White Paper

Assessing library quality using high-throughput microfluidic electrophoresis

The QIAxcel® Advanced system for library quality control in the NGS workflow

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Introduction

Emerging high-throughput next-generation sequencing (NGS) workflows, which typically involve library preparation, sequencing and data analysis, showcase superior sequencing speed and accuracy, thanks to an advent of innovative technologies. Accurate quality control (QC) during library preparation is fundamental to optimizing data yields while reducing operational costs across sequencing platforms (1).

Library preparation involves normalization and pooling of DNA or RNA templates at desired concentrations. Depending on the sequencing platform, templates may be fragmented to an optimal size before adapter ligation for PCR amplification or sequencing. An essential component of the library quality assessment is determination of nucleic acid concentration and fragment size for molarity calculation. Assessing both individual and pooled library quality confirms successful library construction and concentration prior to sequencing and, if optimized, can result in the generation of millions to billions of high-quality reads from a single run. Therefore, laboratories need to promptly utilize QC instrumentation that is reliable, reproducible and cost-effective to maximize sequencing throughput.

Library quality can be evaluated using a range of techniques, each providing different advantages for specific NGS workflows. Traditionally, ultrathin slab-gels were coupled with UV-Vis spectrophotometry assays to quantify library fragment sizes for Sanger sequencing (2, 3). However, these techniques either overestimated or underestimated library quantities, leading to inconsistent sequencing results that were uneconomical. Current NGS workflows use a combination of qPCR, fluorometry and microfluidic electrophoresis systems to provide accurate and cost-effective library quantification (4–7).

Microfluidic electrophoresis platforms, unlike fluorometry or qPCR techniques, calculate nucleic acid concentrations and visually display library fragment sizes, presenting a standalone library QC solution to calculate sample molarity. Visualizing library traces, particularly for low-input samples, is an important quality assessment, as library fragment sizes can directly influence data quality and sequencing output (8). Hence, microfluidic electrophoresis systems have emerged as a cost-effective fragment separation method, providing higher sample resolution and sensitivity. When combined with other assays, such as fluorometry, these electrophoresis systems provide accurate library quantification, while minimizing manual handling and analysis time (7).

The QIAxcel Advanced system is a versatile microfluidic electrophoresis instrument, ideal for high-throughput NGS workflows. This fragment analyzer can analyze up to 96 samples without any pre-sample preparation. A range of 12-channel cartridge kits facilitate quality control of RNA, gDNA, and the wide variety of sequencing technologies. Each capillary runs independently, using an inner alignment marker to unify samples, and results are displayed in real time.

Materials and methods

Library preparation

Total genomic DNA was extracted from fecal material using the DNeasy® 96 PowerSoil® Pro QIAcube® HT Kit (QIAGEN; cat. no. 47021) on the QIAcube HT automation system (QIAGEN; cat. no. 9001896.) The extraction was conducted according to manufacturer's recommendations with a modification to the initial homogenization, to include a bead-based lysis in a 2 ml deep-well plate containing 0.1 mm zirconia beads. The Quant-iT™ dsDNA Assay Kits (Thermo Fisher Scientific®; cat. no. Q33120) was used to quantify resulting DNA.

Sequencing libraries were prepared using the DNA Prep (M) Tagmentation (Illumina®; cat. no. 2001870) and Nextera™ XT DNA Library Preparation kits (Illumina; cat. no. FC-131-1096) with IDT for Illumina DNA/RNA UD Index sets A-D (Illumina; cat. no. 2002721, 20027214, 20042666, 20042667). Standard manufacturer's processes were followed at a fifth of the reaction volume to accommodate the high sample throughput. ATAC-seq libraries were prepared following the methods described in Buenrostro, J. et al. (9).

