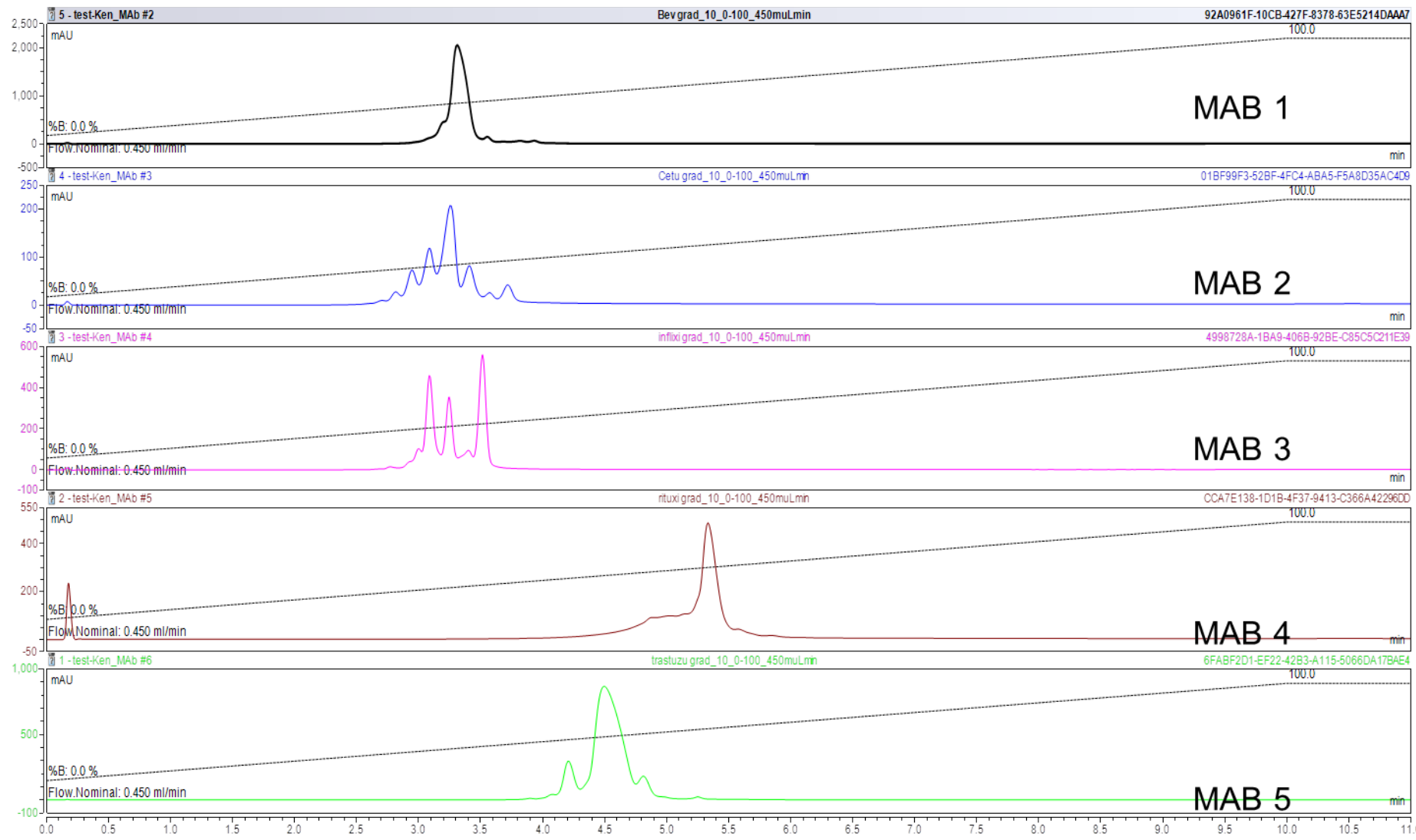
	Buffer A	Buffer B
pH	5.6	10.2
Form	Liquid	Liquid
Concentrate	10X	10X
Shipping condition	Room Temp	Room Temp
Storage condition	4 ~ 8 °C	4 ~ 8 °C

*pH gradient platform method for charge variant analysis*

# mAb Standards Using Linear pH Gradient

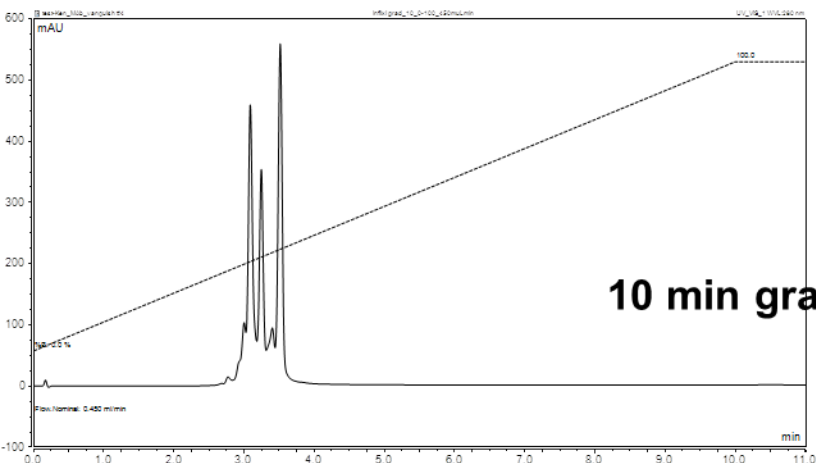


# Fast, Generic and Linear pH Gradient – Vanquish UHPLC

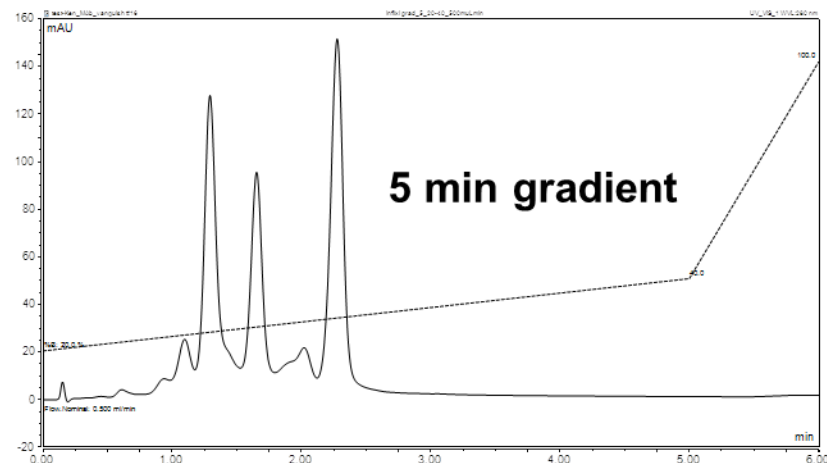


**pH 5.6 to 10.2 in 10 minutes, MABPac SCX-10, 2 x 50 mm**

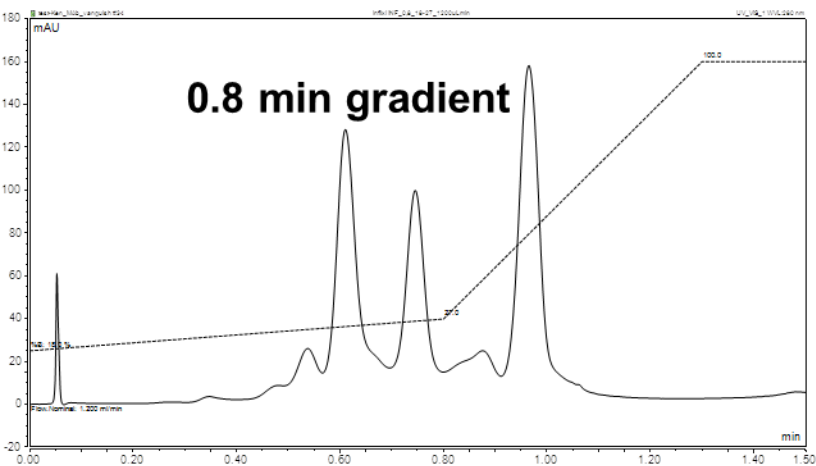
# Infliximab – Vanquish System Ultra-fast Gradients



