Application Note

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An Improved Workflow for Profiling and Quantitation of Sialic Acids in Biotherapeutics

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Abstract

This application note describes use of the Agilent AdvanceBio Sialic Acid profiling and quantitation kit (p/n GS24-SAP) to profile and perform absolute quantitation of sialic acids present in biotherapeutic glycoproteins as well as the NISTmAb. This kit uses a new and improved high-throughput workflow for the preparation, separation, and detection of sialic acids labeled with 1,2-diamino-4,5-methylenedioxybenzene (DMB). Sialic acid capping at the non-reducing terminal of N- or O-glycans can serve a key role in mediating the effectiveness of biotherapeutic glycoproteins.[1]

The workflow described here demonstrates the AdvanceBio Sialic Acid profiling and quantitation kit for release of terminal sialic acid by acid hydrolysis, followed by DMB labeling and both qualitative and quantitative analysis. DMB-labeled sialic acids from samples and standards are separated by reversed-phase liquid chromatography (LC) and quantitated using fluorescence detection (FLD) and structurally confirmed by mass spectrometry (MS).

Introduction

The composition of glycans present on biotherapeutic glycoproteins can affect immunogenicity, pharmacokinetics, and pharmacodynamics.[2] Glycans are carbohydrates composed of monosaccharides arranged into many different possible oligosaccharide structures based on composition and linkage position. Depending on the molecule and the application, terminal sialic acid may reduce the rate of clearance, reduce antibody-dependent cellular cytotoxicity (ADCC) activity, or can be anti-inflammatory.[3–5] Two forms commonly found in biotherapeutics are N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc). Neu5Ac is usually the predominant species, while Neu5Gc is not synthesized by humans and its presence on biotherapeutics can be immunogenic. Therefore, it is essential to monitor the absolute quantity of sialic acid, as well as the levels of different sialic acid species present in therapeutic glycoproteins.

Presented here is a new high-throughput workflow based on a 96-well plate format for the release, labeling, and analysis of sialic acids from therapeutic glycoproteins using rituximab, etanercept, NISTmAb, and cetuximab as examples. Sialic acid residues are released then labeled with 1,2-diamino-4,5-methylenedioxybenzene (DMB) in a two-step procedure. DMB-labeled sialic acids are then separated and analyzed using a rapid 10-minute method based on reversed-phase ultrahigh-performance liquid chromatography (UHPLC) with FLD detection for quantitation and optional MS detection for mass analysis. The workflow offers both qualitative characterization of Neu5Ac, Neu5Gc, and other sialic acid species using a sialic acid reference panel (SARP), as well as absolute quantitation with picomole level sensitivity using included Neu5Ac and Neu5Gc quantitative standards. The workflow enables reliable and reproducible high-throughput sample preparation for the profiling and quantitation of sialic acids. This kit provides a broad detection range and improved sensitivity for molecules with low levels of sialylation.

Experimental

Sample preparation

Samples were prepared using a using a 96-well plate format. Sialic acids were released from rituximab (Rituxan, lot number M190170), etanercept (Enbrel, lot number M190088), NISTmAb (lot number 14HB-D-002), and Erbitux (cetuximab, lot number MI60886) through an acid hydrolysis reaction. The method eliminates the need for a dry-down step, thereby decreasing overall sample preparation time by 1 to 2 hours. The sample amount is typically 200 µg of glycoprotein with low-level sialylation and 5 µg of highly sialylated glycoprotein. Serial dilutions of sialic acid reference standards Neu5Ac and Neu5Gc were used to prepare a standard curve and to determine the limit of quantitation (LOQ) and limit of detection (LOD) for the assay. Released sialic acids, SARP, and standards were then derivatized with DMB following the workflow illustrated in Figure 1, release and labeling steps were carried out in a thermocycler.

LC/FLD/MS analysis of DMB-labeled sialic acids

DMB-labeled sialic acids from Rituxan, Enbrel, NISTmAb, and Erbitux were analyzed using reversed-phase (RP) separation with an Agilent 1290 Infinity II LC system with fluorescence detection (FLD) for quantitation. All RP-UHPLC separations were conducted under the conditions described in Table 1.

