



# An Overview of a Novel Analytical Affinity Chromatography Column

## TSKgel<sup>®</sup> FcR-III A-NPR<sup>™</sup>

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**Manager, Technical Service / Sr. Application Scientist**

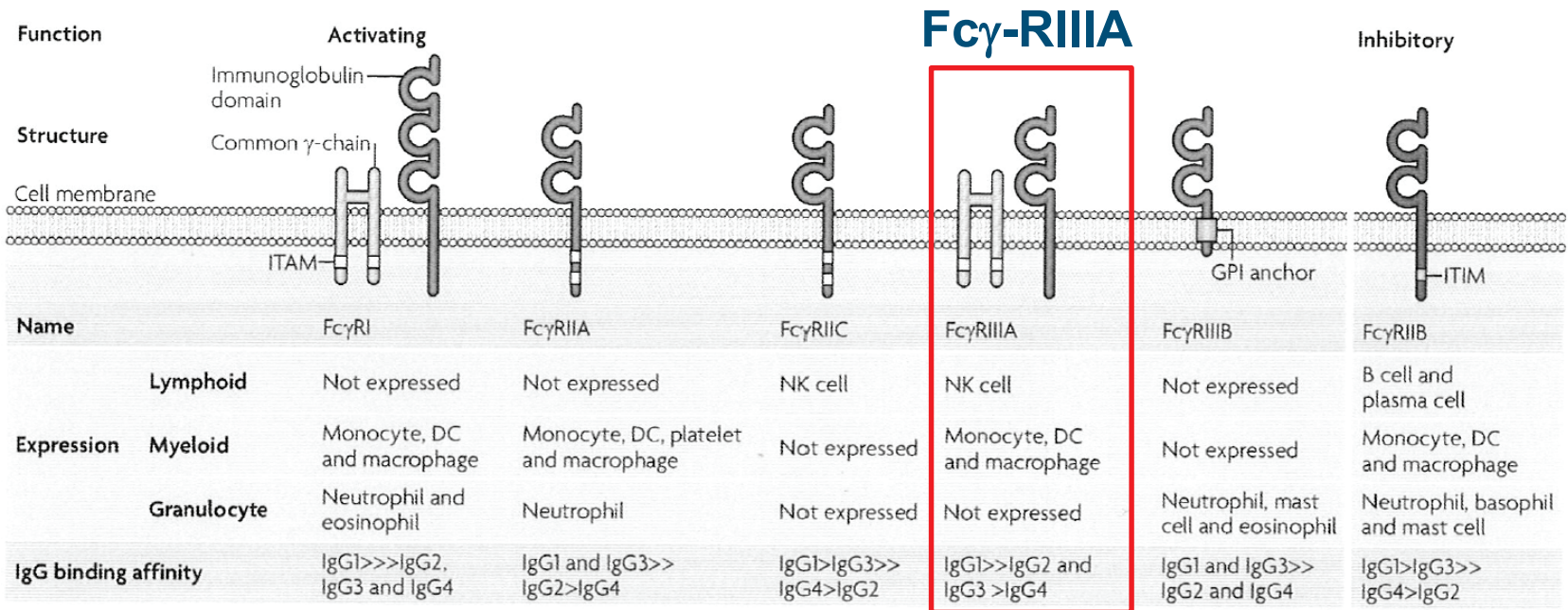
**Tosoh Bioscience LLC, King of Prussia, PA**



# Content

- Introduction on development of novel FcR ligand
- Characteristics of the FcR column
- Applications
- Method Development aspects
- Conclusions

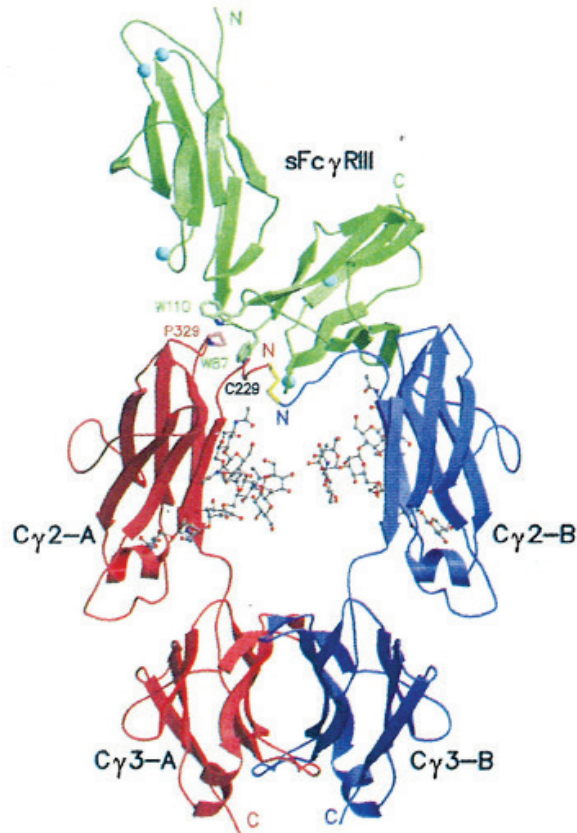
# Fcγ receptors (FcRs) and their functions



Kenneth G. C. Smith and Menna R. Clatworthy, *Nature Reviews Immunology* 10, 328-343 (May 2010)



# Tosoh's rFcγRIIIA ligand (Green)



Modified Recombinant, 20 kDa

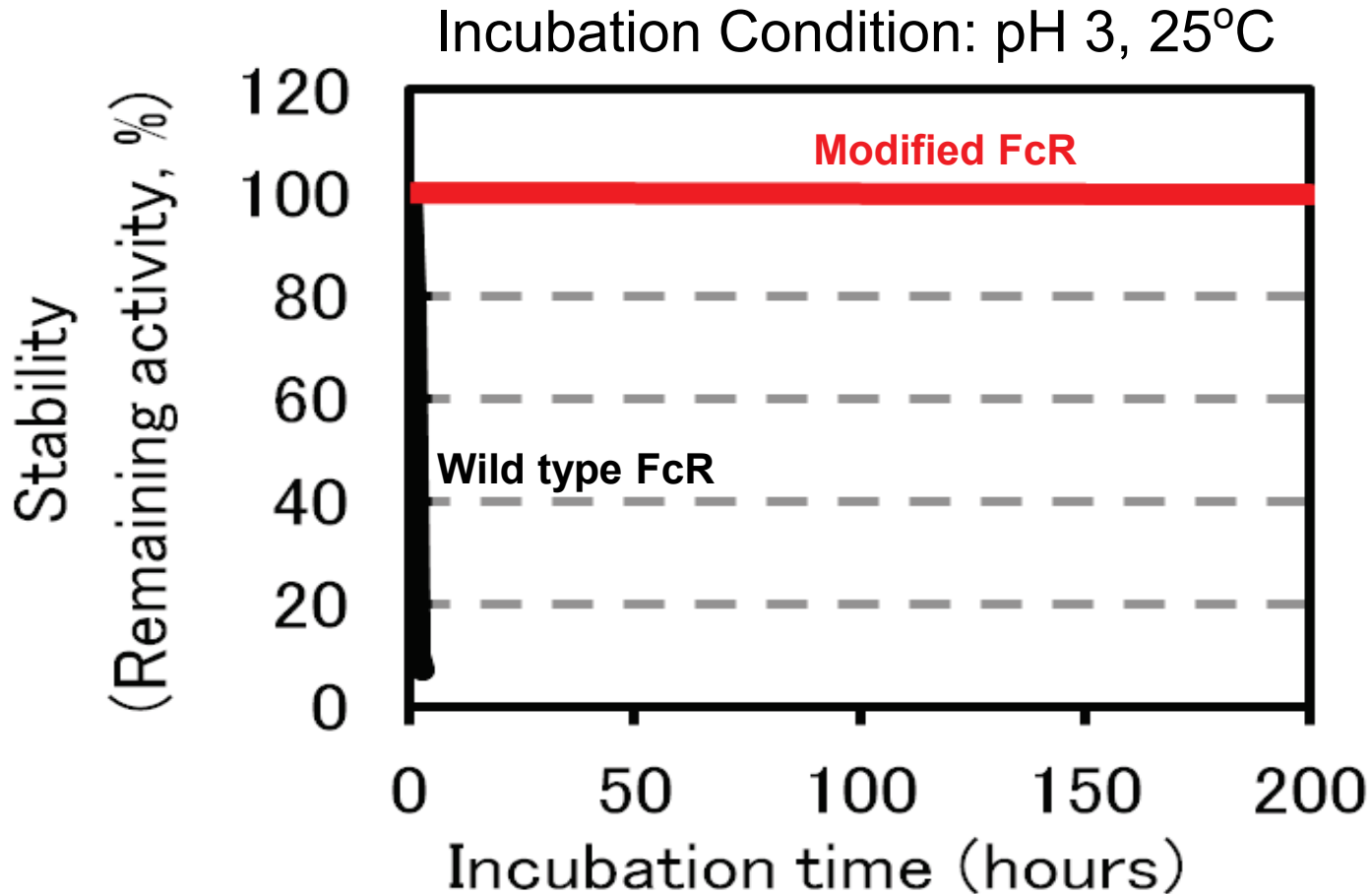
E. Coli expression system, non-glycosylated

