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Consumables Workflow Ordering Guide

N-Glycan Analysis of Biotherapeutic Glycoproteins using AdvanceBio Gly-X InstantPC Sample Preparation and LC/FLD/MS

N-glycan analysis productivity simplified and standardized

The location and structure of N-linked glycans can play a critical role in the pharmacology of therapeutic proteins, potentially affecting immunogenicity, pharmacokinetics and pharmacodynamics. Agilent AdvanceBio Gly-X is a next generation N-glycan sample preparation platform[1] that provides a simplified in-solution workflow using InstantPC dye for rapid glycan labeling and high signal for fluorescence detection (FLD) and mass spectrometry (MS) along with an efficient vacuum plate cleanup step to remove excess label and denaturant. Labeled N-glycan samples are ready for UHPLC/FLD/MS in 60 minutes or less using the AdvanceBio Glycan Mapping column for hydrophilic interaction liquid chromatography (HILIC), followed by relative quantitation. In addition, a wide range of InstantPC-labeled N-glycan standards are available to calibrate N-glycan separations and identify N-glycan species.

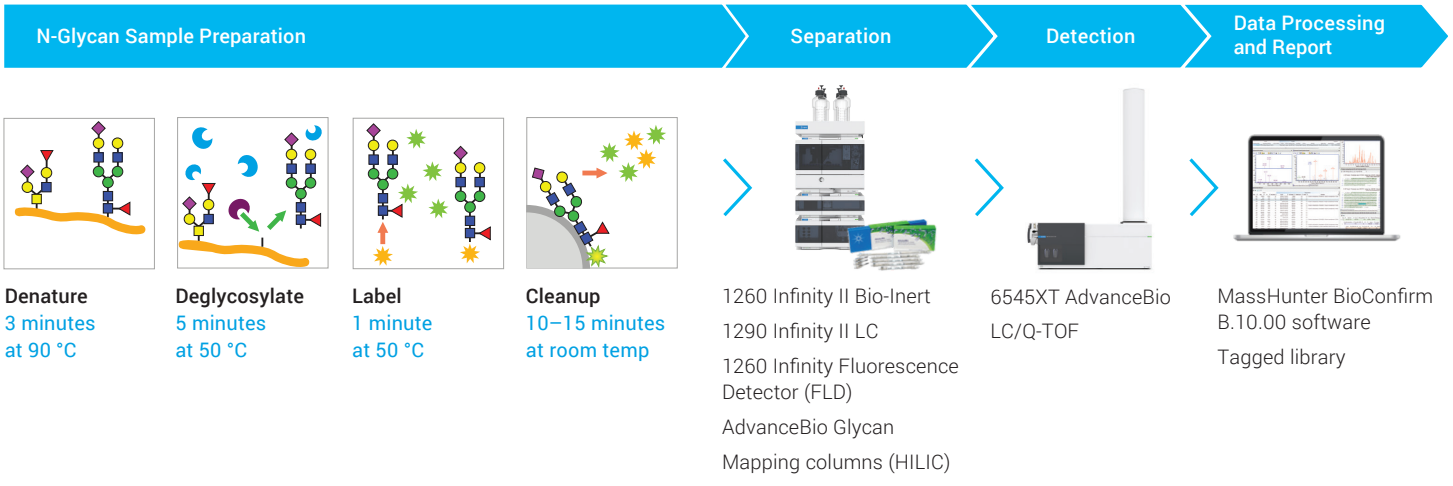


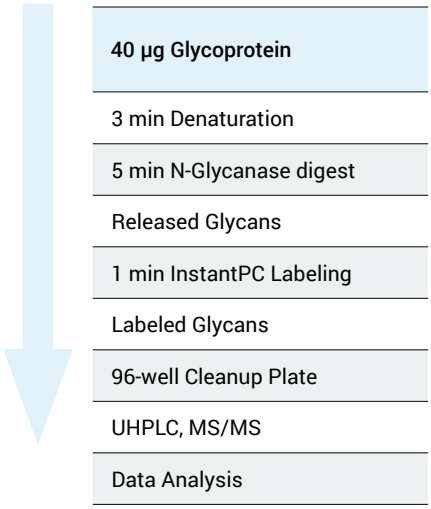
Figure 1. Released N-glycan analysis workflow using Gly-X InstantPC sample preparation with LC/FLD/MS.