Microfluidic electrophoresis

The average library size and quality were assessed using three microfluidic electrophoresis systems. The QIAxcel Advanced instrument was run using the QIAxcel DNA High Resolution Kit (QIAGEN; cat. no. 929002) with a 10 µl library sample using the method OM500-AM10s, 20 seconds injection time, QX DNA Size Marker 50–800 bp v2.0 (QIAGEN; cat. no. 929561) and QX Alignment Marker 15 bp/5 kb (QIAGEN; cat. no. 929524).

Libraries were also run on two alternative microfluidic electrophoresis systems (Supplier T and Supplier BA) through external vendors following standard manufacturer's processes.

Results

Reliable and reproducible quantification of library fragments

Library fragment sizes may vary depending on the specimen type, sample quality, concentration and the library preparation method. To determine sample reproducibility and system sensitivity, a library prepared using DNA Prep, (M) Tagmentation (Illumina) was run in technical replicates (n=6) on all three microfluidic electrophoresis systems and the resulting electrophoretic profiles were compared.

The average library fragment sizes varied between the systems. Fragment sizes averaged 460 bp for the QIAxcel Advanced, 450 bp for Supplier T and 400 bp for Supplier BA (Figure 1 A). The library traces for each replicate were noticeably more defined in the QIAxcel Advanced compared to Suppliers T and BA (Figures 1 B–D). QIAxcel Advanced generated the lowest coefficient of variation (CV) between the average fragment sizes at 0.56% as compared to the competitor systems at 0.83% (Supplier T) and 0.81% (Supplier BA), suggesting it is a highly reproducible system (Table 1).

Highly precise and sensitive library quantification even at low concentrations

Library QC methods must be reliable across a range of input amounts. To compare system capabilities, varying concentrations of a serially diluted Illumina DNA Prep (M) Tagmentation library were run as technical replicates (n=3) on all three systems.

Library fragments were successfully detected throughout the dilution series on all three systems (Figures 2 A–C). Notably, the electrophoretic profiles from the QIAxcel Advanced system displayed the highest precision at low concentrations compared to Suppliers T and BA. In fact, the QIAxcel Advanced system displayed the lowest intra-replicate variability across all five library concentrations, with a CV ranging from 0% to 0.32% (Table 2).

