Automation of Sample Preparation for Accurate and Scalable Quantification and Characterization of Biotherapeutic Proteins Using the Agilent AssayMAP Bravo Platform

Application Note

Automated Protein Sample Preparation for LC/MS

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Abstract

The Agilent AssayMAP Bravo platform automates a wide variety of LC/MS sample prep workflows on a single instrument that is easy to use, gives highly reproducible results, reduces hands-on time, and can simultaneously process 8–96 samples. We report results from two workflows that are routinely used to quantify and characterize biotherapeutic proteins. Peptide quantification was performed using the AssayMAP Bravo to purify 96 antibody samples in parallel with PG-W (immobilized Protein G) cartridges, digest the antibodies, and clean up the peptides with C18 cartridges. LC/MS analysis indicated that the workflow was highly reproducible with CVs < 10 %. Sequence coverage characterization was performed using the AssayMAP Bravo to purify antibody samples, digest the antibodies with three different proteases in parallel, and clean up the peptides. LC/MS analysis indicated that the combined sequence coverage from all three enzymes was 99 % and 100 % for the heavy and light chains, respectively. Finally, we report analytical figures of merit for antibody purification using PG-W and PA-W (immobilized Protein A) cartridges and the AssayMAP Affinity Purification application. Antibodies were purified with CVs less than 5 %, recovery greater than 90 %, and elution volumes of less than 10 µL.

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Introduction

An ever-increasing number of potential biotherapeutic proteins must be rapidly and accurately characterized so data-driven decisions can be made to move the best candidates efficiently through the drug discovery and development process. This has resulted in a sample processing bottleneck that demands more rapid, scalable, and reproducible methods to supplant current low-throughput and laborious manual sample prep methods for common workflows such as protein purification and quantification, whole protein mass determination, sequence coverage analysis, and characterization of post translational modifications (PTMs).

The Agilent AssayMAP Bravo platform addresses these challenges by enabling the parallel processing of samples through a variety of LC/MS sample prep workflows, with low variability and minimal hands on time. The AssayMAP Bravo platform consists of easy-to-use yet flexible software optimized for LC/MS sample prep workflows, the Bravo liquid handler fitted with a 96-channel AssayMAP syringe head, and disposable 5 µL packed-bed cartridges that mate with the syringes. The liquid handling of the AssayMAP Bravo provides precise flow control through the cartridges, thereby allowing chromatography to be performed in a highly parallel format that enables high reproducibility, excellent sample recovery, efficient washing, and very low elution volumes. This Application Note demonstrates the utility of the platform for widely-used bioprocess workflows and benchmarks analytical figures-of-merit for automated antibody purification.

Experimental

AssayMAP applications

The AssayMAP Bravo is controlled by stand-alone automated operations called applications. Every application can be used independently or combined with other applications to execute workflows. The common workflows are delivered with the component applications bundled together. Each application is optimized to give maximum flexibility with the minimum number of inputs to ensure ease-of-use. The workflows characterized in this Application Note use three different AssayMAP applications; Affinity Purification, In-Solution Digestion[1], and Peptide Cleanup[1]. While the applications have different user-selectable features, reagents, and labware options, they maintain a consistent organization with three sections: **Application Settings**, **Deck Layout**, and **Labware Table** (Figure 1). Before a run is executed, the application evaluates all entry fields and alerts the user of any incompatible settings that could lead to costly and time-consuming mistakes.

Antibody purification

The Affinity Purification application uses Agilent PA-W and PG-W cartridges, which are packed with immobilized protein A and protein G resin, respectively. The buffers and solutions used for this application note are listed in Table 1, but their composition may be varied to meet different experimental design and purity requirements. The main steps conducted by this application are:

- Priming the cartridges to wet them and remove entrapped air
- Equilibrating the cartridge to establish optimal conditions for target binding
- Loading samples on cartridges
- Washing the cartridges with up to two wash solutions
- Eluting the purified antibody

