Saliva testing

A comparative study of saliva collection devices using Axiom human genotyping arrays

Introduction

High-density DNA microarrays play an important role in research focused on identifying the genetic basis of complex human diseases using genome-wide association studies (GWAS). Microarrays enable accurate, cost-effective genotyping of both common and rare variants that include single-nucleotide polymorphisms, insertions or deletions, and multiallelic polymorphisms. These studies routinely use hundreds of thousands of samples or more, so there is a need for efficient, cost-effective laboratory workflows that span from sample collection and nucleic acid extraction to downstream genotyping with microarrays. Applied Biosystems™ Axiom™ human genotyping arrays currently use multiple sources of human genomic DNA (gDNA) from sample types including blood, saliva, buccal cells, and cell lines, at an input mass of 100–200 ng gDNA per sample per array. Genotyping performance consistently achieves an average sample call rate of ≥99.0%, average sample concordance to independent DNA genotype information (HapMap) of ≥99.5%, and intra- and inter-run reproducibility of ≥99.8%. In large-scale genotyping studies, saliva has been shown to be a high-quality substitute for existing collection methods and sample types like whole blood and buccal swabs, as it is noninvasive and plentiful. Sequencing and genotyping applications that use saliva have shown equivalency with those that use buccal swabs and whole blood [1–3]. A previous study comparing DNA yield and quality from matched saliva and buccal swab samples indicated high yields of intact gDNA suitable for downstream applications such as qPCR and next-generation sequencing (NGS) at various collection times [1]. As a noninvasive sample type that has minimal storage requirements, saliva can be an excellent option to streamline workflows in a cost-effective manner. However, questions surrounding bacterial content of saliva and its impact on downstream sequencing and genotyping applications remain.

Here we evaluate the quantity, quality, and impact of microbial DNA in human samples from various saliva collection devices using a high-density Axiom SNP genotyping array.

Materials and methods

Sample collection

Raw saliva was collected from 24 donors in 50 mL conical tubes and stored at ambient temperature. The same day of collection, the saliva was mixed thoroughly and manually dispensed at recommended volumes into 4 collection devices: Thermo Scientific™ SpeciMAX™ Stabilized Saliva Collection Kit, Thermo Scientific™ SpeciMAX™ Saliva Collection Kit, Oragene™-Discover (OGR-600) device (DNA Genotek), and SDNA-1000 Saliva Collection Device (Spectrum Solutions, research-only version of the product). This resulted in 96 individual samples that were all stored at ambient temperature.

Nucleic acid extraction

Three days after sample collection, gDNA was extracted from all 96 samples using the Applied Biosystems™ MagMAX™ DNA Multi-Sample Ultra 2.0 Kit on the Thermo Scientific™ KingFisher™ Apex Purification System using the 200 µL saliva workflow. For devices that stabilize samples, including the SpeciMAX Stabilized device, OGR-600 device, and SDNA-1000 device, 200 µL of sample was dispensed directly into the sample plate, according to the MagMAX DNA Multi-Sample Ultra 2.0 Kit user guide. For raw saliva samples collected using the SpeciMAX Saliva Collection Kit, 1X PBS was used to make a 1:1 dilution before extraction using the MagMAX kit. All samples were eluted with a 50 µL elution volume. The same extraction process was repeated at 14 days after sample collection.

Quality evaluation

To determine DNA yields, the Invitrogen™ Qubit™ dsDNA HS Assay Kit and Invitrogen™ Qubit™ fluorometer were used. A Thermo Scientific™ NanoDrop™ spectrophotometer was used to obtain purity and quality information of the extracted nucleic acids. The Applied Biosystems™ TaqMan™ RNase P Copy Number Reference Assay and Applied Biosystems™ TaqMan™ 16S bacterial assay were used to quantify human and bacterial gDNA, respectively. Human gDNA and *E. coli* gDNA standard curves with a 7-point serial dilution were used for quantification. The remaining eluate was used for genotyping with the Applied Biosystems™ Axiom™ Precision Medicine Diversity Array Kit, 96-format workflow, using 4 HapMap controls for each array. General array quality metrics and resolution for two *APOE* markers were characterized. Figure 1 details the experimental workflow to identify the impact of bacterial growth in saliva on the downstream microarray application. The Agilent™ Bioanalyzer™ and TapeStation™ systems were used to assign a DNA integrity number (DIN), a measure of gDNA integrity. This value ranges from 1 to 10, where 1 indicates highly degraded gDNA and 10 represents highly intact gDNA.

