# Glycosylation and Sialic Acid Analysis of Biotherapeutic Glycoproteins

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# Outline

Importance of N-glycan analysis for biotherapeutic development

Dye options for released N-glycan analysis

- InstantPC for HILIC (rapid, high FLD and MS signal)
- 2-AB for HILIC (well-established)

HILIC separations of labeled N-glycans

- FLD and MS detection

Sialic acid quantitation and analysis workflow

- Total sialic acid quantitation (plate based)
- Sialic acid species profiling (LC/FLD/MS)

# N-Glycans on Biotherapeutics

1/5$^{th}$ of all proteins in SwissProt are glycoproteins

More than 60% of biotherapeutics are glycosylated

N-Glycan structure can affect pharmacokinetics, pharmacodynamics, and immunogenicity

Glycosylation can be a critical quality attribute (CQA)

Even if not a CQA, glycosylation still monitored as a product quality attribute

Monoclonal antibody (mAb) and antibody-derived biotherapeutic proteins are glycosylated

Other molecules including blood factors (EPO, Factor VIII) also glycosylated

Khoury, et al., *Nat. Sci. Rep.* 1 (90), doi:10.1038/srep00090 (2011)
Planinc et al., *Anal. Chim. Acta* 921, 13–27 (2016)
Walsh, *Nat. Biotechnol.* 32(10), 992–1000 (2014)
Liu, *J. Pharm. Sci.* 104 (6), 1866–1884 (2015)

# Top Selling Pharmaceuticals (2010)

| | Trade name | Type | Main Indications | Company | Sales (USD millions/year) |
|---|---|---|---|---|---|
| 1 | Lipitor | Small molecule | Hypercholesterolemia, Dyslipidemia | Pfizer Inc. | 7,244 |
| 2 | Nexium | Small molecule | Symptomatic Gastroesophageal Reflux Disease | AstraZeneca Pharmaceuticals LP | 6,309 |
| 3 | Plavix | Small molecule | Reduction of atherosclerotic events | Bristol-Myers Squibb Company | 6,129 |
| 4 | Advair Diskus | Small molecule | Chronic obstructive pulmonary disease | GlaxoSmithKline | 4,711 |
| 5 | Abilify | Small molecule | Bipolar Mania | Bristol-Myers Squibb Company | 4,551 |
| 6 | Seroquel | Small molecule | Bipolar Depression | AstraZeneca Pharmaceuticals LP | 4,349 |
| 7 | Singulair | Small molecule | Asthma | Merck & Co., Inc. | 4,072 |
| 8 | Crestor | Small molecule | Hypercholesterolemia, dyslipidemia | AstraZeneca Pharmaceuticals LP | 3,758 |
| 9 | Actos | Small molecule | Type 2 diabetes | Takeda | 3,534 |
| 10 | Epogen | Biologic | Anemia | Amgen | 3,323 |

Glycosylated

- Only 1 glycosylated molecule in 2010 Top 10

*IMS Institute for Healthcare Informatics. The use of medicines in the United States: review of 2010.

# Top Selling Pharmaceuticals (2018)

| Rank | Drug | Trade names | Type | Main indications | Company | Sales (USD billions/year) |
|---|---|---|---|---|---|---|
| 1 | adalimumab | Humira | Biologic | Autoimmune | AbbVie | 19.936 |
| 2 | apixaban | Eliquis | Small molecule | Anticoagulation | Bristol-Myers Squibb/Pfizer | 9.872 |
| 3 | lenalidomide | Revlimid | Small molecule | MDS, multiple myeloma, MCL | Celgene | 9.685 |
| 4 | nivolumab | Opdivo | Biologic | Cancer (anti-PD-1 immunotherapy | Bristol-Myers Squibb/Ono Pharmaceutical | 7.570 |
| 5 | pembrolizumab | Keytruda | Biologic | Cancer (anti-PD-1 immunotherapy) | Merck & Co. | 7.171 |
| 6 | etanercept | Enbrel | Biologic | Autoimmune including RA | Amgen/Pfizer | 7.126 |
| 7 | trastuzumab | Herceptin | Biologic | Breast cancer | Roche (Genentech) | 6.981 |
| 8 | bevacizumab | Avastin | Biologic | Cancer types, AMD | Roche (Genentech) | 6.847 |
| 9 | rituximab | Rituxan, MabThera | Biologic | Autoimmune, cancer types | Roche (Genentech)/Biogen | 6.750 |
| 10 | rivaroxaban | Xarelto | Small molecule | Anticoagulation | Bayer/J&J | 6.589 |

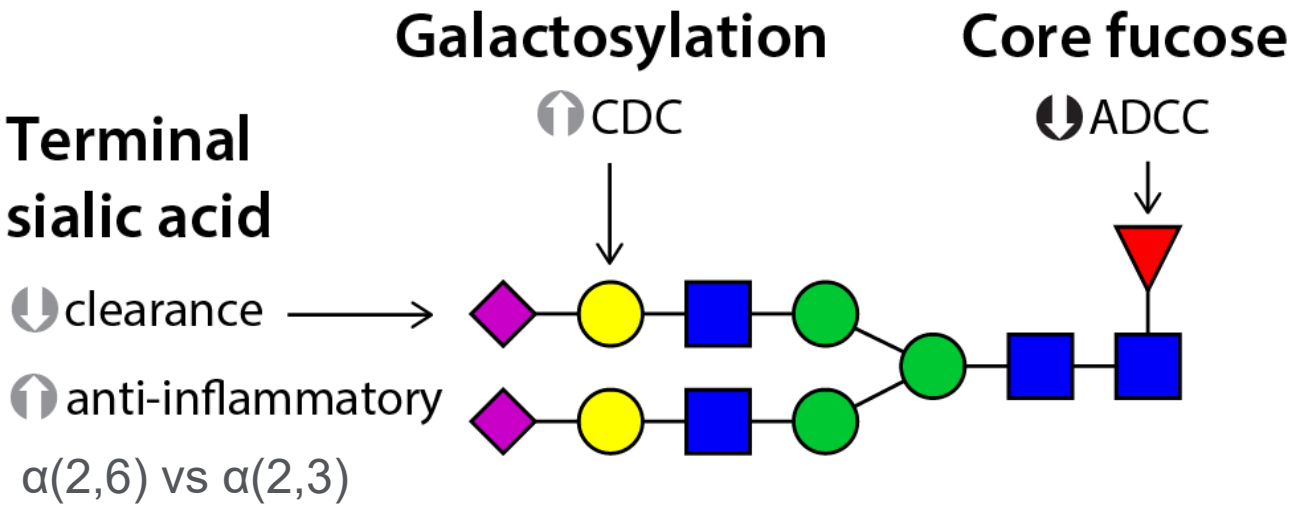
Glycosylated

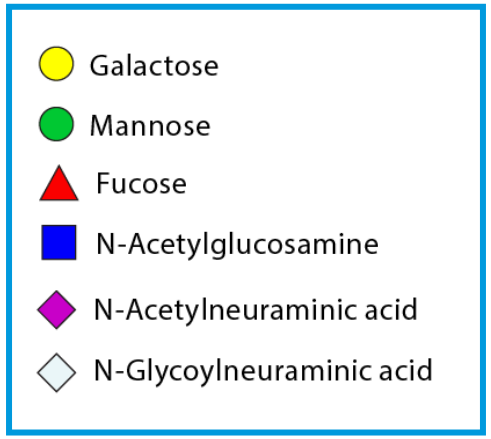
- 7/10 glycosylated biotherapeutics

Top-selling drugs are ranked based on sales or revenue reported for 2018 by bio/pharma companies in press announcements, annual reports, investor materials, and/or conference calls.

https://www.genengnews.com/a-lists/top-15-best-selling-drugs-of-2018/

# Effect of N-Glycan Structure on Biotherapeutics




- ADCC: Antibody-Dependent Cell-mediated Cytotoxicity
- CDC: Complement-Dependent Cytotoxicity

Reviewed in:

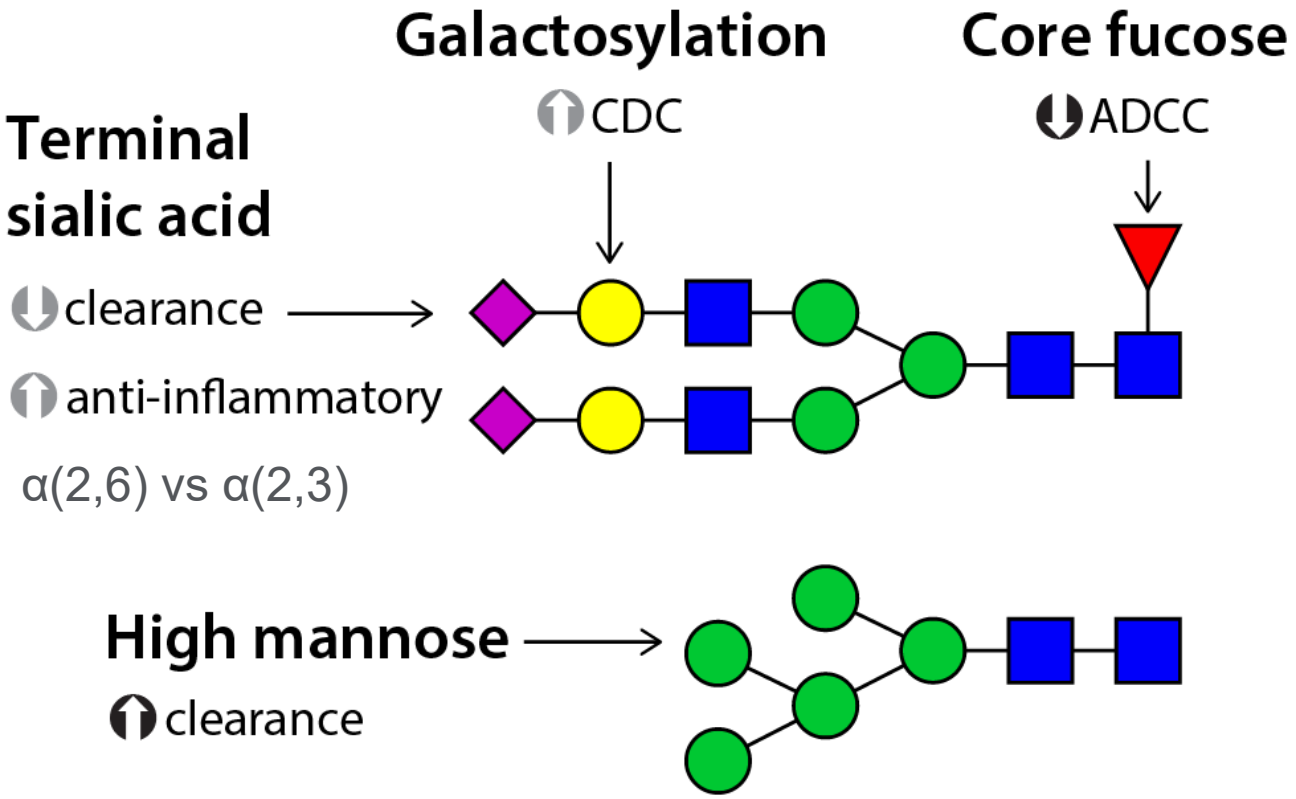

Jones, *BioPharm Intl*. 2017; 30:20-25

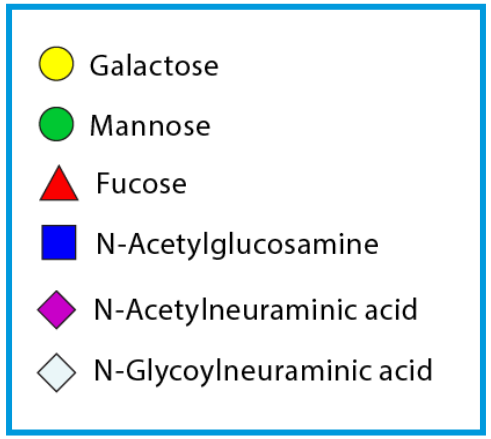
Higel et al., *Eur J Pharm Biopharm.* 2016; 100: 94

Liu, *J Pharm Sci.* 2015; 104: 1866-1884

Abes & Teillaud, *Pharmaceuticals* 2010; 3:146-57

# Effect of N-Glycan Structure on Biotherapeutics

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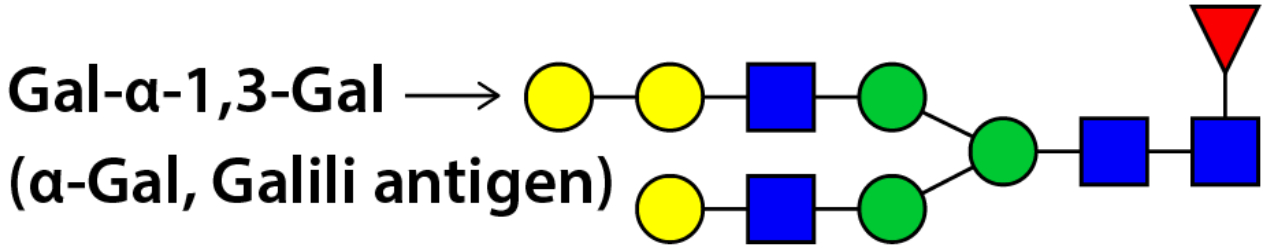
# Non-Human/Immunoreactive N-Glycans

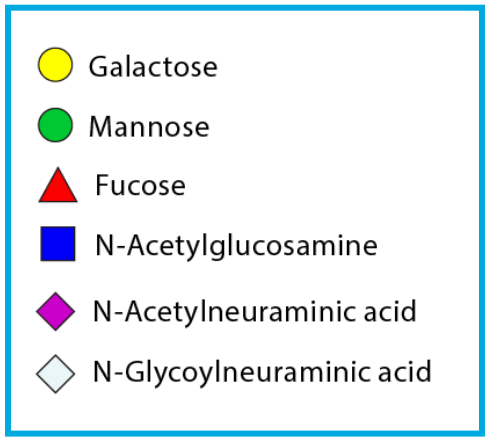



Cetuximab - N-glycans with α-Gal on Fab region

- Associated with adverse immunological responses

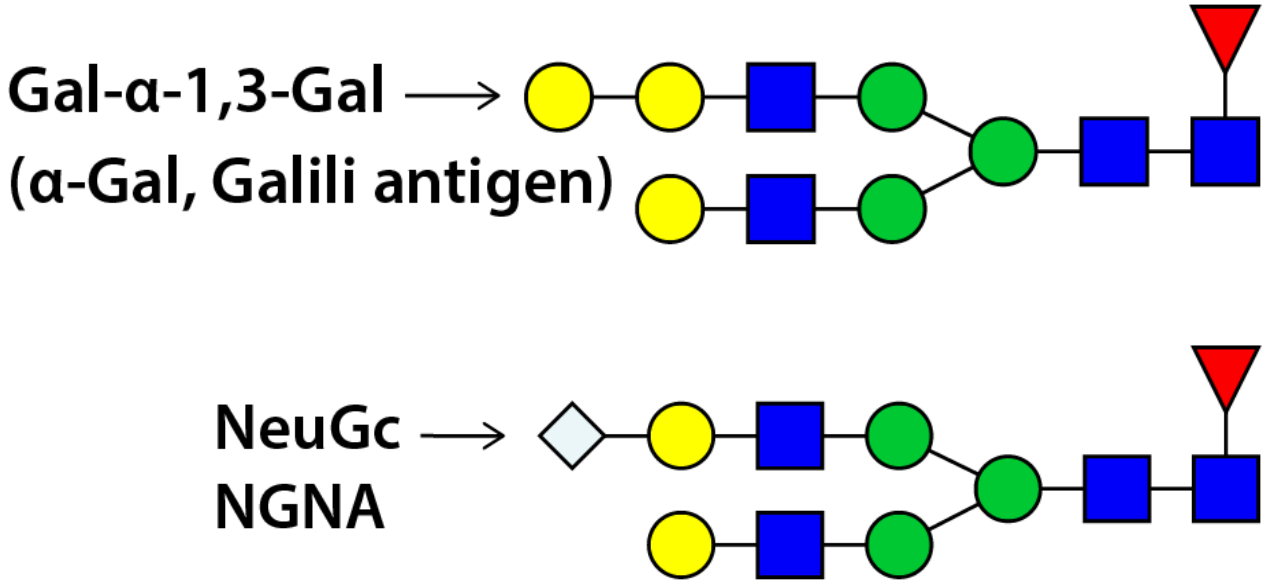
Abatacept - N-glycans with α-Gal on Fc region

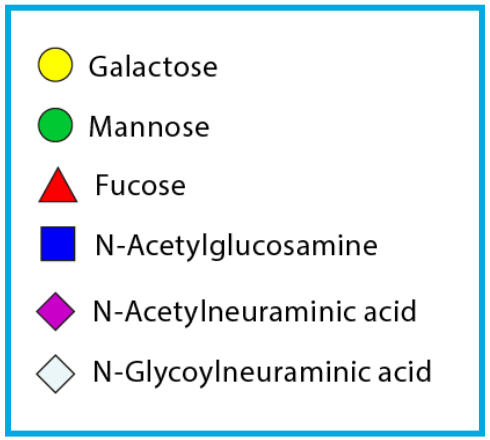
- No reported adverse effects




Macher & Galili, *Biochim Biophys Acta.* 2010; 1780 (2): 75-88
Van Bueren et al., *Nat Biotechnol.* 2011; 29: 574-576
Bosques et al., *Biotechnol.* 2010; 28: 1153-1156
Chung et al., *N Eng J Med.* 2008; 358(11): 1109-1117

# Non-Human/Immunoreactive N-Glycans

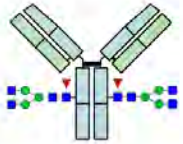
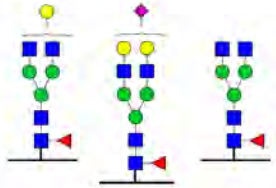
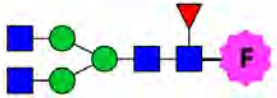
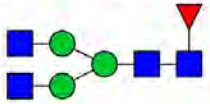



