Application Note

Pharma and Biopharma

A streamlined drug-to-antibody ratio determination workflow for intact and deglycosylated antibody-drug conjugates

Using the Agilent AssayMAP Bravo and Agilent 6545XT AdvanceBio accurate-mass quadrupole time-of-flight LC/MS system

Authors

Jerry Han, Zach Van Den Heuvel, and Steve Murphy
Agilent Technologies, Inc.

Introduction

Antibody drug conjugates (ADCs), a fast-growing class of biomolecule, comprise a monoclonal antibody (mAb) conjugated to a small molecule drug through synthetic linkers. The ratio of the conjugated drug to mAb (drug-to-antibody ratio or DAR) is one of the critical quality attributes for ADC development because it can affect efficacy and safety[1,2]. Monitoring the changes in DAR distribution *in vivo* can provide important insights into the biotransformation of ADCs (for example, drug deconjugation)[3,4]. However, measurement of average DAR and DAR distribution in circulation by LC/MS requires tedious and error-prone sample preparation procedures such as affinity capture of ADCs from complex matrices (for example, serum/plasma) and deglycosylation of the purified antibodies. To address the challenge of this complex sample preparation workflow (Figure 1), this Application Note automates affinity purification and deglycosylation on the Agilent AssayMAP Bravo platform[5,6]. To enable isolation of ADCs with all DAR species (0 to N) in serum, affinity purification was performed using the target antigen. Deglycosylation was used to simplify the mass spectrum of the purified antibodies by eliminating the various glycoforms, and to improve the detection limit. The whole automated process, including antigen immobilization, affinity purification, and on-cartridge deglycosylation, can be completed in approximately five hours, with minimal hands-on time.

This Application Note demonstrates a streamlined ADC DAR workflow using the AssayMAP Bravo, with an Agilent 6545XT AdvanceBio LC/Q-TOF, and Agilent BioConfirm DAR Calculator software. The workflow is simple to implement, highly reproducible, scalable, and minimizes hands-on time.

Experimental

Materials

Recombinant human HER2 extracellular domain (ECD) was purchased from ACRO Biosystems (Newark, DE; HE2-H5225). Trastuzumab emtansine (T-DM1) was manufactured by Genentech. EZ-Link Sulfo-NHS-LC-Biotin, Pierce Biotin Quantitation Kit, and Zeba Spin Desalting Columns, 7K MWCO, 0.5 mL were from Thermo Fisher Scientific (Grand Island, NY; 21327, 28005 and 89882). Rat serum was from Sigma-Aldrich (St. Louis, MO; R9759). Rapid PNGase F was from New England BioLabs (Ipswich, MA; P0711). AssayMAP Streptavidin cartridges were from Agilent Technologies (Santa Clara, CA; SA-W, G5496-60010). 10X HEPES buffer, 100 mM, 1.5 M NaCl, pH 7.4 (HBS-N) was from GE Healthcare Life Sciences (Marlborough, MA; BR100670). 5 M NaCl was from Promega (PAV4221). 1 M Tris-HCl, pH 8.0 was from Quality Biological (351-007-101). All other chemicals were from Sigma-Aldrich (St. Louis, MO).

Biotinylation of HER2 ECD

To obtain a final concentration of 2 mg/mL, 1 mg of lyophilized recombinant HER2 ECD was reconstituted in 500 µL of Milli-Q water. Half (0.5 mg in 250 µL) of the reconstituted HER2 ECD was biotinylated using EZ-Link Sulfo-NHS-LC-Biotin per the manufacturer's instructions. The molar fold excess of biotin was 250, and the incubation was one hour at room temperature. After labeling, the extra Sulfo-NHS-LC-Biotin and Sulfo-NHS (by-product) were removed by gel filtration using Zeba Spin Desalting Columns. The biotinylation levels in the labeled HER2 ECD were estimated using a Pierce Biotin Quantitation Kit, per the manufacturer's instructions.

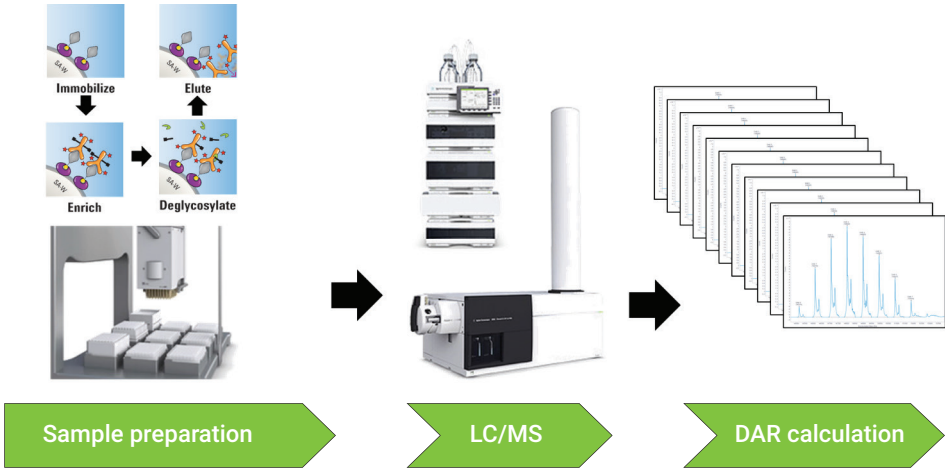


Figure 1. ADC DAR characterization workflow.