Additional inline analysis using an Agilent 6545XT AdvanceBio LC/Q-TOF mass spectrometer (Table 2) was also performed to confirm elution order of the DMB-labeled sialic acid species present in the SARP. A fixed flow splitter was used post-FLD, diverting approximately 50% of the flow to waste and 50% to the MS. The data was analyzed with Agilent OpenLab CDS 2.3 and MassHunter Qualitative Analysis 10.0 software. Neu5Gc and Neu5Ac were quantified using the calibration curves.

Materials

Acetonitrile (LC/MS grade, Honeywell Burdick & Jackson) was purchased from VWR. Methanol (Optima LC/MS grade) was purchased from Fisher Scientific. Nanopure water generated in-house was used for all experiments.

Instrumentation

DMB-labeled sialic acid samples were separated using an Agilent InfinityLab Poroshell 120 EC-C18 column (2.1 × 75 mm, 2.7 µm; p/n 697775-902) using the method details in Table 1, on an Agilent LC/MS setup composed of:

- Agilent 1290 Infinity II high-speed pump (G7120A)
- Agilent Infinity multisampler (G7167B)
- Agilent 1290 Infinity II multicolumn thermostat (G7116B)
- Agilent 1260 Infinity fluorescence detector (G1321B)
- Agilent 6545XT AdvanceBio LC/Q-TOF (parameters in Table 2)