Potentially immunogenic

Ghaderi et al., *Biotechnol Genet Eng Rev.* 2012; 28:147-75

# N-Glycan Analysis Options

| Material | Structure | Analytical Methods |
|---|---|---|
| Intact glycoproteins |  | Electrophoresis, lectin arrays,<br>LC–ESI–MS: HRAM (+ reduction) |
| Glycopeptides |  | LC–MS, MALDI, CE |
| Released glycans | | LC–FLR, LC–MS, CE–LIF |
| ▸ Derivatized |  | Exoglycosidases (linkage assignment) |
| ▸ Underivatized |  | MALDI–TOF, HPAE–PAD |
| Monosaccharides | | |
| ▸ Derivatized |  | RP LC–FLR |
| ▸ Underivatized |  | HPAE–PAD |

Jones, *BioPharm Intl*. 2017; 30:20-25

# N-Glycan Analysis Options

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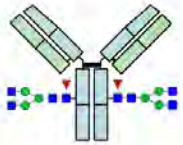
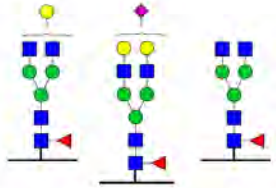
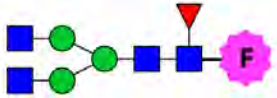
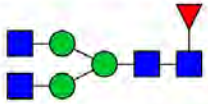
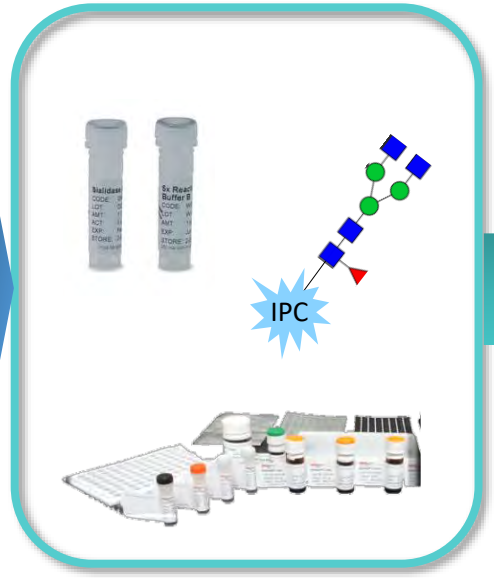
| Intact glycoproteins |  | Electrophoresis, lectin arrays,<br>LC–ESI–MS: HRAM (+ reduction) |
| Glycopeptides |  | LC–MS, MALDI, CE |
| Released glycans<br>▸ Derivatized |  | LC–FLR, LC–MS, CE–LIF<br>Exoglycosidases (linkage assignment) |
| ▸ Underivatized |  | MALDI–TOF, HPAE–PAD |
| Monosaccharides<br>▸ Derivatized |  | RP LC–FLR |
| ▸ Underivatized |  | HPAE–PAD |

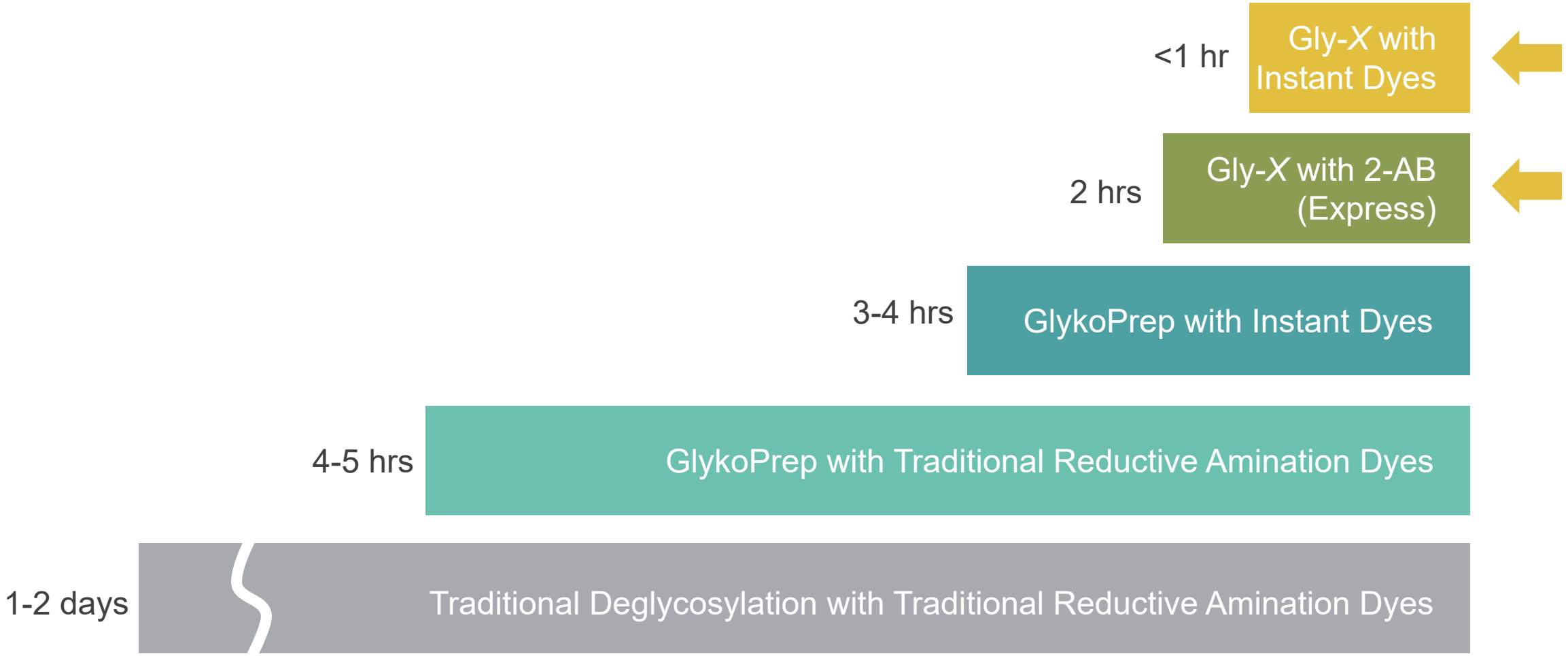
Relative % area of glycan species

Sialic acid quantitation

# Agilent Released N-Glycan Workflow

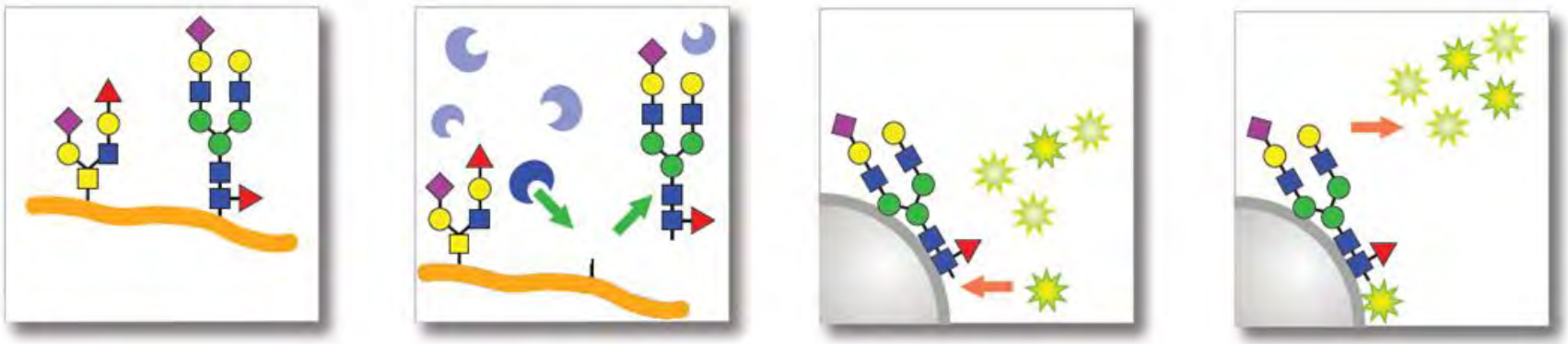
| Automation | Sample Prep | Separation | Detection | Data Processing & Report |
|---|---|---|---|---|
|  |  |  |  |  |
| AssayMAP Bravo Liquid Handler<br>GlykoPrep<br>InstantPC, InstantAB 2-AB, APTS | Glycan analysis reagents<br>Gly-X InstantPC<br>Gly-X 2-AB Express<br>Enzymes<br>Standards | 1260 Infinity II Bio-Inert<br>1290 Infinity II LC System<br>AdvanceBio Glycan Mapping Columns (HILIC) | 6545XT AdvanceBio<br>Q-TOF<br>(or equivalent MS) | MassHunter BioConfirm B.10.00 Software<br>Tagged Library<br>Dedicated Released Glycan (IPC) Workflow |

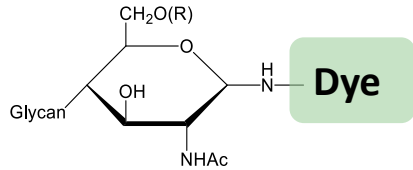
# N-Glycan Sample Prep Evolution



# Gly-*X* N-Glycan Sample Prep Technology

1-40 µg glycoprotein sample*, 0.05-2 mg/ml






Denature
3 min at 90 °C

Deglycosylate
PNGase F, 5 min at 50 °C

Label
InstantDye, 1 min
*InstantPC, InstantQ, InstantAB*
Reductive amination dye, 60 min
(no dry down)
*2-AB, APTS*

Clean up
15-20 min at RT

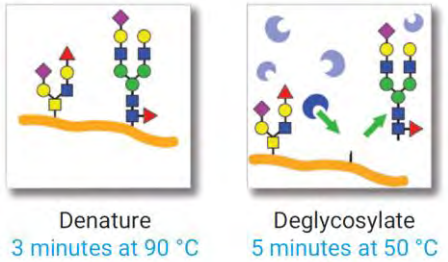
Labeled N-Glycans
LC/FLD, LC/MS, CE

Workflow time: 45 min – 120min (16 samples)

*Loading range depends on protein, can go up to 100 µg for mAbs e.g. Rituxan

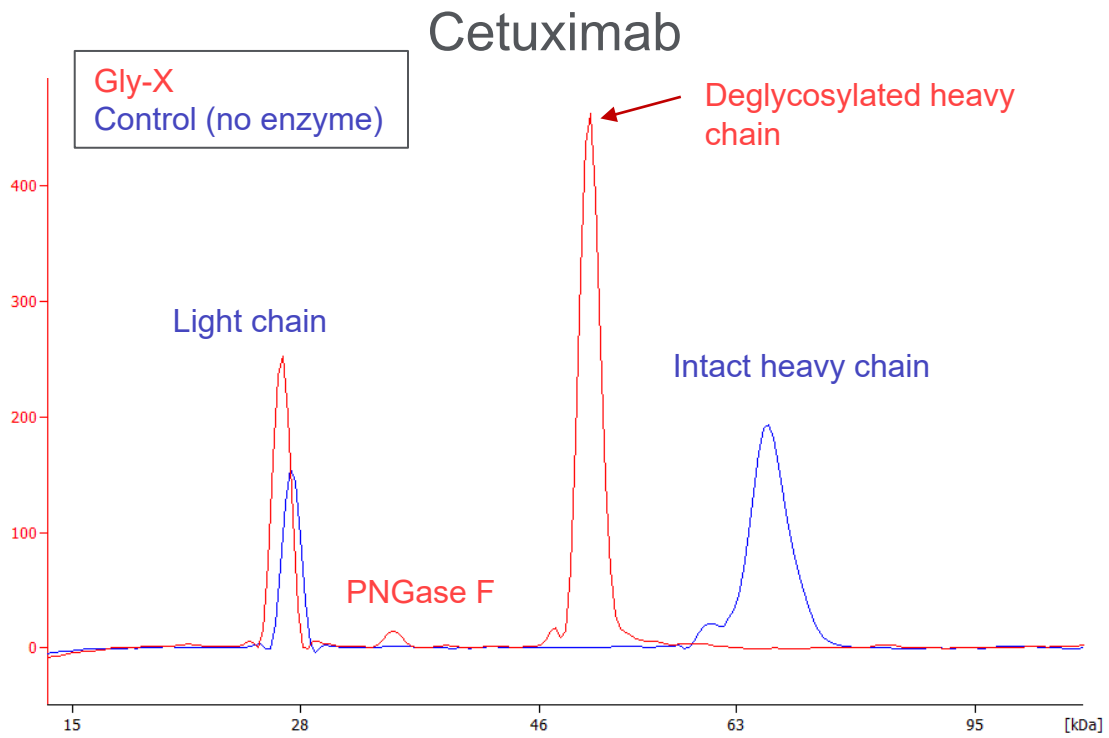
# Novel In-Solution Enzymatic Protein Denaturation & Deglycosylation

Gly-X in-solution 5-minute deglycosylation with PNGase F achieves > 99% N-glycan release for most proteins tested



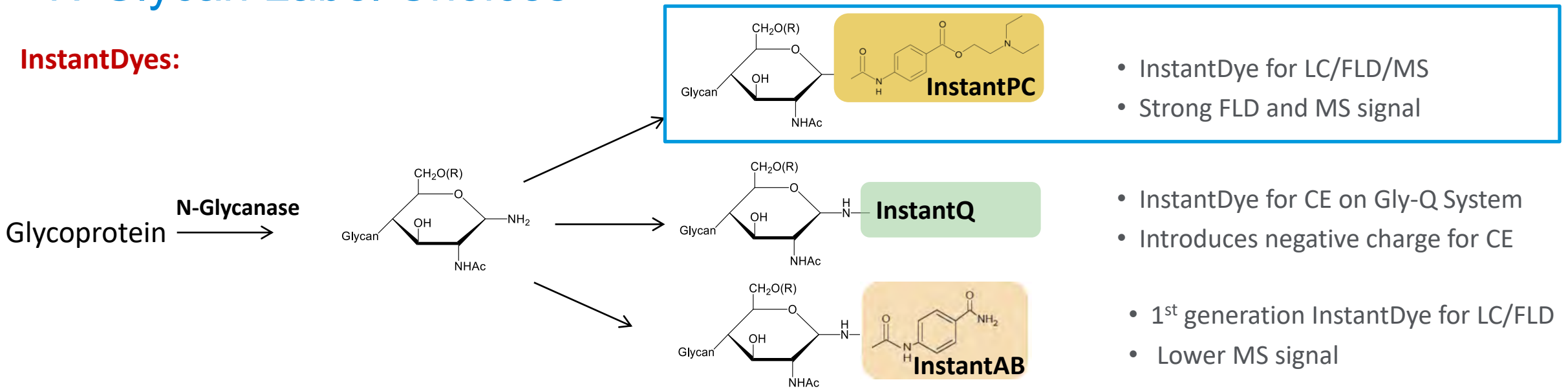

| Protein | % Deglycosylation |
|---|---|
| Rituximab | >99% |
| Cetuximab | >99% |
| Enbrel | >99% |
| Zaltrap | >99% |

Analytical Method:
Agilent 2100 Bioanalyzer, Protein 230 Kit
(electropherograms not normalized)



