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Consumables Workflow Ordering Guide

N-Glycan Analysis of Biotherapeutic Glycoproteins Using AdvanceBio Gly-X 2-AB Express Sample Preparation and LC/FLD/MS

N-glycan analysis simplified and standardized

The location and structure of N-linked glycans plays a critical role in the pharmacology of therapeutic proteins, potentially affecting immunogenicity, pharmacokinetics, and pharmacodynamics.

2-AB (2-aminobenzamide) is a well-established tag that has been used to generate N-glycan data for more than 20 years. Agilent AdvanceBio Gly-X 2-AB Express is a high-performance N-glycan sample preparation platform[1] with a simplified workflow, using a five-minute in solution deglycosylation step followed by 2-AB labeling on a solid-state matrix. Excess dye is washed away with acetonitrile before eluting labeled samples with DI water without requiring sample drying. Samples are ready for UHPLC/FLD/MS in 2 hours or less using the AdvanceBio Amide HILIC column for hydrophilic interaction liquid chromatography (HILIC) followed by relative quantitation. In addition, a wide range of 2-AB-labeled N-glycan standards are available to calibrate N-glycan separations and help identify N-glycan species.

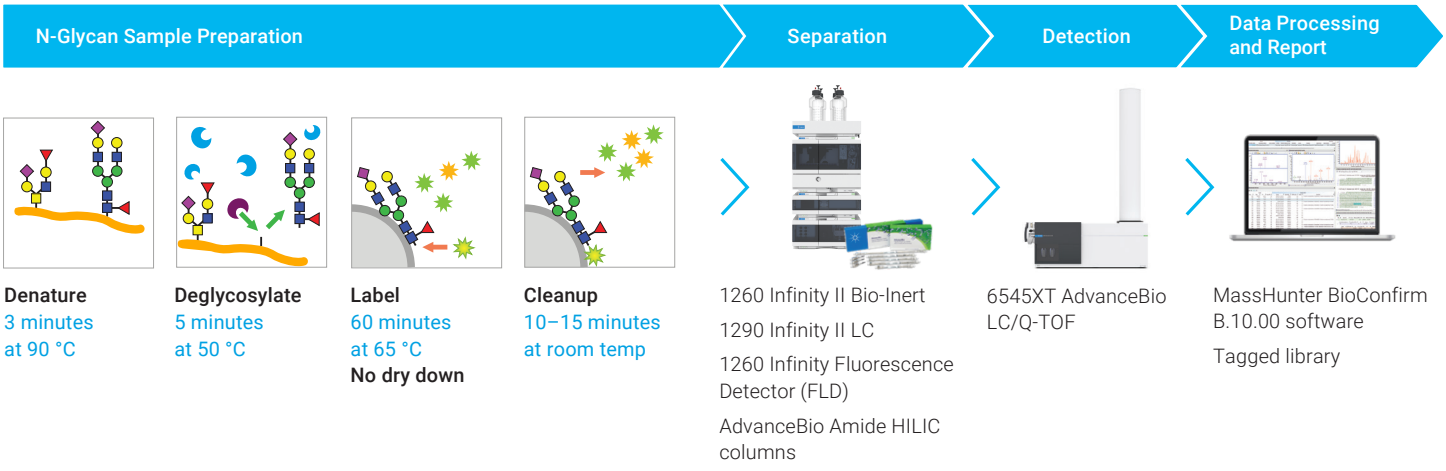


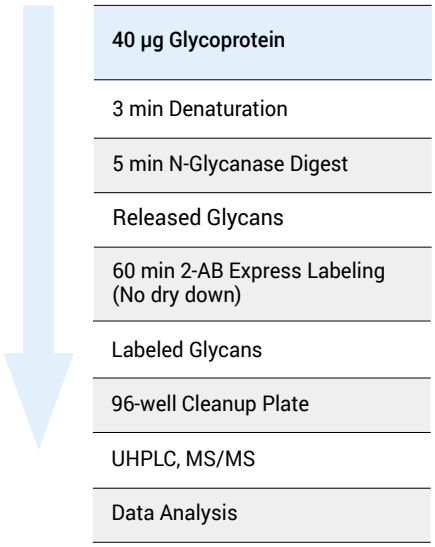
Figure 1. Released N-Glycan Analysis workflow using Gly-X 2-AB Express sample preparation with LC/FLD/MS.