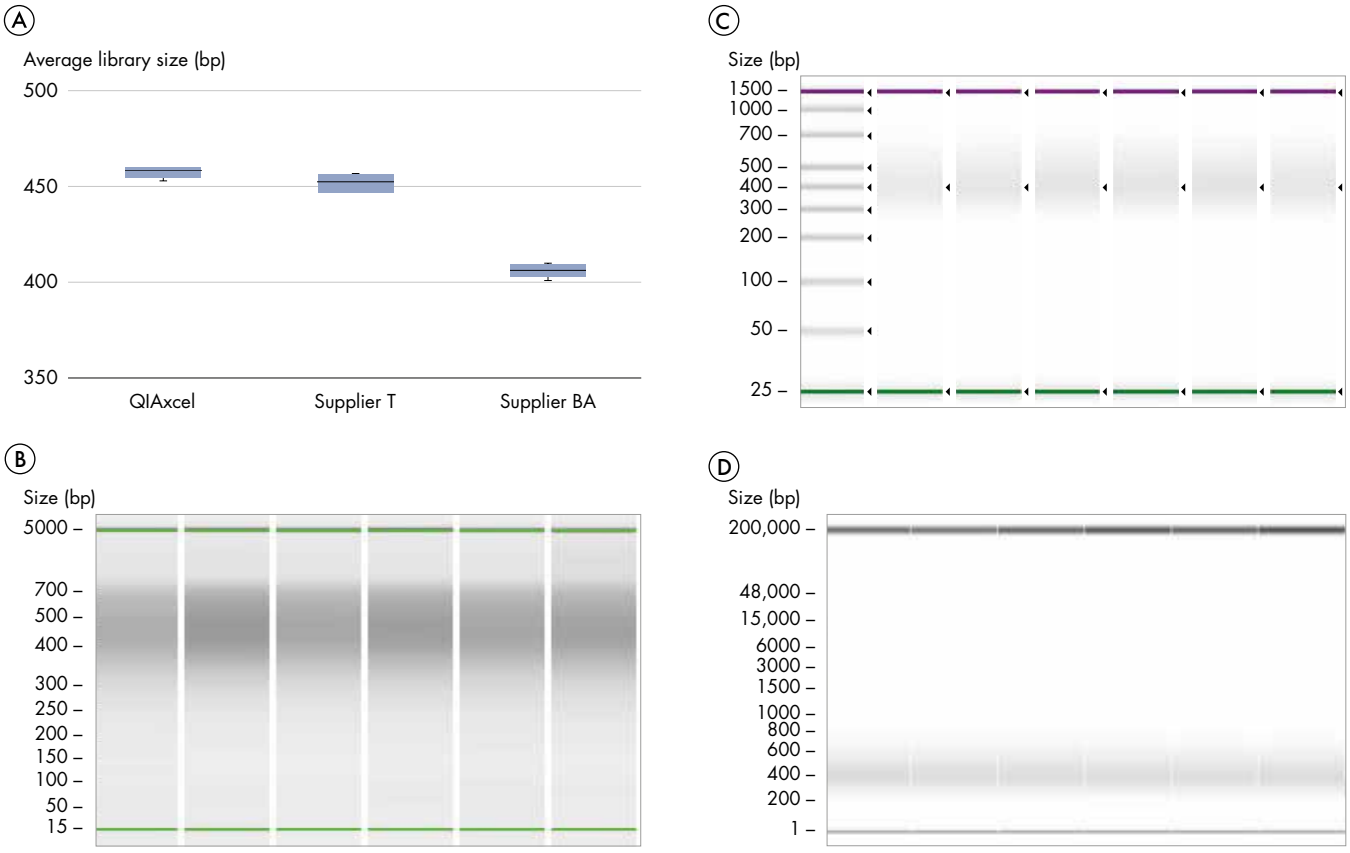


Figure 1. Comparison of reproducibility between the microfluidic electrophoresis platforms. Average fragment size (bp) was determined using a single Illumina DNA Prep (M) Tagmentation library, performed in technical replicates (n=6). **A:** Box and whisker plot showing the comparison of average fragment sizes (bp) between the QIAxcel Advanced, Supplier T and Supplier BA. **B:** QIAxcel gel image. **C:** Supplier T gel image. **D:** Supplier BA gel image.

Table 1. A comparison of the coefficient of variation (CV) between microfluidic electrophoresis platforms.

	QIAxcel DNA High Resolution Kit	Supplier T	Supplier BA
Replicate 1	453	455	410
Replicate 2	459	456	408
Replicate 3	460	454	408
Replicate 4	457	451	404
Replicate 5	456	447	404
Replicate 6	459	448	401
CV (%)	0.56	0.83	0.81

Table 2. Variability comparison – average fragment size (bp), standard deviation and coefficient of variance (CV) – between the microfluidic electrophoresis platforms.

		QIAxcel DNA High Resolution Kit	Supplier T	Supplier BA
Serial dilution 1	Average (bp)	325	329	281
	Standard deviation	0	4.24	3.46
	CV (%)	0	1.29	1.23
Serial dilution 2	Average (bp)	325	346	278
	Standard deviation	0.58	3.21	4.16
	CV (%)	0.18	0.93	1.5
Serial dilution 3	Average (bp)	323	335	278
	Standard deviation	1	5.57	2.65
	CV (%)	0.31	1.66	0.95
Serial dilution 4	Average (bp)	322	345	284
	Standard deviation	0.58	13.58	7.51
	CV (%)	0.18	3.94	2.65
Serial dilution 5	Average (bp)	314	345	278
	Standard deviation	1	4.04	4.36
	CV (%)	0.32	1.17	1.57