Results

Percentage of bacterial gDNA and DIN

To determine the approximate percentages of human gDNA compared to bacterial gDNA, two standard curves were generated using human gDNA or *E. coli* gDNA (Figure 2). R^2 values were greater than 99% for both. Extracted nucleic acid from saliva specimens at 3 and 14 days post-collection were run with the same assays and compared against the standard curves in Figure 2 to determine the percentages of human and bacterial gDNA. Figure 3 shows high DIN scores obtained from all three kits for saliva stabilization, indicating that stabilization buffer is needed during sample collection to preserve the integrity of DNA. DNA extracted using the kit for raw collection resulted in lower DIN values than those for stabilized DNA. DIN values were not significantly different between stabilized samples for days 3 and 14 post-collection.

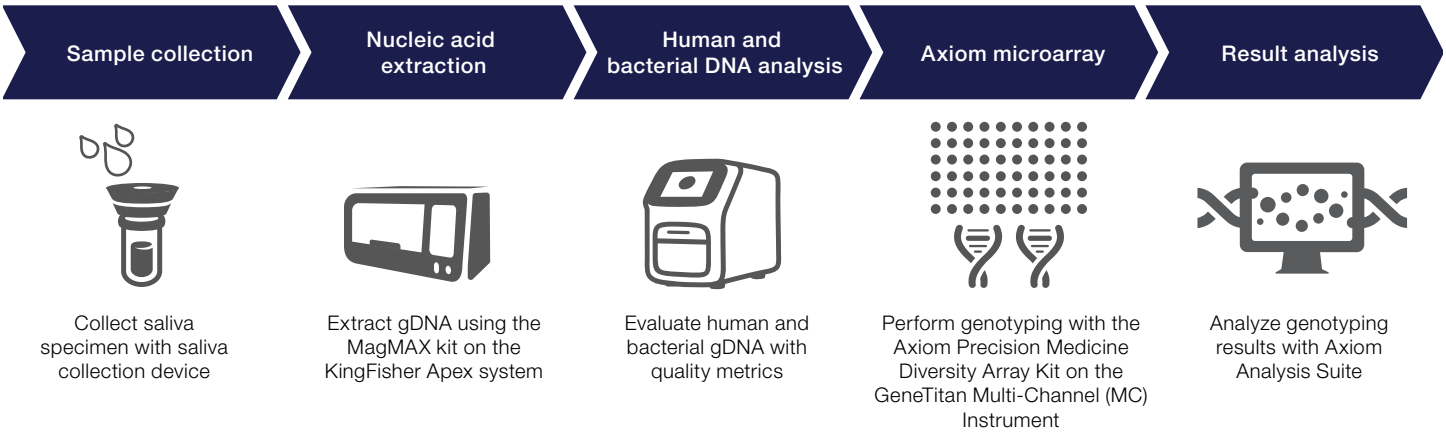


Figure 1. Workflow used to assess the impact of microbial DNA on Axiom microarray applications using gDNA extracted from saliva.