Software

- Agilent OpenLab CDS 2.3
- Agilent MassHunter Qualitative Analysis 10.0

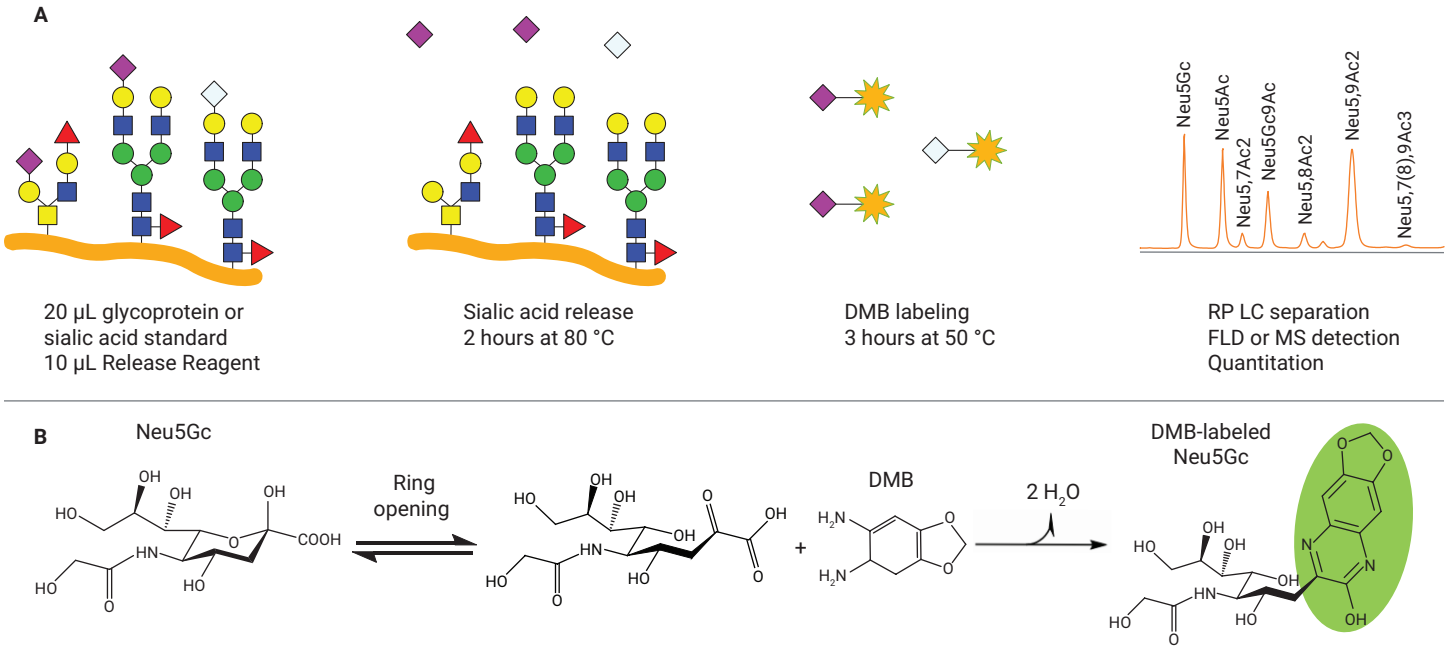


Figure 1. Sialic acid release and DMB labeling workflow (A) overview (B) DMB labeling mechanism of sialic acid Neu5Gc.

Table 1. Reversed-phase UHPLC conditions.

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

Table 2. Agilent 6545XT AdvanceBio LC/Q-TOF parameters.

6545XT AdvanceBio LC/Q-TOF	
Source	Dual AJS ESI
Gas Temperature	350 °C
Drying Gas Flow	11 L/min
Nebulizer	15 psi
Sheath Gas Temperature	400 °C
Sheath Gas Flow	12 L/min
Vcap	1,400 V
Nozzle Voltage	1,800 V
Fragmentor	120 V
Skimmer	65 V
Mass Range (MS)	*m/z* 400 to 1,000
Mass Range (MS/MS)	*m/z* 100 to 550
Acquisition Mode	High resolution (4 GHz)

Results and discussion

LC/FLD/MS analysis of DMB-labeled sialic acids

RP-UHPLC analysis of DMB-labeled SARP results in the separation and detection of seven sialic acid derivatives: Neu5Gc, Neu5Ac, Neu5,7Ac2, Neu5Gc,9Ac, Neu5,8Ac2, Neu5,9Ac2, and Neu5,7(8),9Ac3. While differences in retention times may be observed with different columns, flow rate, solvents, or laboratory conditions, the elution order of DMB-derivatized sialic acids remains consistent. The reference panel is used to evaluate the resolution and accuracy of the chromatographic system at the beginning of the sample sequence. A typical FLD chromatogram of DMB-labeled SARP is shown in Figure 2A. Identification of the DMB-sialic acid derivatives was confirmed by mass spectrometry (Figure 2B).

Analysis of sialic acid content of biotherapeutics and NISTmAb

DMB-labeled sialic acids identified by applying the workflow to Rituxan, Enbrel, NISTmAb, and Erbitux are shown in Figure 3. Both Rituxan (Figure 3A) and Enbrel (Figure 3B) contain primarily Neu5Ac while NISTmAb (Figure 3C) and Erbitux (Figure 3D) contain primarily Neu5Gc. Mass spectra of major peaks in DMB-labeled samples from Enbrel and Erbitux confirm their identities as Neu5Ac and Neu5Gc, respectively (Figure 4).