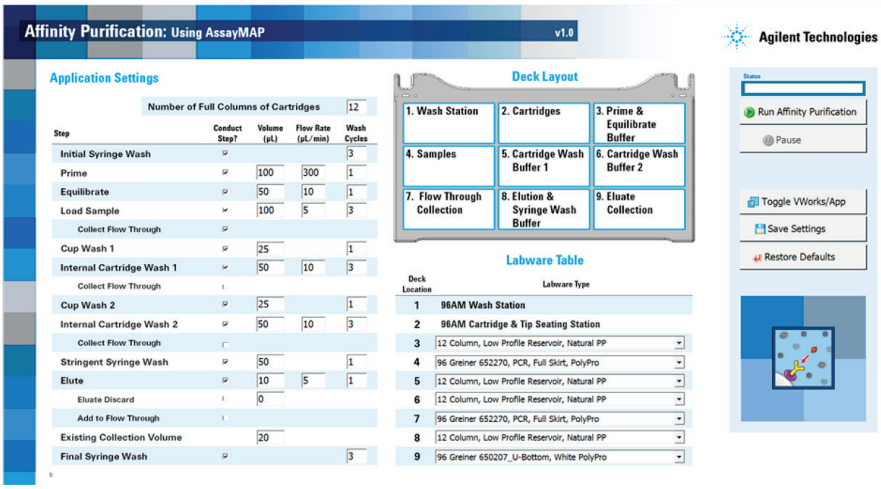


Figure 1. Affinity Purification application user interface. The user modifies default Application Settings and Labware selections as appropriate for the run, places consumables at the indicated deck locations, and clicks **Run Affinity Purification**. The settings shown were used for the experiment in Figure 3. User Guides provide detailed explanations of all steps.

Cartridge performance was benchmarked using antibody isotypes that suit the respective binding selectivity of protein G or protein A; human IgG (hIgG) for PG-W (protein G strongly binds all four subtypes present in the polyclonal antibody preparation) and subtype hIgG1 for PA-W (protein A does not bind hIgG3).

Quantitative recovery was examined by serially diluting hIgG1 (1–100 µg) into 25 µL samples of particulate-free Chinese hamster ovary (CHO) cell culture supernatant (CCS), loading the sample onto PA-W and PG-W cartridges in triplicate, and washing the cartridges. Bound antibody was eluted with 10 µL of 5 % acetic acid into an existing collection volume of 10 µL of 5 % acetic acid for a total of 20 µL (Figure 2). Eluted sample from this type of experiment is routinely quantified using a UV/Vis plate reader, but for this experiment quantification was done using an Agilent 1260 Bio-inert LC System with an Agilent Poroshell 300SB-C18 column (2.1 × 75 mm, 5 µm) for greater sensitivity. The relationship between loaded and eluted mass was plotted.

Reproducibility was examined by serially diluting hIgG (12.5, 25, 50, and 100 µg) into 100 µL of CCS, loading the samples onto PG-W cartridges, and washing the cartridges. A full plate of 96 samples (24 at each load mass) was purified and eluted with 50 µL of 5 % acetic acid into a half-area UV plate for quantification using a plate reader. A relatively large elution volume was used in this experiment to minimize variability introduced by the plate reader. The mass recovered in each sample was plotted, and CVs were calculated for each set of 24 replicates.

Table 1. Samples, reagents, and analytical instrumentation.