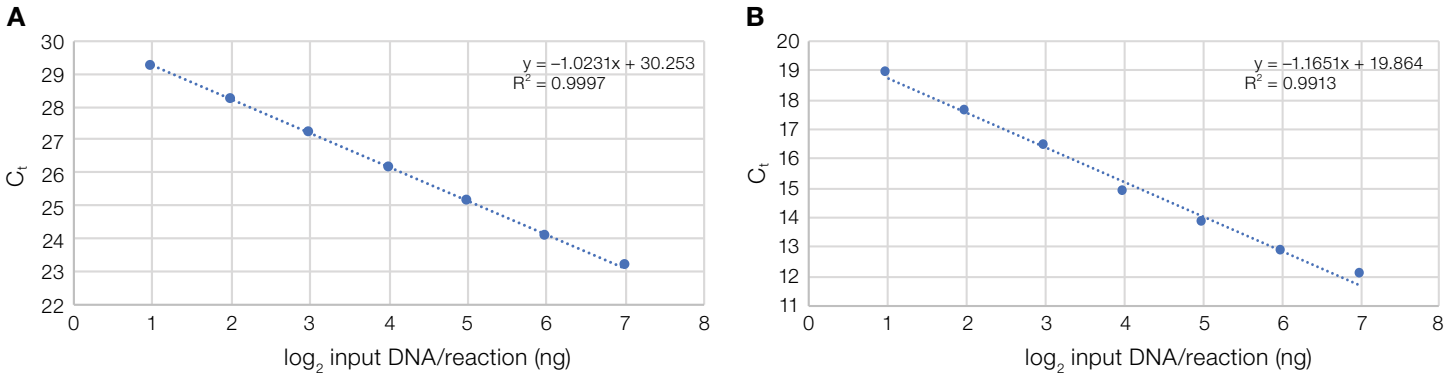


Figure 2. Standard curves generated from qPCR data. Serial dilution of **(A)** human gDNA (RNase P assay) and **(B)** *E. coli* gDNA (16S rRNA gene assay) were used to create standard curves for the determination of human and bacterial gDNA content in extracted samples.

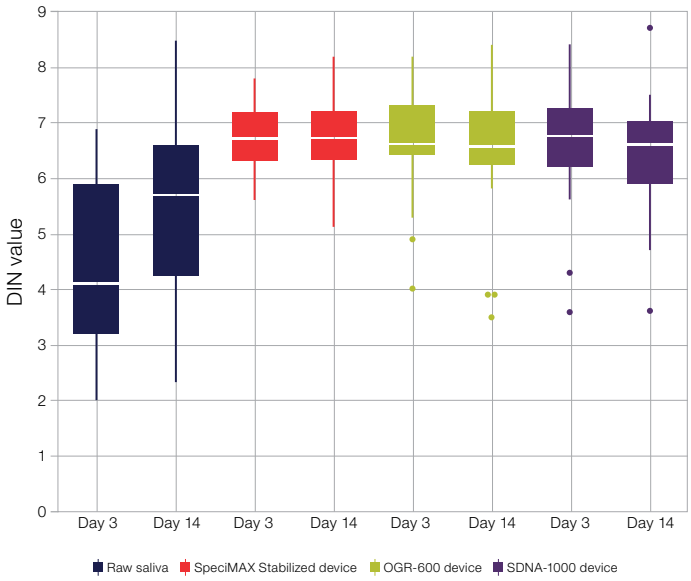
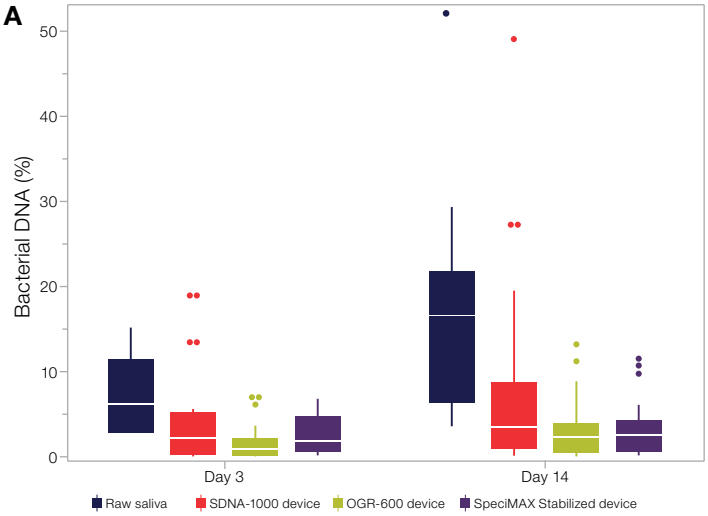


Figure 3. DIN values of gDNA samples.

The ratio of bacterial gDNA to human gDNA increased over a 14-day period in all saliva collection devices but is most prominent in the raw saliva collection device (Figure 4). The total percentage of bacterial gDNA increased by almost 10% from day 3 to day 14 post-collection. All stabilized saliva collection devices had minor increases in total bacterial gDNA content between days 3 and 14 post-collection, with average increases of less than 4%. The SDNA-1000 device on average contained approximately 4% bacterial gDNA and 96% human gDNA for day 3, increasing to approximately 8% bacterial gDNA and 92% human gDNA at day 14 post-collection. Next, the OGR-600 device on average contained ~1.7% bacterial gDNA on day 3 post-collection, which then increased to ~3.1% in 11 days of ambient storage. Finally, for the SpeciMAX Stabilized device, there was ~3% bacterial DNA and ~97% human DNA at day 3 post-collection, which remained approximately the same through to day 14. Large standard deviations are to be expected, because differing oral microbiomes are anticipated across 24 different donors. All bacterial and human gDNA percentages of total gDNA yields were averaged and approximated using the standard curves in Figure 2.