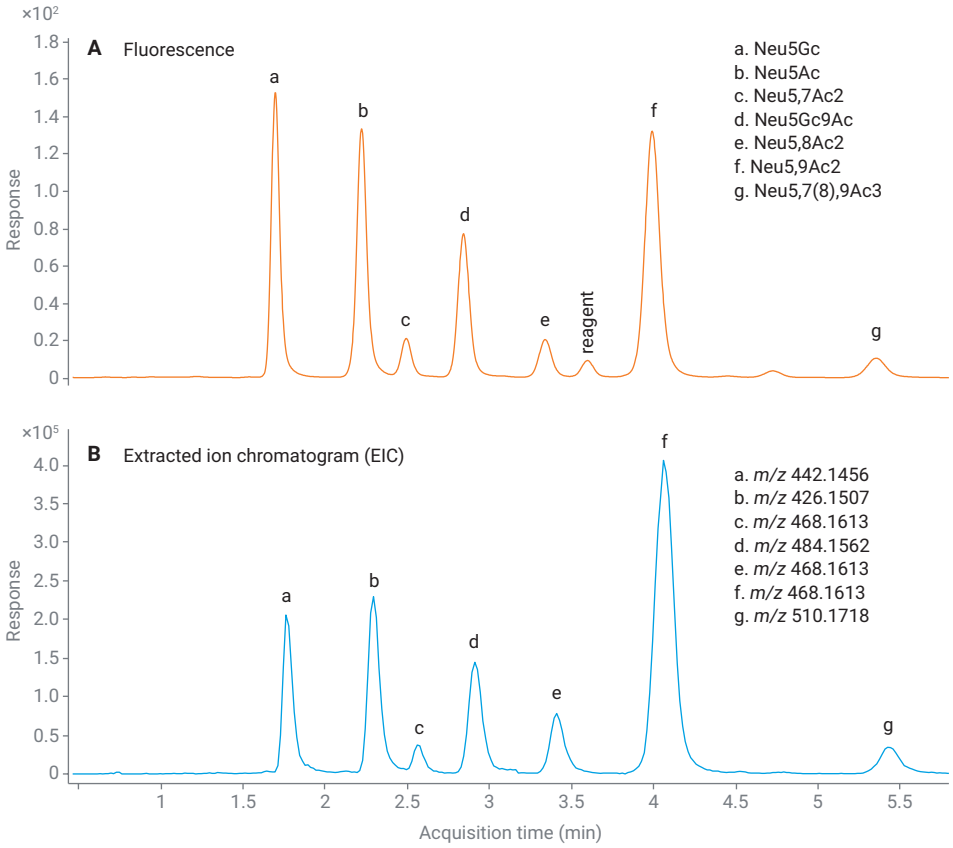


Figure 2. UHPLC chromatogram of DMB-labeled SARP. (A) fluorescence; (B) extracted ion chromatogram of DMB-labeled sialic acid species, $[M+H]^{1+}$.

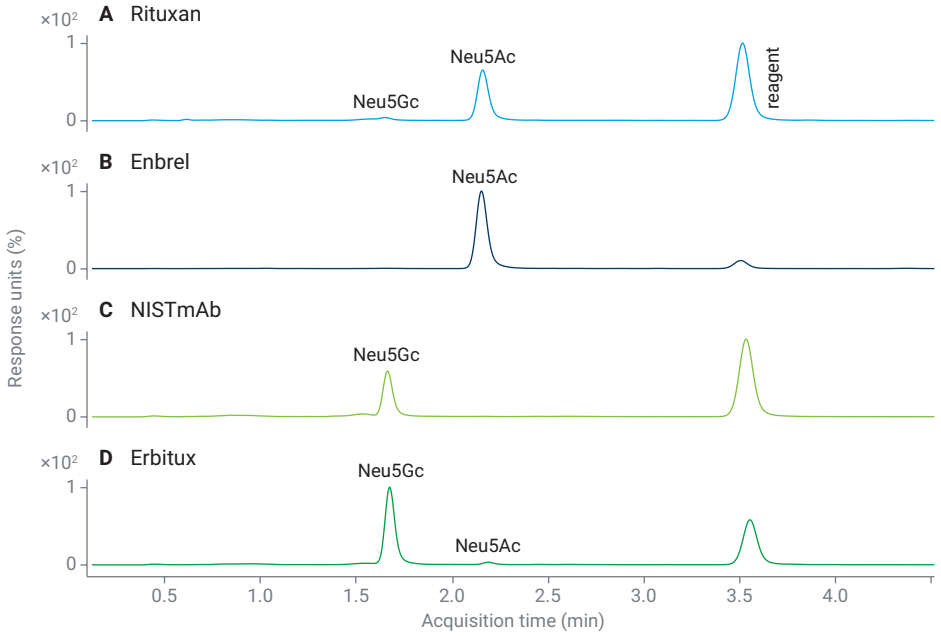


Figure 3. UHPLC fluorescence profiles of DMB-labeled sialic acids from different glycoproteins (A) Rituxan; (B) Enbrel; (C) NISTmAb; and (D) Erbitux.