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 2-AB N-glycan sample preparation and analysis. Many were used to analyze N-glycans on rituximab (Rituxan, a monoclonal antibody or mAb) and etanercept (Enbrel, an Fc fusion protein), demonstrating the entire workflow including sample preparation, separation, and data interpretation.[2]

This Gly-X 2-AB Express N-Glycan analysis workflow guide includes ordering information for:

- The sample preparation kit – samples are prepared and ready for analysis in 2 hours using AdvanceBio Gly-X 2-AB Express technology.
- 2-AB labeled N-glycan standards – these well-characterized individual standards and libraries are essential when profiling N-glycan species that 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.
- Concentrated mobile phase ready for dilution.



Steps that enhance productivity:

- 5 minute PNGase F digestion at 50 ˚C that provides unbiased N-glycan release.
- 2-AB Express labeling that is performed while glycans are immobilized on the cleanup matrix, eliminating dry down and reducing total sample preparation time.
- The use of an established 2-AB labeling dye that assures data continuity for ongoing projects.
- A modular kit format for flexible use.

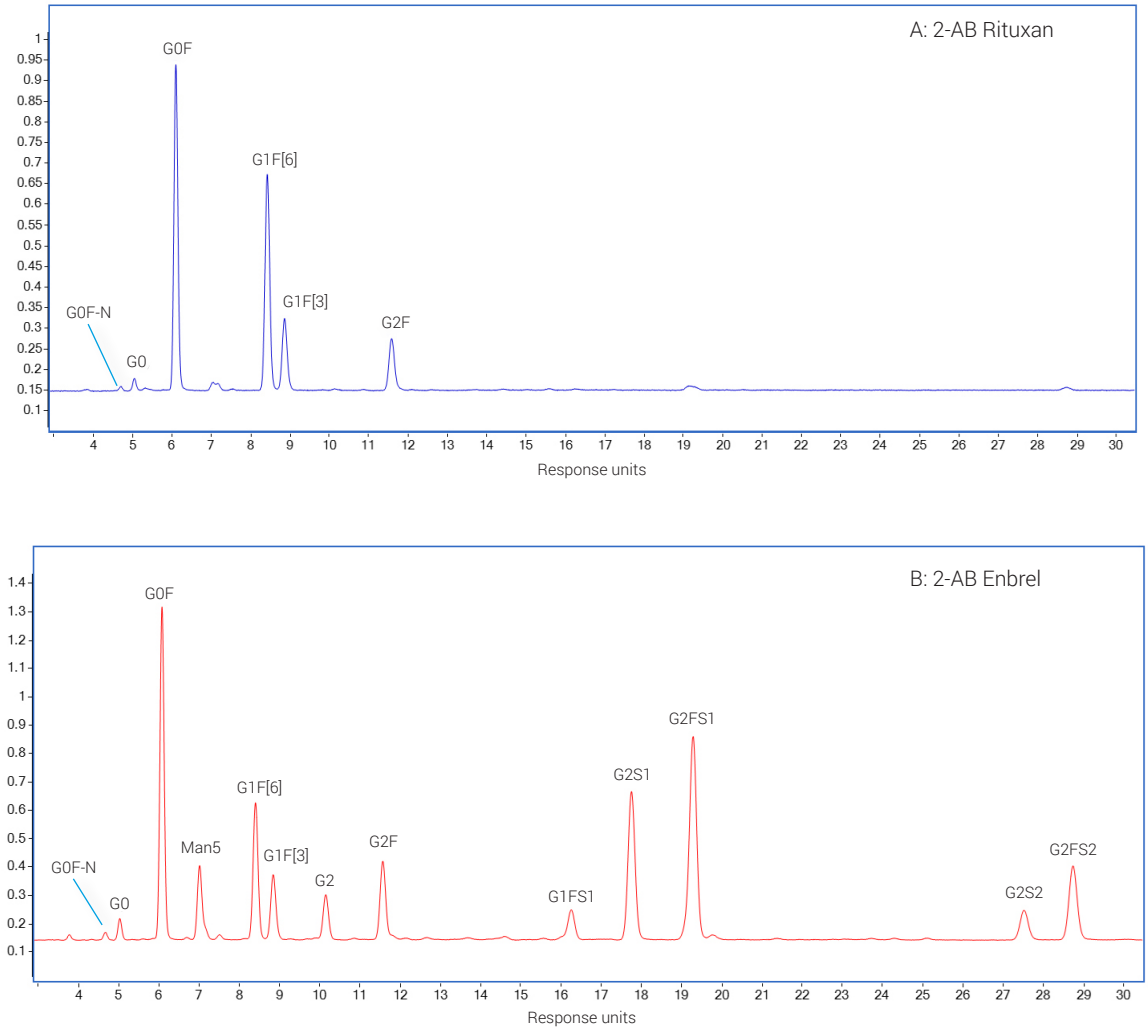


Figure 2. HILIC-UHPLC fluorescence profile of (A) Rituxan and (B) Enbrel N-glycans labeled with 2-AB. N-Glycan relative percent areas are shown in Tables 1 and 2, n = 4. UHPLC conditions and Q-TOF parameters are shown in Tables 3 and 4.

Table 1. Relative % area, SD, and %CV values for Rituxan N-glycans labeled with 2-AB, n = 4.

	Average Rel % Area	Standard Deviation	%CV
G0F-N	0.523	0.030	5.715
G0	1.423	0.041	2.891
G0F	42.780	0.052	0.122
G1F[6]	33.760	0.077	0.229
G1F[3]	11.853	0.040	0.340
G2F	9.660	0.140	1.447

Table 2. Relative % area, SD, and %CV values for Enbrel N-glycans labeled with 2-AB, n = 4.

