Technical note

CONCATENATING AMPLICONS USING PACBIO KINNEX KITS TO INCREASE THROUGHPUT

Overview

This technical note describes the principles and advantages of concatenating amplicons using PacBio® Kinnex™ kits.

Note: This technical note is intended as a guide for best practices and to report typical example results (see Appendix). PacBio does not guarantee the success of concatenating amplicons that are not officially supported by the Kinnex kits, which currently include:

- **Full-length cDNA** generated using the **Iso-Seq® express 2.0 kit** with the *Kinnex full-length RNA kit*
- **Full-length 16S** sequences generated according to **official protocol** with the *Kinnex 16S rRNA kit*
- **Single-cell cDNA** generated using **compatible single-cell platforms** with the *Kinnex single-cell RNA kit*

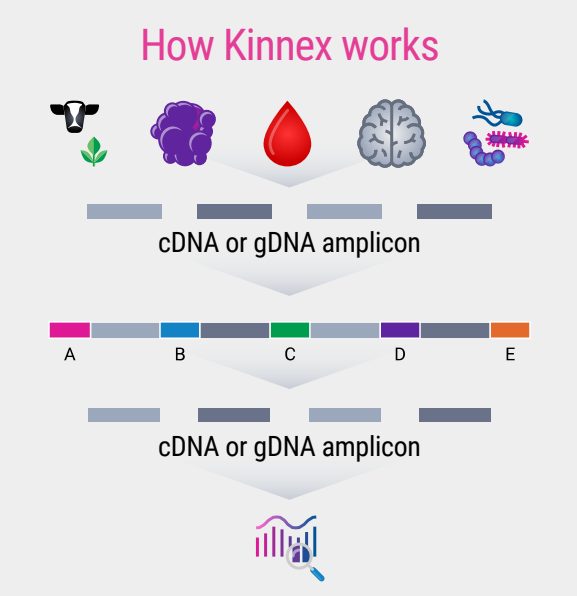

Figure 1. How Kinnex works. Watch the **Kinnex animation** to learn more.

What is Kinnex?

The Kinnex kits are based on the method developed by **Al'Khafaji et al.** (2023). Originally termed multiplexed array isoform sequencing (MAS-ISO-seq, or MAS-Seq), this method was developed **to concatenate cDNAs into longer fragments suitable for long-read sequencing**. It takes advantage of the disparity between optimal DNA fragment lengths for **HiFi sequencing**, 15–20 kb, and smaller sizes of transcript cDNA (1–10 kb with typical estimated **average length of 2 kb for a human transcriptome**). The original MAS-ISO-Seq method concatenated single-cell cDNA from the 10x platform, which has an average read length of 600–800 bp, and used a 15-fold concatenation array to increase throughput. PacBio commercialized the MAS-Seq method and adjusted the concatenation factors according to different average amplicon sizes (Table 1).

Average amplicon size	Example	Recommended kit	Expected library insert size
600–1000 bp	10x single-cell cDNA	Kinnex single-cell RNA kit (16-fold)	10–16 kb
1–2 kb	Full-length 16S rRNA	Kinnex 16S rRNA kit (12-fold)	~19 kb
2–3 kb	Bulk cDNA	Kinnex full-length RNA kit (8-fold)	15–20 kb
>3 kb	Not recommended for Kinnex concatenation		

Table 1. Recommended Kinnex kit based on average amplicon sizes.

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When is Kinnex concatenation appropriate?

Kinnex concatenation is a method that increases sequencing throughput for smaller amplicons. The balance between the amplicon size and the concatenation factor, as well as additional Kinnex library generation cost, needs to be taken into consideration. HiFi sequencing produces optimal yield for inserts between 15–20 kb; therefore, the throughput advantage plateaus for larger amplicon sizes exceeding 3 kb.

You can consider concatenating amplicons using Kinnex kits if:

- The amplicons have an average size between 200 bp – 3 kb.
- The amplicons have molecular ends that are either directly compatible or can be re-amplified to establish Kinnex compatibility.

Benefits of Kinnex concatenation include:

- Increased throughput on PacBio long-read sequencers
- Retained HiFi accuracy despite throughput increase
- No change to secondary analysis – once reads are de-concatenated into S-reads, the S-reads represent the original, pre-concatenated amplicon and can be analyzed with established pipelines. For example, de-concatenated 16S amplicons can be run through the HiFi-16S-workflow pipeline, while de-concatenated bulk cDNA amplicons can use the Iso-Seq workflow.