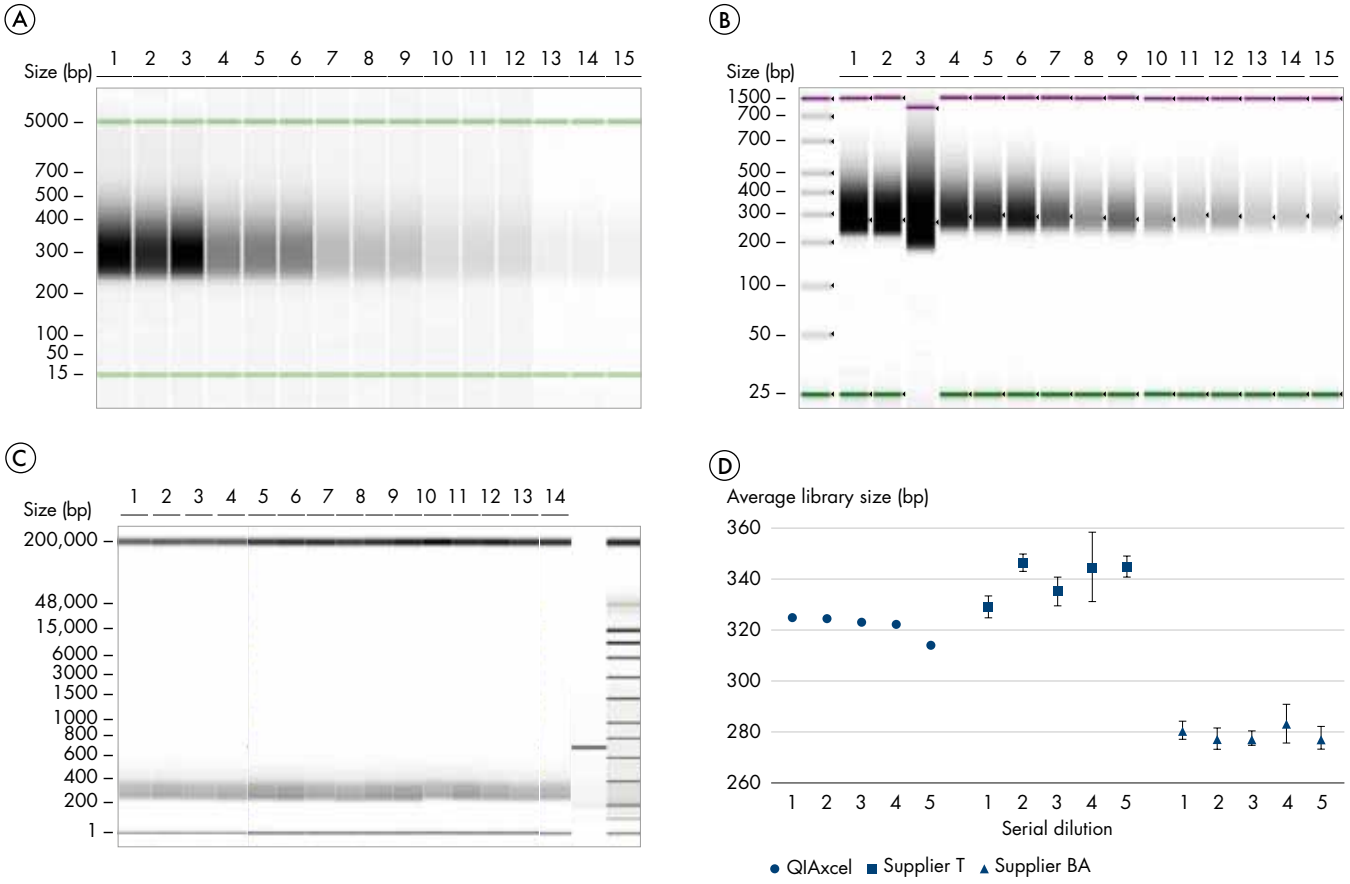


Figure 2. Comparison of sensitivity between the microfluidic electrophoresis platforms using library serial dilution. Average library fragment size (bp) was determined for 5 serial dilutions of an Illumina DNA Prep (M) Tagmentation library with each dilution performed in technical replicates (n=3). Final library concentrations were 160 nM (lanes labeled 1–3), 80 nM (lanes labeled 4–6) and 40 nM (lanes labeled 7–9). **A:** QIAxcel DNA High Resolution gel image. **B:** Supplier T gel image. **C:** Supplier BA gel image. **D:** Box and whisker plot comparing the average fragment sizes (bp) between the QIAxcel Advanced system (●) and Suppliers T (■) and BA (▲).

High fidelity recognition of library fragments

To enable accurate pooling and optimal sequencing outcomes, it is important to determine specific library fragment fractions. Libraries prepared using Illumina DNA Prep, (M) Tagmentation, Illumina Nextera XT DNA Library Preparation Kit and ATAC-seq were run in technical replicates (n=3) on all three systems to assess precision over a broad range of fragment fractions.

Electrophoretic profiles from the QIAxcel Advanced system demonstrate the ability to accurately distinguish distinct fragment sizes for different library preparations between replicates in comparison to Suppliers T and BA (Figures 3 A, C, E). This was particularly evident in the ATAC-seq library trace comparison where the QIAxcel Advanced system was able to detect and clearly display varying fragment sizes ranging from 100 bp to 1100 bp in a single sample (Figures 3 B, D, F). This high level of precision and reproducibility are essential in high throughput workflows and will ultimately improve the efficacy of the sequencing run.

Versatile fragment detection accommodating a range of sizes

Abnormal sample fragmentation during library preparation can lead to undertagmentation or overtagmentation, potentially impacting sequencing results. The QIAxcel Advanced system was assessed using an undertagmented library with longer fragments (using Illumina Nextera XT library) and an overtagmented library with smaller

fragments (using Illumina DNA Prep, (M) Tagmentation Library). Library fragments from both the overtagmented and undertagmented samples were clearly distinguishable in the electrophoretic profiles from the QIAxcel Advanced system (Figure 4 B-C).