To evaluate the impact of bacterial gDNA from 3 and 14 days post-collection on microarrays, the Axiom Precision Medicine Diversity Array (PMDA) was hybridized with Axiom 2.0 assay DNA target prepared with the extracted nucleic acids from raw and stabilized saliva collection devices across all 24 donors, along with HapMap controls to ensure quality of microarray runs. Dish QC, median absolute pairwise distribution (MAPD), and waviness-SD were determined for all samples at 3 and 14 days post-collection to evaluate quality of the extracted nucleic acids before performing genotyping analysis.

A



B

Bacterial gDNA content			
Day	**Device**	**Mean bacterial gDNA (%)**	**Standard deviation (%)**
3	SpeciMAX Saliva Collection Kit (raw saliva)	6.91	4.79
	SDNA-1000 device	4.25	5.82
	OGR-600 device	1.71	2.15
	SpeciMAX Stabilized Saliva Collection Kit	2.49	2.19
14	SpeciMAX Saliva Collection Kit	16.90	10.90
	SDNA-1000 device	7.88	11.80
	OGR-600 device	3.09	3.58
	SpeciMAX Stabilized Saliva Collection Kit	3.24	3.26
Bacterial gDNA increases between 3 and 14 days post-collection (%)			
Difference of 11 days	SpeciMAX Saliva Collection Kit	9.99	10.40
	SDNA-1000 device	3.63	6.52
	OGR-600 device	1.38	1.57
	SpeciMAX Stabilized Saliva Collection Kit	0.75	1.60

Figure 4. Amount of bacterial gDNA measured from different collection devices. (A) Graph of percentages of bacterial gDNA from raw saliva and the 3 different stabilized saliva collection devices. **(B)** Table of percentages of bacterial gDNA on days 3 and 4 post-collection and increases in bacterial gDNA during 11-day storage.

Array analysis QC—dish QC

Dish QC (DQC), based on intensities of probe sequences for non-polymorphic genome locations, indicate high-quality samples if the contrast distribution is well-resolved, or as the DQC value approaches zero there is minimal resolution between distributions of AT and GC probe contrast values [4]. One of 24 donor samples was removed from the array analysis to allow for control samples on the array, leaving 23 donor samples. DQC analysis for raw saliva across all donors at day 3 post-collection indicated a large range, with the bulk of the samples resolving between a DQC value of 0.6 and 0.9. In contrast, all stabilized saliva collection devices showed a much higher-quality DQC value between 0.9 and 1.0, with minor variations. For all stabilized saliva devices at day 3, three matched sample outliers had lower DQC values, with a fourth outlier for the OGR-600 device. At day 14 post-collection, there was a significant decrease in the DQC of the raw saliva samples, with the majority of samples yielding a DQC value below 0.5. In contrast, the mean DQC value for samples preserved using stabilized devices remained closer to approximately 1.0, with just a few outliers (Figure 5).

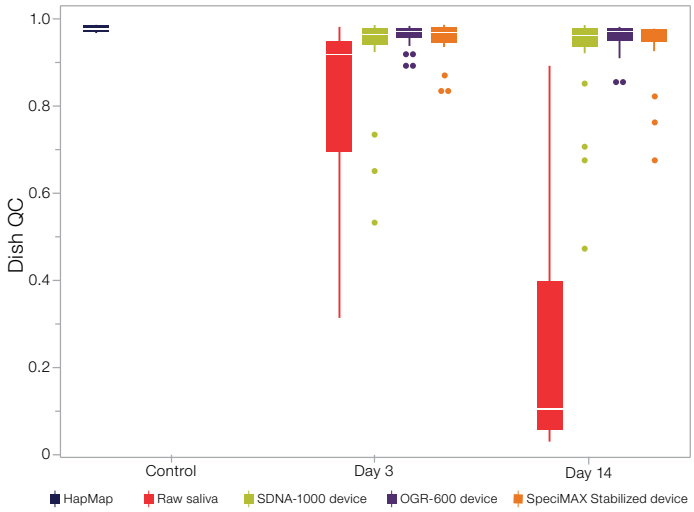


Figure 5. DQC of 24 matched samples for raw saliva and stabilized collection devices on day 3 and day 14 post-collection.