	Average Rel % Area	Standard Deviation	%CV
G0F-N	0.428	0.005	1.170
G0	1.155	0.006	0.500
G0F	19.783	0.026	0.133
Man5	5.225	0.034	0.654
G1F[6]	9.468	0.029	0.303
G1F[3]	4.663	0.056	1.193
G2	3.280	0.008	0.249
G2F	6.223	0.019	0.304
G1FS1	3.083	0.039	1.253
G2S1	14.400	0.029	0.204
G2FS1	20.523	0.039	0.188
G2S2	3.415	0.053	1.540
G2FS2	8.350	0.014	0.169

Table 3. Agilent 1290 Infinity II UHPLC HILIC/FLD conditions for 2-AB labeled N-glycans.

Parameter	Value		
Column	Agilent AdvanceBio Amide HILIC, 2.1 x 150 mm, 1.8 µm (p/n 859750-913)		
Column Temp	60 °C		
Mobile Phase	A) 50 mM ammonium formate, pH 4.4 B) Acetonitrile		
Gradient Program	Time (minutes)	%B	Flow rate (mL/min)
	0	74	0.6
	50	54	0.6
	51	40	0.6
	52	40	0.6
	54	74	0.6
	64	74	0.6
Injection Volume	1 µL		
Fluorescence Detection	Agilent 1260 Infinity II FLD 2-AB: λEx 260 nm, λEm 430 nm		

Table 4. Agilent 6545XT Q-TOF parameters for 2-AB 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	2,500 V
Nozzle Voltage	500 V
Fragmentor	120 V
Skimmer	65 V
Mass Range	*m/z* 300 to 3,000
Scan Rate	1 spectrum/sec
Acquisition Mode	Extended dynamic range (2 GHz)

Getting started with Gly-X 2-AB Express: Tips for optimal results

Glycoprotein sample preparation considerations

Glycoprotein samples should be prepared at a maximum concentration of 2 mg/mL in a low salt neutral buffer free of detergents. 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, 20 µL of each 2 mg/mL solution). Higher quantities of protein could be used for mAbs (up to 100 µg) but data linearity should be assessed when loading more than 40 µg.
- Buffer: Low salt (~150 mM) neutral buffer without detergents. Sample can be diluted with water or 50 mM HEPES, pH 7.9.
- A 10 kDa molecular weight cut-off spin centrifugal filter is recommended when the sample salt concentration is higher than 150 mM.

