PacBio

Technical overview

Kinnex library preparation using Kinnex 8-fold prep kit

Vega system ICS v1.2

Revio system ICS v13.5

SMRT Link v26.2

PN 103-916-000 Rev 01 | October 2026

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Technical overview

Kinnex library preparation using Kinnex 8-fold prep kit

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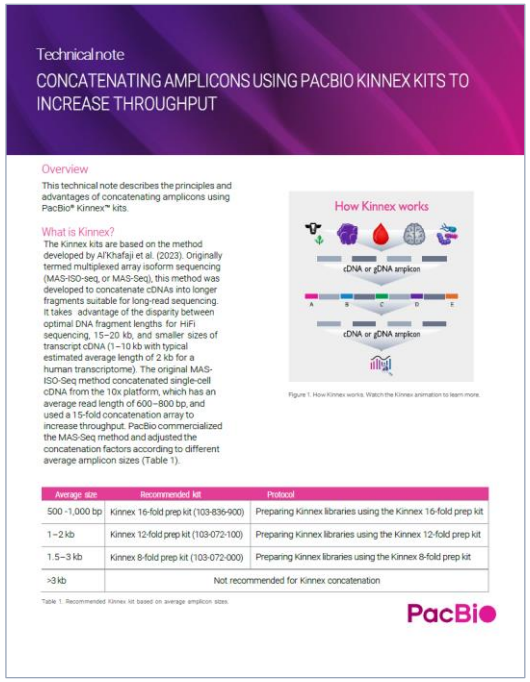
Kinnex library preparation using Kinnex 8-fold prep kit: Getting started

Application-specific literature → **Application-specific protocol** → **Application-specific technical overview** → **Library preparation, sequencing & analysis**



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.

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).

How Kinnex works

cDNA or gDNA amplicon

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

Average size	Recommended kit	Protocol
500 -1,000 bp	Kinnex 16-fold prep kit (103-836-900)	Preparing Kinnex libraries using the Kinnex 16-fold prep kit
1–2 kb	Kinnex 12-fold prep kit (103-072-100)	Preparing Kinnex libraries using the Kinnex 12-fold prep kit
1.5–3 kb	Kinnex 8-fold prep kit (103-072-000)	Preparing Kinnex libraries using the Kinnex 8-fold prep kit
>3 kb	Not recommended for Kinnex concatenation	

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

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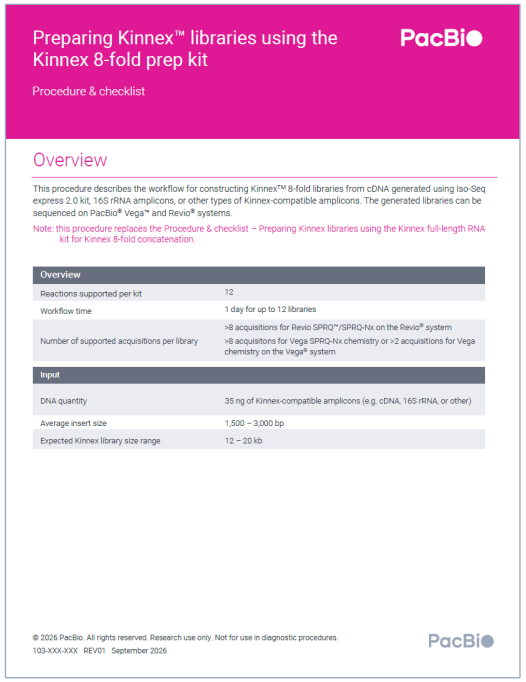
Technical note – Concatenating cDNA or gDNA amplicons using Kinnex kits to increase throughput (102-326-636)

Summary overview of application-specific Kinnex library preparation and data analysis workflow recommendations.



Preparing Kinnex™ libraries using the Kinnex 8-fold prep kit

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Procedure & checklist

Overview

This procedure describes the workflow for constructing Kinnex™ 8-fold libraries from cDNA generated using Iso-Seq express 2.0 kit, 16S rRNA amplicons, or other types of Kinnex-compatible amplicons. The generated libraries can be sequenced on PacBio® Vega™ and Revio® systems.

Note: this procedure replaces the Procedure & checklist – Preparing Kinnex libraries using the Kinnex full-length RNA kit for Kinnex 8-fold concatenation.

Overview	
Reactions supported per kit	12
Workflow time	1 day for up to 12 libraries
Number of supported acquisitions per library	>8 acquisitions for Revio SPRQ™/SPRQ-Nx on the Revio® system >8 acquisitons for Vega SPRQ-Nx chemistry or >2 acquisitions for Vega chemistry on the Vega® system
Input	
DNA quantity	35 ng of Kinnex-compatible amplicons (e.g. cDNA, 16S rRNA, or other)
Average insert size	1,500 – 3,000 bp
Expected Kinnex library size range	12 – 20 kb

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Procedure & checklist – Preparing Kinnex libraries using Kinnex 8-fold prep kit (103-920-900)

Technical documentation containing application-specific Kinnex library preparation protocol details.

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Technical overview

Kinnex library preparation using Kinnex 8-fold prep kit

Vega system ICS v1.2
Revio system ICS v13.5
SMRT Link v26.2

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Example sequencing performance for Kinnex full-length RNA libraries prepared with human cDNA [Revio system + SPRQ chemistry[1]]

Kinnex full-length RNA library for universal human reference RNA (UHRR) sample

Raw Data Report | HiFi Read Length | Read Segmentation Metrics

Raw Base Yield	1,442 Gb
Mean Polymerase Read Length	71.9 kb
P0	20%
P1	80%
P2	1%

HiFi Reads	10.4 M
HiFi Base Yield	146.6 Gb
Mean HiFi Read Length	14.0 kb
Median HiFi Read Quality	Q32
HiFi Read Mean # of Passes	7

Input HiFi Reads	10,404,037
Segmented reads (5-reads)	81,012,120
Mean length of 5-reads	1,758 bp
Percent of reads with full arrays	94.55%
Mean array size (concentration factor)	7.79

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Technical Overview – Kinnex library preparation using Kinnex 8-fold prep kit (103-916-000)

Technical overview presentations describe sample preparation details for constructing concatenated HiFi libraries using Kinnex prep kits. Example sequencing performance data for Kinnex libraries are also summarized.

Kinnex-compatible amplicon generation
(Application-specific procedure)

Follow application-specific protocols to generate amplicons with the required Kinnex PCR handle sequences

↓

Kinnex library preparation
(Kinnex prep kit)

Use Kinnex-compatible amplicons to generate Kinnex library containing arrayed DNA segments

↓

HiFi sequencing
(Revio & Vega systems)

Perform ABC[1] and sequence Kinnex libraries on PacBio long-read systems

↓

Data analysis
(SMRT Link)

Use SMRT Link Read Segmentation data utility to split arrayed HiFi reads

Use SMRT Link or other tools to perform downstream secondary analysis

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[1] ABC = Anneal primer / Bind polymerase / Clean up bound complex

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Kinnex library concatenation method overview

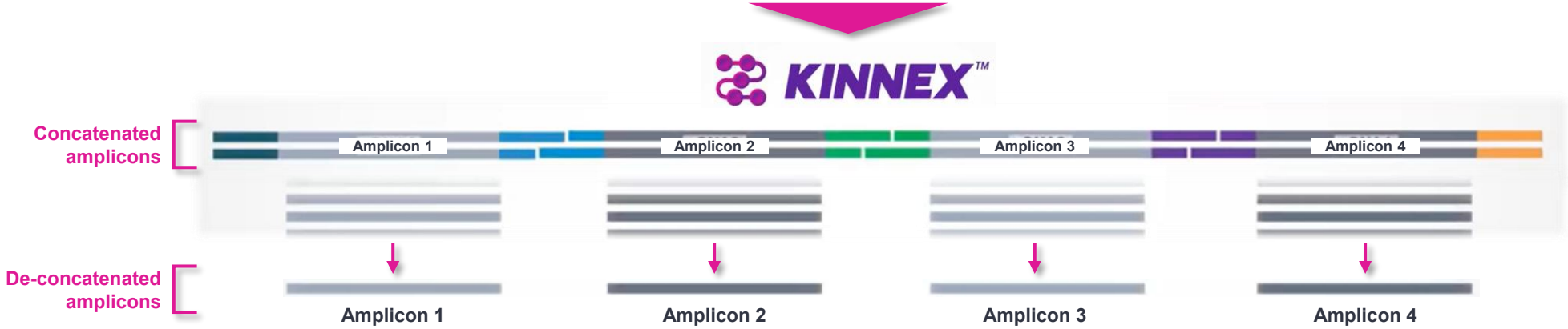
What is Kinnex?

Kinnex is a general amplicon concatenation method for increasing HiFi sequencing data yield

- PacBio **Kinnex kits** are based on the MAS-Seq (Multiplexed Arrays Isoform Sequencing) method[1] originally developed by Al'Khafaji et al. (2023) to concatenate cDNAs into longer fragments suitable for long-read sequencing
- PacBio Kinnex method takes advantage of the disparity between optimal DNA fragment lengths for HiFi sequencing, 15–20 kb, and smaller amplicon sizes (e.g., <3 kb)
- By concatenating amplicons into larger fragments, **PacBio Kinnex method increases HiFi data throughput per run** (i.e., per SMRT Cell acquisition)[2]

Large insert HiFi libraries are ideal for whole genome sequencing…

…but for smaller amplicon sizes, the extra sequencing capacity is wasted



Kinnex method concatenates smaller amplicons into larger fragments to be sequenced with HiFi

→ HiFi reads can be bioinformatically de-concatenated to represent the original amplicon molecules

[1] 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. See Al'Khafaji, A.M. et al. (2024) High-throughput RNA isoform sequencing using programmable cDNA concatenation. Nature biotechnology. [Link]

[2] Watch the Kinnex animation to learn more about how the PacBio Kinnex method works.

When is Kinnex concatenation appropriate?

Kinnex concatenation is generally suitable for amplicons with an average insert size <3 kb

- Kinnex concatenation is a method that increases sequencing throughput for **smaller amplicons** (e.g., cDNA or other gDNA amplicons <3 kb)
- The balance between the amplicon size and the concatenation factor, as well as additional Kinnex library generation cost, needs to be taken into consideration[1]
- HiFi sequencing produces optimal yield for libraries between 15 – 20 kb; therefore, the throughput advantage with the Kinnex concatenation method plateaus for larger amplicon sizes exceeding 3 kb.

You can consider concatenating amplicons using Kinnex kits if:

- ☑ The amplicons have an **average size <3 kb**[2]
- ☑ The amplicons have molecular ends that are either directly compatible or can be re-amplified to establish **Kinnex compatibility**

KINNEX v2.0

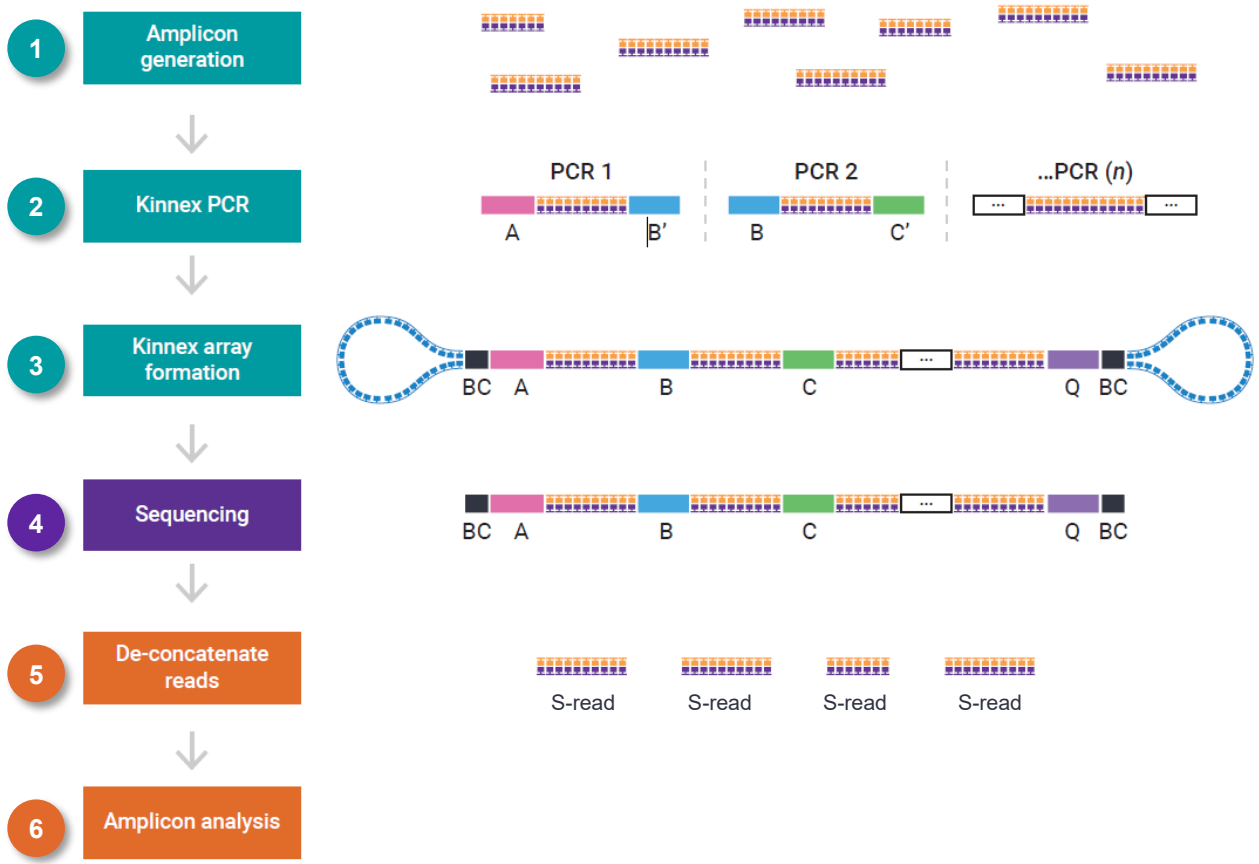
Benefits of Kinnex concatenation include:

- Increased throughput on PacBio long-read sequencers
- Retention of HiFi accuracy levels in conjunction with increased throughput levels
- No change to secondary analysis – once HiFi reads are de-concatenated into segmented reads (S-reads), the S-reads represent the original, pre-concatenated amplicon and can be analyzed with already-established pipelines.
    - For example, de-concatenated 16S amplicons can be analyzed using the PacBio HiFi-16S-workflow pipeline, while de-concatenated bulk cDNA amplicons can be analyzed using the PacBio Iso-Seq workflow

[1] See ***Technical note – Concatenating cDNA or gDNA amplicons using Kinnex kits to increase throughput*** (102-326-636) for a description of the principles and advantages of concatenating amplicons using PacBio Kinnex kits
[2] Guidance is based on peak or average insert size distribution. Targeted amplicons over 3 kb are not supported.

Kinnex concatenation workflow overview

Key steps in Kinnex concatenation workflow include amplicon generation, Kinnex PCR, and Kinnex array formation



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.

Kinnex concatenation workflow key steps[1]

1. **Amplicon generation:** Amplicons are required to have Kinnex-compatible molecular ends (see later section)
   - Once these are generated, choose the appropriate Kinnex kit based on the average amplicon size (or target application) and proceed with Kinnex PCR
2. **Kinnex PCR:** Run parallel PCR reactions per sample [i.e., 8, 12, or 16 reactions based on the Kinnex kit chosen] using premixed Kinnex primer pairs
   - This step generates amplified DNA products containing programmable sequences at both ends
3. **Kinnex array formation:** Library inserts containing programmable ends are assembled to generate a linear array
   - Ligation of barcoded Kinnex terminal adapters results 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 HiFi sequencing yields
4. **Sequencing:** Kinnex terminal adapters are different from standard SMRTbell adapters and hence require the Kinnex sequencing primer (103-179-000) during the “Annealing, Binding, and Cleanup (ABC)” step for optimal sequencing results on Revio and Vega systems.
5. **De-concatenate reads:** Once HiFi reads are generated, run SMRT Link Read Segmentation data utility to produce segmented reads (S-reads) that represent the original unconcatenated amplicons
6. **Amplicon analysis:** Use SMRT Link or command line tools to perform downstream secondary analysis of recovered amplicon segment reads

[1] See ***Technical note – Concatenating cDNA or gDNA amplicons using Kinnex kits to increase throughput*** (102-326-636) for Kinnex concatenation workflow overview; refer to the relevant Kinnex library prep protocols and Kinnex technical overview training documentation for specific details regarding Kinnex PCR and Kinnex array formation steps.

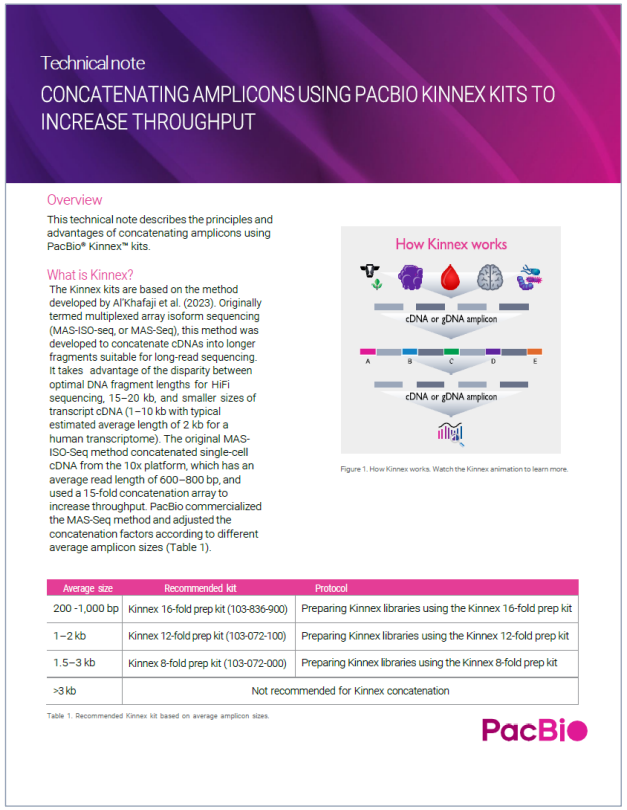
Experimental design considerations for concatenating amplicons to increase HiFi sequencing data throughput

Kinnex v2.0 workflows enable flexibility for choosing different Kinnex prep kits based on input amplicon average insert size

Kinnex prep kit recommendations for different input amplicon size ranges

- HiFi sequencing produces optimal data yield for inserts between 15–20 kb; therefore, the data throughput advantage with Kinnex concatenation methods plateaus for larger amplicon sizes exceeding 3 kb
- You can consider concatenating amplicons using Kinnex kits if the amplicons have an **average size <3 kb**[1]

Input amplicon average insert size[1]	Default recommended Kinnex prep kit	Expected throughput (Segmented reads or 'S-reads')
<1 kb	Kinnex 16-fold prep kit (103-836-900)	Revio (SPRQ-Nx): 100 – 150 M Vega (SPRQ-Nx): 80 – 100 M
1 – 2 kb	Kinnex 12-fold prep kit (103-072-100)	Revio (SPRQ-Nx): 70 – 100 M Vega (SPRQ-Nx): 50 – 60 M
1.5 – 3 kb	Kinnex 8-fold prep kit (103-072-000)	Revio (SPRQ-Nx): 50 – 70 M Vega (SPRQ-Nx): 40 – 50 M
>3 kb	**Not recommended for Kinnex concatenation**	



Technical note

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How Kinnex works

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

Average size	Recommended kit	Protocol
200 -1,000 bp	Kinnex 16-fold prep kit (103-836-900)	Preparing Kinnex libraries using the Kinnex 16-fold prep kit
1–2 kb	Kinnex 12-fold prep kit (103-072-100)	Preparing Kinnex libraries using the Kinnex 12-fold prep kit
1.5–3 kb	Kinnex 8-fold prep kit (103-072-000)	Preparing Kinnex libraries using the Kinnex 8-fold prep kit
>3 kb	Not recommended for Kinnex concatenation	

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

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Technical note – Concatenating cDNA or gDNA amplicons using Kinnex kits to increase throughput (102-326-636).

***Technical note – Concatenating cDNA or gDNA amplicons using Kinnex kits to increase throughput* (102-326-636)** provides guidance on selecting an appropriate Kinex concatenation level based on input amplicon size range

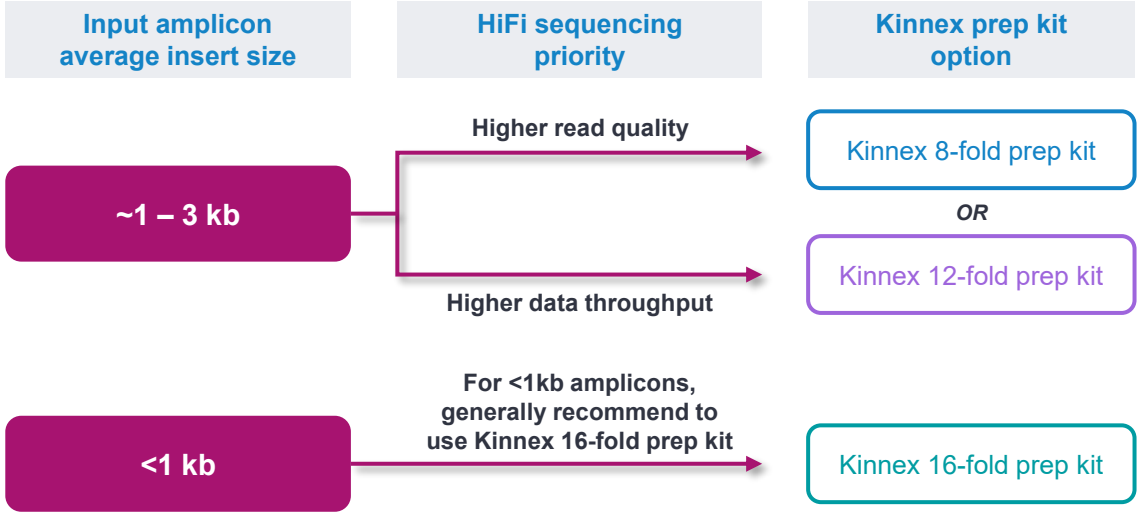
[1] Guidance is based on peak or average insert size distribution. Targeted amplicons over 3 kb are not supported for Kinnex concatenation procedures.