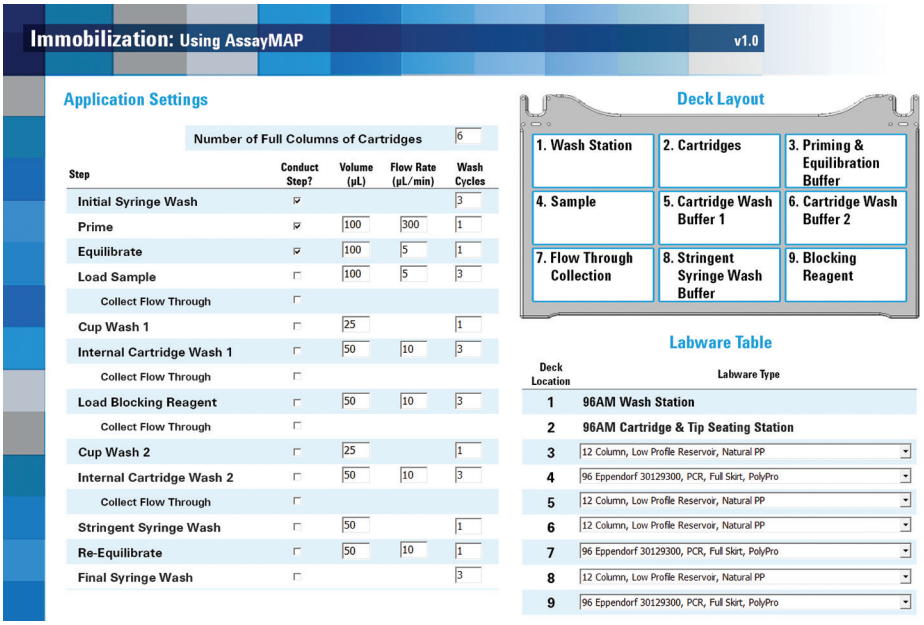
Automated antigen immobilization, affinity capture, and on-cartridge deglycosylation

Automation setup: The biotinylated antigen immobilization, ADC purification, and ADC deglycosylation were carried out using the Immobilization, Affinity Purification, and On-Cartridge Reaction Applications on the AssayMAP Bravo platform. Figures 2–4 show the deck layouts and labware used for these procedures. Table 1 summarizes the samples and reagents. The solution volumes required for each application were determined using their respective Reagent Volume Calculator. To prepare the platform for the Immobilization Application, 48 (six columns) of streptavidin (SA-W) cartridges were transferred from the cartridge rack (deck 6 on the AssayMAP Bravo) to the seating station (on deck 2) using the Cartridge Transfer v1.0 utility (Table 2B).

SA-W cartridge conditioning: SA-W cartridges were primed with 100 µL of 1 % formic acid (FA) at a flow rate of 300 µL/min to clear any air entrapped in the resin. This was followed by an equilibration step with 100 µL of 1 % FA at a flow rate of 5 µL/min. This step removes any streptavidin that is weakly bound to the cartridge resin and would come off in the elution step, where it could interfere with downstream analysis. Figure 2A shows the application settings. The SA-W cartridge conditioning and the biotinylated antigen immobilization runs can be combined into a single run (see Wu; *et al.*[7]), but were separated here for simplicity.

Biotinylated HER2 ECD immobilization: To generate the affinity (bait) cartridges for affinity purification, 3 µg of the biotinylated HER2 ECD were immobilized on each of the 48 individual streptavidin cartridges (SA-W) using the Immobilization v1.0 application. Figure 2B shows the application settings. The cartridges primed in the SA-W condition step were equilibrated with 10 bed volumes of binding buffer (50 µL, 10 mM HEPES, 150 mM NaCl, pH 7.4) at 10 µL/min. Then, 3 µg of the biotinylated HER2 ECD in 100 µL of binding buffer were loaded into each equilibrated cartridge at a flow rate of 5 µL/min. After the biotinylated HER2 ECD was loaded, the cartridges were washed with 10 bed volumes (50 µL) of binding buffer at 10 µL/min. The cartridges were then ready for the affinity purification step.

A



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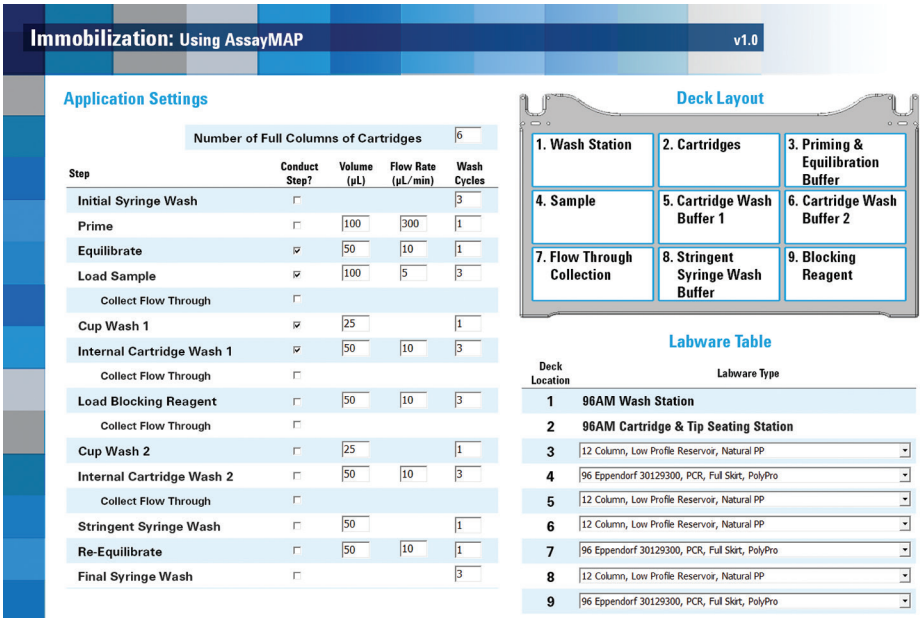

Figure 2. Immobilization application settings. A) Conditioning SA-W cartridges; (B) immobilization of biotinylated HER2 ECD on SA-W cartridges.