Protein-based ligand

**Ref:** Peter Sondermann *et al.*, *Nature*, vol. 406, 20 July 2000, 267-273

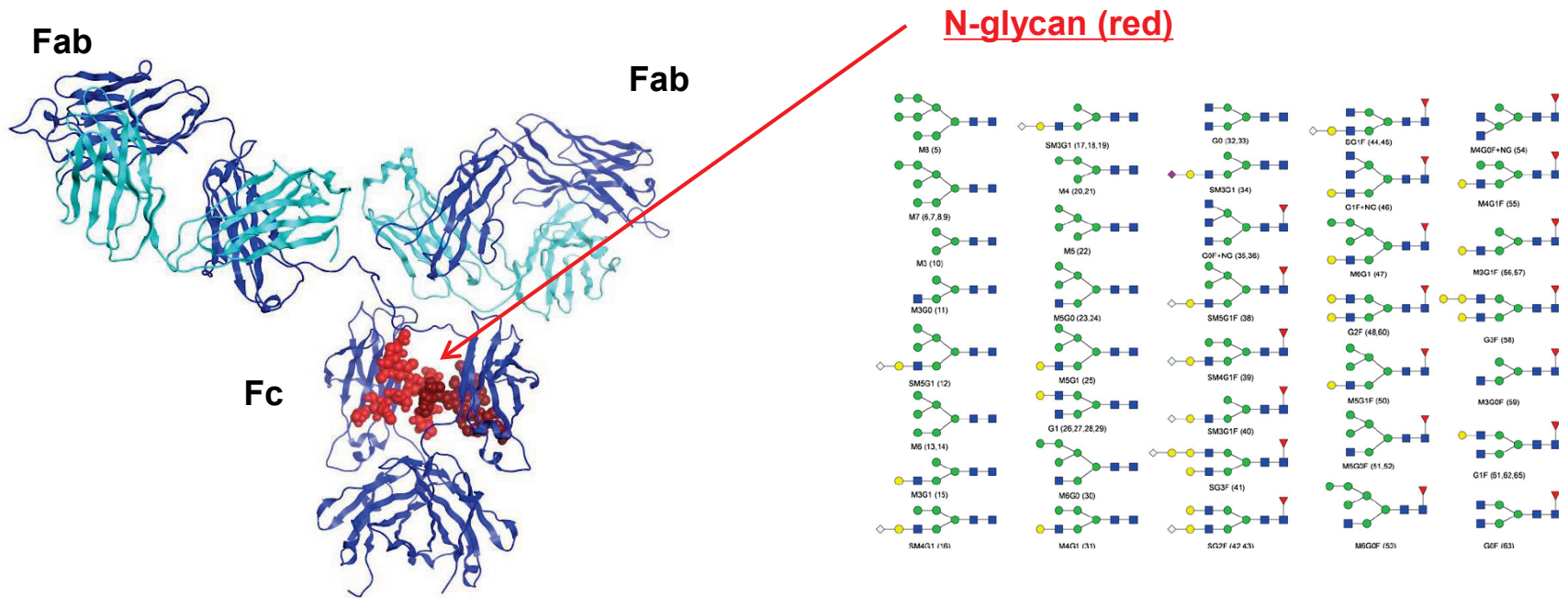


# Acid Stability of Tosoh's FcγRIII ligand



**Stability of wild type FcγRIIIA is not enough for its use as affinity ligand**

# Conserved N-Glycosylation site of mAb

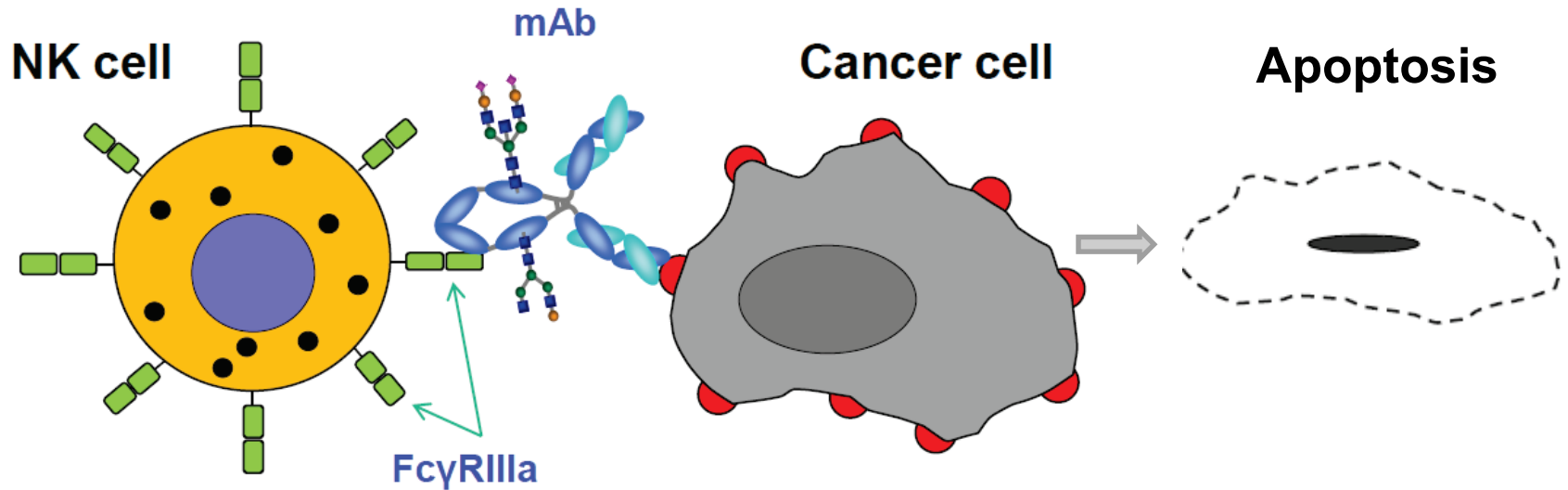


**Structure of N-glycan attached to Asn297 of Fc region affects the drug efficacy**

Ref:

1. Shinkawa T et al., J.Biol. Chem. **278**, 3466-3473 (2003).
2. Pablo Umana et al., Nature Biotech. **17 FEB**, 176-180 (1999).
3. Saphire, E.O et.al, Science 293:1155-1159 (2001)

# Role of Fc $\gamma$ RIIIA in ADCC activity



**Types of N-Glycans at Asn297 of Fc receptor affect the binding affinity, thus ADCC activity**

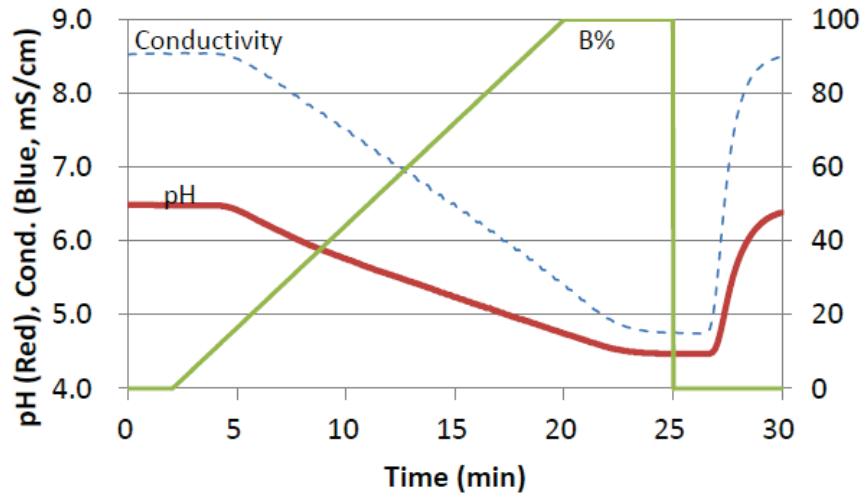


# Column Attributes

Attributes	
Ligand	Modified recombinant FcγRIIIA ( <i>E. coli</i> expression system, non-glycosylated)
Base matrix	nonporous resin, 5 μm
Column	4.6 mm ID x 7.5 cm, PEEK
Sample mass	5 – 50 μg of IgG (recommended)
Standard flow rate	1.0 mL/min
Temperature	15 °C - 25 °C
pH range	4 - 8
Recommended buffer system	A: 50 mmol/L citrate buffer, 150 mmol/L NaCl, pH 6.5 B: 50 mmol/L citrate buffer, 150 mmol/L NaCl, pH 4.5
Maximum pressure	9 MPa

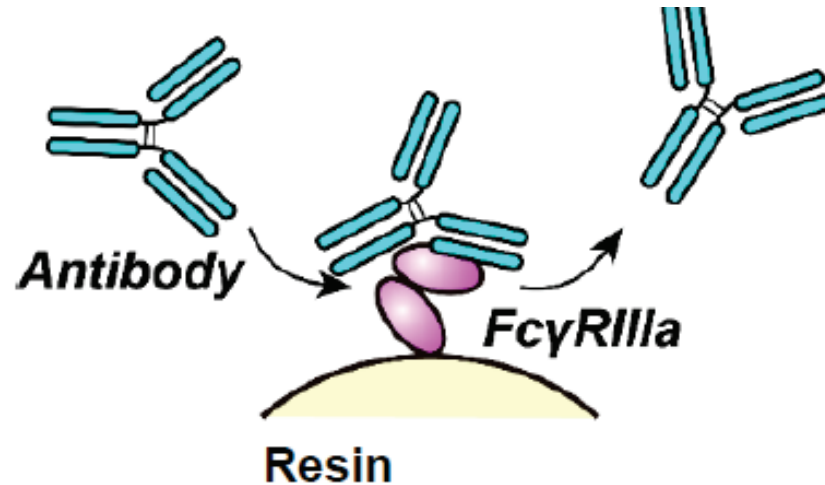


# Standard Chromatographic Condition



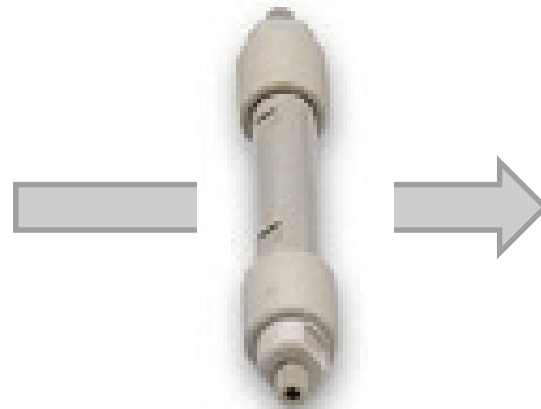
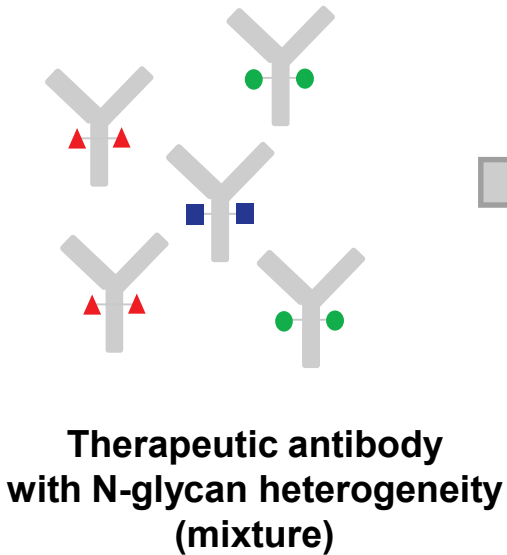
## Condition

System, Tosoh LC-8020  
 Buffer A, 50 mM Citrate pH6.5  
 Buffer B, 50 mM Citrate pH4.5  
 Gradient, B0-100% (2-20min, liner)  
 Flow rate, 1.0 mL/min  
 Temperature, 15 – 25 degree C (Column oven)  
 Detection, ABS 280 nm