Incubation and cleanup hardware

During the Gly-X 2-AB Express sample preparation workflow, samples are heated to 90 °C for protein denaturation, 50 °C for PNGase F digestion, and 65 °C for 2-AB labeling. We recommend using a thermocycler or dry block heater when heating the samples in the 96-well plate provided using the suggestions below.

The workflow employs a simple, vacuum-driven cleanup. If you wish to use an equivalent heater, vacuum manifold or pump other than the models suggested in this table, validation may be needed. Please contact Agilent for further assistance.

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
Compact Digital Dry Bath/Block Heater (Thermo Fisher Scientific)	88871001
Vacuum manifold (Millipore)	MSVMHTS00
Vacuum pump (Millipore)	WP6211560, 110 V WP6122050, 220V

HILIC best practices

- Small injection volumes of 1 µL (aqueous) 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 solvent for injection volumes > 1 µL, please consult the Gly-X 2-AB user manual.[2]
- Agilent AdvanceBio Ammonium Formate Mobile Phase concentrate (p/n G3912-00000) can be used to prepare 1 L of 50 mM ammonium formate, pH 4.4.
- 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 60 °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 2-AB to calibrate LC/FLD/MS systems used for released glycan analysis.[3] Glycan standards are critical to help identify glycan isomers and co-eluting peaks. Potential co-eluters include G0F/Man5, Man5/G1, G1FS1/G2F.

Data analysis and reporting

The data were analyzed using Agilent MassHunter BioConfirm Qualitative Analysis Software with Personal Compound Database.

Note: Agilent now offers MassHunter 11 with OpenLab ECM XT data analysis software are 21 CFR Part 11 compliant.

Easy selection and ordering information

To order items listed in the following tables from the Agilent online store, add items to your Favorite Products list by clicking on the MyList link in the header. You can then enter the quantities for the products you need, add the products to your 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 be ordered online by clicking on the individual part numbers or through your regular sales and distributor channels.

MyList 1 Gly-X 2-AB Express N-glycan sample preparation, AdvanceBio Amide HILIC column, solvents, and sample containment.

Description	Part No.
N-glycan Sample Preparation	
AdvanceBio Gly-X 2-AB Express kit, 96-ct	GX96-2AB
AdvanceBio Gly-X 2-AB Express kit, 24-ct	GX24-2AB*
AdvanceBio Gly-X 2-AB Express starter pack	GX400
AdvanceBio Gly-X deglycosylation and 2-AB Express labeling module set, 24-ct	GX24-401AB*
Columns, Fittings and Connectors	
AdvanceBio Amide HILIC, 2.1 x 150 mm, 1.8 µm	859750-913
Agilent InfinityLab Quick Connect Fitting (column inlet)	5067-5965
Agilent InfinityLab Quick Turn Fitting (column outlet)	5067-5966
Solvents & Reagents	
InfinityLab ultrapure LC/MS acetonitrile, 1 L	5191-4496
InfinityLab ultrapure LC/MS standard, water, 1 L	5191-4498
AdvanceBio Ammonium Formate Mobile Phase concentrate, 10 mL	G3912-00000
MS solution, formic acid, 10 mL	US-700002341
Solvent Filtration Supplies**	
InfinityLab solvent filtration assembly	5191-6776
InfinityLab solvent filtration flask, glass, 2 L	5191-6781
Filter membrane, nylon 47 mm, 0.2 µm, 100/pk	5191-4341
Filter membrane, regenerated cellulose 47 mm, 0.2 µm, 100/pk	5191-4340
Solvent bottle glass filter, solvent inlet, 20 µm	5041-2168
Solvent Handling Supplies	
InfinityLab Stay Safe cap starter kit	5043-1222
InfinityLab solvent bottle, clear, 1 L	9301-6524
InfinityLab solvent bottle, amber, 1 L	9301-6526
Solvent bottle, clear, 2 L	9301-6342
Solvent bottle, amber, 2 L	9301-6341
InfinityLab Stay Safe purging bottle	5043-1339
InfinityLab waste can, GL45, 6 L with Stay Safe cap§	5043-1221
InfinityLab charcoal filter with time strip, 58 g§	5043-1193
Vials & Caps†	
Vial, screw style, 2 mL, polypropylene, 100/pk‡	5191-8150
Vial insert, 300 µL, polypropylene, polymer feet, 100/pk‡	5182-0549
9 mm, screw style clear polypropylene cap, 100/pk	5191-8151

