Workflows for N-Glycan Analysis of Biotherapeutics Using LC/FLD/MS

John Yan, Ph.D.
Applications Chemist

Agilent LC and LC/MS Chemistries and Supplies Webinar Series

Tuesday, April 21st 2020

DE.7095833333

04/21/2020

Outline

Importance of N-glycan analysis for biotherapeutic development

Choosing a dye for released N-glycan analysis

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

HILIC separations of labeled N-glycans

- FLD and MS detection

Sialic acid quantitation workflow for biotherapeutics

- Total sialic acid quantitation (plate based)

Agilent

N-Glycans on Biotherapeutics

1/5th 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.

Agilent

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/

Agilent

Top Selling Biopharmaceutical Products (2017)

Rank	Product	Sales, 2017 ($ billions)[a]	Cumulative sales, 2014–2017 ($ billions)	Year first approved	Company	Patent expiry[b]	Biosimilar version(s) approved
1	Humira (adalimumab; anti-TNF)	18.94	62.6	2002	AbbVie, Eisai	2016 (US) 2018 (EU)	Halimatoz/Hefiya/Hyrimoz, Amgevita/Amjevita/Solymbic, Cyltezo, Imraldi
2	Enbrel (etanercept; anti-TNF)	8.34	35.4	1998	Amgen, Pfizer, Takeda Pharmaceuticals	2015 (EU) 2028 (US)	Erelzi, Benepali
3	Rituxan/MabThera (rituximab; anti-CD20)	7.78	29.1	1997	Roche, Biogen Idec	2013 (EU) 2016 (US)	Blitzima/Truxima, Ritemvia, Rituzena, Rixathon/Riximyo
4	Remicade (infliximab; anti-TNF)	7.77	35.6	1998	Johnson & Johnson, Merck, Mitsubishi Tanabe Pharma	2015 (EU) 2018 (US)	Zessly, Ixifi, Renflexis/Flixabi, Inflectra/Remsima
5	Herceptin (trastuzumab; anti-HER2)	7.39	27.1	1998	Roche	2014 (EU) 2019 (US)	Herzuma, Kanjinti, Trazimera, Ogivri, Ontruzant
6	Avastin (bevacizumab; anti-VEGF)	7.04	27.0	2004	Roche	2017 (US) 2019 (EU)	Mvasi
7	Lantus (insulin glargine)	6.72	27.4	2000	Sanofi	2014 (EU & US)	Semglee, Lusduna, Abasaglar/Basaglar
8	Eylea (aflibercept; anti-VEGF)	5.93	18.0	2011	Regeneron, Bayer	2020 (EU) 2021 (US)	
9	Opdivo (nivolumab; anti-PD-1 receptor)	5.79	11.4	2014	Bristol-Myers Squibb, Ono Pharmaceutical	2027 (US) 2026 (EU)	
10	Neulasta (pegfilgrastim)	4.53	20.1	2002	Amgen, Kyowa Hakko Kirin	2014 (US) 2015 (EU)	Fulphila
11	Stelara (ustekinumab; anti-IL-12 & IL-23)	4.01	12.2	2009	Janssen Cilag (Johnson & Johnson)	2023 (US) 2024 (EU)	
12	Keytruda (pembrolizumab, anti-PD-1)	3.81	5.7	2014	Merck	2036 (US) 2028 (EU)	
13	Prolia/Xgeva (denosumab, anti-RANKL)	3.54	11.6	2010	Amgen	2025 (US) 2022 (EU)	
14	Lucentis (ranibizumab; anti-VEGF) *	3.38	14.3	2006	Roche, Novartis	2016 (EU & US)	
15	Novolog/Novorapid (insulin aspart)	3.31	11.7	1999	Novo Nordisk	2015 (EU & US)	

☐ Glycosylated

☐ Glycosylated biosimilars

- 11 of the top 15: glycosylated

- Biopharmaceutical – any drug manufactured in or extracted from biological sources
- Biosimilar – drug that is a copy of an original or innovator biopharmaceutical
- As patent expiry approaches, biopharma companies will focus on production of potential biosimilars

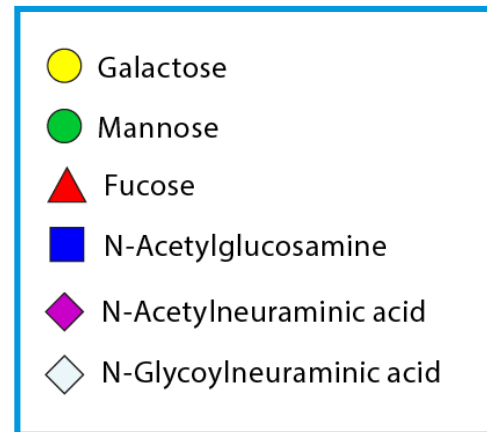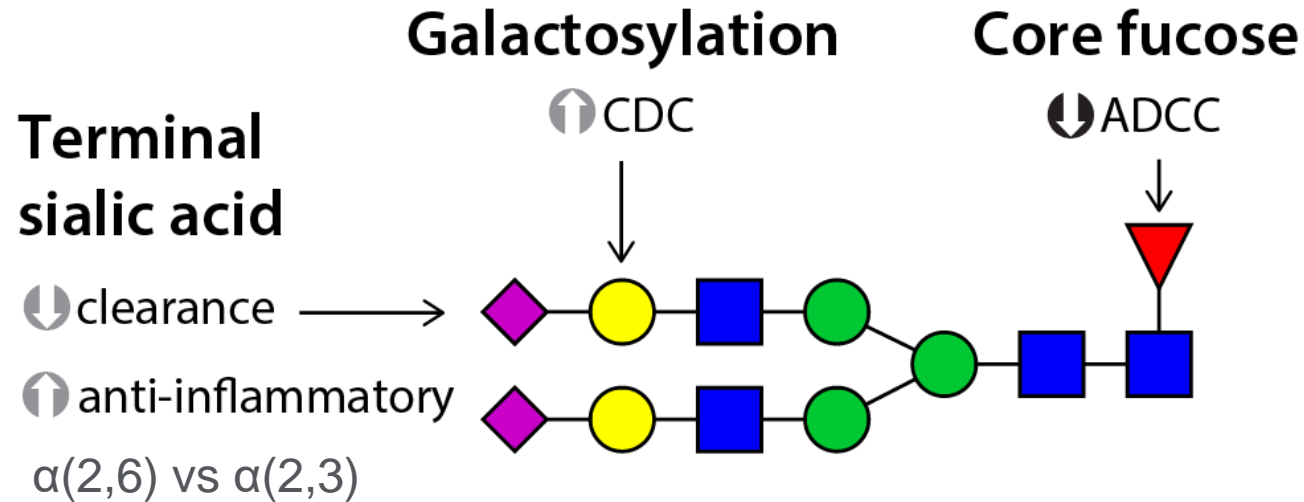* Lucentis (ranibizumab) is a Fab fragment made in E. coli, not glycosylated

Walsh G, Nat Biotechnol. 2018 Dec 6;36(12):1136-1145

Agilent

Fc N-Linked Glycosylation at N297
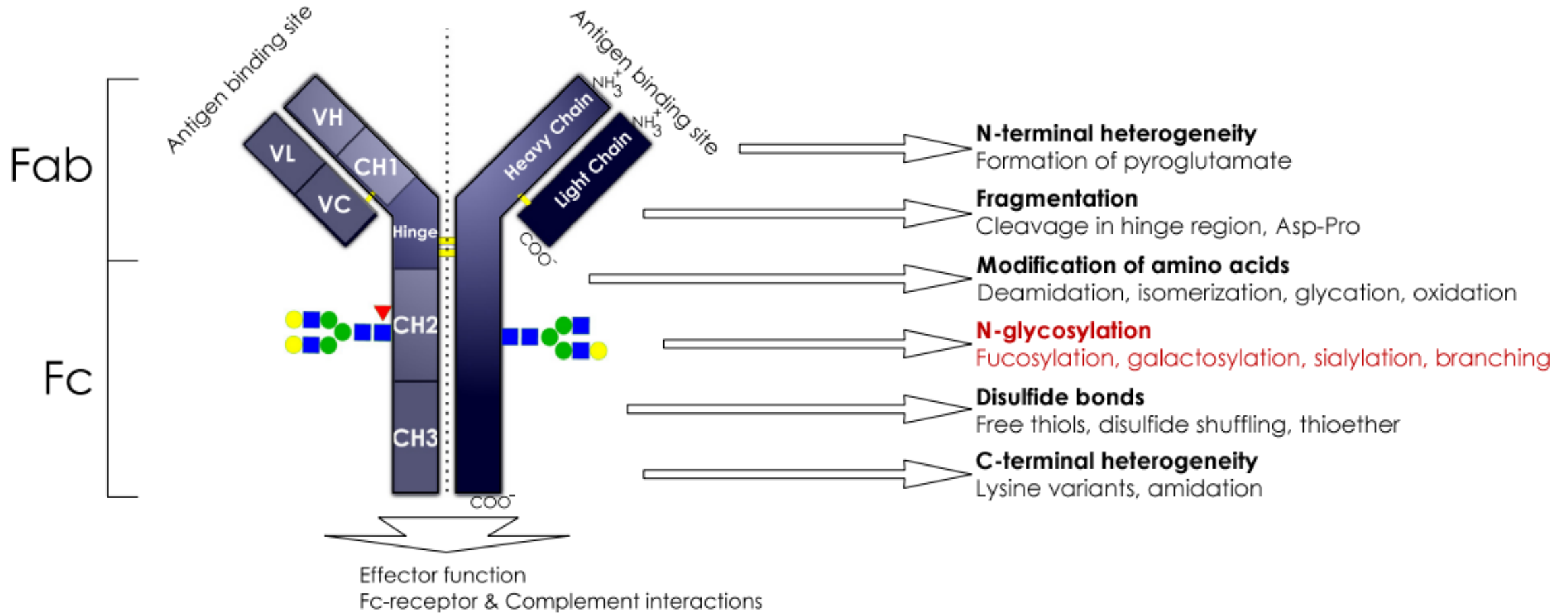
Higel et al., *Eur J Pharm Biopharm.* 2016; 100: 94

Effect of N-Glycan Structure on Biotherapeutics

Galactosylation ⬆CDC

Core fucose ⬇ADCC

Terminal sialic acid

⬇clearance

⬆anti-inflammatory

α(2,6) vs α(2,3)

Legend:
- ○ Galactose
- ● Mannose
- ▲ Fucose
- ■ N-Acetylglucosamine
- ◆ N-Acetylneuraminic acid
- ◇ N-Glycoylneuraminic acid

- 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

Agilent

Effect of N-Glycan Structure on Biotherapeutics
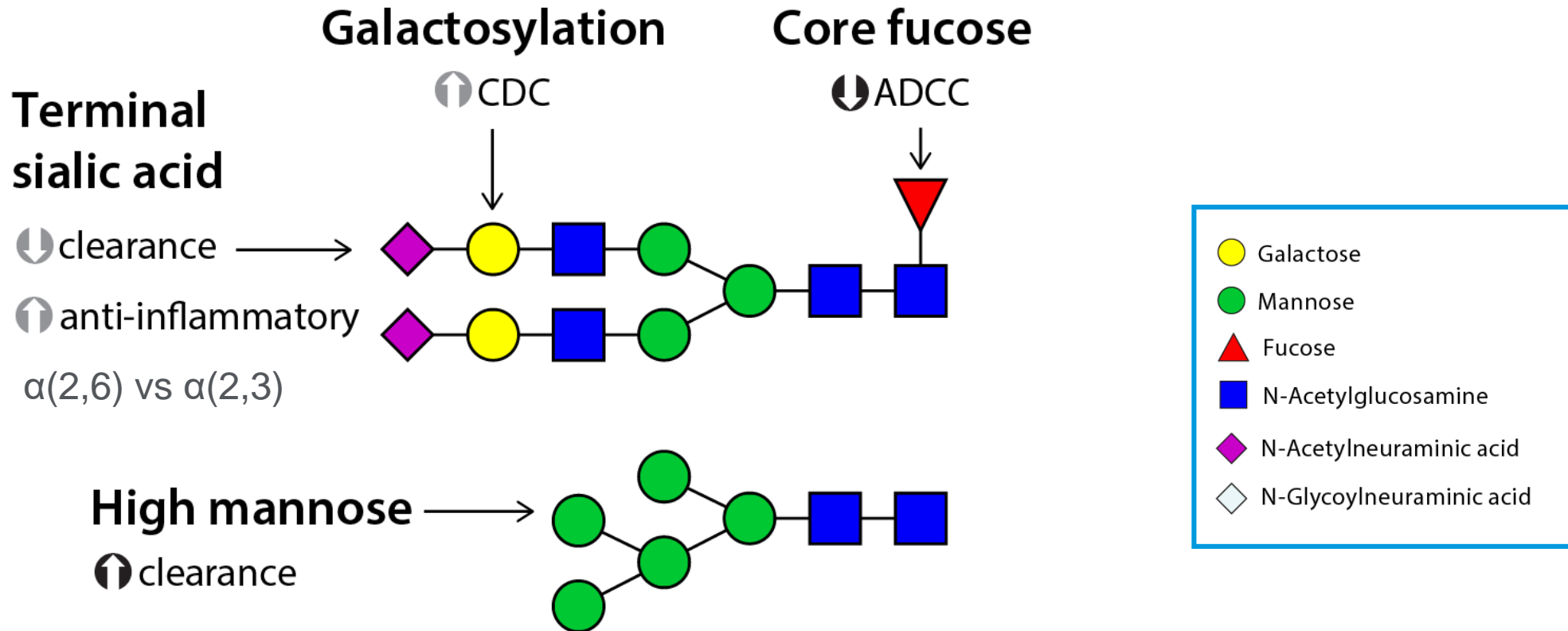
Reviewed in:

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

Non-Human/Immunoreactive N-Glycans

Gal-α-1,3-Gal →
(α-Gal, Galili antigen)
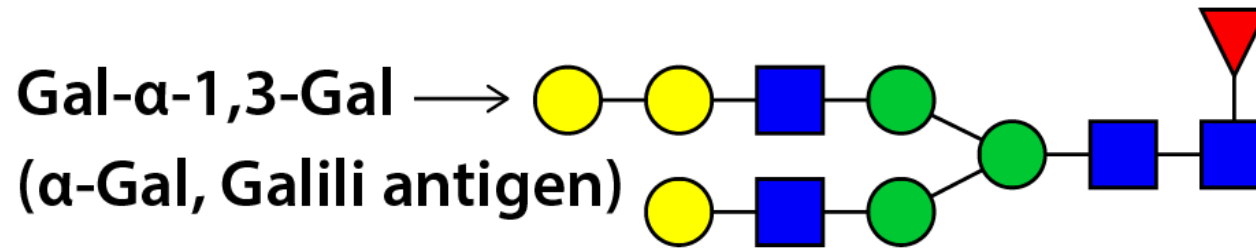
Cetuximab - N-glycans with α-Gal on Fab region
• Associated with adverse immunological responses

Abatacept - N-glycans with α-Gal on Fc region
• No reported adverse effects

Legend:
- ○ Galactose (yellow circle)
- ● Mannose (green circle)
- ▲ Fucose (red triangle)
- ■ N-Acetylglucosamine (blue square)
- ◆ N-Acetylneuraminic acid (purple diamond)
- ◇ N-Glycoylneuraminic acid (open diamond)
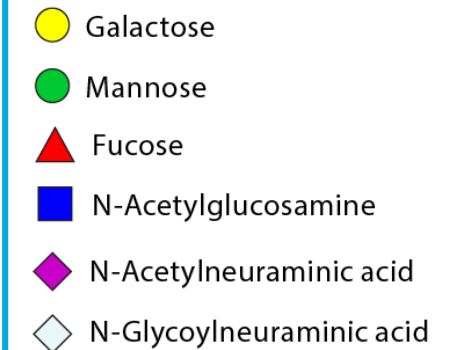
Macher & Galili, *Biochim Biophys Acta.* 2010; 1780 (2): 75-88
Van Bueren et al., *Nat Biotechnol.* 2011; 29: 574-576
Bosques et al., *Biotechnol.* 2010; 28: 1153-1156
Chung et al., *N Eng J Med.* 2008; 358(11): 1109-1117
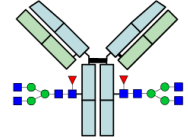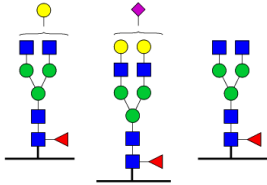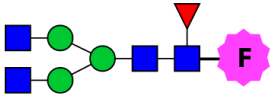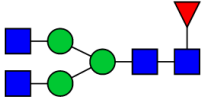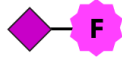
Non-Human/Immunoreactive N-Glycans
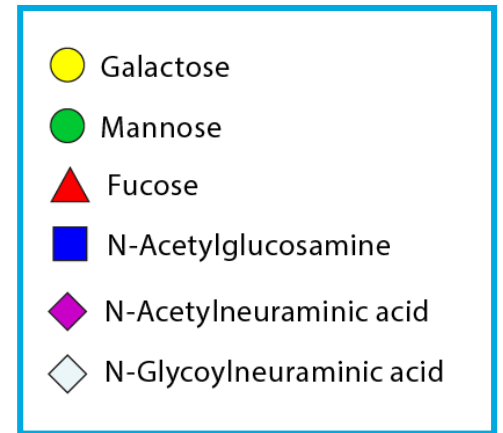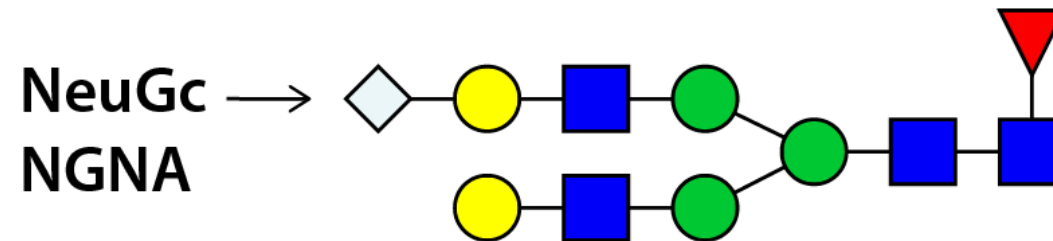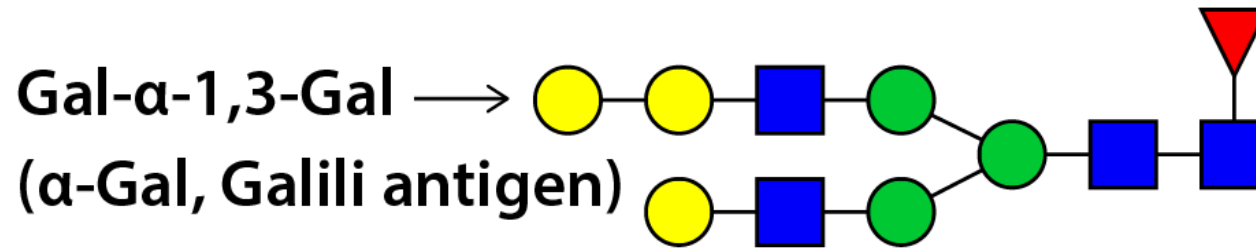
Potentially immunogenic
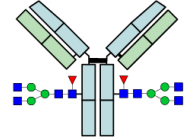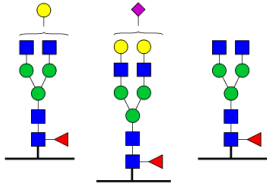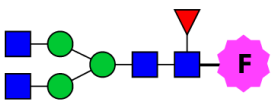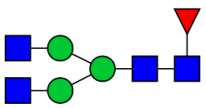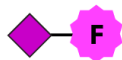
Ghaderi et al., *Biotechnol Genet Eng Rev.* 2012; 28:147-75

N-Glycan Analysis Options

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

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

Agilent

N-Glycan Analysis Options

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

Relative % area of glycan species

Sialic acid quantitation

Agilent

Agilent Released N-Glycan Workflow

Automation	Sample Prep	Separation	Detection	Data Processing & Report

IPC

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

For Research Use Only. Not for use in diagnostic procedures.

Agilent

N-Glycan Sample Prep Evolution

<1 hr — **Gly-*X* with Instant Dyes**

2 hrs — **Gly-*X* with 2-AB (Express)**

3-4 hrs — **GlykoPrep with Instant Dyes**

4-5 hrs — **GlykoPrep with Traditional Reductive Amination Dyes**

1-2 days — **Traditional Deglycosylation with Traditional Reductive Amination Dyes**

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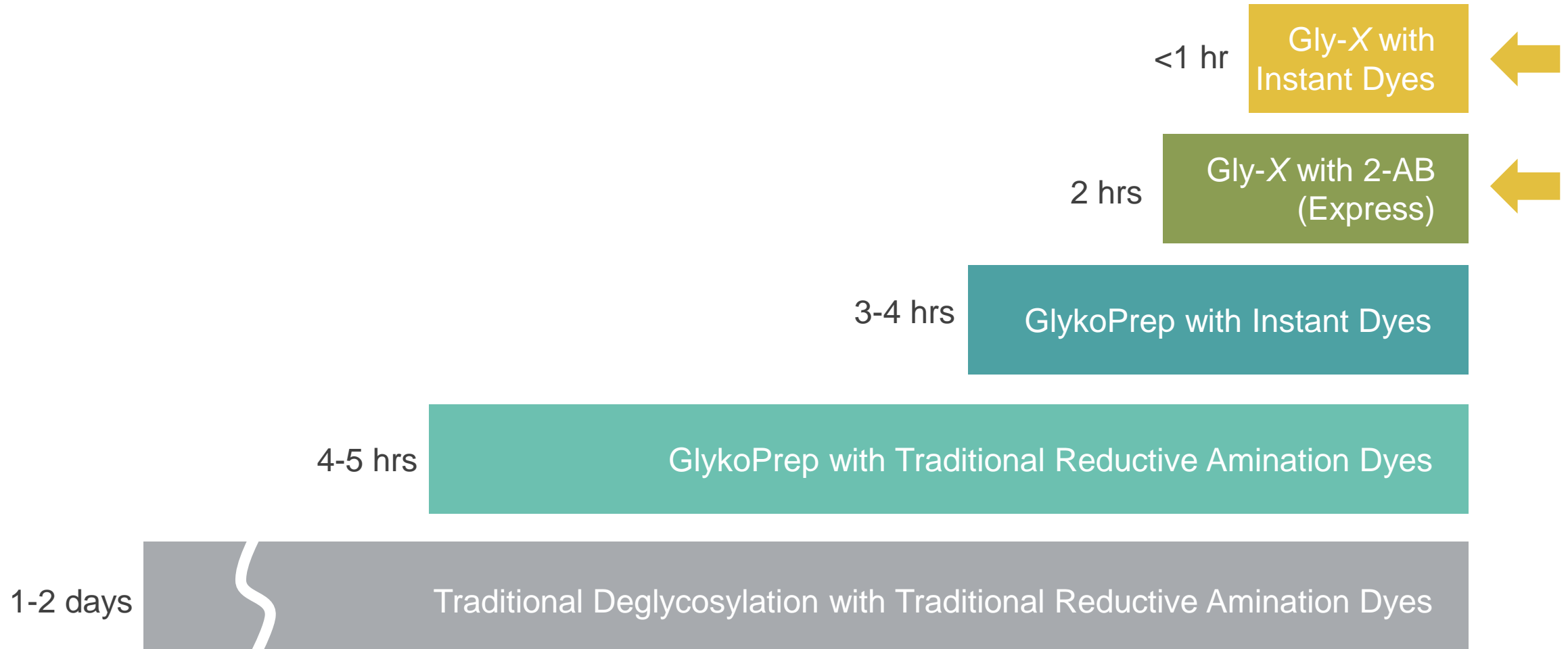
Gly-*X* N-Glycan Sample Prep Technology

1-40 µg glycoprotein sample*, 0.05-2 mg/ml
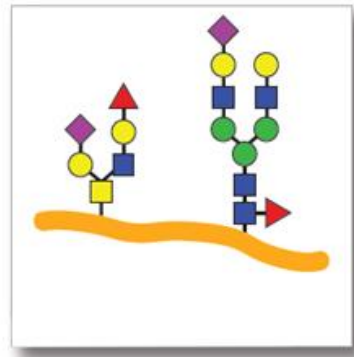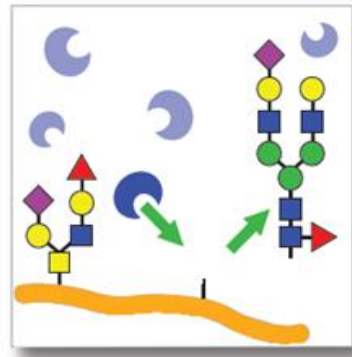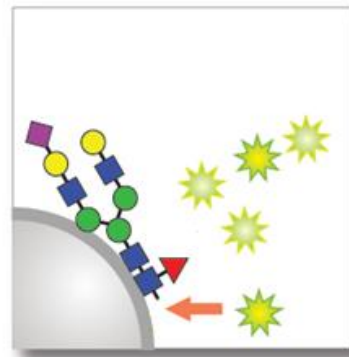
Denature	Deglycosylate	Label	Clean up	Labeled N-Glycans
3 min at 90 °C	PNGase F, 5 min at 50 °C	InstantDye, 1 min *InstantPC, InstantQ, InstantAB* **Reductive amination dye, 60 min (no dry down)** *2-AB, APTS*	15-20 min at RT	LC/FLD, LC/MS, CE
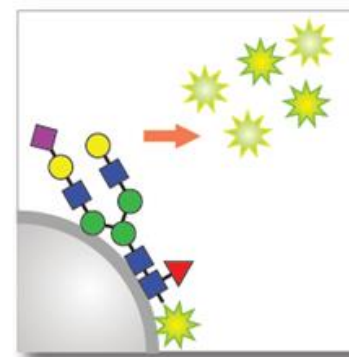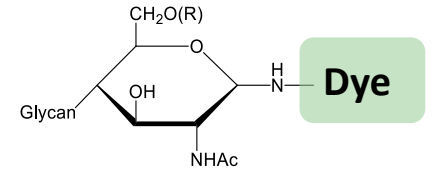
Workflow time: 45 min – 120min (16 samples)

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

Agilent

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

Denature
3 minutes at 90 °C

Deglycosylate
5 minutes at 50 °C
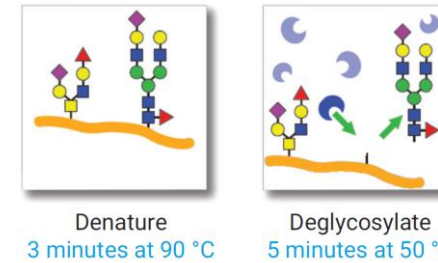
Protein	% Deglycosylation
Rituximab	>99%
Cetuximab	>99%
Enbrel	>99%
Zaltrap	>99%