**10 min gradient**



**5 min gradient**



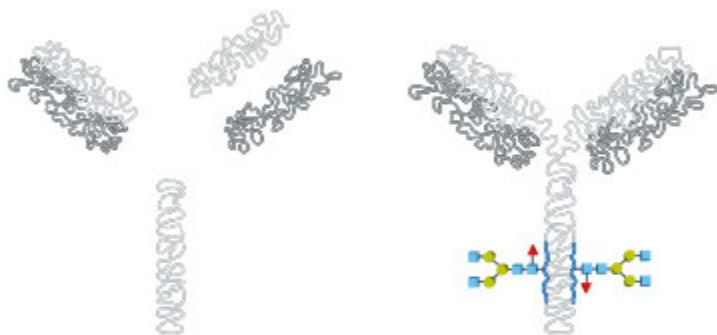
**0.8 min gradient**

**3 steps method development**

- 1.** 10 minutes 0→100% B in 10 minutes
- 2.** 20→40% B in 5 minutes
- 3.** 18→27% B in 0.8 minutes

***Resolution and number of charge variants maintained in sub-minute gradients***

# Intact & Native Protein Workflow



Protein or reduced protein

Protein A, IEC pH-gradient, HIC, SEC



UHPLC Orbitrap MS

## 1. Preparation

- Protein A, Enzymatic reduction, IEC pH-gradient, HIC, SEC,

## 2. Chemistries

- Multi-dimension analysis
- IEC pH-gradient, HIC, SEC

## 3. Separations & Detection

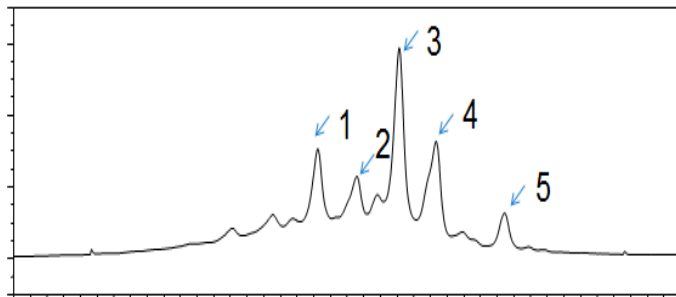
- Vanquish Flex UHPLC or UltiMate 3000 BioRS UHPLC system
- Automated desalting on MSPac DS-10 de-salter cartridge

## 4. Characterization

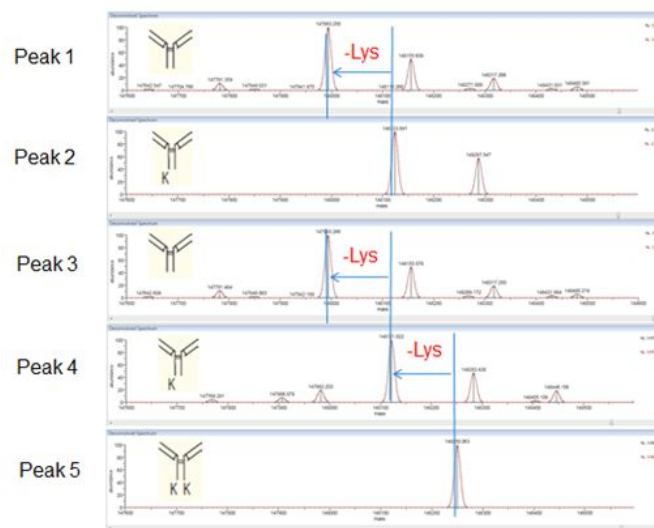
- Exactive Plus or Q Exactive Plus Mass Spectrometer
- BioPharma Finder Software
- Full scan method, intact mass deconvolution

# In-depth HRAM Charge Variant Characterization

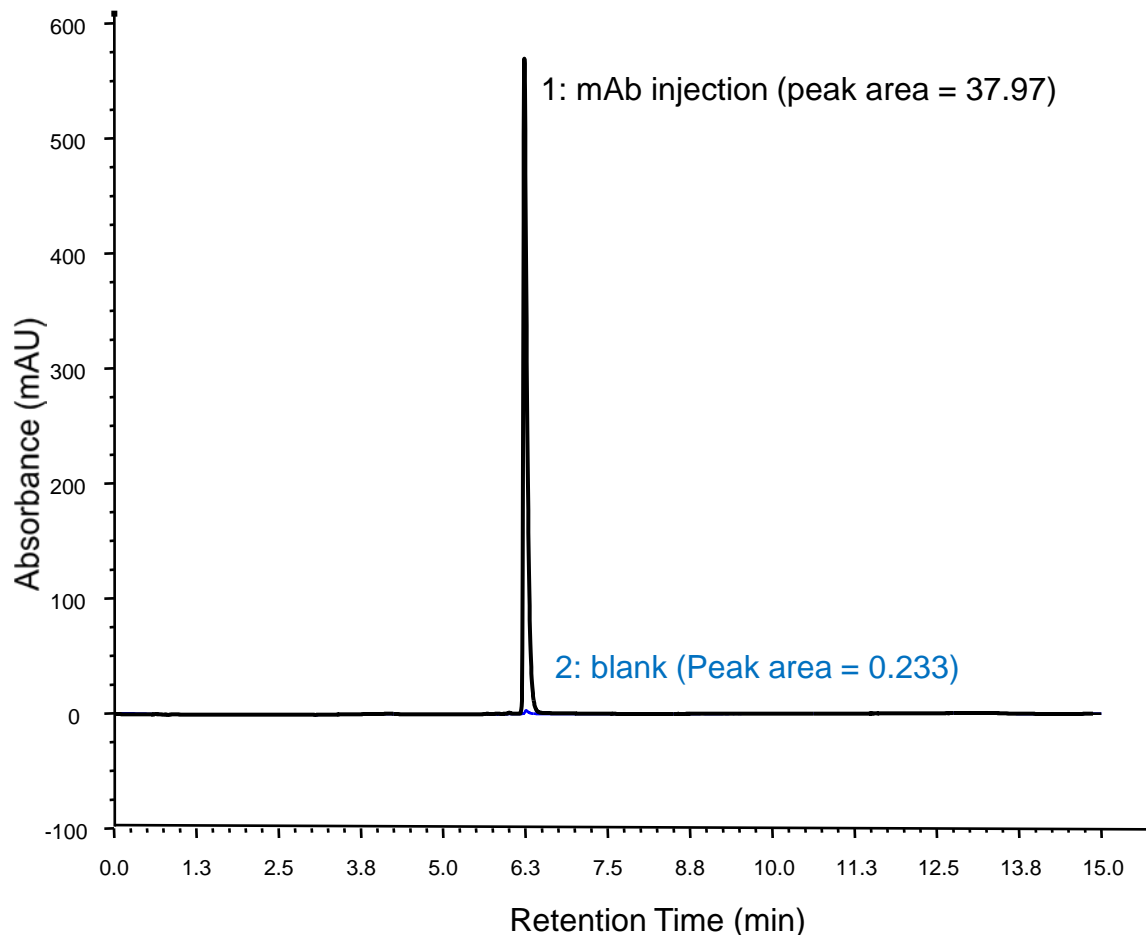
1<sup>st</sup> dimension: IEX pH gradient + fraction collection