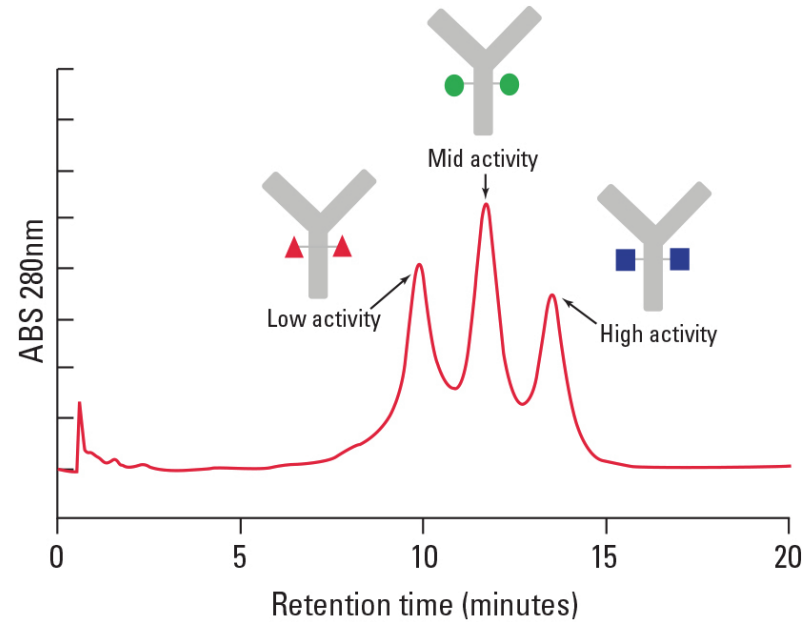




# FcR Column Chromatography

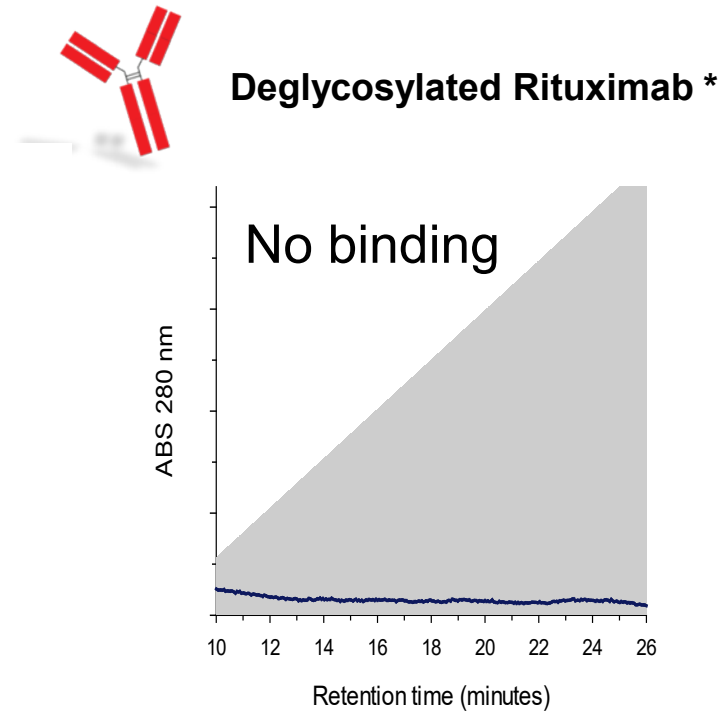
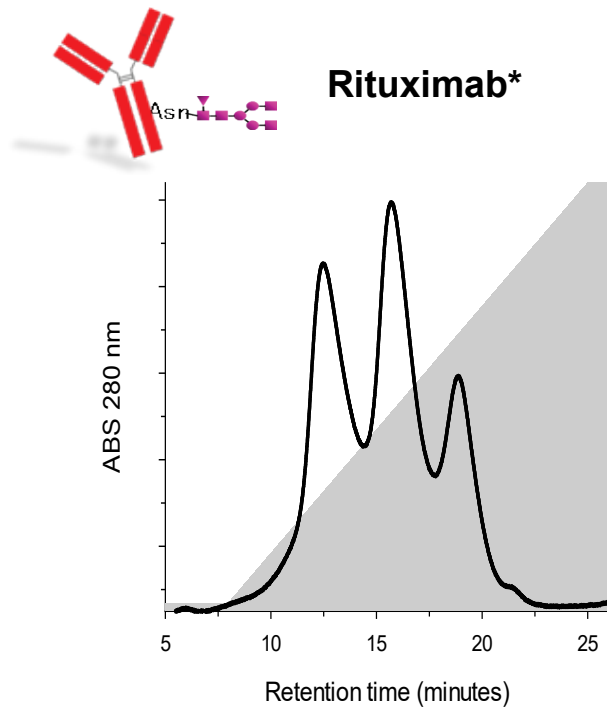


FcR column



mAb components are separated based on the correlation between N-glycan structure and ADCC activity.

# Removal of N-Glycans Prevent mAb Binding to FcR Receptor

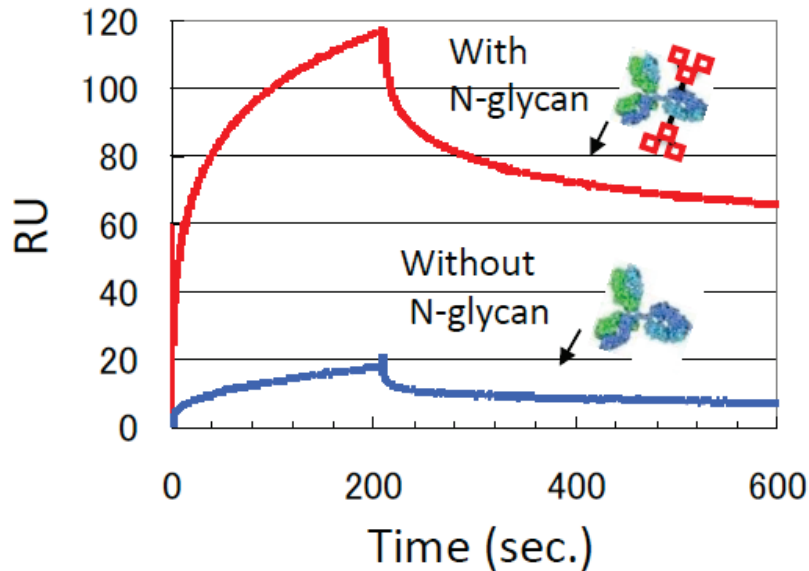


Column: TSKgel FcR-III A-NPR, 5  $\mu$ m  
Mobile Phase: A: 50 mmol/L Citrate, pH 6.5 ; B: 50 mmol/L Citrate, pH 4.5  
Flow rate: 1 mL/min  
Injection: 30  $\mu$ L  
Sample: Rituximab (1  $\mu$ g/ $\mu$ L)

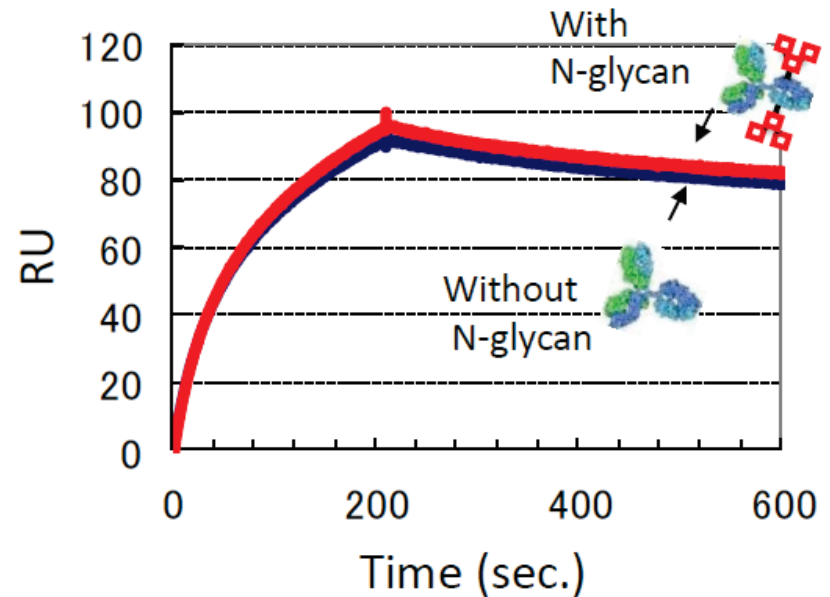
\* Data: Master Thesis of Leila Ghaleh, TU Darmstadt  
Rituximab kindly provided by Rentschler Biopharma

# Selectivity of the modified FcγRIIIA ligand

## Modified FcγRIIIA

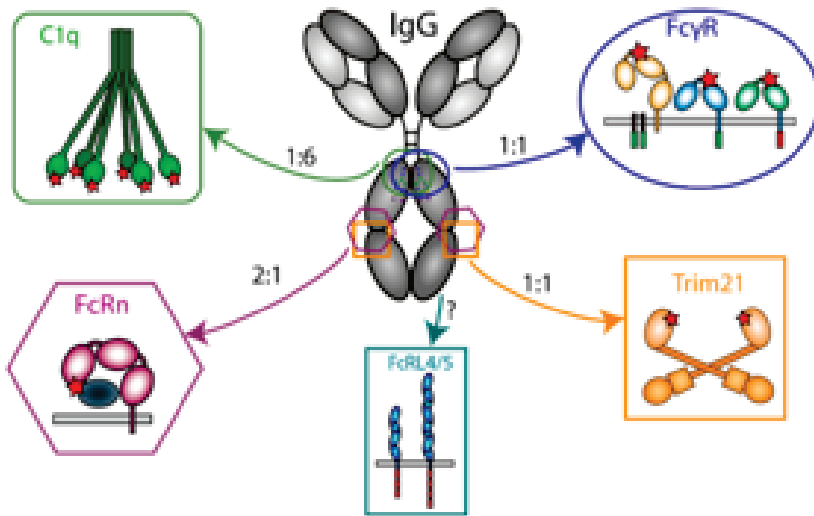


## Protein A

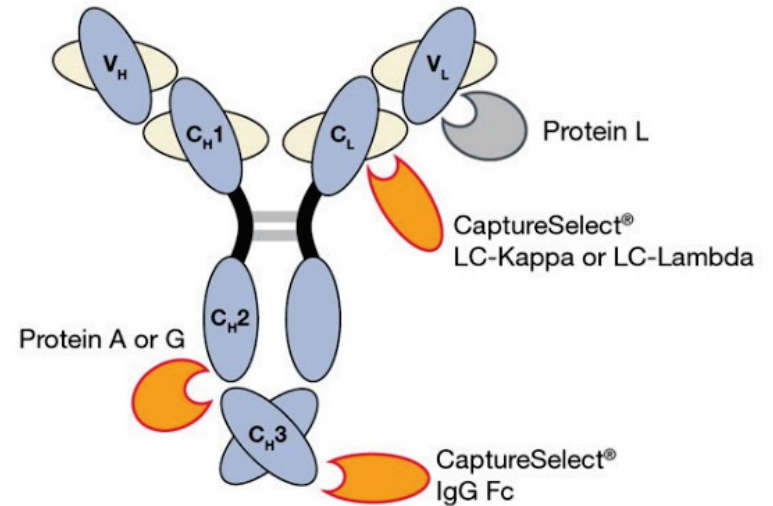


**The modified FcR ligand does not bind to mAb without N-glycan.**

# Binding Sites: FcγRIIIA ligand vs. Protein A



Ref: 1. Antibodies 2019, 8(2), 30;  
<https://doi.org/10.3390/antib8020030>

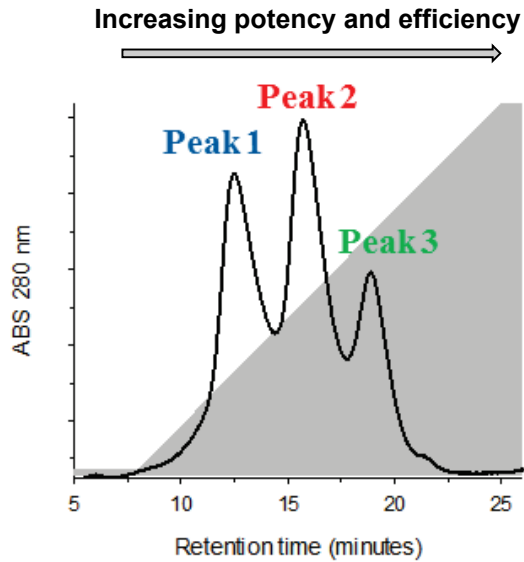


Ref: 2: ThermoFisher.com

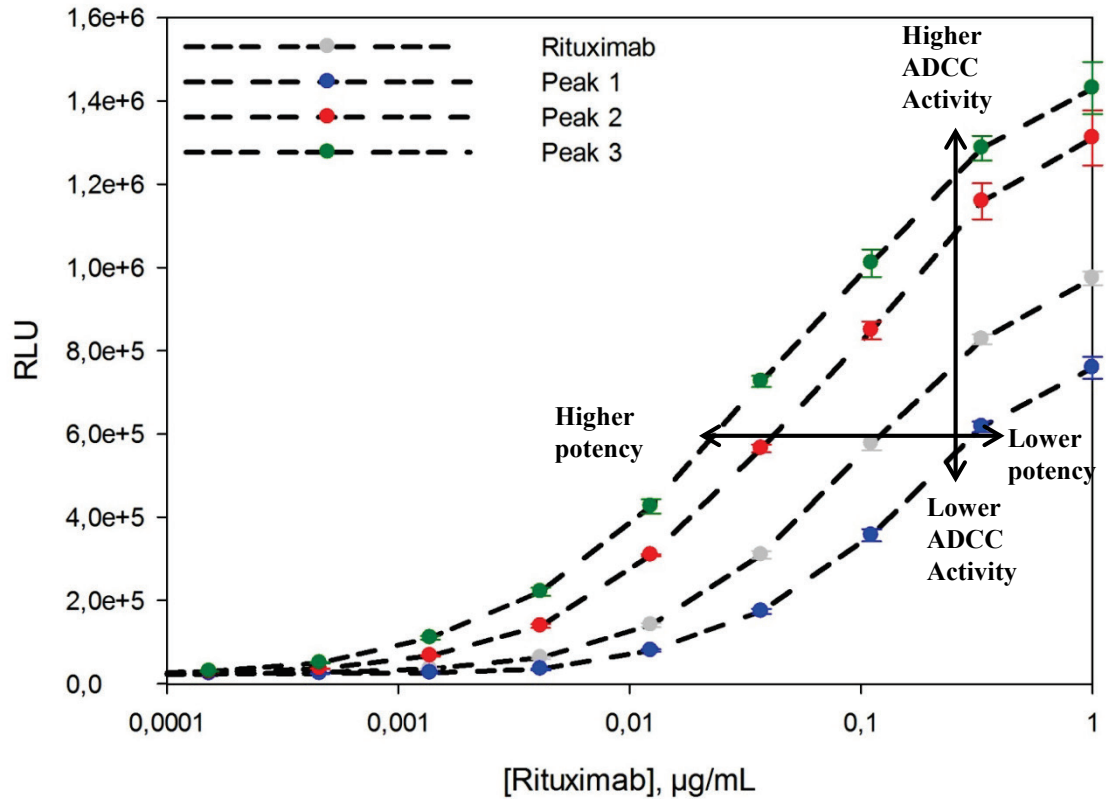
**Fcγ receptor binding site is located near the hinge region of IgG**



