Element Biosciences

Technical Note

Accurate Quantification of Circular Libraries for Sequencing on the Element AVITI™ System

Quantification and quality control methods for libraries prepared with the Adept™ Standard or Adept Rapid PCR-Free Protocol

Introduction

The Element AVITI System employs rolling circle amplification (RCA) to amplify each library into a polony that contains hundreds of copies of the original library. This strategy avoids magnifying amplification errors and drives high-quality sequencing. RCA requires a circular library. Depending on the library prep protocol, the library is circularized manually on-bench or automatically onboard the AVITI System. Accurate quantification of a library circularized on-bench with Element Adept Library Compatibility Workflow is key to a successful run.

Quantification results help balance sample representation in a pool and inform the target loading concentration to optimize polony density on the flow cell to minimize the variation that can cause underloading or overloading. The right polony density improves data quality, output, and run performance. This technical note assesses quantification methods for circular libraries and summarizes quality control (QC) options (Table 1).

Circular library workflow

Two of the protocols in the Adept Workflow generate circular libraries: Adept Standard and Adept Rapid PCR-Free. Both protocols require preparing a linear library with a third-party kit and circularizing the library with the Element Adept Library Compatibility Kit v1.1 (catalog # 930-00007) (Figure 1).

Following third-party recommendations for measuring the quantity and average length of the input linear library lays the groundwork for successful circularization with the Adept Workflow. Quantification provides the data necessary to dilute the final library to the target loading concentration for sequencing. Element strongly recommends a spike-in of PhiX Control Library, Adept (Element Biosciences catalog # 830-00004) or other positive control, such as a library previously quantified and sequenced on the AVITI System, to enable error rate reporting.

Method	Detection	Limit of Detection	Time to Process 20 Libraries
Quantitative PCR (qPCR)	Circular library	pM	> 1 hour
Fluorometer (Qubit and PicoGreen)	Circular library and linear ssDNA	> 0.2 ng total DNA[a]	~15 minutes
Microfluidics-based automated electrophoresis (Bioanalyzer, Fragment Analyzer, and TapeStation)	Circular library and linear ssDNA	50 pg[b]	> 30 minutes

[a] Based on the Qubit ssDNA Assay Kit (Thermo Fisher Scientific catalog # Q10212)
[b] Based on the Agilent RNA 6000 Pico Kit (Agilent part # 5067-1513)

Table 1. Summary of quantification and QC methods for circular libraries

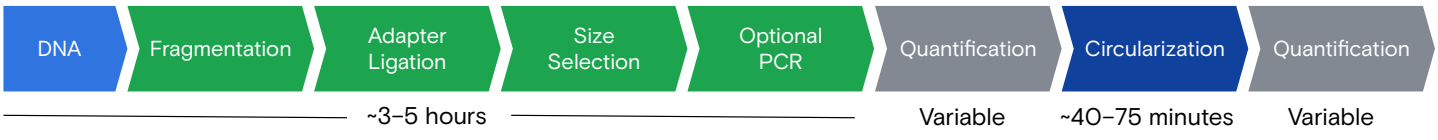

Figure 1. Preparing a circular library for the AVITI System starts with DNA input. Linear library prep fragments the DNA, adds adapters, and performs size selection and optional amplification. Quantification and QC validates the linear library that is then circularized and rechecked.

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Quantification options

qPCR quantification

The Adept Standard and Adept Rapid PCR-Free protocols include a qPCR procedure that detects only circularized libraries, which are required for capture on the flow cell (Figure 2). The procedure selectively amplifies the ligated junction by applying primers that anneal to the outer library primers facing the ligation junctions. Linear libraries, incomplete libraries, and single-stranded oligos are not quantified. This design amplifies the ligated junction fragments, so each amplified qPCR product is the same size regardless of linear library length. Adjusting the quantification result based on linear library sizes is unnecessary.

The Element Adept Library Compatibility Kit v1.1 includes a qPCR standard and primer that is specific to both the kit and kit version. A mismatch of primers and standards or the use of components from third-party qPCR assays leads to inaccurate quantification. Additionally, the Adept qPCR procedure recommends SYBR Green PCR Master Mix (Thermo Fisher Scientific catalog # 4364346) for convenience and performance. With testing that demonstrates comparable quantification results, an alternative qPCR master mix can replace SYBR Green PCR Master Mix.

Fluorometric quantification

Qubit, PicoGreen, and other fluorometric quantification methods employ fluorescence-based dyes that selectively bind to DNA or RNA. These methods are often faster than qPCR but measure all DNA, which risks overestimating the concentration of a circular library. Secondary structures, incomplete library fragments, linear library that failed to circularize due to ligation failure, residual primer dimers from PCR, partially digested splint oligos, and partially digested linear library and other partially constructed fragments can all influence the measured DNA (Figure 2B, Figure 3).