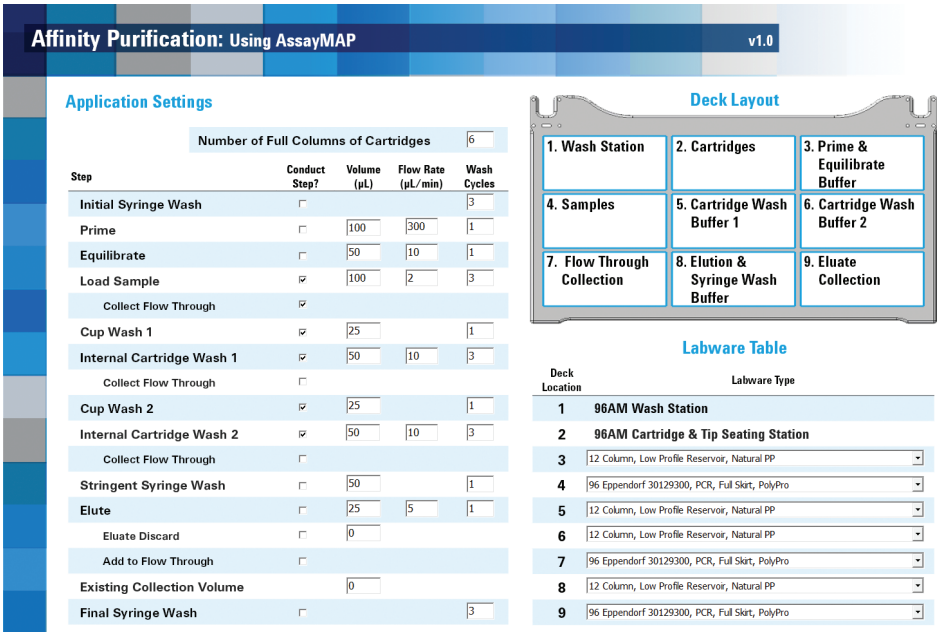

Figure 3. Affinity purification application settings.

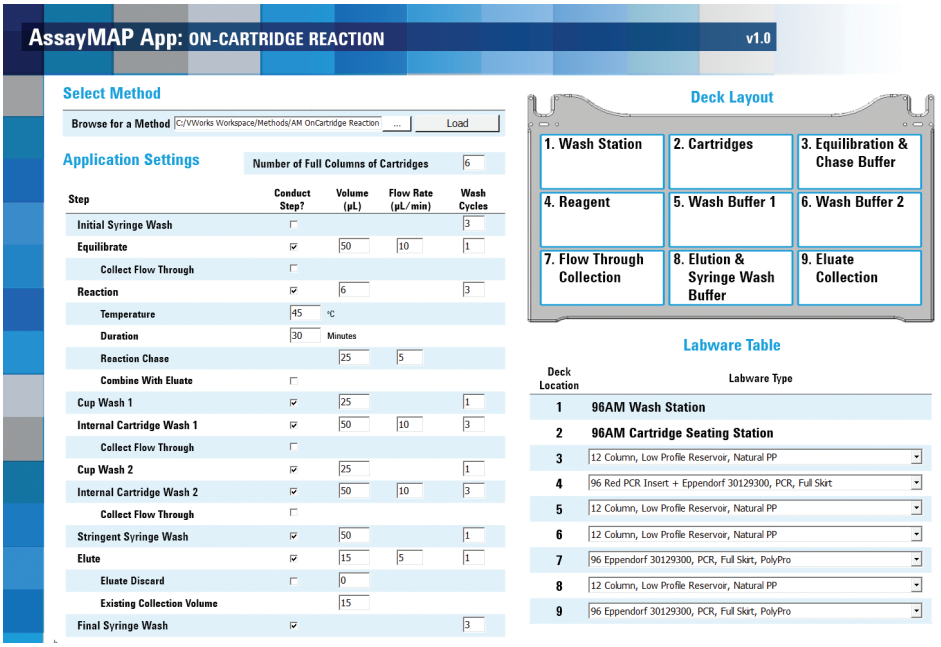

Figure 4. On-cartridge reaction application settings.

ADC affinity purification: To obtain different concentrations of T-DM1 in sera from 20 to 0.3125 µg/mL, plus a no-T-DM1 control (Table 2A), various amounts of T-DM1 (Trastuzumab emtansine, an ADC that targets HER2 ECD) were spiked into clarified rat sera. The dilution set was also prepared in water for direct injection onto the LC/MS. For affinity capture, the serum samples were further diluted 1:1 with binding buffer (10 mM HEPES, 150 mM NaCl, pH 7.4) immediately before loading them onto the biotinylated HER2 ECD-SA-W cartridges. Affinity purification was carried out using the Affinity Purification v1.0 application. Figure 3 shows the application settings. Briefly, 100 µL of diluted rat serum containing T-DM1 was passed through the affinity cartridge at a flow rate of 2 µL/min. Each row of cartridges (n = 6) was loaded with a different concentration of T-DM1 (see Table 2B for plate layout). After the samples were loaded, the cartridges were sequentially washed with 10 bed volumes (50 µL) of the high-salt HEPES buffer (50 µL, 10 mM HEPES, 1 M NaCl, pH 7.4), then 10 bed volumes (50 µL) of binding buffer at a flow rate of 10 µL/min. The cartridges were then ready for deglycosylation.

Deglycosylation: To compare DAR analysis with and without glycans present, half of the cartridges with purified T-DM1 were incubated with PNGase F (columns 1–3), and the other half of the cartridges (columns 4–6) were incubated with deglycosylation buffer without PNGase F (no deglycosylation controls) (Table 2B).

Table 1. Samples and reagents.

Samples and reagents	
Wash station	Milli-Q water, for all protocols
Immobilization v1.0	
SA-W cartridge conditioning	
Cartridges	SA-W (G5496-60010)
Prime	1 % FA
Equilibrate	1 % FA
Biotinylated HER2 ECD immobilization	
Cartridges	Conditioned SA-W
Equilibrate and cartridge wash buffer	10 mM HEPES, 150 mM NaCl, pH 7.4
Sample	Biotinylated HER2 ECD
Affinity purification v1.0	
Cartridges	Biotinylated HER2 ECD-SA-W
Sample	Rat sera spiked with different concentrations of ADC
Cartridge wash 1	10 mM HEPES, 1 M NaCl, pH 7.4
Cartridge wash 2	10 mM HEPES, 150 mM NaCl, pH 7.4
On-cartridge reaction v1.0	
Cartridges	ADC-bound biotinylated HER2 ECD-SA-W
Equilibrate and chase buffer	20 mM Tris, pH 8.0
Deglycosylation enzyme	PNGase F
Cartridge wash 1	10 mM HEPES, 1 M NaCl, pH 7.4
Cartridge wash 2	0.003 % FA
Elution buffer	1 % FA
Neutralization solution	0.5 % ammonium hydroxide

Table 2. Experimental design. (A) Serial dilutions and loading amounts and (B) plate layout for affinity purification and deglycosylation.

