Element Biosciences

Technical Note

Sequencing Short Insert Libraries

with Cloudbreak Freestyle™

Highlights

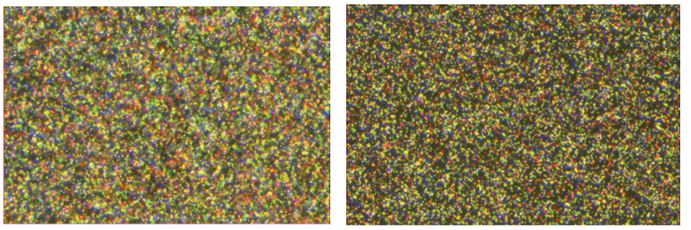
- Insert size can impact sequencing metrics and analysis
- Using the short insert recipe improves quality and density for short insert libraries

Introduction

Cloudbreak Freestyle streamlines library conversion and enables linear library loading and circularization on the AVITI™ System without hands-on library conversion steps, while providing the high performance of avidite base chemistry (ABC).

Cloudbreak Freestyle is compatible with a wide range of next-generation sequencing (NGS) applications from whole genome sequencing to single-cell studies. However, one consideration that is more critical with Cloudbreak Freestyle than Adept Cloudbreak chemistry with various applications is library insert size. Some NGS applications, such as miRNA sequencing and some proteomics applications, target small molecules in the preparation method, pushing insert sizes to less than 100 base pairs (bp) long. Other preparation methods, such as RNA-seq and whole exome sequencing, tend to target sizes in a more medium range (from ~100–350 bp) but can also be impacted by sample input type (e.g., FFPE samples tend to result in smaller libraries due to degradation). Regardless of the preparation, insert size must be considered when selecting read length and other aspects of sequencing, as it can have an effect on sequencing metrics and downstream analysis.

Element provides a short insert recipe specifically for sequencing short insert libraries. Using this recipe produces equivalent or better performance for libraries with insert sizes less than 300 bp as compared to the standard recipe with only a small increase in run time (~2 hours). This document demonstrates the performance of sequencing short insert libraries of various insert sizes with Cloudbreak Freestyle and the short insert recipe.



(A) Standard recipe (B) Short insert recipe

Figure 1. Polony morphology of miRNA libraries sequenced with **(A)** standard recipe and **(B)** short insert recipe.

Sequencing quality and density

Some NGS library applications, such as miRNA sequencing, use very short library insert sizes. Short insert libraries sequenced with the standard recipe can produce less uniform polony sizing and brightness, negatively impacting density and quality (Figure 1A). The short insert recipe recovers density and improves polony morphology and quality for these libraries (Figure 1B). Recommendations for using this recipe are based on testing a variety of library types and insert sizes. The following sections describe the benefits of the short insert recipe for libraries with insert sizes less than 100 bp, equivalent performance for libraries with insert sizes 100–300 bp, and slightly reduced performance for libraries with insert sizes greater than 300 bp.

Library insert Size	Recommendation
< 100 bp	Short insert recipe required
100–300 bp	Short insert recipe recommended
300–750 bp	Standard recipe recommended
> 750 bp	Long insert recipe required

Table 1. Recommendations for using the short insert recipe.

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Insert sizes less than 100 bp

For libraries with aligned insert sizes of less than 100 bp, the short insert recipe is required to improve polony morphology, resulting in better quality and equivalent or better density in the sequencing run. Libraries tested in this size range include proteomics libraries (insert size < 20 bp), synthetic libraries (insert size 20 bp), and miRNA libraries (insert size 20–50 bp). For all three library types, there is a marked improvement in quality, with all types showing an increase of at least 2.5% in %Q30 across the sequencing run (Figure 2A).

For the smallest libraries (insert size 20 bp or less) density also improves significantly, with proteomics and synthetic libraries showing density improvements of more than 30% with the short insert recipe (Figure 2B).