The Qubit ssDNA Assay Kit referenced in Table 1 quantifies single-stranded DNA (ssDNA) or oligonucleotides, but is not specific to the circular, single-stranded libraries that the Adept Workflow generates. To prevent secondary structures from overestimating concentrations, denature the library before Qubit quantification and include a positive control—such as a previously quantified and sequenced library—in the assay. *Heat-Denature a Circular Library* on page 3 provides thermal cycler-based instructions to denature a library for Qubit quantification. NaOH is an alternative denaturation method for libraries with high GC content or sites without an available thermal cycler.

Compatibility considerations

Element maintains a list of third-party preps compatible with the standard and rapid PCR-free protocols at go.elembio.link/compatible. When using an unlisted prep for the first time, follow the Adept qPCR procedure to confirm successful circularization and compatibility. Incompatible libraries typically quantify close to background measures of yield. To establish the correlation between qPCR and fluorometry, quantify the same library type with both methods before committing exclusively to fluorometry.

The exception to Qubit quantification as a qPCR alternative is reduced linear library input. The limit of detection for the Qubit ssDNA Assay Kit is > 0.2 ng. For libraries circularized from input < 0.5 pmol, qPCR offers superior sensitivity and accuracy.

Comparison of quantification methods

To compare quantification methods, ~560 bp circular libraries were prepared from triplicates of five different input amounts: 0.05, 0.1, 0.25, 0.5, and 0.75 pmol. Both the Qubit ssDNA Assay Kit and a qPCR reaction attempted to quantify each circular library. At very low input of 0.05 pmol—the supported input range for the Adept Workflow is 0.2–0.5 pmol—two of the three samples did not meet the Qubit limit of detection and did not report a reading.

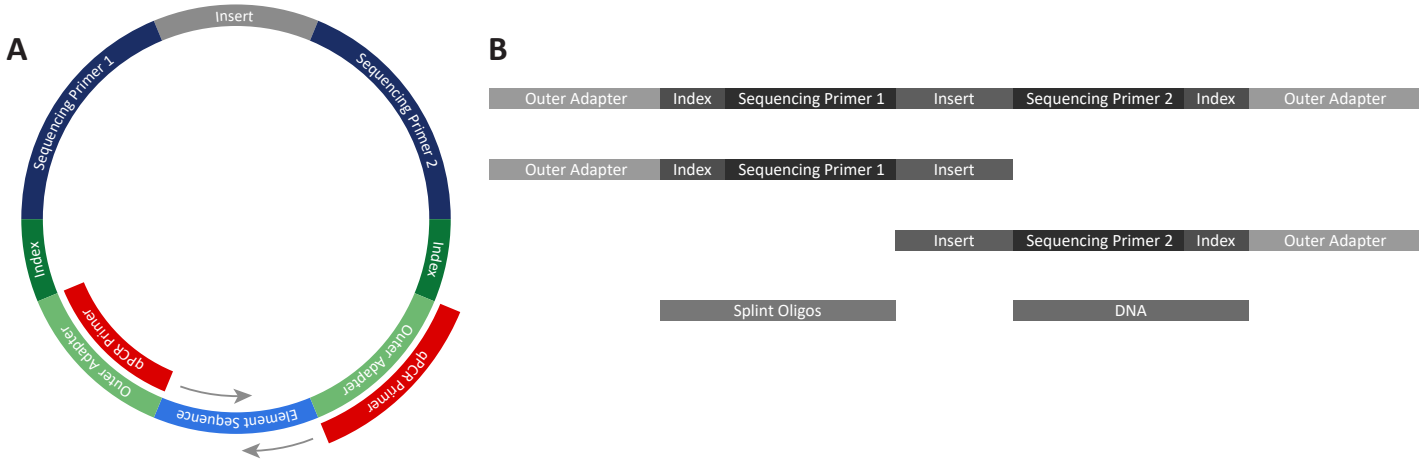

Figure 2. The Adept qPCR procedure targets the area over the junction of the circular library to quantify only the circular ligated product (A) and omit other material (B) from the reading. Overall, qPCR offers greater sensitivity, quantifying libraries that are below the Qubit limit of detection.

The remaining results show similar readings among libraries of the same size with the same input concentration, indicating that Qubit can be a reasonable alternative to qPCR for circular libraries prepared from ≥ 0.1 pmol linear library. Before switching to any fluorometric quantification method, quantify libraries with qPCR to ensure accurate results for the library type and conditions. The relationship between the two methods is variable so different library sizes can impact Qubit results. A 560 bp library correlates well with qPCR, but the trend might not hold for other sizes. Figure 4 presents results for 560 bp libraries.

Heat-denature a circular library

Denaturing a circular library for Qubit quantification minimizes the secondary structures that skew results. The following instructions heat-denature the circular library to prepare for quantification with the Qubit ssDNA Assay. Exact volumes are crucial for staying above the limit of detection.