* *The 24-ct kit (GX24-2AB) contains a 96-well cleanup plate and 24-ct 2-AB Labeling module. Store the cleanup module at room temperature and order 24 ct refills of Gly-X 2-AB Express Deglycosylation and Labeling module set (GX24-401AB).*

** *Use the InfinityLab Solvent Filtration assembly prior to analysis if using solvents other than those listed in this table.*

† *2-AB 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.*

‡ *Agilent recommends using a 250 µL vial insert with the 2 mL polypropylene vial to minimize dead volume.*

§ *Charcoal filter not included with waste can, order 5043-1221 and 5043-1193 together.*

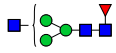
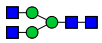
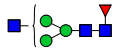
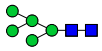
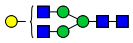
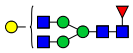
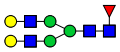
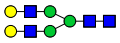
MyList 2 Additional configurations of Gly-X 2-AB Express N-glycan sample preparation kits and modules.

Description	Part No.
AdvanceBio Gly-X deglycosylation module, 96-ct	GX96-100
AdvanceBio Gly-X 2-AB Express labeling module, 96-ct	GX96-401
AdvanceBio Gly-X 2-AB Express cleanup module, 96-ct	GB96-402
AdvanceBio Gly-X deglycosylation module, 24-ct	GX24-100
AdvanceBio Gly-X 2-AB Express labeling module, 24-ct	GX24-401
AdvanceBio Gly-X deglycosylation and 2-AB Express labeling module set, 96-ct	GX96-401AB

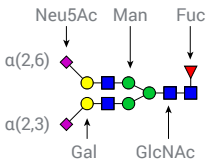
Glycan standards

For a full list of Agilent labeled N-glycan standards, please see our Glycan Standards Technical Flyer.[3]

MyList 3 2-AB labeled N-glycan standards for glycans that appear in rituximab and etanercept.[2] These standards can be used as controls in N-glycan separation and to differentiate co-eluting peaks.

Description	CFG Structure	Part No.
G0F-N / F(6)A1		GKSB-402
G0 / A2		GKSB-301
G0F / FA2		GKSB-302
Man5 / M5		GKSB-103
G1 / A2G1		GKSB-317
G1F / FA2G1		GKSB-316
G2F / FA2G2		GKSB-305
G2 / A2G2		GKSB-304*

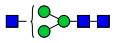
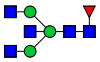
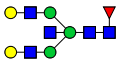
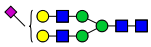
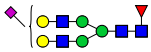
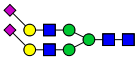
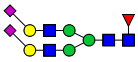
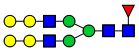
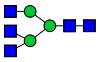
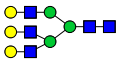
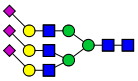
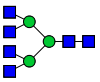
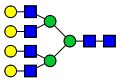
** GKSB-304 2AB labeled N-glycan standard for Etanercept only. Does not occur in rituximab.*



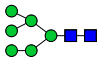
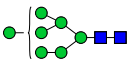
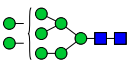
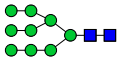
Neu5Ac = N-acetylneuraminic acid
Man = mannose
GalNAc = N-acetylgalactosamine
Fuc = fucose
Gal = galactose
GlcNAc = N-acetylglucosamine
Asn = asparagine
Ser = serine
Thr = threonine

Figure 3. Glycan cartoons follow the recommendations of the Consortium for Functional Glycomics[5] (CFG) and were drawn using GlycoWorkbench 2.14.10.