# N-Glycan Label Choices

## InstantDyes:

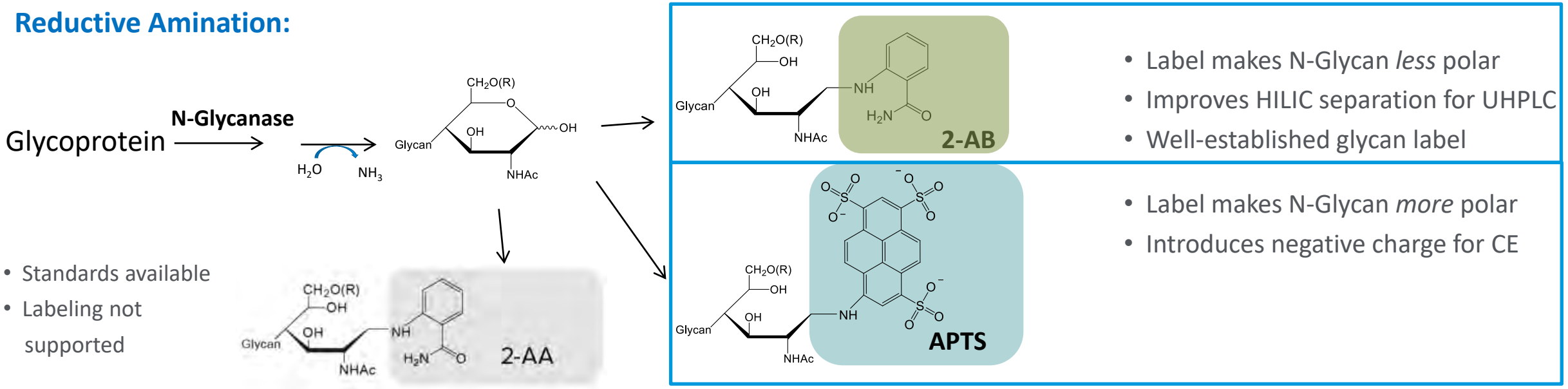



- InstantDye for LC/FLD/MS
- Strong FLD and MS signal

- InstantDye for CE on Gly-Q System
- Introduces negative charge for CE

- 1st generation InstantDye for LC/FLD
- Lower MS signal

## Reductive Amination:

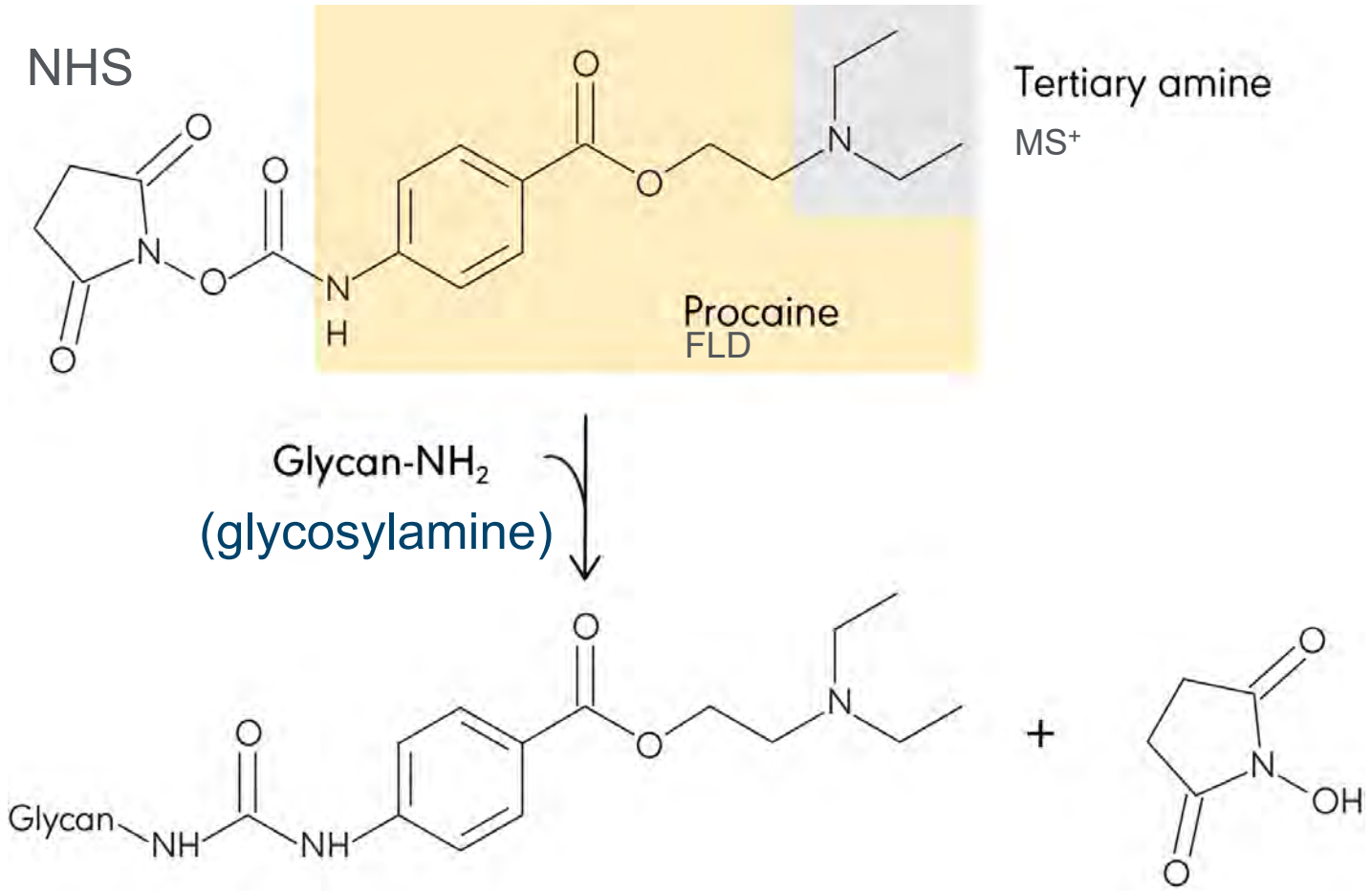


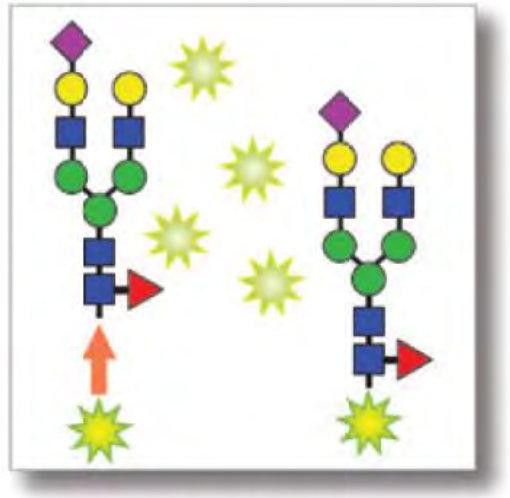

- Label makes N-Glycan *less* polar
- Improves HILIC separation for UHPLC
- Well-established glycan label

- Label makes N-Glycan *more* polar
- Introduces negative charge for CE

- Standards available
- Labeling not supported

# InstantPC Dye (IPC)

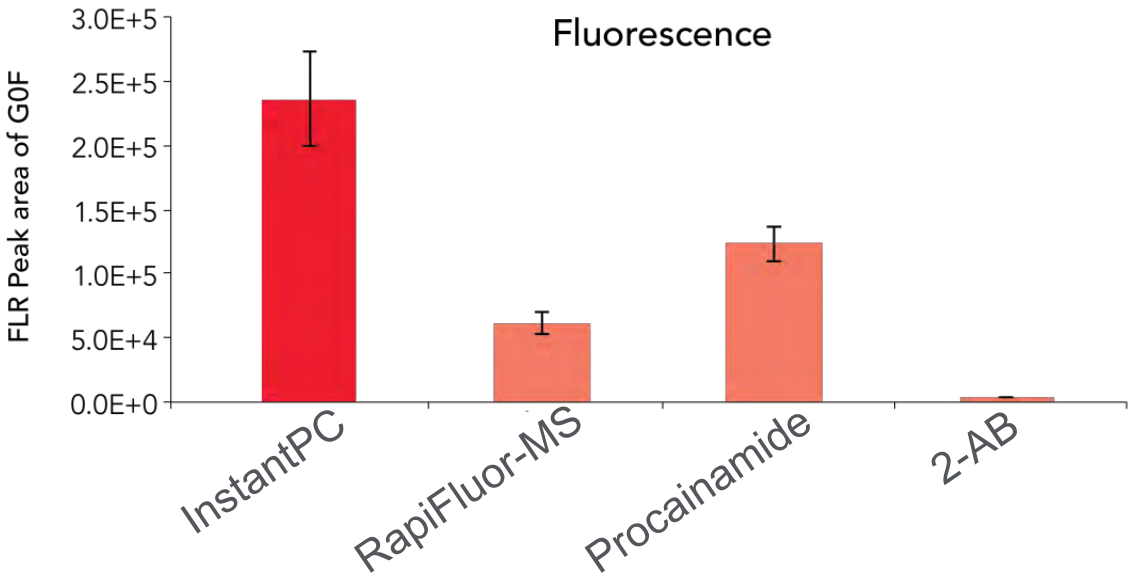


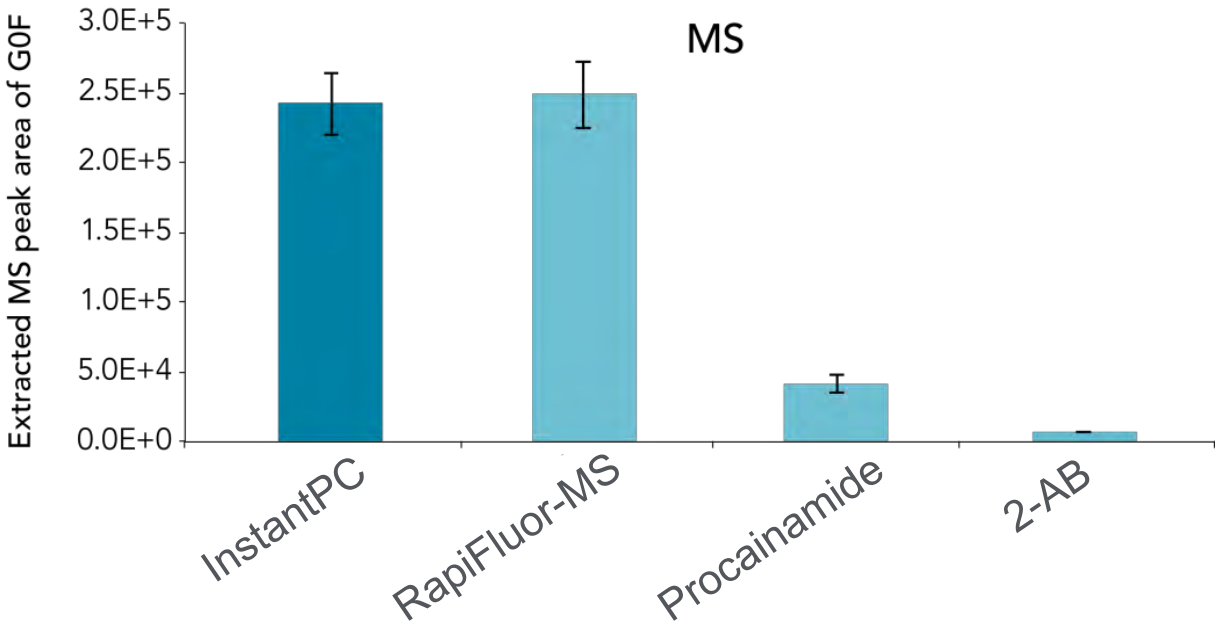



Label
1 minute at 50 °C

Workflow time: 45 min (16 samples), 100 min (96 samples)

# FLD and MS Response Comparison




Labeling uses same amount of released glycans from GlykoPrep digestion

*Reference: ProZyme Poster Presentation at ASMS, St. Louis, MO, May 31 - June 4, 2015*

# UHPLC-HILIC Separation of Labeled N-Glycans

Separation

UHPLC

Detection

Fluorescence

- InstantPC: ex/em 285/345 nm
- 2-AB: ex/em 260/430 nm

Q-TOF

- LC/MS mass confirmation for glycan ID
- Positive mode

## Amide HILIC column

- 1.8 µm fully porous for speed and performance (1200 bar)
- 2.7 µm superficially porous for resolution at lower pressures (600 bar)
- Unique hydrophilic bonding
- MS and FLD compatibility

## HILIC: hydrophilic interaction liquid chromatography Example Method: 1 hour

| Time (min) | %A | %B | Flow (ml/min) |
|---|---|---|---|
| 0.00 | 20.0 | 80.0 | 0.50 |
| 2.00 | 25.0 | 75.0 | 0.50 |
| 48.00 | 38.0 | 62.0 | 0.50 |
| 49.00 | 60.0 | 40.0 | 0.50 |
| 51.50 | 60.0 | 40.0 | 0.50 |
| 52.00 | 18.0 | 82.0 | 0.50 |
| 60.00 | 18.0 | 82.0 | 0.50 |

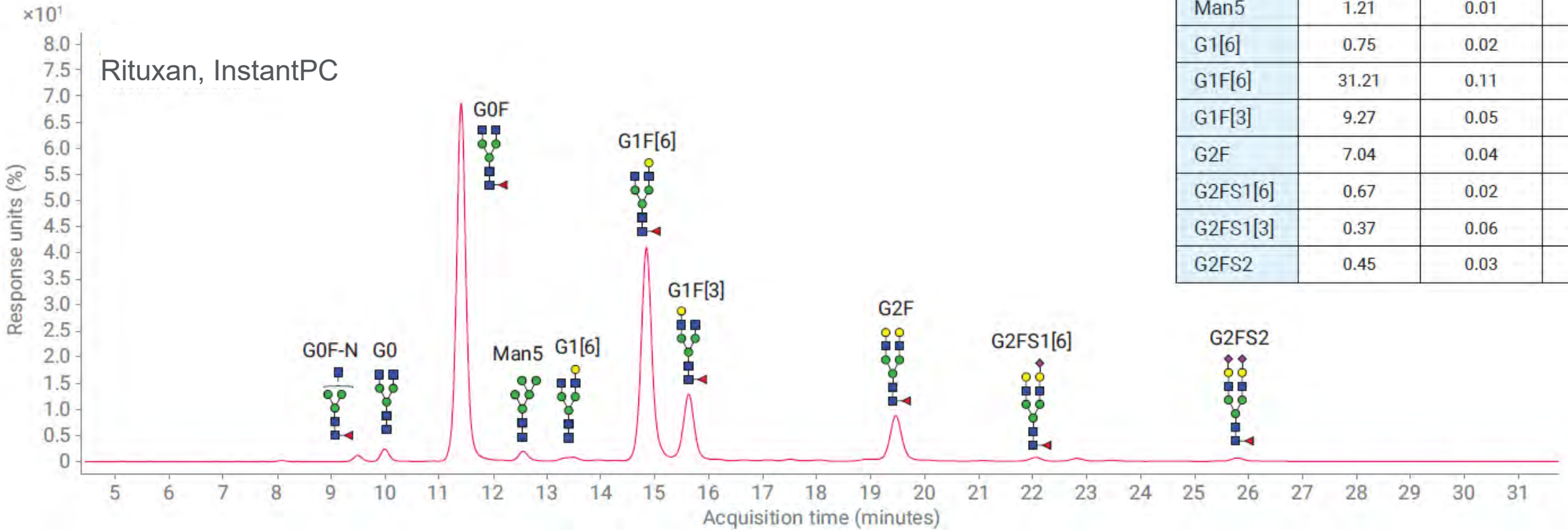
AdvanceBio Glycan Mapping Column
2.1 x 150 mm, 1.8 µm

A: 50 mM ammonium formate, pH 4.5
B: Acetonitrile

Column temperature: 40 °C

# Rituximab InstantPC Glycans

| | Average Rel % Area | Standard Deviation | %CV |
|---|---|---|---|
| G0F-N | 0.75 | 0.01 | 1.55 |
| G0 | 1.47 | 0.02 | 1.18 |
| G0F | 46.82 | 0.07 | 0.15 |
| Man5 | 1.21 | 0.01 | 0.83 |
| G1[6] | 0.75 | 0.02 | 2.67 |
| G1F[6] | 31.21 | 0.11 | 0.35 |
| G1F[3] | 9.27 | 0.05 | 0.54 |
| G2F | 7.04 | 0.04 | 0.51 |
| G2FS1[6] | 0.67 | 0.02 | 2.29 |
| G2FS1[3] | 0.37 | 0.06 | 15.98 |
| G2FS2 | 0.45 | 0.03 | 6.67 |




UHPLC-HILIC, 60 minute method

AdvanceBio Glycan Mapping column

2.1 x 150 mm, 1.8 µm

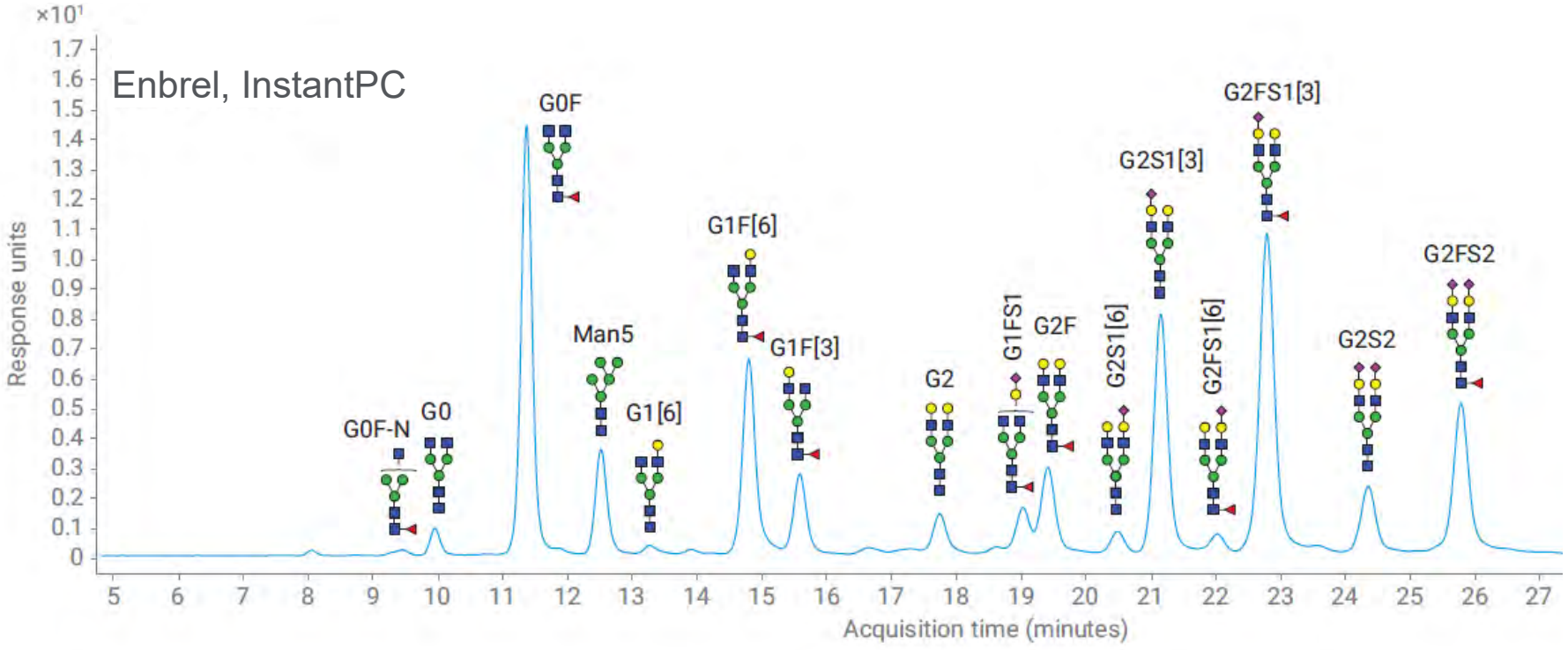
FLD: 285/345 nm

1 µl injection (equivalent to glycans from 0.4 µg protein)

