

Impact of Truncated Library Molecules on Cloudbreak Freestyle™

Highlights

- Poor quality adapters or truncated adapters can negatively impact circularization and sequencing
- An end-polish PCR or indexing spike-in can improve polony density for libraries with truncated adapters

Introduction

Cloudbreak Freestyle enables direct linear library loading of your own third-party or Elevate™ libraries on the AVITI™ System, eliminating hands-on circularization steps while still using avidite base chemistry (ABC) to deliver high performance and excellent sequencing quality. Although Cloudbreak Freestyle is directly compatible with most libraries, the adapters used in library preparation can affect how efficiently libraries are circularized and sequenced on the instrument.

Indexing primers with adapter sequences are a key piece of many library preparation workflows for multiplexing and capture of library molecules on a flow cell. For third-party workflows, these synthetic oligonucleotides include an indexing sequence and an outer adapter sequence. Because the reactions used to produce these oligonucleotides are not perfectly efficient, some percentage of each batch contains internal deletions or truncated sequences, which can impact downstream preparation and sequencing. Indexing primers also tend to be long, which further compounds issues that arise from imperfect synthesis.

Various levels of purification to address imperfect synthesis are available from vendors for these oligonucleotides. High-performance liquid chromatography (HPLC) purification is often the preferred method for oligonucleotide purification as it provides higher resolution and purity of full-length nucleotides, while maintaining high yield and compatibility with downstream applications. Truncated sequences can occur

more frequently with less stringent forms of purification, such as desalt purification. If truncated sequences are severe and frequent enough, they can impact circularization and sequencing and result in a lower polony density than expected.

One mitigation for this phenotype is to perform an end-polish PCR. Performed after normal library prep using a third-party kit, this PCR ‘polishes’ the ends of library fragments to ensure they have blunt ends, resulting in a higher percentage of full-length adapters. These libraries can then circularize more efficiently and produce the expected polony density. However, this process requires an additional PCR and SPRI bead cleanup.

This technical note describes an alternative mitigation to improve polony density with only a minor modification to indexing PCR. This mitigation adds third-party outer adapter primers to the indexing primer mixes before indexing PCR to result in a similar ‘polishing’ of blunt ends without additional PCR and cleanup steps.

Mitigation: Indexing Spike-in

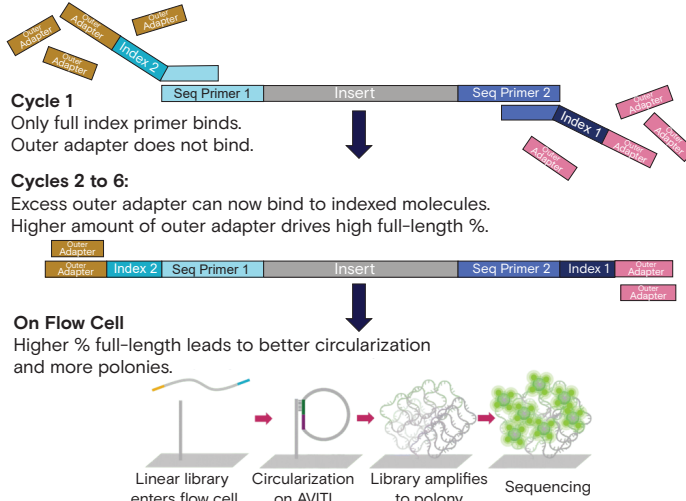


Figure 1. Schematic depicting the indexing spike-in mitigation during PCR cycles 1–6.

The indexing spike-in mitigation uses the existing indexing PCR with standard indexing primers that already contain third-party outer adapter sequences, and adds a high proportion (95%) of additional third-party outer adapter primers, reducing indexing primers to just 5% of the total primer mix in each reaction. If additional library complexity is desired, this percentage can be increased to 10% indexing primer with 90% outer adapter sequence.

While the first cycle of the PCR proceeds as normal where only the full indexing primers bind to the library fragments, subsequent cycles contain excess third-party outer adapter primers from the spike-in that now bind to the newly indexed molecules and ‘polish’ the ends of the fragments. The result is a higher full-length percentage of the adapters that have been annealed to the libraries (Figure 1).

Methods

To create controlled levels of truncation in indexing primers for testing the spike-in mitigation strategy, two batches of indexing primers with identical index sequences were used: one standard set and one intentionally truncated set. The truncated set was designed to be incompatible with Cloudbreak Freestyle. Both sets of indexing primers received standard desalt purification by the oligonucleotide vendor. Before conducting any indexing PCR, both sets of indexing primers were mixed in preset ratios of truncated to standard (0:100, 20:80, 40:60, 60:40, 80:20, and 100:0). A PhiX control library was then indexed using the premixed primer sets with two conditions: a standard indexing PCR with no mitigation for truncation, and an indexing PCR with the spike-in mitigation applied.