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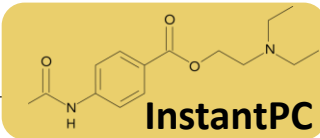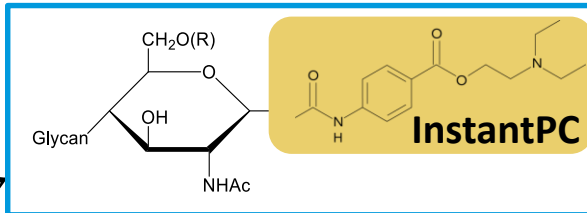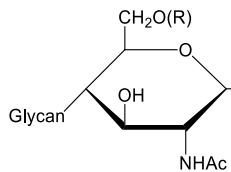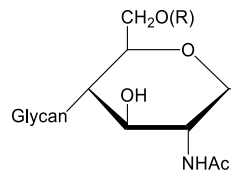Agilent 2100 Bioanalyzer, Protein 230 Kit
(electropherograms not normalized)

Cetuximab

Gly-X
Control (no enzyme)

Deglycosylated heavy chain

Light chain

Intact heavy chain

PNGase F

Agilent
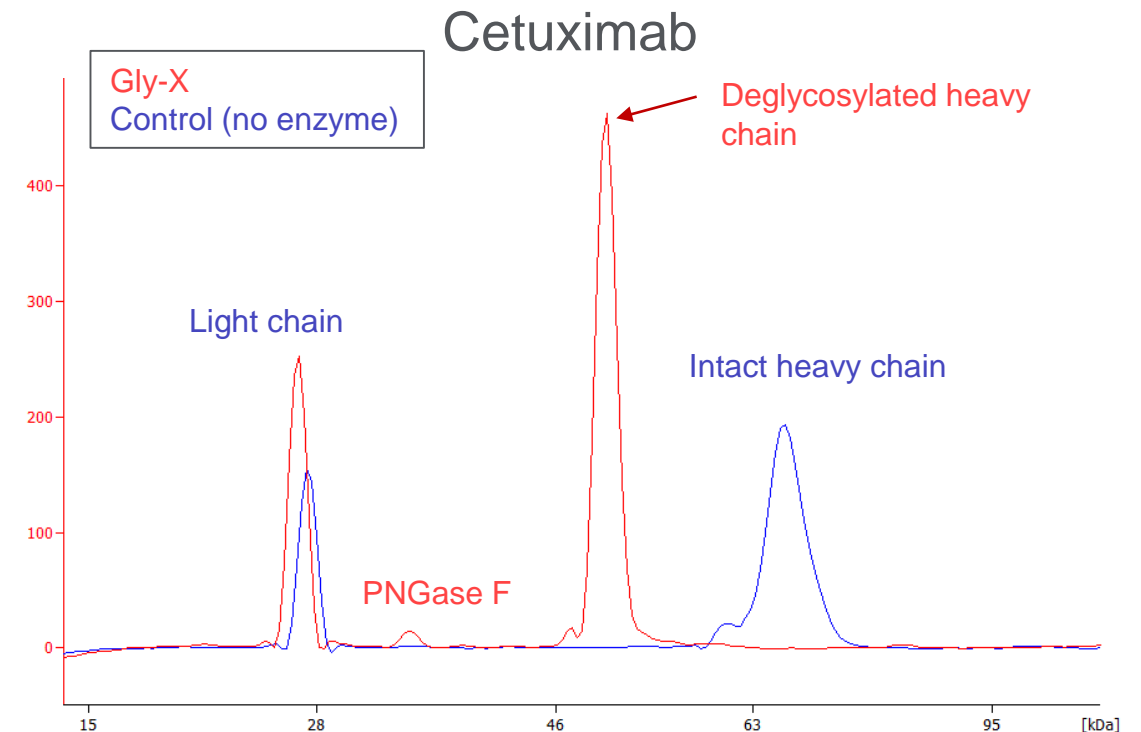
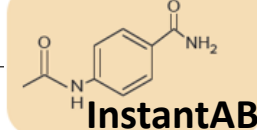# N-Glycan Label Choices

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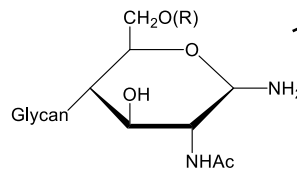
- InstantDye for LC/FLD/MS
- Strong FLD and MS signal

InstantPC

InstantQ

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

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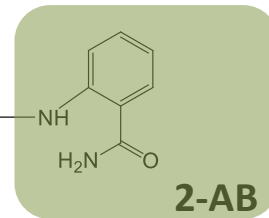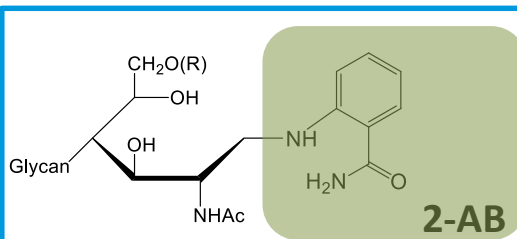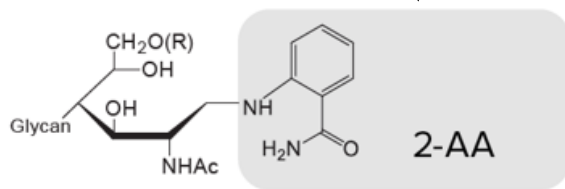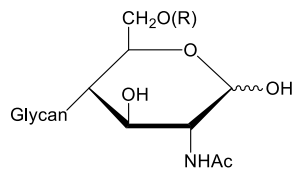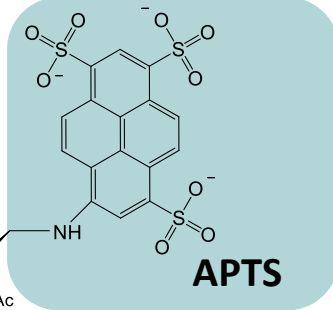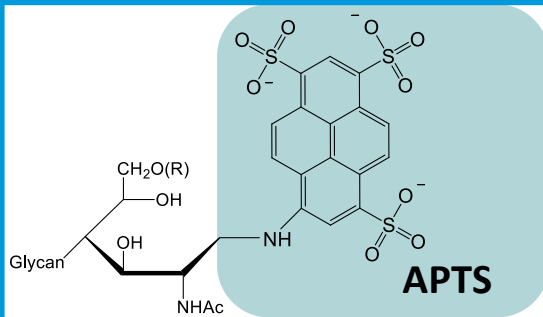
- 1st generation InstantDye for LC/FLD
- Lower MS signal

Glycoprotein $\xrightarrow{\text{N-Glycanase}}$

Reductive Amination:
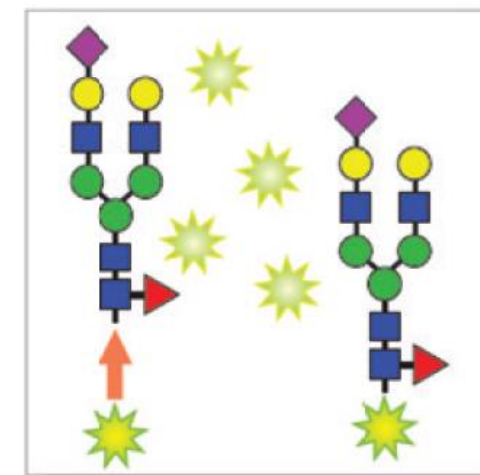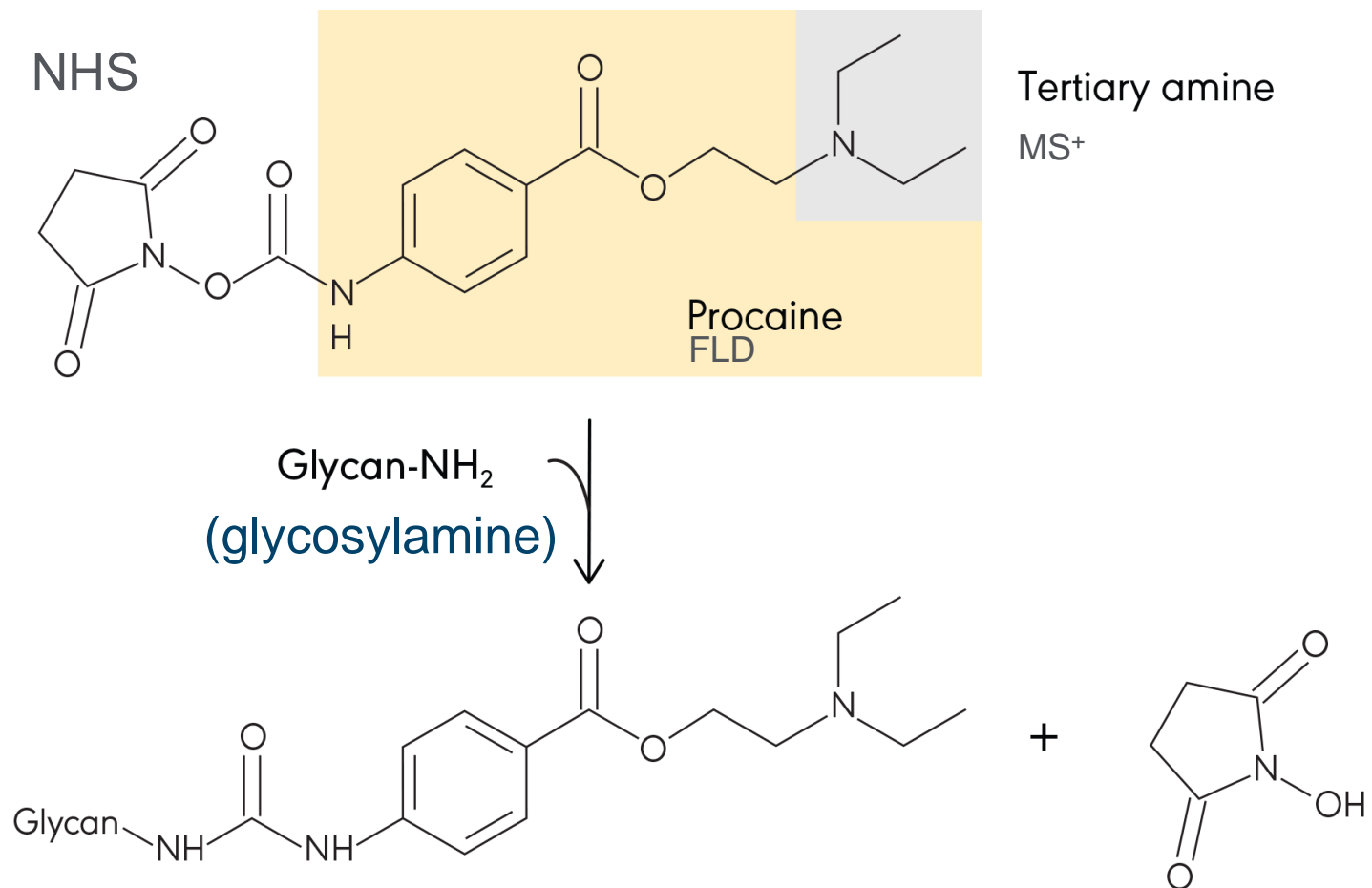
Glycoprotein $\xrightarrow{\text{N-Glycanase}}$

H_2O NH_3

2-AB

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

2-AA

- Standards available
- Labeling not supported

APTS

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

Agilent

InstantPC Dye (IPC)