N = 4

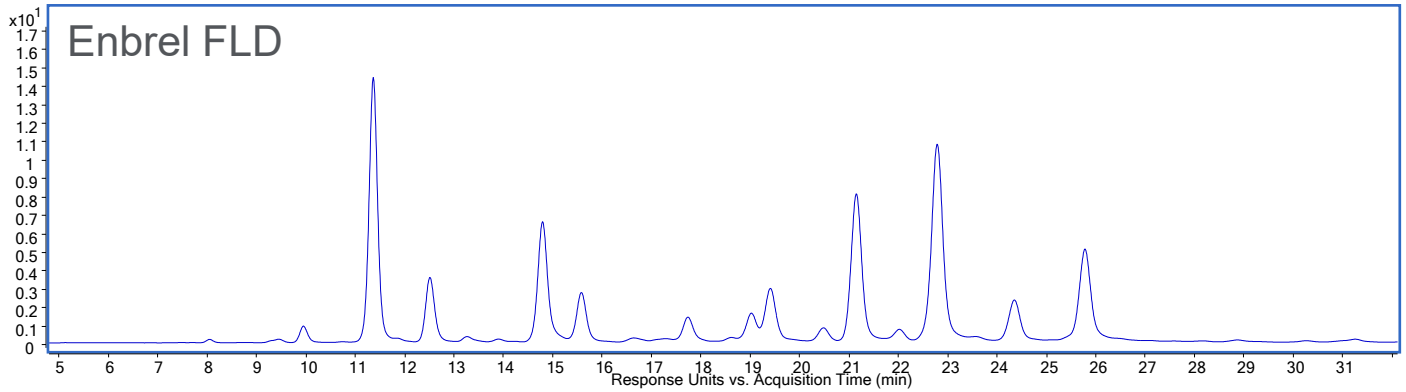
# Etanercept InstantPC N-glycans

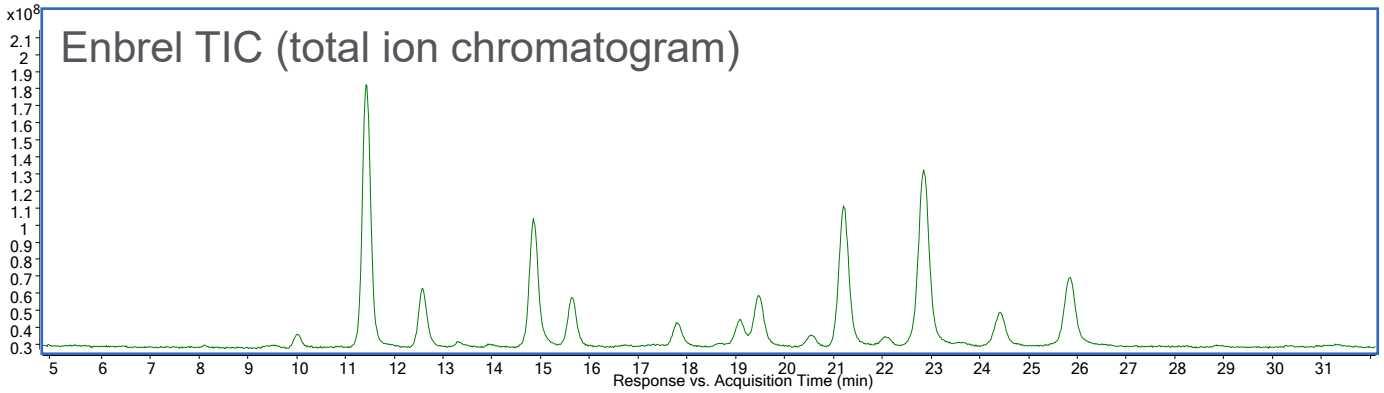
- Etanercept: Fc fusion protein
- Tumor necrosis factor receptor (TNFR) fused to Fc of IgG1
- 3 N-glycan sites: 1 in Fc, 2 in receptor
- N = 4




| | Average Rel % Area | Standard Deviation | %CV |
|---|---|---|---|
| G0 | 1.10 | 0.02 | 2.09 |
| G0F | 19.36 | 0.16 | 0.84 |
| Man5 | 5.08 | 0.03 | 0.52 |
| G1[6] | 0.48 | 0.00 | 0.00 |
| G1F[6] | 10.48 | 0.04 | 0.39 |
| G1F[3] | 3.97 | 0.01 | 0.25 |
| G2 | 2.08 | 0.01 | 0.55 |
| G1FS1 | 1.84 | 0.05 | 2.49 |
| G2F | 4.26 | 0.09 | 1.99 |
| G2S1[6] | 1.18 | 0.01 | 0.49 |
| G2S1[3] | 13.91 | 0.04 | 0.31 |
| G2FS1[6] | 0.89 | 0.00 | 0.00 |
| G2FS1[3] | 20.54 | 0.08 | 0.37 |
| G2S2 | 4.26 | 0.01 | 0.14 |
| G2FS2 | 10.54 | 0.08 | 0.78 |

# MS of InstantPC N-Glycans from Enbrel




Fluorescence

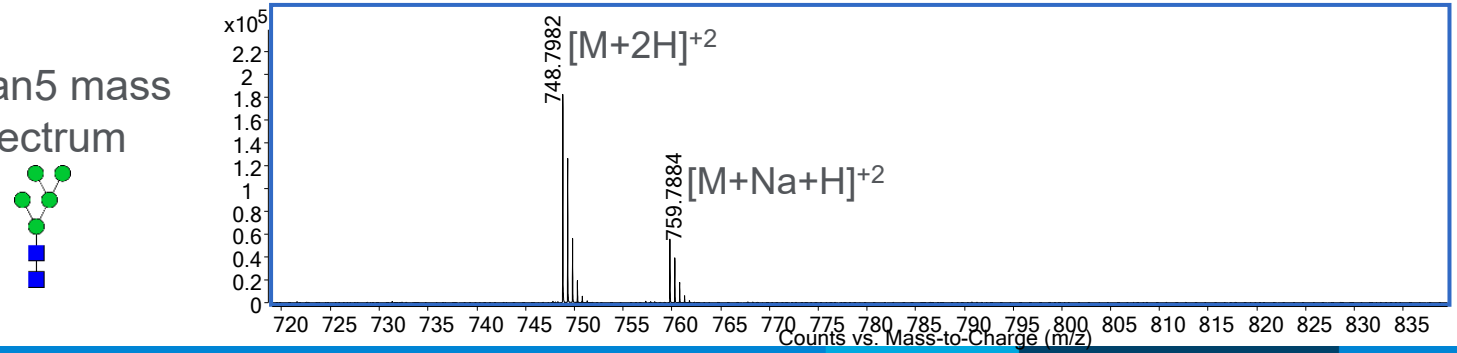
Q-TOF 6545XT

(or equivalent MS)

Positive mode MS:

Most biantennary InstantPC N-glycans $[M+2H]^{2+}$, larger sialylated will be majority $[M+3H]^{3+}$

Man5 mass spectrum



# InstantPC N-Glycan Standards & Libraries

## Individual N-Glycans

| Product Code | Description |
|---|---|
| GKPC-401 | G0-N |
| GKPC-301 | G0 |
| GKPC-402 | G0F-N |
| GKPC-302 | G0F |
| GKPC-317 | G1 |
| GKPC-316 | G1F |
| GKPC-304 | G2 |
| GKPC-305 | G2F |
| GKPC-329 | G1S1 (α2,3) |
| GKPC-319 | G1S1 (α2,6) |
| GKPC-330 | G1FS1 (α2,3) |
| GKPC-320 | G1FS1 (α2,6) |
| GKPC-321 | A1 (α2,3) |

| Product Code | Description |
|---|---|
| GKPC-311 | A1 (α2,6) |
| GKPC-325 | A1F (α2,3) |
| GKPC-315 | A1F (α2,6) |
| GKPC-322 | A2 (α2,3) |
| GKPC-312 | A2 (α2,6) |
| GKPC-323 | A2F (α2,3) |
| GKPC-313 | A2F (α2,6) |
| GKPC-103 | Man5 |
| GKPC-104 | Man6 |
| GKPC-105 | Man7 |
| GKPC-106 | Man8 |
| GKPC-107 | Man9 |

***New*** Alpha Gal

## N-Glycan Libraries

| Product Code | Description |
|---|---|
| GKPC-005 | Human IgG N-Linked Glycan Library |
| GKPC-503 | Glucose Homopolymer |
| GKPC-233 | α(2-3) Sialylated Triantennary Library |
| GKPC-263 | α(2-6) Sialylated Triantennary Library |
| GKPC-234 | α(2-3) Sialylated Tetraantennary Library |
| GKPC-264 | α(2-6) Sialylated Tetraantennary Library |

***New*** CHO mAb glycoprotein & N-glycan library

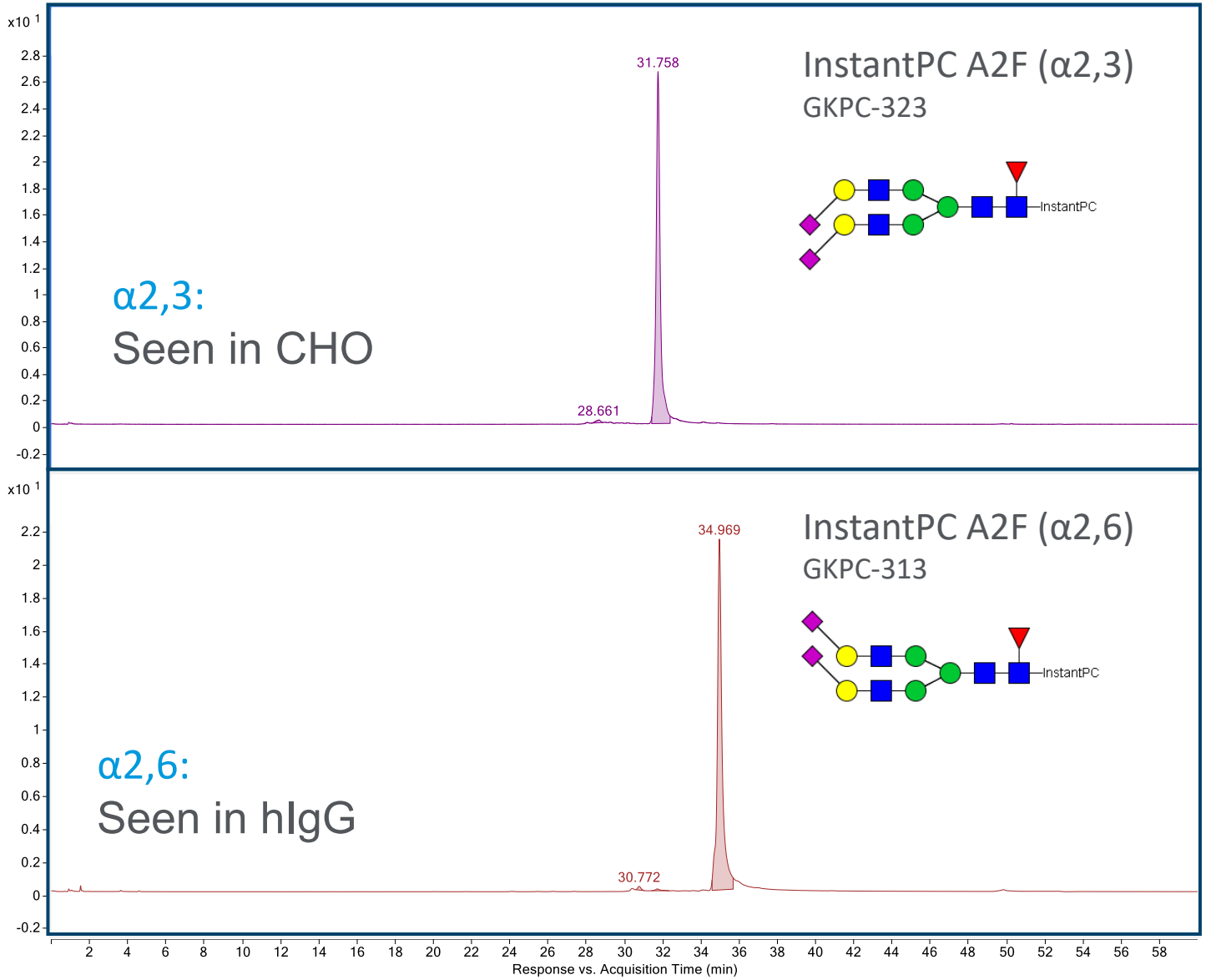
**Labeled Glycan Standards**

2-AB, 2-AA, APTS, InstantPC, InstantAB, InstantQ

Learn more: **www.agilent.com/chem/glycananalysis**

Glycan Standards Flier 5994-0999EN

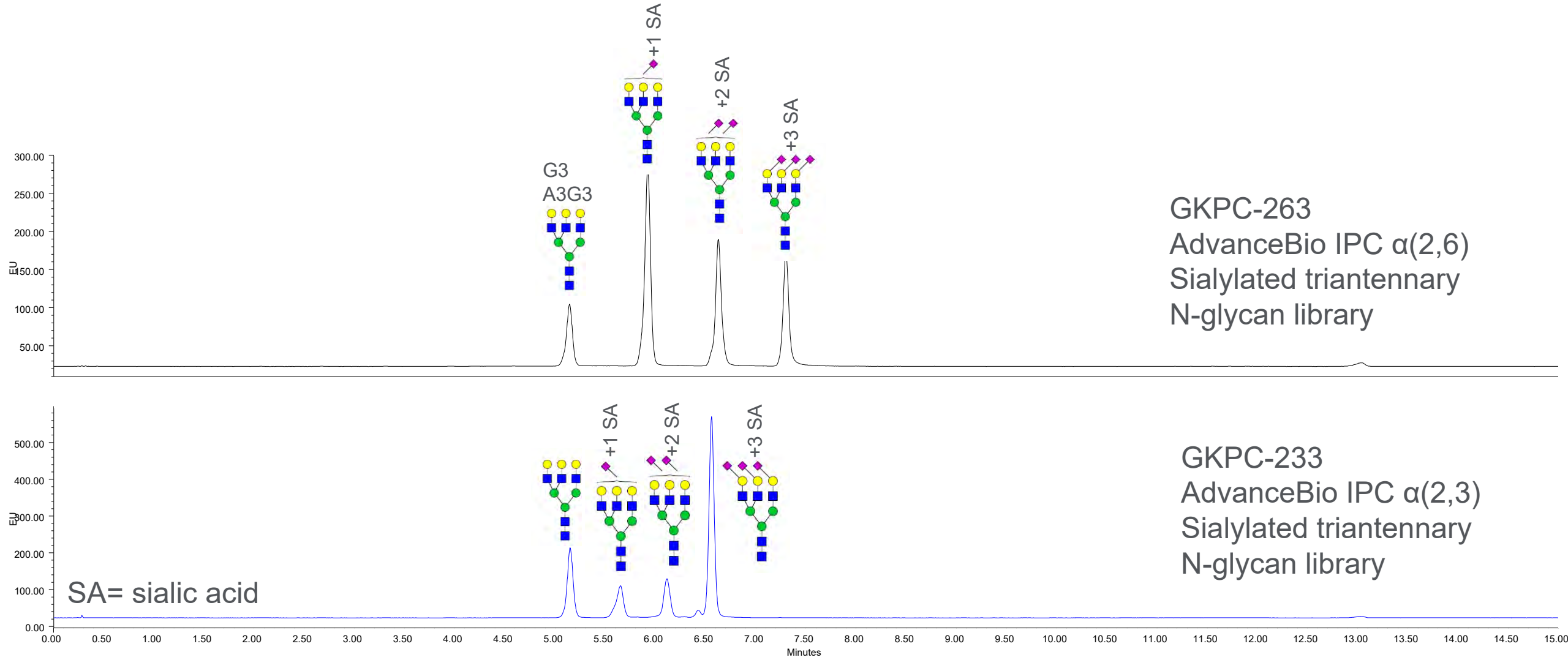
# InstantPC Sialylated N-Glycan Standards




- **CHO glycoproteins**: α(2,3)-linked sialic acid [1]
- **Human IgG**: α(2,6)-linked sialic acid [2]
- **α(2,3)**-sialylated N-glycans have a shorter HILIC retention time than isomeric N-glycans with **α(2,6)** sialic acid linkages [3]

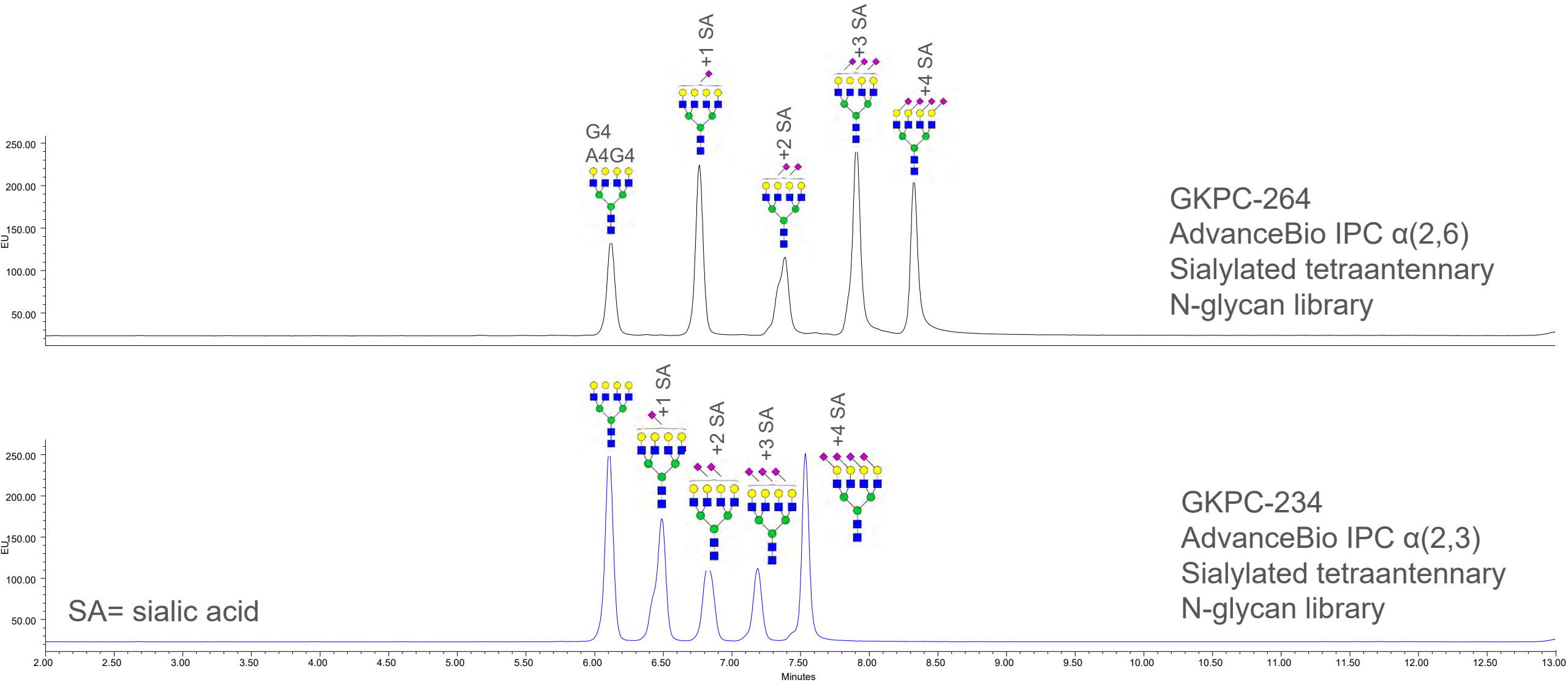

1. Lee EU et al., *J Biol Chem.* 1989;264(23):13848-55.
2. Anthony RM et al. *Science.* 2008;320(5874):373-6.
3. Raymond C et al., *mAbs.* 2015;7(3):571-83.