Array analysis QC—MAPD and waviness-SD

The absolute values of pairwise differences between $\log_2$ ratios for the 24 samples were evaluated with MAPD and waviness-SD QC metrics. Raw saliva from day 3 post-collection had much higher MAPD and waviness-SD values compared to SDNA-1000, OGR-600, and SpeciMAX Stabilized devices. On day 14 post-collection, raw saliva did not even register MAPD and waviness-SD values, which most likely indicates the samples were of low quality. As stated in the Axiom copy number analysis technical note [5], MAPD measures the variability in the $\log_2$ ratios between adjacent probes and is indicative of short-range variation across the genome. Elevated MAPD can impact the accuracy of CN calls, and it is recommended to exclude samples with high MAPD from analysis. On the other hand, waviness-SD is a measure of long-range variation in the probe intensities on the array. An elevated waviness-SD may indicate that sample or processing effects have caused excessive noise. SDNA-1000, OGR-600, and SpeciMAX Stabilized devices show consistent MAPD and waviness-SD at days 3 and 14 post-collection (Figure 6).

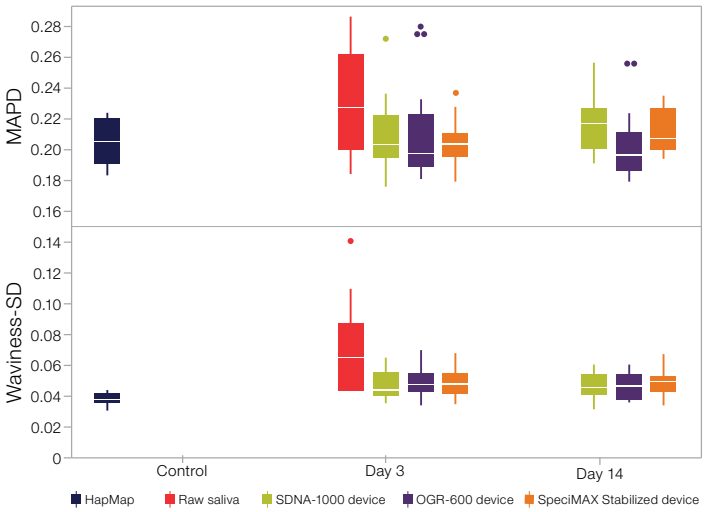


Figure 6. MAPD and waviness-SD of 24 matched samples across raw saliva and stabilized collection devices on day 3 and day 14 post-collection.

Array analysis QC—call rate and passing

In addition to quality metrics such as DQC, MAPD, and waviness-SD, QC call rate (CR) was evaluated for the stabilized devices for both day 3 and day 14 post-collection. Raw saliva was not evaluated by this metric because of the poor DQC, MAPD, and waviness-SD quality. Axiom PMDA analysis was conducted by individual clustering of SpeciMAX Stabilized, OGR-600, and SDNA-1000 devices for all 23 donors (Table 1). The number of input samples reads 46 because it includes both days 3 and 14 post-collection. Of the 46 samples collected with the SpeciMAX Stabilized device, 43 had a DQC value of at least 0.82; this was 40/46 for the SDNA-100 device and 46/46 for the OGR-600 device. Overall, the 3 stabilized collection devices provided a high passing QC CR of ~99% along with a high average sample CR. All stabilized devices had more than 95% of markers passing filtering. Although the OGR-600 device rescued 3 low-performing samples that failed the genotyping thresholds for a passing sample of DQC ≥0.82, the inclusion of these samples in the genotype clustering may explain the slight decrease in average sample call rate of the OGR-600 device as compared to the SpeciMAX Stabilized device.

APOE genotyping performance comparison using different saliva collection devices

APOE mutations have been found to be associated with differential risk of Alzheimer's disease and cardiovascular disease with clinical significance [6]. However, while *APOE* genotypes have excellent performance for blood samples (100% concordance to imputed genotypes), they are difficult to resolve for raw saliva samples. Bacterial contamination is usually high in raw saliva samples, hindering good resolution of the two probesets for *APOE*. Saliva extraction devices are designed to stabilize and reduce the amount of bacterial contamination in raw saliva samples, which could improve genotyping performance of the *APOE* probesets. Thus, the performance of two *APOE* probesets was compared across collection devices.