On-deck samples and reagents	
Wash station	Deionized water, for all protocols
Affinity Purification v1.0	
Cartridges	PA-W (G5496-60000), PG-W (G5496-60008)
Prime and equilibrate buffer	Phosphate-buffered saline (PBS)
Samples	hIgG , hIgG1, or sequenced monoclonal antibody
Cartridge Wash 1	1 M NaCl in PBS
Cartridge Wash 2	PBS
Elution and syringe wash buffer	5 % acetic acid or 12 mM HCl plus 0.1 M NaCl
Existing collection solution	Nothing, 5 % Acetic acid, 12 mM HCl plus 0.1 M NaCl, or 550 mM Tris base, 9 M urea, 15 mM TCEP
In-Solution Digestion v1.0	
Wash plate	50 mM NaOH
Alkylant	120 mM iodoacetamide (20 mM final)
Diluent mixture	3 mm TCEP (4 mM final)
Protease	trypsin, 0.075 µg/µL in 50 mM acetic acid (1:20 final)
	chymotrypsin, 0.075 µg/µL in 1 mM HCl (1:20 final)
	Glu-C, 0.075 µg/µL in purified water (1:20 final)
Peptide Cleanup v2.0	
Cartridges	C18 Cartridges (5190-6532)
Priming and syringe wash	0.1 % TFA, 50 % acetonitrile
Equilibration and cartridge wash buffer	0.1 % TFA
Elution buffer	0.1 % TFA, 50 % acetonitrile
Eluate collection plate	0.1 % TFA
Analytical instrumentation	
Automation	
Core automation platform	Agilent AssayMAP Bravo (G5542A)
Agilent AssayMAP Bravo accessories	Risers, 146 mm (G5498B#055), Peltier Thermal Station with STC controller (G5498B#035), Custom Plate Nest (G5498B#017), PCR Plate Insert (G5498B#013), Orbital Shaking Station w/control Unit (G5498B#033)
Additional accessories	Agilent PlateLoc Thermal Microplate sealer (G5402A)
LC/MS	
Mass spectrometer	Agilent 6550 iFunnel Q-TOF LC/MS, Dual Agilent Jet Stream ESI
LC systems and columns	Agilent 1290 Infinity LC System
	Agilent 1260 Infinity Bio-Inert LC System
	Agilent AdvanceBio Peptide Mapping columns, C18: Analytical - 2.1 × 250 mm, 2.7 µm (p/n 651750-902) Guard - 2.1 mm Fast Guard (p/n 851725-911) Agilent Poroshell 300SB-C18: Analytical - 2.1 × 75 mm, 5 µm (p/n 660750-902)

Minimal elution volume was examined for two eluents with different stringencies by loading 50 µg of antibody in duplicate onto PA-W and PG-W cartridges and washing the cartridges using the Affinity Purification application. To examine differences in the elution profiles, antibodies were eluted from each cartridge type with 5 % acetic acid or 12 mM HCl, 0.1 M NaCl (pH 2), using a custom protocol that collected the eluate in 2 or 3 µL fractions. These small volume fractions were eluted into wells containing the same eluent for a total volume of 50 µL. Quantification was performed using an Agilent 1260 HPLC as described above. Duplicates were averaged, and eluted mass per fraction was plotted with respect to cumulative elution volume.

Protein characterization workflows

The peptide quantification workflow used the Affinity Purification, In-Solution Digestion, and Peptide Cleanup applications. Ninety-six samples containing 15 µg of a sequenced monoclonal antibody (mAb) in 100 µL CCS were purified using the Affinity Purification application with PG-W cartridges. The antibody was eluted with 10 µL of 5 % acetic acid directly into 20 µL of existing collection volume containing concentrated denaturant and reductant in Tris buffer (Table 1) for a final composition of 365 mM Tris pH 8.1, 6.0 M urea, 10 mM TECP. The eluates were incubated at 60 °C for 30 minutes. The AssayMAP Bravo deck was reconfigured and the samples were alkylated, diluted, and Agilent proteomics grade trypsin was added at 1:20 (enzyme:substrate) using the In-Solution Digestion application. Reactions (180 µL each) were incubated overnight at 37 °C, followed by acidification with 20 µL of 10 % trifluoroacetic acid (TFA). The deck was rearranged for the Peptide Cleanup application and the samples were loaded on C18 reversed-phase cartridges and washed with 0.1 % TFA. Peptides were eluted off the C18 cartridge with 15 µL of 0.1 % TFA in 50 % acetonitrile and directly mixed into 135 µL of 0.1 % TFA. A 10 µL (1 µg) amount of each sample was analyzed using an Agilent 1290 HPLC and an Agilent 6550 iFunnel Q-TOF LC/MS, Dual Agilent Jet Stream ESI. The resulting data were analyzed with MassHunter BioConfirm software.

An antibody sequence coverage workflow used the Affinity Purification, In-Solution Digestion, and Peptide Cleanup applications. The workflow was run essentially as described above for 15 µg samples of a sequenced mAb in 100 µL CCS, except three different proteases were used in parallel in the In-Solution Digestion application. The proteases used were trypsin (Agilent), chymotrypsin (Promega Corp.), and Glu-C (Promega Corp.). Ten microliters (1 µg) of each sample was analyzed using the 1290 HPLC and 6550 iFunnel Q-TOF LC/MS, Dual Agilent Jet Stream ESI. The resulting data were analyzed with SpectrumMill software.