End-to-end N-glycan analysis workflow solution designed and manufactured by Agilent

In this guide, you'll find the consumables you need to get started with InstantPC N-glycan analysis. Many of the consumables were tested and their results reported in the application note 5994-1348EN.[2] This study assessed the N-glycans of rituximab (Rituxan, a monoclonal antibody or mAb) and etanercept (Enbrel, an Fc fusion protein) and demonstrated that InstantPC labeled N-glycan analysis shows significantly higher fluorescence signal and greater MS ionization efficiency compared with 2-AB glycans, allowing detection of low abundance glycan species.

This Gly-X InstantPC N-Glycan analysis workflow guide includes ordering information for:

- Sample preparation kit – Gly-X InstantPC technology, specifically developed and optimized for strong fluorescence signal in LC/FLD and enhanced ionization for MS analysis.
- InstantPC-labeled N-glycan standards – these well-characterized individual standards and libraries play an essential role in profiling N-glycan species which can impact the safety and efficacy of biotherapeutic drug products.
- Liquid chromatography columns for separation of glycans by HILIC.
- Solvents and reagents.
- Vials and caps.
- Data analysis and reporting.



Here's how you boost N-glycan analysis productivity

- Samples ready for UHPLC/FLD or LC/MS in less than 1 hour.
- 5-min PNGase F digestion provides unbiased N-glycan release.
- InstantPC dye for high UHPLC/FLD and MS signal.
- Simple, ambient stable 96-well cleanup plate.
- Supports rapid and high resolution analysis.
- Modular format supports flexible sample throughput and eliminates waste.

Figure 2 shows examples of HILIC/FLD data for released N-glycans from rituximab and etanercept prepared with Gly-X InstantPC. Further information including MS data for N-glycan structure assignment is included in Application Note 5994-1348EN.