A

Serial dilutions and loading amounts			
ADC concentration in rat serum (µg/mL)	**Serum volume used (µL)**	**Mass loaded on cartridge (ng)**	**Mass loaded on LC column (ng)**
20	50	1000	200
10	50	500	100
5	50	250	50
2.5	50	125	25
1.25	50	62.5	12.5
0.625	50	31.25	6.25
0.3125	50	15.625	3.125
0	50	0	0

B

Plate layout for affinity purification and deglycosylation (ADC concentration in rat serum (µg/mL))						
	1	**2**	**3**	**4**	**5**	**6**
A	20	20	20	20	20	20
B	10	10	10	10	10	10
C	5	5	5	5	5	5
D	2.5	2.5	2.5	2.5	2.5	2.5
E	1.25	1.25	1.25	1.25	1.25	1.25
F	0.625	0.625	0.625	0.625	0.625	0.625
G	0.3125	0.3125	0.3125	0.3125	0.3125	0.3125
H	0	0	0	0	0	0

Deglycosylation | No deglycosylation

On-cartridge deglycosylation was carried out using the On-Cartridge Reaction v1.0 application. Figure 4 shows the application settings. Briefly, the T-DM1-bound cartridges were equilibrated with 10 bed volumes (50 µL) of the deglycosylation buffer (20 mM Tris, pH 8.0) at a flow rate of 10 µL/min. For the reaction step, 4 µL of the heated PNGase F (columns 1–3) or deglycosylation buffer (columns 4–6) was aspirated from deck position 4 (a Peltier heater/cooler) onto each T-DM1-bound cartridge. This step was followed by a 2-µL slow aspiration over 30 minutes. The software always loads the first 4 µL of the reaction volume at 10 µL/min, then loads the remaining volume (2 µL in this example) over the duration time set on the form. During the reaction, the cartridge tips were in contact with the solution in the wells to maintain an elevated temperature inside the cartridge by conductance. To give a temperature of approximately 37 °C inside the cartridges (reaction temperature), the Peltier temperature was set at 45 °C. Although only 6 µL of solution was aspirated through the cartridges, 12 µL of 1:12 diluted PNGase F (columns 1–3) and deglycosylation buffer alone (columns 4–6) was aliquoted into the PCR plate at position 4 at the beginning of the run. Volume more than the amount aspirated through the cartridge is necessary due to volume loss from evaporation, and the need to have at least 3 µL per well throughout the course of the run to maintain contact between the bottom of the well and the tip of the cartridge for heat transfer. The amount of excess volume required depends on the temperature setting and the reaction time. To collect the released glycans remaining in the cartridges after the reaction, 25 µL of deglycosylation buffer (reaction chase) was aspirated through each cartridge into the syringe probes at a flow rate of 5 µL/min. The combined flow-throughs (reaction chase, released N-glycans, and enzyme solution) collected in the syringes were then dispensed into the empty flow-through collection plate. The cartridges were washed with 10 bed volumes of the high-salt HEPES buffer (50 µL, 10 mM HEPES, 1 M NaCl, pH 7.4) at a flow rate of 10 µL/min. This was followed by a low stringency wash with 10 bed volumes of 0.003 % FA at the same flow rate to remove high-salt HEPES buffer (not compatible with MS) and some nonspecifically bound entities. Deglycosylated (columns 1–3) or intact (columns 4–6) T-DM1 immobilized on the cartridges were eluted with 15 µL of 1 % FA at a flow rate of 5 µL/min into the elution plate that contained 15 µL of 0.5 % ammonium hydroxide per well (existing collection volume) to immediately neutralize the samples. The final volume of each sample was then 30 µL. Finally, the diluted unprocessed T-DM1 samples in water were added to the empty wells along with the processed samples, and the plate was sealed using an Agilent PlateLoc. The samples were then ready for LC/MS analysis.

LC/MS analysis

Prepared samples were analyzed on an Agilent 6545XT AdvanceBio accurate-mass quadrupole time-of-flight LC/MS system coupled with an Agilent 1290 Infinity II UHPLC system. The LC/MS parameters were set as shown in Tables 3A and 3B. For each sample, 6 µL out of 30 µL (20 %) were injected for analysis.

Table 3. LC and MS parameters. A) LC parameters used to separate ADCs. B) MS parameters used to acquire MS data for intact ADCs.

	Parameter	Value
A	**Agilent 1290 Infinity II UHPLC system**	
	Column	Agilent PLRP-S 1000Å 8 µm 150 × 2.1 mm (PL1912-3802)
	Sample thermostat	5 °C
	Mobile phase A	0.1 % FA in water
	Mobile phase B	0.1 % FA in acetonitrile
	Gradient (segmented)	Time (min) %B 0–1 25–25 1–2 25–37 2–4 37–37 4–4.5 37–50 4.5–5.5 50–50 5.5–6 50–25 6–8.5 25–25
	Stop time	8.5 minutes
	Column temperature	60 °C
	Flow rate	0.4 mL/min
B	**Agilent 6545XT AdvanceBio accurate-mass quadrupole time-of-flight MS system**	
	Ion mode	Positive ion mode
	Source	Agilent Dual Jet Stream
	Drying gas temperature	350 °C
	Drying gas flow	12 L/min
	Sheath gas temperature	400 °C
	Sheath gas flow	11 L/min
	Nebulizer	60 psi
	Capillary voltage	5,500 V
	Nozzle	2,000 V
	Fragmentor voltage	380 V
	Skimmer	140 V
	Oct RF Vpp	750 V
	Acquisition parameters MS mode	High (30,000 *m/z*) mass range Extended mass range (2 GHz) MS only mode Mass Range 1,000–5,000 *m/z*

MS data analysis

Raw data files were analyzed by Agilent MassHunter BioConfirm software. Average spectra were extracted and deconvoluted from the total ion chromatograms (TICs) at a retention time between 3.05 and 3.85 minutes. Table 4 shows the deconvolution parameters. For DAR analysis, the deconvoluted spectra were exported and analyzed by Agilent MassHunter DAR Calculator. The default parameters in DAR Calculator were used to determine the DAR value for intact (nonreduced), lysine-linked ADCs, except the max DAR peak number was set at 7. To assess reproducibility and performance of quantitative analysis, extracted ion chromatograms (EICs) in the *m/z* range 2,000–4,000 were extracted and integrated using Agilent MassHunter Qualitative Analysis.