Experimental design considerations for concatenating amplicons to increase HiFi sequencing data throughput (cont.)

Users may consider choosing a higher-fold Kinnex prep kit for increased data throughput or a lower-fold Kinnex prep kit for increased read quality

Kinnex prep kit options to achieve either higher data throughput or higher read quality

- Selecting a higher-fold Kinnex prep kit can enable higher data throughput (i.e., higher number of segmented reads ("S-reads") per acquisition) [**Note:** The final Kinnex concatenated HiFi library size should not greatly exceed the optimal range of 15 – 20 kb.]
- Selecting a lower-fold Kinnex prep kit can enable higher read quality (i.e., higher mean HiFi read QV score)



Note: Kinnex concatenated HiFi library size should not greatly exceed ~20 kb

Example:
1.5 kb amplicon Kinnex library prepared with Kinnex 8-fold prep kit *versus* Kinnex 12-fold prep kit

HiFi sequencing performance results obtained for a 1.5 kb amplicon Kinnex library prepared with Kinnex 8-fold prep kit and Kinnex 12-fold prep kit (Revio system + SPRQ-Nx chemistry)

Kinnex prep kit	*P1*	HiFi yield	HiFi reads	HiFi read length	HiFi read quality	Q30+ bases	S-reads
Kinnex 12-fold prep kit	69%	147 Gb	7.8 M	18.8 kb	Q34	93.9%	**93.4 M**
Kinnex 8-fold prep kit	74%	120 Gb	9.5 M	12.6 kb	**Q37**	95.4%	75.9 M

Higher read quality

Higher S-read yield

IMPORTANT: Final data throughput (yield) will depend on **application-specific downstream analysis procedures and quality filter settings used**[3]

- For some applications using secondary analysis tools that require high read quality, datasets generated with Kinnex 8-fold prep kit may retain a higher yield of useable reads after quality filtering (despite lower raw S-read throughput) compared to datasets generated with Kinnex 12-fold prep kit

[1] Example: For Kinnex 16s rRNA applications using secondary analysis tools that require high read quality (e.g., DADA2), 16S datasets generated with Kinnex 8-fold prep kit may retain a higher yield of reads after 16S quality filtering (despite lower raw S-read throughput) compared to datasets generated with Kinnex 12-fold prep kit.

Experimental design considerations for concatenating amplicons to increase HiFi sequencing data throughput (cont.)

Use the recommended input amplicon DNA quantity for Kinnex concatenation to generate sufficient amounts of final library material for HiFi sequencing

Input amplicon mass requirements for Kinnex library preparation

- Refer to Kinnex Procedure & checklist library prep protocol documentation for the latest recommended amplicon input mass requirements

Kinnex prep kit	Procedure & checklist protocol reference	Recommended input amplicon length range	Recommended input amplicon DNA quantity	Expected Kinnex HiFi library size	# of supported acquisitions per library
Kinnex 8-fold prep kit	Procedure & checklist – Preparing Kinnex libraries using the Kinnex 8-fold prep kit (103-920-900)	1,500 – 3,000 bp	• **35 ng** of Kinnex-compatible amplicons for input into Kinnex PCR step	~12 – 20 kb	• >8 acquisitions for Revio SPRQ/SPRQ-Nx chemistry • >8 acquisitions for Vega SPRQ-Nx chemistry • >2 acquisitions for Vega v1 chemistry
Kinnex 12-fold prep kit	Procedure & checklist – Preparing Kinnex libraries using the Kinnex 12-fold prep kit (103-921-000)	1,000 – 2,000 bp	• **35 ng** of Kinnex-compatible amplicons for input into Kinnex PCR step	~12 – 20 kb	• >8 acquisitions for Revio SPRQ/SPRQ-Nx chemistry • >8 acquisitions for Vega SPRQ-Nx chemistry • >2 acquisitions for Vega v1 chemistry

Kinnex 16-fold prep kit	Procedure & checklist – Preparing Kinnex libraries using the Kinnex 16-fold prep kit (103-885-300)	<1 kb	• For cDNA amplicons generated via template-switch oligo (TSO) method (e.g., 10x Genomics):
			  • **>15 ng** of cDNA amplicons for input into TSO PCR step and **25 ng** of post-TSO removal amplicons for input into Kinnex PCR step
			• For other amplicon types and cDNA synthesized without TSO method:
			  • **25 ng** of Kinnex-compatible amplicon DNA/cDNA for input into Kinnex PCR step
				~10 – 17 kb	• >4 acquisitions for Revio SPRQ/SPRQ-Nx chemistry
					• >4 acquisitions for Vega SPRQ-Nx chemistry
					• >1 acquisitions for Vega v1 chemistry

Experimental design considerations for concatenating amplicons to increase HiFi sequencing data throughput (cont.)

Amplicons must be generated or amplified to have Kinnex-compatible molecular ends before proceeding with Kinnex library prep procedures[1]