For the standard indexing PCR, a total of 2 μ M of indexing primers were used per reaction using the same set of premixed primers as the spike-in condition. For the indexing spike-in, third-party outer adapter primers from the KAPA HiFi kit were added into the indexing PCR at a ratio of 95% outer adapter primers to 5% indexing primers, with the total primer amount remaining constant at 2 μ M total per reaction. All other parameters of the reaction, including the amount of KAPA HiFi MasterMix and input amount of the control library (0.05 pmole), were held constant. Both conditions received six cycles of PCR with the program shown in Table 1. After the indexing PCR, a 1X SPRI cleanup was completed before library QC.

Temperature (°C)	Time (seconds)	Cycles
98	45	1
98	15	
60	30	6
72	30	
60	72	1
Hold	4	N/A

Table 1. Thermal cycler program used for both standard indexing and indexing spike-in mitigation PCRs.

Libraries were pooled before sequencing according to the indexing condition, with or without the spike-in mitigation. The Individually Addressable Lanes add-on was used to sequence both pools on a single flow cell on the AVITI System. The pool without the mitigation was loaded in one lane and the pool with the mitigation in the other lane. Both pools were loaded at 11 pM with 0.1 pM of a separate PhiX control library for sequencing run QC, and then sequenced with a 2 x 150 Cloudbreak Freestyle High Output Kit.

Results: Improved polony density

Both indexing conditions, with and without the indexing spike-in mitigation, have comparable quality of 94–96% Q30. However, polony density differs considerably between them. The spike-in mitigation increases total polony density regardless of the proportion of truncated primers used in the library prep (Figure 2), and the effect of this mitigation is more pronounced as the proportion of truncated primers in the prep increases.

At medium levels of truncation (40–60% of indexing primers truncated), per-sample polony density is nearly doubled when the spike-in mitigation is applied. In a worst-case 100% truncated scenario where none of the indexing primers contain full-length adapters, a sample that would otherwise fail to circularize and sequence achieves a per-sample density of 51 million polonies with the spike-in mitigation.

At low levels of truncation (0–20%), polony density still increases by 4–10 million paired-end polonies per sample with the spike-in mitigation. This result is likely because the 0% truncated condition refers to the percentage of intentionally truncated index primer in the mix (0%), rather than the true non-full-length percentage of primer present. Because all conditions used standard desalt purification of the oligonucleotide primers and oligonucleotide synthesis is not 100% efficient, it is likely that a small percentage of non-full-length primers are present in the 0% condition. This small percentage appears to have been improved by the spike-in mitigation, resulting in the observed small density boost.

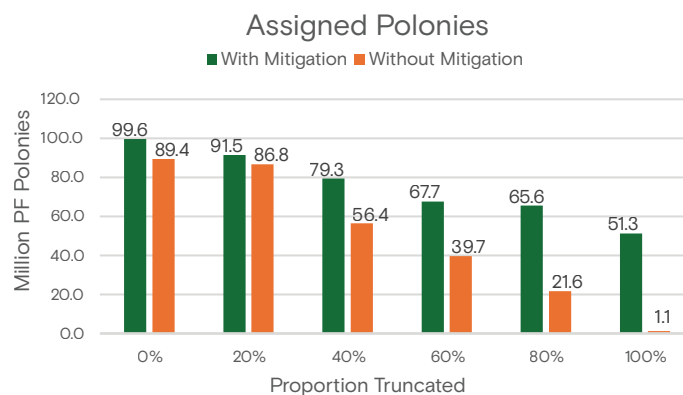


Figure 2. Million assigned pass-filter polonies per sample with (shown in green) and without (shown in orange) indexing spike-in mitigation.

Considerations and implementation

The indexing spike-in mitigation can be customized for an existing third-party library preparation without additional PCR and cleanup steps. The ratio of third-party outer adapter primers to indexing primers can be adjusted up or down as needed. For example, increasing the ratio of third-party outer adapter primers beyond 95% of the total primers in the indexing reaction can result in a higher percentage of full-length adapters that have annealed to library fragments, but at the cost of reduced library yield and complexity. The number of PCR cycles can be adjusted to fit an existing workflow or to change the final yield from the indexing PCR.

Conclusion

The indexing spike-in mitigation increases total polony density for libraries prepared using desalt-purified and truncated adapters. This mitigation requires minimal modifications to existing library preparation workflows without additional PCR or cleanup steps. HPLC purification of adapters used in library preparation can also improve density by increasing the percentage of full-length adapters in resulting libraries. Improvements in density were observed for all conditions using the spike-in mitigation without sacrificing sequencing quality and without increasing library preparation time.

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