Genotype calling was performed according to the Best Practices Workflow described in the Axiom Genotyping Solution Data Analysis user guide [4]. SNP cluster plots were generated for the two *APOE* probesets (Figure 7A for AX-95861335, Figure 7B for AX-59878593). Given that the samples used in this study were collected from donors, consensus imputed genotypes were used as reference truth for comparing concordance and cluster properties between different saliva collection devices. To obtain the imputed reference genotypes, genotype calls from all recommended probesets on chromosome 19 on the array (approximately 20,000 probesets), from each sample that passed QC, including multiple replicates of each donor, were uploaded to the Michigan Imputation Server [7–9] for imputation. Consensus was then taken for each marker across the imputed genotypes for all replicates of the same donor. For the two *APOE* markers, all replicates of the same donor have the same imputed genotypes; thus consensus was reached without setting thresholds. Genotyping concordance was computed against the imputed reference (Table 2).

Table 1. Axiom PMDA analysis of stabilized saliva collection devices for 24 matched samples on day 3 and day 14 post-collection.

Axiom PMDA analysis (by kit clustering)	SpeciMAX Stabilized device	OGR-600 device	SDNA-1000 device
Number of input samples	46	46	46
Samples with DQC ≥0.82	43	46	40
DQC—passed samples with QC CR ≥97.0%	41	46	38
DQC median	0.969	0.968	0.963
Average passing QC CR	99.28%	99.10%	99.17%
Average sample CR	99.77%	99.68%	99.79%
Markers passing filtering	96.95%	95.01%	95.62%

Table 2. Genotyping concordance for gDNA extracted from stabilized saliva collection devices for two *APOE* markers.

	Genotyping concordance		
Probeset ID	SpeciMAX Stabilized device	OGR-600 device	SDNA-1000 device
AX-95861335	100% (95% CI: 91–100%)	61% (95% CI: 45–76%)	82% (95% CI: 66–92%)
AX-59878593	48% (95% CI: 32–64%)	64% (95% CI: 48–78%)	63% (95% CI: 46–78%)

For the genotyping algorithm to make high-confidence calls, samples with the same genotype should resolve in a single and isolated cluster, not sharing an intensity profile with samples of a different genotype. In Figure 7A for probeset AX-95861335, samples collected with the SpeciMAX Stabilized device show clear separation in the contrast space between the two genotype clusters. Samples with the same genotypes share similar intensity values, which resolved with minimal variation in the genotype clusters. Samples that had the same genotypes and were stabilized with the OGR-600 and SDNA-1000 devices have more divergent intensity profiles. The two genotype clusters appear to have slightly overlapping contrast intensities, which resulted in a few samples receiving the wrong genotype call. For this *APOE* marker, the genotyping concordance for samples stabilized with the SpeciMAX Stabilized device is 100%. Samples stabilized with OGR-600 and SDNA-1000 devices have concordance of 61% and 82%, respectively.