Establishing Kinnex-compatible molecular ends

- To be compatible with Kinnex workflows, **amplicons must be generated with sequence-defined ends** (**Kinnex handles**) as depicted in the underlined portion in the figure below
- **Optional barcodes** (e.g., 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** (.e.g., Kinnex bulk Iso-Seq cDNA amplicons, Kinnex 16S amplicons, 10x Single Cell Gene Expression libraries) 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
```

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.

Resources for Kinnex library prep[2]

For using Kinnex 8-fold prep kit (103-072-000) for 8-fold concatenation:

- Procedure & checklist – Preparing Kinnex libraries using the Kinnex 8-fold prep kit (103-920-900)
- Technical overview – Kinnex library preparation using Kinnex prep kits (TBD)

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

- Procedure & checklist – Preparing Kinnex libraries using 16S rRNA amplicons (103-921-000)
- Technical overview – Kinnex library preparation using Kinnex prep kits (TBD)

For using Kinnex 16-fold prep kit (PN: 103-836-900) for 16-fold concatenation:

- Procedure & checklist – Preparing Kinnex libraries using Kinnex 16-fold prep kit (103-885-300)
- Technical overview – Kinnex library preparation using Kinnex prep kits (TBD)

[1] Refer to ***Technical note – Concatenating cDNA or gDNA amplicons using Kinnex kits to increase throughput*** (102-326-636) for guidance on how to establish Kinnex-compatible molecular ends with custom amplicon sample types and for general Kinnex concatenation troubleshooting tips.
[2] Refer to the relevant Kinnex library prep protocols and Kinnex technical overview training documentation for specific details on concatenating cDNA or gDNA amplicons using Kinnex kits

Default Kinnex library prep workflow recommendations based on input amplicon average insert size

Kinnex workflows enable flexibility for choosing different Kinnex library prep kits based on different input amplicon average size ranges[1]

Input amplicon average insert size[1]	Default recommended Kinnex prep kit	Expected throughput (Segmented reads or 'S-reads')	Kinnex application notes
<1 kb	Kinnex 16-fold prep kit (103-836-900)	Revio (SPRQ-Nx): 100 – 150 M Vega (SPRQ-Nx): 80 – 100 M	• Default kit for Kinnex single-cell RNA and short custom amplicons <1 kb
1 – 2 kb	Kinnex 12-fold prep kit (103-072-100)	Revio (SPRQ-Nx): 70 – 100 M Vega (SPRQ-Nx): 50 – 60 M	• Default kit for most Kinnex bulk RNA applications that enables higher data throughput and better recovery of shorter (< 1kb) transcripts
1.5 – 3 kb	Kinnex 8-fold prep kit (103-072-000)	Revio (SPRQ-Nx): 50 – 70 M Vega (SPRQ-Nx): 40 – 50 M	• Default kit for Kinnex 16S rRNA applications • Optional kit for Kinnex bulk RNA applications when retaining largest transcripts is the priority
>3 kb	**Not recommended for Kinnex concatenation**		

[1] Guidance is based on peak or average insert size distribution. Targeted amplicons over 3 kb are not supported. Refer to ***Technical note – Concatenating cDNA or gDNA amplicons using Kinnex kits to increase throughput*** (102-326-636) for latest recommendations on Kinnex library prep kit selection based on average amplicon insert size.

Default Kinnex library prep workflow recommendations based on HiFi sequencing application

Default Kinnex workflow recommendations provide a starting point for achieving an optimal balance between HiFi sequencing data quality and overall throughput

Kinnex application	Default recommended Kinnex prep kit	Expected throughput (Segmented reads or 'S-reads')	Kinnex application notes
Single-cell RNA	Kinnex 16-fold prep kit (103-836-900)	Revio (SPRQ-Nx): 100 – 150 M Vega (SPRQ-Nx): 80 – 100 M	• Perform single-cell cDNA amplicon generation using 10x Genomics system or other PacBio Compatible Partner products[1] • Kinnex 16-fold library prep workflow uses unified Kinnex concatenation kit 2.0 and reduces hands-on time for 10x single-cell RNA applications
Bulk rRNA	Kinnex 12-fold prep kit (103-072-100)	Revio (SPRQ-Nx): 70 – 100 M Vega (SPRQ-Nx): 50 – 60 M	• Perform cDNA amplicon generation using Iso-Seq express 2.0 kit + 1.1x cDNA cleanup[2] • 1.1x cDNA cleanup + Kinnex 12-fold library prep workflow provides higher S-read yield and better recovery of <1 kb transcripts • Can optionally consider using Kinnex 8-fold prep kit (+ 0.9x cDNA cleanup) for Kinnex bulk RNA applications when retaining largest transcripts is the priority
16S rRNA	Kinnex 8-fold prep kit (103-072-000)	Revio (SPRQ-Nx): 50 – 70 M Vega (SPRQ-Nx): 40 – 50 M	• Perform 16S amplicon generation using Kinnex-compatible barcoded 16S primers[3] • Kinnex 8-fold library prep workflow generally provides higher read quality and generates more usable reads after downstream data filtering using 16S analysis tools
Custom amplicons	**Insert size-dependent**	Depends on selected Kinnex library prep method	• Generally, recommend to use Kinnex 16-fold prep kit for amplicons <1 kb; Kinnex 12-fold pre kit for amplicons 1 – 2 kb; and Kinnex 8-fold prep kit for amplicons 1.5 – 3 kb • For overlapping amplicon size ranges, can select appropriate Kinnex library prep kit based on consideration of HiFi sequencing performance priorities (i.e., higher read quality versus higher throughput)

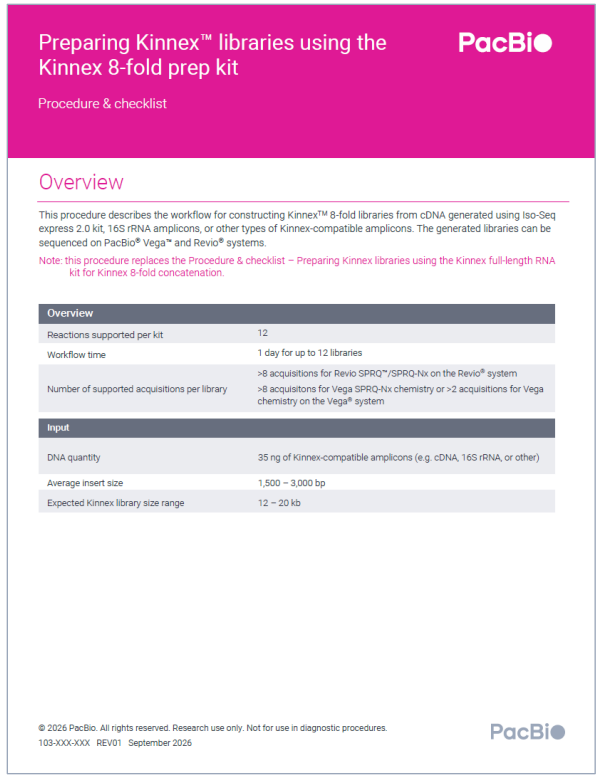
[1] Refer to specific Application Note literature for compatibility with third-party products such as ArgenTag and Parse Biosciences single-cell kits.
[2] Refer to ***Procedure & checklist – Preparing Kinnex-compatible cDNA using Iso-Seq Express 2.0 kit*** (102-396-000).
[3] Refer to ***Procedure & checklist – Preparing 16S rRNA amplicons for Kinnex*** (103-885-200) for recommended 16S gene-specific forward and reverse PCR primer sequences to order.

Kinnex 8-fold library preparation procedure description

Procedure & checklist – Preparing Kinnex libraries using Kinnex 8-fold prep kit (103-920-900) describes the workflow for constructing Kinnex 8-fold libraries from cDNA generated using Iso-Seq express 2.0 kit, 16S rRNA amplicons, or other types of Kinnex-compatible amplicons. The generated libraries can be sequenced on PacBio Vega and Revio systems.

Overview	
Reactions supported per kit	12
Workflow time	1 day for up to 12 libraries
Number of supported acquisitions per library	>8 acquisitions for Revio SPRQ™/SPRQ-Nx on the Revio® system >8 acquisitions for Vega SPRQ-Nx chemistry or >2 acquisitions for Vega chemistry on the Vega® system

Input	
DNA quantity	35 ng of Kinnex-compatible amplicons (e.g. cDNA, 16S rRNA, or other)
Average insert size	1,500 – 3,000 bp
Expected Kinnex library size range	12 – 20 kb



Preparing Kinnex™ libraries using the Kinnex 8-fold prep kit

PacBio

Procedure & checklist

Overview

This procedure describes the workflow for constructing Kinnex™ 8-fold libraries from cDNA generated using Iso-Seq express 2.0 kit, 16S rRNA amplicons, or other types of Kinnex-compatible amplicons. The generated libraries can be sequenced on PacBio® Vega™ and Revio® systems.

Note: this procedure replaces the Procedure & checklist – Preparing Kinnex libraries using the Kinnex full-length RNA kit for Kinnex 8-fold concatenation.

Overview	
Reactions supported per kit	12
Workflow time	1 day for up to 12 libraries
Number of supported acquisitions per library	>8 acquisitions for Revio SPRQ™/SPRQ-Nx on the Revio® system >8 acquisitons for Vega SPRQ-Nx chemistry or >2 acquisitions for Vega chemistry on the Vega® system
Input	
DNA quantity	35 ng of Kinnex-compatible amplicons (e.g. cDNA, 16S rRNA, or other)
Average insert size	1,500 – 3,000 bp
Expected Kinnex library size range	12 – 20 kb

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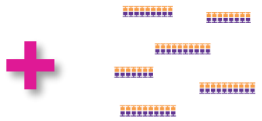
103-XXX-XXX REV01 September 2026

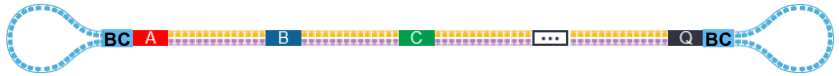
PacBio

PacBio Documentation (103-920-900)

Kinnex 8-fold prep kit
103-072-000 (12 rxn)



Kinnex-compatible amplicons[1]



Kinnex 8-fold library template (~12–20 kb)
Contains 8 concatenated amplicon segments

Refer to **separate** protocols[1] for generating Kinnex-compatible amplicons to be used as input for this Kinnex library prep procedure

[1] To generate Kinnex compatible cDNA refer to protocol, ***Preparing Kinnex-compatible cDNA using Iso-Seq Express 2.0 kit*** (102-396-000). To generate full-length 16S rRNA amplicons refer to protocol, ***Preparing Kinnex-compatible 16S rRNA amplicons*** (103-885-200). To generate other Kinnex-compatible amplicons refer to ***Technical Note: Concatenating Amplicons Using PacBio Kinnex Kits to Increase Throughput*** (102-326-636) for the input, size, and PCR requirements.

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Kinnex 8-fold library preparation workflow

Use Kinnex 8-fold prep kit to generate 8-segment Kinnex concatenation library

Kinnex 8-fold prep kit bundle (103-072-000)

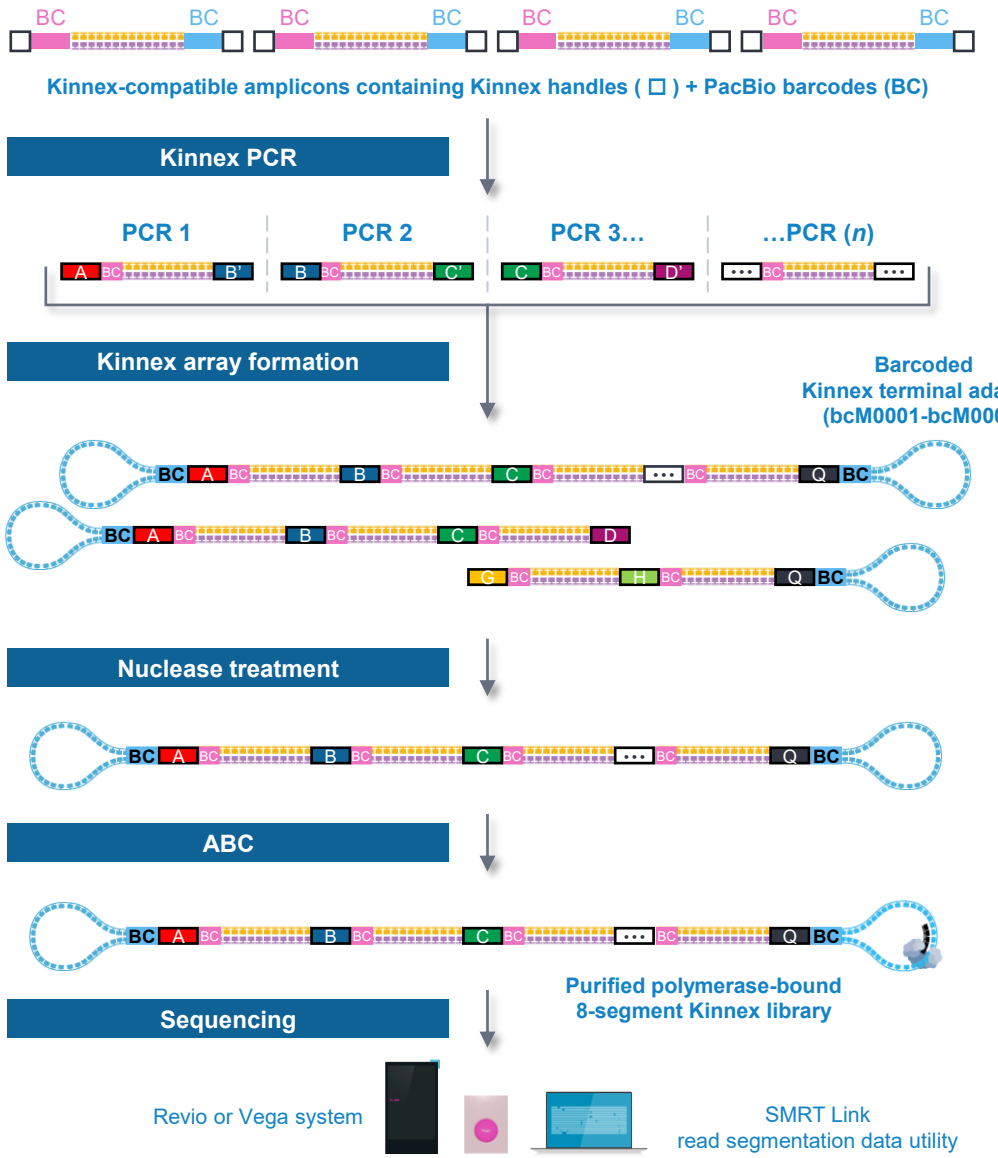
Includes Kinnex PCR kit, Kinnex concatenation and ancillary DNA cleanup reagents needed for incorporation of Kinnex segmentation adapters and Kinnex array formation for generating Kinnex libraries.

Kinnex 8-fold prep kit components

Component		Description
1		**Kinnex PCR 8-fold kit (12 rxn)** • Contains reagents for Kinnex PCR to incorporate segmentation adapters
2		**Kinnex concatenation kit 2.0 (12 rxn)** • Contains reagents for Kinnex array formation and SMRTbell template construction • Includes barcoded Kinnex adapter mixes (bcM0001 – bcM0004)
3		**SMRTbell cleanup beads** • For DNA cleanup
4		**Elution buffer** • For DNA cleanup

Kinnex 8-fold library prep, sequencing & analysis

Procedure & checklist – Preparing Kinnex libraries using Kinnex 8-fold prep kit (103-920-900)



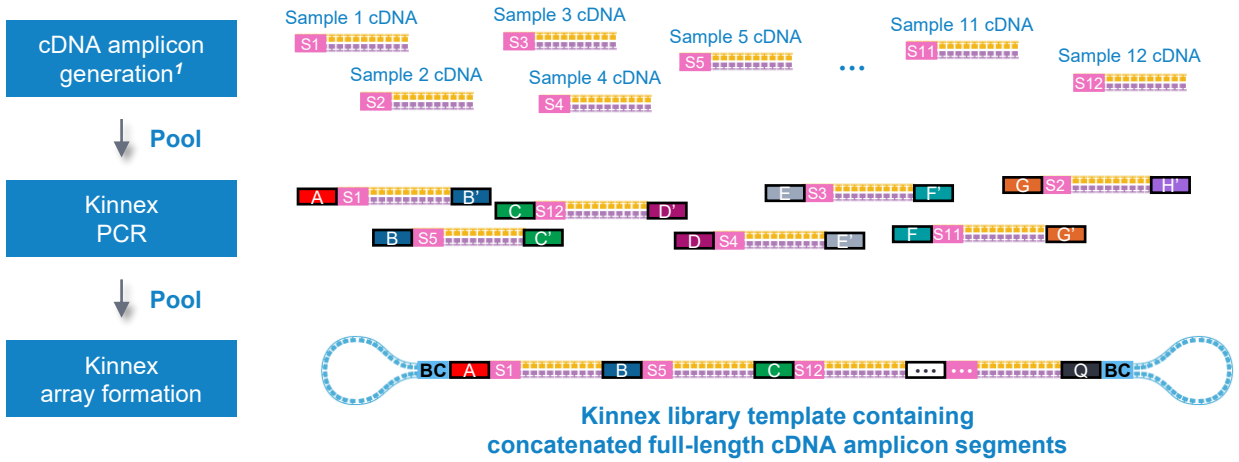
Kinnex library prep barcoding options for sample multiplexing

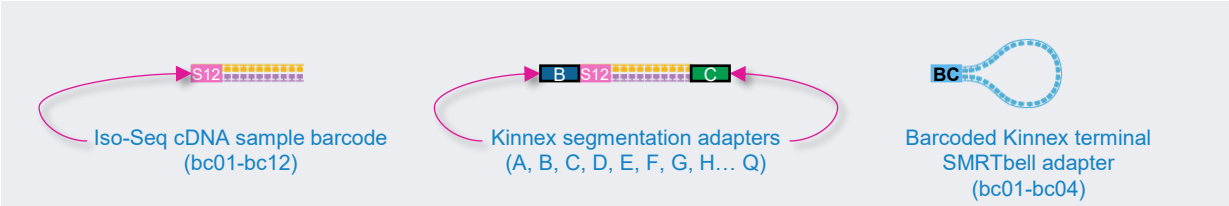
Kinnex library prep kits support sample multiplexing through combined use of amplicon-level sample barcodes + Kinnex library-level barcodes

Kinnex library preparation procedure supports sample multiplexing through combined use of:

- **Amplicon sample barcodes** incorporated during amplicon generation step using, for example, PacBio barcoded Iso-Seq cDNA PCR primers bc01–12[1], PacBio barcoded 16S PCR primers Fwd_01 – 12 + Rev_13 – 44,[2] etc.
- **Kinnex library barcodes** incorporated during Kinnex array formation step using PacBio barcoded Kinnex terminal SMRTbell adapters (bc01 – bc04)[3]

Example 1: Workflow to generate 48-plex Kinnex bulk RNA library using (12) barcoded Iso-Seq cDNA primers + (4) barcoded Kinnex SMRTbell adapters





Kinnex bulk RNA sample multiplexing can be achieved with one of the three following methods:

1. **Barcoded target-specific PCR primers**
   - Use up to **12** different barcoded Iso-Seq primers (bc01–12) in PacBio cDNA generation protocol[1] to amplify samples.
   - Amplified barcoded Iso-Seq cDNA samples can be pooled (up to 12-plex) and brought through a single Kinnex PCR reaction in Kinnex library prep protocol.
2. **Barcoded Kinnex adapters**
   - Use up to **4** different Kinnex barcoded adapters (bc01–04) in Kinnex array formation step in Kinnex library prep protocol workflow.
3. **A combination of the above two approaches** to achieve up to **48-plex**.

[1] To generate Kinnex compatible cDNA refer to protocol, ***Preparing Kinnex-compatible cDNA using Iso-Seq Express 2.0 kit*** (102-396-000).
[2] To generate full-length 16S rRNA amplicons refer to protocol, ***Preparing Kinnex-compatible 16S rRNA amplicons*** (103-885-200).
[3] Kinnex adapter barcode sequences can be downloaded from SMRT Link Data Management module.

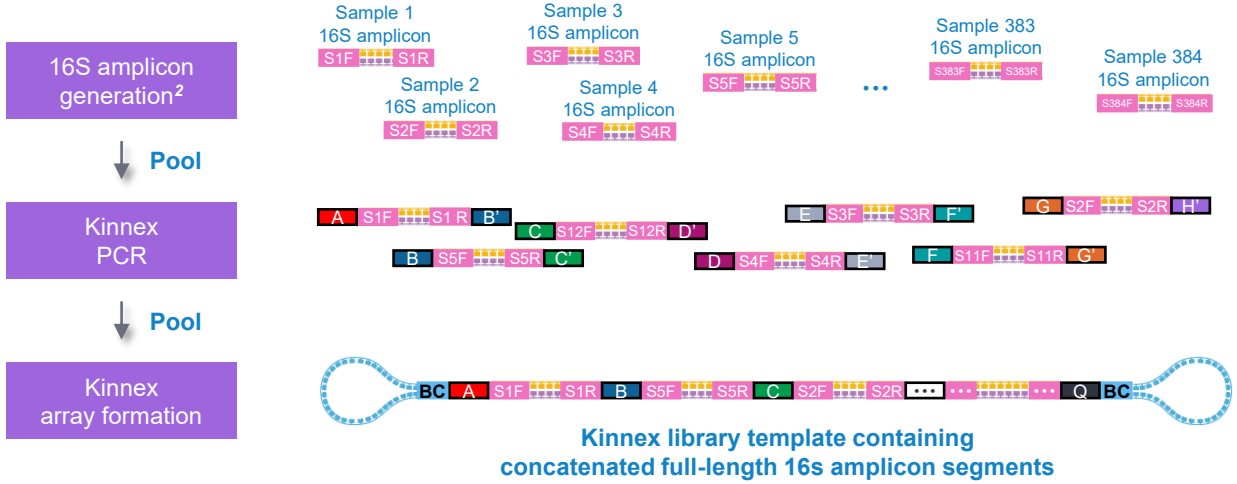
Kinnex library prep barcoding options for sample multiplexing (cont.)

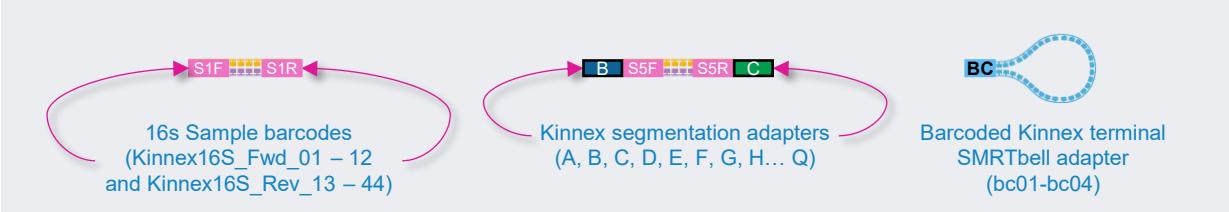
Kinnex library prep kits support sample multiplexing through combined use of amplicon-level sample barcodes + Kinnex library-level barcodes

Kinnex library preparation procedure supports sample multiplexing through combined use of:

- **Amplicon sample barcodes** incorporated during amplicon generation step using, for example, PacBio barcoded Iso-Seq cDNA PCR primers bc01–12[1], PacBio barcoded 16S PCR primers Fwd_01 – 12 + Rev_13 – 44,[2] etc.
- **Kinnex library barcodes** incorporated during Kinnex array formation step using PacBio barcoded Kinnex terminal SMRTbell adapters (bc01 – bc04)[3]

Example 2: Workflow to generate 1,536-plex Kinnex 16s rRNA library using (12) barcoded forward PCR primers + (32) barcoded reverse PCR primers + (4) barcoded Kinnex SMRTbell adapters





Kinnex 16s rRNA sample multiplexing can be achieved with one of the three following methods:

1. **Barcoded target-specific PCR primers**
   - Use up to **12** different barcoded 16S Forward PCR primers (Fwd_01 – 12) + **32** different barcoded 16S Rerverse PCR primers (Rev_13 – 44) in PacBio 16S amplicon generation protocol[2] to amplify samples.
   - Amplified barcoded 16S amplicon samples can be pooled (up to 384-plex) and brought through a single Kinnex PCR reaction in Kinnex library prep protocol.
2. **Barcoded Kinnex adapters**
   - Use up to **4** different Kinnex adapters (bc01–04) in Kinnex array formation step in Kinnex library prep protocol workflow.
3. **A combination of the above two approaches** to achieve up to **1,536-plex**.

[1] To generate Kinnex compatible cDNA refer to protocol, ***Preparing Kinnex-compatible cDNA using Iso-Seq Express 2.0 kit*** (102-396-000).
[2] To generate full-length 16S rRNA amplicons refer to protocol, ***Preparing Kinnex-compatible 16S rRNA amplicons*** (103-885-200).
[3] Kinnex adapter barcode sequences can be downloaded from SMRT Link Data Management module.

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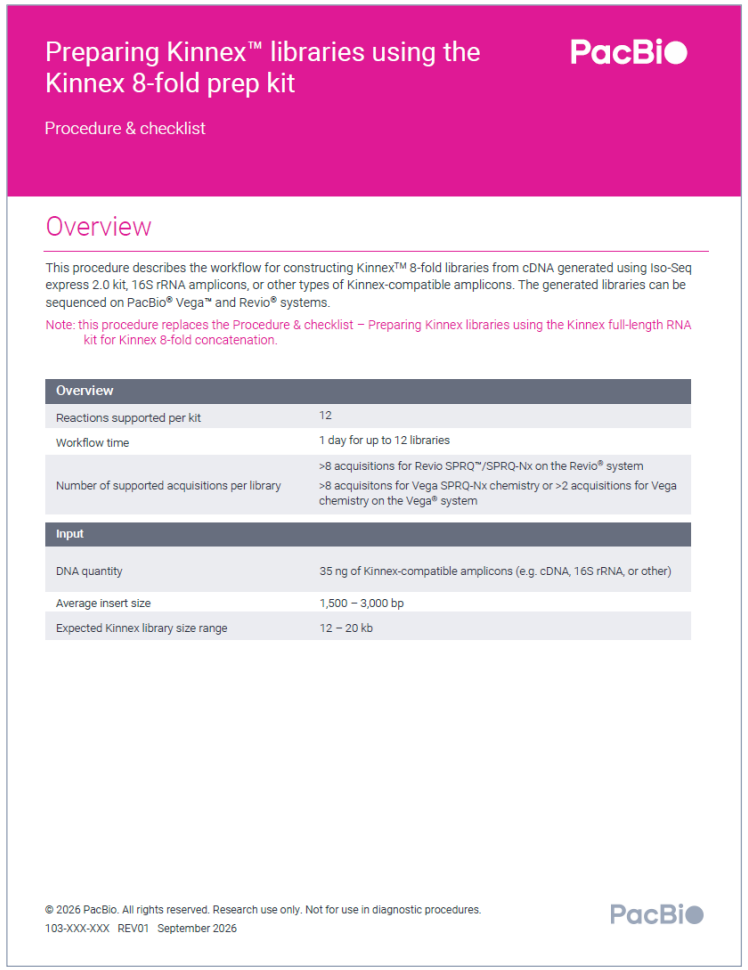
Kinnex 8-fold library preparation workflow

Procedure & checklist – Preparing Kinnex libraries using Kinnex 8-fold prep kit (103-920-900)

Procedure & checklist 103-920-900 describes the workflow for constructing Kinnex 8-fold libraries from cDNA generated using Iso-Seq express 2.0 kit, 16S rRNA amplicons, or other types of Kinnex-compatible amplicons. The generated libraries can be sequenced on PacBio Vega and Revio systems.