# InstantPC Sialylated Triantennary N-Glycan Library




Shorter retention of α(2,3)-sialylated N-glycans vs α(2,6) sialic acid linkages

# InstantPC Sialylated Tetraantennary N-Glycan Library




Shorter retention of α(2,3)-sialylated N-glycans vs α(2,6) sialic acid linkages

# Additional Resources for InstantPC N-Glycan LC/FLD & MS Analysis

**Streamlined Workflows for N-Glycan Analysis of Biotherapeutics Using InstantPC with LC/FLD/MS**
*John Yan, Andres Guerrero, Ace G. Galermo, Ted Haxo, Sergey Vlasenko, Justin Hyche,Tom Rice and Aled Jones*

ASMS 2019 poster ThP697

**A Comprehensive Approach for Monoclonal Antibody N-linked Glycan Analysis from Sample Preparation to Data Analysis**
*David Wong, Oscar Potter, Jordy Hsaio, Te-Wei Chu*

Technical Note 5991-8550EN

**Analysis of Monoclonal Antibody N-glycans by Fluorescence Detection and Robust Mass Selective Detection Using the Agilent LC/MSD XT**
*Oscar Potter, Gregory Staples, Jordy Hsaio, Te-Wei Chu*

Technical Note 5991-8071EN

**Comparison of Relative Quantification of Monoclonal Antibody N-Glycans Using Fluorescence and MS Detection**
*Oscar Potter and Greg Staples, Agilent Technologies*

Technical Note 5991-6958EN

**Comparison of Common Fluorescent Labels for LC/MS Analysis of Released N-Linked Glycans**
*John Yan, Andres Guerrero, Steven Mast, Ted Haxo, Aled Jones*

Technical Note 5994-0942EN

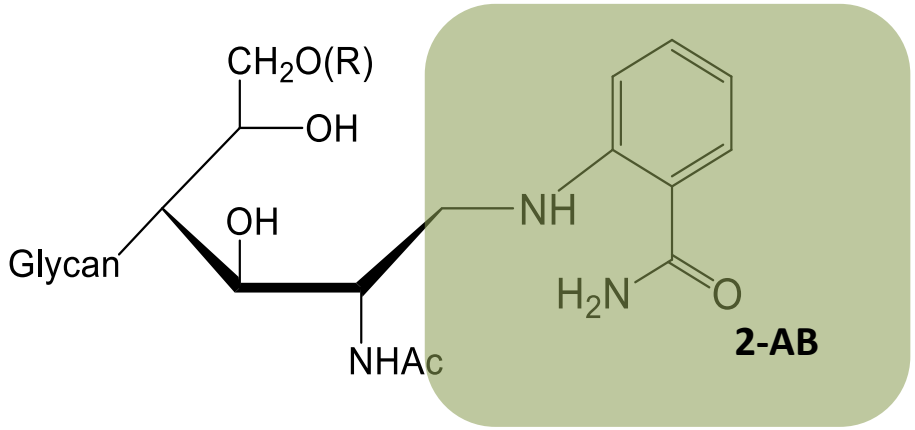
**Automation of Gly-X N-Glycan Sample Prep with InstantPC and InstantQ Dyes**
*Loredana Serafini*, Ted Haxo†, Emily Dale†, Adele Taylor†, Katherine M. Brendza* (*Gilead, †ProZyme)*

ProZyme Technical Note (copy available upon request)

**Development of an Instant Glycan Labeling Dye for High Throughput Analysis by Mass Spectrometry**
*Michael Kimzey, Zoltan Szabo, Vaishali Sharma, Alexander Gyenes, Samnang Tep, Adele Taylor, Aled Jones, Justin Hyche, Ted Haxo, Sergey Vlasenko*

ProZyme Technical Note (copy available upon request)

# 2-AB Or Not 2-AB….




Label makes N-glycan less polar

Traditionally used for HILIC separations of N-glycans with fluorescence detection

Lower FLD and MS signal than InstantPC

2-AB (2-aminobenzamide) dye has been used to generate released N-glycan data for more than 20 years

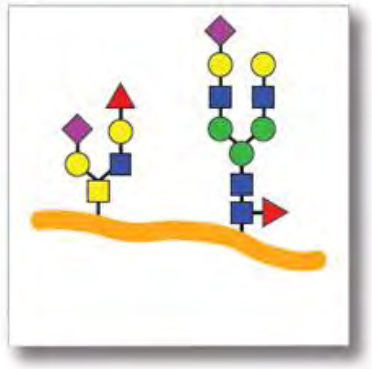
Well established in many laboratories

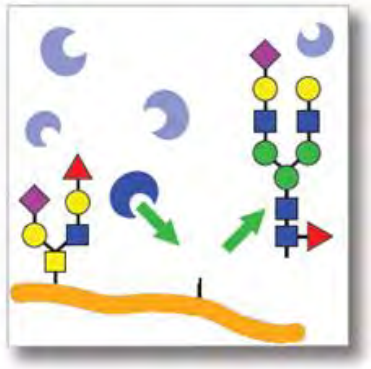
May need to match historic data using 2-AB

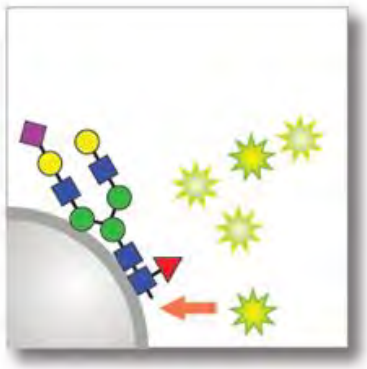
# Gly-X 2-AB Express Workflow

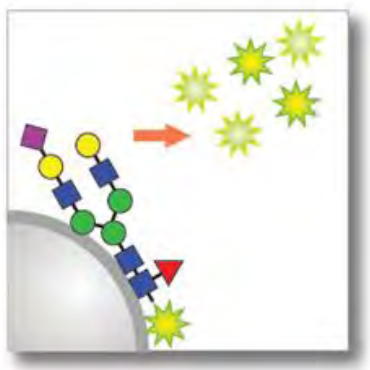
1-40 µg
0.05-2 mg/ml

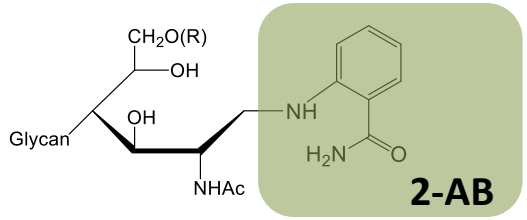
Reductive Amination











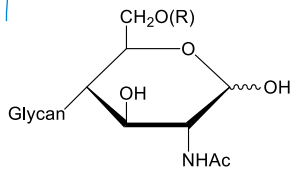

Denature
3 min at 90°C

Deglycosylate
5 min at 50°C

Label
60 min at 65°C
No Dry Down

Clean up
15-20 min at RT

Labeled N-Glycans




Workflow time: 120 min

2-AB

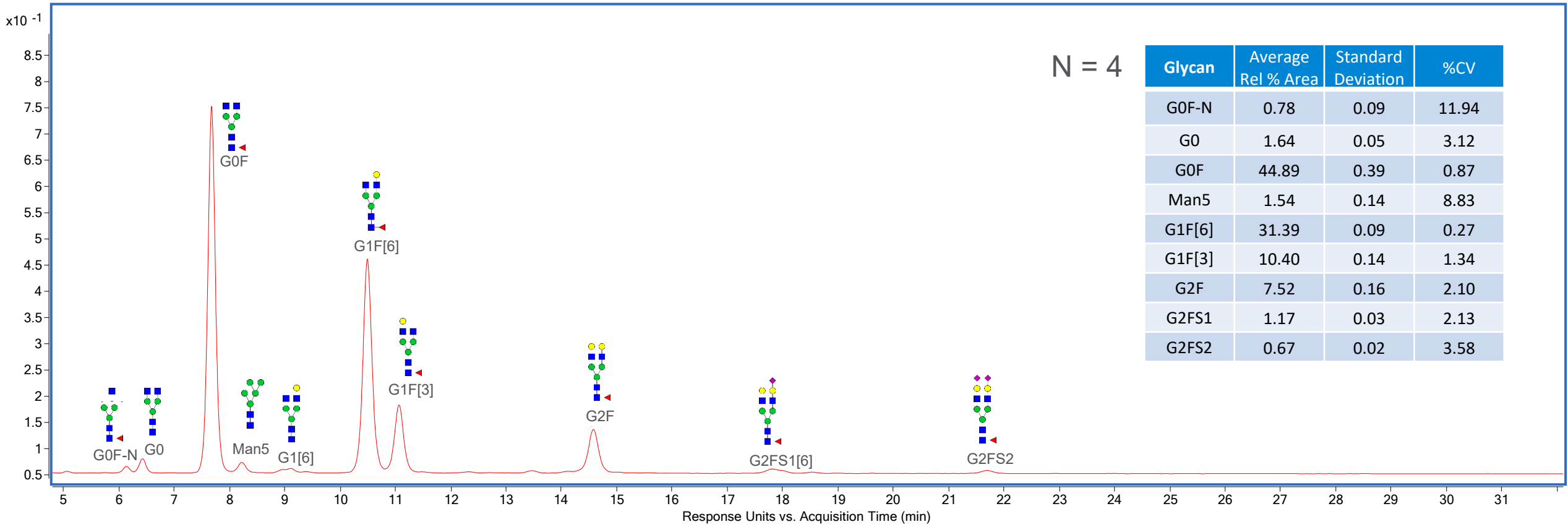
On-matrix labeling

# Rituximab Gly-X 2-AB Express N-Glycans

## Analytical Method:

UHPLC-HILIC, 60 minute method, 1 µl injection (equivalent to glycans from 0.4 µg protein), FLD: 260/430 nm

AdvanceBio Glycan Mapping column, 2.1 x 150 mm, 1.8 µm

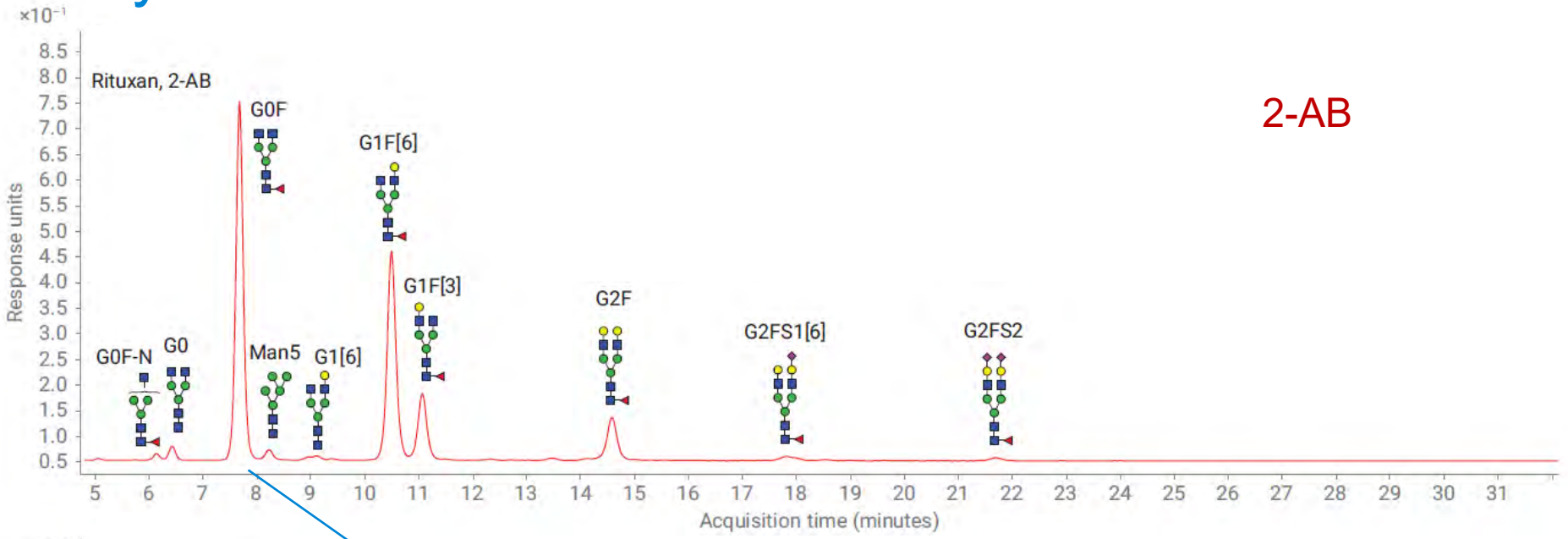



N = 4

| Glycan | Average Rel % Area | Standard Deviation | %CV |
|---|---|---|---|
| G0F-N | 0.78 | 0.09 | 11.94 |
| G0 | 1.64 | 0.05 | 3.12 |
| G0F | 44.89 | 0.39 | 0.87 |
| Man5 | 1.54 | 0.14 | 8.83 |
| G1F[6] | 31.39 | 0.09 | 0.27 |
| G1F[3] | 10.40 | 0.14 | 1.34 |
| G2F | 7.52 | 0.16 | 2.10 |
| G2FS1 | 1.17 | 0.03 | 2.13 |
| G2FS2 | 0.67 | 0.02 | 3.58 |