2<sup>nd</sup> dimension: Polymer RP-LC/MS



# Carryover : Polymeric MAbPac RP



Column: MAbPac RP, 4  $\mu$ m  
Format: 3  $\times$  50 mm  
Mobile phase A: H<sub>2</sub>O/TFA (99.9 : 0.1 v/v)  
Mobile phase B: MeCN/ H<sub>2</sub>O/TFA (90: 9.9 :0.1 v/v/v)

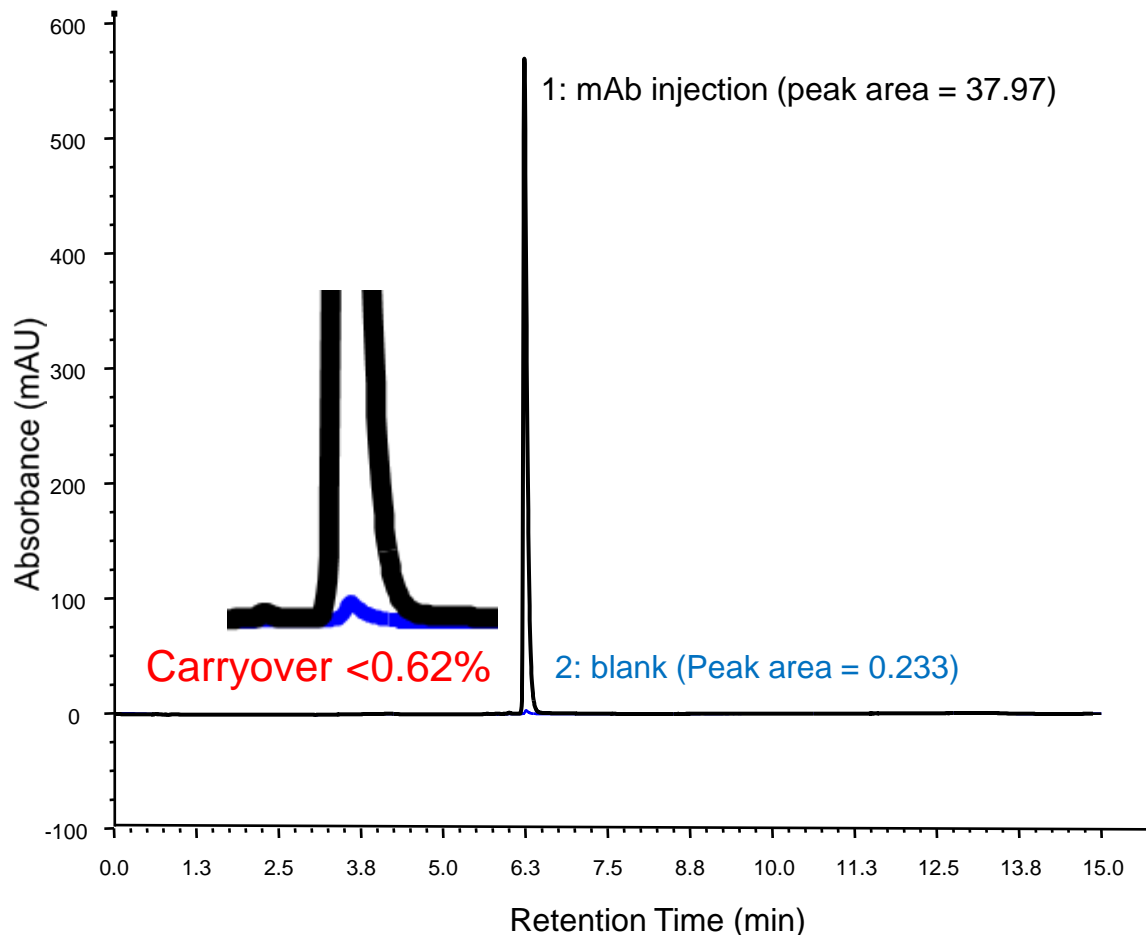
Gradient:

Time (min)	%A	%B
0.0	100	0
1.0	100	0
11.0	0	100
12.0	0	100
14.0	100	0
15.0	0	100

Temperature: 80  $^{\circ}$ C  
Flow rate: 0.5 mL/min  
Inj. volume: 5  $\mu$ L  
Detection: UV (280 nm)  
Sample: mAb (5 mg/mL)



# Carryover : Polymeric MAbPac RP



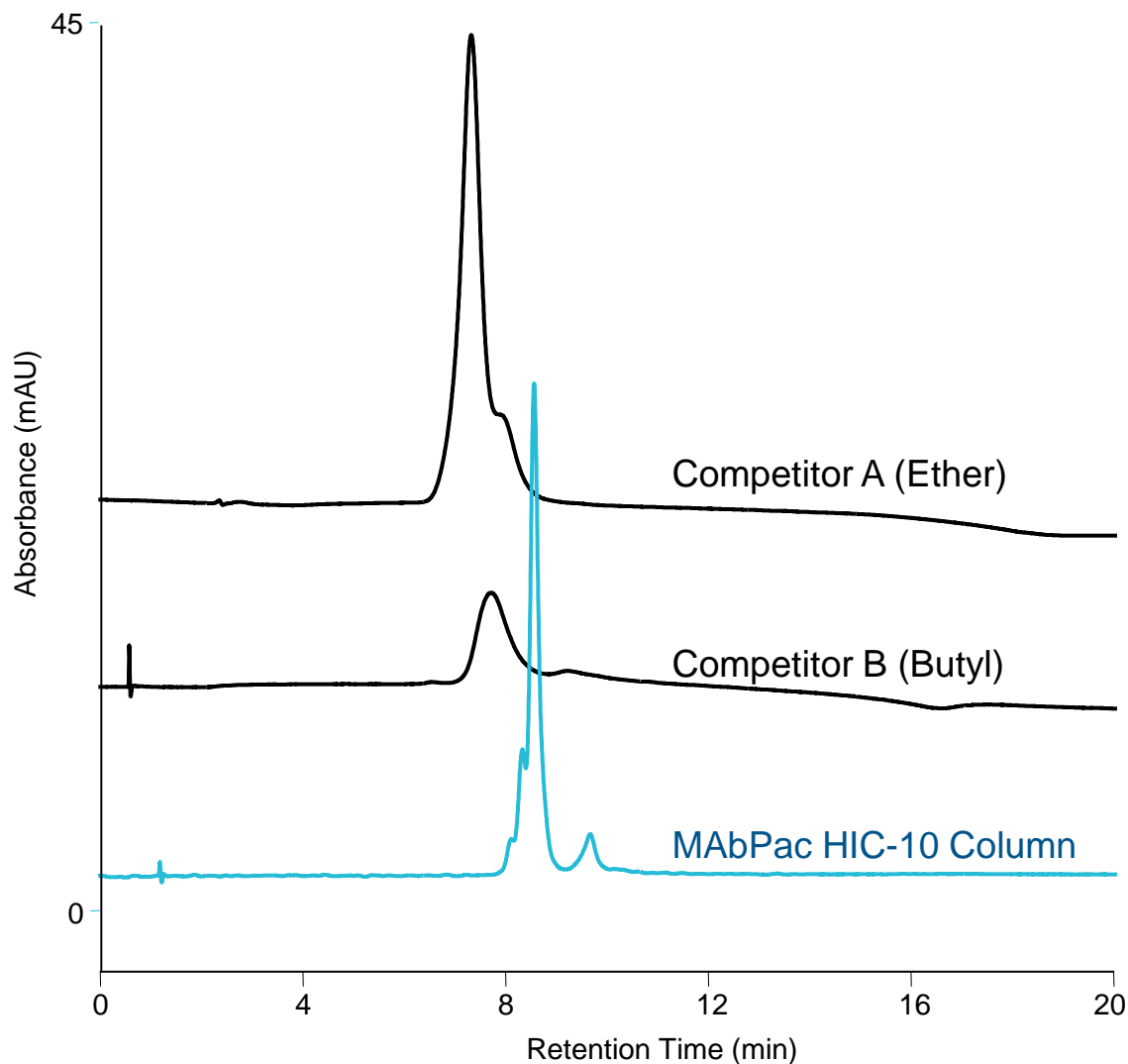
Column: MAbPac RP, 4  $\mu$ m  
Format: 3  $\times$  50 mm  
Mobile phase A: H<sub>2</sub>O/TFA (99.9 : 0.1 v/v)  
Mobile phase B: MeCN/ H<sub>2</sub>O/TFA (90: 9.9 :0.1 v/v/v)