Procedure & checklist contents

1. Kinnex-compatible amplicon input QC recommendations and general best practices for reagent & sample handling.
2. Enzymatic workflow steps for construction of 8-segment Kinnex arrays from Kinnex-compatible amplicons.
3. Enzymatic workflow steps for nuclease treatment of Kinnex libraries.
4. Workflow steps for final cleanup of Kinnex libraries using SMRTbell cleanup beads.
5. Sample setup ABC[1] workflow steps to prepare Kinnex libraries for sequencing on Revio and Vega systems.



Preparing Kinnex™ libraries using the Kinnex 8-fold prep kit

PacBio

Procedure & checklist

Overview

This procedure describes the workflow for constructing Kinnex™ 8-fold libraries from cDNA generated using Iso-Seq express 2.0 kit, 16S rRNA amplicons, or other types of Kinnex-compatible amplicons. The generated libraries can be sequenced on PacBio® Vega™ and Revio® systems.

Note: this procedure replaces the Procedure & checklist – Preparing Kinnex libraries using the Kinnex full-length RNA kit for Kinnex 8-fold concatenation.

Overview	
Reactions supported per kit	12
Workflow time	1 day for up to 12 libraries
Number of supported acquisitions per library	>8 acquisitions for Revio SPRQ™/SPRQ-Nx on the Revio® system >8 acquisitons for Vega SPRQ-Nx chemistry or >2 acquisitions for Vega chemistry on the Vega® system
Input	
DNA quantity	35 ng of Kinnex-compatible amplicons (e.g. cDNA, 16S rRNA, or other)
Average insert size	1,500 – 3,000 bp
Expected Kinnex library size range	12 – 20 kb

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PacBio Documentation (103-920-900)

[1] ABC = Anneal primer / Bind polymerase / Clean up bound complex

Kinnex 8-fold library preparation and sequencing workflow key steps

Kinnex library construction steps can be completed within 1 day[1]

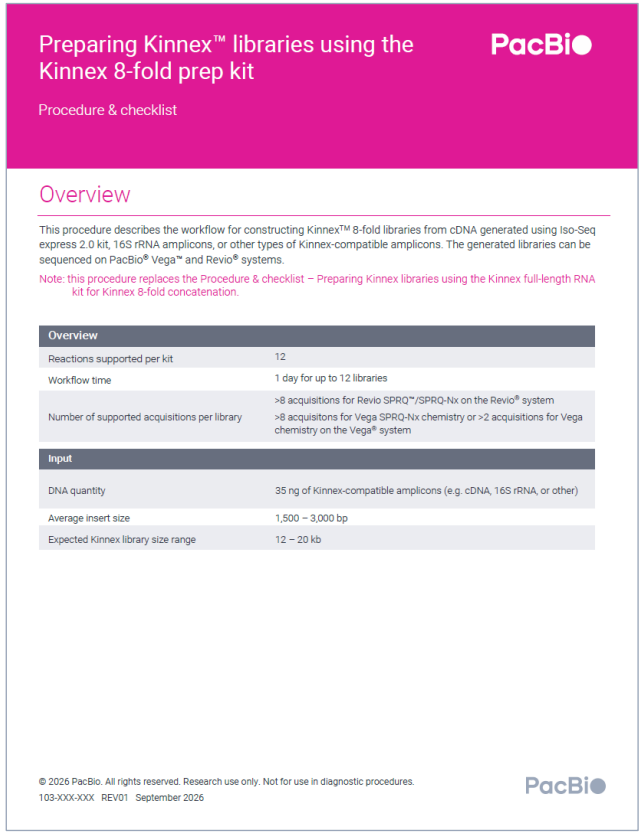
KCAG + QC	Kinnex-compatible amplicon generation[2]	Example: Bulk cDNA 16S amplicons	PacBio or other third-party reagents
KCAG + QC	1 Input quality control	0.5 hrs	Qubit 4 fluorometer system (ThermoFisher Sci. Q33238) DNA sizing QC tool (Agilent TapeStation or other tool)
	Safe stop		
Kinnex library prep	2 Kinnex PCR + cleanup	2 hrs	
	Safe stop		
Kinnex library prep	3 Kinnex array formation + cleanup	2 hrs	Kinnex 8-fold prep kit (103-072-000)
	Safe stop		
Kinnex library prep	4 Nuclease treatment + cleanup	0.5 hrs	
	Safe stop		
ABC	5 Annealing, binding & cleanup (ABC)	1 hr	Revio SPRQ polymerase kit (103-496-900) *or* Vega SPRQ polymerase kit (103-794-300)

1 DAY

Refer to appropriate PacBio or other third-party protocol documenation[2,3]

Kinnex 8-fold library prep protocol reference

Procedure & checklist – Preparing Kinnex libraries using Kinnex 8-fold prep kit (103-920-900)



Preparing Kinnex™ libraries using the Kinnex 8-fold prep kit

PacBio

Procedure & checklist

Overview

This procedure describes the workflow for constructing Kinnex™ 8-fold libraries from cDNA generated using Iso-Seq express 2.0 kit, 16S rRNA amplicons, or other types of Kinnex-compatible amplicons. The generated libraries can be sequenced on PacBio® Vega™ and Revio® systems.

Note: this procedure replaces the Procedure & checklist – Preparing Kinnex libraries using the Kinnex full-length RNA kit for Kinnex 8-fold concatenation.

Overview	
Reactions supported per kit	12
Workflow time	1 day for up to 12 libraries
Number of supported acquisitions per library	>8 acquisitions for Revio SPRQ™/SPRQ-Nx on the Revio® system >8 acquisitons for Vega SPRQ-Nx chemistry or >2 acquisitions for Vega chemistry on the Vega® system
Input	
DNA quantity	35 ng of Kinnex-compatible amplicons (e.g. cDNA, 16S rRNA, or other)
Average insert size	1,500 – 3,000 bp
Expected Kinnex library size range	12 – 20 kb

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[1] Example workflow step times shown are for manually processing up 12 Kinnex libraries using Kinnex 8-fold prep kit.
[2] To generate Kinnex compatible cDNA refer to protocol, ***Preparing Kinnex-compatible cDNA using Iso-Seq Express 2.0 kit*** (102-396-000).
[3] To generate full-length 16S rRNA amplicons refer to protocol, ***Preparing Kinnex-compatible 16S rRNA amplicons*** (103-885-200).

General best practices recommendations for preparing Kinnex 8-fold libraries

Reagent and sample handling

Kinnex library prep reagents

- Room temperature is defined as any temperature in the range of 18–23°C for this protocol
- Thaw Kinnex array and repair buffer, nuclease buffer, Kinnex adapter mix, and primers at room temperature
- Briefly vortex reagent buffers and Kinnex adapters prior to use. Enzyme mixes do not require vortexing
- Quick-spin all reagents in a microcentrifuge to collect liquid at tube bottom prior to use
- Keep all temperature-sensitive reagents on ice
- Bring SMRTbell cleanup beads and Qubit 1X dsDNA HS reagents to room temperature for 30 minutes prior to use
- Ensure SMRTbell cleanup beads are thoroughly mixed to a homogenous suspension before accurately pipetting the required volume, as small changes in bead volume can significantly alter the size distribution of your sample
- During bead binding and elution steps, pipette-mixing until beads are distributed evenly in solution
- Mix each reaction by pipetting up and down 10 times or until fully resuspended. Avoid introducing bubbles.
- In Kinnex PCR, keep sample(s) on ice until thermal cycler lid has reached 105°C to avoid digestion of primers by polymerase exonuclease activity
- The workflow takes approximately 1 day to complete
    - If a stop is necessary, refer to the workflow for safe stopping points

Kinnex 8-fold prep kit (103-072-000)

Temperature-sensitive reagents Kinnex PCR 8-fold kit 103-071-600	
Tube color	Reagent
Green	Kinnex PCR mix 103-107-700
Orange	Kinnex primer mix A 103-107-800
	Kinnex primer mix B 103-107-900
	Kinnex primer mix C 103-108-000
	Kinnex primer mix D 103-108-100
	Kinnex primer mix E 103-108-200
	Kinnex primer mix F 103-108-300
	Kinnex primer mix G 103-108-400
	Kinnex primer mix HQ 103-108-500

Temperature-sensitive reagents Kinnex concatenation kit 2.0 103-836-400	
Tube color	Reagent
Red	Kinnex enzyme 103-110-400
Yellow	Kinnex ligase 103-110-500
White	Kinnex array and repair buffer 103-110-300
Green	Kinnex repair mix 103-837-500
Light Purple	Nuclease buffer 103-110-200
Light Green	Nuclease mix 103-110-100
Blue	Kinnex adapter bc01 mix 103-109-600
	Kinnex adapter bc02 mix 103-109-700
	Kinnex adapter bc03 mix 103-109-800
	Kinnex adapter bc04 mix 103-109-900

General best practices recommendations for preparing Kinnex 8-fold libraries (cont.)

Reagent and sample handling

Sequencing prep reagents (for sample setup ABC)

- Once thawed, place reaction buffers and sequencing primer on-ice prior to making master mix. The Loading buffer should be left at room-temperature.
- **Note:** The Loading buffer is light sensitive and should be protected from light when not in use.
- **Keep the following reagents on a cold block or ice:**
  - Sequencing polymerase
  - Sequencing control
- **Bring the following reagents up to room temperature 30 minutes prior to use:**
  - Loading buffer
  - SMRTbell cleanup beads

Thaw the following reagents at room temperature:

Component	Tube color
Annealing buffer	Light blue
Kinnex sequencing primer	Light purple
Polymerase buffer	Yellow
Loading buffer	Green
Dilution buffer	Blue

Input quality control

Input quality control is highly recommended before proceeding to the Kinnex PCR step

Procedure & checklist – Preparing Kinnex libraries using Kinnex 8-fold prep kit (103-920-900)

1 Input quality control
↓
2 Kinnex PCR + cleanup
↓ Safe stop
3 Kinnex array formation + cleanup
↓
4 Nuclease treatment + cleanup
↓ Safe stop
5 Annealing, binding & cleanup (ABC)

- The recommended input for Kinnex 8-fold PCR is 35 ng of Kinnex-compatible cDNA or other types of amplicons[1] with an average size distribution between 1.5 – 3 kb
- Prior to Kinnex library preparation, evaluate the size distribution of the input amplicons on an Agilent Bioanalyzer system using the high-sensitivity DNA kit or on an Agilent TapeStation using the D5000/High Sensitivity D5000 ScreenTape Assay to determine whether it is suitable for the protocol
  - The recommended peak or average amplicon size should be between 1.5 and 3 kb for Kinnex library preparation using the Kinnex 8-fold prep kit
  - Smaller cDNA or amplicons may be used if desired. Kinnex 12-fold and 16-fold kits are generally recommended when concatenating smaller amplicons

Step 1
procedural summary

DNA sizing QC

Amplicon QC

↓

Proceed to **Step 2**

[1] To generate Kinnex-compatible cDNA please refer to protocol, ***Preparing Kinnex-compatible cDNA using Iso-Seq Express 2.0 kit*** (102-396-000). To generate Kinnex-compatible 16S rRNA amplicons please refer to protocol, ***Preparing Kinnex-compatible 16S rRNA amplicons*** (103-885-200). To generate other Kinnex-compatible amplicons please refer to the technical note, ***Concatenating amplicons using PacBio Kinnex kits to increase throughput*** (102-326-636) for the input, size, and PCR requirements.

Kinnex PCR

In this step, incorporate programmable Kinnex segmentation adapter sequences into amplified DNA products

Procedure & checklist – Preparing Kinnex libraries using Kinnex 8-fold prep kit (103-920-900)

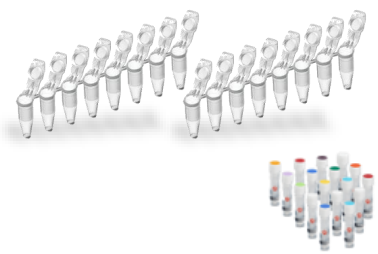
1. Input quality control
2. Kinnex PCR + cleanup
   - Safe stop
3. Kinnex array formation + cleanup
4. Nuclease treatment + cleanup
   - Safe stop
5. Annealing, binding & cleanup (ABC)

Step 2 procedural description

Set up 8 parallel Kinnex PCR reactions per sample with premixed Kinnex primers to generate amplified DNA products containing programmable sequences at both ends

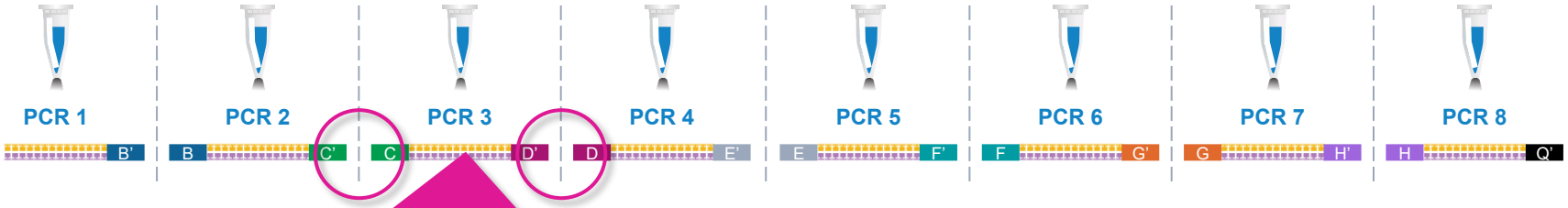
Input amplicon sample

Distribute input amplicon sample + Kinnex PCR reaction mix into 8 PCR tubes or 8 wells in a 96-well plate



OR

Perform Kinnex PCR by adding premixed Kinnex primer pair solutions



Example: Amplified products from Kinnex PCR 3 contain flanking segmentation adapter sequences that are **complementary** to the ends of amplified products from Kinnex PCR 2 & Kinnex PCR 4

Pool 8 Kinnex PCR products together and perform 1.05X SMRTbell bead cleanup

Kinnex PCR-amplified amplicon sample ready for Kinnex array formation step

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Kinnex PCR (cont.)

Procedural summary

Step 2 procedural summary

Procedure & checklist – Preparing Kinnex libraries using Kinnex 8-fold prep kit (103-920-900)

1. Input quality control
2. Kinnex PCR + cleanup
   - Safe stop
3. Kinnex array formation + cleanup
4. Nuclease treatment + cleanup
   - Safe stop
5. Annealing, binding & cleanup (ABC)

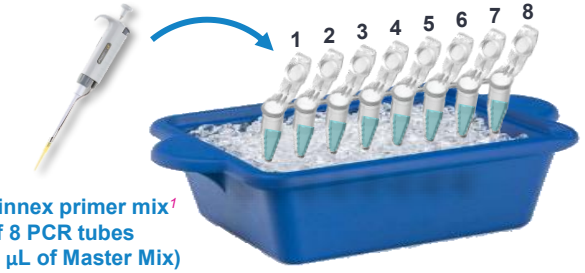
2.1 Kinnex PCR

X = 35 (ng) / input amplicon concentration (ng/µL)

88 - X µL PCR-grade water + 110 µL Kinnex PCR mix + X µL 35 ng of input amplicon = 198 µL master mix → 198 µL master mix

Aliquot 22.5 µL of Master Mix into each of the 8 PCR tubes (for 8X concatenation) on ice

Kinnex primer mix



Add 2.5 µL of Kinnex primer mix[1] into each of 8 PCR tubes (containing 22.5 µL of Master Mix)

Kinnex PCR Rx tube	8X concatenation	Tube color
1 [25 µL Rx volume]	Kinnex primer mix A	Orange
2 [25 µL Rx volume]	Kinnex primer mix B	
3 [25 µL Rx volume]	Kinnex primer mix C	
4 [25 µL Rx volume]	Kinnex primer mix D	
5 [25 µL Rx volume]	Kinnex primer mix E	
6 [25 µL Rx volume]	Kinnex primer mix F	
7 [25 µL Rx volume]	Kinnex primer mix G	
8 [25 µL Rx volume]	Kinnex primer mix HQ	

25 µL PCR Rx vol.

Run Kinnex PCR program (9 cycles)

2.2 Pooling of 8 Kinnex PCR products and 1.05X SMRTbell cleanup

Kinnex PCR 1: 23 µL + Kinnex PCR 2: 23 µL + Kinnex PCR 3: 23 µL + ... + Kinnex PCR 8: 23 µL → **Pooled Kinnex PCR reactions**: 184 µL total pooled vol.

184 µL Pooled Kinnex PCR reactions + 193 µL 1.05X SMRTbell cleanup beads → 40 µL purified Kinnex PCR sample

Proceed to **Step 3**

[1] Kinnex PCR 8-fold kit (103-071-600) contains eight different Kinnex primer mixes (Kinnex primer mix A/B/C/D/E/F/G/HQ).

Kinnex PCR (cont.)

Procedural notes

Procedure & checklist – Preparing Kinnex libraries using Kinnex 8-fold prep kit (103-920-900)

1. Input quality control
2. Kinnex PCR + cleanup
   - Safe stop
3. Kinnex array formation + cleanup
4. Nuclease treatment + cleanup
   - Safe stop
5. Annealing, binding & cleanup (ABC)

2.1 Kinnex PCR

✔	Step	Instructions
	2.1.1	Thaw primers. The entire volume of primers can be transferred to an 8-tube strip for ease of use with a multi-channel pipette.

	8X concatenation	Tube color	PN
1	Kinnex primer mix A	Orange	103-107-800
2	Kinnex primer mix B		103-107-900
3	Kinnex primer mix C		103-108-000
4	Kinnex primer mix D		103-108-100
5	Kinnex primer mix E		103-108-200
6	Kinnex primer mix F		103-108-300
7	Kinnex primer mix G		103-108-400
8	Kinnex primer mix HQ		103-108-500

2.1.2 Briefly vortex to mix, then quick-spin to collect liquid and place the primer mixes on ice.

2.1.3 Thaw the following components, briefly vortex to mix, then quick-spin to collect liquid and place on ice. Add the below components to a LoBind tube, pulse vortex to mix and quick-spin. Place master mix on ice.

Reaction Mix 1 (RM1):

Master mix components	Volume for 8X concatenation*
PCR-grade water	88-X µL
Kinnex PCR mix	110 µL
35 ng of Kinnex-compatible amplicon	X µL
Total volume	198 µL

X = 35 (ng)/input concentration (ng/µL), where the input can be from a single-plex or multiplex sample.
*10% overage included

2.1.4. Aliquot **22.5 µL** of **RM1** into each of the 8 PCR tubes (for 8X concatenation) **on ice.**

2.1.5 Add **2.5 µL** of the appropriate Kinnex primer mix into each respective PCR tube on ice.

2.1.6 Thoroughly mix by pipetting up and down 10 times. Quick-spin to collect all liquid.

Run the Kinnex PCR program and keep samples on ice until thermal cycler lid has heated to 105°C. The duration of PCR is approximately 1 hour.

Kinnex PCR program

Heated lid set at 105°C. Reaction volume set to 25 µL.