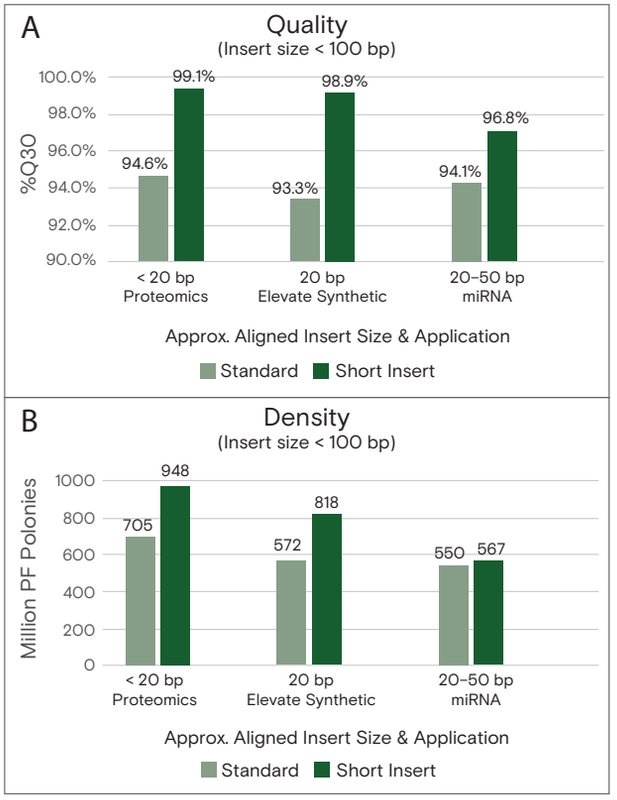


Figure 2. (A) Quality (%Q30) and **(B)** density (million pass-filter polonies) of libraries with insert sizes less than 100 bp sequenced with standard and short insert recipes.

The miRNA libraries, which have a slightly larger size range than the other tested libraries in this category (20–50 bp), showed a minor density improvement. Based on these findings, the short insert recipe is recommended for all libraries with insert sizes less than 100 bp when sequencing with Cloudbreak Freestyle.

Insert sizes 100–300 bp

Using the short insert recipe for libraries with insert sizes 100–300 bp results in modest improvements or equivalent performance compared to libraries sequenced with the standard recipe. Both third-party and Elevate libraries prepared for whole-exome sequencing (WES) were tested in this size range, as well as third-party and Elevate RNA-seq libraries. Libraries tested in this size range had comparable quality between the standard recipe and the short insert recipe: %Q30 was within 1% for either setting (Figure 3A). All libraries tested in this size range yielded some improvement in density, though the increase is generally smaller than what is expected for libraries with insert sizes less than 100 bp (Figure 3B).

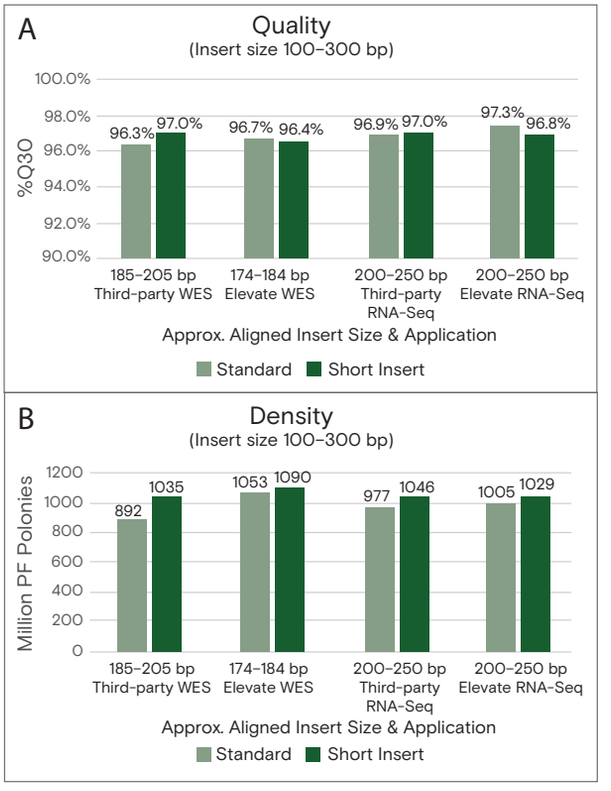


Figure 3. (A) Quality (%Q30) and **(B)** density (million pass-filter polonies) of libraries with insert sizes of 100–300 bp sequenced with standard and short insert recipes.