Results and Discussion

Affinity purification characterization

The quantitative recovery range for PA-W and PG-W cartridges was examined using the Affinity Purification application. A serial dilution of 1–100 µg hIgG or hIgG1 was loaded onto PG-W or PA-W cartridges, respectively. The cartridges were washed, and bound antibodies were eluted and quantified. Recovery exceeded 90 % of the loaded mass for each cartridge type across the entire range. Figure 2 illustrates the relationship between loaded and eluted antibody mass with the identity line shown in black. The left panel shows the entire range, while the right panel shows the 0 to 10 µg region.

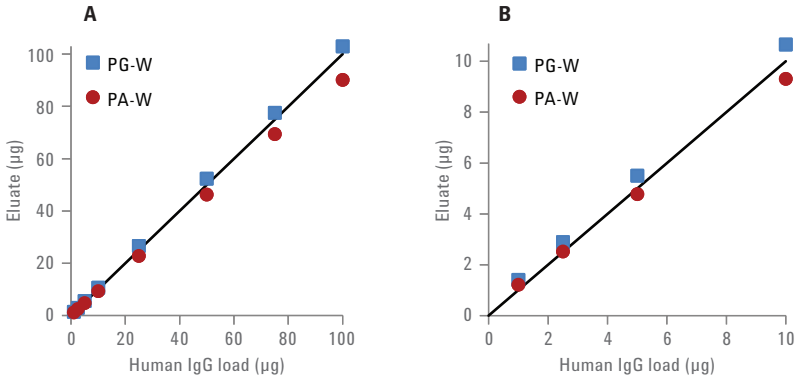


Figure 2. Affinity Purification quantitative recovery using PG-W and PA-W cartridges.

Reproducibility across an entire plate of 96 samples was tested by parallel purification of hIgG in CCS, using PG-W cartridges. Twenty-four cartridges were used at each of four antibody mass loads. The cartridges were washed, and eluted antibody was quantified (Figure 3). Percent recovery and CVs were calculated (Table 2).

Minimum elution volume

Precise flow rate control combined with small cartridge bed volumes enables quantitative elution in very small volumes, but eluent formulation and stringency must also be evaluated to ensure success. The elution profiles of PG-W and PA-W cartridges with two eluents of different stringencies were examined. Fifty micrograms of hIgG or hIgG1 was loaded onto PG-W or PA-W cartridges, respectively. The cartridges were washed, and bound antibodies were eluted with either 5 % acetic acid (Figure 4A) or 12 mM HCl and 0.1 M NaCl (Figure 4B), using 2 µL or 3 µL fraction collection, respectively. Eluted antibody mass for each fraction was plotted with respect to cumulative elution volume.

Figure 4A shows maximum recovery in less than 10 uL of 5 % acetic acid for both PG-W and PA-W cartridges. This eluent exhibits high stringency and is a good choice for keeping the sample volume low at the beginning of a multistep workflow that culminates in LC/MS analysis. Figure 4B shows maximum recovery in 21 µL or less for PA-W, or 30 µL or less for PG-W, when eluting with 12 mM HCl, 0.1 M NaCl. This eluent is a good choice when a gentler elution solution is required. The first fraction (void volume) contained no protein for either eluent, which increased the volume and lowered the purified antibody concentration. The Affinity Purification application allows the user to specify the start and end points for eluate collection, and automatically discard the void volume, if desired.

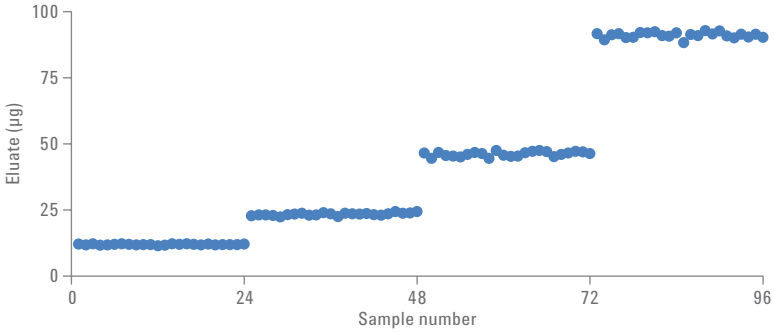


Figure 3. Affinity Purification reproducibility. The results from the purification of 96 hIgG samples using the Affinity Purification application.

Table 2. Calculated recoveries and % CV.