1. For each library being quantified, add 16 µl nuclease-free water to a new PCR tube or PCR plate well.
2. Transfer 4 µl library to the tube or well to prepare 20 µl of 1:5 diluted library.
3. Cap the tube or seal the plate with a PCR-grade seal.
4. Place the diluted libraries into a thermal cycler.
5. Run the following program with the lid set to 100–110°C:
   - 5 minutes at 80°C
   - 2 minutes at 4°C
   - Hold at 4°C
6. Remove the libraries from the thermal cycler.
7. Briefly vortex and centrifuge the tubes or plate to collect condensation.
8. Place the tubes or plate on the benchtop and allow the libraries to reach room temperature.
9. Follow supplier instructions for the Qubit ssDNA Assay Kit to quantify the room-temperature libraries. Use all 20 µl of the denatured library.

QC options

The Bioanalyzer, Fragment Analyzer, TapeStation, and other microfluidics-based automated electrophoresis systems can visualize a circular library for QC purposes. Circular libraries are single-stranded, so RNA chips provide the most accurate results. Denaturing the library before QC prevents secondary structures from affecting library migration: heat the library at 80°C for 5 minutes, cool to 4°C, and load the library onto the chip (Figure 5).

If digestion is incomplete after ligation, the Bioanalyzer and Fragment Analyzer can detect peaks < 150 bp (Figure 6). A TapeStation system does not always detect incomplete digestion because the lower marker overlaps with the region. In general,

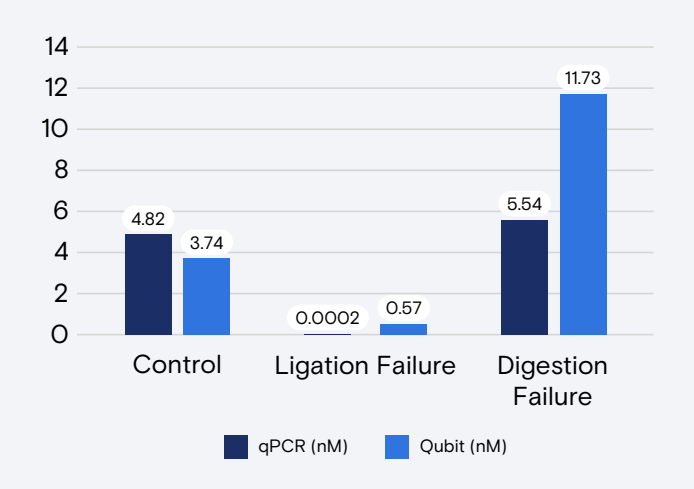

Figure 3. Quantification results for libraries that experienced ligation or digestion failure show inconsistent readings between qPCR and Qubit methods. Each tested library was 550 bp long and prepared from 0.5 pmol input.

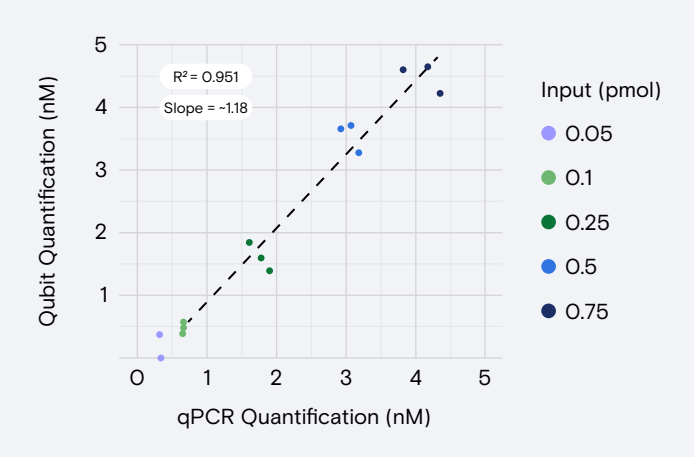

Figure 4. Circular libraries, each 560 bp long, prepared from different input amounts and quantified with qPCR and Qubit show similar results among input amounts. Qubit cannot detect libraries prepared from input < 0.1 pmol.

Element does not recommend any of these systems for quantification of circular libraries or size estimation.

Summary

Accurate quantification of circular libraries is essential to achieving the optimal polony density for a successful run. Checking the linear library ensures proper input for circularization and checking the circular library ensures appropriate input for sequencing. Although qPCR offers the benefit of detection for thorough, robust quantification of circular libraries, Qubit is also effective with proper testing and modifications. Multiple electrophoresis-based systems enable visualization, with the Bioanalyzer and Fragment Analyzer providing greater sensitivity than TapeStation.