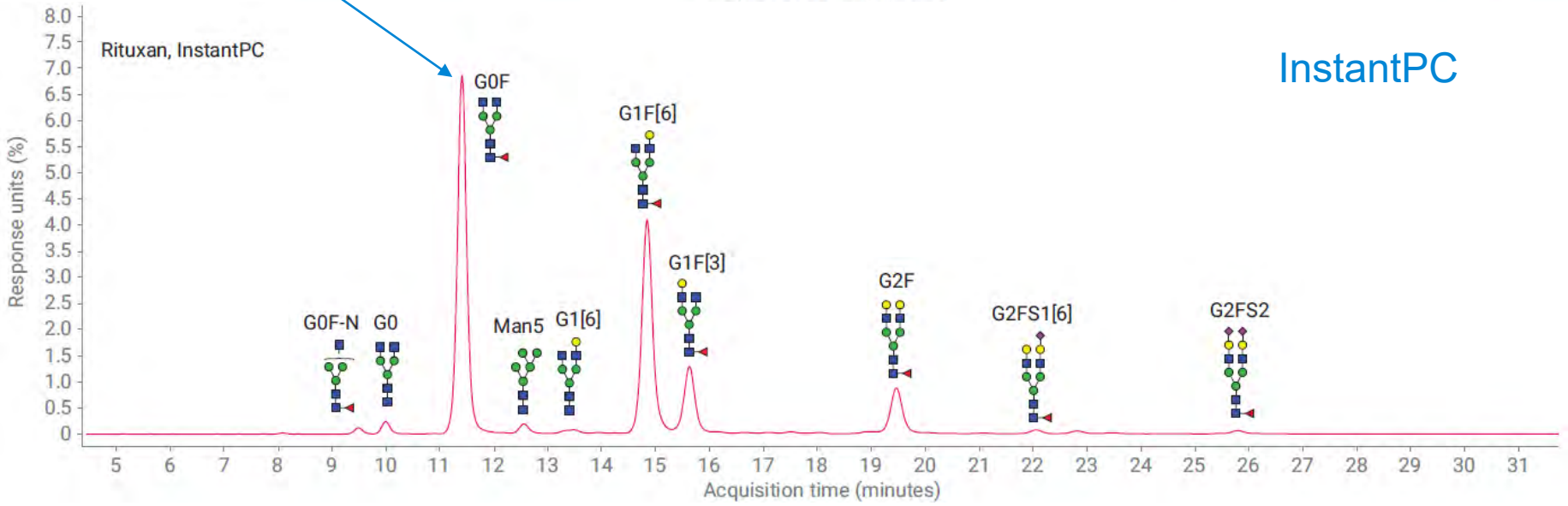
# Gly-X 2-AB vs InstantPC - rituximab

N=4




2-AB

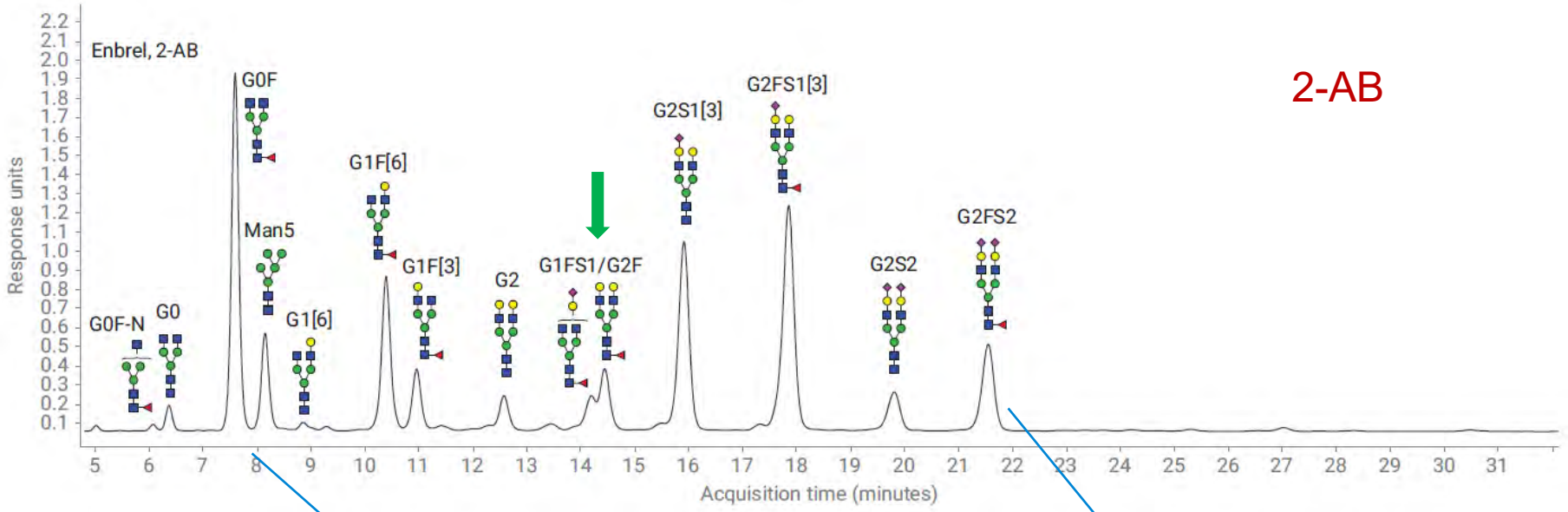
| | Average Rel % Area | Standard Deviation | %CV |
|---|---|---|---|
| G0F-N | 0.78 | 0.09 | 11.94 |
| G0 | 1.64 | 0.05 | 3.12 |
| G0F | 44.89 | 0.39 | 0.87 |
| Man5 | 1.54 | 0.14 | 8.83 |
| G1F[6] | 31.39 | 0.09 | 0.27 |
| G1F[3] | 10.40 | 0.14 | 1.34 |
| G2F | 7.52 | 0.16 | 2.10 |
| G2FS1 | 1.17 | 0.03 | 2.13 |
| G2FS2 | 0.67 | 0.02 | 3.58 |




InstantPC

| | Average Rel % Area | Standard Deviation | %CV |
|---|---|---|---|
| G0F-N | 0.75 | 0.01 | 1.55 |
| G0 | 1.47 | 0.02 | 1.18 |
| G0F | 46.82 | 0.07 | 0.15 |
| Man5 | 1.21 | 0.01 | 0.83 |
| G1[6] | 0.75 | 0.02 | 2.67 |
| G1F[6] | 31.21 | 0.11 | 0.35 |
| G1F[3] | 9.27 | 0.05 | 0.54 |
| G2F | 7.04 | 0.04 | 0.51 |
| G2FS1[6] | 0.67 | 0.02 | 2.29 |
| G2FS1[3] | 0.37 | 0.06 | 15.98 |
| G2FS2 | 0.45 | 0.03 | 6.67 |

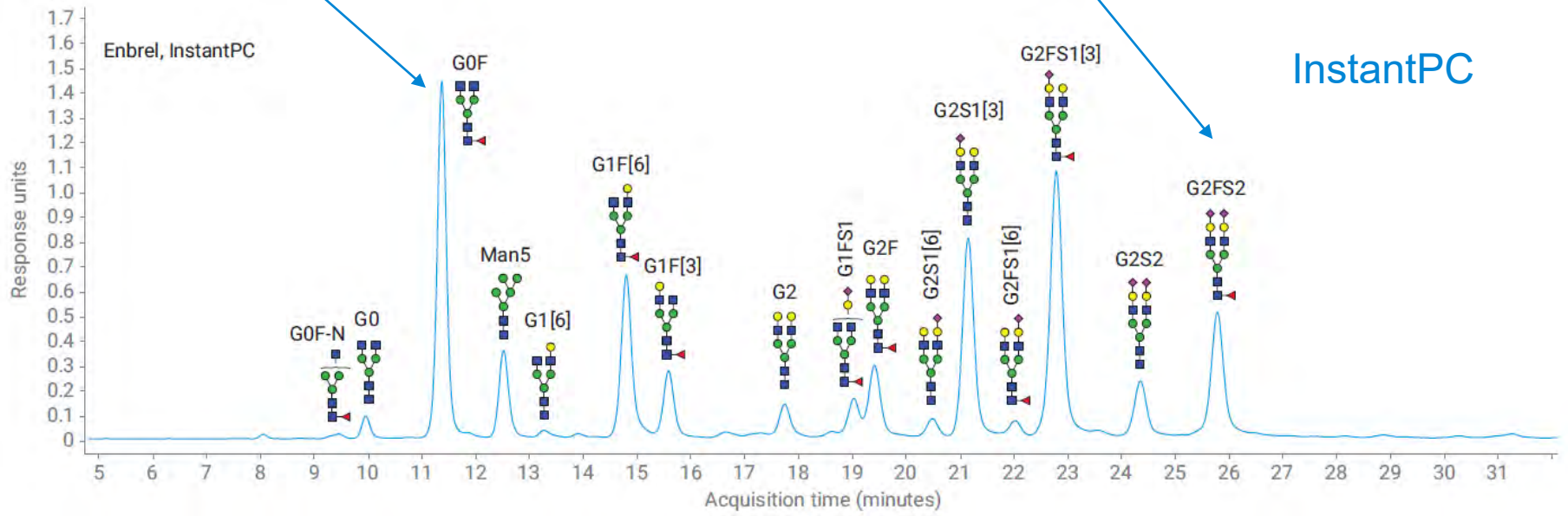
# Gly-X 2-AB vs InstantPC - etanercept




2-AB

| | Average Rel % Area | Standard Deviation | %CV |
|---|---|---|---|
| G0F-N | 0.32 | 0.02 | 7.44 |
| G0 | 1.27 | 0.07 | 5.34 |
| G0F | 20.18 | 0.45 | 2.22 |
| Man5 | 5.50 | 0.34 | 6.17 |
| G1[6] | 0.45 | 0.02 | 3.89 |
| G1F[6] | 10.35 | 0.33 | 3.18 |
| G1F[3] | 3.92 | 0.17 | 4.39 |
| G2 | 2.21 | 0.15 | 6.78 |
| G2F/G1FS1 | 7.00 | 0.25 | 3.63 |
| G2S1 | 15.19 | 0.17 | 1.09 |
| G2FS1 | 20.10 | 0.32 | 1.59 |
| G2S2 | 4.19 | 0.25 | 5.95 |
| G2SF2 | 9.35 | 0.74 | 7.93 |

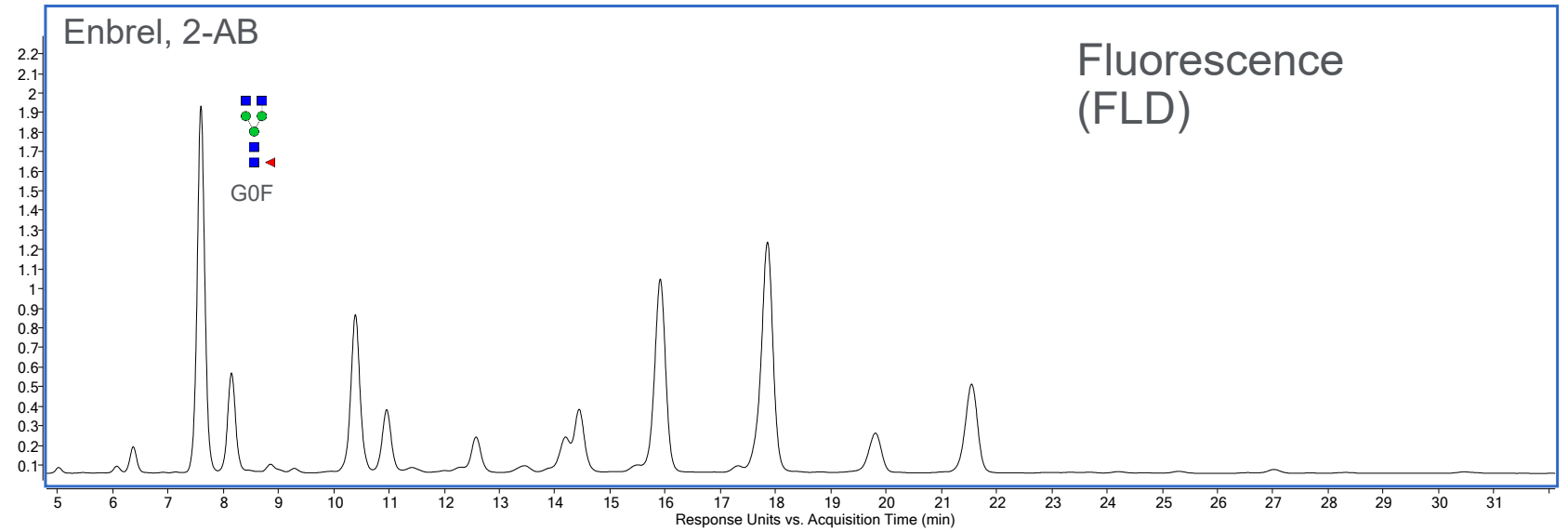
N=4

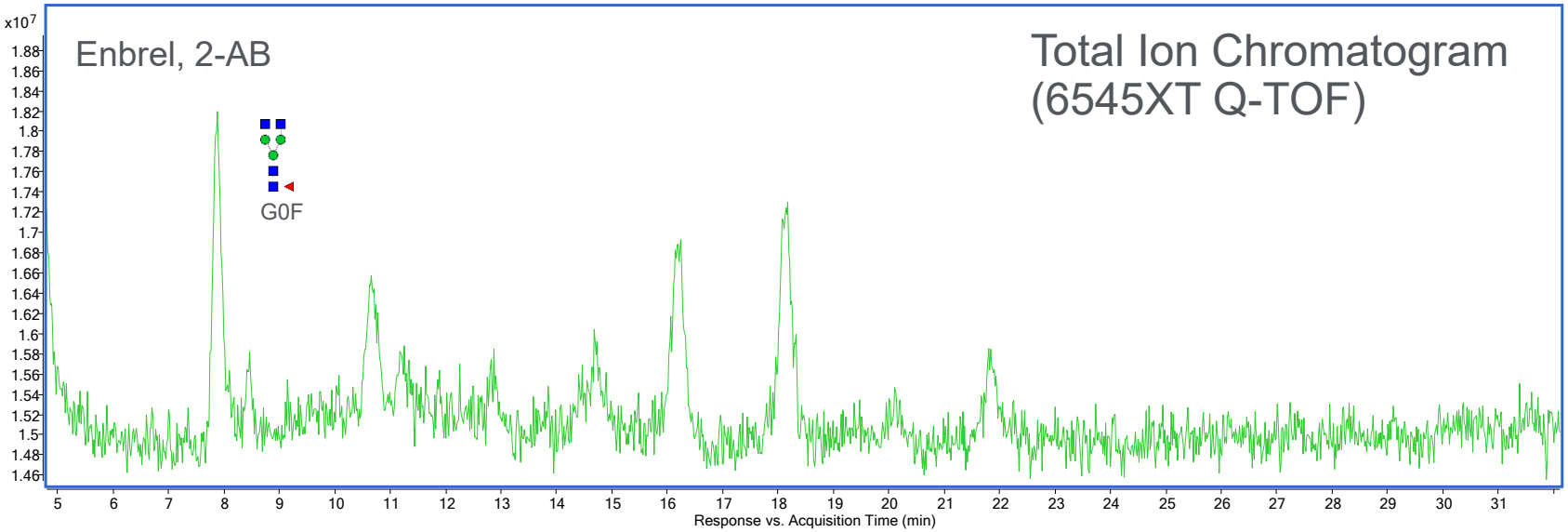



InstantPC

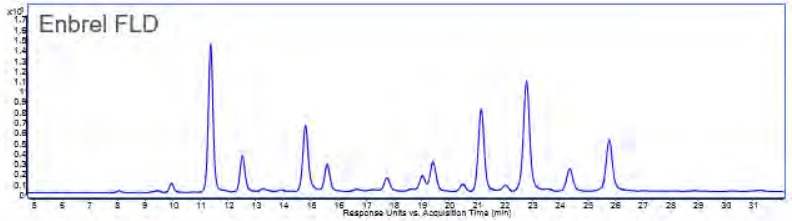
| | Average Rel % Area | Standard Deviation | %CV |
|---|---|---|---|
| G0 | 1.10 | 0.02 | 2.09 |
| G0F | 19.36 | 0.16 | 0.84 |
| Man5 | 5.08 | 0.03 | 0.52 |
| G1[6] | 0.48 | 0.00 | 0.00 |
| G1F[6] | 10.48 | 0.04 | 0.39 |
| G1F[3] | 3.97 | 0.01 | 0.25 |
| G2 | 2.08 | 0.01 | 0.55 |
| G1FS1 | 1.84 | 0.05 | 2.49 |
| G2F | 4.26 | 0.09 | 1.99 |
| G2S1[6] | 1.18 | 0.01 | 0.49 |
| G2S1[3] | 13.91 | 0.04 | 0.31 |
| G2FS1[6] | 0.89 | 0.00 | 0.00 |
| G2FS1[3] | 20.54 | 0.08 | 0.37 |
| G2S2 | 4.26 | 0.01 | 0.14 |
| G2FS2 | 10.54 | 0.08 | 0.78 |

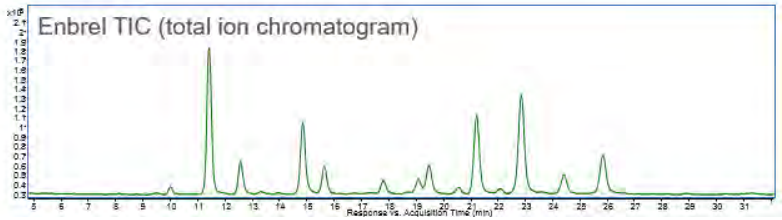
# 2-AB N-Glycans: MS




*(InstantPC)*



# 2-AB N-Glycan Standards & Libraries

## Individual N-Glycans

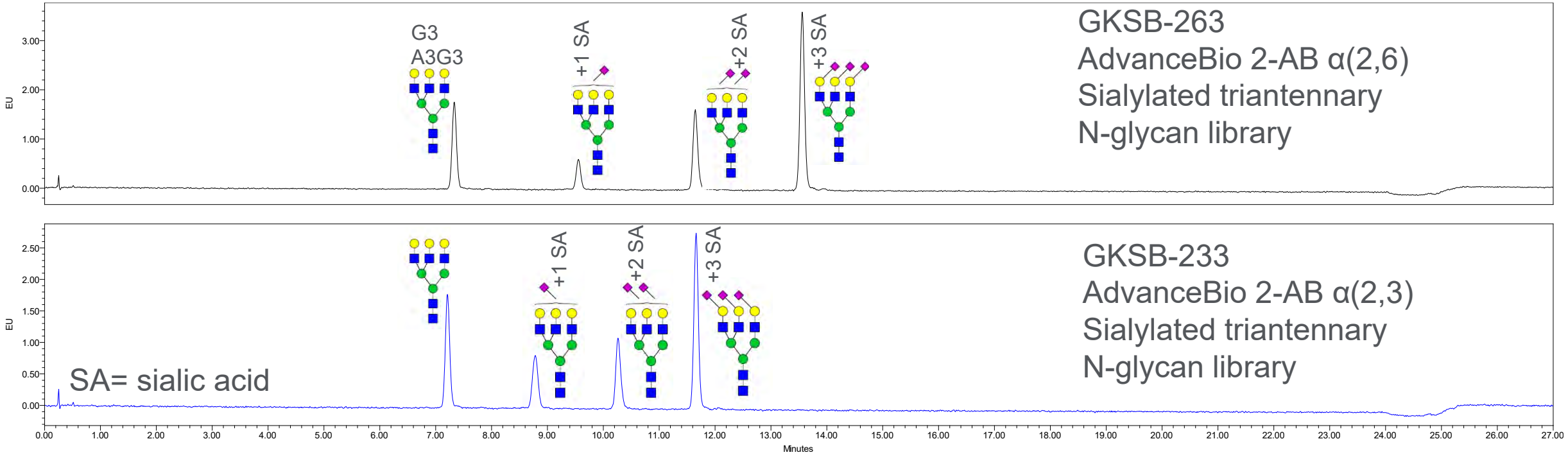
| Product Code | Description |
|---|---|
| GKSB-401 | G0-N |
| GKSB-301 | G0 |
| GKSB-402 | G0F-N |
| GKSB-302 | G0F |
| GKSB-303 | G0FB |
| GKSB-317 | G1 |
| GKSB-316 | G1F |
| GKSB-304 | G2 |
| GKSB-305 | G2F |
| GKSB-306 | G2FB |
| GKSB-318 | NA2Ga2F |
| GKSB-311 | A1 |
| GKSB-315 | A1F |
| GKSB-312 | A2 |
| GKSB-313 | A2F |
| GKSB-307 | NGA3 |

| Product Code | Description |
|---|---|
| GKSB-308 | G3 |
| GKSB-314 | A3 |
| GKSB-309 | NGA4 |
| GKSB-310 | G4 |
| GKSB-111 | HYBR |
| GKSB-100 | NN |
| GKSB-101 | Man3 |
| GKSB-102 | Man3F |
| GKSB-103 | Man5 |
| GKSB-104 | Man6 |
| GKSB-105 | Man7 |
| GKSB-106 | Man8 |
| GKSB-107 | Man9 |
| GKSB-201 | GalGalNAc |
| GKSB-203 | 3'-SLN |
| GKSB-204 | 6'-SLN |