- Can transfer entire volume of primers to PCR tubes for ease of use with multi-channel pipettes (8 primer mix tubes)

- Set up Kinnex PCR reactions **ON ICE**
- PCR polymerase 3'→5' exonuclease activity negatively impacts amplification yield if prepared at room temp.

- **Critical step! Correct setup of all 8 Kinnex PCR reactions is required** – any missing/incorrect Kinnex primer pairs will result in no/low SMRTbell yield

- Set up on ice and add PCR reaction to thermal cycler after lid has preheated to 105°C to avoid digestion of primers by polymerase exonuclease activity

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Kinnex PCR (cont.)

Procedural notes

Procedure & checklist – Preparing Kinnex libraries using Kinnex 8-fold prep kit (103-920-900)

1 Input quality control

↓

2 Kinnex PCR + cleanup

↓ Safe stop

3 Kinnex array formation + cleanup

↓

4 Nuclease treatment + cleanup

↓ Safe stop

5 Annealing, binding & cleanup (ABC)

2.2 Pooling of 8 Kinnex PCR products and 1.05X SMRTbell cleanup

✔	Step	Instructions
	2.2.1	Add exactly **23 µL** from each of the 8 PCR reactions into a 1.5 mL tube for a total volume of **184 µL**.
	2.2.2	Add 193 µL (**1.05X** v/v) of resuspended, room-temperature SMRTbell cleanup beads to a tube of pooled Kinnex PCR amplicon. **The correct ratio of beads to pooled sample is critical at this step.**
	2.2.3	Pipette-mix the beads until evenly distributed.
	2.2.4	Quick-spin the tube in a microcentrifuge to collect liquid.
	2.2.5	Incubate at **room temperature** for **10 minutes** to allow the DNA to bind beads
	2.2.6	Place the tube in a magnetic separation rack until the beads separate fully from the solution.
	2.2.11	Remove the tube from the magnetic rack. **Immediately** add **40 µL** of **elution buffer** to the tube and resuspend the beads by pipetting 10 times or until evenly distributed.
	2.2.12	Quick-spin the tube in a microcentrifuge to collect liquid.
	2.2.13	Incubate at **room temperature** for **5 minutes** to elute DNA.
	2.2.14	Place tube in a magnetic separation rack until beads separate fully from the solution.
	2.2.15	Slowly aspirate the cleared eluate without disturbing the beads. Transfer 40 µL of the eluate to a new PCR tube strip. Discard old tube with beads.
	2.2.16	Evaluate sample concentration. • Take a 1 µL aliquot from each tube, dilute with 9 µL of elution buffer. Using 1 µL of the dilution, measure DNA concentration with a Qubit fluorometer using the 1x dsDNA HS kit. Yield typically ranges from 5–9 µg.
	2.2.17	Proceed to the next step of the protocol if sample quantity is acceptable (maximum input: 5 µg). Do not proceed if less than 3 µg is available. Note: If the Kinnex PCR yield is less than 3 µg, repeat Kinnex PCR and quantifying each individual tube with 1X dsDNA HS kit for low yield troubleshooting.

SAFE STOPPING POINT -- Store at 4°C or -20°C for long-term storage

- Pool **exactly 23 µL** from each Kinnex PCR reaction in a clean 1.5 mL DNA LoBind tube for a total combined volume of 184 µL

- Add **193 µL** of SMRTbell cleanup beads (1.05X)
- Kinnex PCR mix significantly increases stringency of SMRTbell clean up beads, **so accurate pipetting is critical**

- Perform DNA concentration QC using Qubit dsDNA HS kit
- Typical yield of Kinnex PCR products is **5–9 µg**

- Proceed to the next step of the protocol if sample quantity is acceptable (maximum input: 5 µg)
- **Do not proceed if less than 3 µg is available**
- **Note:** If Kinnex PCR yield is less than 3 µg, repeat Kinnex PCR and quantify each individual tube with Qubit 1X dsDNA HS assay for low yield troubleshooting

[1] DNA concentration QC measurements for individual Kinnex PCR reactions prior to pooling are not required.

Kinnex array formation

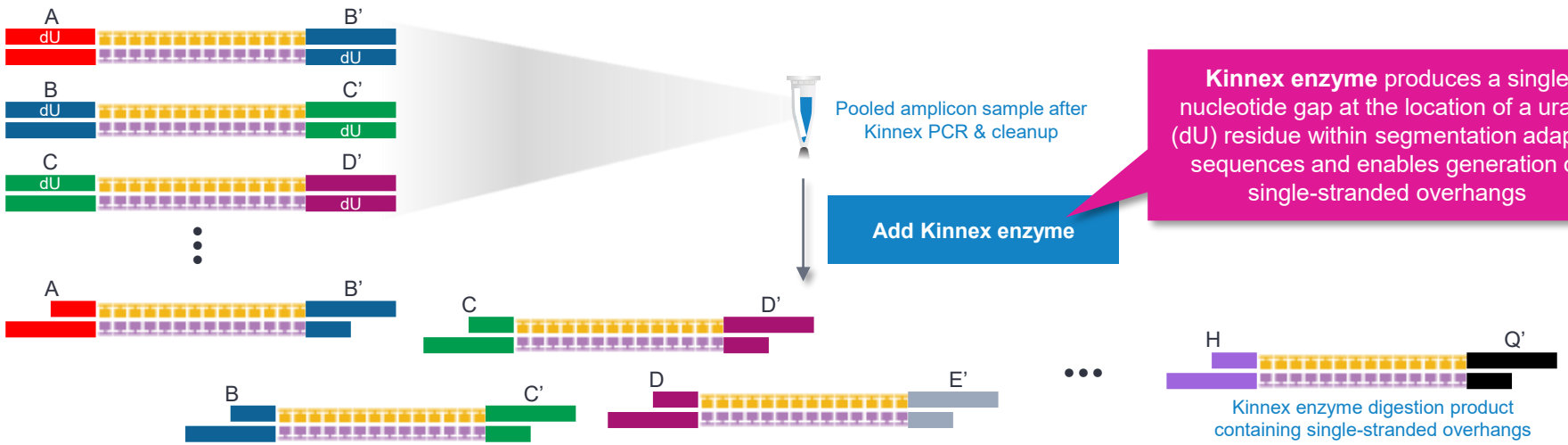
In this step, assemble amplicon DNA “segments” containing programmable ends into a linear array

Procedure & checklist – Preparing Kinnex libraries using Kinnex 8-fold prep kit (103-920-900)

1. Input quality control
2. Kinnex PCR + cleanup
   - Safe stop
3. **Kinnex array formation + cleanup**
4. Nuclease treatment + cleanup
   - Safe stop
5. Annealing, binding & cleanup (ABC)

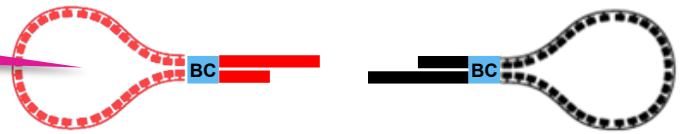
Step 3 procedural description

Treat pooled Kinnex PCR products with Kinnex enzyme to create single-stranded overhangs to enable subsequent directional assembly of amplicon inserts into a linear, ordered array



Kinnex enzyme produces a single nucleotide gap at the location of a uracil (dU) residue within segmentation adapter sequences and enables generation of single-stranded overhangs

Barcoded Kinnex terminal adapters[1] are ligated to specific overhang sequences at array ends



Barcoded forward Kinnex terminal adapter

Barcoded reverse Kinnex terminal adapter

Add Kinnex adapters + Kinnex ligase

Add **Kinnex ligase** to enable directional assembly of amplicon DNA segments containing “sticky ends” into a linear, ordered array

Complete 8-segment transcript array molecule containing 2 Kinnex terminal adapters

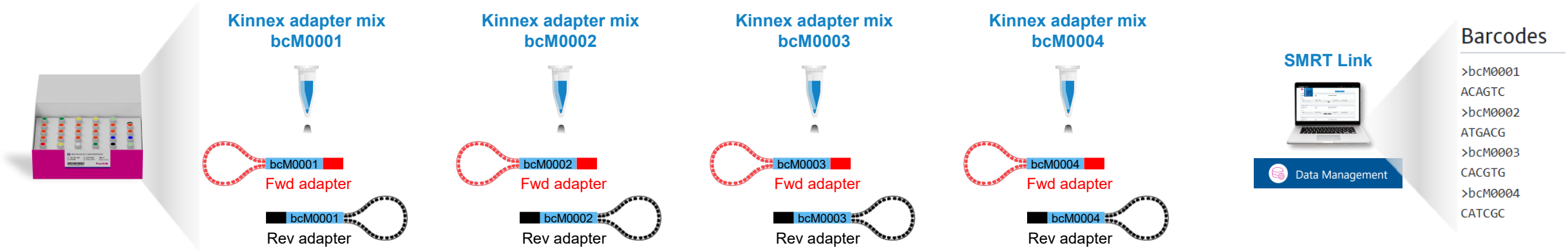
Kinnex library prep workflow supports incorporation of PacBio barcodes at the array formation step to enable up to **4-plex sample multiplexing at the Kinnex library level**

[1] **Note:** Four barcoded terminal Kinnex adapters (Kinnex adapter bcM0001-M0004) are available for Kinnex array formation step. **Note:** Kinnex concatenation workflow is not compatible with standard SMRTbell adapters from SMRTbell prep kit 3.0 and is also not compatible with SMRTbell adapter index plate 96A/B/C/D.

Kinnex array formation (cont.)

Kinnex terminal adapters incorporate barcode sequences to enable up to 4-plex sample multiplexing at the Kinnex library level

- Kinnex adapters contain barcode sequences[1] to enable (optional) sample multiplexing at the Kinnex (SMRTbell) library level (up to 4-plex)
  - Forward and reverse Kinnex adapter pairs are pre-mixed in Kinnex concatenation kits
  - Kinnex concatenation kits contain a total of 4 barcoded Kinnex adapter mixes (bcM0001-bcM0004) to enable multiplexing of up to 4 samples per SMRT Cell



Example complete Kinnex library molecule containing barcoded Kinnex terminal adapters at both ends

Barcoded forward Kinnex terminal adapter

Both forward & reverse Kinnex terminal adapters contain the same barcode sequence

Barcoded reverse Kinnex terminal adapter

[1] Kinnex adapter barcode sequences can be downloaded from SMRT Link Data Management module.

Kinnex array formation (cont.)

Kinnex terminal adapters use a new design that enables improved HiFi sequencing performance

- Kinnex adapters enable longer polymerase read length performance and improved sample loading efficiency
  - → Improved overall HiFi data yield

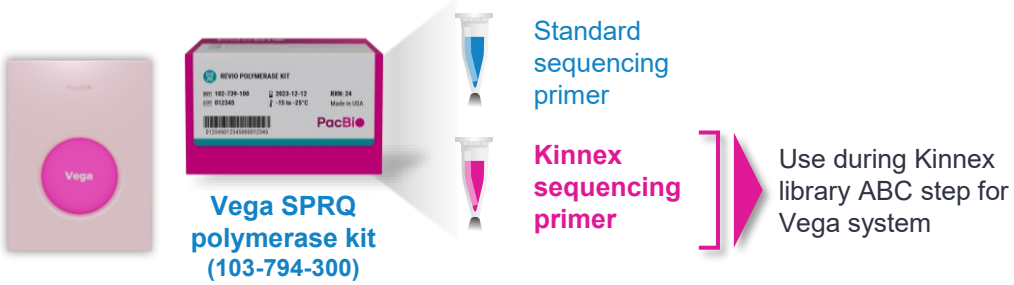
Forward and reverse Kinnex terminal adapters have **different structures**

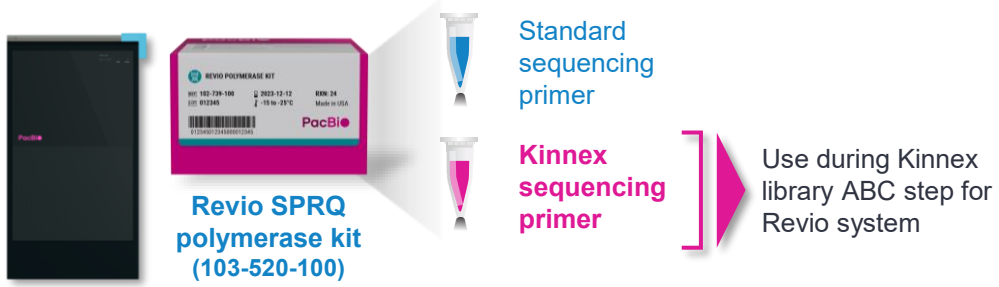
Annealed & bound Kinnex library molecule

Barcoded forward Kinnex terminal adapter

Barcoded reverse Kinnex terminal adapter

- Kinnex adapters require **Kinnex sequencing primer**[1] (instead of Standard sequencing primer) for the annealing reaction during sample setup ABC step





[1] **Note:** Revio SPRQ polymerase kit and Vega SPRQ polymerase kit contain both the Standard sequencing primer and the Kinnex sequencing primer.

Kinnex array formation (cont.)

Procedural summary

Procedure & checklist – Preparing Kinnex libraries using Kinnex 8-fold prep kit (103-920-900)

1. Input quality control
2. Kinnex PCR + cleanup
   - Safe stop
3. **Kinnex array formation + cleanup**
4. Nuclease treatment + cleanup
   - Safe stop
5. Annealing, binding & cleanup (ABC)

Step 3 procedural summary

3.1 Kinnex array formation

5 µg in 39 µL (128 ng/µL) Kinnex PCR sample from Step 4 + 2 µL Barcoded Kinnex adapter[1] +

Master Mix (17 µL): 7 µL Kinnex array and repair buffer + 4 µL Kinnex enzyme + 6 µL Kinnex ligase

→ 58 µL total Rx vol. → Run Kinnex primer digestion/ligation program (45°C 60 min, Hold at 4°C)

→ 58 µL Kinnex array sample + 2 µL Kinnex repair mix → 60 µL total Rx vol. → Run DNA damage repair program (37°C 15 min, 45°C 15 min, Hold at 4°C)

3.2 1X SMRTbell cleanup

60 µL Kinnex library + 60 µL 1X SMRTbell cleanup beads → 40 µL purified Kinnex library

Proceed to **Step 4**

[1] Kinnex concatenation kit 2.0 contains four different barcoded Kinnex adapter mixes (bcM0001–bcM0004) → Select one barcode to use per Kinnex library preparation reaction.

Kinnex array formation (cont.)

Procedural notes

Procedure & checklist – Preparing Kinnex libraries using Kinnex 8-fold prep kit (103-920-900)

1 Input quality control

↓

2 Kinnex PCR + cleanup

↓ Safe stop

3 Kinnex array formation + cleanup

↓

4 Nuclease treatment + cleanup

↓ Safe stop

5 Annealing, binding & cleanup (ABC)

3.1 Kinnex array formation

✔	Step	Instructions
	3.1.1	In a 0.2 mL PCR tube, add 5 µg of pooled Kinnex PCR sample from Section 2 in 39 µL of volume (128 ng/µL). Dilute with elution buffer going into this step if the sample is too concentrated.
		Add 2 µL of Kinnex adapter (bc01–04; select one barcode per library preparation).

Tube color	Components	Volume
Blue	Kinnex barcoded adapter	2 µL

	3.1.2	Add the following components in the listed order. If processing multiple samples, make a master mix with 10% overage. Pipette mix maste… Reaction Mix 2 (RM2):

Tube color	Components	Volume

		Add **17 µL** of **RM2** to the PCR tube containing sample and Kinnex barcoded adapter for a final volume of 56 µL. Pipette-mix and run the Kinnex primer digestion/ligation program.
	3.1.3	**Kinnex primer digestion/ligation program** Heated lid set at ≥55°C. Reaction volume set to 60 µL

Step	Temperature	Duration
1	45°C	60 min
2	4°C	Hold

	3.1.4	After running the Kinnex primer digestion/ligation program, add 2 µL of Kinnex repair mix directly to the Kinnex primer digestion/ligation sample.

Tube color	Components	Volume
Green	Kinnex repair mix	2 µL

	3.1.5	Thoroughly mix by pipetting up and down 10 times and then quick spin to collect all liquid.
		Run the Kinnex repair program. **Kinnex repair program** Heated lid set at ≥55°C. Reaction volume set to 60 µL.
	3.1.6	

Step	Temperature	Duration

- Recommended input amount to proceed with Kinnex array formation is **5 µg** of Kinnex PCR amplicons (from Step 2)
  - Proceeding with <3 µg is **not recommended** since lower input amounts may lead to insufficient final library yields to enable optimal sequencing results

- **IMPORTANT:** If combining multiple barcoded Kinnex libraries for sequencing, make sure each library uses one of the 4 different Kinnex barcoded adapters

- Add **Kinnex repair mix** to repair nicked / damaged DNA sites within newly formed Kinnex array products

- After running **Kinnex repair program**, perform 1X SMRTbell bead cleanup (Step 3.2)

PacBio

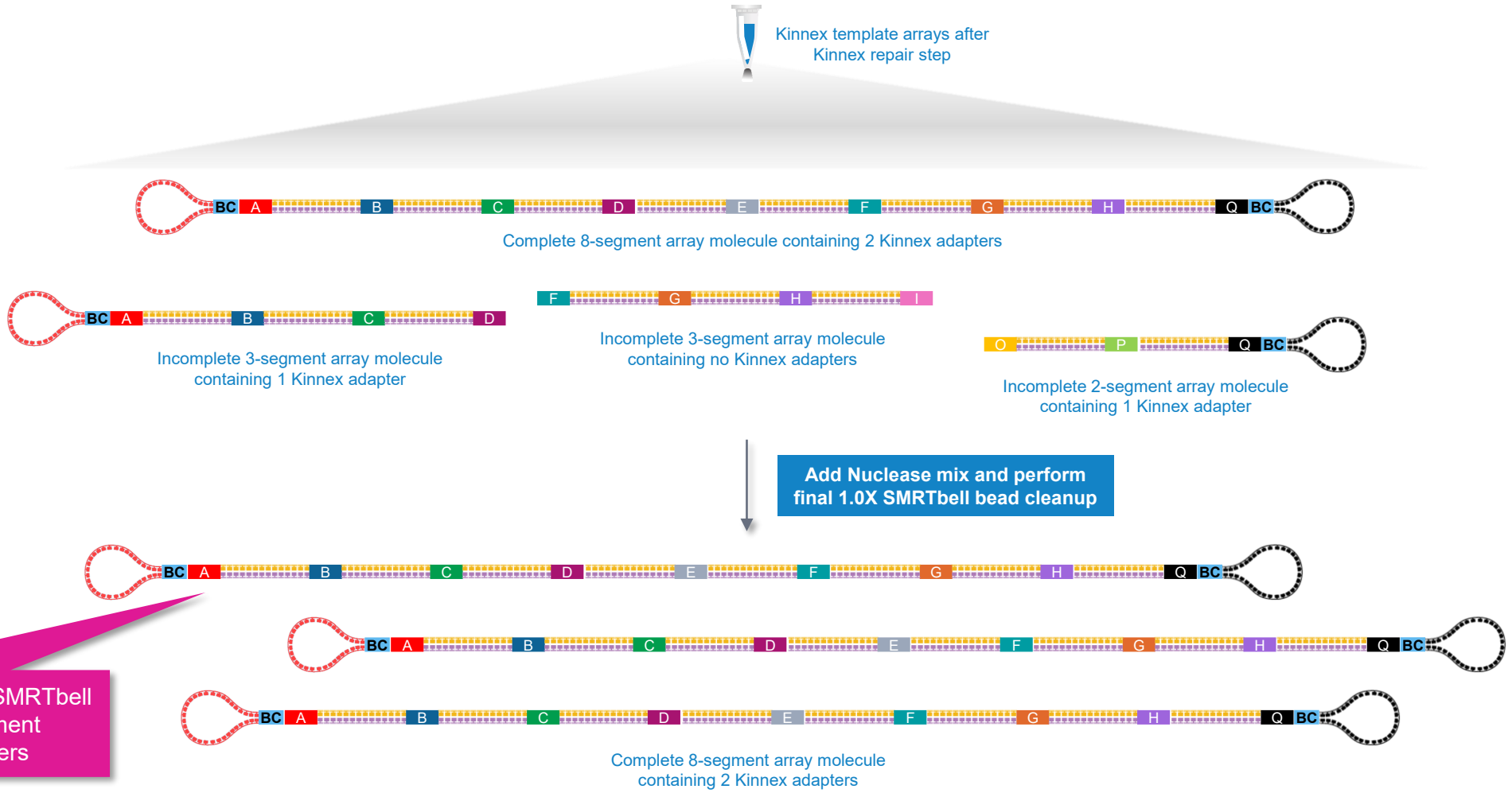
Nuclease treatment & cleanup

Perform nuclease treatment and final SMRTbell bead cleanup to remove incomplete SMRTbell template arrays

Procedure & checklist – Preparing Kinnex libraries using Kinnex 8-fold prep kit (103-920-900)

1. Input quality control
2. Kinnex PCR + cleanup
   - Safe stop
3. Kinnex array formation + cleanup
4. **Nuclease treatment + cleanup**
   - Safe stop
5. Annealing, binding & cleanup (ABC)

Step 4 procedural description



- After nuclease treatment, most remaining SMRTbell templates are complete (full-length) 8-segment array molecules capped with Kinnex adapters

PacBio

Nuclease treatment & cleanup (cont.)

Procedural summary

Procedure & checklist – Preparing Kinnex libraries using Kinnex 8-fold prep kit (103-920-900)

1. Input quality control
2. Kinnex PCR + cleanup
   - Safe stop
3. Kinnex array formation + cleanup
4. Nuclease treatment + cleanup
   - Safe stop
5. Annealing, binding & cleanup (ABC)

Step 4 procedural summary

4.1 Nuclease treatment

40 µL Kinnex library sample from Step 5 + Master Mix (10 µL): 5 µL Nuclease buffer + 5 µL Nuclease mix → 50 µL total Rx vol. → Run nuclease treatment program (37°C 15 min, Hold at 4°C)

4.2 1X SMRTbell cleanup

50 µL Nuclease-treated Kinnex library + 50 µL 1X SMRTbell cleanup beads → 25 µL final purified Kinnex library → Final purified Kinnex library → DNA quantification QC → DNA sizing QC

Expect ~10–30% recovery of the starting Kinnex-PCR product

Kinnex 8-fold library size is typically ~12–20 kb

Proceed to **ABC**[1]

[1] Follow **Step 5** instructions in **Procedure & checklist** to prepare the Kinnex SMRTbell library (25 µL) for sequencing on the Revio system or Vega system.

Nuclease treatment & cleanup (cont.)

Procedural notes

Procedure & checklist – Preparing Kinnex libraries using Kinnex 8-fold prep kit (103-920-900)

1. Input quality control
2. Kinnex PCR + cleanup
   - Safe stop
3. Kinnex array formation + cleanup
4. Nuclease treatment + cleanup
   - Safe stop
5. Annealing, binding & cleanup (ABC)

4.1 Nuclease treatment

✔	Step	Instructions
	4.1.1	Add the following components to a new microcentrifuge tube. Adjust component volumes for the number of samples being prepared, plus 10% overage. Pipette mix master mix. For individual preps, add components directly to each sample from the previous step in the order and volume listed below.

Reaction Mix 3 (RM3):

Tube	Component	Volume
Light purple	Nuclease buffer	5 µL
Light green	Nuclease mix	5 µL
Total volume		10 µL

	4.1.2	Add 10 µL of **RM3** to each sample. The total volume should be 50 µL.
	4.1.3	Thoroughly mix by pipetting up and down 10 times and then quick-spin to collect all liquid.
	4.1.4	Run the nuclease treatment program.

Nuclease treatment program

Heated lid set at ≥47°C. Reaction volume set to 50 µL.

Step	Temperature	Duration
1	37°C	15 min
2	4°C	Hold

- Perform nuclease treatment for **15 min**

Nuclease treatment & cleanup (cont.)

Procedural notes

Procedure & checklist – Preparing Kinnex libraries using Kinnex 8-fold prep kit (103-920-900)

1 Input quality control

↓

2 Kinnex PCR + cleanup

↓ ○ Safe stop

3 Kinnex array formation + cleanup

↓

4 Nuclease treatment + cleanup

↓ ○ Safe stop

5 Annealing, binding & cleanup (ABC)

4.2 Final cleanup with 1X SMRTbell cleanup beads

✓	Step	Instructions