Overview of Kinnex library workflow and analysis

To establish Kinnex compatibility, amplicons are required to have Kinnex-compatible molecular ends (see next section). Once these are generated, choose the appropriate Kinnex kit based on the recommended concatenation factor listed in Table 1 and proceed with Kinnex PCR. The Kinnex PCR steps consist of parallel PCR reactions per sample – 8, 12, or 16 reactions based on the Kinnex kit chosen – using premixed Kinnex primer pairs. The resulting PCRs generate amplified DNA products containing programmable sequences at both ends. Refer to the Kinnex PCR section in the Kinnex protocols and technical training decks at the end of this document for details.

In the Kinnex array formation step, library inserts containing programmable ends are assembled to generate a linear array. Further, the addition of barcoded Kinnex terminal adapters result in the formation of complete, full-length array SMRTbell® templates along with partial arrays. Subsequent nuclease treatment removes partial arrays to retain only full-array SMRTbell templates for achieving optimal sequencing yield. Refer to the Appendix for additional details and tips.

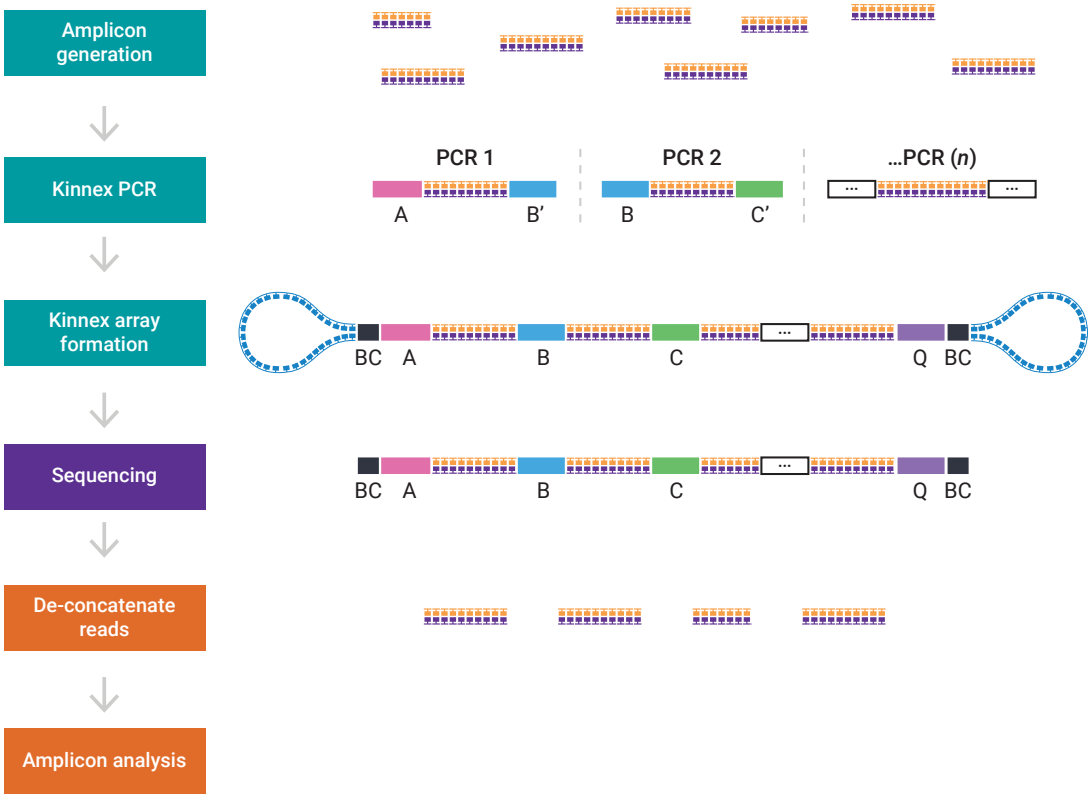

Figure 2. Kinnex concatenation workflow. **Amplicons must be generated or amplified to have Kinnex-compatible molecular ends before continuing to the Kinnex PCR and array formation step**. Kinnex libraries should be sequenced with the appropriate sequencing chemistry and run configurations. Once de-concatenated using Read Segmentation in SMRT® Link, the individual amplicons can be analyzed using amplicon-specific workflows.

Establishing Kinnex-compatible molecular ends

To be compatible with the Kinnex workflow, amplicons must be generated with sequence-defined ends as depicted in the underlined portion in Figure 3. Optional barcodes (such as sample indices, UMIs and single cell barcodes) should be placed internally between the Kinnex handles and the amplicon-specific primers. Kinnex handles may be present already in certain amplicons, such as 10x Single Cell Gene Expression libraries or the Kinnex 16S amplicons, or can be added by PCR amplification, such as for **Parse Evercode single cell libraries**.