Gradient:

Time (min)	%A	%B
0.0	100	0
1.0	100	0
11.0	0	100
12.0	0	100
14.0	100	0
15.0	0	100

Temperature: 80  $^{\circ}$ C  
Flow rate: 0.5 mL/min  
Inj. volume: 5  $\mu$ L  
Detection: UV (280 nm)  
Sample: mAb (5 mg/mL)

# Hydrophobic Interaction Chromatography



Column: MAbPac HIC-10, 4.6 × 100 mm  
Competitor A (Ether), 7.5 × 75 mm  
Competitor B (Butyl), 4.6 × 100 mm

Mobile phase A: 2.0 M ammonium sulfate, 100 mM sodium phosphate, pH 7.0

Mobile phase B: 100 mM sodium phosphate, pH 7.0

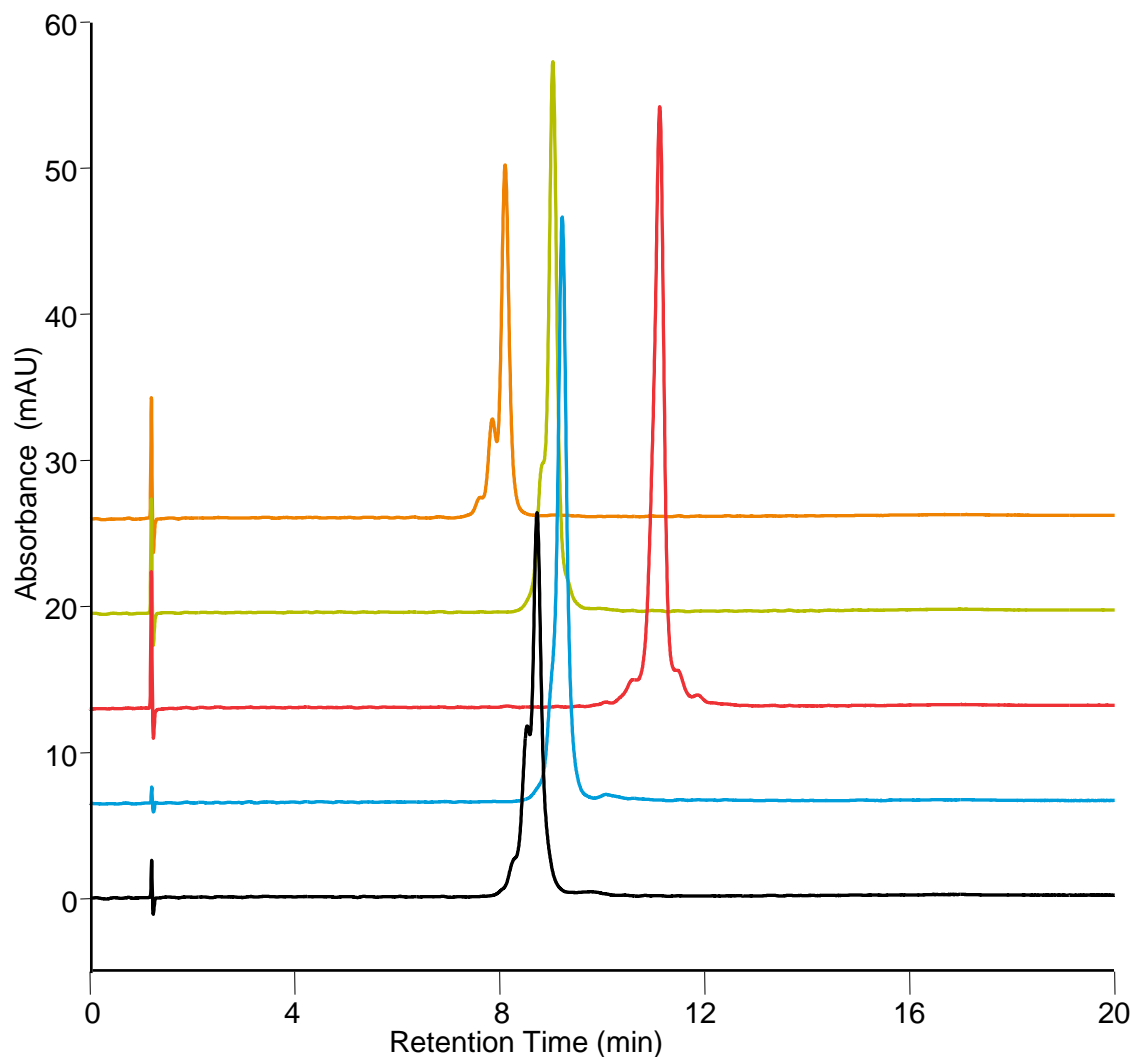
Gradient:

Time (min)	%A	%B
-5.0	60	40
0.0	60	40
1.0	60	40
15.0	0	100
20.0	0	100

Temperature: 30 °C  
Flow rate: 1.0 mL/min  
Inj. volume: 2 µL (4 mg/mL)  
Competitor A (Ether): 4 µL

Detection: UV (280 nm)  
Sample: mAb

# Global Analysis of Native mAbs



Column: MAbPac HIC-10, 5  $\mu$ m  
Format: 4.6  $\times$  100 mm  
Mobile phase A: 2.0 M ammonium sulfate, 100 mM sodium phosphate, pH 7.0  
Mobile phase B: 100 mM sodium phosphate, pH 7.0