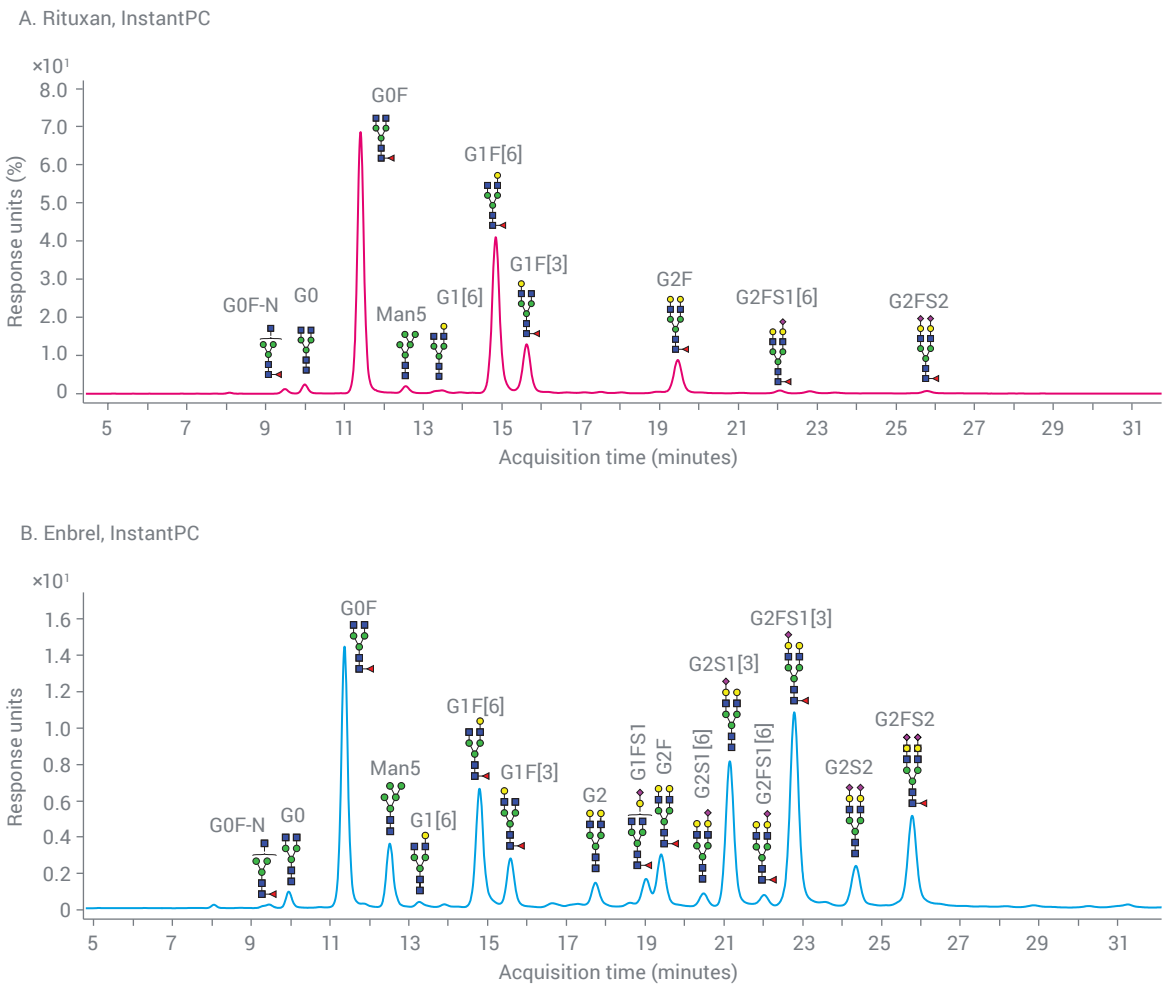


Figure 2. HILIC-UHPLC fluorescence profile of A) Rituxan and B) Enbrel N-glycans labeled with InstantPC. N-Glycan relative percent areas are shown in Tables 1 and 2, n = 4. Data is from Application Note 5994-1348EN. UHPLC conditions and Q-TOF parameters are shown in Tables 3 and 4.

Table 1. Relative % area, SD, and %CV values for Rituxan N-glycans labeled with InstantPC, n = 4.

	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

Table 2. Relative % area, SD, and %CV values for Rituxan N-glycans labeled with InstantPC, 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

Table 3. Agilent 1290 Infinity II UHPLC HILIC/FLD conditions for InstantPC labeled N-glycans.

Parameter	Value		
Column	Agilent AdvanceBio Glycan Mapping, 2.1 × 150 mm, 1.8 µm (p/n 859700-913)		
Column Temp	40 °C		
Mobile Phase	A) 50 mM ammonium formate, pH 4.5 B) Acetonitrile		
Gradient Program	InstantPC labeled N-glycans		
	Time (minutes)	%B	Flow rate (mL/min)
	0	80	0.5
	2	75	0.5
	48	62	0.5
	49	40	0.5
	51.5	80	0.5
	52	80	0.5
	60	80	0.5
Injection Volume	1 µL (equivalent to glycans from 0.4 µg protein)		
Fluorescence Detection	Agilent 1260 Infinity II FLD InstantPC: λEx 285 nm, λEm 345 nm		

Table 4. Agilent 6545XT Q-TOF parameters for InstantPC labeled N-glycans.

Agilent 6545XT Q-TOF	
Source	Dual AJS ESI
Gas Temperature	150 °C
Drying Gas Flow	9 L/min
Nebulizer	35 psi
Sheath Gas Temperature	300 °C
Sheath Gas Flow	10 L/min
Vcap	3,000 V
Nozzle Voltage	500 V
Fragmentor	120 V
Skimmer	65 V
Mass Range	m/z 600 to 3,000
Scan Rate	1 spectra/sec
Acquisition Mode	High resolution (4 GHz)

Getting started with Gly-X InstantPC

Glycoprotein sample prep considerations

Glycoprotein samples should be prepared to a maximum of 2 mg/mL in a low salt neutral buffer free of detergents and nucleophiles such as amines. Higher concentration samples should be diluted in water or 50 mM HEPES, pH 7.9.

- Maximum concentration: 2 mg/mL.
- Maximum amount of protein per reaction: 40 µg (for example, 2 µL of each 2 mg/mL solution). Higher quantity of protein could be used for mAbs, up to 100 µg, but data linearity should be assessed.
- Buffer: Low salt (~150 mM) neutral buffer without detergents and nucleophiles such as amines. Sample can be diluted with water or 50 mM HEPES, pH 7.9.