Table 4. Agilent MassHunter BioConfirm parameters for deconvolution.

Parameter	Agilent MassHunter BioConfirm Deconvolute (MS): Protein
Deconvolution algorithm	Maximum entropy
Deconvolution settings	Mass Range: 140–160 KDa Mass Step: 1.0 Da
Use limited *m/z* range	2,400–3,600 *m/z*
Baseline	Subtract baseline Baseline factor: 7.00
Adduct	Proton
Isotope width	Automatic

Results and discussion

ADC DAR characterization

To address the challenges of complex sample preparation for ADC DAR characterization in serum using the Agilent AssayMAP Bravo platform, a complete workflow was developed. The automated sample preparation workflow includes:

- Immobilization of biotinylated target antigens on AssayMAP streptavidin cartridges (SA-W)
- Purification of ADCs from complex matrices
- Deglycosylation of the purified ADCs on-cartridge

Two result plates were generated:

- A flow-through plate containing the soluble reaction products, in this case N-glycans, which were not analyzed in this study
- The elution plate, containing purified and desalted intact and deglycosylated ADCs, which were analyzed by LC/MS

Three application protocols in the Protein Sample Prep WorkBench were used consecutively: Immobilization v1.0, Affinity Purification v1.0, and On-Cartridge Reaction v1.0. Using the application settings in this study (Figures 2–4), the approximate instrument time for immobilization was one hour, affinity purification 1.2 hours, and on-cartridge deglycosylation 1.3 hours. The entire automated sample process can be completed in approximately 4.5 hours. AssayMAP makes it possible to complete the preparation of up to 96 samples simultaneously, and begin LC/MS analysis in one day with minimal hands-on time.

Preparation of biotinylated antigen and antigen-coupled cartridges

Recombinant human HER2 ECD (Accession number AAA75493, extracellular domain: amino acids Thr23–Thr652), containing the target binding site for T-DM1, was used as the bait for affinity purification. To avoid nonspecific endogenous antibody capture, a biotinylated antigen affinity purification approach was used with streptavidin rather than using a protein A or G cartridge. All DAR species (0 to N) can be isolated by antigen purification. As the first step to couple the antigen to AssayMAP streptavidin cartridges, HER2 ECD was labeled with Sulfo-NHS-LC-Biotin through primary amines. There are 16 potential primary amine labeling sites in HER2 ECD: 15 lysine residues and the protein N-terminal end. It was determined that 250-fold molar excess resulted in labeling approximately nine biotin groups per HER2 ECD molecule in one hour at room temperature. In this study, 3 µg of bait antigens (~6-fold molar excess compared to the maximum amount of ADCs to be captured) was used for immobilization on each cartridge. This mass of affinity purification ligand was far below the binding capacity of the SA-W cartridge but was chosen to minimize the consumption of affinity ligand while still enabling high levels of target recovery.

Affinity purification of ADCs from serum

To mimic the kinds of *in vivo* ADC samples that are often analyzed during ADC development, serially diluted T-DM1 was spiked into rat serum to final concentrations of 20, 10, 5, 2.5, 1.25, 0.625, 0.3125, and 0 µg/mL (Table 2A). The key parameters for efficient binding during the loading step are the molar excess of affinity ligand to target and residence time for binding. The residence time is controlled by the loading flow rate. We optimized both parameters to use the lowest molar excess of affinity ligand to minimize cost, while using the highest flow rate possible to minimize run time. The compromise used was to have a molar excess of approximately 6-fold and a loading flow rate of 2 µL/min. These variables may need to be optimized for other binding partners.

On-cartridge deglycosylation

For ADC DAR characterization, multiple glycoforms complicate the MS spectrum and reduce the accuracy of the DAR calculation. Therefore, a deglycosylation step is commonly applied to remove glycans before LC/MS analysis. In this study, a newly developed on-cartridge reaction approach was carried out to deglycosylate ADCs after affinity purification on the cartridge. Compared with the traditional deglycosylation reaction in solution, on-cartridge deglycosylation streamlines the entire process of protein purification, deglycosylation, and buffer exchange. PNGase F is separated from ADCs during this process, thereby avoiding ion suppression in the LC/MS analysis[8]. The deglycosylation was performed at 37 °C to preserve ADC integrity and minimize evaporation while achieving complete deglycosylation in 30 minutes or less. The N-glycans are available for labeling, if desired.

Evaluation of sample preparation performance by LC/MS

T-DM1 samples prepared using an Agilent AssayMAP Bravo platform, as described above, and unprocessed T-DM1 controls were separated on a PLRP-S column with an Agilent 1290 Infinity II UHPLC system, and analyzed on an Agilent 6545XT AdvanceBio accurate-mass quadrupole time-of-flight MS system. TICs, EICs, extracted spectra, deconvoluted spectra, and DAR values were generated from the raw data using the MassHunter software suite (see MS data analysis for detail). Figure 5 shows LC/MS data from 200 ng of control and purified T-DM1 (on-column assuming 100 % recovery during sample preparation). The TICs showed a high degree of purity for the samples spiked into serum and good recovery compared to the control T-DM1 (Figures 5A–C). The extracted spectra and deconvoluted spectra of the control, affinity purified, and affinity purified plus deglycosylated T-DM1 samples indicate that the affinity purification of ADCs did not introduce bias. It also showed that on-cartridge