Sample number	Load (µg)	% Recovery	% CV
1–24	12.5	95.4	1.3
25–48	25	93.5	1.8
49–72	50	92.3	1.8
73–96	100	91.0	1.1

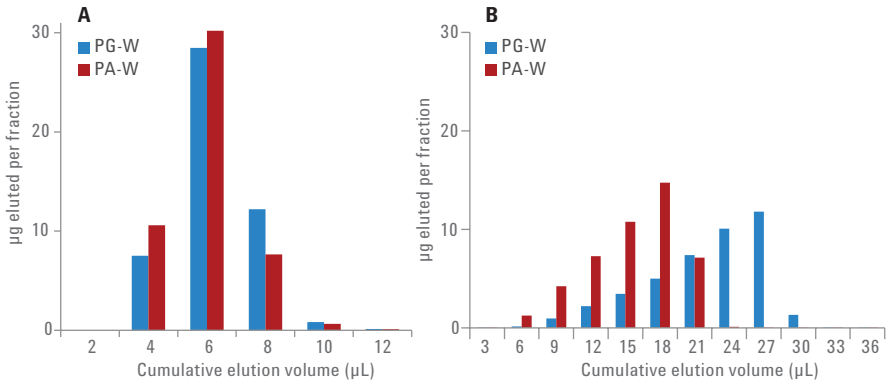


Figure 4. Minimum elution volume for antibody purification. Fifty micrograms of bound antibody was eluted in 2- or 3-µL fractions and mixed into a total volume of 50 µL for quantification. The eluted mass for each fraction was plotted with respect to cumulative elution volume.

Workflow for mAb peptide quantification by LC/MS

A sample preparation workflow, illustrated in Figure 5, was performed on the AssayMAP Bravo in which a full plate of 96 samples each containing 15 µg of mAb in CCS was processed using the Affinity Purification, In-Solution Digestion, and Peptide Cleanup applications described above. LC/MS analysis was done and extracted ion chromatograms (EIC) were generated for six proteotypic peptides, from which relative protein abundance could be inferred. CVs of 5–8 % were calculated from EIC peak areas for all 96 samples, which indicated that reproducibility of the entire workflow was very high. An overlay of the EICs from the first row of 12 samples is shown in Figure 6.

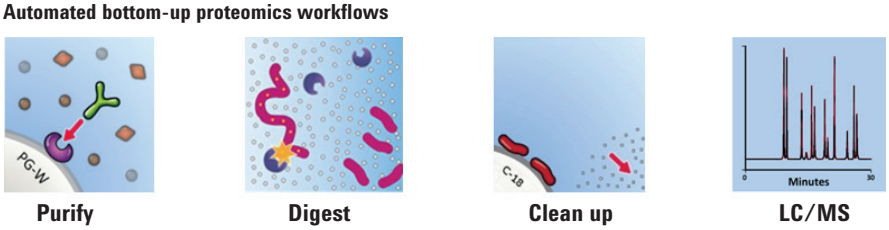
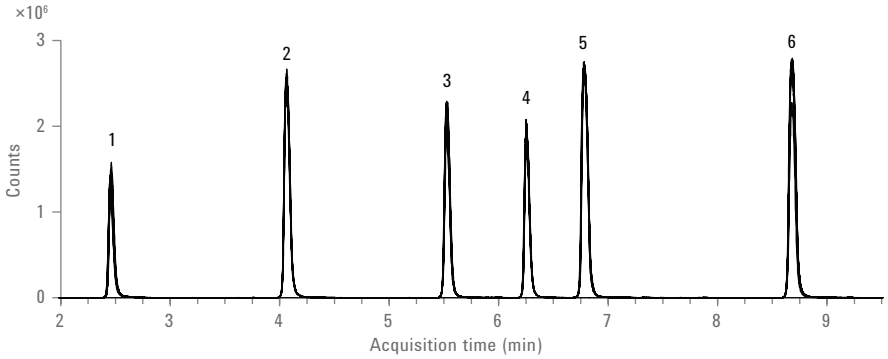


Figure 5. Workflow diagram for biotherapeutic antibody sample preparation. For this study, replicates of mAb in particulate-free CCS were purified in parallel using the Affinity Purification application, digested using the In-Solution Digestion application, and then the resulting peptides were purified and concentrated on C18 reversed-phase cartridges using the Peptide Cleanup application, resulting in samples ready for LC/MS analysis.