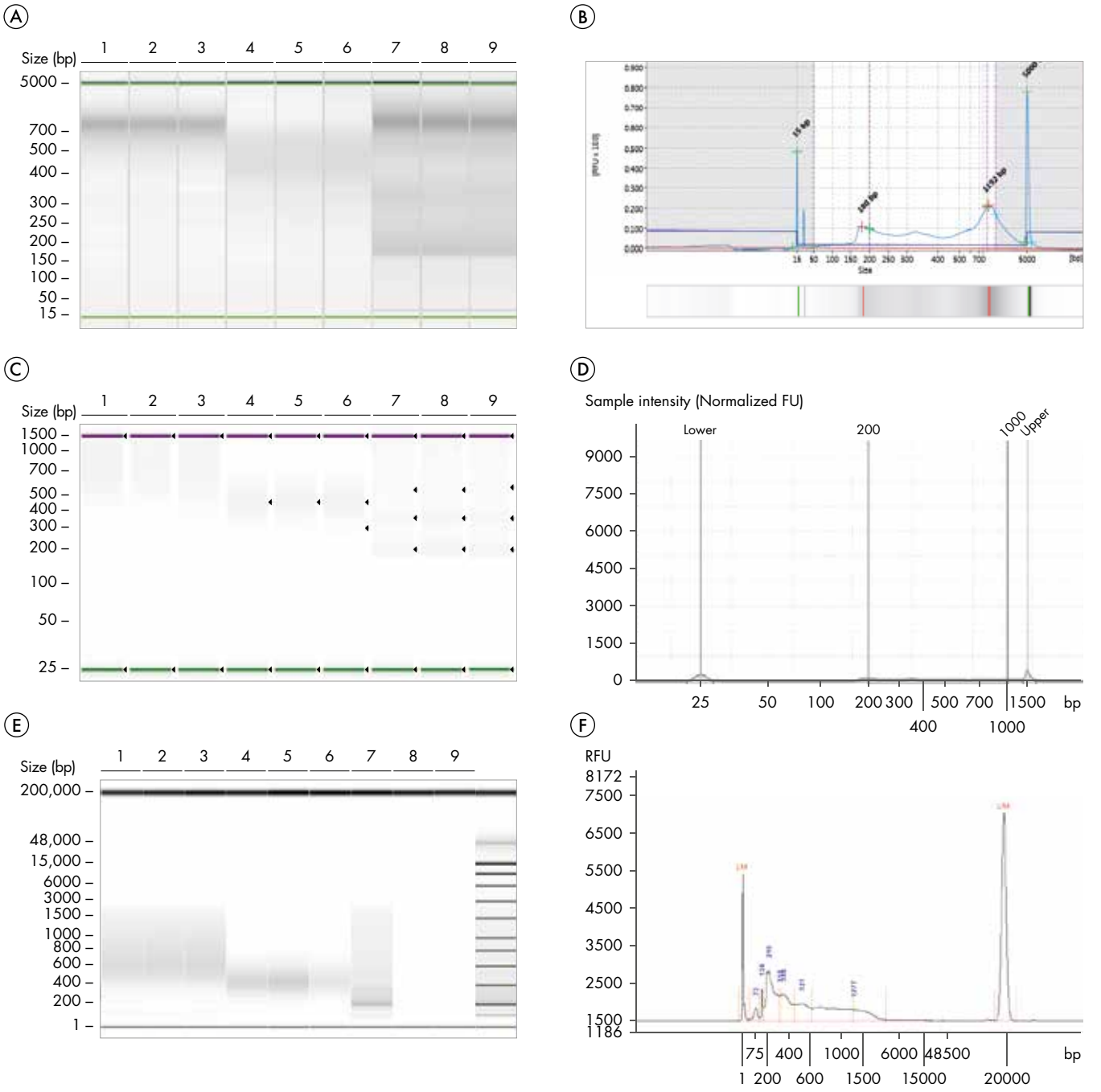


Figure 3. Comparison of resolution between QIAxcel and two other microfluidic electrophoresis platforms. Libraries prepared using Illumina Nextera XT (lanes labeled 1–3), Illumina DNA prep (M) Tagmentation (lanes labeled 4–6), and ATAC-seq (lanes labeled 7–9) were run in technical replicates (n=3) using the three microfluidic electrophoresis platforms. **A:** QIAxcel gel image of all three library types. **B:** QIAxcel trace image of the ATAC-seq library. **C:** Supplier T gel image of all three library types. **D:** Supplier T trace image of the ATAC-seq library. **E:** Supplier BA gel image of all three library types. **F:** Supplier BA trace image of the ATAC-seq library.