	4.2.1	Add **50 µL** (1X v/v) of resuspended, room temperature **SMRTbell cleanup beads** to each sample from the previous step. Pipette-mix the beads until evenly distributed.
	4.2.2	Quick-spin the tube strip in a microcentrifuge to collect all liquid.
	4.2.3	Incubate at **room temperature** for **10 minutes** to allow DNA to bind the beads.
	4.2.4	Place the tube strip in a magnetic separation rack until the beads separate fully from the solution.
	4.2.5	Slowly remove the cleared supernatant without disturbing the beads and discard the supernatant.
	4.2.6	Slowly dispense **200 µL**, or enough to cover the beads, of **freshly prepared 80% ethanol** into each tube. Remove the 80% ethanol and discard.
	4.2.7	Repeat the previous step.
	4.2.8	Remove residual 80% ethanol: • Remove the tube strip from the magnetic separation rack. • Quick-spin the tube strip in a microcentrifuge. • Place the tube strip back in a magnetic separation rack until beads separate fully from
	4.2.14	Recommended: Evaluate sample concentration and size. • Take a 1 µL aliquot from each tube, dilute with **4 µL** of elution buffer. • Using 1 µL of the dilution, measure DNA concentration with a Qubit fluorometer using the 1x dsDNA HS kit. **Expect 10-30% recovery of the starting Kinnex-PCR product.** • Recommended: Further dilute each aliquot to 250 pg/µL with the Femto Pulse dilution buffer. Check the final Kinnex library size distribution with a Femto Pulse system (see Figure 1 in the Appendix for an example).
	4.2.15	**If required, normalize 25 µL of library to <60 ng/µL.** Using a concentration above 60 ng/µL into ABC will result in poor sequencing performance.
	4.2.16	Proceed to Section 5 to prepare library for sequencing with Vega, Vega SPRQ-Nx or Revio (+SPRQ/SPRQ-Nx) chemistry.
	4.2.17	Store Kinnex libraries at 4°C if sequencing within the week. Long-term storage should be at -20°C. Minimize freeze-thaw cycles when handling Kinnex libraries.

SAFE STOPPING POINT - Store at 4°C or -20°C for long-term storage

- Perform **DNA concentration QC** on final purified Kinnex RNA library using a Qubit dsDNA HS assay
  - Typical final SMRTbell library yield from 5 µg of input DNA into Kinnex array formation is **~10 – 30%** – a much higher observed yield might suggest incomplete digestion of partial SMRTbell templates
  - **Troubleshooting tip:** If SMRTbell library yield is higher than expected and sample loading is lower than expected, consider repeating nuclease treatment step

- Perform **DNA sizing QC** on final purified Kinnex library using a Femto Pulse system (expected final library insert size is **~12 – 20 kb**)

- Kinnex final SMRTbell library concentration must be **≤60 ng/ µL** to proceed with sample setup ABC [Step 5]
  → Using a concentration above 60 ng/µL will result in lower loading during sequencing

PacBio

Kinnex 8-fold library prep inputs & expected step yields

Final Kinnex library yield is typically sufficient to support ≥8 SMRT Cell acquisitions on Revio & Vega systems

Kinnex 8-fold prep kit

Procedure & checklist – Preparing Kinnex libraries using Kinnex 8-fold prep kit (103-920-900)

Kinnex PCR

Kinnex PCR → **SMRTbell bead cleanup**

IN: 35 ng of amplicon insert (single or multiplex)

OUT: 5-9 µg of amplified products after pooling

Kinnex array formation

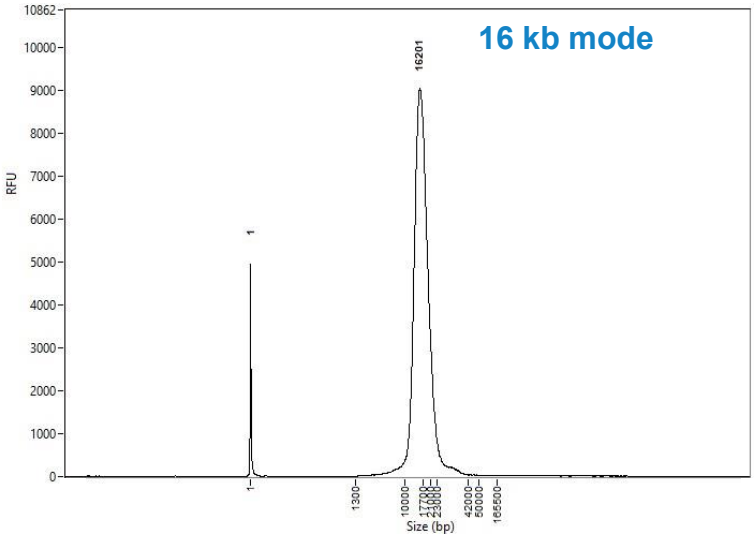
Kinnex digestion / Ligation → **DNA damage repair** → **SMRTbell cleanup** → **Nuclease treatment** → **SMRTbell bead cleanup**

IN: 5 µg of pooled Kinnex PCR products

OUT: 80% recovery from Kinnex digestion Rx input

IN: 40 µL of purified Kinnex repair reaction products

OUT: 10-30% recovery from Kinnex digestion Rx input



Example Femto pulse DNA sizing QC analysis results for final Kinnex 8-fold library prepared with cDNA amplicons generated from universal human reference RNA (UHRR) sample.

Example Kinnex 8-fold library prep yields

Amplicon input for Kinnex 8-fold array formation	5000 ng
Post-nuclease treatment & final library cleanup yield (%)[1]	1000 ng (20%)

[1] **Post-nuclease treatment & final cleanup yields typically ranged from ~10% to ~30% when using UHRR total RNA samples for Kinnex 8-fold library construction.**

Final Kinnex library yield is typically sufficient to load the following number of SMRT Cells:

- >8 acquisitions for Revio system with SPRQ-Nx chemistry
- >8 acquisitions for Vega system with SPRQ-Nx chemistry

PacBio

Kinnex sequencing preparation workflow

Sample setup and run design recommendations for Kinnex libraries

Key sample setup and run design setup parameters for Revio and Vega systems (**SMRT Link v26.2**)

Workflow	Key setup parameters	Vega system v1.2+ (SPRQ-Nx) recommended settings	Revio system v13.5+ (SPRQ-Nx) recommended settings
Sample setup	**Library type**	Kinnex	Kinnex
	Primer	Kinnex sequencing primer	Kinnex sequencing primer
	Polymerase kit	Vega SPRQ polymerase kit	Revio SPRQ polymerase kit
	Concentration on plate	80 – 130 pM	130 – 160 pM
Run design	**Use Adaptive Loading**	N/A	YES
	Application Type[2]	Kinnex full-length RNA; or Kinnex 16S rRNA; or Kinnex single-cell RNA	Kinnex full-length RNA; or Kinnex 16S rRNA; or Kinnex single-cell RNA
	Library Type[3]	Kinnex	Kinnex
	Movie Acquisition Time	24 hrs	24 hrs
	Add Analysis	YES	YES
	Analysis workflow	Read segmentation data utility or other application-specific analysis workflow	Read segmentation data utility or other application-specific analysis workflow
	Segmentation adapter set	MAS-Seq Adapter v1 (MAS16); or MAS-Seq Adapter v2 (MAS12); or MAS-Seq Adapter v3 (MAS8)	MAS-Seq Adapter v1 (MAS16); or MAS-Seq Adapter v2 (MAS12); or MAS-Seq Adapter v3 (MAS8)

Runs

Refer to **Procedure & checklist protocol documentation** for the latest sample setup (ABC[1]) recommendations for Kinnex libraries

[1] ABC = Anneal sequencing primer / Bind polymerase / Clean up bound complexes

[2] **Note:** A "Kinnex generic" run design application type option for running Kinnex custom amplicon libraries will be available in a future version of SMRT Link.
[3] **Note:** SMRT Link Run design **Library Type** field determines which adapter finding algorithm is used during post-primary analysis. When sequencing a Kinnex library sample, if 'Standard' library type is mistakenly selected instead of 'Kinnex' then a higher missing adapter rate (> 95%) and a slight degradation in barcode demultiplexing performance (~93-96% barcoded HiFi read yield) will be observed.

Sample setup and run design recommendations for Kinnex libraries (cont.)

Key sample setup and run design setup parameters for Revio and Vega systems (**SMRT Link v26.2**)

Workflow	Key setup parameters	Vega system v1.2+ (SPRQ-Nx) recommended settings	Revio system v13.5+ (SPRQ-Nx) recommended settings
Sample setup	**Library type**	Kinnex	Kinnex
	Primer	Kinnex sequencing primer	Kinnex sequencing primer
	Polymerase kit	Vega SPRQ polymerase kit	Revio SPRQ polymerase kit
	Concentration on plate	80 – 130 pM	130 – 160 pM
Run design	**Use Adaptive Loading**	N/A	YES
	Application Type[2]	Kinnex full-length RNA; or Kinnex 16S rRNA; or Kinnex single-cell RNA	Kinnex full-length RNA; or Kinnex 16S rRNA; or Kinnex single-cell RNA
	Library Type[3]	Kinnex	Kinnex
	Movie Acquisition Time	24 hrs	24 hrs
	Add Analysis	YES	YES
	Analysis workflow	Read segmentation data utility or other application-specific analysis workflow	Read segmentation data utility or other application-specific analysis workflow
	Segmentation adapter set	MAS-Seq Adapter v1 (MAS16); or MAS-Seq Adapter v2 (MAS12); or MAS-Seq Adapter v3 (MAS8)	MAS-Seq Adapter v1 (MAS16); or MAS-Seq Adapter v2 (MAS12); or MAS-Seq Adapter v3 (MAS8)

Runs

IMPORTANT!

Refer to **Procedure & checklist protocol documentation** for the latest sample setup (ABC[1]) recommendations for Kinnex libraries

[1] ABC = Anneal sequencing primer / Bind polymerase / Clean up bound complexes

NOTE: **SMRT Link v26.2 dropdown menu does not contain a "Kinnex generic" application type option**[2]

To set up a Kinnex generic (custom) amplicon library run design in SMRT Link v26.2, users can perform the following:

1. Select "**Kinnex 16S rRNA**" application type option as a placeholder.
2. Manually specify the correct **Segmentation adapter set** used
   - e.g., select "MAS-Seq Adapter v3 (MAS8)" if Kinnex 8-fold prep kit was used for Kinnex library construction
3. Manually **specify any other analysis options if applicable**
   - **Note:** By default, **Add Analysis = YES** and **Analysis workflow = Read segmentation** ***if*** "**Kinnex 16S rRNA**" application type is specified)

[2] **Note:** A "Kinnex generic" run design application type option for running Kinnex custom amplicon libraries will be available in a future version of SMRT Link.

[3] **Note:** SMRT Link Run design **Library Type** field determines which adapter finding algorithm is used during post-primary analysis. When sequencing a Kinnex library sample, if 'Standard' library type is mistakenly selected instead of 'Kinnex' then a higher missing adapter rate (> 95%) and a slight degradation in barcode demultiplexing performance (~93-96% barcoded HiFi read yield) will be observed.

Example run design setup for application-specific Kinnex libraries

v26.2

Follow SMRT Link run design instructions to prepare Kinnex libraries for sequencing

Select **desired Kinnex application type** in SMRT Link run design page

Runs

After specifying your application type, **SMRT Link auto-fills** selected Run Design parameter fields with default recommended values

Kinnex single-cell RNA

Whole genome sequencing
- Human WGS
- Microbial assembly
- Other WGS

RNA sequencing
- Iso-Seq method
- MAS-Seq single cell
- Kinnex single-cell RNA
- Kinnex full-length RNA

Viral sequencing
- Adeno-associated virus

Metagenomics
- Kinnex 16S rRNA
- Full-length 16S rRNA sequencing

Kinnex full-length RNA

Whole genome sequencing
- Human WGS
- Microbial assembly
- Other WGS

RNA sequencing
- Iso-Seq method
- MAS-Seq single cell
- Kinnex single-cell RNA
- Kinnex full-length RNA

Viral sequencing
- Adeno-associated virus

Metagenomics
- Kinnex 16S rRNA
- Full-length 16S rRNA sequencing

Kinnex 16S rRNA *OR* other (custom) amplicon

Whole genome sequencing
- Human WGS
- Microbial assembly
- Other WGS

RNA sequencing
- Iso-Seq method
- MAS-Seq single cell
- Kinnex single-cell RNA
- Kinnex full-length RNA

Viral sequencing
- Adeno-associated virus

Metagenomics
- Kinnex 16S rRNA
- Full-length 16S rRNA sequencing

If running a **Kinnex custom amplicon library**, users can select "**Kinnex 16S rRNA**" as a placeholder in run design[1]

[1] **Note:** A "Kinnex generic" run design application type option for running Kinnex custom amplicon libraries will be available in a future version of SMRT Link.

Example run design setup for application-specific Kinnex libraries (cont.)

v26.2

Sample and run information

	Kinnex single-cell RNA	Kinnex full-length RNA	Kinnex 16S rRNA *OR* other (custom) amplicon
Plate 1, Well A01	Kinnex single-cell RNA library demo	Kinnex full-length RNA library demo	Kinnex 16S rRNA library demo
Application (Required)	Kinnex single-cell RNA	Kinnex full-length RNA	Kinnex 16S rRNA
Plate Well (Required)	Plate 1, Well A01	Plate 1, Well A01	
Well Name (Required)			
Well Comment			
Library Type (Required)	Kinnex	Kinnex	Kinnex
Insert Size (bp) (Required)	15000	16000	17000
Library Concentration (pM) (Required)			
Movie Acquisition Time (hours)	24	24	24

Select **application type** to autofill Library Type & Movie Acquisition Time recommended settings

Default Library Type = **Kinnex** (instead of Standard or AAV)[1]

Default Movie Time = **24 hrs**

If running a **Kinnex custom amplicon library**, users can select "**Kinnex 16S rRNA**" as a placeholder in run design

Standard SMRTbell library type containing standard SPK3 barcoded terminal adapters

Forward and reverse standard terminal adapters have the **same structure**

Kinnex SMRTbell library type containing Kinnex barcoded terminal adapters

Forward and reverse Kinnex terminal adapters have **different structures**

Library Type field determines which adapter finding algorithm is used during post-primary analysis[1]

[1] **Note:** When sequencing a Kinnex library sample, if 'Standard' library type is mistakenly selected instead of 'Kinnex' then a higher missing adapter rate (> 95%) and a slight degradation in barcode demultiplexing performance (~93-96% barcoded HiFi read yield) will be observed.

Example run design setup for application-specific Kinnex libraries (cont.)

v26.2

Sample indexing (barcoding) information

Kinnex single-cell RNA

Samples

Sample is indexed: YES / NO

Indexes (Required): MAS SMRTbell Barcoded Adapters (v2)

Biosample names (Required): Interactively | From a File

Kinnex full-length RNA

Sample is indexed: YES / NO

Indexes (Required): MAS SMRTbell Barcoded Adapters (v2)

Biosample names (Required): Interactively | From a File

Kinnex 16S rRNA *OR* other (custom) amplicon

Samples

Sample is indexed: YES / NO

Indexes (Required): MAS SMRTbell Barcoded Adapters (v2)

Biosample names (Required): Interactively | From a File

Sample is indexed field is automatically set to **YES** for all Kinnex application types

Default **Indexes** FASTA for Kinnex libraries = **MAS SMRTbell Barcoded Adapters (v2)**

plete Kinnex library molecule containing barcoded Kinnex terminal MAS adapters[1] at both ends

Barcoded forward Kinnex terminal adapter

Both forward & reverse Kinnex terminal adapters contain the same barcode sequence

Barcoded reverse Kinnex terminal adapter

Example interactive biosample name specification

Barcode Selector and Sample Name Editor

Available Barcodes: Filter...

Barcode
bcM0001--bcM0001
bcM0002--bcM0002
bcM0003--bcM0003
bcM0004--bcM0004

Included Barcodes: Filter...

Barcode	Bio Sample

Barcode Selector and Sample Name Editor

Available Barcodes: Filter...

Barcode
bcM0003--bcM0003
bcM0004--bcM0004

Included Barcodes: Filter...

Barcode	Bio Sample
bcM0001--bcM0001	Kinnex adapter-barcoded library 1
bcM0002--bcM0002	Kinnex adapter-barcoded library 2

SMRT Link

Data Management

MAS SMRTbell barcoded adapter indexes