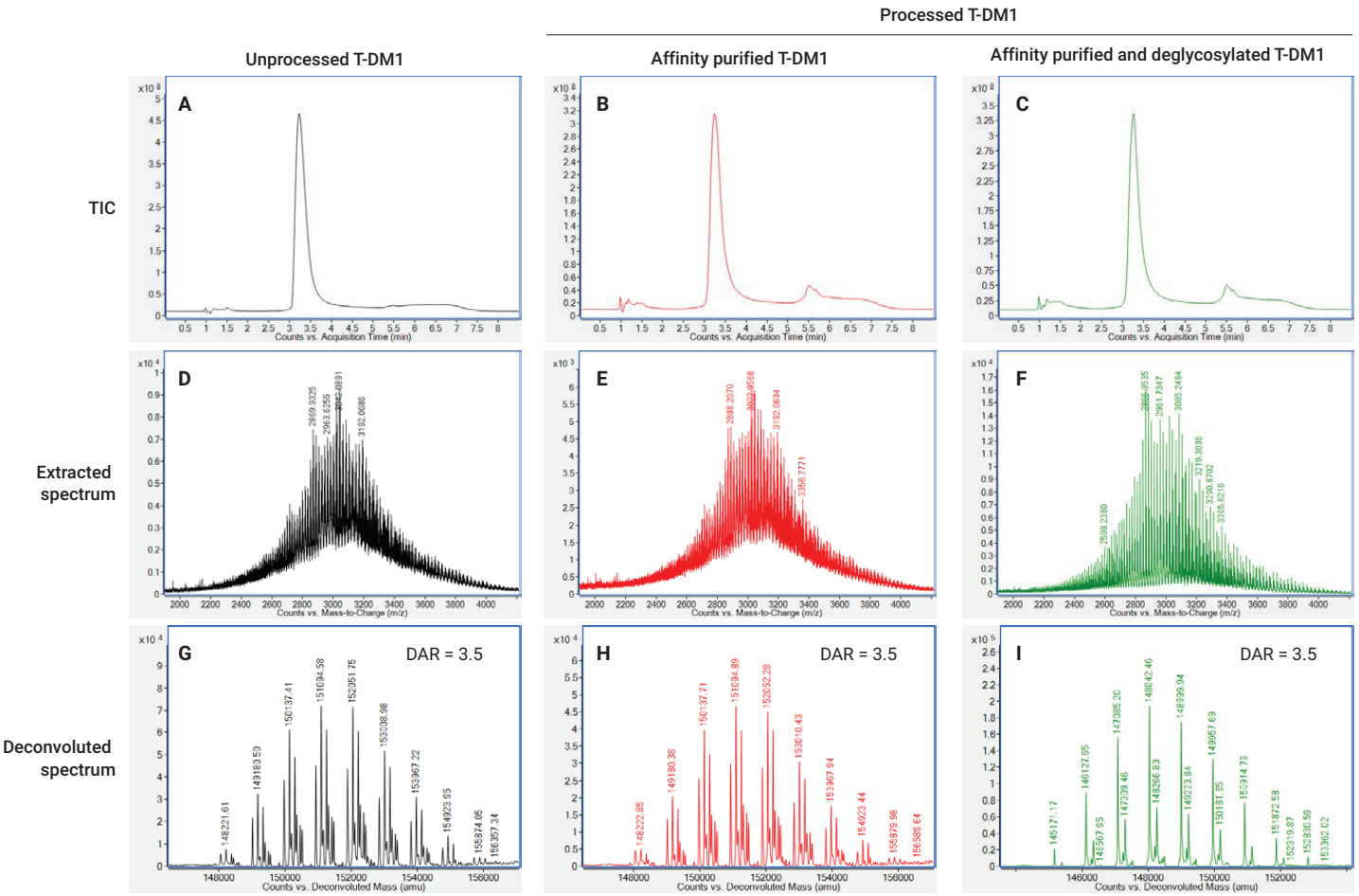

Figure 5. Representative TICs, extracted spectra, and deconvoluted spectra from 200 ng of T-DM1 before and after sample preparation. TIC of unprocessed T-DM1 (A), affinity purified intact T-DM1 (B), affinity purified and deglycosylated T-DM1 (C); extracted spectrum of unprocessed T-DM1 (D), affinity purified intact T-DM1 (E), affinity purified and deglycosylated T-DM1 (F); deconvoluted spectrum of unprocessed T-DM1 (G), affinity purified intact T-DM1 (H), and affinity purified and deglycosylated T-DM1 (I).

deglycosylation simplified the deconvoluted spectrum and increased signal intensity, as expected, by removing most glycoforms (Figure 5D–I).

To evaluate the reproducibility of this workflow, EIC-based quantification was applied to both purified and purified/deglycosylated T-DM1 from rat serum spiked with various concentrations of T-DM1, as described above. The EICs were extracted and integrated over the range of 2,000–4,000 *m/z*. The area under the curve (AUC) was used for relative quantification. Figure 6 shows the overlaid EICs and %CV between test replicates (n = 3). All %CV values were below 10, except the 3.125 ng of the glycosylated T-DM1 samples (CV % = 14.50) (Figure 6). The overall low CVs and the tightly overlapped EICs demonstrate the excellent reproducibility of this workflow.