Kinnex FWD primer CTACACGACGCTCTTCCGATCT – [optional barcodes] – [amplicon specific FWD primers]

Kinnex REV primer AAGCAGTGGTATCAACGCAGAG – [optional barcodes] – [amplicon specific REV primers]

Example 1. Kinnex 16S forward and reverse primer sequences

Kinnex 16S FWD01 CTACACGACGCTCTTCCGATCT – GATCGAGTCA – AGRGTTYGATYMTGGCTCAG

Kinnex 16S REV13 AAGCAGTGGTATCAACGCAGAG – TCATCGACGT – RGYTACCTTGTTACGACTT

Example 2. Iso-Seq express 2.0 forward and reverse primer sequences

IsoSeqX bc01 FWD CTACACGACGCTCTTCCGATCT – ACTACAC – GCAATGAAGTCGCAGGGTTGGG

IsoSeqX REV AAGCAGTGGTATCAACGCAGAGTAC

Figure 3. Schematic for Kinnex-compatible primers. Kinnex handles (5' to 3') are shown in black underline and must be present at the ends of the amplicons to be compatible with Kinnex concatenation. Optional barcodes can be included internally. Amplicon-specific primers (and optional internal barcode sequences) must be designed to avoid strong secondary structures in the context of Kinnex handles.

Recommended materials + equipment

- Refer to the specific Kinnex protocols based on the kit selected (see Resources section at the end of this document) for the required materials & equipment.
- Custom primers might be required to establish Kinnex compatibility (Figure 3).
- To QC library size before and after concatenation, the Agilent FemtoPulse, TapeStation, or Fragment Analyzer (or other sizing tools that can measure fragments between 10–20 kb) are recommended.

Sequencing and analysis

Regardless of the concatenated amplicon, the resulting library should be treated as a Kinnex library. Importantly, the Kinnex terminal adapters are different from standard SMRTbell adapters in that they allow the sequencing polymerase to bind to only one end of the SMRTbell library, and hence require the Kinnex sequencing primer (103-179-000) during the "Annealing, Binding, and Cleanup (ABC)" step for optimal sequencing results.

To specify Kinnex libraries in Sample Setup menu:

- Select the appropriate Kinnex application based on the Kinnex kit used. The library type should be "Kinnex".

To specify Kinnex libraries in Run menu:

- Select the appropriate Kinnex application based on the Kinnex kit used. The library type should be "Kinnex".
- Select "Read Segmentation" in Analysis Workflow

Once HiFi reads are generated, Read Segmentation will produce the segmented reads (S-reads) that represent the original unconcatenated amplicons, which can be used for further analysis.

Resources

For using Kinnex full-length RNA kit (PN: 103-072-000) for 8-fold concatenation:

- Procedure & checklist—Preparing Kinnex libraries using the Kinnex full-length RNA kit
- Technical overview—Kinnex library preparation using Kinnex full-length RNA kit

For using Kinnex 16S rRNA kit (PN: 103-072-100) for 12-fold concatenation:

- Procedure & checklist—Preparing Kinnex libraries using 16S rRNA amplicons
- Technical overview—Kinnex library preparation using Kinnex 16S rRNA kit

For using Kinnex single-cell RNA kit (PN: 103-072-200) for 16-fold concatenation:

- Procedure & checklist—Preparing Kinnex libraries using Kinnex single-cell RNA kit
- Technical overview—Kinnex library preparation using Kinnex single-cell RNA kit

Appendix 1. Amplicon concatenation examples and troubleshooting tips

Successful Kinnex concatenation should yield a library that reflects the size increase by the concatenation factor. For example, a 16S rRNA amplicon with a 12-fold concatenation using the Kinnex 16S rRNA kit would have a library size of ~19 kb (see **Kinnex 16S rRNA technical overview** and Figure 4), while a bulk Iso-Seq library with an average size of ~1.9 kb will have a library size of ~16 kb after 8-fold concatenation (see **Kinnex full-length technical deck**). Quantitative sizing is best done with the FemtoPulse or FragmentAnalyzer (with accurate sample concentration). The TapeStation and other equivalent QC tools can qualitatively inform the sizing but have limited resolution above 10 kb.