Quantitative analysis of sialic acid content

Based on the chromatographic separation and fluorescence response of DMB-labeled Neu5Ac and Neu5Gc standards, a quantitative calibration curve was generated (Figure 5). The LOD and LOQ were calculated using the noise determined by OpenLab CDS 2.3 using P2P noise calculation. The detectable mole quantities of Neu5Gc and Neu5Ac from Rituxan, Enbrel, NISTmAb, and Erbitux was determined based on integrated peak areas and listed in Table 3. Total sialic acid quantitation results are consistent with those obtained from the AdvanceBio Total Sialic Acid quantitation kit (p/n GS48-SAQ) (Table 4). The kit also shows improved performance compared to an older DMB labeling workflow (p/n GKK-407) (Table 5) by allowing an increased concentration of glycoprotein per sample well as a decrease in sample dilution prior to analysis, resulting in an increase in fluorescence signal for DMB labeled sialic acids.

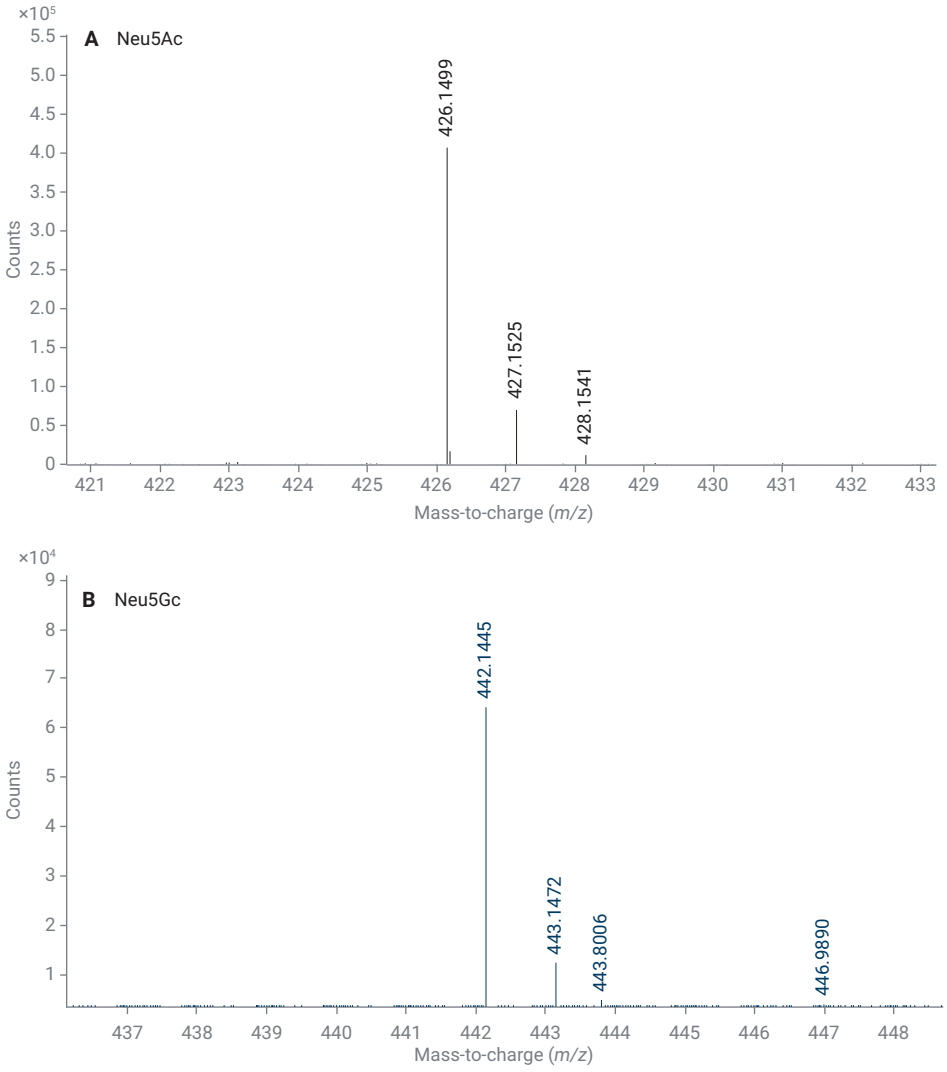
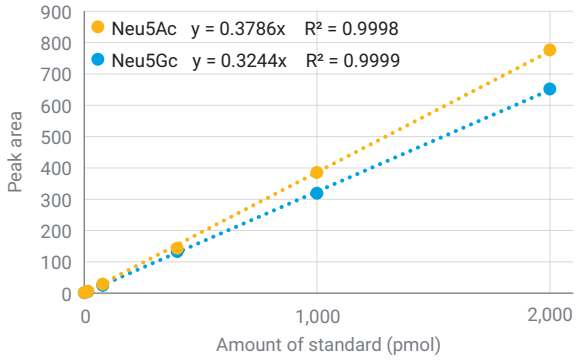