Secondary metrics analyzed for the WES libraries showed that recipe choice (standard or short insert) had little impact on these metrics (on-target rate and fold-80 examples shown in Figure 4). For libraries with 100–300 bp insert sizes, the short insert recipe is considered optional. Before deciding to use the short insert recipe for your specific case, weigh the importance of factors like total run time (~2 hour increase), polony density (modest improvement), and quality.

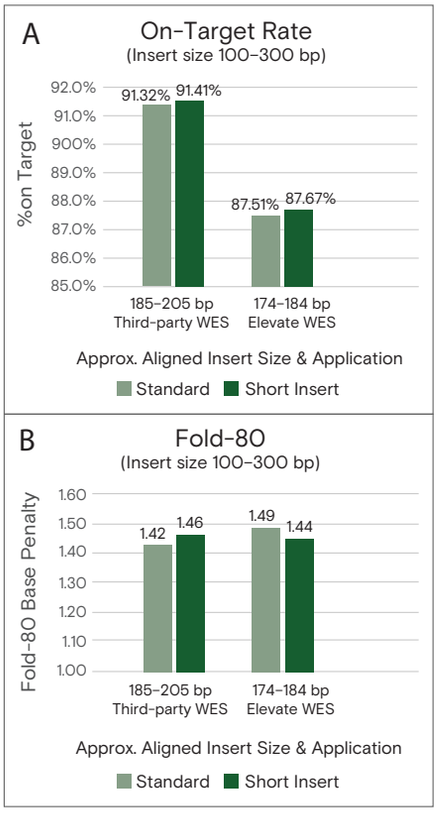


Figure 4. **(A)** On-target rate and **(B)** Fold-80 base of WES libraries with insert sizes 170–205 bp, sequenced with standard and short insert recipes.

Insert sizes greater than 300 bp

The short insert recipe is not recommended for larger libraries (> 300 bp) as quality and density can be reduced. While considerable improvements in polony morphology contribute to improved density and quality in libraries with smaller insert sizes, longer insert libraries might be impacted by lower intensity, contributing to reduced overall quality and lower total polony density.

Testing of WGS libraries with insert sizes of ~355 bp show overall quality reductions of > 2% with a greater impact in Read 2 (Figure 5A), as well as density reductions of more than 100 million paired-end polonies when the short insert recipe was applied (Figure 5B). As a result, the short insert recipe is not recommended for libraries with insert sizes greater than 300 bp.

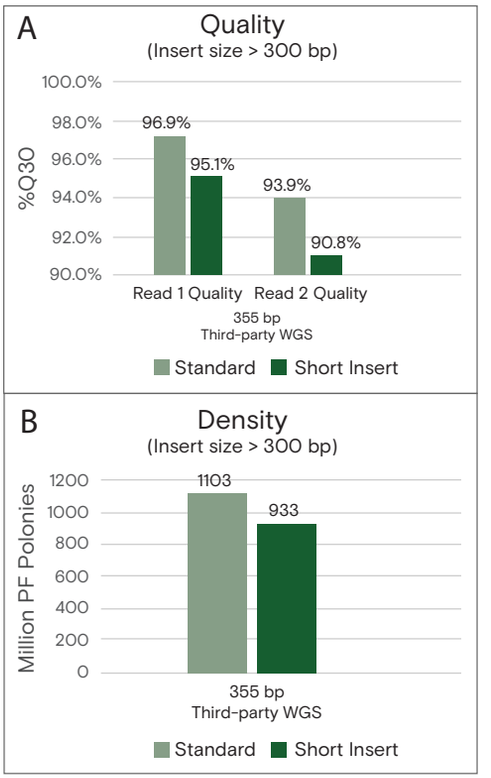


Figure 5. **(A)** Quality (%Q30) and **(B)** density (million pass-filter polonies) of libraries with insert sizes greater than 300 bp sequenced with standard and short insert sequencing recipes.