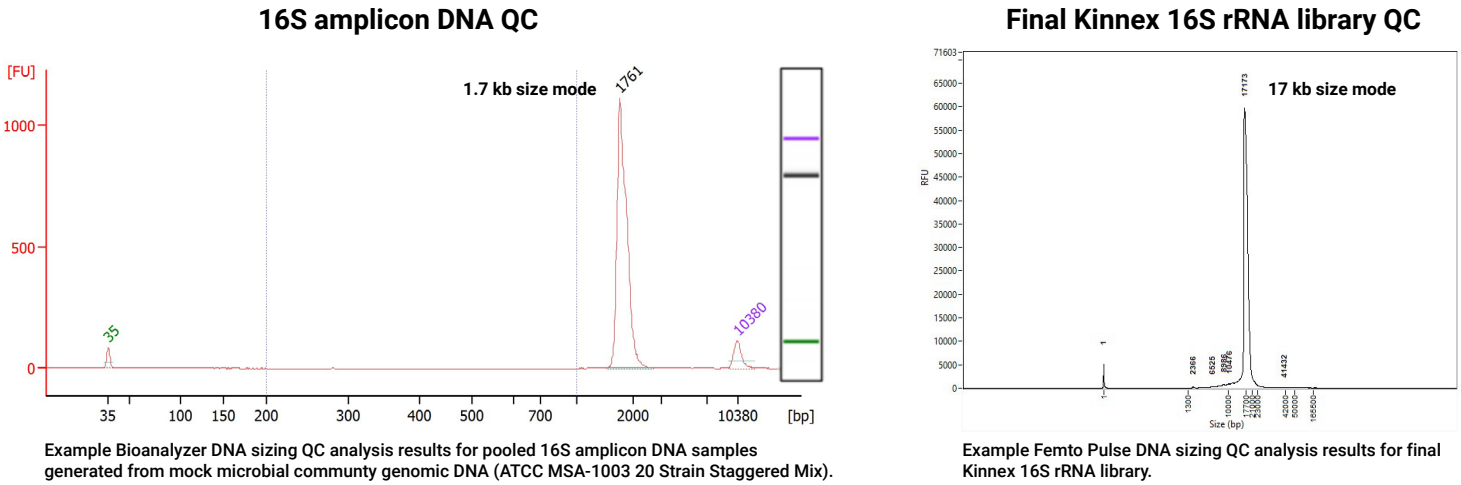

Figure 4. QC example from the Kinnex **16S rRNA technical deck**. Before concatenation, the 16S amplicons have an average size of ~1.7 kb. After concatenation, the Kinnex library has a size mode of 17 kb.

Figure 5 shows an example of array formation using 1 kb amplicons with Kinnex handles by PCR, resulting in 16 kb concatenated SMRTbell libraries:

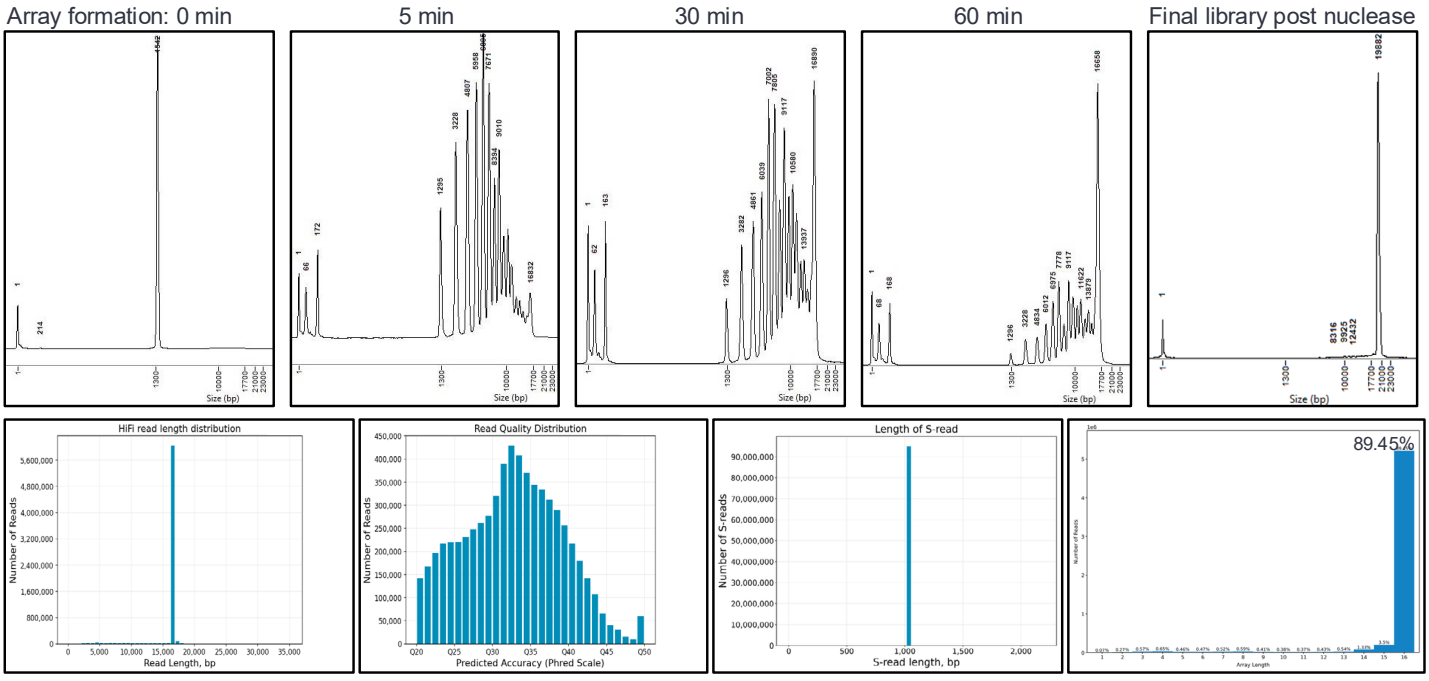

Figure 5: A progressive 16-fold concatenation of a 1 kb amplicon is demonstrated using the Kinnex single-cell RNA kit (103-072-200). After nuclease digestion, a 16 kb library is obtained and sequenced on the Revio® system. The sequencing result shows the expected 16 kb HiFi read length with a median QV = 33, and 1 kb original read length after segmentation in SMRT Link. Minor size discrepancies are expected with the Agilent Femto Pulse System.

Figure 6 shows an array formation using 2 kb amplicons with Kinnex handles by PCR, resulting in 16 kb concatenated SMRTbell libraries.

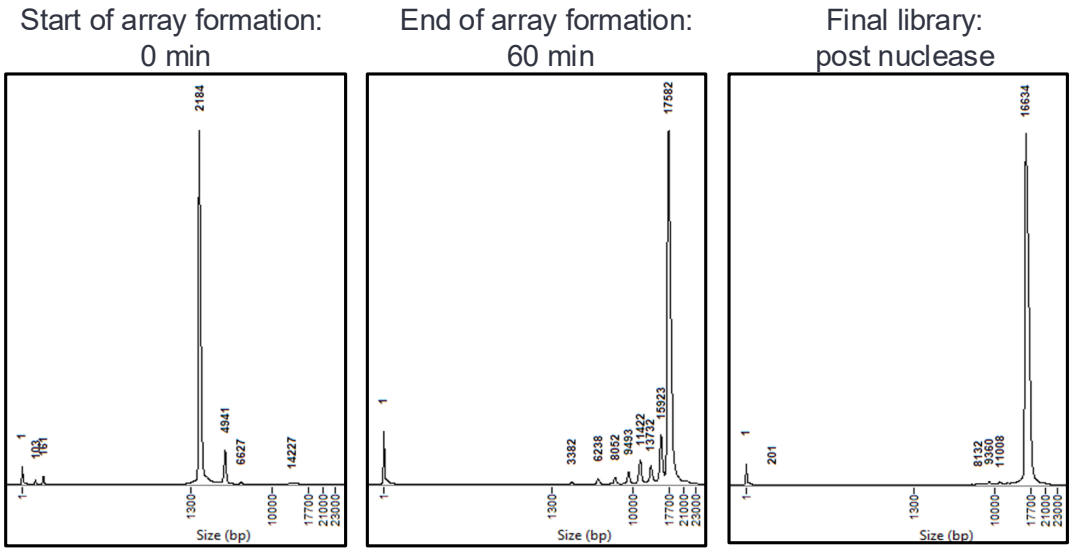

Figure 6: An 8-fold array formation of a 2 kb amplicon using the Kinnex full-length RNA kit (103-072-000), sized by Agilent Femto Pulse system. A 16 kb library is obtained after nuclease digestion.

Note that certain reagents, such as DNA Damage Repair mix, need to be properly stored on ice during usage and immediately closed after use to retain potency. Using expired or mishandled DNA Damage Repair, for example, can result in low library yield and high amounts of incomplete arrays which can sometimes be seen in the sizing plots. If sequenced, the sequencing performance might be compromised by lower loading, shorter mean polymerase read length, and lower HiFi yield.

It is also important to use the Kinnex sequencing primer during the "Annealing, Binding, and Cleanup (ABC) step" and designate the Kinnex library type in the Run menu. Using an incorrect sequencing primer will result in lower HiFi yield and selecting the incorrect library type can lead to reduced demultiplexing rate. Nevertheless, if the S-reads from the HiFi reads correctly represent the original amplicons, then the concatenation has been successful. Users may need additional optimization to improve library quality and sequencing yield.