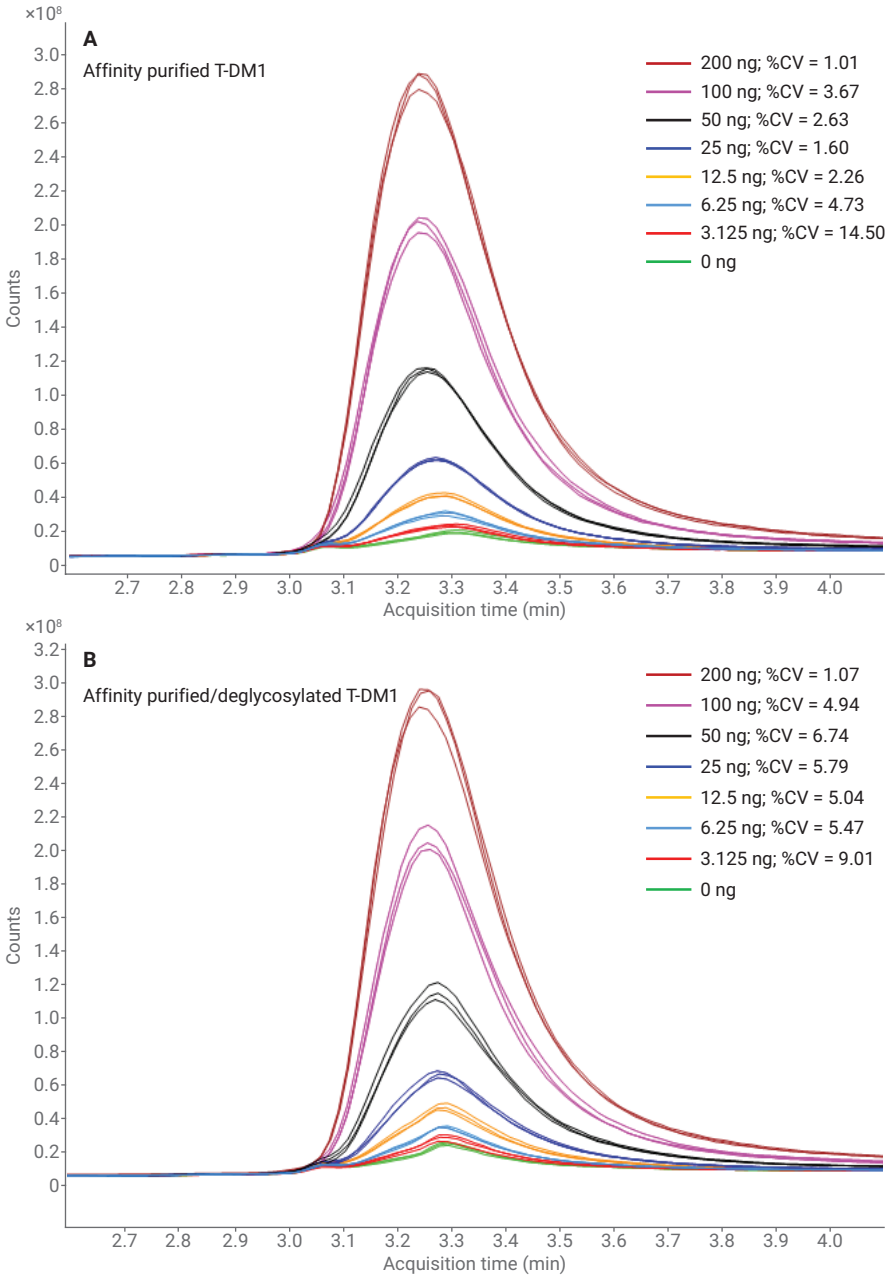

Figure 6. The workflow reproducibility evaluated by the EICs of the affinity purified T-DM1. The ADC T-DM1 was spiked in rat serum at concentrations of 20 to 0.3125 µg/mL and recovered by affinity purification with or without deglycosylation, followed by intact LC/MS analysis. The column loading masses of T-DM1, based on the initial sample inputs, are indicated (see Table 2A). The replicate traces are overlaid and shown in the same color. The %CV between replicates was calculated based on the integrated AUCs (n = 3).

The performance of this workflow for the quantification of affinity purified T-DM1 at the intact protein level was further evaluated based on the linear dynamic range of the assay. The AUCs for the purified and purified/deglycosylated T-DM1 were plotted (Figure 7). Regression analysis indicated excellent linear responses for both purified (R^2 = 0.9987) and purified/deglycosylated (R^2 = 0.9996) T-DM1 from serum samples with 0.3125–10 µg/mL T-DM1 (3.125 to 100 ng of column loading amounts based on initial spiked amount). Note that only 20 % of the eluate was injected for this analysis. These results demonstrated the feasibility of using the AssayMAP Bravo platform to isolate ADCs from complex matrix for quantification analysis.

To determine DAR values, the deconvoluted spectra from control, purified, and purified/deglycosylated T-DM1 samples, described above (Table 2A), were analyzed using Agilent DAR Calculator. DAR0-7 were used to determine the average DAR. The average DAR values from three replicates were calculated to be approximately 3.5 ±0.1 across all samples (Figure 8), which are consistent with the reported DAR value[9]. Based on the results of the control T-DM1, the limit of DAR measurement is 3.125 ng on-column T-DM1. The lowest serum concentration of T-DM1 that could reliably be measured after affinity purification on the AssayMAP platform was 0.3125 µg/mL, although the spectrum was noisy at this level. Deglycosylation of the purified T-DM1 samples simplified the mass spectrum, allowing a more confident measurement of DAR at 0.3125 µg/mL serum concentration (Figure 8).

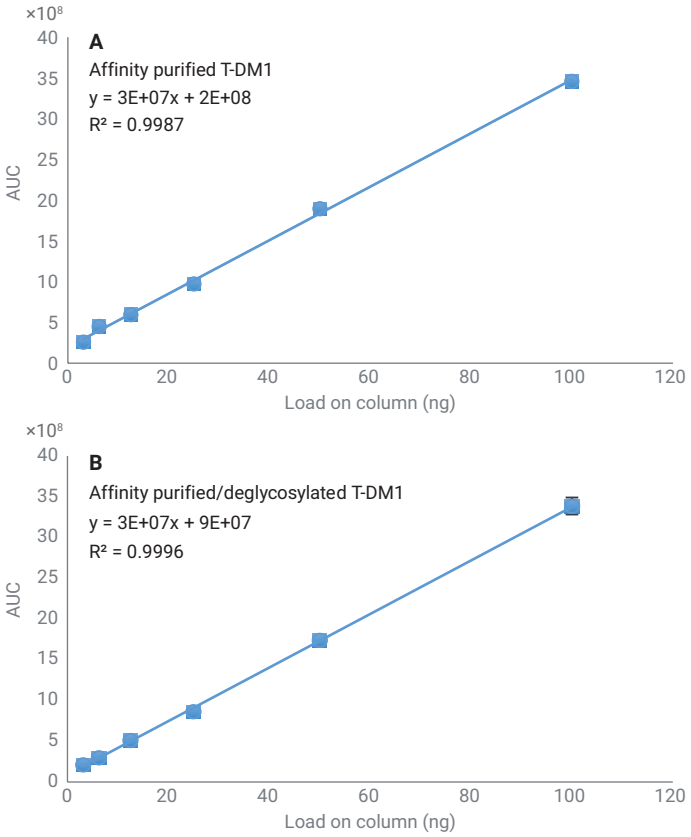

Figure 7. Linear dynamic range analysis of affinity purified and affinity purified/deglycosylated T-DM1 from serum samples. The T-DM1 was spiked into rat serum to a final concentration of 0.3125 to 10 ng/µL. T-DM1 from these samples were affinity purified with or without deglycosylation, followed by intact LC/MS analysis. The EICs in the 2,000-4,000 *m/z* range were extracted and integrated. The AUCs, after removing background, were plotted against the on-column T-DM1 loading amounts from these samples (see Table 2A). Linear regression analysis was carried out to assess the linearity.