Other considerations:

- Sample in amine buffers (for example, Tris, arginine, glycine, histidine) components should follow a buffer exchange step before deglycosylation. A 10 kDa molecular weight cut-off spin centrifugal filter is recommended.
- 0.1% formic acid should be used as an eluent when samples are prepared by protein A affinity chromatography.
- PBS is not recommended.
- Please consult the Gly-X InstantPC manual for further details.[5]

Incubation and cleanup hardware

During the Gly-X InstantPC sample prep, the samples are heated to 90 °C during protein denaturation, and to 50 °C for PNGase F digestion and InstantPC labeling. For heating the samples in the 96 well plate provided, we recommend using a thermocycler, or a dry block heater, and suggestions are provided below. The cleanup process is driven by vacuum. If you wish to use a vacuum manifold and pump other than the Millipore model suggested, please contact Agilent.

Heating and Vacuum Hardware (non-Agilent)	Part No.
96-well Thermocycler (Corning)	THERM-1001, 110V THERM-1000, 230V
Dry Block Heater, 4 Block, HB4DG, US (Qt: 2) (Troemner)	HB4DG
Modular Heating Blocks (Qt: 2) (VWR)	VWR 13259-260
Vacuum manifold (Millipore)	MSVMHTS00
Vacuum pump (Millipore)	WP6211560, 110 V WP6122050, 220V

HILIC separation best practices

Small injection volumes of 1 µL labeled glycans are most convenient for HILIC separations. Aqueous injection volumes > 1 µL will compromise peak shape and resolution. For instructions on sample dilution with organic solvents for injection volumes > 1 µL, please consult the Gly-X InstantPC user manual, 5994-1231EN, page 14.

Users should optimize their HPLC systems to minimize dead volume. Optimal column life is achieved by operating only up to 80% of the maximum pressure.

The typical operating temperature is 40 °C. Higher temperatures can be used, but will shorten column lifetime.

Glycan standards

Agilent offers a broad range of released N-glycan standards and libraries labeled with InstantPC which enables calibration of LC/FLD/MS systems used for released glycan analysis. For a complete list of labeled N-glycan standards, please see our Glycan Standards Technical Flier, 5994-2202EN. Glycan standards are critical to help identify glycan isomers and co-eluting peaks. Potential co-elutions include G0F/Man5, Man5/G1, G1FS1/G2F.

Easy selection and ordering information

To order items listed in the tables below from the Agilent online store, add items to your *Favorite Products* list by clicking on the MyList # header links. Then, enter the quantities for the products you need, Add to Cart and proceed to checkout. Your list will remain under *Favorite Products* for your use with future orders.

If this is your first time using *Favorite Products*, you will be asked to enter your email address for account verification. If you have an existing Agilent account, you will be able to log in. However, if you don't have a registered Agilent account, you will need to register for one. This feature is valid only in regions that are e-commerce enabled. All items can also be ordered through your regular sales and distributor channels.

MyList 1 Gly-X InstantPC N-glycan sample preparation, AdvanceBio Glycan Mapping HILIC column used in 5994-1348EN, solvents, and sample containment.

Description	Part No.
Sample Preparation	
AdvanceBio Gly-X N-glycan prep with InstantPC kit, 96-ct	GX96-IPC
AdvanceBio Gly-X N-glycan prep with InstantPC kit, 24-ct*	GX24-IPC*
Gly-X Vacuum Manifold Spacer	GX100
HILIC Column	
AdvanceBio Glycan Mapping 300Å, 2.1 x 150 mm, 1.8 µm	859700-913
Reagents	
InfinityLab ultrapure LC/MS acetonitrile (1L)	5191-4496
MS solution, formic acid, 10 mL	US-700002341
InfinityLab ultrapure LC/MS standard, water	5191-4498
Vials & Caps**	
Screw-top vials, 250 µL, 100/pk	5190-2242
Cap, snap, blue, PTFE/silicone septa, 100/pk. Cap size: 11 mm	5182-0541

** 24-ct kit (GX24-IPC) contains a 96-well cleanup plate. Store the cleanup plate at room temp, and order 24-ct refills of Gly-X InstantPC Deglycosylation and Labeling Modules (GX24-201PC).*

*** InstantPC labeled glycans are eluted into a 96 well plate. Users may either inject samples from the plate onto LC directly, or transfer to sample vials.*

MyList 2 Additional configurations of Gly-X InstantPC N-glycan sample preparation kits and modules.