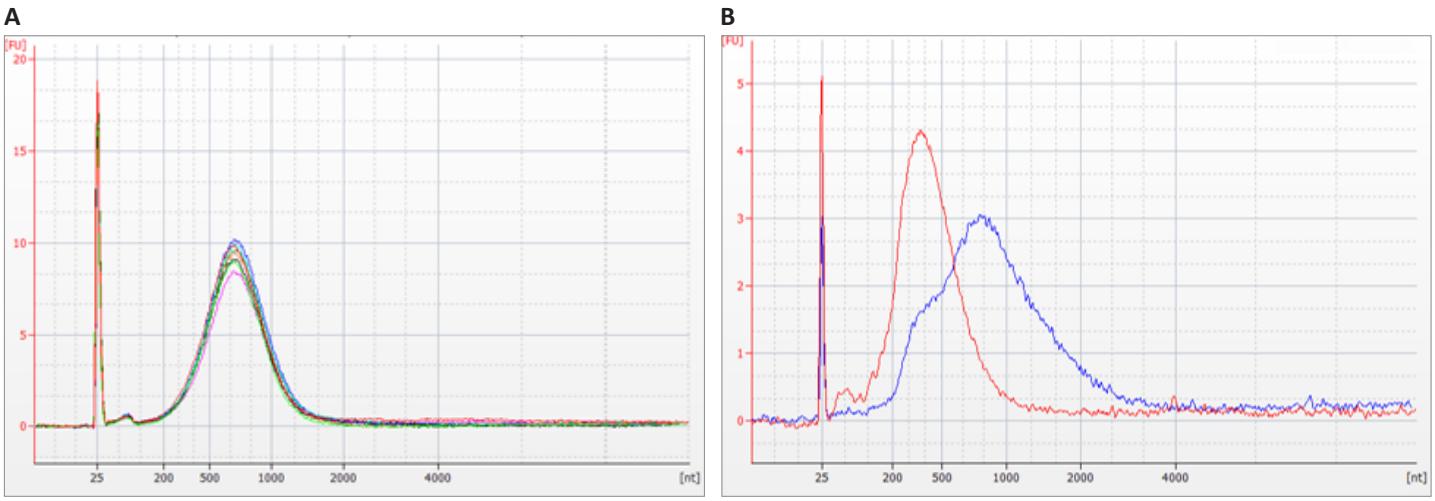

Figure 5. Example Bioanalyzer traces showing typical results for circular libraries (A) and a comparison between a heat-denatured library depicted in red and an untreated library depicted in blue (B). Peaks for the untreated library deviate from all other peaks, demonstrating the importance of denaturing the library for accurate QC.

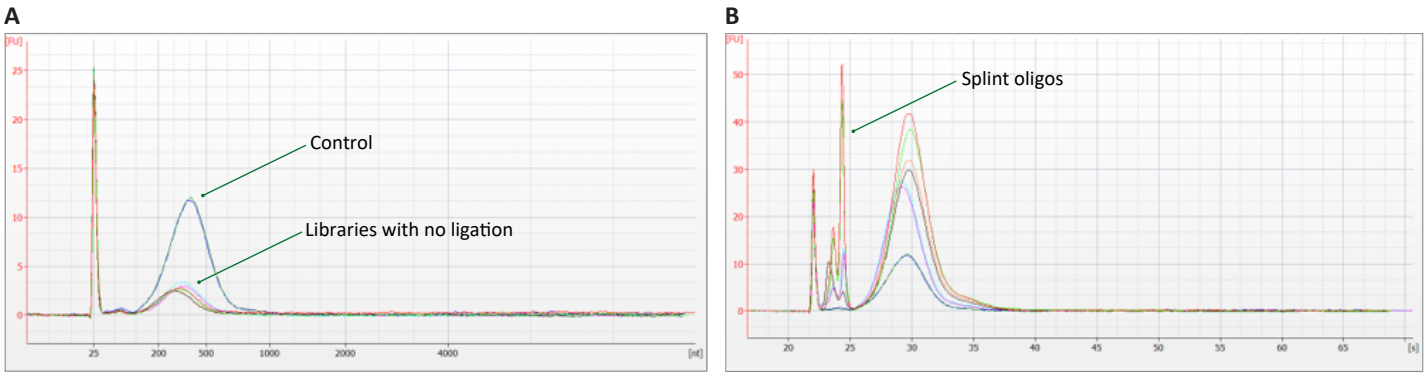

Figure 6. Example Bioanalyzer traces showing ~560 bp libraries with ligation failure (A) and incomplete or failed digestion (B). Smaller libraries can have peaks similar to splint oligos and cannot use this method to evaluate quality.

References

1. Element Biosciences, *Element Adept Library Compatibility Workflow User Guide for the Standard Protocol*, October 2023, doc. no. MA-00001.
2. Element Biosciences, *Element Adept Library Compatibility Workflow User Guide for the Rapid PCR-Free Protocol*, October 2023, doc. no. MA-00033.

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