```
>bcM0001
ACAGTC
>bcM0002
ATGACG
>bcM0003
CACGTG
>bcM0004
CATCGC
```

[1] Four barcoded terminal Kinnex adapters (Kinnex adapter bcM0001-bcM0004) are available for Kinnex array formation step. Kinnex adapter barcode sequences can be downloaded from SMRT Link Data Management module.

Example run design setup for application-specific Kinnex libraries (cont.)

v26.2

Data options

Kinnex single-cell RNA

Default = **NO** for **Include Base Kinetics**

Data Options

Include Base Kinetics	YES NO
Consensus Mode	MOLECULE STRAND
Assign Data To Project	General Project

Default **Consensus Mode = MOLECULE**[1]

Kinnex full-length RNA

Data Options

Include Base Kinetics	YES NO
Consensus Mode	MOLECULE STRAND
Assign Data To Project	General Project

Kinnex 16S rRNA *OR* other (custom) amplicon

Data Options

Include Base Kinetics	YES NO
Consensus Mode	MOLECULE STRAND
Assign Data To Project	General Project

Can leave Include Base Kinetics and Consensus Mode fields at their default settings for Kinnex library samples

[1] Molecule Consensus Mode → generates a single circular consensus sequencing read for a given library insert molecule. Strand Consensus Mode → generates a consensus sequence for each strand (forward & reverse) of a given library insert molecule.

Example run design setup for application-specific Kinnex libraries (cont.)

SMRT LINK v26.2

Analysis options

Kinnex single-cell RNA

Analysis Options

Default = **YES** for **Add Analysis**

Add Analysis: YES / NO

Analysis Name (Required): Kinnex_Single-Cell_RNA_Demo_Analysis_Job_Nam

Select Analysis Workflow (Required): Read Segmentation and Single-Cell Iso-Seq

Segmentation Adapter Set: MAS-Seq Adapter v1 (MAS16)

Primer Set (Required): 10x Chromium single cell 3' cDNA primers

Reference Set (Required): Human Genome hg38, with Gencode v39 annotations

Kit Type: 10X 3' KIT / 10X 5' KIT

Advanced Parameters

Kinnex full-length RNA

Analysis Options

Analysis: YES / NO

Name (Required): Kinnex_Full-Length_RNA_Demo_Analysis_Job_Name

Select Analysis Workflow (Required): Read Segmentation and Iso-Seq

Segmentation Adapter Set: MAS-Seq Adapter v3 (MAS8)

Primer Set (Required): Iso-Seq v2 Barcoded cDNA Primers

Reference Set: Human Genome hg38, with Gencode v39 annotations

Cluster of Barcoded Samples: Pool reads and cluster together

Advanced Parameters

Kinnex 16S rRNA *OR* other (custom) amplicon

Analysis Options

Add Analysis: YES / NO

Analysis Name (Required): Kinnex_Full-Length_16S_rRNA_Demo_Analysis_Job_Name

Select Analysis Workflow (Required): Read Segmentation

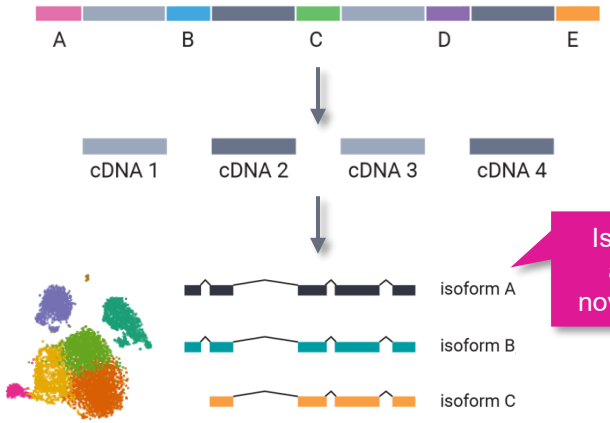
Segmentation Adapter Set: MAS-Seq Adapter v2 (MAS12)

Advanced Parameters

Analysis Workflow is automatically filled in

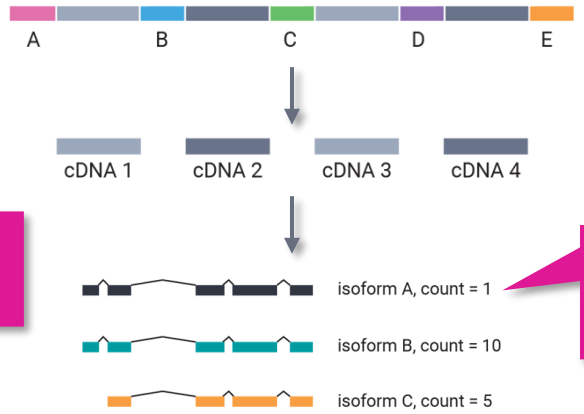
Split arrayed HiFi reads at adapter positions, generating segmented reads (S-reads)

Read Segmentation and Single-Cell Iso-Seq



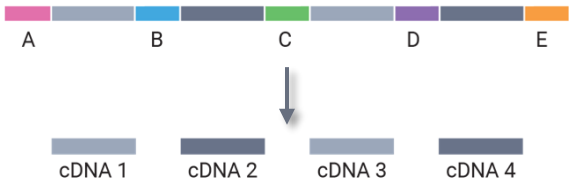
Isoform-classification analysis to identify novel genes & isoforms

Read Segmentation and Iso-Seq



Isoform-classification analysis to identify novel genes & isoforms with abundance information (raw counts & normalized counts per million)

Read Segmentation



Example run design setup for application-specific Kinnex libraries (cont.)

SMRT LINK v26.2

Analysis options

Kinnex single-cell RNA

Analysis Options

Default Segmentation Adapter Set = **MAS-Seq Adapter v1 (MAS16)** for Kinnex single-cell RNA application type

Add Analysis	
Analysis Name Required	
Select Analysis Workflow Required	Read Segmen… …gle-Cell Iso-Seq
Segmentation Adapter Set	MAS-Seq Adapter v1 (MAS16)
Primer Set Required	10x Chromium single cell 3' cDNA primers
Reference Set Required	Human Genome hg38, with Gencode v39 annotations
Kit Type	10X 3' KIT / 10X 5' KIT

Advanced Parameters

Kinnex full-length RNA

Analysis Options

Default Segmentation Adapter Set = **MAS-Seq Adapter v3 (MAS8)** for Kinnex full-length RNA application type

Add Analysis	
Analysis Name Required	
Select Analysis Workflow Required	Read Segme… …Seq
Segmentation Adapter Set	MAS-Seq Adapter v3 (MAS8)
Primer Set Required	Iso-Seq v2 Barcoded cDNA Primers
Reference Set	Human Genome hg38, with Gencode v39 annotations
Cluster of Barcoded Samples	Pool reads and cluster together

Advanced Parameters

Kinnex 16S rRNA *OR* other (custom) amplicon

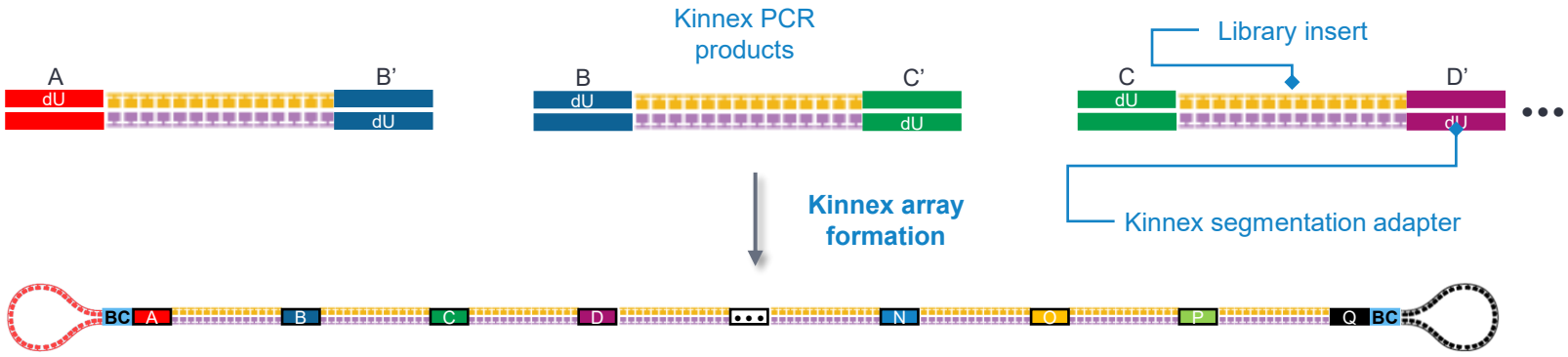
Analysis Options

Default Segmentation Adapter Set = **MAS-Seq Adapter v2 (MAS12)** for Kinnex 16S rRNA application type

Add Analysis	
Analysis Name Required	
Select Analysis Workflow Required	Read …
Segmentation Adapter Set	MAS-Seq Adapter v2 (MAS12)

Advanced Parameters

IMPORTANT! If needed, manually specify the correct **Segmentation Adapter Set** that corresponds to the Kinnex library prep concatenation method **actually used**



Complete array molecule containing concatenated library insert segments

Kinnex library prep kit used	Segmentation Adapter Set to specify in SMRT Link v26.2 run design Analysis Options
Kinnex 8-fold prep kit (103-072-000)	MAS-Seq Adapter v3 (MAS8)
Kinnex 12-fold prep kit (103-072-100)	MAS-Seq Adapter v2 (MAS12)
Kinnex 16-fold prep kit (103-836-900)	MAS-Seq Adapter v1 (MAS16)

Example run design setup for application-specific Kinnex libraries (cont.)

SMRT LINK v26.2

Analysis options

Kinnex single-cell RNA

Analysis Options

Add Analysis	YES / NO
Analysis Name Required	Kinnex_Single-Cell_RNA_Demo_Analysis_Job_Name
Select Analysis Workflow Required	Read Segmentation and Single-Cell Iso-Seq
Segmentation Adapter Set	MAS-Seq Adapter v1 (MAS16)
Primer Set Required	10x Chromium single cell 3' cDNA primers
Reference Set Required	Human Genome hg38, with Gencode v39 annotations
Kit Type	10X 3' KIT / 10X 5' KIT
	Advanced Parameters

Specify **Primer Set** used for cDNA amplification

10x Forward (F) PCR primer

10x Reverse (R) PCR primer

5' [CBC][UMI]TTTTTTTTTT LIBRARY INSERT CCC TSO 3'
3' [CBC][UMI]AAAAAAAAAA LIBRARY INSERT GGG TSO 5'

For Kinnex single-cell 3' RNA analysis, select '10x Chromium single cell 3' cDNA primers'

OR

5' [CBC][UMI][TSO]GGG LIBRARY INSERT AAAAAAAAAA 3'
3' [CBC][UMI][TSO]CCC LIBRARY INSERT TTTTTTTTTT 5'

For Kinnex single-cell 5' RNA analysis, select '10x Chromium single cell 5' cDNA primers'

Kinnex full-length RNA

Analysis Options

Add Analysis	YES / NO
Analysis Name Required	Kinnex_Full-Length_RNA_Demo_Analysis_Job_Name
Select Analysis Workflow Required	Read Segmentation and Iso-Seq
Segmentation Adapter Set	MAS-Seq Adapter v3 (MAS8)
Primer Set Required	Iso-Seq v2 Barcoded cDNA Primers
Reference Set	Human Genome hg38, with Gencode v39 annotations
Cluster of Barcoded Samples	Pool reads and cluster together
	Advanced Parameters

Specify **Reference** Set. Default sets are:

- Human Genome hg38, with Gencode v39 annotations
- Mouse Genome mm39, with Gencode vM28 annotations

Forward (F) PCR primer (Barcoded Iso-Seq primer bcXX[2]) — BC TSO

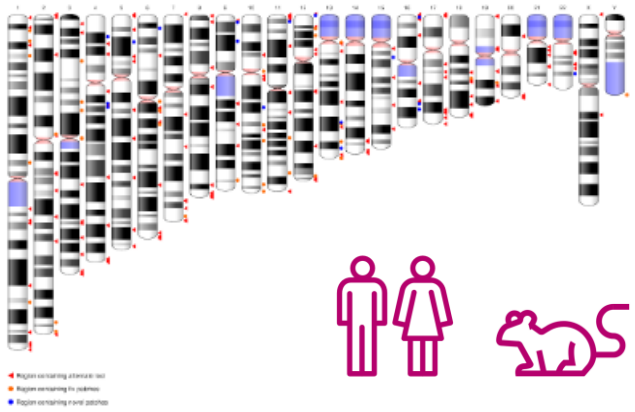
Reverse (R) PCR primer (Iso-Seq cDNA amplification primer)

5' BC TSO GGG LIBRARY INSERT AAAAAAAAAA 3'
3' BC TSO CCC LIBRARY INSERT TTTTTTTTTT 5'

For Kinnex full-length RNA analysis, select 'Iso-Seq v2 Barcoded cDNA primers'

Specify primer sequence file in FASTA format to **identify cDNA primers for removal** (include the 5' and 3' cDNA primers)

Specify reference genome & annotation sets to align high quality isoforms to, and to collapse isoforms mapped to the same genomic loci.



Example run design setup for application-specific Kinnex libraries (cont.)

Analysis options

Kinnex single-cell RNA

Analysis Options

Add Analysis: YES / NO

Analysis Name (Required): Kinnex_Single-Cell_RNA_Demo_Analysis_Job_Name

Select Analysis Workflow (Required): Read Segmentation and Single-Cell Iso-Seq

Segmentation Adapter Set: MAS-Seq Adapter v1 (MAS16)

Primer Set (Required): 10x Chromium single cell 3' cD

Reference Set (Required): Human Genome hg38, with G v39 annotations

Kit Type: 10X 3' KIT / 10X 5' KIT

Advanced Parameters

Specify **Kit Type** used for single-cell cDNA generation

Specification of Kit Type (10x 3' Kit or 10x 5' Kit) **determines which set of 10x barcode sequences to use**, and also affects **UMI and single-cell barcode design settings**[1]

5' [CBC][UMI]TTTTTTTTTT LIBRARY INSERT CCC TSO 3'
3' [CBC][UMI]AAAAAAAAAA LIBRARY INSERT GGG TSO 5'

For Kinnex single-cell 3' RNA analysis, select '10x 3' Kit'

OR

5' [CBC][UMI][TSO]GGG LIBRARY INSERT AAAAAAAAAA 3'
3' [CBC][UMI][TSO]CCC LIBRARY INSERT TTTTTTTTTT 5'

For Kinnex single-cell 5' RNA analysis, select '10x 5' Kit'

Kinnex full-length RNA

Analysis Options

Add Analysis: YES / NO

Analysis Name (Required)

Select Analysis Workflow (Required)

mentation Adapter Set: MAS-S (MAS8)

Primer Set (Required): v2 Barcoded cDNA Primers

Reference: Human Genome hg38, with Gencode v39 annotations

Cluster of Barcoded Samples: Pool reads and cluster together

Advanced Parameters

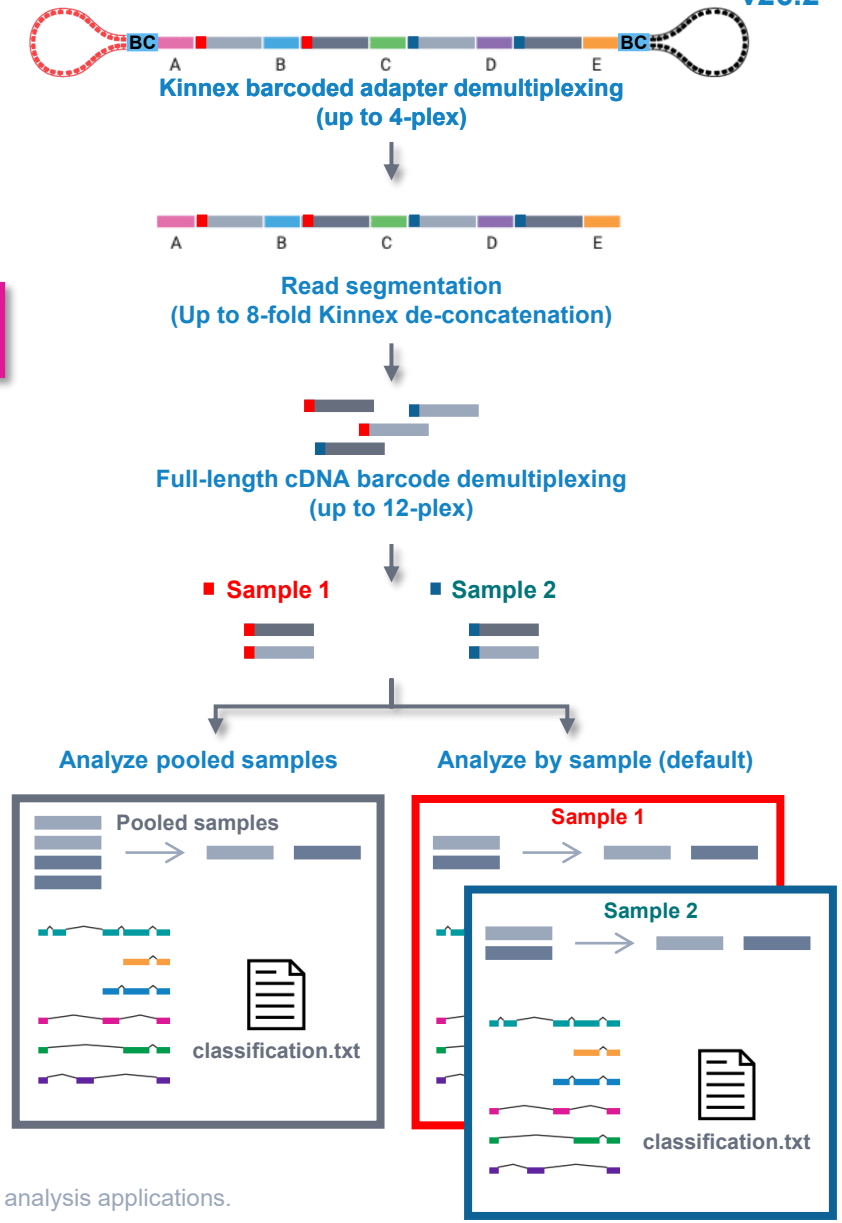
Specify **how to perform read clustering for barcoded samples**

Pool reads and cluster together
-- select --
Cluster reads separately
Pool reads and cluster together

Specification of **Cluster of Barcoded Samples** setting **determines whether all FLNC reads will be pooled for clustering** (Does not apply to non-barcoded samples.)

Clustering options for Kinnex full-length RNA samples

v26.2



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[1] See *SMRT Link User Guide* documentation for detailed descriptions of advanced parameters for Iso-Seq / Single-Cell Iso-Seq analysis applications.

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Kinnex example sequencing performance

Kinnex example performance – Revio system v13.5 + SPRQ-Nx

Kinnex libraries show excellent HiFi sequencing performance across multiple SMRT Cell uses that meets or exceeds Revio system specifications for every use[1]

Kinnex library type									
Kinnex 8-fold prep kit	**Use**	**HiFi yield (Gb)**	**HiFi reads (M)**	**HiFi length (kb)**	**HiFi read QV**	**P1**	**Segmented reads per acquisition**	**Mean S-read length**	**Product specifications[5]**
Kinnex full-length RNA, UHRR[2]	1	137.1	9.5	14.4	27	84.4	72.1 M	1.83 kb	50 – 70 M S-reads per acquisition
	2	135.2	9.3	14.5	29	68.8	71.0 M	1.83 kb	
	3	116.2	7.9	14.6	29	60.2	61.2 M	1.83 kb	
Kinnex 12-fold prep kit	**Use**	**HiFi yield (Gb)**	**HiFi reads (M)**	**HiFi length (kb)**	**HiFi read QV**	**P1**	**Segmented reads per acquisition**	**Mean S-read length**	**Product specifications[5]**
Kinnex 16S rRNA, 384-plex ATCC MSA1002[3]	1	170.5	8.8	19.3	33	78.8	103.4 M	1.61 kb	70 – 100 M S-reads per acquisition
	2	168.2	8.7	19.4	35	65.4	102.1 M	1.61 kb	
	3	139.8	7.2	19.5	36	50.3	84.9 M	1.61 kb	
Kinnex 16-fold prep kit	**Use**	**HiFi yield (Gb)**	**HiFi reads (M)**	**HiFi length (kb)**	**HiFi read QV**	**P1**	**Segmented reads per acquisition**	**Mean S-read length**	**Product specifications[5]**
Kinnex single-cell 3' RNA (10x)[4]	1	153.7	10.9	14.1	29	74	164 M	906 bp	100 – 150 M S-reads per acquisition
	2	143.0	10.0	14.2	29	71	153 M	908 bp	
	3	127.3	8.9	14.4	30	61	136 M	909 bp	

[1] **Note:** HiFi yield is dependent on library quality and sequencing preparation procedures. Specified yields are based on high-quality samples prepared following best practices. Reduced HiFi yield on Use 2 and/or Use 3 is typically caused by lower P1 loading efficiency.
[2] Kinnex full-length RNA UHRR libraries were prepared using Kinnex 8-fold prep kit and run on a Revio system with SPRQ-Nx chemistry (24-hour acquisition time; 140 pM on-plate loading concentration).
[3] Kinnex full-length 16S rRNA, ATCC MSA1002 libraries were prepared with Kinnex 12-fold pre kit and run on a Revio system with SPRQ-Nx chemistry (24-hour acquisition time; 120 pM OPLC).
[4] Kinnex single-cell 3' RNA libraries were prepared with Kinnex 16-fold pre kit and run on a Revio system with SPRQ-Nx chemistry (24-hour acquisition time; 160 pM OPLC).
[5] See ***Revio specifications sheet*** (102-326-552) for expected HiFi yield performance range for different library insert sizes and applications.

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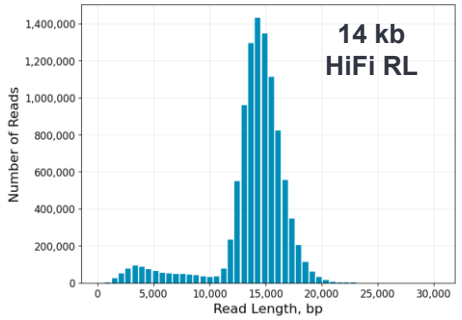
Kinnex example performance – Revio system v13.5 + SPRQ-Nx (cont.)

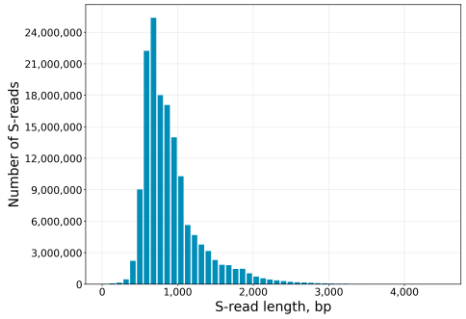
Kinnex single-cell RNA

Kinnex 16-fold prep kit

HiFi read length



S-read length

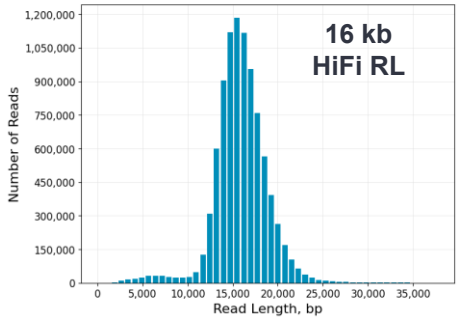


Value	Analysis Metric
10,030,881	Reads
149,910,947	Segmented reads (S-reads)
906	Mean length of S-reads
88.24 %	Percent of reads with full arrays
14.94	Mean array size (concatenation factor)

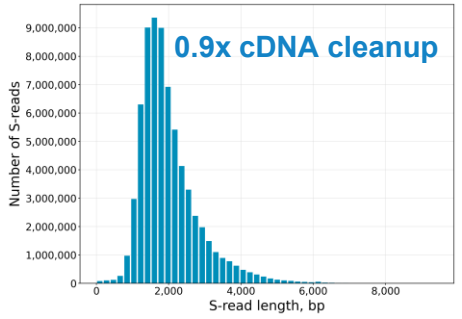
Kinnex full-length RNA

Kinnex 8-fold prep kit

HiFi read length



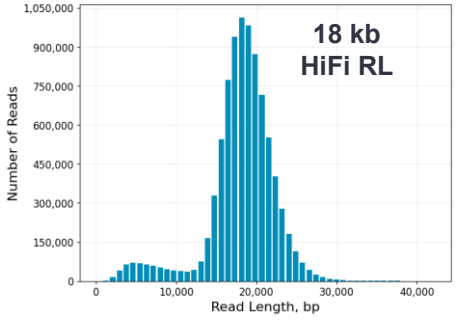
S-read length



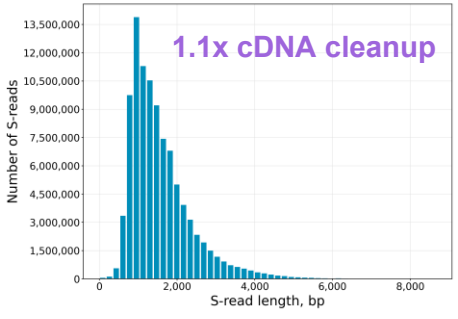
Value	Analysis Metric
9,044,421	Reads
69,407,271	Segmented reads (S-reads)
2,002	Mean length of S-reads
92.67 %	Percent of reads with full arrays
7.67	Mean array size (concatenation factor)

Kinnex 12-fold prep kit

HiFi read length



S-read length

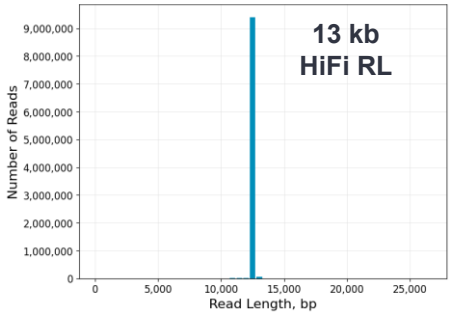


Value	Analysis Metric
8,742,074	Reads
96,912,559	Segmented reads (S-reads)
1,566	Mean length of S-reads
87.77 %	Percent of reads with full arrays
11.09	Mean array size (concatenation factor)

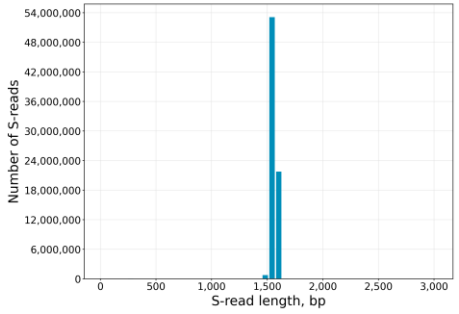
Kinnex 16S rRNA

Kinnex 8-fold Prep kit

HiFi read length



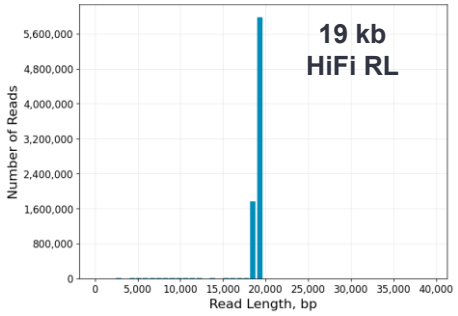
S-read length



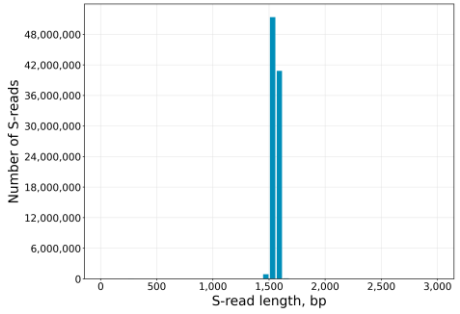
Value	Analysis Metric
9,541,465	Reads
75,949,260	Segmented reads (S-reads)
1,560	Mean length of S-reads
97.77 %	Percent of reads with full arrays
7.96	Mean array size (concatenation factor)

Kinnex 12-fold prep kit

HiFi read length



S-read length



Value	Analysis Metric
7,860,184	Reads
93,421,796	Segmented reads (S-reads)
1,560	Mean length of S-reads
96.94 %	Percent of reads with full arrays
11.89	Mean array size (concatenation factor)

Kinnex example performance – Vega system v1.2 + SPRQ-Nx

Kinnex libraries show excellent HiFi sequencing performance that meets or exceeds Vega system specifications[1]

Kinnex library type								
Kinnex 8-fold prep kit	**HiFi yield (Gb)**	**HiFi reads (M)**	**HiFi length (kb)**	**HiFi read QV**	**Loading level**	**Segmented reads per acquisition**	**Mean S-read length**	**Product specifications[5]**
Kinnex full-length RNA, UHRR[2]	99.9	6.5	15.3	34	63%	47.9 M	1.98 kb	**40 – 50 M S-reads per acquisition**
Kinnex 12-fold prep kit	**HiFi yield (Gb)**	**HiFi reads (M)**	**HiFi length (kb)**	**HiFi read QV**	**Loading level**	**Segmented reads per acquisition**	**Mean S-read length**	**Product specifications[5]**
Kinnex 16S rRNA, 384-plex ATCC MSA1001, 1003[3]	99.9	5.2	19.3	34	67%	60.6 M	1.61 kb	**50 – 60 S-reads per acquisition**
Kinnex 16-fold prep kit	**HiFi yield (Gb)**	**HiFi reads (M)**	**HiFi length (kb)**	**HiFi read QV**	**Loading level**	**Segmented reads per acquisition**	**Mean S-read length**	**Product specifications[5]**
Kinnex single-cell 3' RNA (10x)[4]	99.9	7.1	14.1	29	67%	108.1 M	894.9 bp	**80 – 100 M S-reads per acquisition**

1 **Note:** HiFi yield is dependent on library quality and sequencing preparation procedures. Specified yields are based on high-quality samples prepared following best practices.
2 Kinnex full-length RNA UHRR libraries were run on a Vega system with SPRQ-Nx chemistry (24-hour acquisition time; 100 pM on-plate loading concentration).
3 Kinnex full-length 16S rRNA, ATCC MSA1001 + MSA 1003 libraries were run on a Vega system with SPRQ-Nx chemistry (24-hour acquisition time; 80 pM on-plate loading concentration).
4 Kinnex single-cell 3' RNA libraries (4-plex) were prepared with Kinnex 16-fold pre kit and run on a Vega system with SPRQ-Nx chemistry (24-hour acquisition time; 130 pM OPLC).
5 See ***Vega specifications sheet*** (102-326-625) for expected HiFi yield performance range for different library insert sizes and applications.

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Technical documentation & applications support resources

Technical resources for Kinnex library preparation, sequencing & data analysis

Kinnex library preparation literature & other resources

- **General Kinnex library preparation**
  - Procedure & checklist – Preparing Kinnex libraries using Kinnex 8-fold prep kit (103-920-900)