# Rituximab – FcR Affinity / ADCC



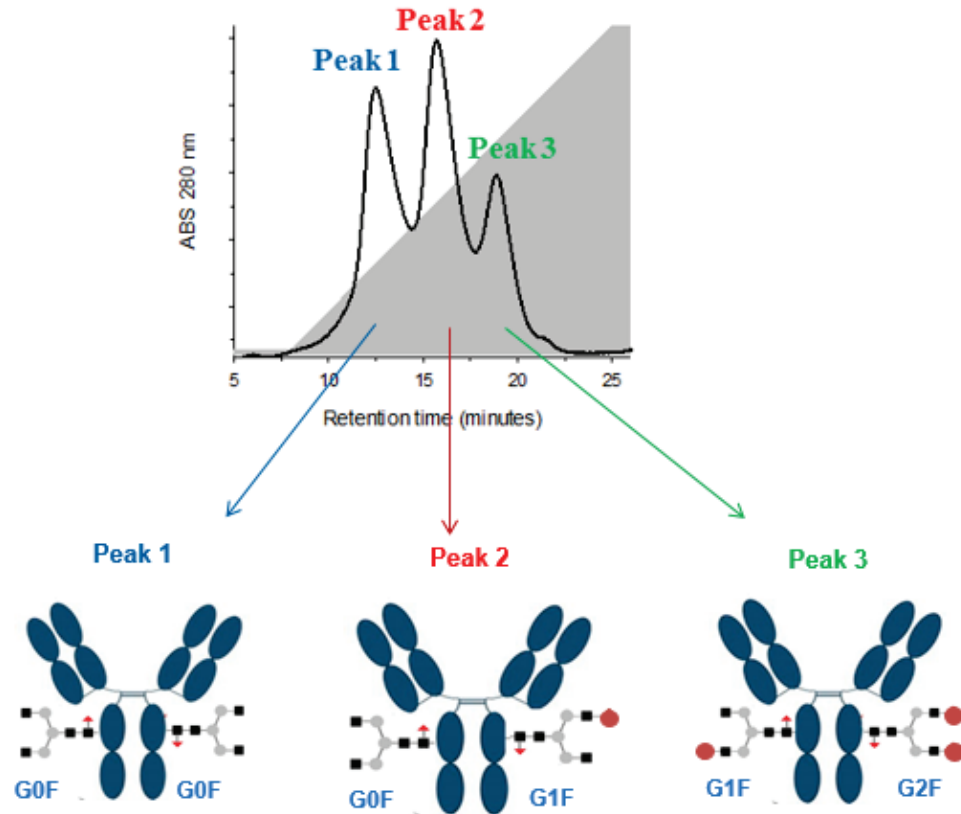
Antibody	EC <sub>50</sub> (µg/ mL)
Rituximab	0.098
Peak 1	0.153
Peak 2	0.072
Peak 3	0.049



## ADCC reporter bioassay (Promega)

\* Data: Master Thesis of Leila Ghaleh, TU Darmstadt  
Rituximab kindly provided by Rentschler Biopharma

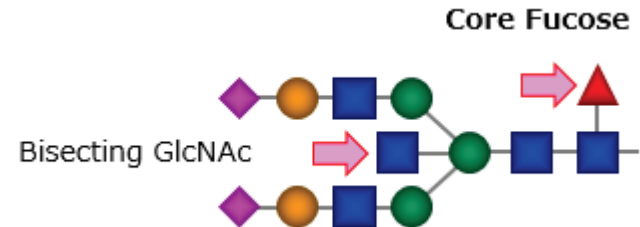
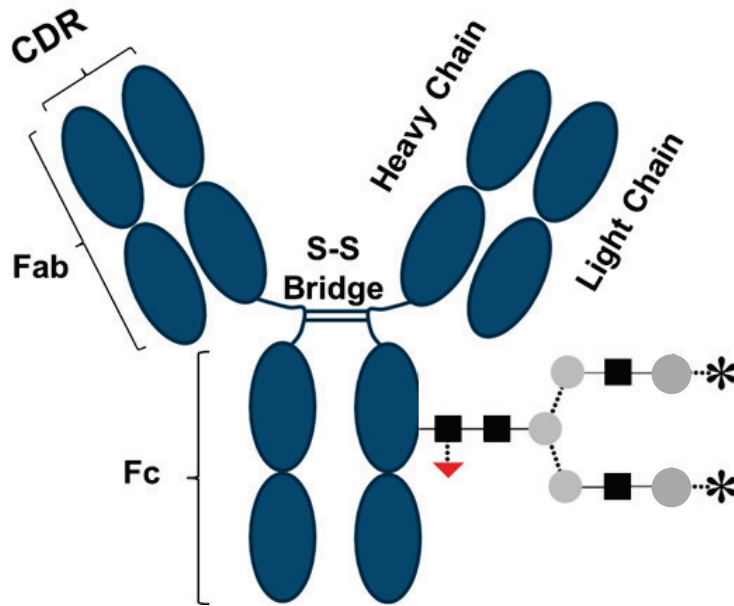
# Rituximab – FcR Affinity / Glycoforms



**The larger the number of terminal galactose,  
the higher the binding affinity.**

\* Data: Master Thesis of Leila Ghaleh, TU Darmstadt  
Rituximab kindly provided by Rentschler Biopharma

# ADCC activity of glycovariants



- N-acetylglucosamine(GlcNAc)
- ▶ Fucose
- Mannose
- ◆ Gal
- \* NeuNAc

**Core fucose decreases ADCC activity**

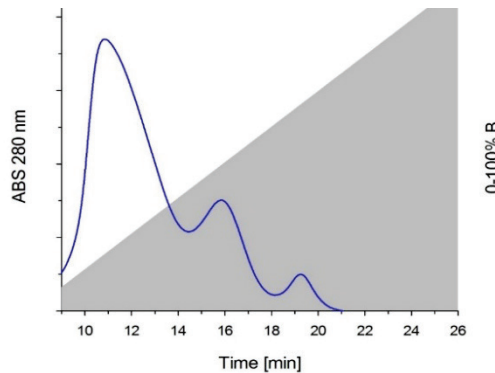
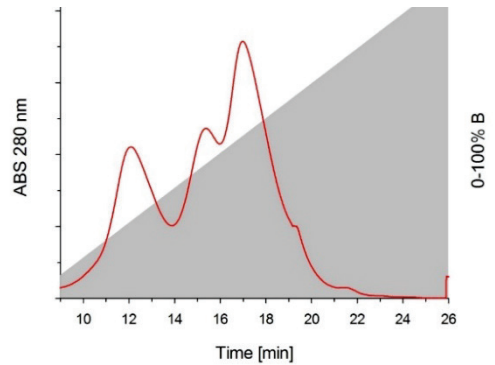




TOSOH

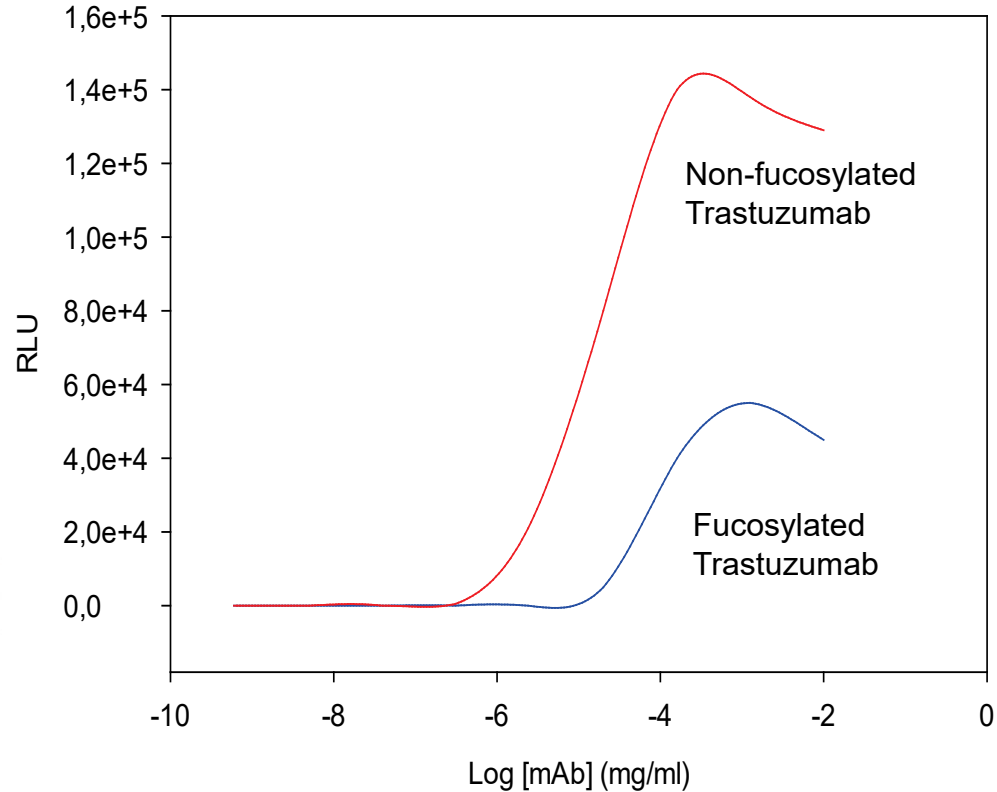
# Impact of Fucosylation

Non-fucosylated Trastuzumab



Fucosylated Trastuzumab

ADCC reporter bioassay (Promega, data Bioceros)



**ADCC activity correlates to the observed FcR affinity**

\*Data: Master Thesis of Leila Ghaleh, TU Darmstadt

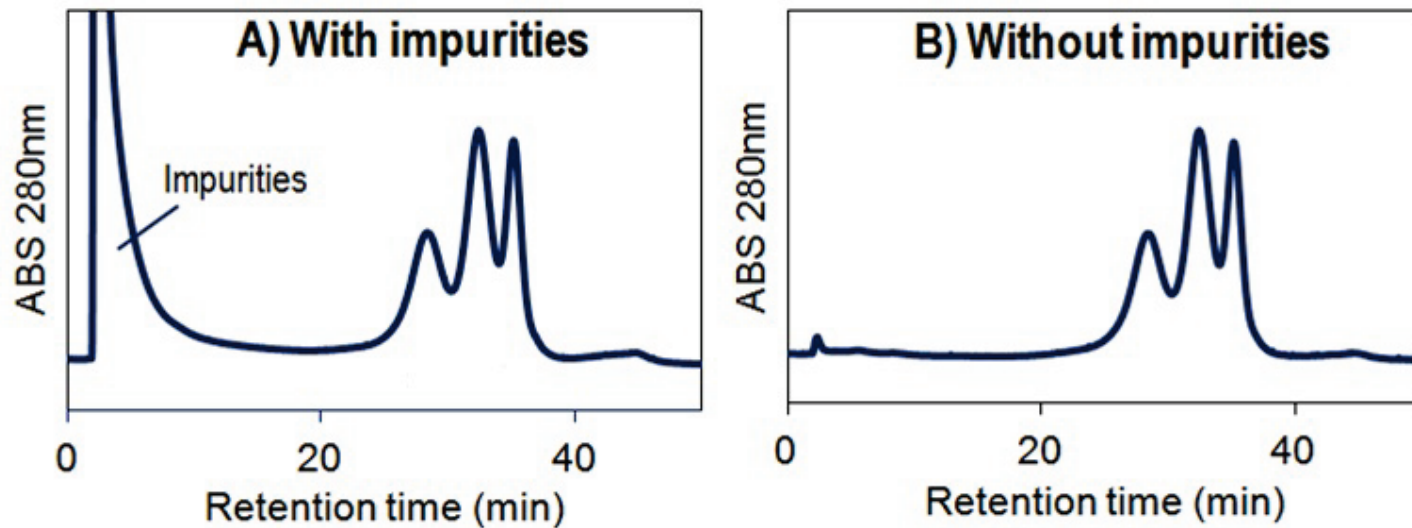
\*\*Fucosylated and non-fucosylated Trastuzumab provided by Bioceros