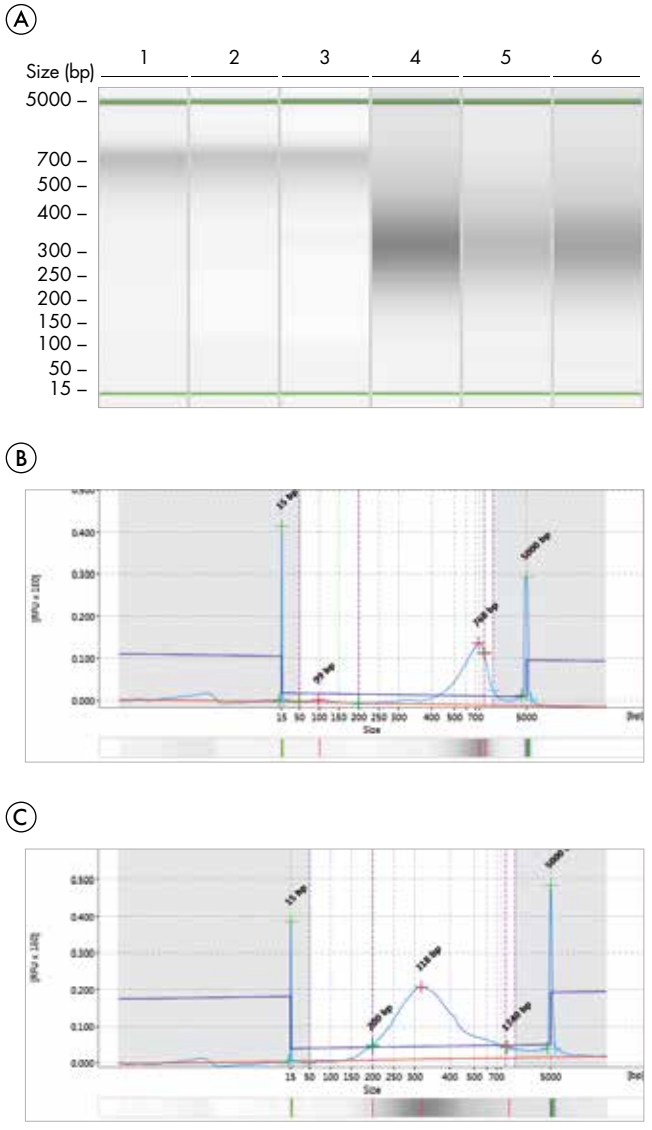


Figure 4. Versatile detection of undertagmented and overtagmented library samples on the QIAxcel Advanced system. Abnormal library preparations were run on the QIAxcel Advanced system. **A:** QIAxcel gel image showing an undertagmented sample library prepared with Illumina Nextera XT (lanes labeled 1–3) and overtagmented sample library prepared with Illumina DNA prep (M) Tagmentation (lanes labelled 4–6). **B:** QIAxcel trace image of the undertagmented Illumina Nextera XT library. **C:** QIAxcel trace image of the overtagmented Illumina DNA prep (M) Tagmentation library.

Discussion

Microfluidic electrophoresis assays show increased sample resolution and sensitivity compared to alternative fragment analysis systems. Achieving high sensitivity during fragment analysis in NGS workflows is critical, as it reflects library quality and enables accurate quantification and visualization of both high and low concentration libraries prior to sequencing. The QIAxcel Advanced system achieves highly precise and sensitive library quantification even at low concentrations.

Compared to alternative microfluidic electrophoretic systems, the high throughput nature of the QIAxcel Advanced system is advantageous in NGS workflows. The system processes up to 96 libraries and mitigates manual intervention errors by employing ready-to-use gel cartridges and preprogrammed run methods.

As little as 1 µl sample can be used, or 10 µl of the purified sample can be loaded directly into the system in plates, which removes tedious sample and reagent preparation, as well as reduces setup and instrument loading times (Table 3). It also removes a source of error because small volume pipetting is not necessary. Furthermore, the system requires little to no maintenance between runs, except proper storage of the cartridge to prevent tips from drying out. Also, new QIAxcel Kits require a one-time intensity calibration to normalize readings between capillaries.

The QIAxcel ScreenGel® software, used for post-run data analysis, provides a clear analysis of library electropherogram traces that can be tailored to specified pass/fail metrics based on individual peak performance. For example, Figure 5 illustrates a sample that has been flagged for review based on a higher-than-expected small-fragment tail. These QC results can be exported as individualized reports and imported into laboratory information management systems (LIMS), typically used in NGS workflows, to track and manage data records during sample processing.