NHS

Tertiary amine
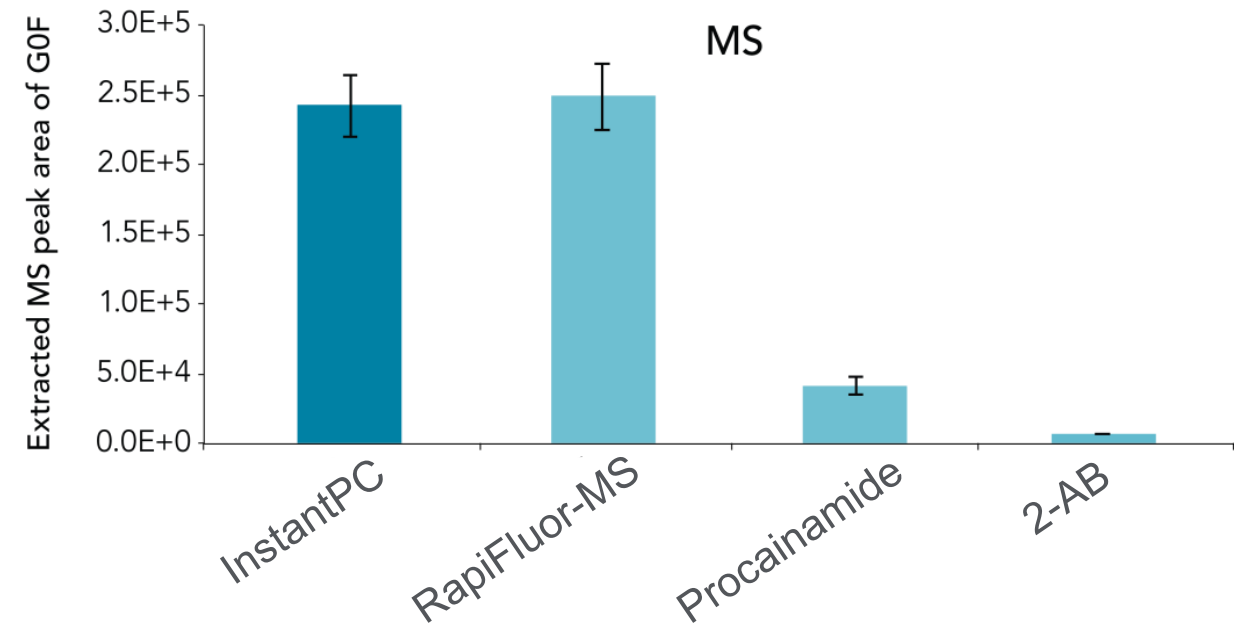
MS+

Procaine
FLD

Glycan-NH$_2$

(glycosylamine)
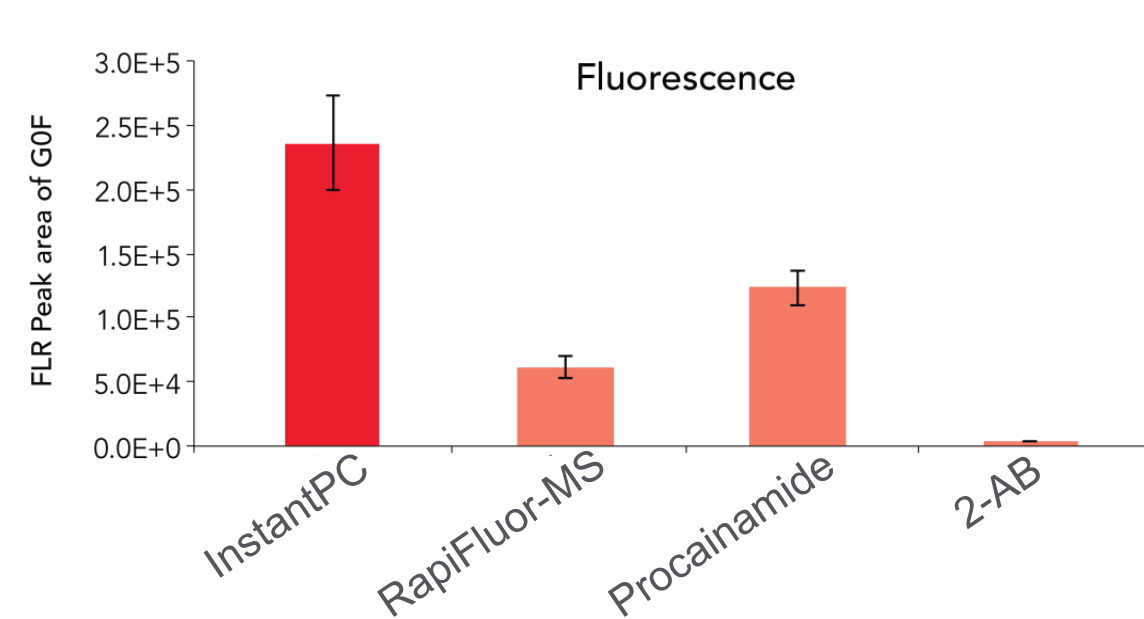
+

Label
1 minute at 50 °C

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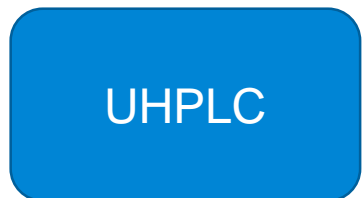
    Agilent

FLD and MS Response Comparison

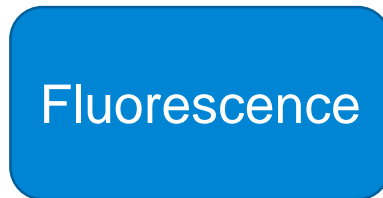Labeling uses same amount of released glycans from GlykoPrep digestion

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

Agilent

UHPLC-HILIC Separation of Labeled N-Glycans

Separation

UHPLC

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

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

HILIC: hydrophilic interaction liquid chromatography
Example Method: 1 hour

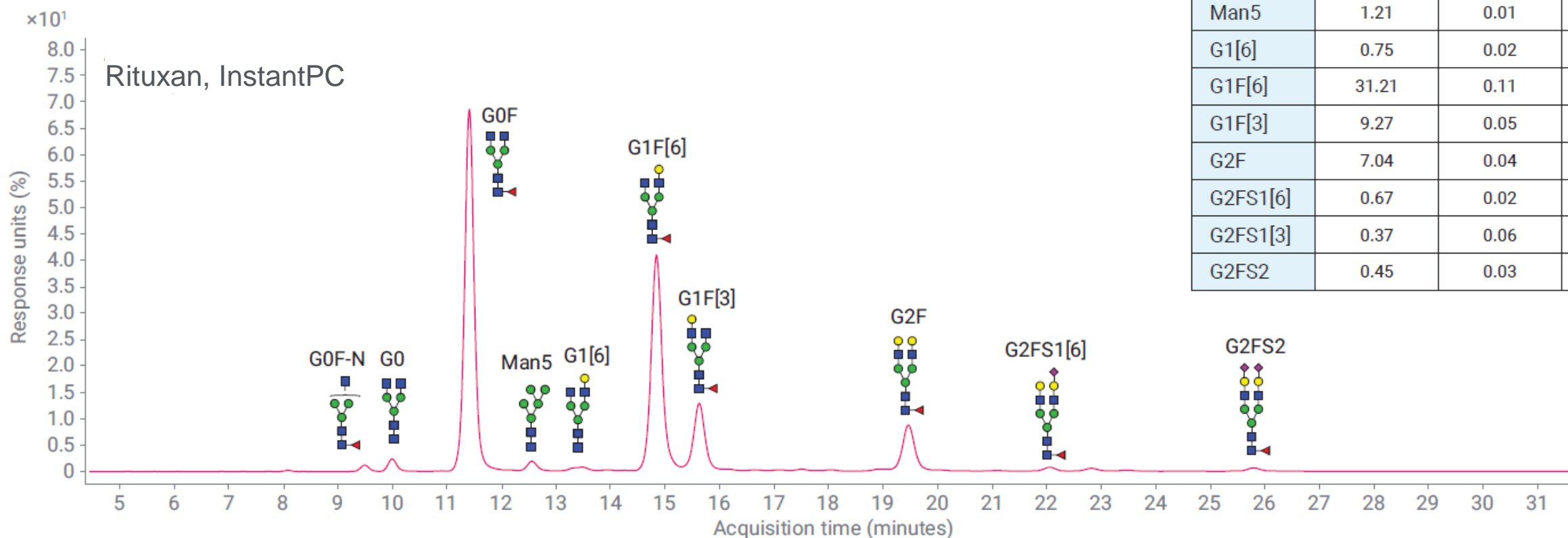| Time (min)	%A	%B	Flow (ml/min)
0.00	20.0	80.0	0.50
2.00	25.0	75.0	0.50
48.00	38.0	62.0	0.50
49.00	60.0	40.0	0.50
51.50	60.0	40.0	0.50
52.00	18.0	82.0	0.50
60.00	18.0	82.0	0.50

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

Agilent

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

Rituxan, InstantPC

UHPLC-HILIC, 60 minute method

AdvanceBio Glycan Mapping column
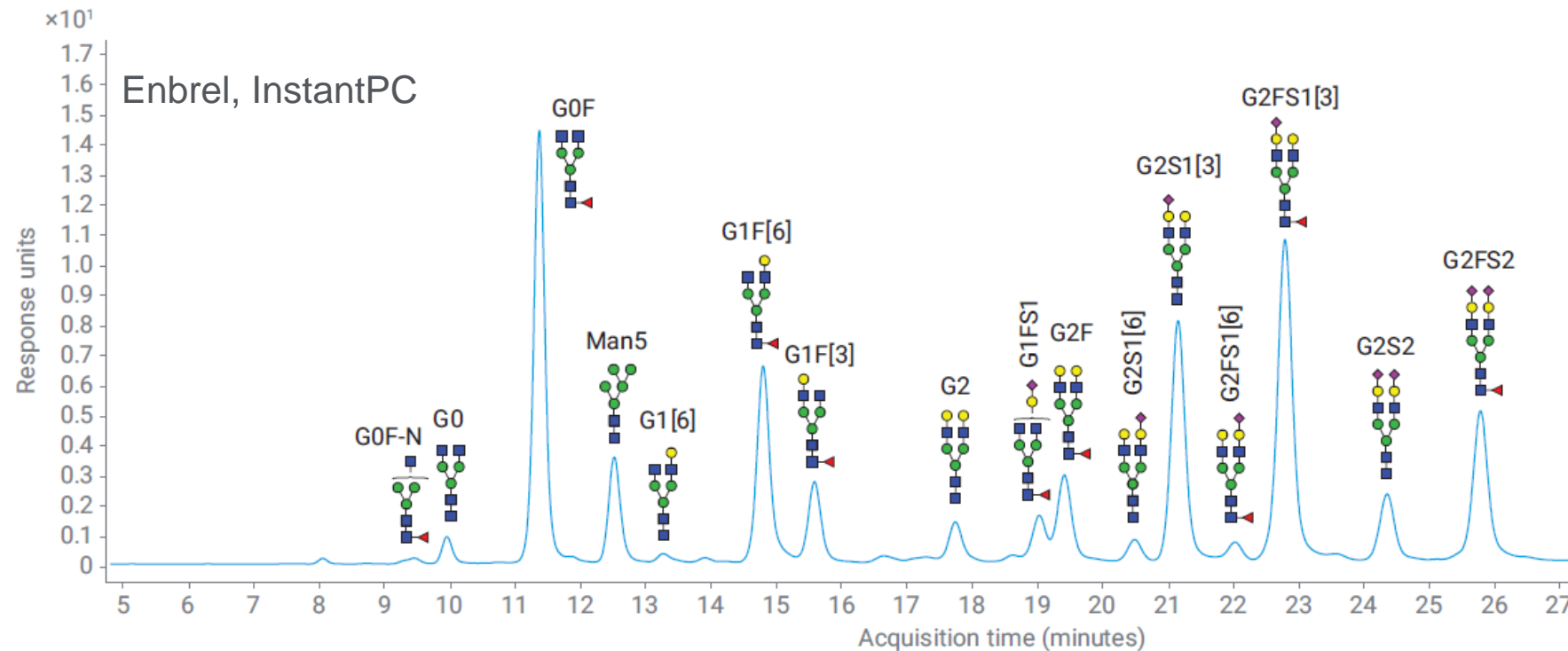
2.1 x 150 mm, 1.8 µm

FLD: 285/345 nm

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

N = 4

Agilent

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

Enbrel, 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

Agilent

MS of InstantPC N-Glycans from Enbrel

Enbrel FLD

Enbrel TIC (total ion chromatogram)
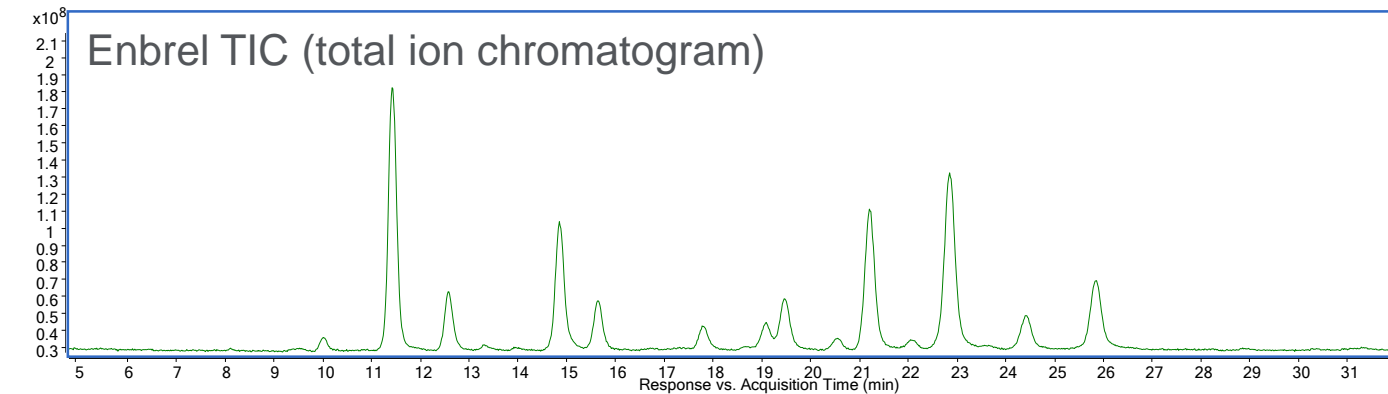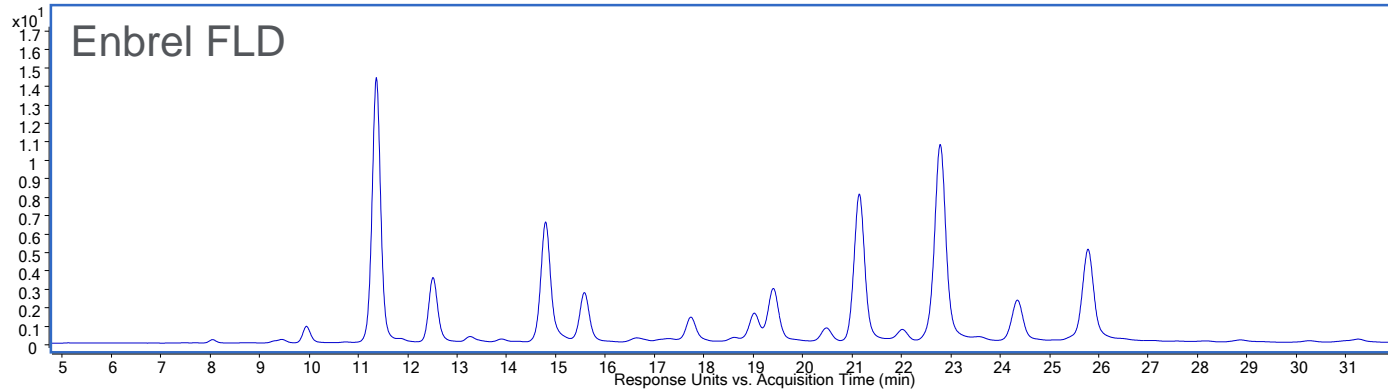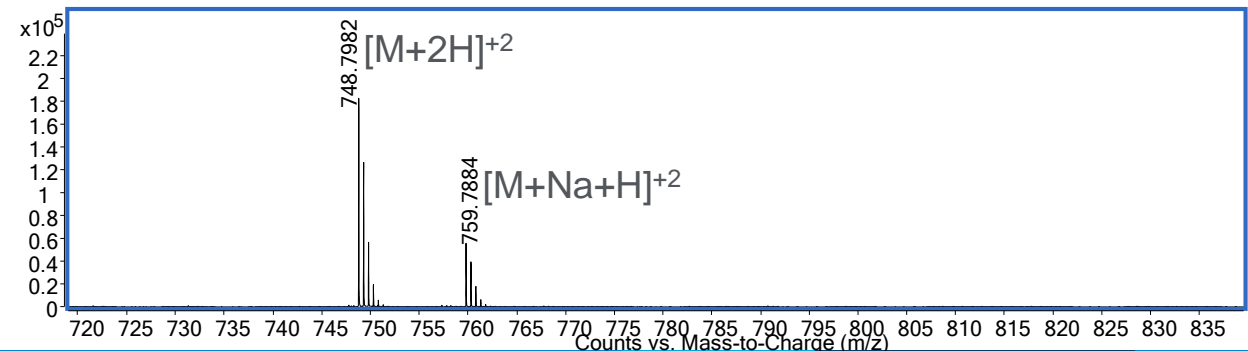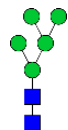
Fluorescence