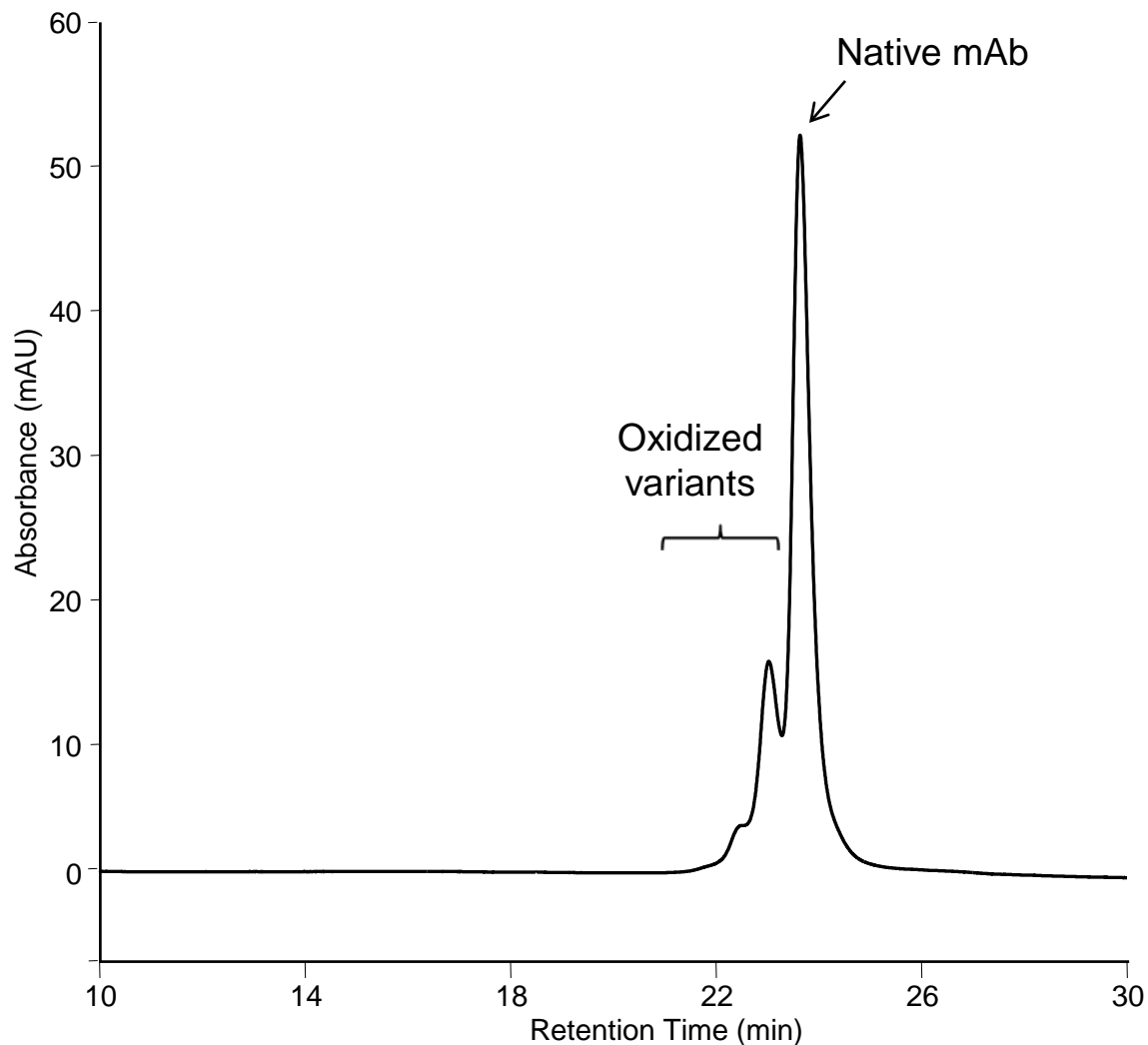
Gradient:

Time (min)	%A	%B
-5.0	60	40
0.0	60	40
1.0	60	40
15.0	0	100
20.0	0	100

Temperature: 30  $^{\circ}$ C  
Flow rate: 1.0 mL/min  
Inj. volume: 2  $\mu$ L (4 mg/mL)  
Detection: UV (280 nm)  
Sample:

mAb1  
mAb2  
mAb3  
mAb4  
mAb5

# Separation of Oxidized mAb on MAbPac HIC-20

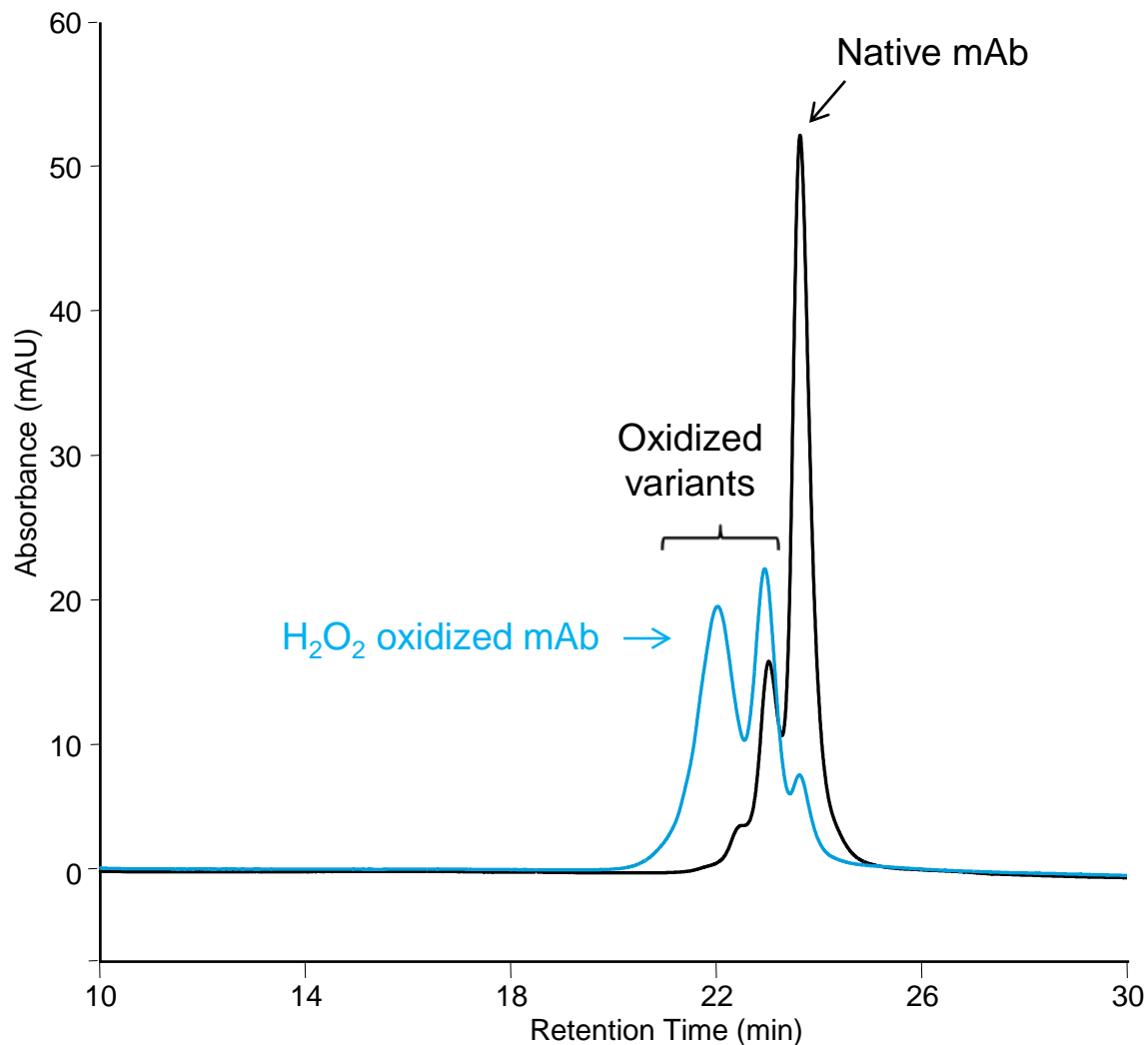


Column: **MAbPac HIC-20, 5  $\mu$ m**  
Format: 4.6  $\times$  250 mm  
Mobile phase A: 2 M ammonium sulfate, 100 mM sodium phosphate, pH 7.0  
Mobile phase B: 100 mM sodium phosphate, pH 7.0  
Gradient:

Time (min)	%A	%B
-6.0	50	50
0.0	50	50
2.0	50	50
30.0	0	100
35.0	0	100

Temperature: 30  $^{\circ}$ C  
Flow rate: 0.5 mL/min  
Inj. volume: Untreated mAb: 20  $\mu$ L (1.25 mg/mL)  
Oxidized mAb: 20  $\mu$ L (1.25 mg/mL)  
Detection: UV (280 nm)  
Sample: Untreated mAb