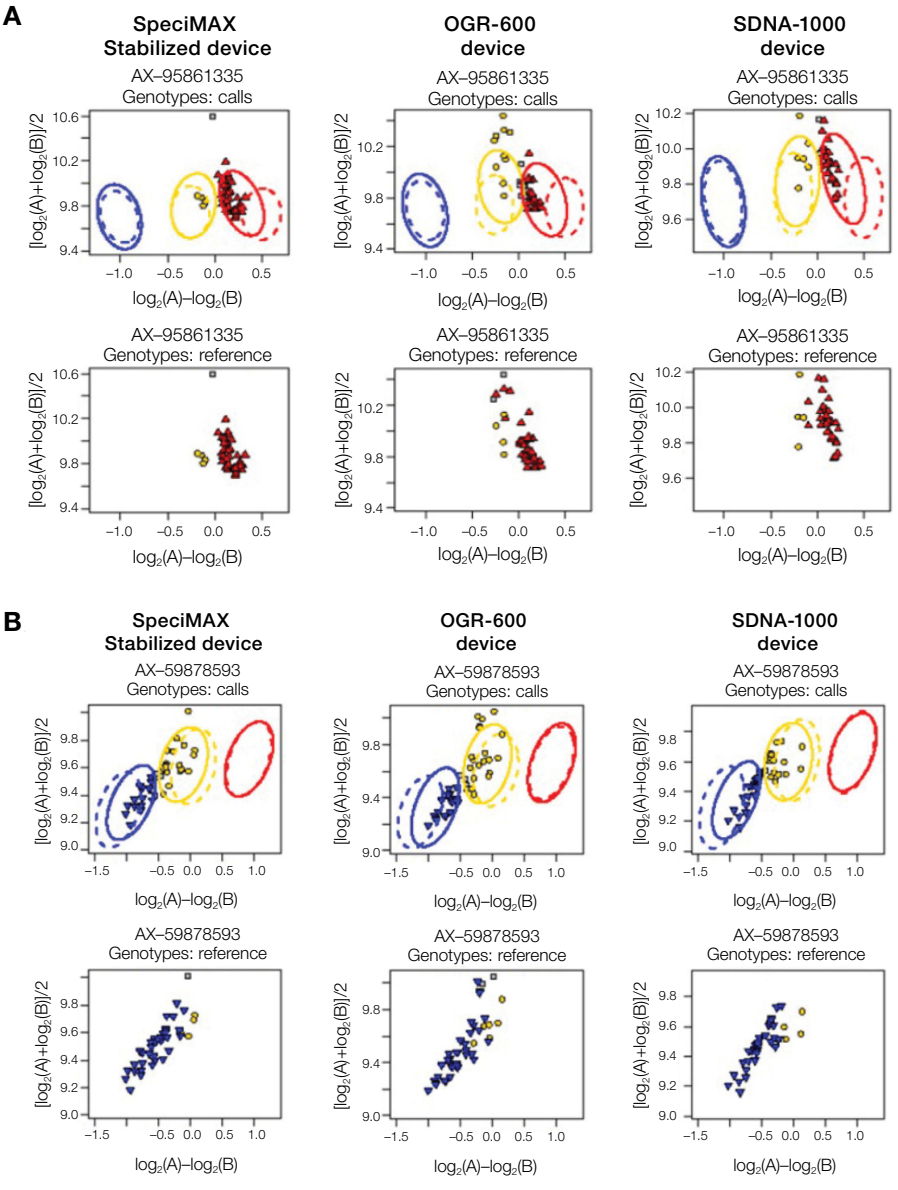


Figure 7. SNP cluster plots for SpeciMAX Stabilized, OGR-600, and SDNA-1000 devices. Genotyping calls are shown for **(A)** probeset ID AX-95861335 and **(B)** probeset ID AX-59878593.

In Figure 7B for probeset AX-59878593, samples stabilized with all three devices show similar intensity profile, with continuous contrast intensities ranging from –1.0 to 0.25 and no clear point of separation between the two genotypes. This means that none of the devices were able to provide good resolution and high-quality genotyping results for this *APOE* marker. The concordance is also comparable amongst the three devices with overlapping confidence intervals. Specifically, the SpeciMAX Stabilized device achieved concordance of 48%, the OGR-600 device achieved concordance of 64%, and the SDNA-1000 device achieved concordance of 63%.

These data show that raw saliva samples tend to have bacterial contamination and require saliva stabilization devices to produce high-quality genotyping results. Three saliva stabilization devices were evaluated to compare genotyping performance for important but difficult-to-genotype marker *APOE*. Out of the three devices evaluated, the SpeciMAX Stabilized device produced good resolution and higher concordance for one of the *APOE* markers as compared to the OGR-600 and SDNA-1000 devices. For the other *APOE* marker, all three devices showed comparable performance but all lack the resolution to yield quality genotyping results.

PGx genotyping performance

Genotyping performance across pharmacogenomics (PGx) markers was also evaluated. The PGx markers are high-value markers from the Clinical Pharmacogenomics Implementation Consortium (CPIC) guidelines, and markers in the PharmGKB™ database with clinical annotations. The marker list of the Axiom PMDA array contains 2,143 probesets for genetic variants of absorption, distribution, metabolism, and excretion (ADME)-related genes that are associated with pharmacokinetic properties of numerous drugs, such as *GSTM1*, *CYP2D6*, *CYP2B6*, and *SULT1A1*. These ADME markers can account for clinically relevant variances as related to drug-response phenotypes.

A summary of genotyping performance for the three analyzed stabilized saliva collection devices is shown in Table 3 (the SpeciMAX collection device with raw saliva was not evaluated for PGx genotyping performance). Several metrics such as average sample call rate and average overall concordance are nearly identical across the three collection devices. However, the SpeciMAX Stabilized device had fewer probeset outliers (called across less than 95% of the samples) than the other two devices (Figure 8).