# Applications of TSKgel FcR-III A-NPR



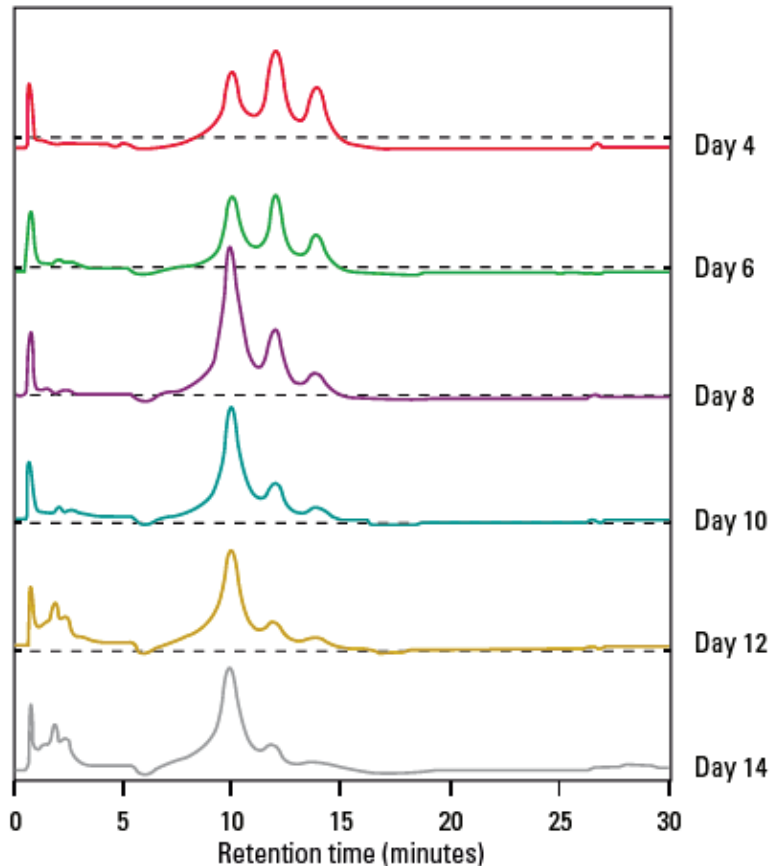
# Application 1: ADCC Activity can be Measured Directly from the Feedstock



- Host cell proteins don't affect the peak profile
- Only 5  $\mu$ g of mAb without pre-processing is enough for analysis to monitor phases of development and production



# Application 2: CHO Cell Culture Screening – Upstream Monitoring



## Conditions

Column; TSKgel FcR-III A-NPR (4.6 mm I.D. x 7.5 cm)  
Elution; Buffer A: 50 mmol/L sodium citrate buffer (pH 6.5)  
Buffer B: 50 mmol/L sodium citrate buffer (pH 4.5)  
Linear gradient (20 min, Buffer B 2-20 min)

Flow rate; 1.0 mL/min  
Detection; UV (280 nm)  
Temperature; 25 degrees C

Sample; mAb, 5 ug

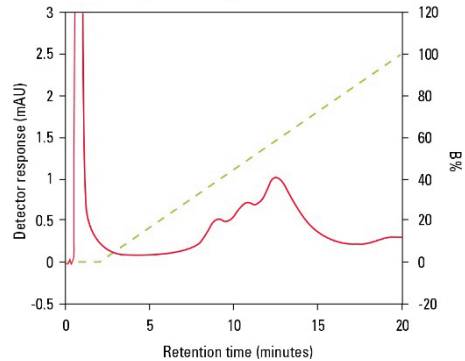
CHO cell culture media\* was filtrated and purified  
by Protein A affinity column.

**Antibody peaks shifted elution volume toward earlier elution, which suggests number of galactose in Fc glycan decreased with increased days of fermentation and suggests lower ADCC activity.**

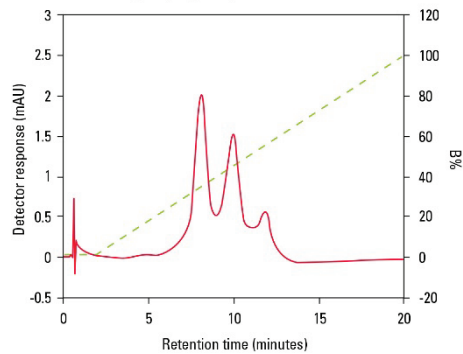


# Application 3: Glycosylation Profiling of Monoclonal Antibodies

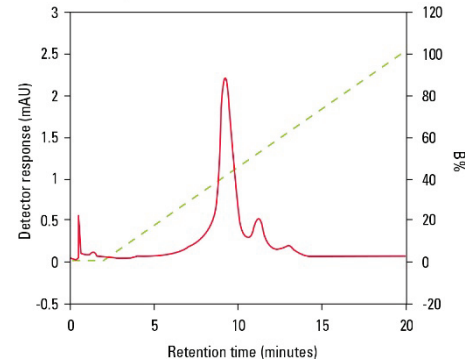
Human IgGs (Sanglorpor)



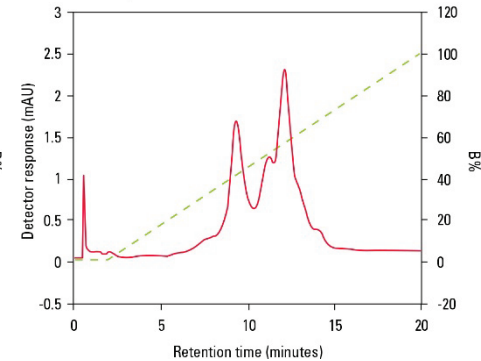
Human IgG<sup>1</sup> (Sigma)



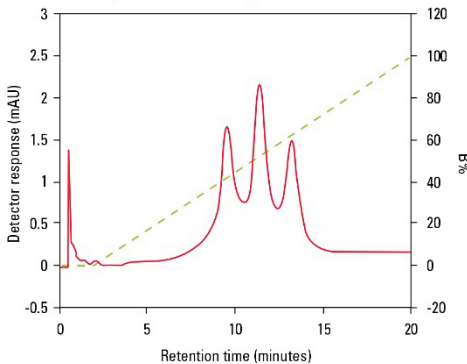
Herceptin (+Fuc)



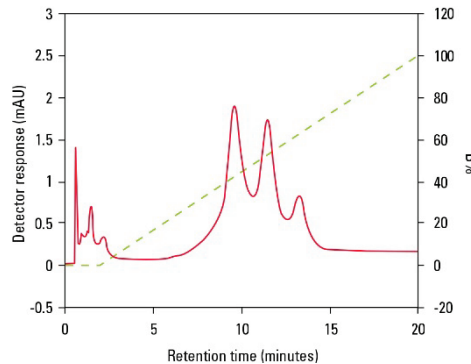
Herceptin (-Fuc)