## N-Glycan Libraries

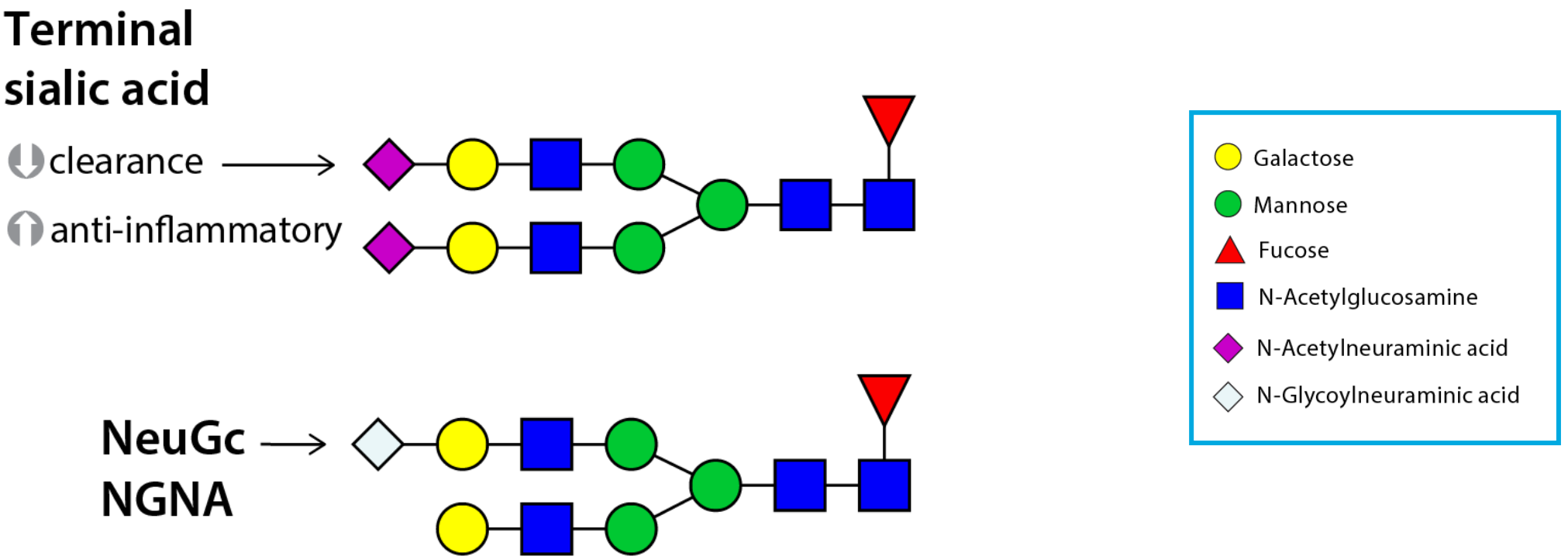
| Product Code | Description |
|---|---|
| GKSB-005 | Human IgG N-Linked Glycan Library |
| GKSB-520 | Biantennary & High Mannose Partitioned Library |
| GKSB-001 | Human α1-acid Glycoprotein N-Linked Glycan Library |
| GKSB-002 | Bovine Fetuin N-linked Glycan Library |
| GKSB-503 | Glucose Homopolymer Standard |
| GKSB-232 | α(2-3) Sialylated Biantennary Library |
| GKSB-262 | α(2-6) Sialylated Biantennary Library |
| GKSB-233 | α(2-3) Sialylated Triantennary Library |
| GKSB-263 | α(2-6) Sialylated Triantennary Library |
| GKSB-234 | α(2-3) Sialylated Tetraantennary Library |
| GKSB-264 | α(2-6) Sialylated Tetraantennary Library |

# 2-AB Sialylated Triantennary N-Glycan Library




Shorter retention of α(2,3)-sialylated N-glycans vs α(2,6) sialic acid linkages

# Importance of Sialic Acid on Biotherapeutics




N-glycolyneuraminic acid (NeuGc/NGNA): Potentially immunogenic

Jones, *BioPharm Intl*. 2017; 30:20-25

# Total Sialic Acid Quantitation

- Plate-based assay (GS48-SAQ, GS96-SAQ)
- Rapid quantitation of total sialic acid released from intact proteins by sialidase A
- Fluorescence plate reader (absorbance an option, lower sensitivity)
- Broad range of detection: 40 – 1,000 pmol fluorescence, 500 – 4,000 pmol absorbance

N-Acetylneuraminic aldolase catalyzes the reversible reaction:

Sialic Acid ↔ Mannosamine + Pyruvic Acid

Then pyruvate oxidase catalyzes the reaction:

Pyruvic Acid → Acetylphosphate + $H_2O_2$

Finally, $H_2O_2$ forms a 1:1 complex with the Dye to form a fluorescent Reporter Dye that may be read by fluorescence or absorbance detection.

Dye + $H_2O_2$ → Reporter Dye

**10 µL Glycoprotein**

30 min Sialidase A Digest

↓

**Released Sialic Acid**

60 min Conversion and Developing

↓

**Read the Plate**

Ex 530 nm / Em 590 nm (FLD)
530 nm (Absorbance)

# Total Sialic Acid Quantitation: Starting Concentrations and Amounts of Glycoprotein

| Glycoprotein | Concentration (mg/mL) | Sample volume (µL) | Sample mass (µg) | MW (kDa) | pmol protein |
|---|---|---|---|---|---|
| Fetuin | 0.25 | 10 | 2.5 | 48 | 52 |
| MabThera | 10 | 10* | 100 | 145 | 690 |
| Enbrel | 0.25 | 10 | 2.5 | 150 | 16.7 |
| Zaltrap | 0.5 | 10 | 5 | 115 | 43 |
| Orencia | 0.5 | 10 | 5 | 92 | 54 |
| EPO alfa | 1 | 10 | 10 | 30.4 | 329 |

**For glycoproteins with lower sialylation such as monoclonal antibodies, up to 30 µL sample may be used with the kit.*

# Intra-Assay Repeatability

- Single operator, 3 replicates per sample
- SA - sialic acid

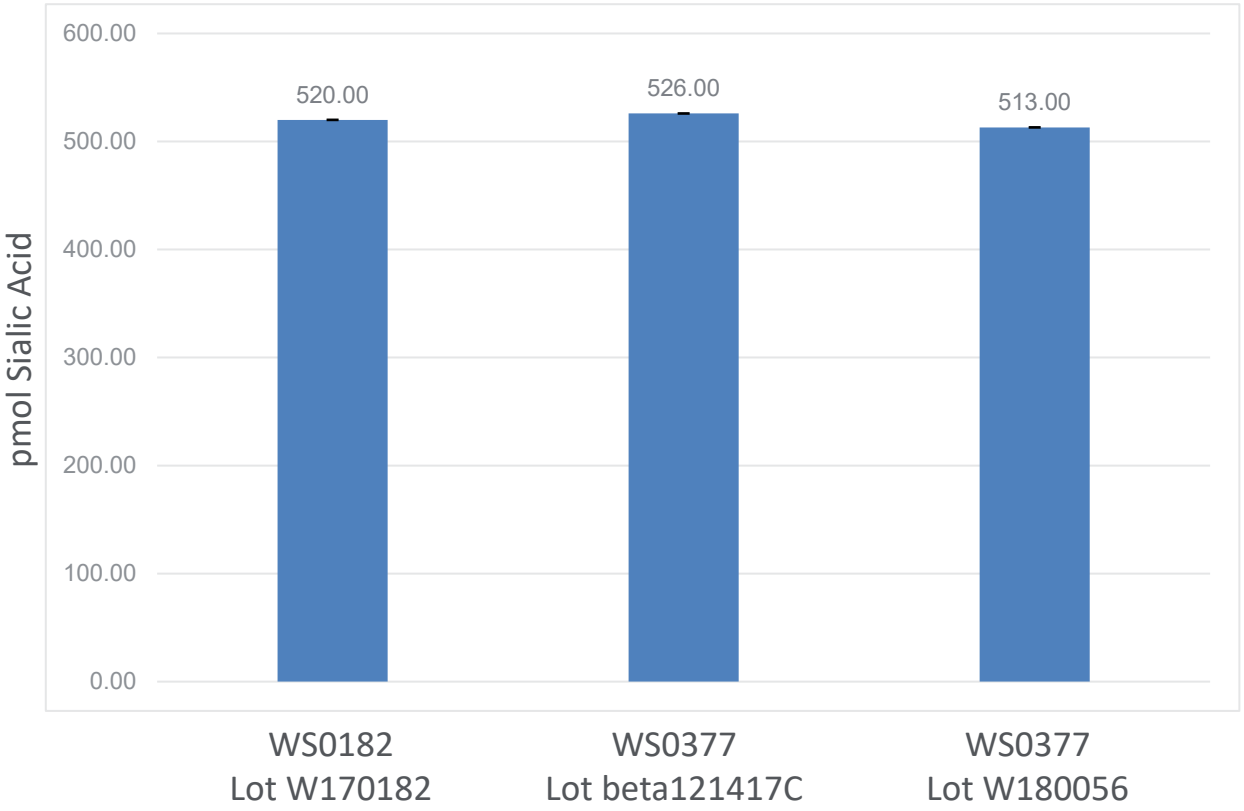
| | Fetuin | MabThera | Enbrel | Zaltrap | Orencia | EPO alfa |
|---|---|---|---|---|---|---|
| Starting concentration (mg/mL) | 0.25 | 10 | 0.25 | 0.5 | 0.5 | 1 |
| MW (kDa) | 48 | 145 | 150 | 115 | 92 | 30.4 |
| nmol SA/10 µL sample | 0.58 | 0.08 | 0.55 | 0.45 | 0.59 | 0.16 |
| nmol SA/mg protein | 232.53 | 0.79 | 220.93 | 90.27 | 117.27 | 15.50 |
| mol SA/mol protein | 11.16 | 0.11 | 33.14 | 10.38 | 10.79 | 0.47 |
| %CV (n=3) | 0.55% | 2.65% | 1.81% | 0.46% | 0.60% | 3.59% |
| SD (mol SA/mol protein) | 0.06 | 0.00 | 0.60 | 0.05 | 0.06 | 0.02 |

# Operator to Operator Repeatability

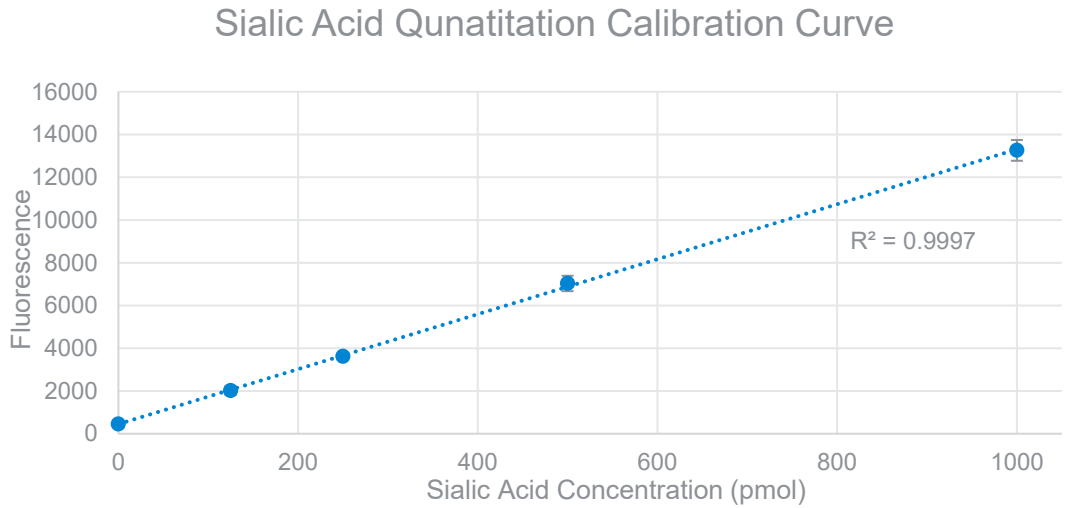
- 6 operators
- Samples: Fetuin, Enbrel, Orencia, Zaltrap, MabThera, EPO alfa
- 3 replicates per sample

| | Fetuin | MabThera | Enbrel | Zaltrap | Orencia | EPO alfa |
|---|---|---|---|---|---|---|
| Operator 1 | 11.35 | 0.18 | 33.24 | 11.41 | 10.56 | 0.45 |
| Operator 2 | 11.16 | 0.11 | 33.14 | 10.38 | 10.79 | 0.47 |
| Operator 3 | 12.07 | 0.18 | 35.58 | 12.65 | 11.52 | 0.49 |
| Operator 4 | 10.34 | 0.17 | 33.76 | 10.92 | 11.17 | 0.49 |
| Operator 5 | 12.08 | 0.16 | 33.20 | 9.91 | 9.50 | 0.46 |
| Operator 6 | 10.44 | 0.17 | 31.66 | 9.74 | 10.32 | 0.45 |
| Average mol SA/mol protein | 11.24 | 0.16 | 33.43 | 10.83 | 10.64 | 0.47 |
| %CV (n=6) | 6.72% | 15.28% | 3.79% | 10.03% | 6.63% | 3.86% |
| SD | 0.76 | 0.02 | 0.02 | 0.71 | 1.09 | 1.27 |

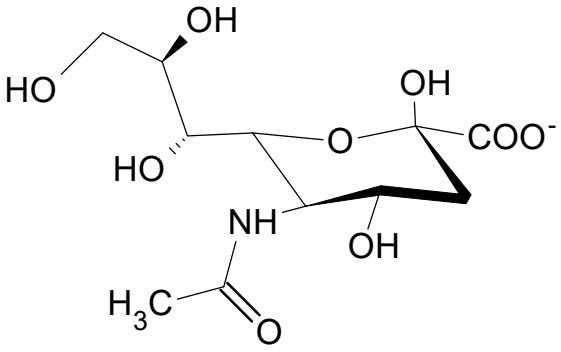
# 100 µM Sialic Acid Standard Lot Comparison (target 500 pmol)



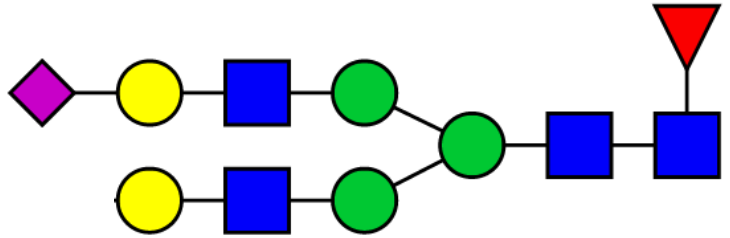

- WS0182: older NANA standard included with GS300/GF57
- WS0377: new NANA standard for GS48/96-SAQ, uses USP material

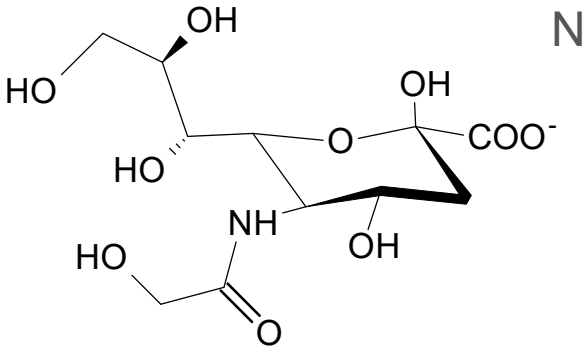


# Neu5Ac and Neu5Gc

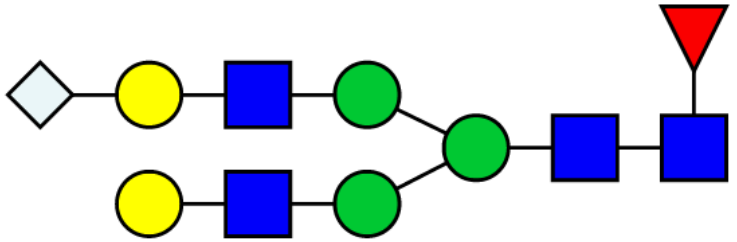


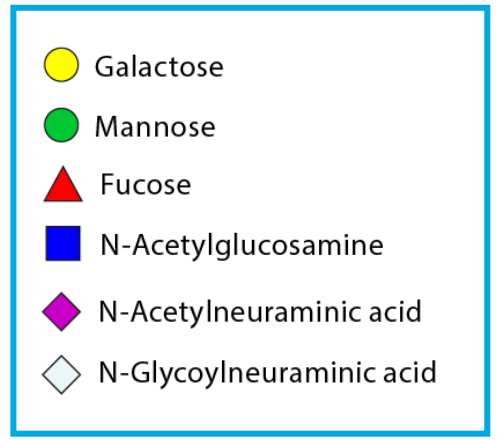

Neu5Ac
NANA






Neu5Gc
NGNA



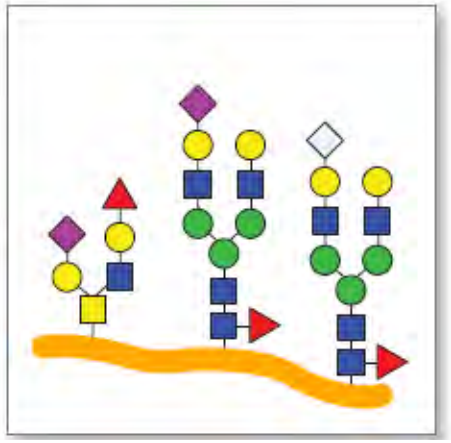



Potentially immunogenic

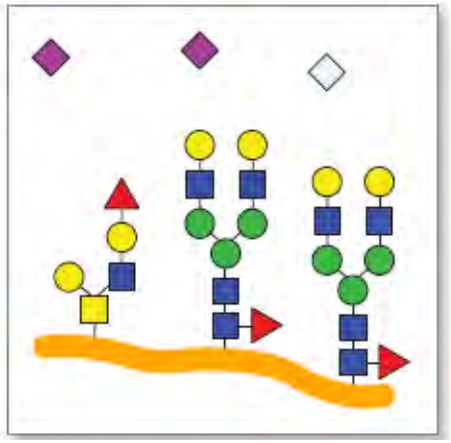
Ghaderi et al., *Biotechnol Genet Eng Rev.* 2012; 28:147-75