↓

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

748.7982 $[M+2H]^{+2}$

759.7884 $[M+Na+H]^{+2}$

For Research Use Only. Not for use in diagnostic procedures.   Agilent

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

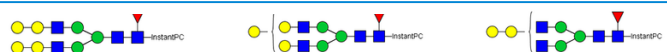| Product Code	Description
GKPC-005	Human IgG N-Linked Glycan Library
GKPC-503	Glucose Homopolymer
GKPC-233	α(2-3) Sialylated Triantennary Library
GKPC-263	α(2-6) Sialylated Triantennary Library
GKPC-234	α(2-3) Sialylated Tetraantennary Library
GKPC-264	α(2-6) Sialylated Tetraantennary Library

New CHO mAb glycoprotein & N-glycan library

Labeled Glycan Standards

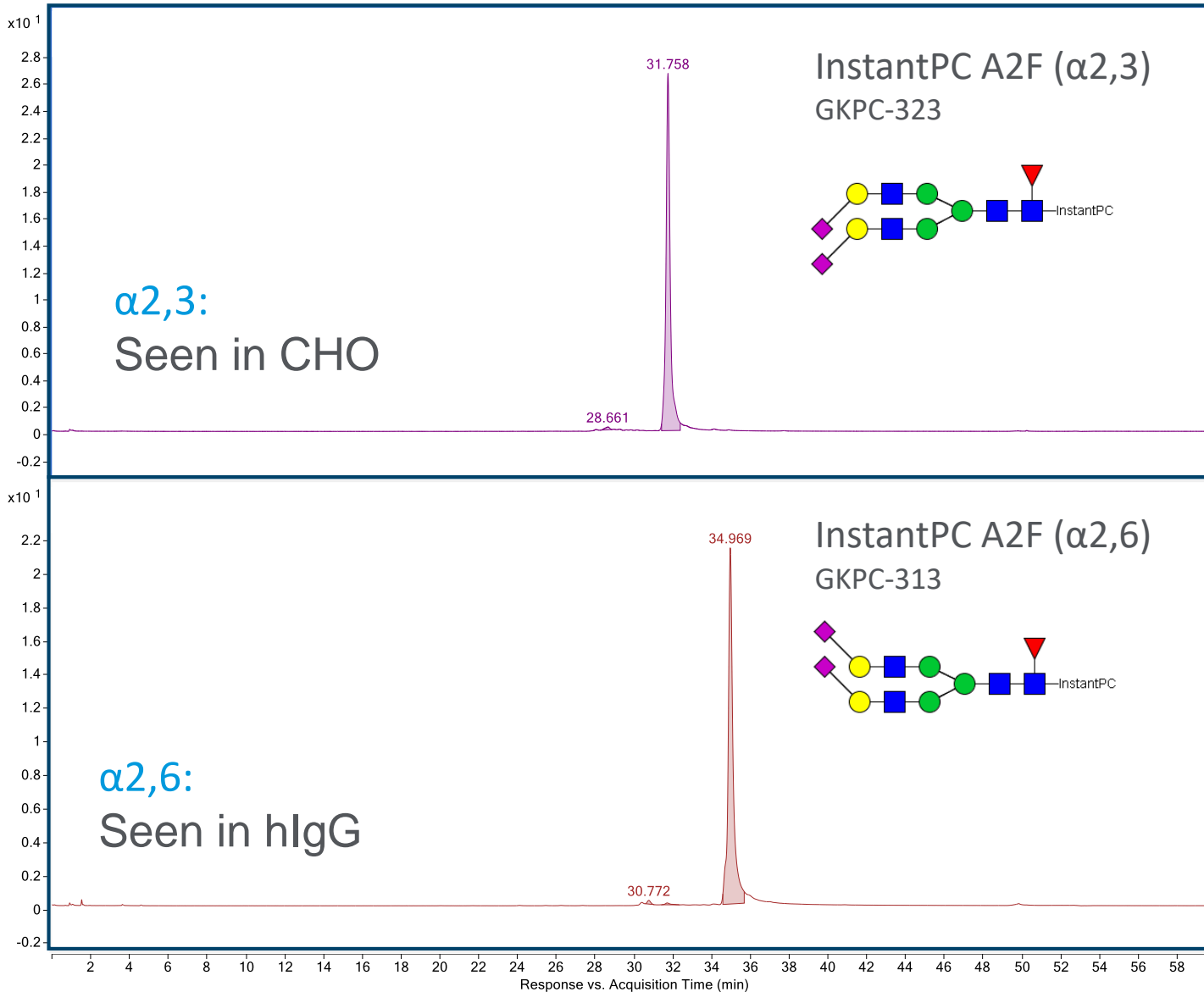2-AB, 2-AA, APTS, InstantPC, InstantAB, InstantQ

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

Glycan Standards Flier 5994-0999EN

Agilent

InstantPC Sialylated N-Glycan Standards

α2,3:
Seen in CHO

InstantPC A2F (α2,3)
GKPC-323

31.758

28.661

α2,6:
Seen in hIgG

InstantPC A2F (α2,6)
GKPC-313

34.969

30.772

Response vs. Acquisition Time (min)

- **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.

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)*
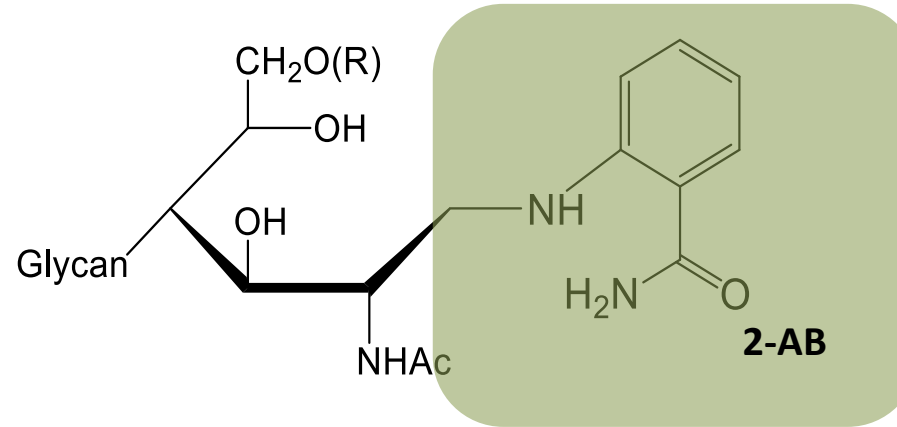
ProZyme Technical Note 4010

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 4003

Agilent

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

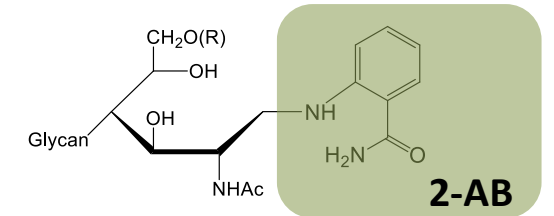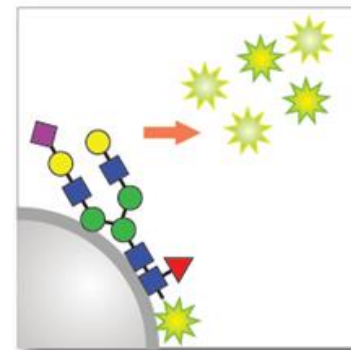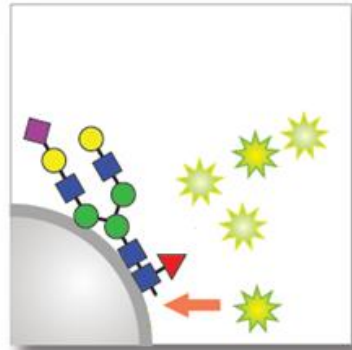2-AB (2-aminobenzamide) dye has been used to generate released N-glycan data for more than 20 years
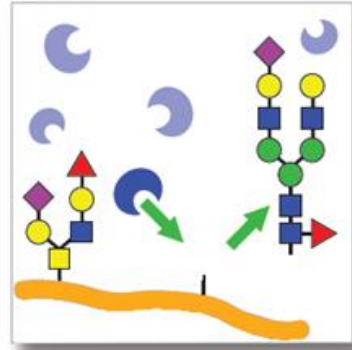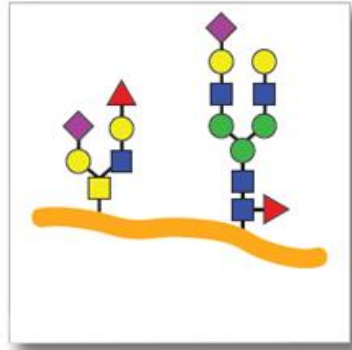
Well established in many laboratories

May need to match historic data using 2-AB

Gly-X 2-AB Express Workflow
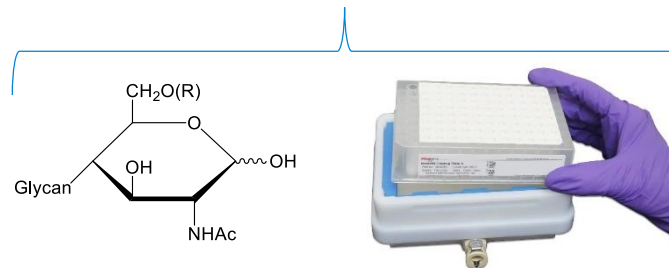
1-40 µg
0.05-2 mg/ml

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
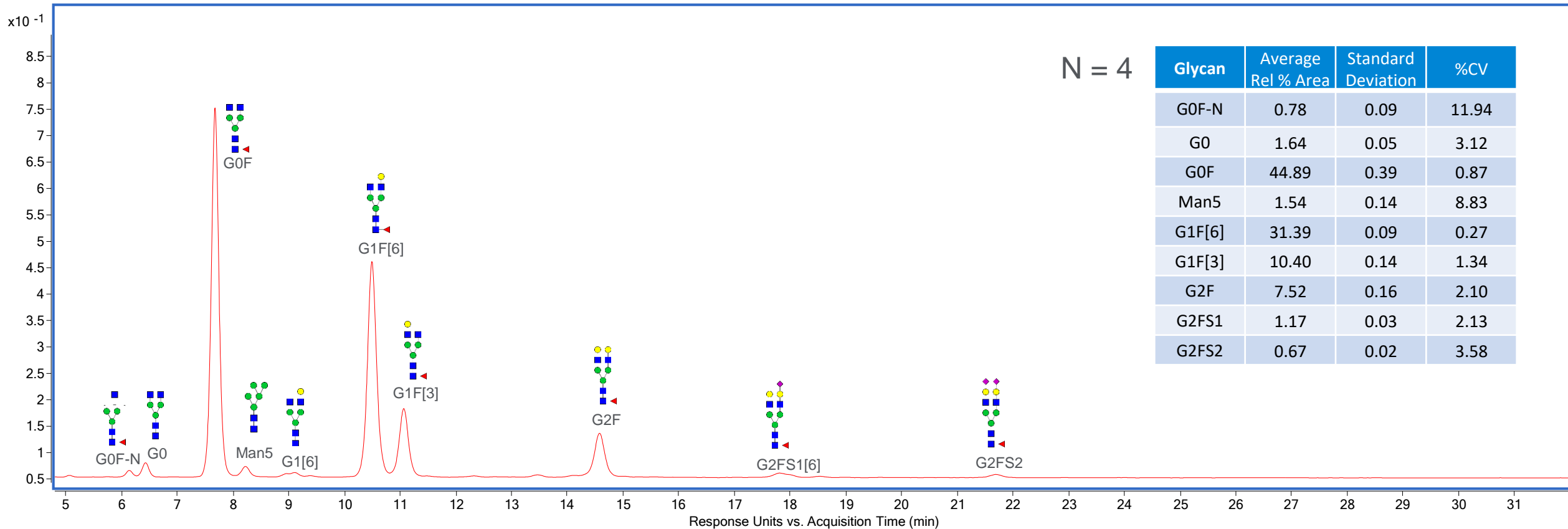
2-AB