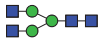
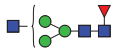
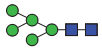
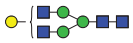
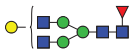
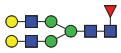
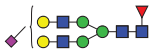
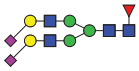
Description	Part No.
AdvanceBio Gly-X N-glycan prep with InstantPC kit, 96-ct	GX96-IPC
AdvanceBio Gly-X N-glycan prep with InstantPC Kit, 24-ct	GX24-IPC
AdvanceBio Gly-X deglycosylation module, 24-ct	GX24-100
AdvanceBio Gly-X InstantPC labeling module, 24-ct	GX24-101
AdvanceBio Gly-X deglycosylation module, 96-ct	GX96-100
AdvanceBio Gly-X InstantPC labeling module, 96-ct	GX96-101
AdvanceBio Gly-X InstantPC cleanup module, 96-ct	GX96-102
AdvanceBio Gly-X deglycosylation and InstantPC labeling module set, 24-ct	GX24-201PC
AdvanceBio Gly-X deglycosylation and InstantPC labeling module set, 96-ct	GX96-201PC
Gly-X Vacuum Manifold Spacer	GX100

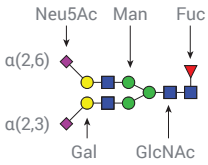
Glycan standards

For a full list of Agilent labeled N-glycan standards, please see our Glycan Standards Technical Flier, 5994-2202EN.

MyList 3 InstantPC labeled N-glycan standards that appear in rituximab.[2] These standards can be used as controls in N-glycan separation and to differentiate coeluting peaks.

MyList 3 (N-Glycans detected in Rituxan)

Description	CFG Structure	Part No.
G0F-N / FA1		GKPC-402
G0 / A2		GKPC-301
G0F / FA2		GKPC-302
Man5 / M5		GKPC-103
G1 / A2G1		GKPC-317
G1F / FA2G1		GKPC-316
G2F / FA2G2		GKPC-305
G2FS1 α(2,3) / FA2G2S(3)1		GKPC-325
G2FS2 α(2,3) / FA2G2S(3)2		GKPC-323

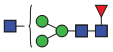
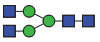
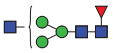
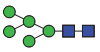
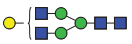
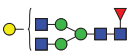
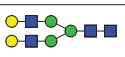
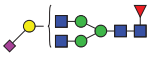
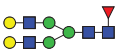
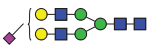
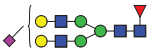
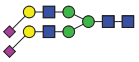
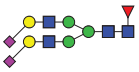


Neu5Ac = N-acetylneuraminic acid
Man = mannose
GalNAc = N-acetylgalactosamine
Fuc = fucose
Gal = galactose
GlcNAc = N-acetylglucosamine
Asn = asparagine
Ser = serine
Thr = threonine

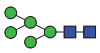
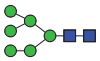
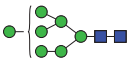
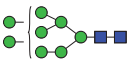
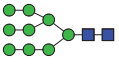
Figure 3. Glycan cartoons follow the recommendations of the Consortium for Functional Glycomics[8] (CFG) and were drawn using GlycoWorkbench 2.14.10.

MyList 4 InstantPC labeled N-glycan standards that appear in etanercept.[2] These standards can be used as controls in N-glycan separation and to differentiate coeluting peaks.

MyList 4 (N-Glycans detected in Enbrel)

Description	CFG Structure	Part No.
G0F-N / FA1		GKPC-402
G0 / A2		GKPC-301
G0F / FA2		GKPC-302
Man5 / M5		GKPC-103
G1 / A2G1		GKPC-317
G1F / FA2G1		GKPC-316
G2 / A2G2		GKPC-304
G1FS1 α(2,3) / FA2G1S(3)1		GKPC-330
G2F / FA2G2		GKPC-305
G2S1 α(2,3) / A2G2S(3)1		GKPC-321
G2FS1 α(2,3) / FA2G2S(3)1		GKPC-325
G2S2 α(2,3) / A2G2S(3)2		GKPC-322
G2FS2 α(2,3) / FA2G2S(3)2		GKPC-323

MyList 5 AdvanceBio InstantPC labeled high mannose N-glycan standards.