# Separation of Oxidized mAb on MAbPac HIC-20

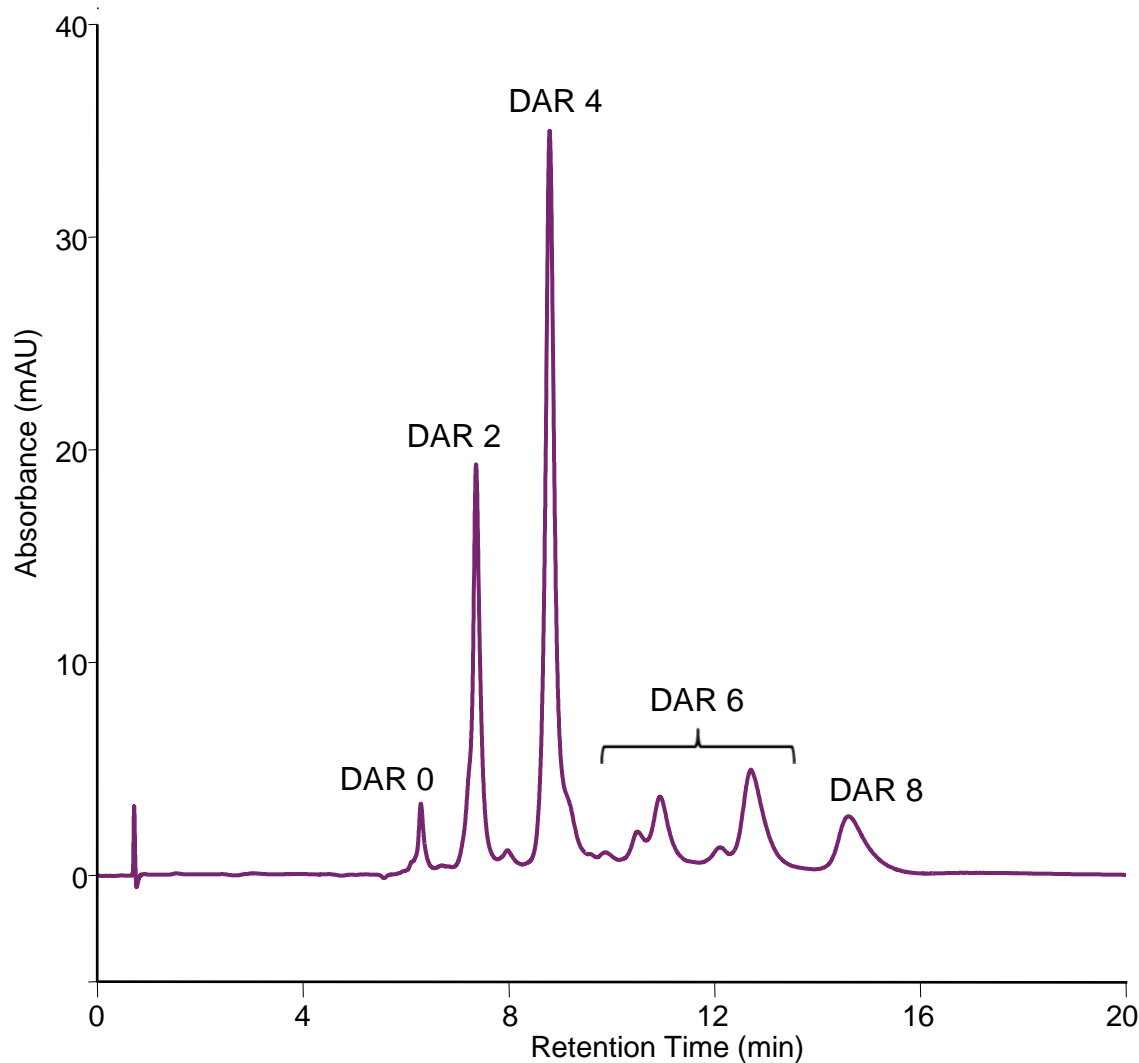


Column: **MAbPac HIC-20, 5  $\mu$ m**  
Format: 4.6  $\times$  250 mm  
Mobile phase A: 2 M ammonium sulfate, 100 mM sodium phosphate, pH 7.0  
Mobile phase B: 100 mM sodium phosphate, pH 7.0  
Gradient:

Time (min)	%A	%B
-6.0	50	50
0.0	50	50
2.0	50	50
30.0	0	100
35.0	0	100

Temperature: 30  $^{\circ}$ C  
Flow rate: 0.5 mL/min  
Inj. volume: Untreated mAb: 20  $\mu$ L (1.25 mg/mL)  
Oxidized mAb: 20  $\mu$ L (1.25 mg/mL)  
Detection: UV (280 nm)  
Sample: Untreated mAb  
H<sub>2</sub>O<sub>2</sub> oxidized mAb

# Separation of Cys-linked ADC



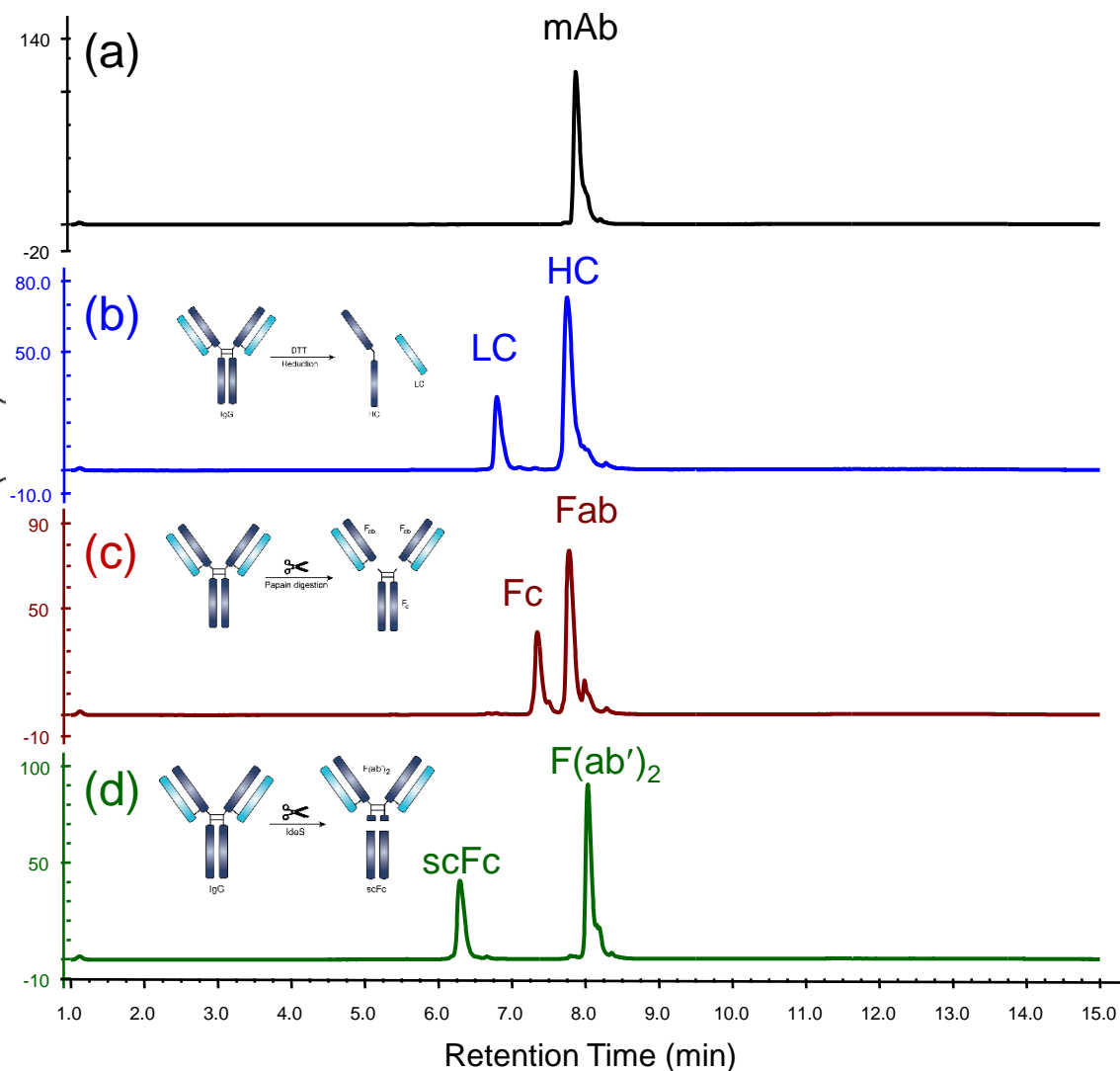
Column: **MABPac HIC-Butyl, 5  $\mu$ m**  
Format: 4.6  $\times$  100 mm  
Mobile phase A: 1.5 M ammonium sulfate, 50 mM sodium phosphate, pH 7.0 / isopropanol (95:5 v/v)  
Mobile phase B: 50 mM sodium phosphate, pH 7.0 / isopropanol (80:20 v/v)