Workflow time: 120 min

2-AB   On-matrix labeling

Agilent

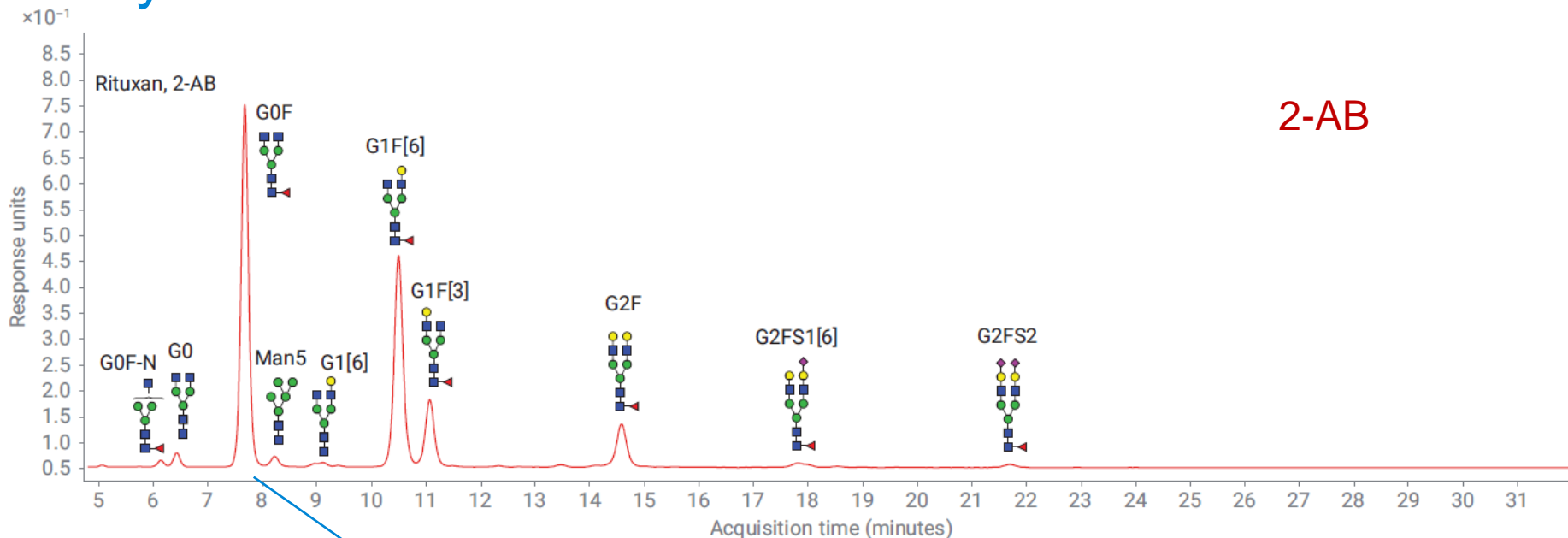# Rituximab Gly-X 2-AB Express N-Glycans

Analytical Method:

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

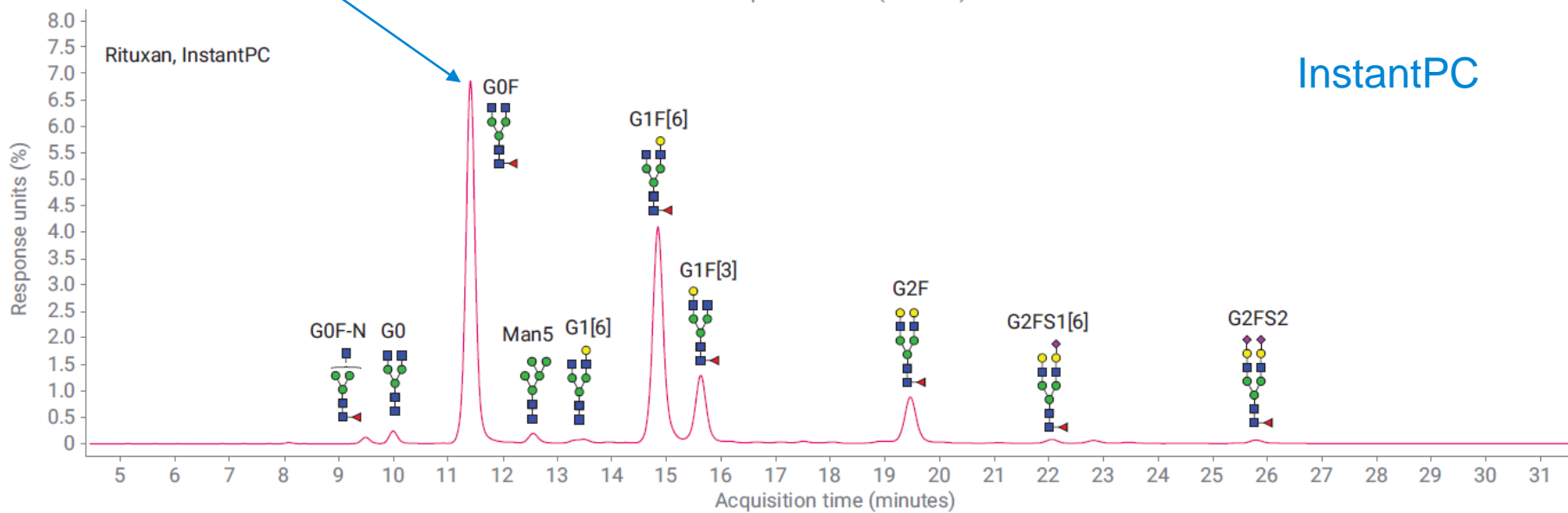AdvanceBio Glycan Mapping column, 2.1 x 150 mm, 1.8 µm

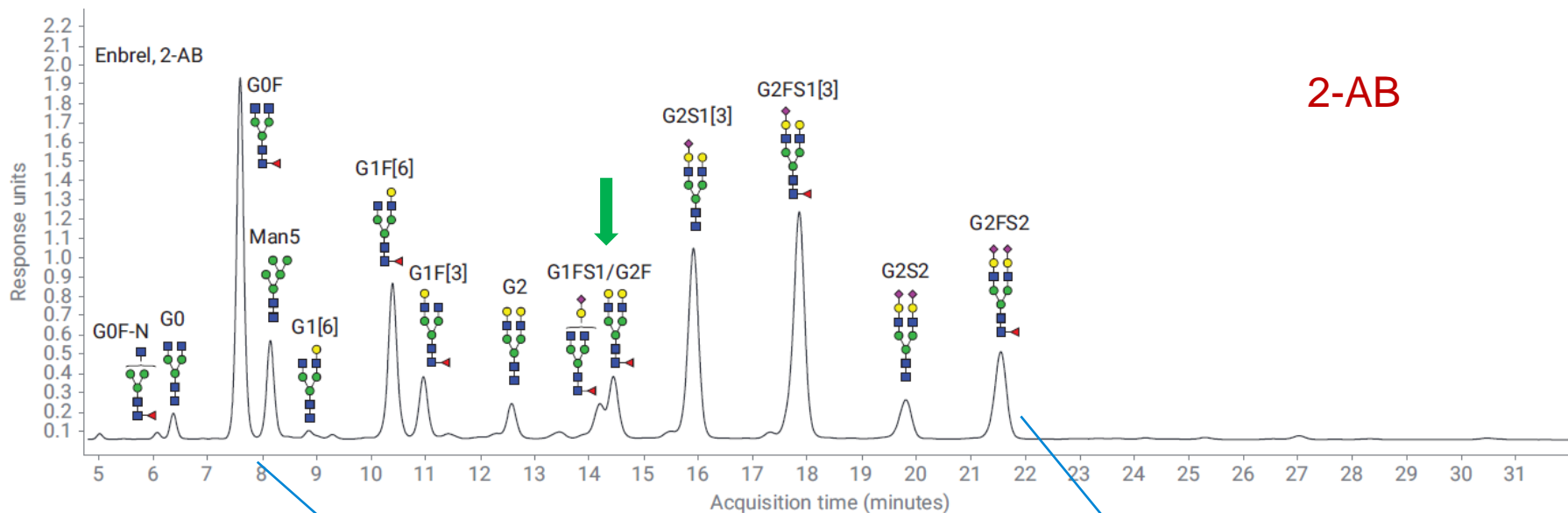| Glycan	Average Rel % Area	Standard Deviation	%CV
G0F-N	0.78	0.09	11.94
G0	1.64	0.05	3.12
G0F	44.89	0.39	0.87
Man5	1.54	0.14	8.83
G1F[6]	31.39	0.09	0.27
G1F[3]	10.40	0.14	1.34
G2F	7.52	0.16	2.10
G2FS1	1.17	0.03	2.13
G2FS2	0.67	0.02	3.58

N = 4

Agilent

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

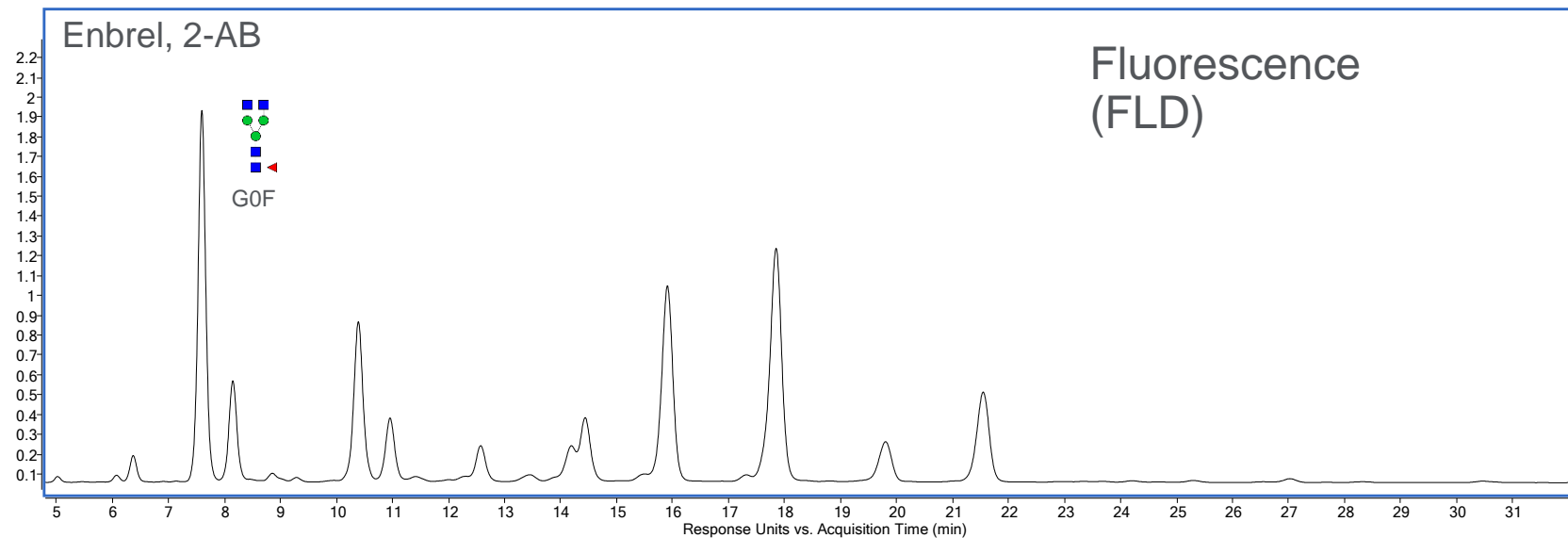# Gly-X 2-AB vs InstantPC - etanercept

2-AB

N=4
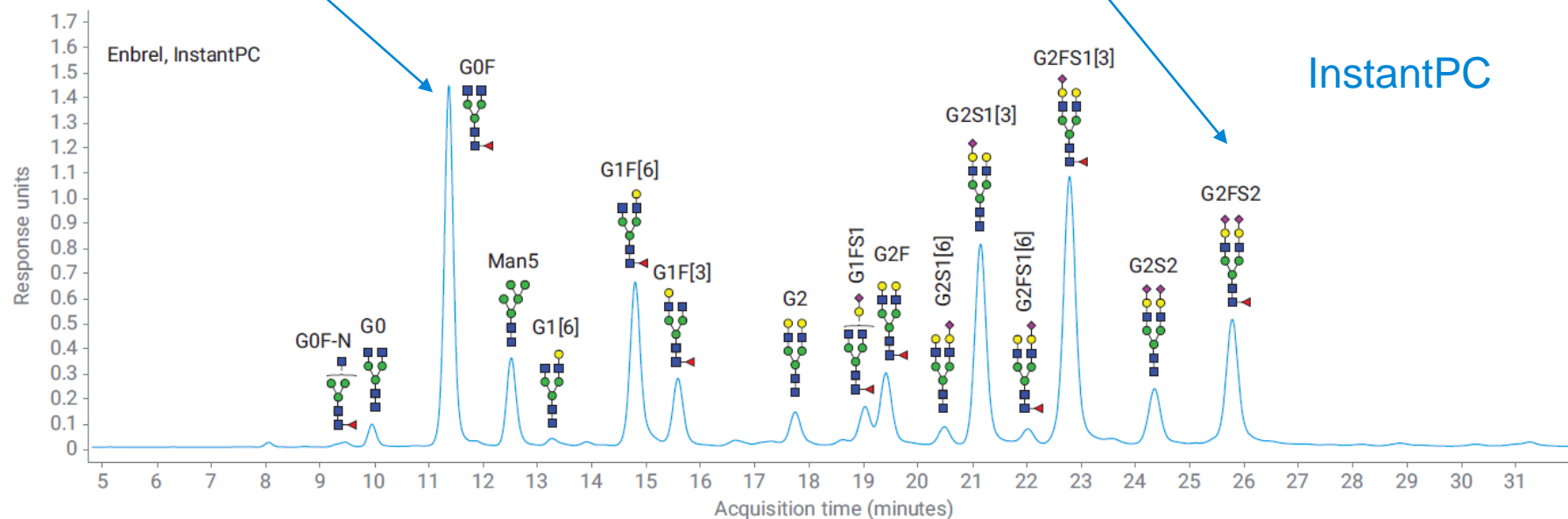
	Average Rel % Area	Standard Deviation	%CV
G0F-N	0.32	0.02	7.44
G0	1.27	0.07	5.34
G0F	20.18	0.45	2.22
Man5	5.50	0.34	6.17
G1[6]	0.45	0.02	3.89
G1F[6]	10.35	0.33	3.18
G1F[3]	3.92	0.17	4.39
G2	2.21	0.15	6.78
G2F/G1FS1	7.00	0.25	3.63
G2S1	15.19	0.17	1.09
G2FS1	20.10	0.32	1.59
G2S2	4.19	0.25	5.95
G2SF2	9.35	0.74	7.93