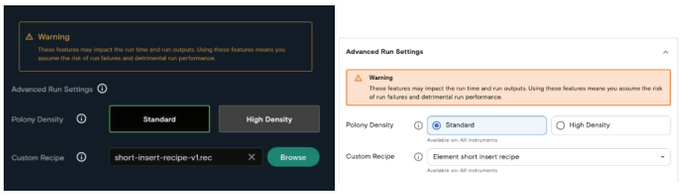
Complex library pools

For complex or broadly distributed library pools, use the standard recipe. Only use the short insert recipe on a case-by-case basis. For example, libraries with a significant population of larger insert sizes (> 450 bp) can be negatively impacted by low intensity, while insert sizes < 300 bp are still likely to sequence well with the short insert recipe. This trend can be seen in the RNA-seq and WES libraries (Figure 3). These libraries have low populations of insert lengths > 450 bp but the majority of the library is smaller with mean insert lengths of ~170–250 bp. These insert lengths likely contribute to the equivalent or improved sequencing performance of these libraries with the short insert recipe. Other library types that might benefit from using the short insert recipe are libraries prepared from cfDNA. These libraries have a smaller, dominant library peak with some larger fragments that can skew the overall distribution outside the recommended range. Knowledge of expected insert sizes and examination of library QC data can help determine whether the short insert recipe is a benefit for a given library pool.

Implementation

You can specify the short insert recipe for your run manually from the AVITI System user interface or when setting up a planned run in ElemBio™ Cloud.

- During a manual run setup, select **Custom Recipe** from the Advanced Settings menu. Then, browse to the short insert recipe preloaded in the recipes list (Figure 6A).
- For a planned run, specify the short insert recipe in the Advanced Run Settings section of the planned run form (Figure 6B).



(A) Manual run setup on AVITI interface (B) Planned run setup on ElemBio Cloud

Figure 6. Short insert sequencing recipe selection using **(A)** manual run setup on the AVITI user interface or **(B)** ElemBio Cloud.

Consider the aligned insert size of your library when deciding whether to use the short insert recipe, noting that the aligned insert size differs from the total library size measured during library QC.

The short insert recipe is compatible with 2 x 75 and 2 x 150 sequencing kits and adds approximately 2 hours to the total sequencing run time.

Conclusion

For applications involving short insert libraries, the short insert sequencing recipe with Cloudbreak Freestyle chemistry provides equivalent or superior sequencing performance as compared to the standard recipe. Libraries with mean insert sizes of less than 300 bp show comparable or better performance with the short insert recipe. The smallest libraries with insert sizes less than 100 bp show the greatest improvements in density and quality compared to the standard recipe.

The short insert recipe is preloaded and easy to access on the AVITI System. The short insert recipe is required for sequencing libraries with insert sizes less than 100 bp and recommended for insert sizes 100–300 bp.

Methods

The following preparation methods were used for libraries of various sizes:

- Customer-provided proteomics libraries (< 20 bp insert length) were sequenced without indexing using a 1 x 67 format on the AVITI System with the standard and short insert recipes.
- MicroRNA libraries (20–50 bp insert length) were prepared with the QIAGEN QIAseq miRNA Library Kit and sequenced using a 1 x 72 format with 10 bp indexing.
- Synthetic Elevate libraries (20 bp insert length) were prepared with the Element Elevate library prep kit and sequenced using a 2 x 20 format with Elevate indexing.
- RNA-seq libraries were prepared with both Elevate and third-party kits and sequenced using a 2 x 75 format with indexing.
- WES libraries were prepared using both Elevate and third-party methods with the Twist for Element Exome Kit and Twist Exome 2.0 kit and sequenced using a 2 x 75 format with indexing.
- WGS libraries (~350 bp insert length) were prepared with a KAPA HyperPrep PCR-free kit and sequenced using a 2 x 150 format with indexing.

After each sequencing run, libraries were demultiplexed using Bases2Fastq. For sequencing runs that include secondary analysis (e.g., WES libraries), analysis was performed using Picard, Sentieon, DeepVariant, and internal pipelines.

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