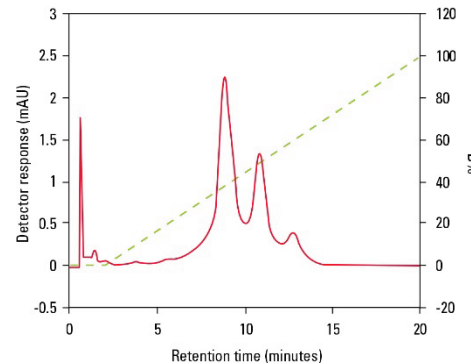
Therapeutic antibody A



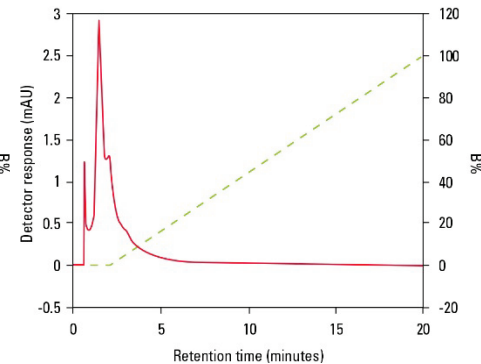
mAb 1



mAb 2



mAb 3



**Mobile phase:** A: 50 mmol/L citrate, pH 6.5

B: 50 mmol/L citrate, pH 4.5

**Gradient:** 0-100% B (2-20 min)

**Flow rate:** 1.0 mL/min

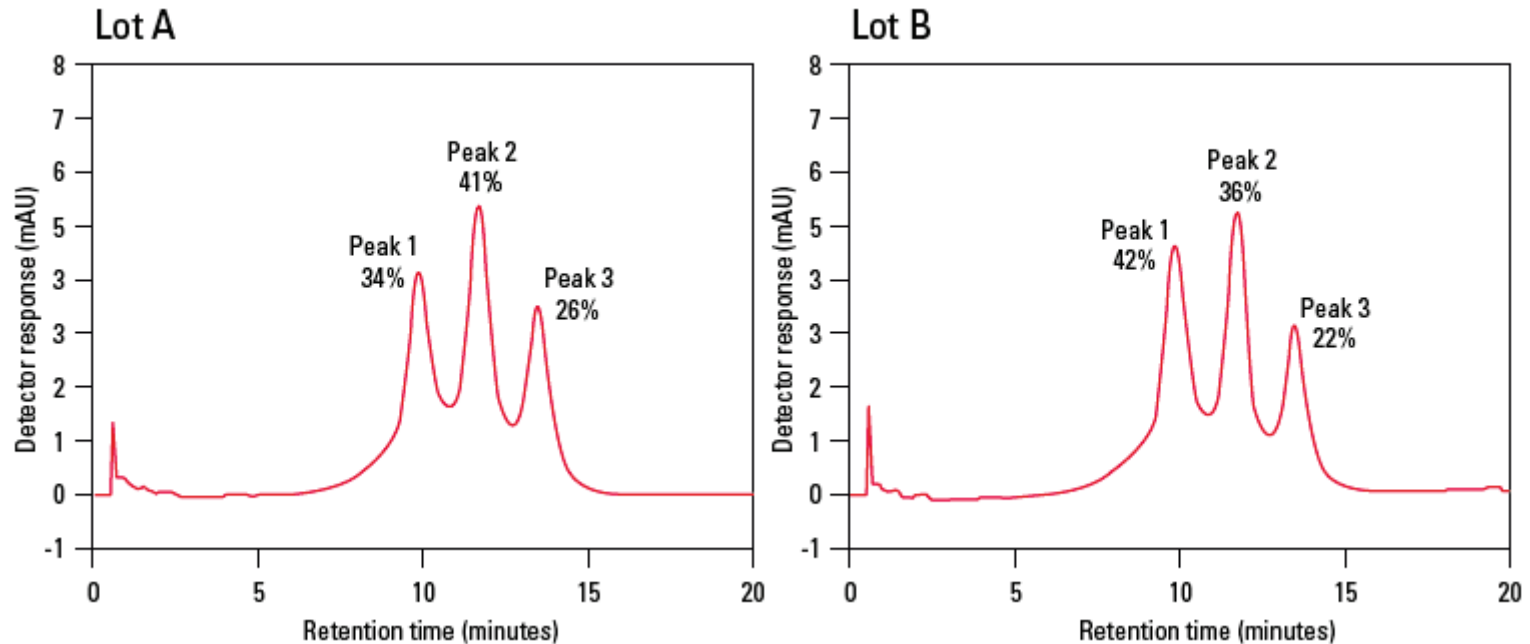
**Temperature:** 25 °C

**Detection:** UV @ 280 nm, 10 Hz

**Injection vol.:** 25 µg on column



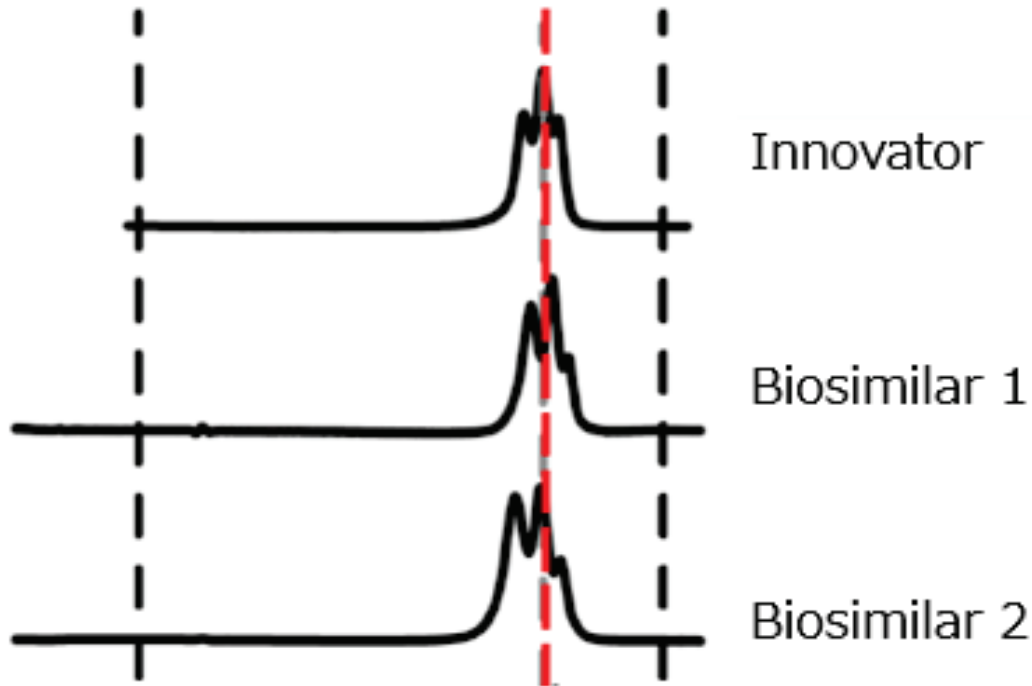
# Application 4: Lot-to-Lot Comparison for Quality Control



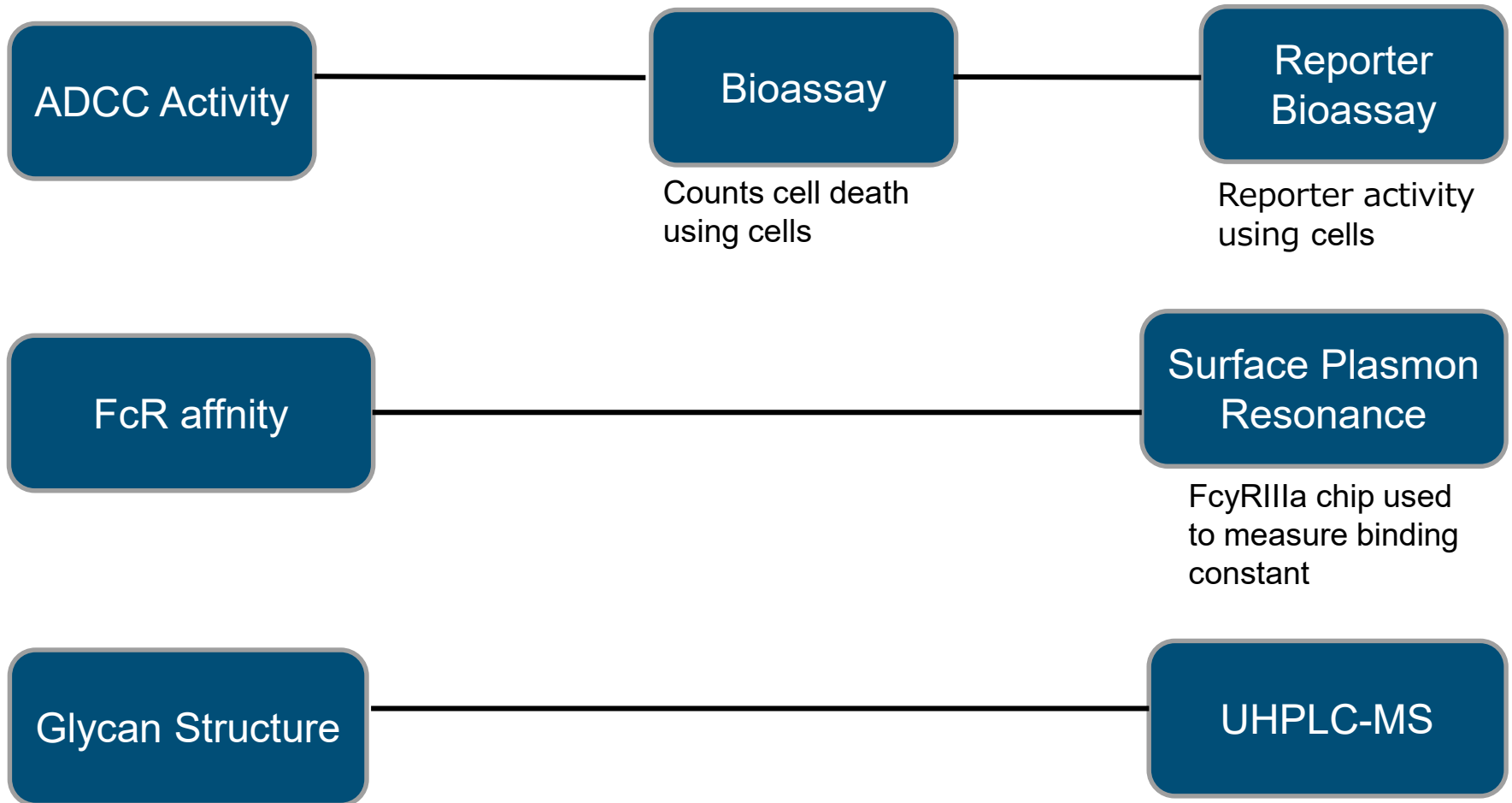
- **N-glycan heterogeneity is main issue in quality control**
- **The TSKgel FcR-III A-NPR analytical column can be used for quality control of a therapeutic antibody.**