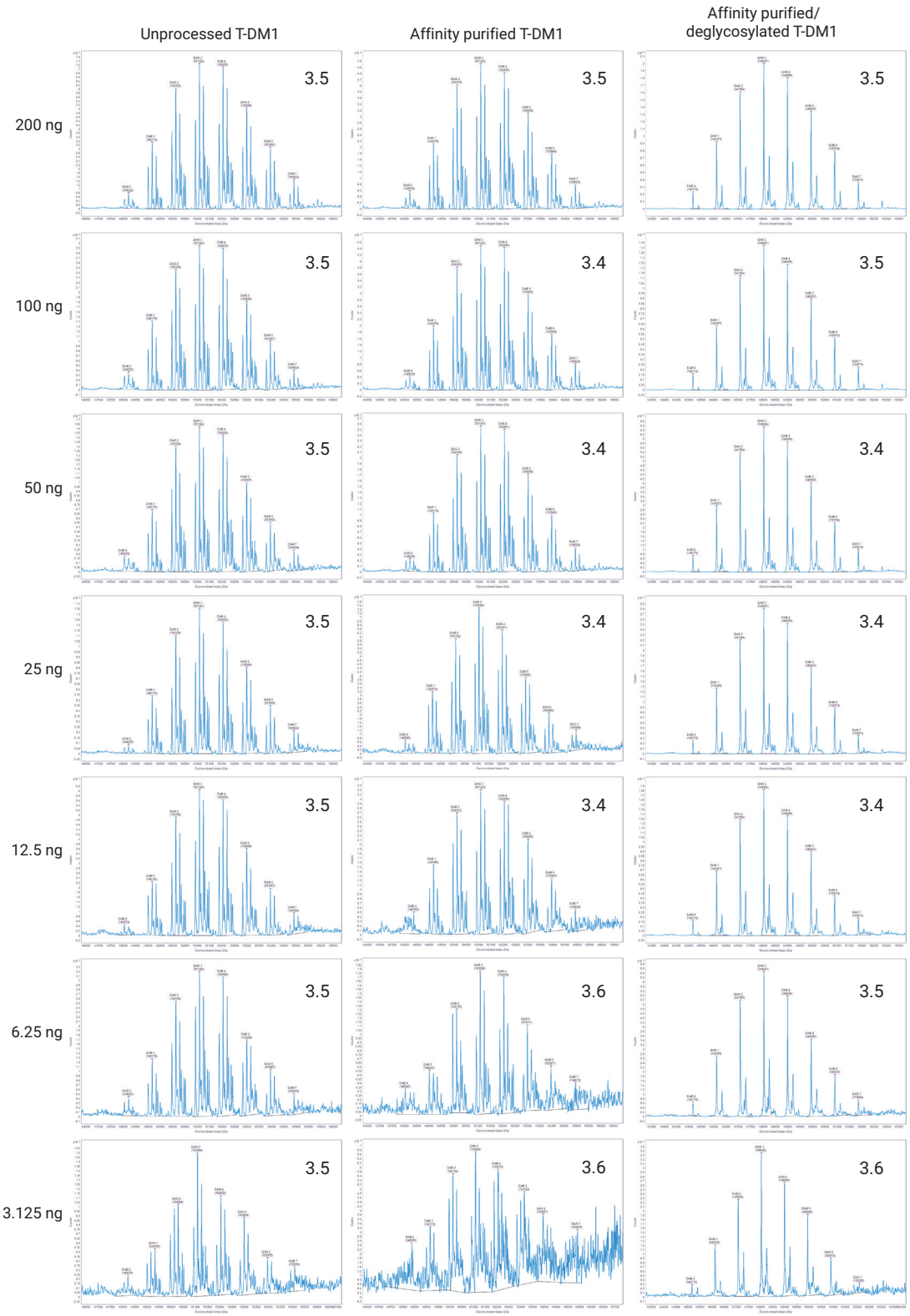

Figure 8. Representative deconvoluted spectra and average DAR values for unprocessed, affinity purified and affinity purified/deglycosylated T-DM1. T-DM1 was spiked into rat serum at different concentrations over the range 0.3125–20 µg/mL and recovered by affinity purification with or without deglycosylation, followed by intact LC/MS analysis. DAR values were determined from deconvoluted spectra using Agilent DAR Calculator. The on-column loading masses based on initial inputs are shown on the left (see Table 2A). The average DAR value averaged from replicates is shown in the upper-right corner in each spectrum (n = 3).

Conclusion

A complete solution was provided to address the challenges of *in vivo* ADC DAR analysis. A typical workflow to recover ADCs from serum followed by deglycosylation before LC/MS analysis was demonstrated with automated procedures using the Agilent AssayMAP Bravo platform. The automated antigen immobilization, affinity purification, and on-cartridge deglycosylation could be completed in approximately 4.5 hours with excellent reproducibility, minimal hands-on time, and the ability to scale as needed. With the high performance of the Agilent 6545XT AdvanceBio accurate-mass quadrupole time-of-flight LC/MS system, the prepared samples can be analyzed seamlessly to yield high-quality data for both qualitative and quantitative studies.

Acknowledgement

We would like to thank Jing Chen for extensive work developing and optimizing the workflow presented here, and Bill Rust, Maryann Shen, and Shuai Wu for advice and critical reading of this Application Note.

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Printed in the USA, September 27, 2019
5991-9010EN

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