Peptide sequence	% CV
1. SLSHSPG	7.8
2. TSTSPIVK	5.6
3. SQVFLK	8.0
4. FTGSGSGTQFSLK	6.6
5. VNSAAFPAPIEK	5.0
6. DVLTITLTPK	7.2

Figure 6. Peptide Quantitation Workflow reproducibility indicated by the overlay of EIC for six mAb peptides from 12 samples, and the CV calculated from the peak area of the same six peptides from 96 samples.

Workflow for mAb sequence coverage

A sample preparation workflow was performed on the AssayMAP Bravo in which samples containing 15 µg mAb in CCS were processed using Affinity Purification, In-Solution Digestion, and Peptide Cleanup applications as described above (Figure 5), with the exception that three proteases (trypsin, chymotrypsin, or Glu-C), were tested in parallel for the In-Solution Digestion step, to determine sequence coverage for a mAb in a single experiment. LC/MS analysis was performed on the desalted peptides to determine sequence coverage.

In Figure 7, sequence coverage is illustrated using colored bars to represent detected peptides from N- to C-terminus (left to right). Upper and lower green bars represent aggregate coverage for the heavy and light chains, respectively, and varying shades of blue represent peptides detected in digests by the individual proteases. White space indicates a lack of sequence coverage for that region. No single enzyme achieved complete coverage of either polypeptide chain. Most notably, a large gap in the dark blue bars is seen for a 70 amino acid region in the heavy chain that lacks trypsin cleavage sites, possibly because the instrumentation was tuned for sensitive detection of much smaller peptides. Digests with chymotrypsin and Glu-C each produced detectable peptides within this and other regions where tryptic digestion left gaps, resulting in coverage of all but six amino acid positions for the heavy chain. Light chain coverage for this antibody was nearly complete using trypsin alone, but digestion with chymotrypsin completed the sequence coverage by filling in the remaining five amino acids.

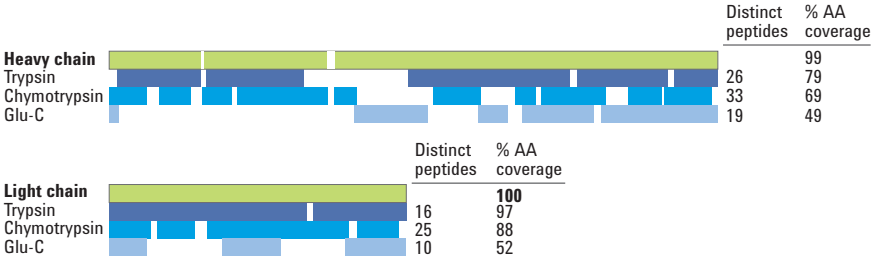


Figure 7. mAb sequence coverage. Green indicates combined amino acid coverage from all three proteases, blue for individual proteases, and white shows lack of coverage. Combined coverage was 433 of 439 heavy chain amino acids, and all 214 light chain amino acids. By contrast, trypsin-only reactions covered only 349 (79 %) for the heavy chain and 209 (97 %) for the light chain. The number of distinct peptides and the percent coverage were determined using SpectrumMill software.

Conclusions

The Agilent AssayMAP Bravo is a flexible and easy-to-use automation platform that allows the execution of workflows for biotherapeutic protein sample preparation prior to LC/MS. The automation of tedious, but critical, liquid handling steps reduces hands-on time and maximizes throughput, with high reproducibility. Characterization of the Affinity Purification cartridges showed a linear range of quantitative binding and recovery from 1–100 µg of loaded antibody, which could be eluted in 10 µL or less, with CVs below 5 %. More importantly, the AssayMAP platform permits users to combine the automation of Affinity Purification, In-Solution Digestion, and Peptide Cleanup into a single workflow that maintains high reproducibility throughout, as demonstrated by CVs less than 10 % for the entire Peptide Quantification workflow presented in this Application Note.

Reference

1. Russell, J.D.; *et al.,* Automation for LC/MS Sample Preparation: High Throughput In-Solution Digestion and Peptide Cleanup Enabled by the Agilent AssayMAP Bravo Platform, *Agilent Technologies Application Note*, publication number 5991-2957EN, **2013**.

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