# Application 5: Comparison Between Biosimilar and Innovator Products



Ref.; M. Kiyoshi et al., Regulatory Science 2017, poster

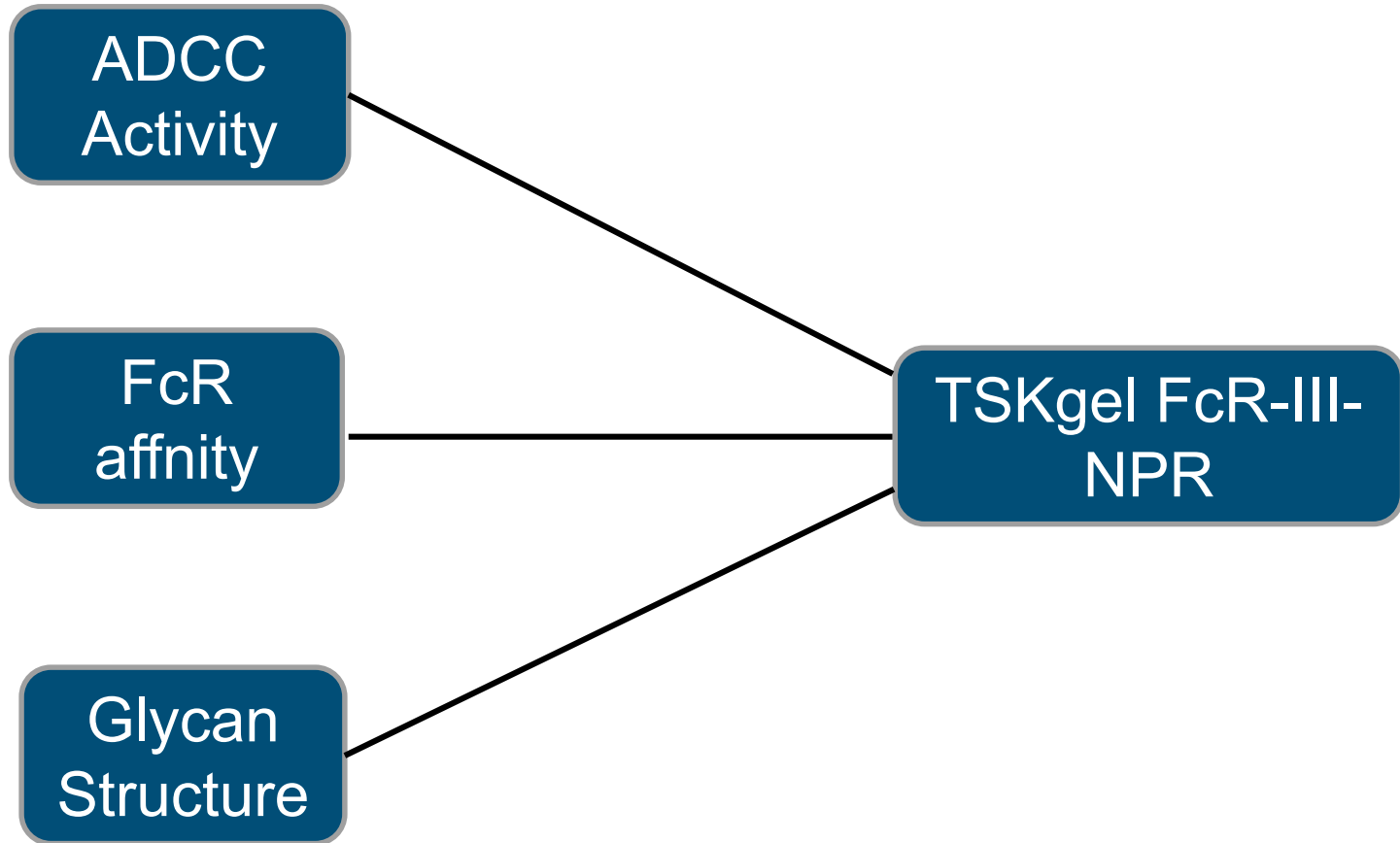


**Three different routes available for three different functions**





# TSKgel FcR-III-NPR Column Relates to All Three Functions



**TSKgel FcR-III-NPR column can be used for all three different functions**



# Applications of TSKgel FcR-III A-NPR

## Summary:

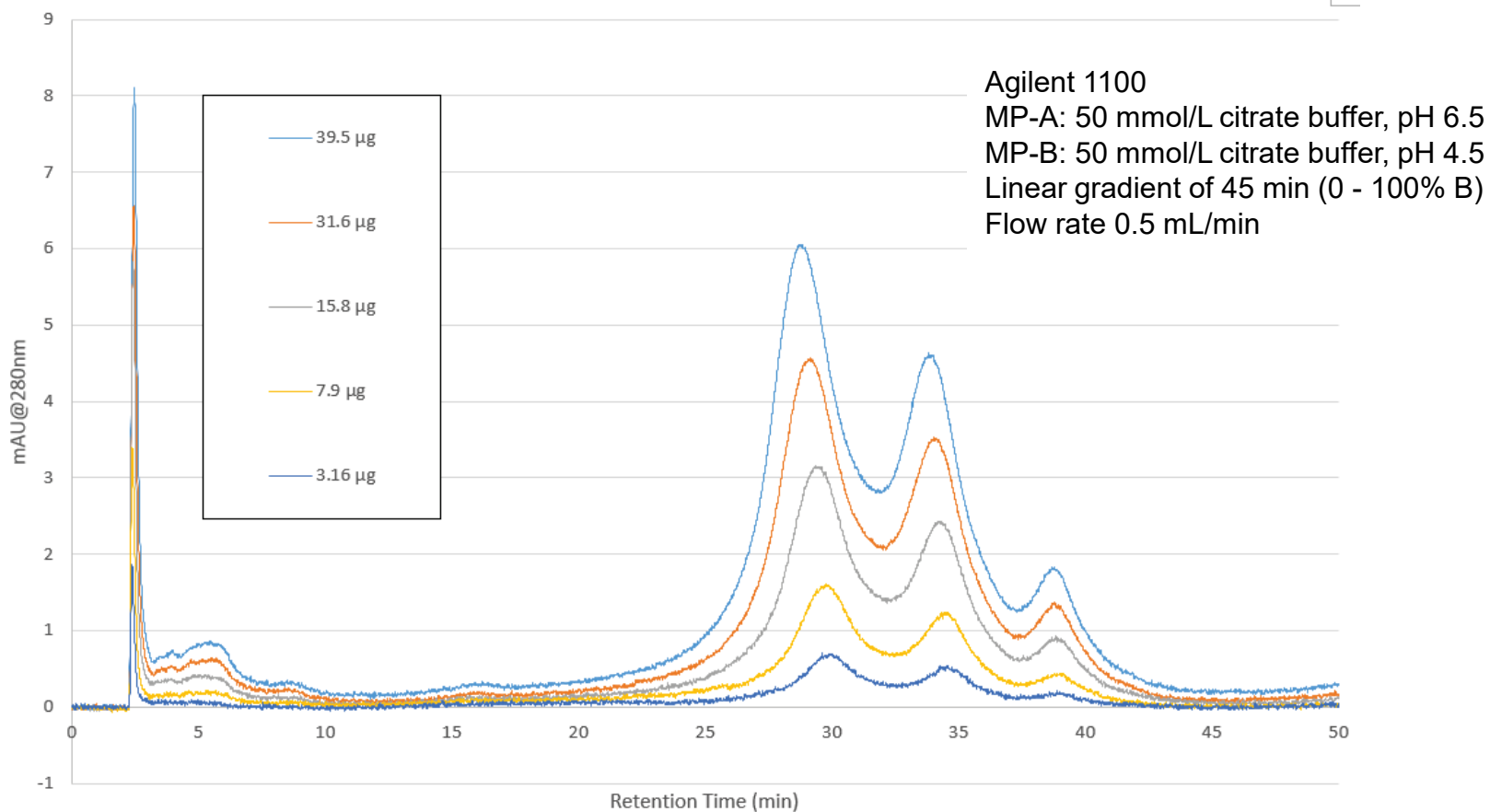
1. To monitor phases of development and production
2. Analysis of purified protein / mAb
3. Upstream monitoring of cell culture supernatant
4. Glycosylation profiling
5. QC analysis of lot-to-lot differences for mAb products
6. CHO cell line screening / to monitor fermentation stage of cell culture media
7. Comparison between biosimilar and innovator products
8. To correlate with ADCC activity



# Method Development

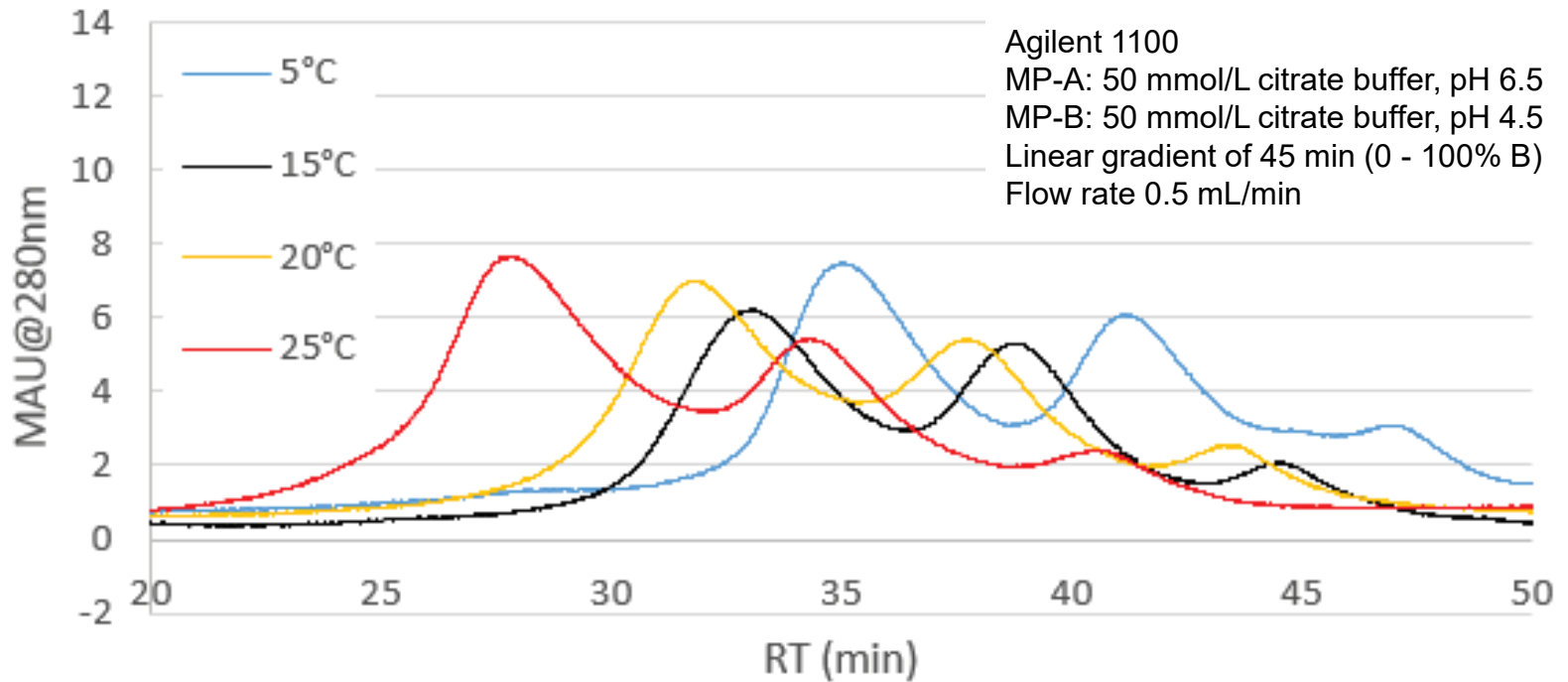


# Effect of Load ( $\mu\text{g}$ ) of mAb on the Separation of the Three Glycoforms



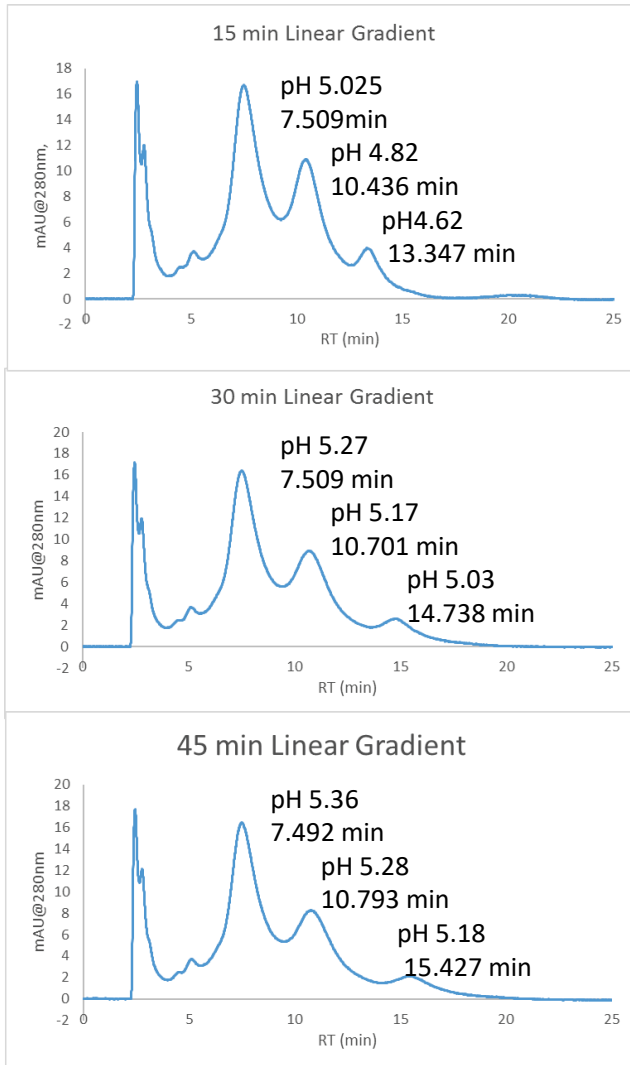


# Effect of Temperature on the Separation using TSKgel FcR-III A-NPR at 0.2 mL/min Flow Rate





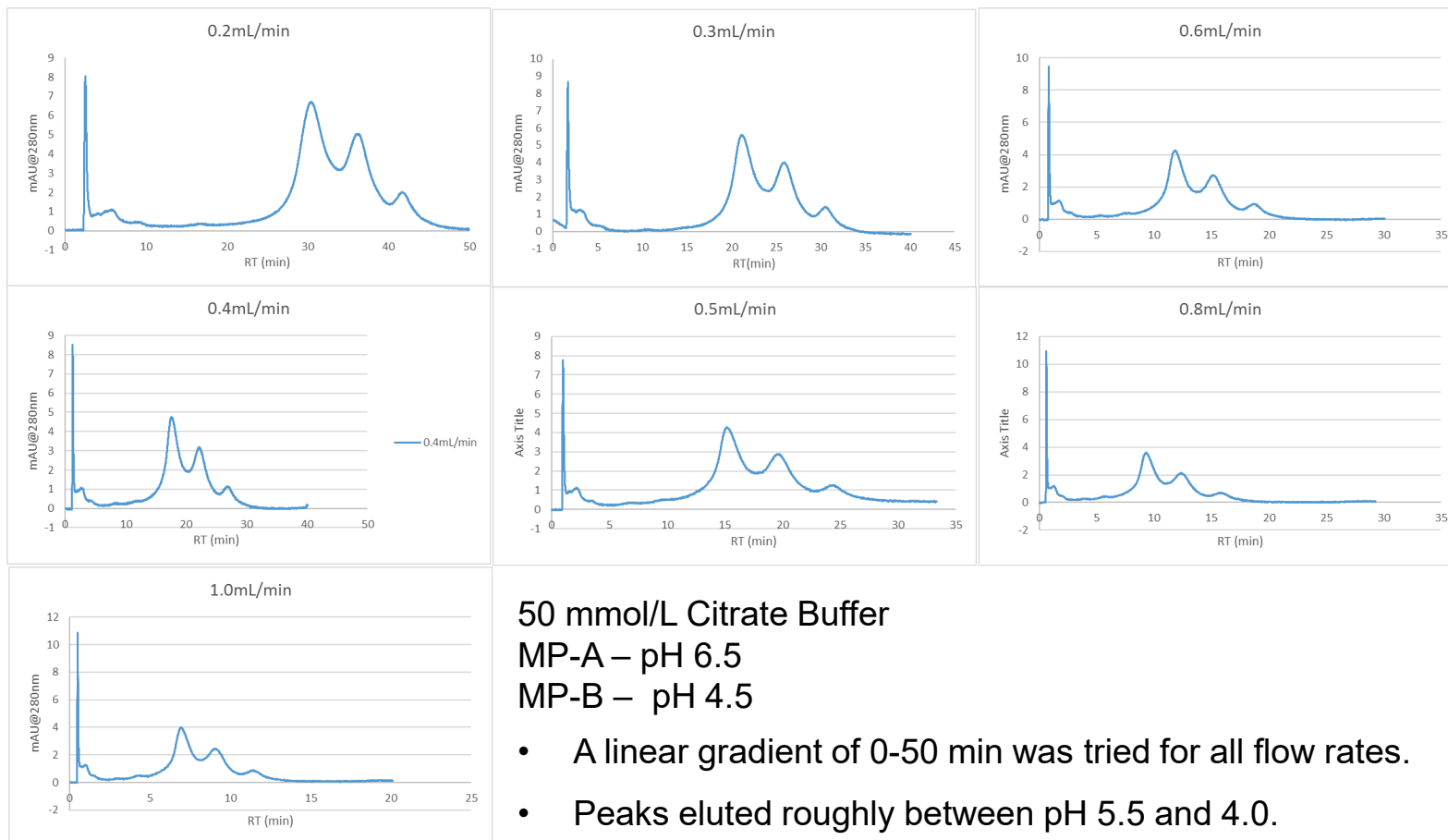
# Effect of Gradient Slope on the Resolution