Table 3. The QIAxcel Advanced system for NGS library assessment – a summary

Instrument properties	Short setup time	• Irregular use: 20 minute room temperature equilibration of cartridge • Regular use: Cartridge stored in QIAxcel system
	Minimal machine and run failures	• Partially dry capillaries could result in DNA peak migration errors • System maintenance purge feature immediately reintroduces moisture to capillaries
	Flexible size range	• Dependent on the QIAxcel Kit; ranges between 20 bp and 20 kb
	Minimal training requirements	• One-day application training provided for new users for immediate operation
	Long-term reagent stability	• Nine months from production
	LIMS integration ready	• Report/export options for different systems
	High cost-effectiveness	• Competitive price per sample
	High sample throughput	• Minimum: 12 samples • Maximum: 96 samples
Sample requirements	Minimal sample volume per preparation	• Around 10 µl to guarantee sample injection • Total sample of 0.1 ng consumed per run • No sample pre-preparation required
	Rapid sample run and analysis	• Duration is 11 minutes per row (12 samples total)

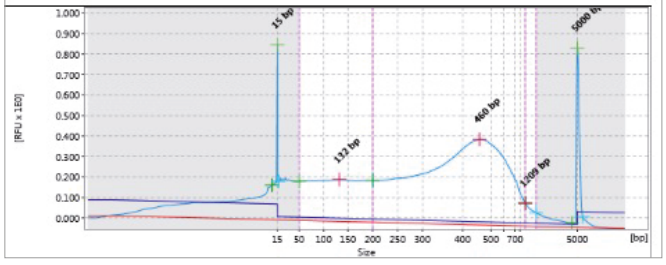
Distribution Analysis Result Table: LP_Microba_v1_150518

C200820008_2021-03-01_10-41-06|R1|E1

Pos	Sample Information	Total Concentration [ng/µl]	Total Molarity [nmol/l]	Sample Quality
A1		3.40	25.42	Passed
A2		11.63	67.75	Passed
A3		14.41	131.43	Review
A4		10.46	38.77	Passed
A5		11.07	134.82	Review
A6		9.43	67.72	Passed
A8		6.14	28.89	Passed

Sample Header

Position:	A5	Run Date:	3/1/2021 10:41:06 AM
Sample Info:			
Sample Comment			
Plate ID:	C200820008_2021-03-01_10-41-06, R: 1, E: 1		



C200820008_2021-03-01_10-41-06|R1|E1

				Small (Aol1)				Library (Aol2)				
Pos	Total Concentration [ng/µl]	Total Molarity [nmol/l]	Sample Quality	Aol Concentration [ng/µl]	Aol Molarity [nmol/l]	Aol Height [S/N]	Aol Height Check	Aol Concentration [ng/µl]	Aol Molarity [nmol/l]	Aol Height [S/N]	Aol Height Check	
A5	11.07	134.82	Review	3.33	48.39	663.84	Not Analyzed	6.31	26.95	1355.76	Passed	

C200820008_2021-03-01_10-41-06|R1|E1

	Large (Aol3)				LP-Small Total		LP-Large Total		
Pos	Aol Concentration [ng/µl]	Aol Molarity [nmol/l]	Aol Height [S/N]	Aol Height Check	Ratio (Mol.)	Ratio Quality	Ratio (Mol.)	Ratio Quality	
A5	0.10	0.10	361.90	Not Analyzed	0.36	Review	0.00	Passed	

Figure 5. Illustration of samples flagged for review from preset profile characteristics including gating, minimum and maximum size, and ratio within these gated parameters.

Summary

The QIAxcel Advanced system has proven valuable for library QC in high-throughput NGS workflows. In comparison to other commercially available platforms, the QIAxcel Advanced system provides higher sensitivity, resolution and precision across multiple library types and concentrations. Its analysis is versatile, enabling the recognition of varying library fragments, whether they have single or multiple peaks, broad smears or a combination of all. It is beneficial not only for the titration of loading concentration for maximum data yield but also for the identification of undesired library fragments. The fast processing, minimal setup and low per-sample cost allow evaluation of each library prior to pooling for rapid sequencing and optimal result generation.

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