Gradient:

Time (min)	%A	%B
-5.0	100	0
0.0	100	0
1.0	100	0
15.0	0	100
20.0	0	100

Temperature: 25  $^{\circ}$ C  
Flow rate: 1.0 mL/min  
Inj. volume: 5  $\mu$ L (5 mg/mL)  
Detection: UV (280 nm)  
Sample: Cys-conjugated ADC mimic

# mAb and mAb Fragments Analysis – Reversed Phase



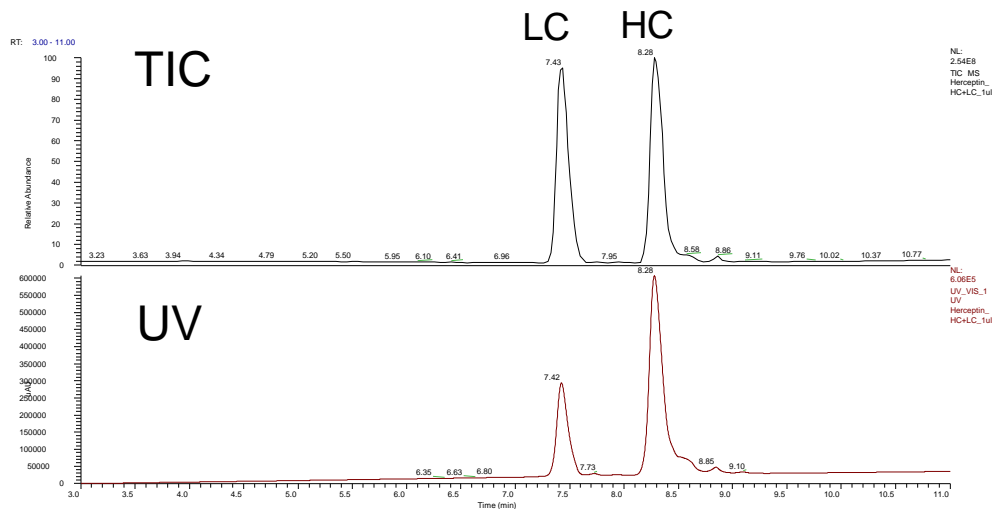
Column: MAbPac RP, 4  $\mu$ m  
 Format: 3  $\times$  50 mm  
 Mobile phase A: H<sub>2</sub>O/FA/TFA (99.88 : 0.1:0.02 v/v/v)  
 Mobile phase B: MeCN/ H<sub>2</sub>O/FA/TFA (90: 9.88 :0.1:0.02 v/v/v/v)

Gradient:

Time (min)	%A	%B
0.0	80	20
1.0	80	20
11.0	55	45
12.0	55	45
14.0	80	20
15.0	80	20

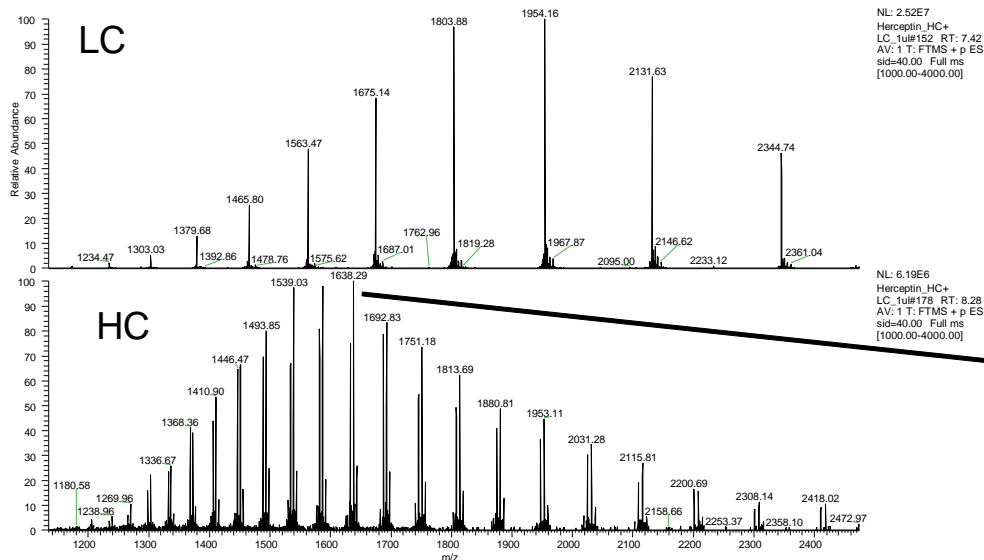
Temperature: 80  $^{\circ}$ C  
 Flow rate: 0.5 mL/min  
 Inj. volume: 5  $\mu$ L  
 Detection: UV (280 nm)  
 Sample:  
 (a) trastuzumab (5 mg/mL)  
 (b) trastuzumab + DTT (4 mg/mL)  
 (c) trastuzumab + Papain (2 mg/mL)  
 (d) trastuzumab + IdeS (2 mg/mL)

# LC/MS Analysis of Reduced mAb

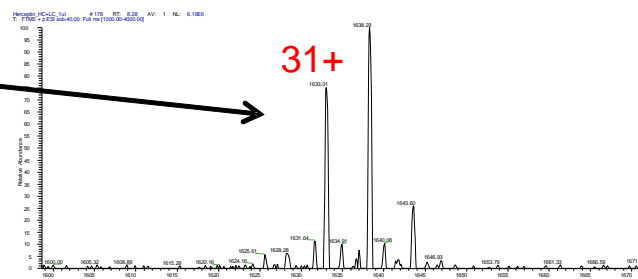


Column: **MABPac RP, 4 μm**  
 Format: 3 × 50 mm  
 Mobile phase A: H<sub>2</sub>O/FA/TFA (99.88 : 0.1:0.02 v/v/v)  
 Mobile phase B: MeCN/ H<sub>2</sub>O/FA/TFA (90: 9.88 :0.1:0.02 v/v/v/v)  
 Gradient:

Time (min)	%A	%B
0.0	80	20
1.0	80	20
11.0	55	45
12.0	55	45
14.0	80	20
15.0	80	20



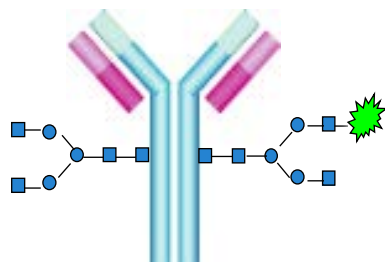
Temperature: 80 °C  
 Flow rate: 0.5 mL/min  
 Inj. volume: 1 μL  
 UV Detection: 280 nm  
 MS Detection: positive-ion mode  
 Mass Spec: Q Exactive Plus  
 Sample: reduced trastuzumab (4 mg/mL)



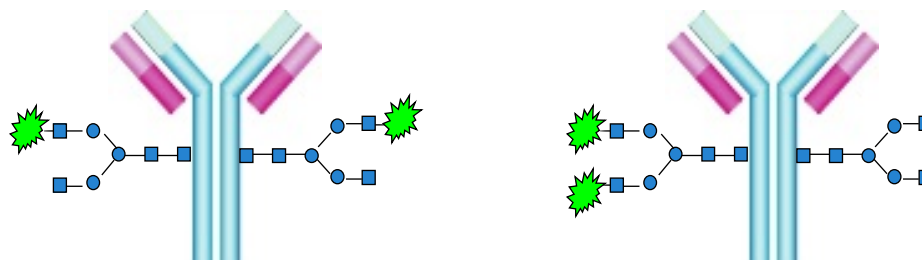


# Heterogeneity of SiteClick™ N-glycan Labeling of Antibody

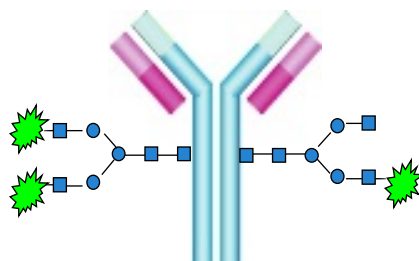
DAR 1



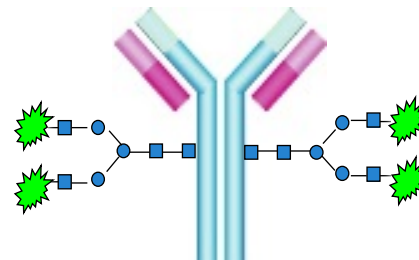
DAR 2



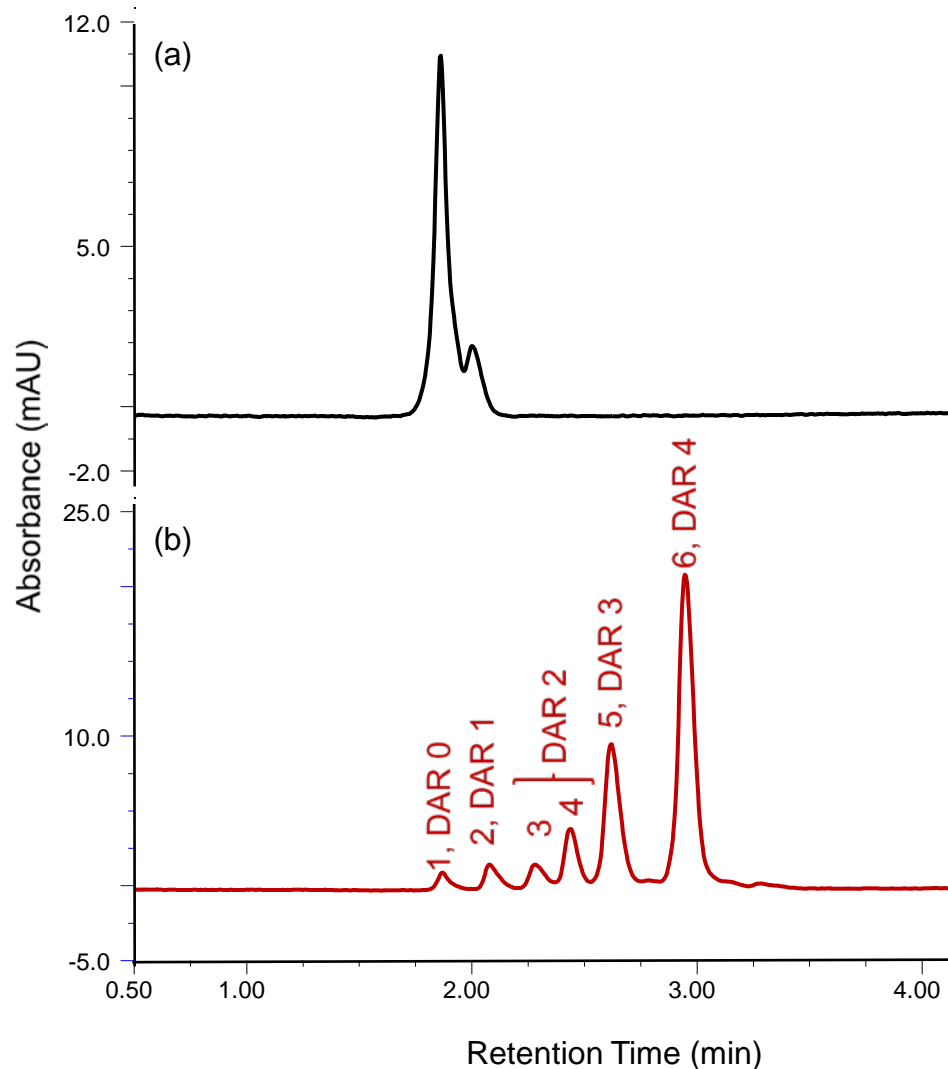
DAR 3



DAR 4

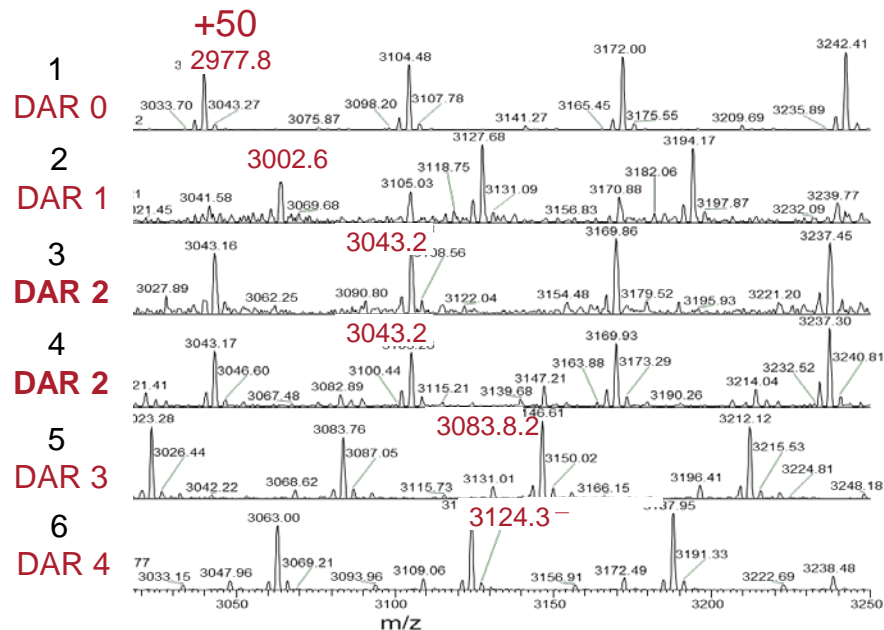


# RP Separation of Unmodified mAbs and ADCs



Column: MAbPac RP, 4  $\mu$ m  
 Format: 2.1  $\times$  50 mm  
 Mobile phase A: H<sub>2</sub>O/TFA (99.9 : 0.1 v/v)  
 Mobile phase B: MeCN/ H<sub>2</sub>O/TFA (90: 9.9 : 0.1 v/v/v)

## MS raw data



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