Agilent 1200  
MP-A: 50 mmol/L citrate buffer, pH 5.53  
MP-B: 50 mmol/L citrate buffer, pH 4.5  
Linear gradient over 15, 30 and 45 minutes  
Flow rate 0.2 mL/min



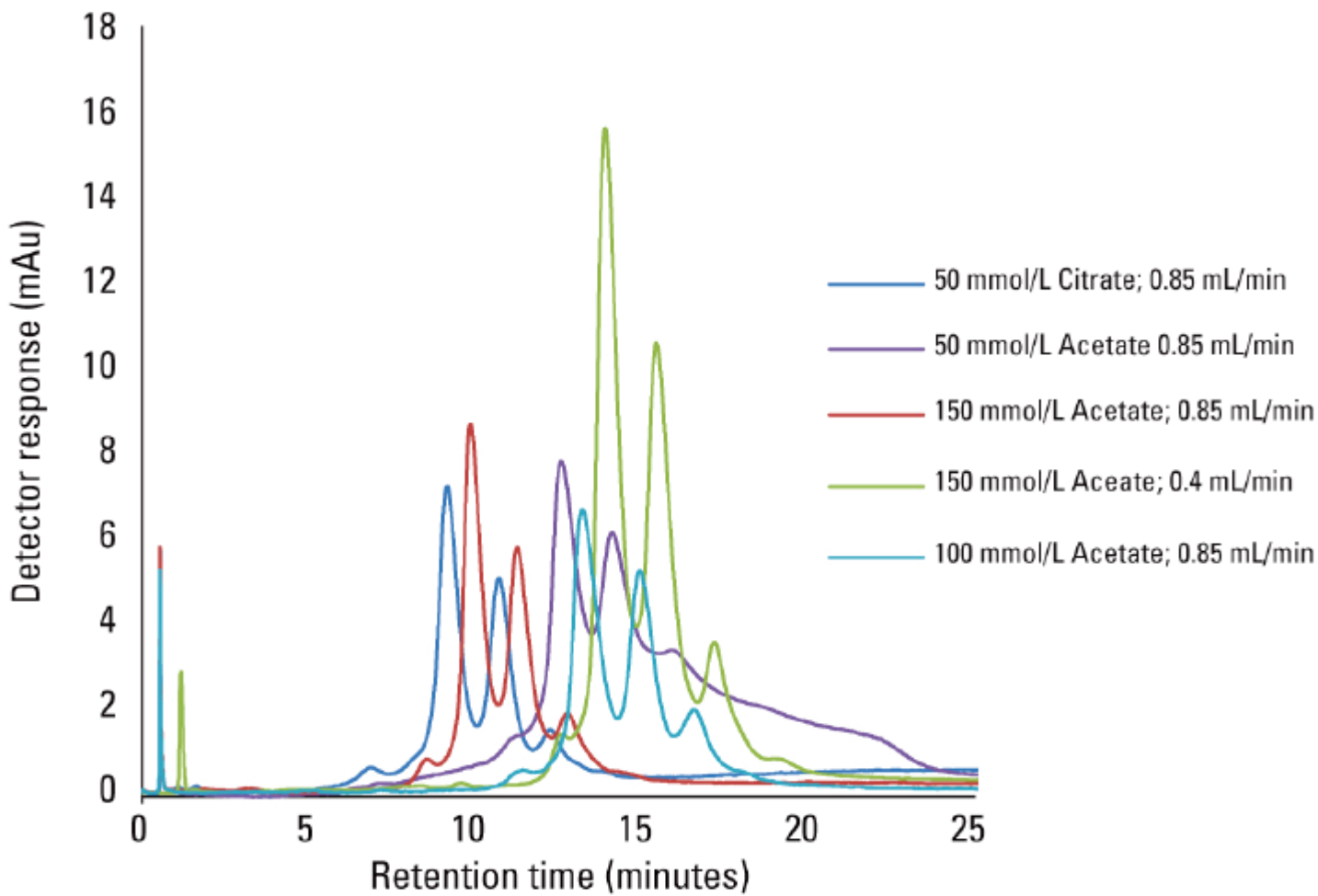
# Effect of Flow Rate



**All the 3 glycoform peaks eluted by 67% B with approximately same % recovery**



# Effect of Mobile Phase Conditions



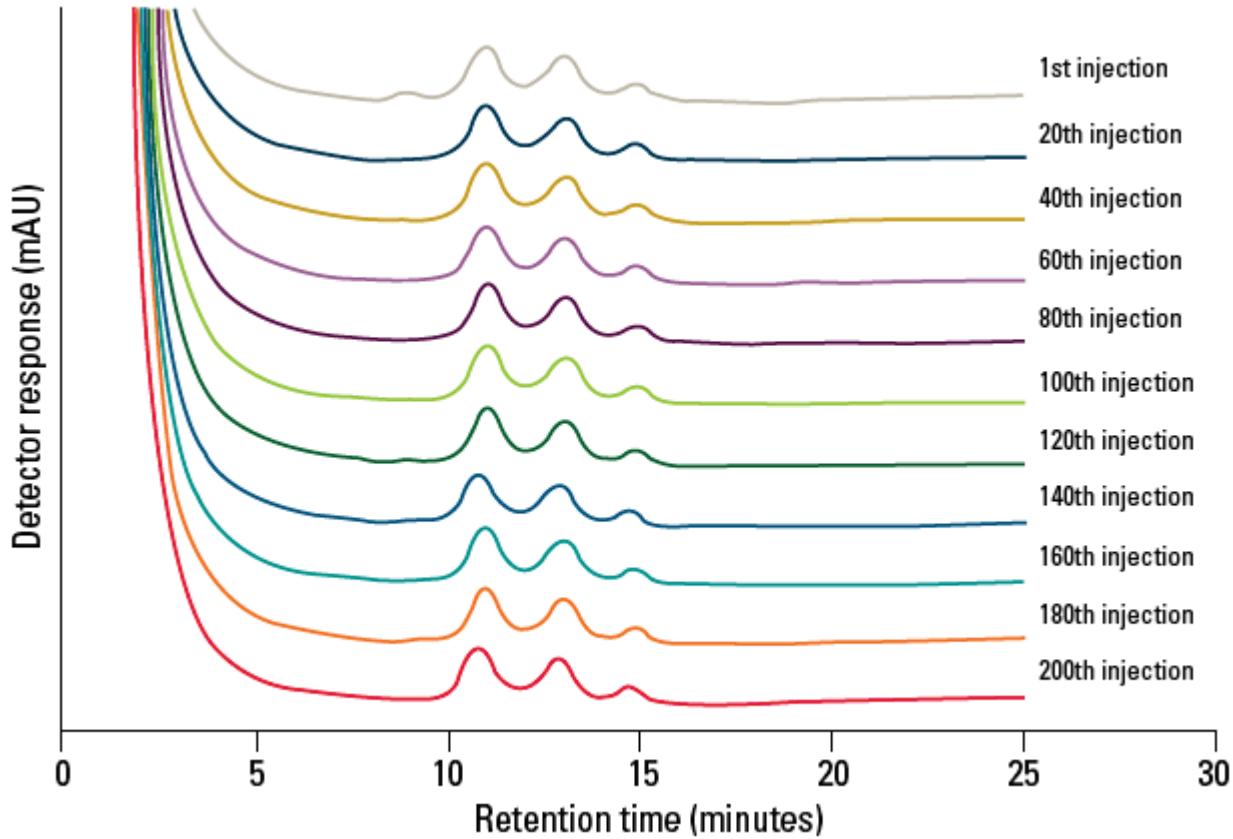




# Reproducibility

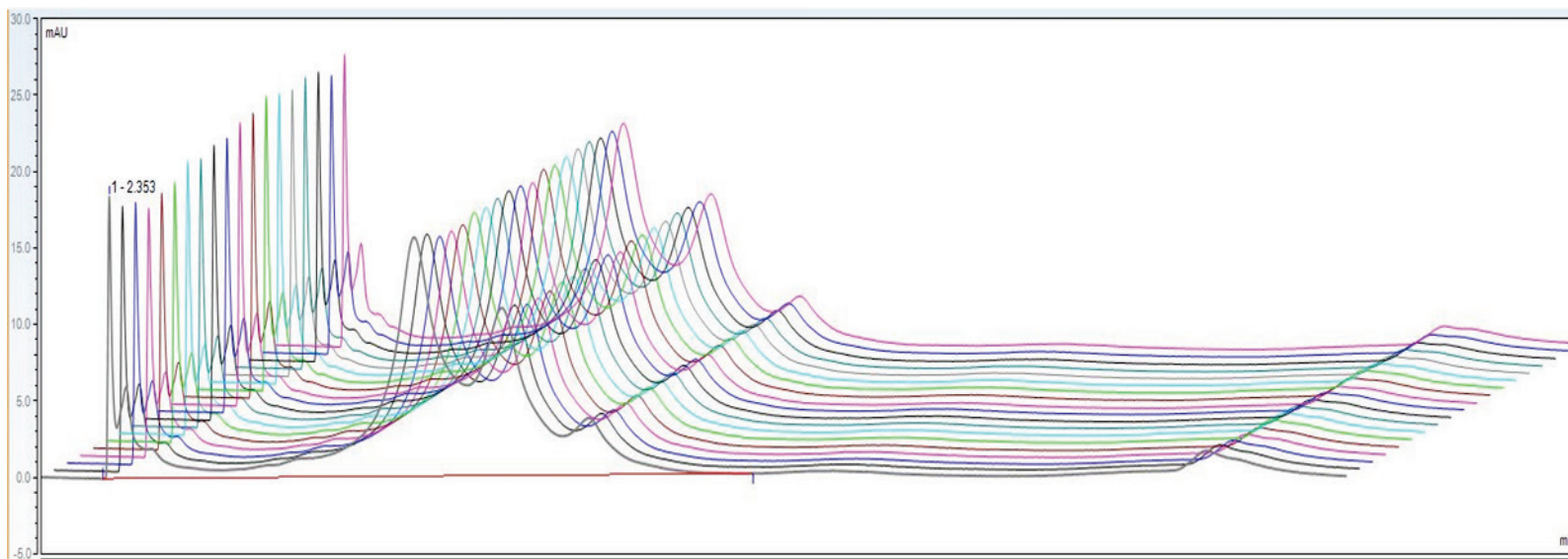
TOSOH

## CHO cell culture Supernatant





# Reproducibility



Inj #	Total Peak Area (mAU*min)
1	67.4902
10	67.747
20	68.0227
30	68.0441
40	66.823
50	68.0164
60	68.1039
70	67.7407
80	67.7407
90	66.8689
100	67.563
Average	67.651
STDEV	0.446
<b>%RSD</b>	<b>0.659</b>



TOSOH

# Conclusions



# Conclusions

- The TSKgel FcR-III A-NPR column offers an easy and rapid analysis method for therapeutic antibodies.
- A qualitative analysis of the TSKgel FcR-III A-NPR stationary phase confirms the elution profile and order mimic the results of cell based ADCC assay.
- The stationary phase is selective for N-glycosylation of the Fc region, which may be useful in other assessments of PTMs and primary structure.
- The TSKgel FcR-III A-NPR column is useful for quality control and process analysis.