Description	CFG Structure	Part No.
Man5 / M5		GKPC-103
Man6 / M6		GKPC-104
Man7 / M7		GKPC-105
Man8 / M8		GKPC-106
Man9 / M9		GKPC-107

MyList 6 InstantPC labeled tri- and tetraantennary N-glycan libraries for studying sialylated glycoproteins. Glycan structures are shown on Certificates of Analysis.

Description	Part No.
InstantPC α(2,3) Sialylated Triantennary N-Glycan Library	GKPC-233
InstantPC α(2,6) Sialylated Triantennary N-Glycan Library	GKPC-263
InstantPC α(2,3) Sialylated Tetraantennary N-Glycan Library	GKPC-234
InstantPC α(2,6) Sialylated Tetraantennary N-Glycan Library	GKPC-264

MyList 7 N-Glycan libraries and control glycoproteins. Glycan structures are shown on the Certificates of Analysis.

Description	Part No.
Human IgG N-Glycan Library consists of complex biantennary oligosaccharides consistent with N-glycans on normal human IgG, including some bisecting GlcNAc N-glycans, labeled with InstantPC	GKPC-005
CHO mAb N-Glycan Library consists of neutral fucosylated complex biantennary N-glycans and high mannose N-glycan Man5 present in many CHO derived monoclonal antibodies (mAbs), labeled with InstantPC	GKPC-020
CHO mAb N-Glycan Library plus CHO mAb Glycoprotein consists of complex biantennary and high mannose N-glycans present in many CHO-derived therapeutic glycoproteins. The source glycoprotein for the library is included for inclusion in sample preparation as a control.	GKPC-020-P
AdvanceBio InstantPC Maltodextrin ladder. May be used as a ladder standard for generating glucose unit (GU) values [7]	GKPC-503
Agilent-NISTmAb*, 25 µL	5191-5744
Agilent-NISTmAb*, 4 x 25 µL	5191-5745

* *Data showing InstantPC N-glycans from NISTmAb is available in Agilent Application Note 5991-8071EN.*

MyList 8 AdvanceBio Glycan Mapping columns for hydrophilic interaction liquid chromatography (HILIC) methods: 2.7 µm superficially porous, for high resolution and lower backpressure, and 1.8 µm for highest resolution. For example, p/n 859700-913 (2.1 × 150 mm,1.8 µm) was used in Application Note 5994-1348EN. Please refer to Agilent Application note 5991-8071EN for 15-, 30- and 37-minute HILIC methods for InstantPC N-glycans using the AdvanceBio Glycan Mapping columns. For further information please visit our website.

Description	Part No.
1.8 mm, 1200 bar maximum pressure, 40 °C maximum temperature	
AdvanceBio Glycan Mapping 300Å, 2.1 x 150 mm, 1.8 µm	859700-913
AdvanceBio Glycan Mapping 300Å, 2.1 x 100 mm, 1.8 µm	858700-913
AdvanceBio Glycan Mapping 300Å, 2.1 x 5 mm, 1.8 µm, guard, 3/pk	821725-905
2.7 mm, 600 bar maximum pressure, 40 °C maximum temperature	
AdvanceBio Glycan Mapping 120Å, 2.1 x 100 mm, 2.7 µm	685775-913
AdvanceBio Glycan Mapping 120Å, 2.1 x 150 mm, 2.7 µm	683775-913
AdvanceBio Glycan Mapping 120Å, 2.1 x 250 mm, 2.7 µm	651750-913
AdvanceBio Glycan Mapping 120Å, 2.1 x 5 mm, 2.7 µm, guard, 3/pk	821725-906
AdvanceBio Glycan Mapping 120Å, 4.6 x 100 mm, 2.7 µm	685975-913
AdvanceBio Glycan Mapping 120Å, 4.6 x 150 mm, 2.7 µm	683975-913
AdvanceBio Glycan Mapping 120Å, 4.6 x 250 mm, 2.7 µm	680975-913
AdvanceBio Glycan Mapping 120Å, 4.6 x 5 mm, 2.7 µm, guard, 3/pk	820750-905

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