Figure 4. Mass spectrum of DMB-labeled sialic acid (A) Neu5Ac from Enbrel; and (B) Neu5Gc from Erbitux.



Sialic Acid	LOD (pmol)	LOQ (pmol)
Neu5Ac	0.016	0.053
Neu5Gc	0.012	0.040

Figure 5. Neu5Ac and Neu5Gc calibration curves, n = 2. LOD and LOQ for Neu5Ac and Neu5Gc are shown in the table.

Table 3. Average pmol/µg of Neu5Ac and Neu5Gc for each glycoprotein is shown in the table, n = 3. ND = not detected.

	Concentration (mg/ml)	Sample Mass (µg)	Neu5Ac (pmol/µg)	%CV	Neu5Gc (pmol/µg)	%CV
Rituxan	10	200	0.60	4.2%	0.02	1.8%
Enbrel	0.25	5	228	6.9%	ND	-
NIST mAb	10	200	ND	-	0.36	1.8%
Erbitux	2	40	0.12	10.9%	3.72	7.1%

Table 4. Total sialic acid (Neu5Ac and Neu5Gc) with the Agilent AdvanceBio Sialic Acid profiling and quantitation kit (p/n GS24-SAP) in comparison to the values obtained with the Agilent AdvanceBio Total Sialic Acid quantitation kit (p/n GS48-SAQ), n = 3.

	Agilent AdvanceBio Sialic Acid Profiling and Quantitation Kit		Agilent AdvanceBio Total Sialic Acid Quantitation Kit	
	pmol/µg	%CV	pmol/µg	%CV
Rituxan	0.62	4.17%	0.47	5.04%
Enbrel	220	1.65%	210	12.34%
Erbitux	3.80	7.26%	3.49	0.69%
Fetuin	226	4.45%	232	7.39%

Table 5. Quantitation of Neu5Ac and Neu5Gc (pmol/µg) using the Agilent AdvanceBio Sialic Acid profiling and quantitation kit (p/n GS24-SAP) in comparison to the values obtained with the Signal DMB Sialic Acid labeling kit (p/n GKK-407), n = 3. ND = not detected.

		GKK-407		Agilent AdvanceBio Sialic Acid Profiling and Quantitation Kit	
Glycoprotein	Sialic acid	pmol/µg	%CV	pmol/µg	%CV
Rituxan	Neu5Gc	ND	-	0.02	1.76%
	Neu5Ac	0.58	1.12%	0.60	4.25%
Enbrel	Neu5Ac	226	3.57%	223	2.92%
Erbitux	Neu5Gc	ND	-	3.68	1.02%
	Neu5Ac	ND	-	0.12	4.46%
Fetuin	Neu5Gc	ND	-	4.78	4.90%
	Neu5Ac	201	1.47%	222	4.44%

Conclusion

The AdvanceBio Sialic Acid profiling and quantitation kit offers improved sensitivity for proteins with low levels of sialylation such as monoclonal antibodies with a single N-glycosylation site in the Fc region. The updated DMB labeling workflow eliminates the dry down step of samples, decreasing sample preparation time.

This workflow provides a method to determine both absolute and relative quantities of Neu5Ac and Neu5Gc present in biotherapeutics. Sample preparation uses a 96-well plate format for high-throughput sample preparation and is highly reproducible. Quantitative data is comparable to older DMB labeling workflows (GKK-407) and AdvanceBio Total Sialic Acid quantitation kit (GS48-SAQ) results.

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