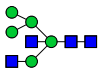
MyList 4 Additional 2-AB labeled complex-type native N-glycan standards.

Description	CFG Structure	Part No.
G0-N		GKSB-401
G0FB		GKSB-303
G2FB		GKSB-306
G2S1 α(2,6)		GKSB-311
G2FS1 α(2,6)		GKSB-315
G2S2 α(2,6)		GKSB-312
G2FS2 α(2,6)		GKSB-313
G2F w/2 α-gal		GKSB-318
A3		GKSB-307
G3		GKSB-308
G3S3 α(2,6)		GKSB-314
A4		GKSB-309
G4		GKSB-310

MyList 5 AdvanceBio Gly-X 2-AB Express labeled high mannose N-glycan standards.

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

MyList 6 2-AB labeled native N-glycan core and hybrid.

Description	CFG Structure	Part No.
Hybrid		GKSB-111
NN		GKSB-100
Man3		GKSB-101
Man3F		GKSB-102

MyList 7 2-AB labeled bi-, tri- and tetraantennary N-glycan libraries for studying sialylated glycoproteins. Glycan structures are shown on Certificates of Analysis.

Description	Part No.
AdvanceBio 2-AB Biantennary and high Manose partitioned library	GKSB-520
AdvanceBio 2-AB α(2,3) Sialylated biantennary N-glycan library	GKSB-232
AdvanceBio 2-AB α(2,6) Sialylated biantennary N-glycan library	GKSB-262
AdvanceBio 2-AB α(2,3) Sialylated triantennary N-glycan library	GKSB-233
AdvanceBio 2-AB α(2,6) Sialylated triantennary N-glycan library	GKSB-263
AdvanceBio 2-AB α(2,3) Sialylated tetraantennary N-glycan library	GKSB-234
AdvanceBio 2-AB α(2,6) Sialylated tetraantennary N-glycan library	GKSB-264

MyList 8 2-AB labeled N-Glycan libraries and control glycoproteins. Glycan structures are shown on the Certificates of Analysis.

Description	Part No.
AdvanceBio 2-AB Human IgG N-glycan library – consists of complex biantennary oligosaccharides consistent with N-glycans on normal human IgG	GKSB-005
AdvanceBio 2-AB Human α-1-acid glycoprotein N-glycan library – consists of a heterogenous mixture of core non-fucosylated bi-, tri-, and tetraantennary glycans with various degrees of sialylation (NeuAc) and some with outer arm fucose residues and lactosamine repeats, consistent with N-glycans previously reported for human α1-acid glycoprotein	GKSB-001
AdvanceBio 2-AB Bovine Fetuin N-glycan library – composed of a mixture of sialylated N-linked glycans (core non-fucosylated), consistent with N-glycans previously reported for bovine fetuin	GKSB-002
AdvanceBio 2-AB Glucose homopolymer standard	GKSB-503
Agilent-NISTmAb*, 25 µL	5191-5744
Agilent-NISTmAb*, 4 x 25 µL	5191-5745

MyList 9 AdvanceBio Amide HILIC columns.

Description	Part No.
1.8 µm, 1200 bar maximum pressure, 80 °C maximum temperature	
AdvanceBio Amide HILIC 300 Å, 2.1 x 150 mm, 1.8 µm	859750-913
AdvanceBio Amide HILIC 300 Å, 2.1 x 100 mm, 1.8 µm	858750-913

References

1. N-Glycan Analysis: Better Together. Agilent Brochure 5994-1647EN.
2. Analysis of labeled Glycans User Manual 5994-1231EN, pg 14.
3. AdvanceBio Glycan Standards Instant PC, 2-AB, 2-AA, APTS, InstantAB, InstantQ, Unlableled 5994-2202EN.
4. Varki A, et al. Symbol Nomenclature for Graphical Representations of Glycans. Glycobiology. 2015 Dec; 25(12): 1323–1324.
5. Development of a Rapid 2-AB Sample Preparation Workflow for N-Glycan Release and Labeling 5994-0945EN.

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