The percentage of recommended probesets (also referred to as percent passing filtering), which is an indication of well-clustered intensities and whose genotypes are recommended for statistical tests in downstream analysis, is highest for the SpeciMAX Stabilized device.

Table 3. Genotyping performance for stabilized saliva collection devices.

Device	n	Average FLD*	Average sample call rate	Average overall concordance	Average heterozygous concordance	Percent passing filtering
SpeciMAX Stabilized device	2,143	10.18	99.76%	99.68%	98.93%	98.13%
OGR-600 device		9.58	99.65%	99.50%	98.70%	96.50%
SDNA-1000 device		10.18	99.72%	99.59%	98.83%	97.20%

* Fisher's linear discriminant (FLD) is a measurement of the genotype cluster resolution of a SNP. High-quality SNPs have well-separated, tightly clustered genotypes that will result in higher FLD values [4].

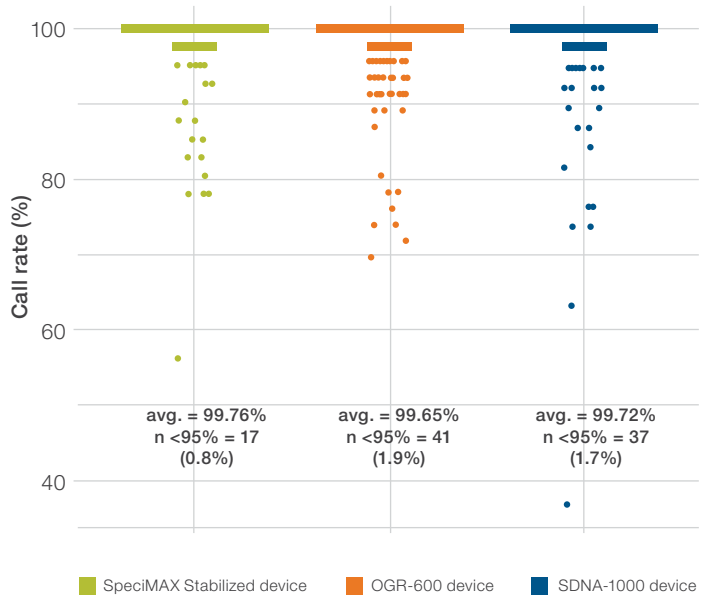


Figure 8. PGx marker call rates for stabilized saliva collection devices.

Conclusions

In evaluating matched saliva specimens collected in raw and stabilized saliva collection devices over a 2-week period, bacterial content was observed to increase over time. The total percentage of bacterial gDNA remained the most consistent from day 3 to day 14 post-collection with the SpeciMAX Stabilized Saliva Collection Kit. This was reflected in the microarray analysis using the Axiom PMDA kit. The results from this study indicate that stabilized saliva specimens processed with the MagMAX DNA Multi-Sample Ultra 2.0 Kit yield high-quality human gDNA suitable for microarray analysis.

From sample collection to downstream microarray, Thermo Fisher Scientific offers a full solution for genotyping analysis. Using the SpeciMAX Stabilized Saliva Collection Kit, samples can be stabilized and stored at ambient temperatures before gDNA is extracted using the MagMAX kit on the KingFisher Apex system. In summary, new workflows utilizing Axiom genotyping arrays that leverage noninvasive samples, such as saliva, can help drive the expansion of microarrays in precision medicine and PGx research, as well as biobank genotyping studies. Scalable lab automation further extends the platform capabilities to enable genotyping of hundreds of thousands of SNPs in a single assay. This scalability allows sample throughput consistent with the needs of both large-scale GWAS and targeted studies being conducted as part of the search for the underlying genetic basis of complex human diseases.

Authors

Yogi Bordiya, Monica Chadha, Lillie Manley, Michelle Staples, Esther Wu, Anu Mittal, and Michael Shapero, Thermo Fisher Scientific

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Ordering information

Product	Cat. No.
SpeciMAX Stabilized Saliva Collection Kit	A50697
MagMAX DNA Multi-Sample Ultra 2.0 Kit	A36570
KingFisher Apex Purification System*	5400930
Axiom Precision Medicine Diversity Array Kit, 96-format	951962
GeneTitan MC Instrument	00-0373

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