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

Agilent

2-AB N-Glycans: MS

Enbrel, 2-AB — Fluorescence (FLD)

G0F

Enbrel, 2-AB — Total Ion Chromatogram (6545XT Q-TOF)

G0F

(InstantPC)

Enbrel FLD

Enbrel TIC (total ion chromatogram)

Column options for 2-AB N-glycans

60 minute method
2.1 x 150 mm, 1.8 µm AdvanceBio Glycan Mapping column

28 minute method
2.1 x 150 mm, 2.7 µm AdvanceBio Glycan Mapping column

Response Units vs. Acquisition Time (min)
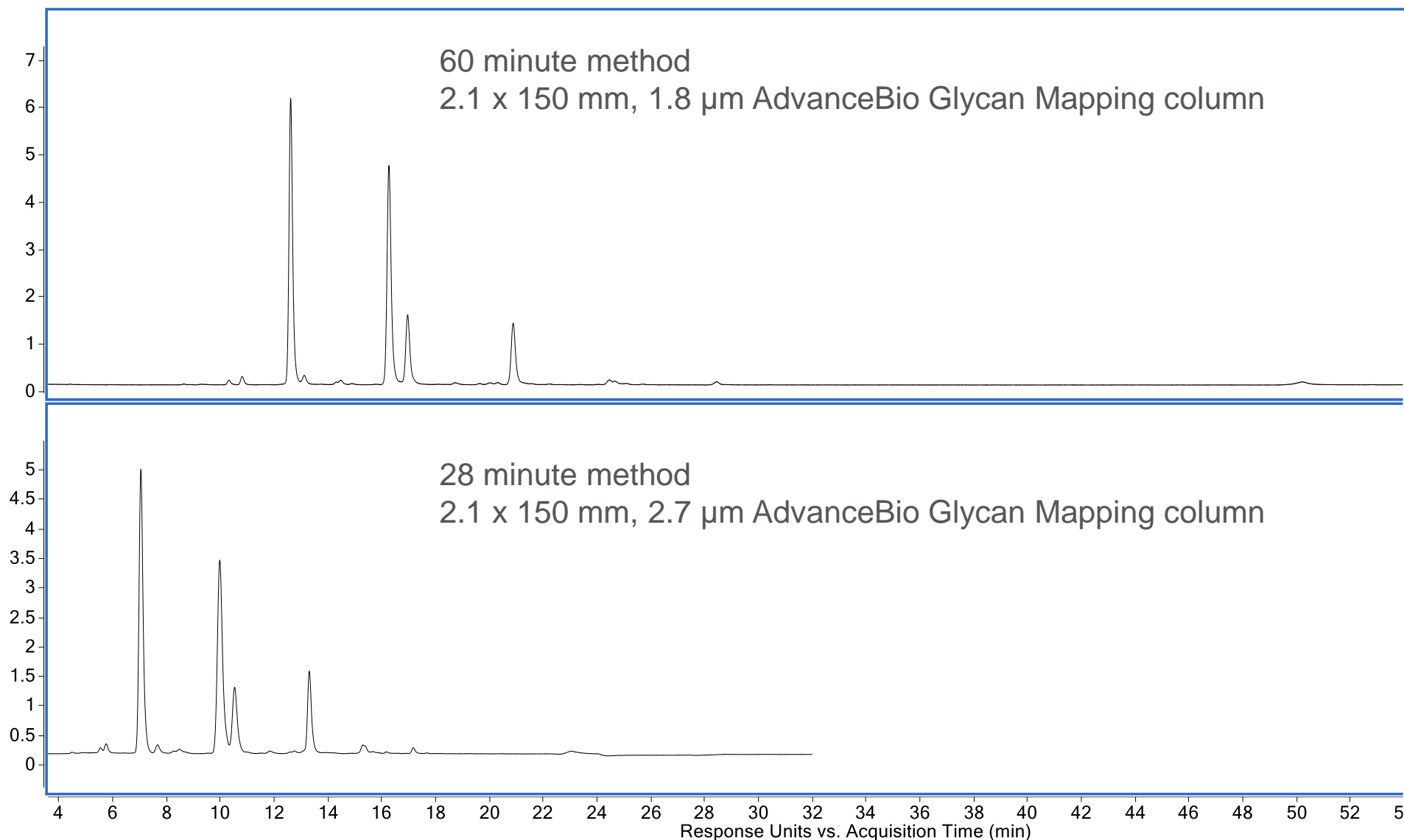
Time (min)	%A	%B	Flow (ml/min)
0.00	26.00	74.00	0.50
1.00	27.50	72.50	0.50
9.00	28.50	71.50	0.50
22.00	40.00	60.00	0.50
22.50	49.00	51.00	0.80
23.50	49.00	51.00	0.70
23.70	26.00	74.00	0.70
25.00	26.00	74.00	0.70
25.50	26.00	74.00	0.80
27.50	26.00	74.00	0.90
28.00	26.00	74.00	0.50

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

Column temperature: 40 °C
Pressure: well below 600 bar

Agilent

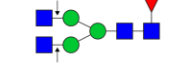# 2-AB N-Glycan Standards & Libraries

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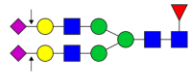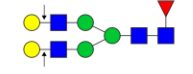
Product Code	Description
GKSB-401	G0-N
GKSB-301	G0
GKSB-402	G0F-N
GKSB-302	G0F
GKSB-303	G0FB
GKSB-317	G1
GKSB-316	G1F
GKSB-304	G2
GKSB-305	G2F
GKSB-306	G2FB
GKSB-318	NA2Ga2F
GKSB-311	A1
GKSB-315	A1F
GKSB-312	A2
GKSB-313	A2F
GKSB-307	NGA3

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

Exoglycosidase Confirmation of Structures

Exoglycosidase [Product Code]	Short Name	Example Glycan	Specificity
Sialidase A (Arthrobacter) [GK80040]	ABS		α (2–3, 6, 8, 9) N-Acetylneuraminic acid linkages
β-Galactosidase (Bovine testis) [GKX-5013]	BTG		β (1–3, 4) Galactose linkages
β-N-Acetylhexoseaminidase (Jack bean) [GKX-5023]	JBH		B (1–2, 3, 4, 6) GlcNAc and GalNAc linkages

◆ N-Acetylneuraminic acid (Neu5Ac) ○ Galactose ■ N-Acetylglucosamine (GlcNAc) ● Mannose ▼ Fucose
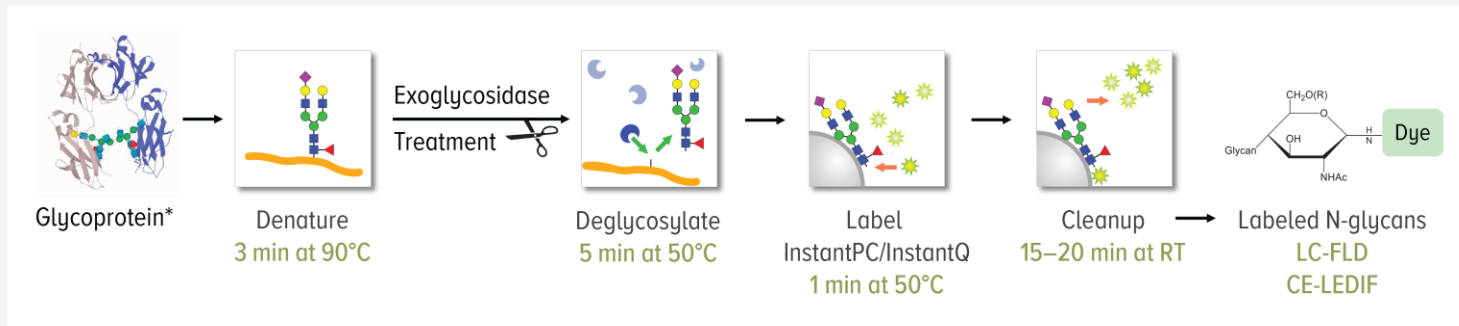
Glycoprotein* → Denature 3 min at 90°C → **Exoglycosidase Treatment** ✂ → Deglycosylate 5 min at 50°C → Label InstantPC/InstantQ 1 min at 50°C → Cleanup 15–20 min at RT → Labeled N-glycans LC-FLD CE-LEDIF

* Glycoprotein structure adopted from PDB entry DOI: 10.2210/pdb5VGP/pdb
PDB structure modified and image genrated with Lite Mol: https://webchemdev.ncbr.muni.cz/LiteMol/

An Intact Protein Approach to Exoglycosidase Sequencing Followed by Released N-Glycan Analysis from Biotherapeutic Glycoproteins (TN4013)
Andres Guerrero, Anna Fong, John Yan, Tom Rice, Justin Hyche, Aled Jones

Agilent

Exoglycosidase Confirmation of Structures: UHPLC-HILIC
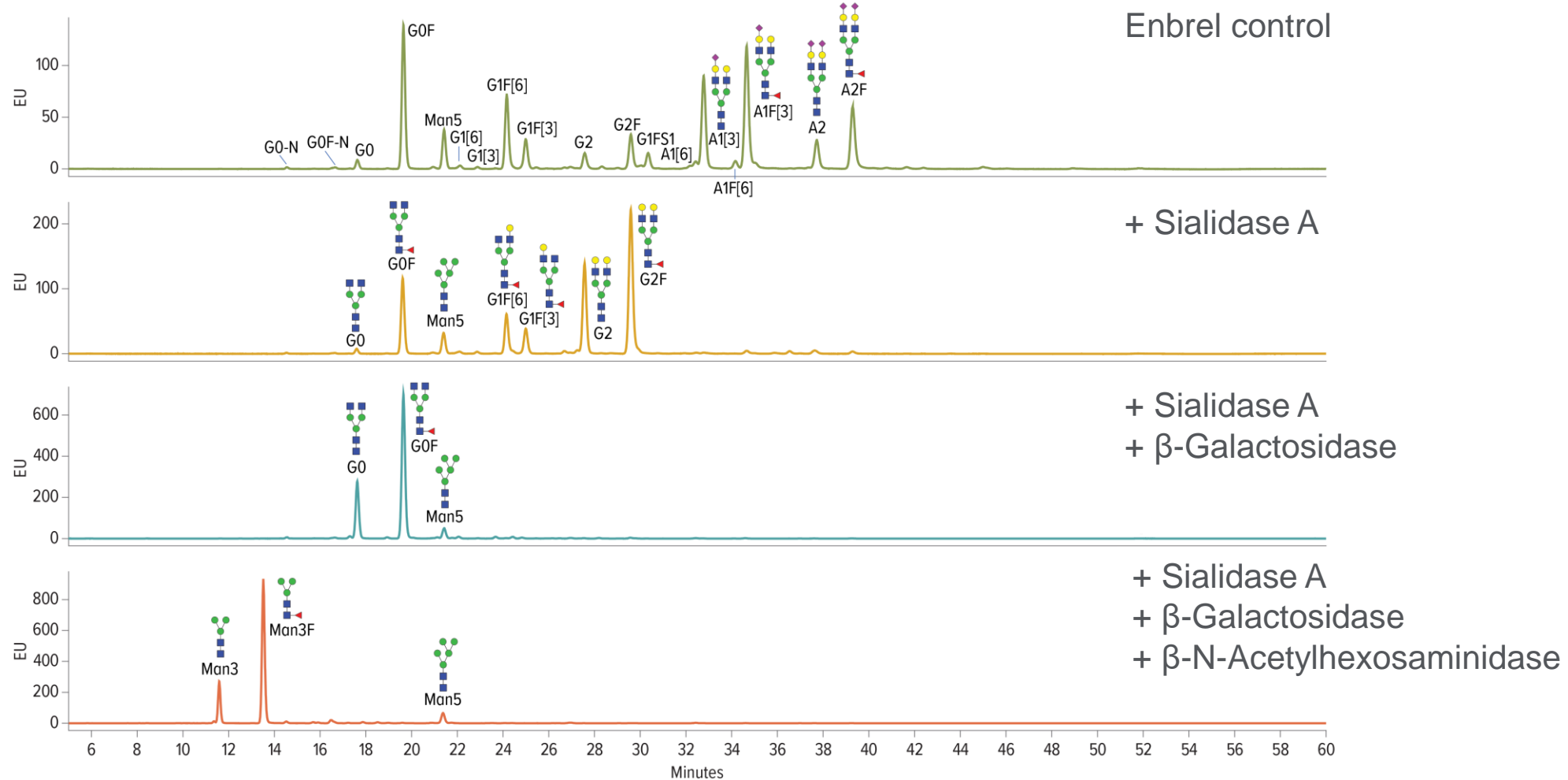
An Intact Protein Approach to Exoglycosidase Sequencing Followed by Released N-Glycan Analysis from Biotherapeutic Glycoproteins (TN4013)
Andres Guerrero, Anna Fong, John Yan, Tom Rice, Justin Hyche, Aled Jones

Gly-X APTS Express: sample prep for capillary electrophoresis (CE)