Jones, *BioPharm Intl*. 2017; 30:20-25

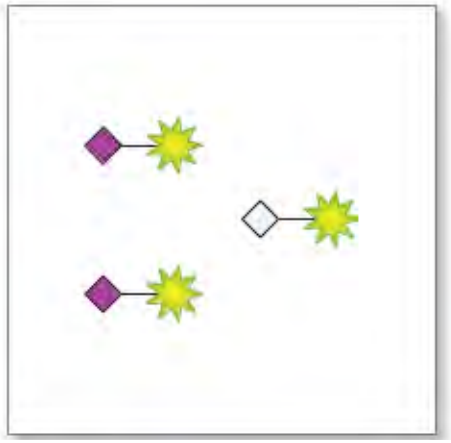
# DMB Labeling for Profiling and Quantitation of Sialic Acid



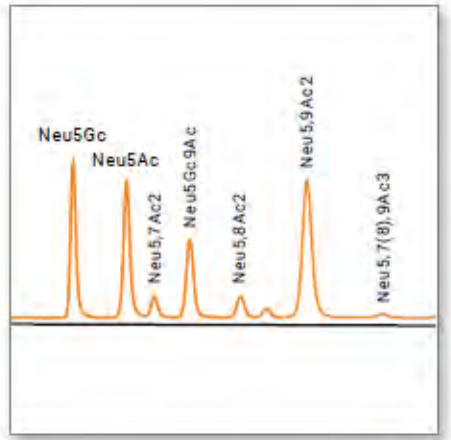
Sialylated glycoprotein



Sialic acid release

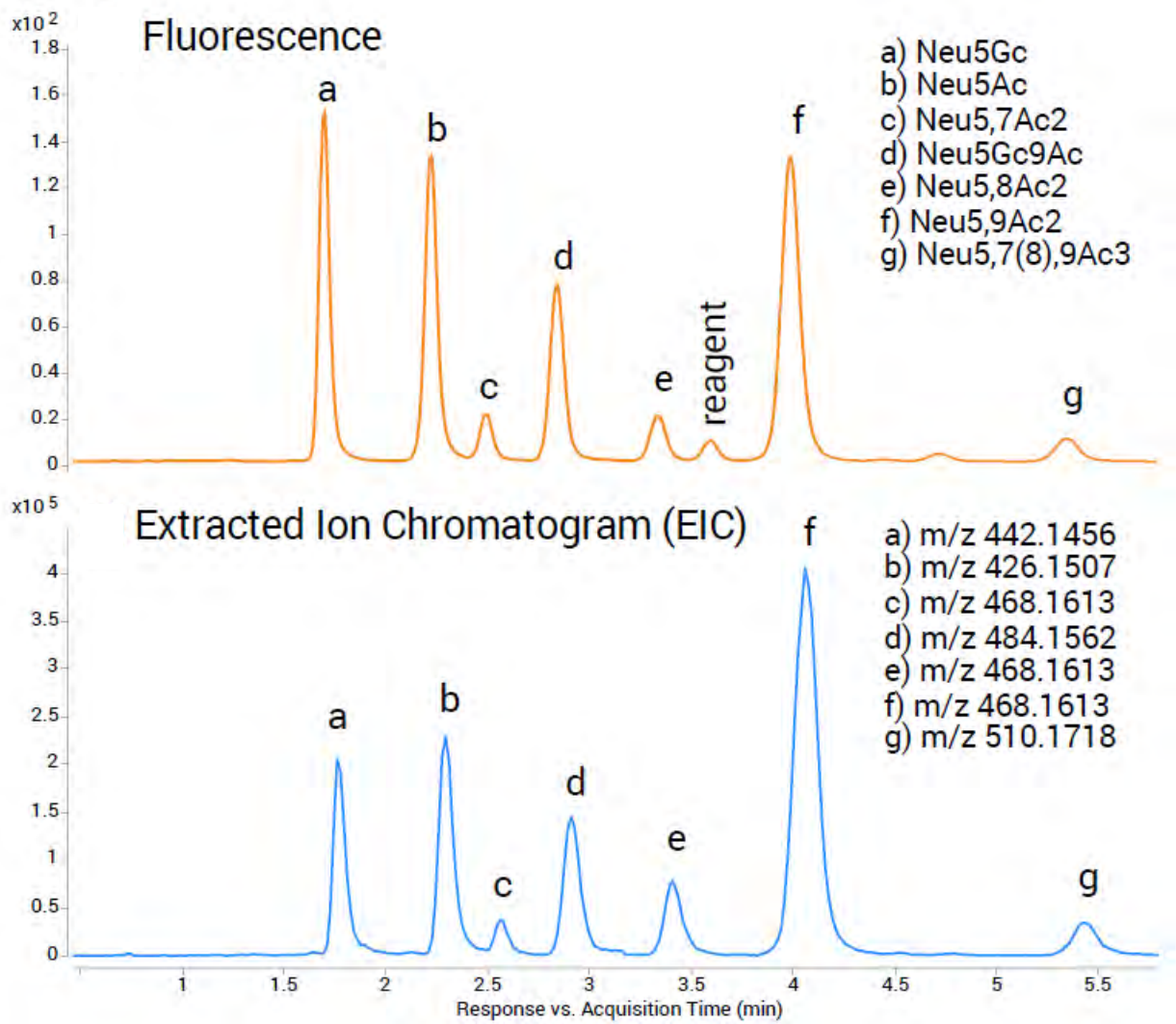


DMB labeling




RPLC separation
FLD or MS detection
Quantitation

# DMB Labeled Sialic Acid Reference Panel (SARP)



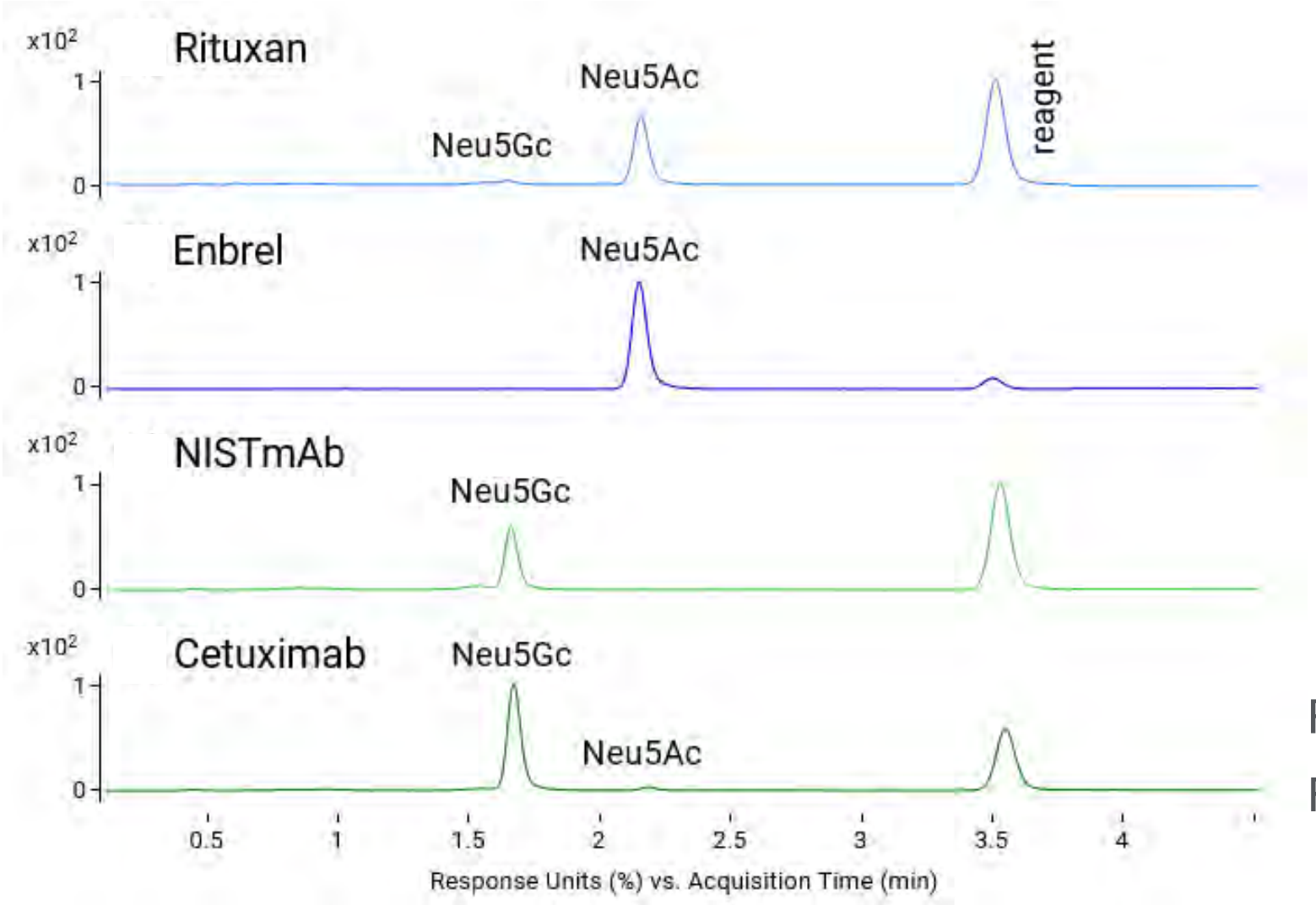

| Parameter | Value |
|---|---|
| Instrument | Agilent 1290 Infinity II LC System |
| Column | Agilent InfinityLab PoroShell 120 EC-C18, 2.1 x 75 mm, 2.7 µm (p/n 697775-902) |
| Column Temp | 30 °C |
| Mobile Phase | A) methanol: acetonitrile: water (4:8:88)<br>B) acetonitrile |
| Gradient Program | Time (min) %A %B Flow rate (mL/min)<br>0.00 100 0 0.4 Isocratic Elution<br>6.00 100 0 0.4<br>6.25 20 80 0.4<br>7.30 20 80 0.4 Wash<br>7.50 100 0 0.4 Re-equilibration<br>10.00 100 0 0.4 |
| Injection Volume | 10 µL |
| Detection | Agilent 1260 Infinity II FLD $\lambda_{Ex}$ 373 nm, $\lambda_{Em}$ 448 nm |

| 6545XT Q-TOF | |
|---|---|
| Source | Dual AJS ESI |
| Gas Temperature | 350 °C |
| Drying Gas Flow | 11 L/min |
| Nebulizer | 15 psi |
| Sheath Gas Temperature | 400 °C |
| Sheath Gas Flow | 12 L/min |
| Vcap | 1400 V |
| Nozzle Voltage | 2800 V |
| Fragmentor | 120 V |
| Skimmer | 65 V |
| Mass Range | m/z 400-1000 |
| Scan Rate | 1 spectra/sec |
| Acquisition Mode | High resolution (4 GHz) |

Pressure > 400 bar

Extracted ion chromatogram of DMB labeled sialic acid species, $[M+H]^{1+}$

# DMB Labeled Sialic Acids of Biotherapeutics & NISTmAb




NISTmAb – available from Agilent

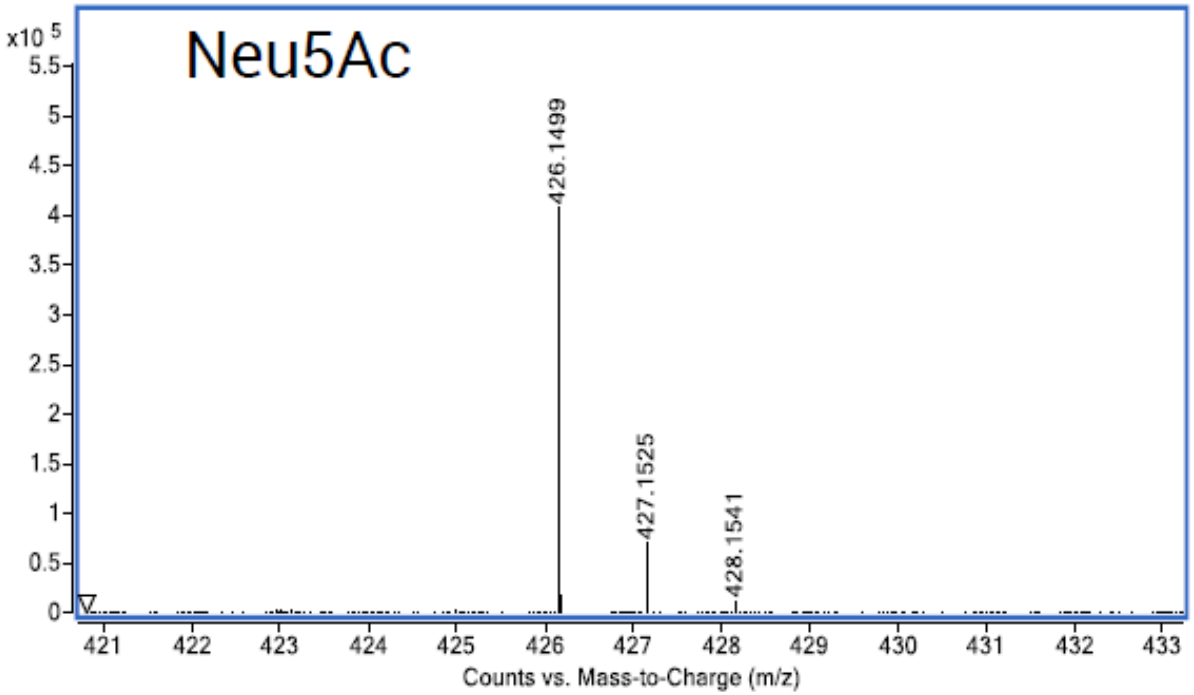
- 25 µL (5191-5744)
- 4 x 25 µL (5191-5745)

RP UHPLC (Poroshell EC-C18)

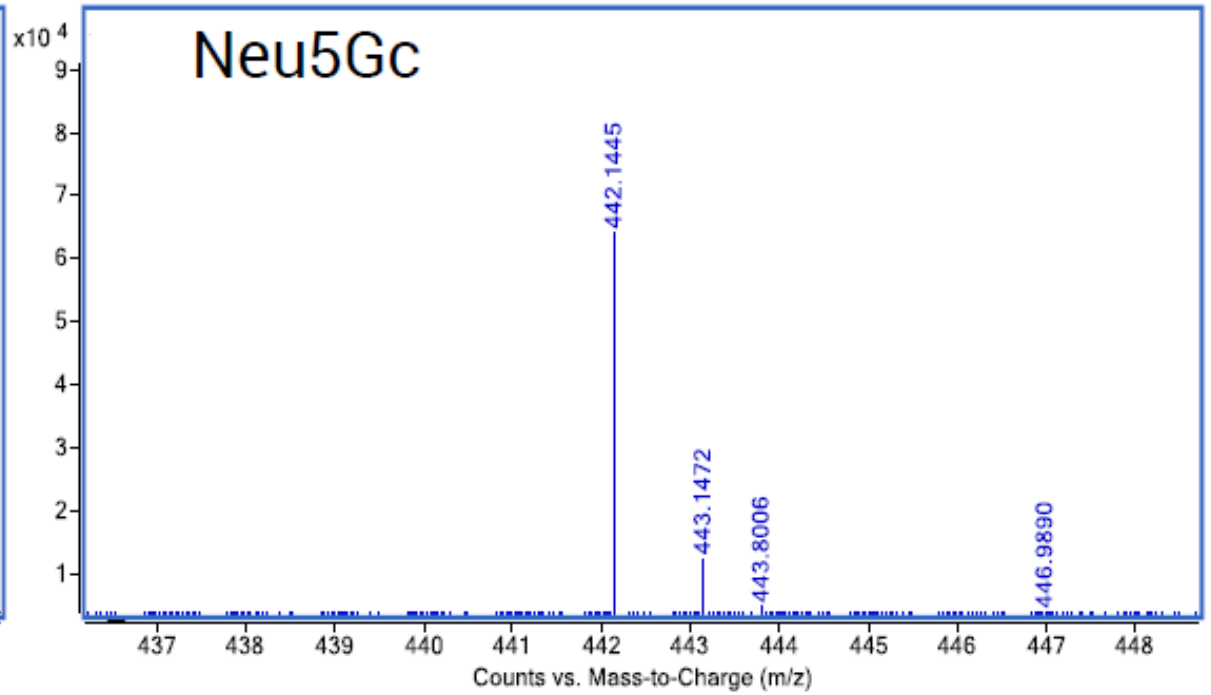
Fluorescence detection

# Mass spectra of DMB NeuAc and NeuGc

Enbrel




Cetuximab



# Summary

Gly-X: rapid, simple, automatable sample preparation for N-glycan analysis

Choice of dyes:

- InstantPC (LC/FLD/MS): >1 hour
- 2-AB Express (LC/FLD/MS): 2 hours

Fluorescence detection for relative % area quantitation

MS detection for the assignment of glycan structures to peaks

Sialylated glycan standards

Sialic acid:

- Total sialic acid quantitation (SAQ), plate based
- Sialic acid profiling (DMB), LC/FLD/MS

# Collaboration

Talk to us about:

- N-Glycan sample prep
- Glycan standards
- Endoglycosidases & Exoglycosidases
- Sialic acid quantitation
- Streptavidin and phycobiliproteins
- Analytical services

Product demos

Beta testing

New technologies

# Acknowledgements

## Agilent Technologies

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Linda Lloyd
Oscar Potter
Suma Ramagiri
Greg Staples