APTS N-Glycan Sample Prep Workflows

GlykoPrep with APTS
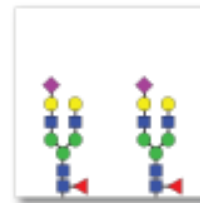Total time ~4–5 hours
On-matrix deglycosylation
In-solution labeling
On-matrix cleanup
Automation-ready format
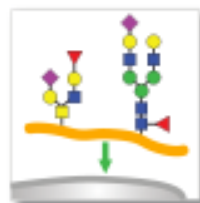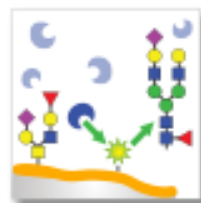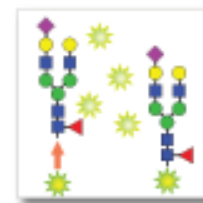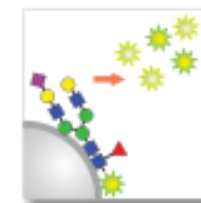
| Denature | Deglycosylate | Dry Down | Label | Clean up |
| 5 min | 15–60 min | 30–60 min | 60 min | 15–30 min |

Gly-X with APTS Express
Total time ~ 2.5 hrs
In-solution deglycosylation
On-matrix labeling & cleanup
Automation-ready format
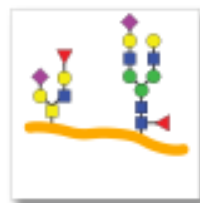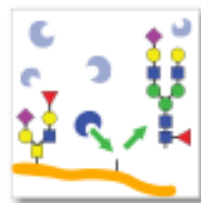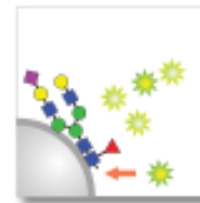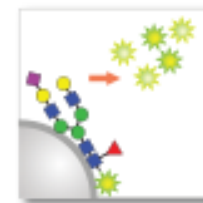
Denature	Deglycosylate	Label	Cleanup	
3 min	5 min	60 min	15–30 min	
		No dry down		

- Solid phase
- Vacuum driven

Development of a Rapid APTS Sample Preparation Workflow for N-Glycan Release and Labeling (5994-0994EN)
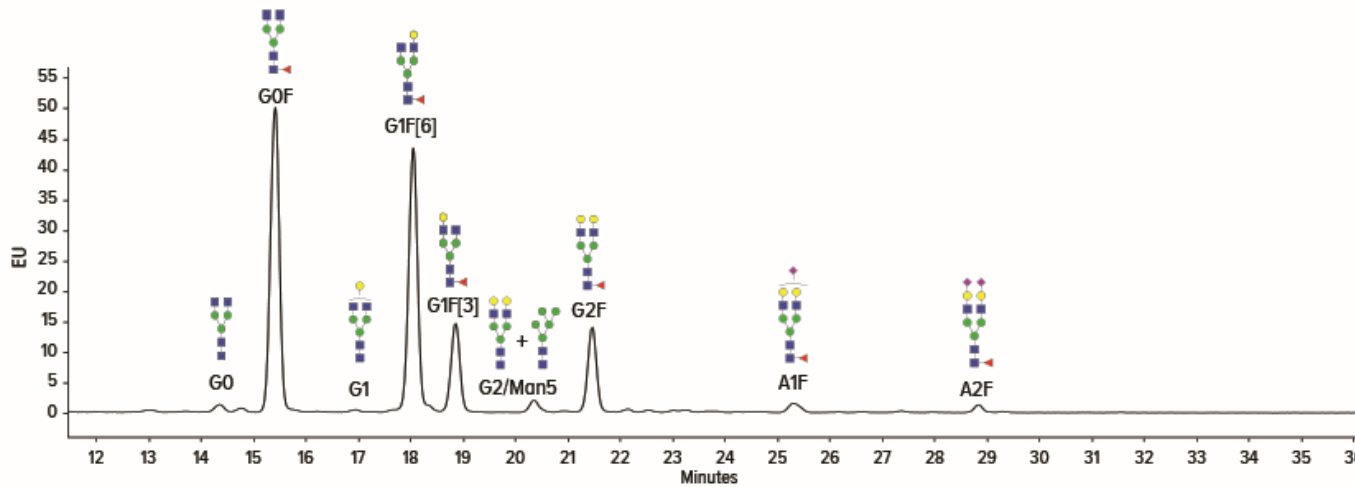
Agilent

Gly-X APTS Express: rituximab

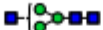MabThera APTS-labeled N-glycans:
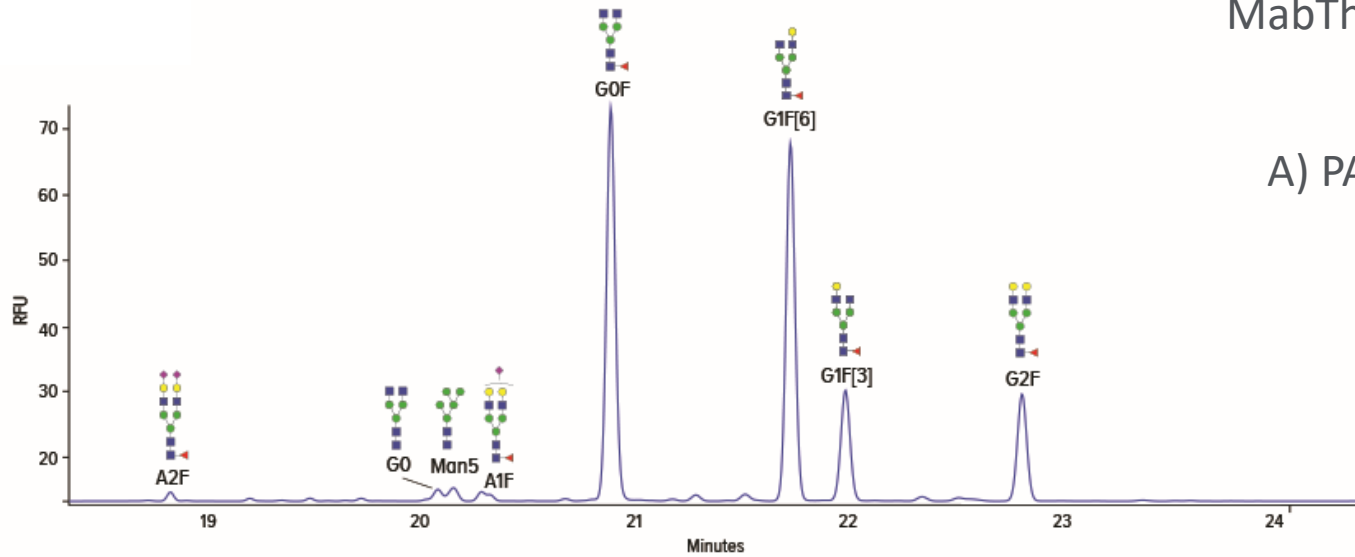
A) PA800 plus (CE)

B) HILIC

Development of a Rapid APTS Sample Preparation Workflow for N-Glycan Release and Labeling
5994-0994EN

Agilent

APTS N-Glycan Standards & Libraries

Individual N-Glycans

Product Code	Description	
GKSP-401	G0-N	
GKSP-301	G0	
GKSP-402	G0F-N	
GKSP-302	G0F	
GKSP-317	G1	
GKSP-316	G1F	
GKSP-304	G2	
GKSP-305	G2F	
GKSP-318	NA2Ga2F	

Product Code	Description	
GKSP-311	A1 (α2,6)	
GKSP-315	A1F (α2,6)	
GKSP-312	A2 (α2,6)	
GKSP-313	A2F (α2,6)	
GKSP-103	Man5	
GKSP-104	Man6	
GKSP-105	Man7	
GKSP-106	Man8	
GKSP-107	Man9	

N-Glycan Libraries

Product Code	Description
GKSP-005	Human IgG N-Linked Glycan Library
GKSP-520	Biantennary & High Mannose Partitioned Library
GKSP-500	APTS Bracketing Standard (dp2 and dp15)
GKSP-503	APTS Maltodextrin Ladder
GKSP-232	α(2-3) Sialylated Biantennary Library
GKSP-262	α(2-6) Sialylated Biantennary Library
GKSP-233	α(2-3) Sialylated Triantennary Library
GKSP-263	α(2-6) Sialylated Triantennary Library
GKSP-234	α(2-3) Sialylated Tetraantennary Library
GKSP-264	α(2-6) Sialylated Tetraantennary Library

Importance of Sialic Acid on Biotherapeutics
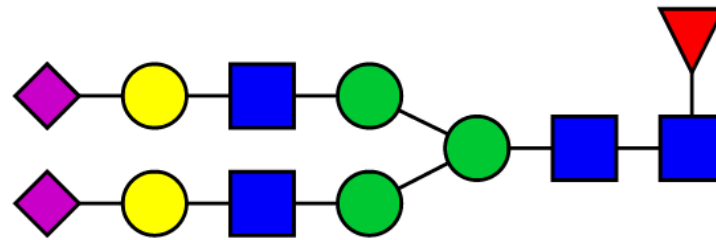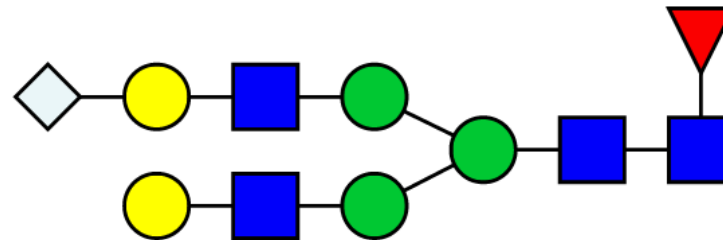
N-glycolyneuraminic acid (NeuGc/NGNA): Potentially immunogenic

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
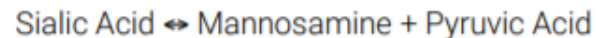
Then pyruvate oxidase catalyzes the reaction:

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
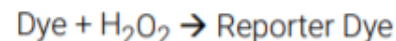
10 µL Glycoprotein

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)

Agilent

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.

Agilent

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

Agilent

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

Agilent

100 µM Sialic Acid Standard Lot Comparison (target 0.5 nmol)
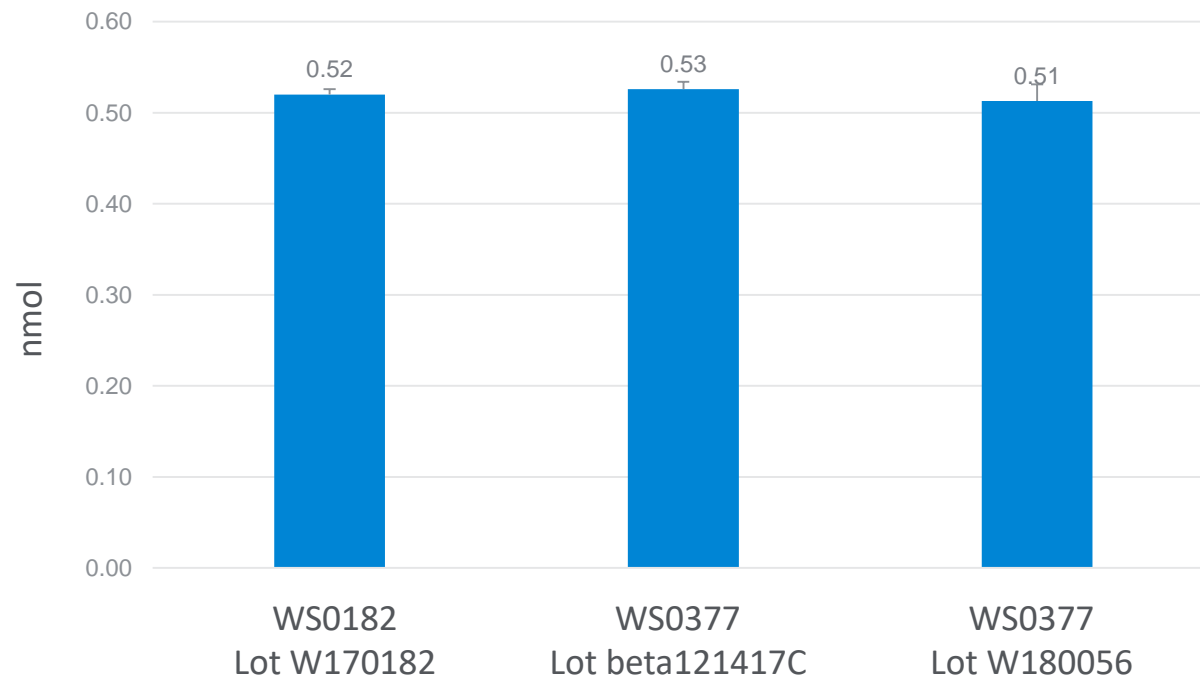
- WS0182: older NANA standard included with GS300/GF57
- WS0377: new NANA standard for GS48/96-SAQ, uses USP material

For Research Use Only. Not for use in diagnostic procedures.   Agilent

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
- APTS Express (for open platform CE) : 2 hours

AdvanceBio Glycan Mapping columns for HILIC separation

Fluorescence detection for relative % area quantitation

MS detection for the assignment of glycan structures to peaks

Sialic acid quantitation:

- Total sialic acid quantitation (SAQ), plate based

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

Agilent

Acknowledgements

Agilent, Hayward CA (formerly ProZyme)

Hamutal Bonen

Archana Datt

Anna Fong

Ace G. Galermo

Ted Haxo

Justin Hyche

Aled Jones

Tom Mannion

Steve Mast

Bethan Morgan

Shiva Pourkaveh

Marco Rhotert

Tom Rice

Jim Torrence

Sergey Vlasenko

**Agilent Technologies
Little Falls DE and Santa Clara CA**

Anne Blackwell

Linda Lloyd

Oscar Potter

Suma Ramagiri

Greg Staples

DE.7095833333

Agilent