  - Procedure & checklist – Preparing Kinnex libraries using Kinnex 12-fold prep kit (103-921-000)
  - Procedure & checklist – Preparing Kinnex libraries using Kinnex 16-fold prep kit (103-885-300)
  - Technical overview – Kinnex library preparation using Kinnex prep kits (103-911-600)
  - Technical overview – Kinnex library preparation using Kinnex 8-fold prep kit (103-916-000)
  - Technical overview – Kinnex library preparation using Kinnex 12-fold prep kit (103-916-100)
  - Technical overview – Kinnex library preparation using Kinnex 16-fold prep kit (103-916-200)
- **Application-specific Kinnex library preparation**
  - Application note – Kinnex full-length RNA kit for isoform sequencing (102-326-591)
  - Application note – Kinnex 16S rRNA kit for full-length 16S sequencing (102-326-601)
  - Application note – Kinnex single-cell RNA for single-cell isoform sequencing (102-326-549)
  - Procedure & checklist – Preparing Kinnex compatible cDNA using Iso-Seq express 2.0 kit (102-396-000)
  - Procedure & checklist – Preparing 16S rRNA amplicons for Kinnex (103-885-200)

Data analysis resources

- **General data analysis**
  - SMRT Link software installation guide [Link]
  - SMRT Link user guide [Link]
  - SMRT Tools reference guide [Link]
- **Application-specific Kinnex data analysis**
  - Application note – Bioinformatics tools for full length isoform sequencing (102-326-593)
  - SMRT Link Kinnex full-length RNA troubleshooting guide (103-552-100)
  - SMRT Link Kinnex single-cell troubleshooting guide (103-516-100)

Technical resources for Kinnex library preparation, sequencing & data analysis (cont.)

Posters & publications

- **Kinnex bulk RNA applications**
  - Ma, J. et al. (2026) Long-read RNA sequencing improves isoform and splicing outlier detection in whole blood from rare disease trios. MedRxiv [Link]
  - Wissel, D. et al. (2026) A systematic benchmark of high-accuracy PacBio long-read RNA sequencing for transcript-level quantification. Genome Biology [Link]
  - Al'Khafaji, A.M. et al. (2024) High-throughput RNA isoform sequencing using programmable cDNA concatenation. Nature biotechnology [Link]
  - Pardo-Palacios, F. et al. (2024) Systematic assessment of long-read RNA-seq methods for transcript identification and quantification. Nature Biotech [Link]
  - Pardo-Palacios, F., et al., (2024) SQANTI3: curation of long-read transcriptomes for accurate identification of known and novel isoforms. Nature Methods [Link]
  - Schertzer, M.D. et al. (2023) Cas13d-mediated isoform-specific RNA knockdown with a unified computational and experimental toolbox. Nature comm [Link]
- **Kinnex 16s rRNA applications**
  - Anderson, M.A.J. et al. (2025) Unlocking River Biofilm Microbial Diversity: A Comparative Analysis of Sequencing Technologies. Molecular Ecology Resources [Link]
  - PacBio PAG poster (2025) – High-resolution microbiome species profiling at scale with the Kinnex kit for full- length 16S rRNA sequencing [Link]
  - PacBio ASM Microbe poster (2024) - Microbiome species profiling at scale with the Kinnex kit for full-length 16S rRNA sequencing [Link]
  - PacBio PAG poster (2024) – Increasing the throughput of full-length 16S sequencing with Kinnex kits [Link]
  - Al'Khafaji, A.M. et al. (2024) High-throughput RNA isoform sequencing using programmable cDNA concatenation. Nature biotechnology [Link]
  - Notario, E. et al. (2023) Amplicon-based microbiome profiling: from second- to third-generation sequencing for higher taxonomic resolution. Genes [Link]
- **Kinnex single-cell RNA applications**
  - Mantri, M. et al. (2026) Single-cell splice isoform usage reveals distinct axes of cellular identity and senescence. BioRxiv. [Link]
  - Wissel, D. et al. (2025) A systematic benchmark of high-accuracy PacBio long-read RNA sequencing for transcript-level quantification. Genome Biology [Link]
  - Al'Khafaji, A.M. et al. (2024) High-throughput RNA isoform sequencing using programmable cDNA concatenation. Nature biotechnology [Link]
  - Deng, E. et al. (2025) Systematic evaluation of single-cell RNA-seq analyses performance based on long-read sequencing platforms. J Advanced Res [Link]

Webinars

- PacBio webinar (2025) – Detection and quantification of transcript isoforms using high-depth Kinnex full-length RNA sequencing [Link]
- PacBio video (2024) – Kinnex explained – how concatenating smaller amplicons increases throughput for PacBio HiFi sequencing [Link]
- PacBio PRISM webinar (2024) – Let's stick together – exploring PacBio Kinnex kits [Link]
- PacBio Iso-Seq social club webinar (2022) – TappAS for isoform differential expression analysis [Link]
- PacBio Iso-Seq social club webinar (2022) – Single-cell Iso-Seq applications in cancer and neurological disorders [Link]

Technical resources for Kinnex library preparation, sequencing & data analysis (cont.)

Example PacBio datasets[1]

Application	Dataset	Data type	PacBio system
Kinnex full-length RNA (Kinnex 8-fold library)	Homo sapiens – Universal human reference RNA (UHRR) [Link]	HiFi long read	Vega system
	Homo sapiens – Universal human reference RNA (UHRR) [Link]	HiFi long read	Revio system – SPRQ chemistry
	Homo sapiens – Universal human reference RNA (UHRR) [Link]	HiFi long read	Sequel II & Revio systems
	Homo sapiens – HG002 [Link]	HiFi long read	Revio system
	Homo sapiens – Heart [Link]	HiFi long read	Revio system
	Homo sapiens – Cerebellum [Link]	HiFi long read	Revio system
	Homo sapiens – Brain [Link]	HiFi long read	Revio system
Kinnex 16S rRNA (Kinnex 12-fold library)	ZymoBIOMICS Fecal Reference with TruMatrix Technology (human) [Link]	HiFi long read	Sequel II & Revio systems
	Mixture: ZymoBIOMICS Gut Microbiome Standard, ZymoBIOMICS Fecal Reference with TruMatrix™ Technology, ATCC 20 Strain Even Mix Genomic Material, ATCC 20 Strain Staggered Mix Genomic Material [Link]	HiFi long read	Sequel II & Revio systems
Kinnex single-cell RNA (Kinnex 16-fold library)	Homo sapiens - PBMC 10x Chromium Single Cell 5’ and 3' libraries [Link]	HiFi long read	Sequel II & Revio systems
	Homo sapiens - HG002 (10x 5’) [Link]	HiFi long read	Revio system

[1] Example PacBio datasets may be downloaded at https://www.pacb.com/connect/datasets/.

www.pacb.com

Revision history (description)	Version	Date
Initial release.	01	October 2026

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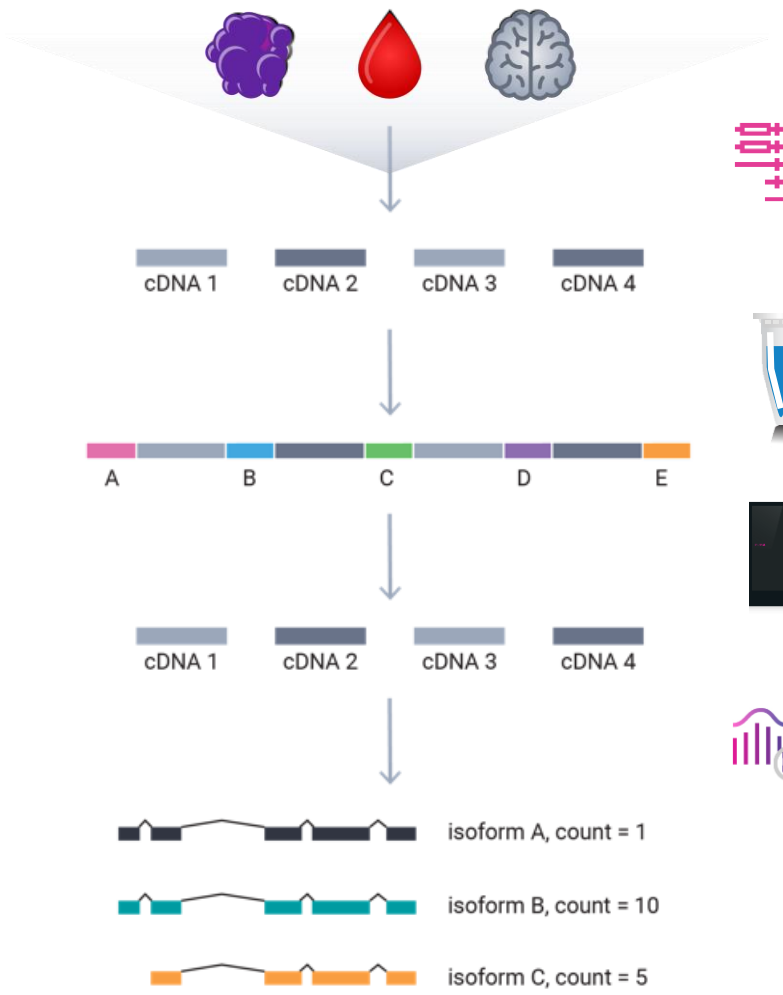
PacBio

APPENDIX 1: Example Kinnex bulk RNA workflow

Kinnex bulk RNA library preparation workflow overview
Kinnex bulk RNA data analysis workflow overview
Kinnex bulk RNA example sequencing performance data
Technical resources for Kinnex bulk RNA library preparation, sequencing & data analysis

Kinnex bulk RNA library preparation workflow overview

Kinnex workflow for bulk RNA sequencing applications



cDNA generation

Procedure & checklist – Preparing Kinnex-compatible cDNA using Iso-Seq Express 2.0 kit (102-396-000)

Kinnex library preparation

Procedure & checklist – Preparing Kinnex libraries using Kinnex 8-fold prep kit (103-238-700); ***OR***

Procedure & checklist – Preparing Kinnex libraries using Kinnex 12-fold prep kit (103-238-800)

PacBio sequencing

Read segmentation + Isoform analysis

SMRT Link user guide (103-891-900)

- 300 ng total RNA (RIN ≥7) input for generating up to 12-plex barcoded cDNA amplicons using Iso-Seq express 2.0 kit (103-071-500)
- 1-day Kinnex library preparation using Kinnex 8-fold prep kit or Kinnex 12-fold prep kit[1]
- SMRT Link Run Design support for 'Kinnex full-length RNA' application type with auto-analysis (read segmentation + isoform an alysis)
- SMRT Link Iso-Seq isoform-classification software to identify novel genes and isoforms with abundance information

[1] See ***Technical note – Targeted enrichment of Kinnex full-length RNA libraries with IDT xGen hybridization and wash kit*** (102-326-632) for modified workflow for performing targeted enrichment using IDT xGen Hyb and Wash kit v3 for preparing PacBio Kinnex bulk RNA libraries.

Kinnex bulk RNA workflow key steps

Full-length cDNA synthesis & amplification

Procedure & checklist – Preparing Kinnex-compatible cDNA using Iso-Seq Express 2.0 kit (102-396-000)

Minimum total RNA input for cDNA synthesis = 300 ng

Poly(A)+ mRNA template

5' AAAAAAAAAA 3'

3' TTTTTTTTTT 5'

Contains Kinnex PCR handle 1

Poly(dT) primer for 1st-strand synthesis

Reverse transcription (1st-strand synthesis) and addition of 'C' bases at transcript 5' end

Template Switch Oligo (TSO)

5' TSO GGG AAAAAAAAAA 3'

3' CCC TTTTTTTTTT 5'

Template switching (from mRNA to TSO) and completion of transcript extension

5' TSO GGG AAAAAAAAAA 3'

3' CCC TTTTTTTTTT 5'

2nd-strand synthesis & cDNA amplification

Forward (F) PCR Primer (Barcoded Iso-Seq primer bcXX[1])

BC TSO

5' TSO 3'

3' TSO CCC TTTTTTTTTT 5'

Reverse (R) PCR Primer (Iso-Seq cDNA amplification primer)

Can barcode up to 12 samples during cDNA amplification step using PacBio barcoded Fwd Iso-Seq primers[1]

Contains Kinnex PCR handle 2

Cleanup & equal-mass pooling

F R

5' BC TSO GGG LIBRARY INSERT AAAAAAAAAA 3'

3' BC TSO CCC LIBRARY INSERT TTTTTTTTTT 5'

Pooled barcoded full-length cDNA for input into Kinnex library construction

Kinnex library prep, sequencing & analysis

Procedure & checklist – Preparing Kinnex libraries using Kinnex 8-fold prep kit (103-238-700); ***OR***

Procedure & checklist – Preparing Kinnex libraries using Kinnex 12-fold prep kit (103-238-800)

F R F R F R F R

BC TSO BC TSO BC TSO BC TSO

Amplified cDNA products containing a PacBio barcode + TSO primer sequence at only one end

Kinnex PCR (parallel PCR Rxs to append Kinnex segmentation adapters)

PCR 1 | PCR 2 | PCR 3… | …PCR (*n*)

A BC B' | B BC C' | C BC D' | ··· BC ···

Pooling & Kinnex array formation

Barcoded Kinnex terminal adapter (bcM0001-bcM0004)[2]

BC A BC B BC C BC ··· BC Q BC

BC A BC B BC C BC D

G BC H BC Q BC

Can also barcode samples during Kinnex array formation using up to 4 different barcoded Kinnex terminal SMRTbell adapters[2]

Kinnex array repair & nuclease Tx

BC A BC B BC C BC ··· BC Q BC

Anneal Kinnex sequencing primer / Bind polymerase / Cleanup (ABC)

BC A BC B BC C BC ··· BC Q BC

Purified polymerase-bound Kinnex library

Long-read sequencing & data analysis

Revio or Vega system

SMRT Link read segmentation & Iso-Seq analysis

[1] Twelve barcoded Iso-Seq primers (Iso-Seq primer bc01–12) are available for cDNA amplification step.

[2] Kinnex adapter barcode sequences can be downloaded from SMRT Link Data Management module.

Kinnex bulk RNA experimental design considerations

Kinnex bulk RNA application use case recommendations for PacBio systems

Example application	Human genetics disease studies	Biopharma for identifying highly expressed targets	Plant & animal whole genome annotation
Experimental goal	Isoform discovery and quantification of moderate-to-rare transcripts	Isoform discovery of high expressed transcripts	Comprehensive transcript annotation in a species
Example study design	Disease vs. normal tissues with multiple replicates	Disease cohort with >20+ samples	Plant or animal with multiple tissue types
Target depth of coverage per sample	10 M reads per sample	5 M reads per sample	5 M reads per sample
Sample multiplexing[1]	**Vega system + SPRQ-Nx:** Up to 4 samples per Vega acquisition (4-plex)	**Vega system + SPRQ-Nx:** Up to 8 samples per Vega SMRT Cell (8-plex)	
	Revio system + SPRQ-Nx: Up to 6 samples per Revio acquisition (6-plex)	**Revio system + SPRQ-Nx:** Up to 12 samples per Revio SMRT Cell (12-plex)	
Expected data throughput (per acquisition)	**Vega system + SPRQ-Nx [Kinnex 8-fold library]:** Up to 50 M reads divided by *N* samples **Vega system + SPRQ-Nx [Kinnex 12-fold library]:** Up to 60 M reads divided by *N* samples		
	Revio system + SPRQ-Nx [Kinnex 8-fold library]: Up to 70 M reads divided by *N* samples **Revio system + SPRQ-Nx [Kinnex 12-fold library]:** Up to 100 M reads divided by *N* samples		
cDNA amplicon generation protocol	Procedure & checklist – Preparing Kinnex-compatible cDNA using Iso-Seq Express 2.0 kit (102-396-000) [Min. input requirement: 300 ng total RNA (RIN ≥7) for 1st-strand cDNA synthesis]		
Kinnex library prep protocol	Procedure & checklist – Preparing Kinnex libraries using the Kinnex 8-fold prep kit (103-920-900); or Procedure & checklist – Preparing Kinnex libraries using the Kinnex 12-fold prep kit (103-921-000)		
SMRT Link data analysis workflows	Read Segmentation and Iso-Seq analysis application with option to “pool reads and cluster together” to get a master isoform classification file with per-sample full-length read counts		
Community data analysis tools	Annotation & quantification: PIGEON, SQANTI3 / Differential analysis: TappAS / Fusion calling: pbfusion / Visualization: SWAN		

[1] Kinnex prep kits can support up to 48-plex Kinnex bulk RNA sample multiplexing through the combined use of 12 different barcoded Iso-Seq cDNA amplification primers (included in Iso-Seq epress 2.0 kit) and 4 different barcoded Kinnex terminal SMRTbell adapters (included in Kinnex concatenation kit 2.0) during Kinnex bulk RNA library construction.

Input RNA quality control for cDNA synthesis

Input RNA quality control is highly recommended before proceeding to the cDNA synthesis step

Procedure & checklist – Preparing Kinnex compatible cDNA using Iso-Seq express 2.0 kit (102-396-000)

1 Input RNA quality control

↓ Safe stop

2 cDNA synthesis + cleanup

↓

3 cDNA amplification + cleanup

Kinnex library prep

Procedure & checklist – Preparing Kinnex libraries using Kinnex 8-fold prep kit (103-920-900)

Procedure & checklist – Preparing Kinnex libraries using Kinnex 12-fold prep kit (103-921-000)

- 300 ng of total RNA per library (minimum concentration 43 ng/µL per library) is required for this procedure
- Sample QC of input total RNA samples should be assessed by measuring RNA Integrity Number (RIN) using a Bioanalyzer 2100 instrument (Agilent Technology) with RNA 6000 Nano kit[1]
  - RIN ≥7.0 (ideally ≥8.0) is sufficient for Kinnex full-length RNA protocol
  - Samples with RIN <7.0 can be processed, but risk of significant underperformance or even failure is greatly increased
- RIN score (1 to 10) is related to ratio of the area under 28s and 18s fragment peaks and also takes into account signal intensity above baseline in the Inter-Region and Fast Region since this is where degradation products appear
- Higher RIN numbers are correlated with better overall sample quality and lower degradation

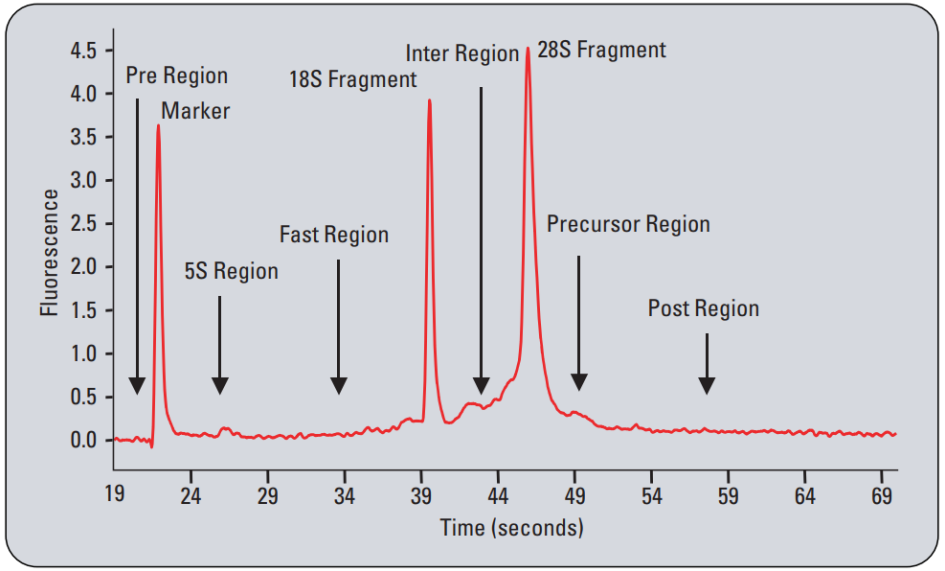
Step 1
procedural summary

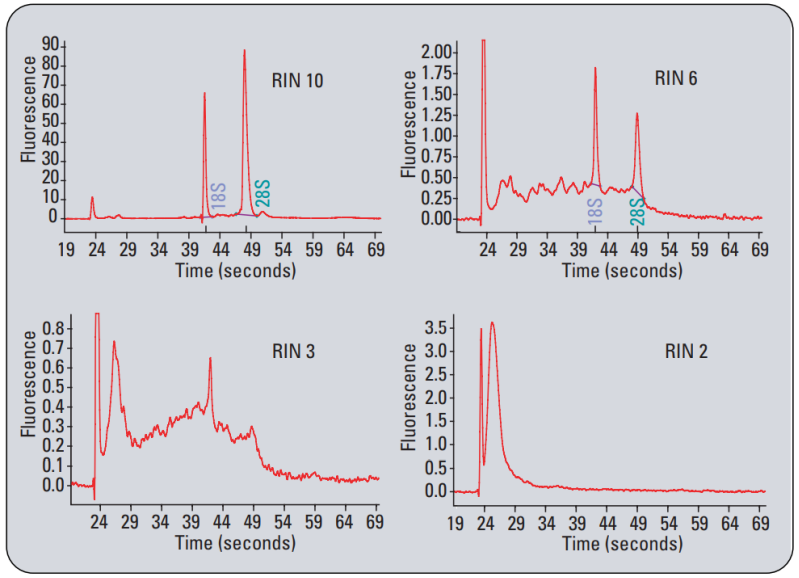
DNA sizing QC

Total RNA RIN ≥ 7

↓

Proceed to **Step 2**





Left: Bioanalyzer electropherogram detailing the regions that are indicative of RNA quality. **Right:** Sample electro-pherograms corresponding to different RNA Integrity Number (RIN) scores. Samples range from intact (RIN 10), to degraded (RIN 2). Images from Agilent Application Note: RNA Integrity Number (RIN) – Standardization of RNA Quality Control (5989-1165EN)

[1] Refer to *Agilent Application Note: RNA Integrity Number (RIN) – Standardization of RNA Quality Control* (5989-1165EN)

cDNA synthesis + cleanup

In this step, total RNA samples are converted to 1st-strand cDNA

Procedure & checklist – Preparing Kinnex compatible cDNA using Iso-Seq express 2.0 kit (102-396-000)

1 Input RNA quality control

↓ Safe stop

2 cDNA synthesis + cleanup

↓

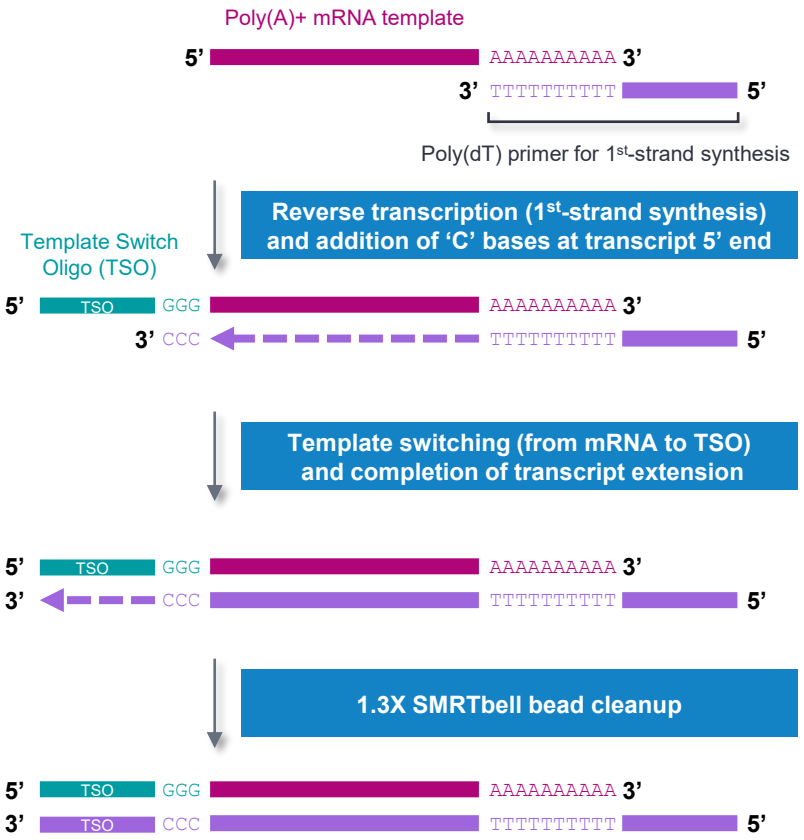
3 cDNA amplification + cleanup

Kinnex library prep

Procedure & checklist – Preparing Kinnex libraries using Kinnex 8-fold prep kit (103-920-900)

Procedure & checklist – Preparing Kinnex libraries using Kinnex 12-fold prep kit (103-921-000)

Minimum total RNA input for cDNA synthesis = 300 ng

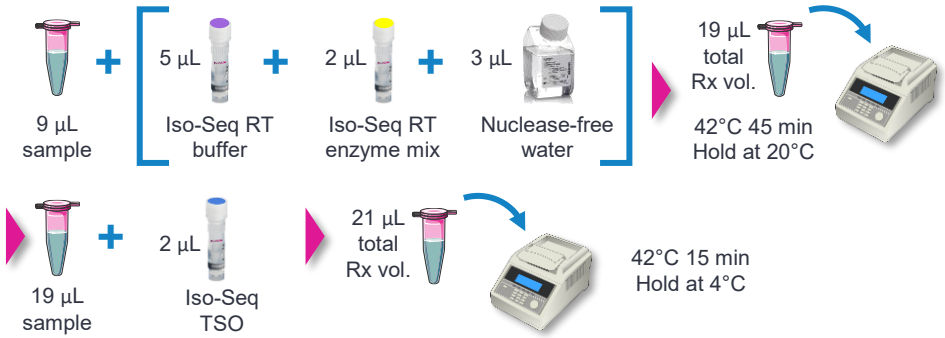


Step 2 procedural summary

2.1 Thawing reagents for first-strand cDNA synthesis

2.2 Primer annealing for first-strand cDNA synthesis

2.3 Reverse transcription and template switching



2.4 1.3X SMRTbell bead cleanup

Proceed to **Step 3**

PacBio

cDNA synthesis + cleanup (cont.)

Procedural notes

Procedure & checklist – Preparing Kinnex compatible cDNA using Iso-Seq express 2.0 kit (102-396-000)

1 Input RNA quality control

↓ ○ Safe stop

2 cDNA synthesis + cleanup

↓

3 cDNA amplification + cleanup

Kinnex library prep

Procedure & checklist – Preparing Kinnex libraries using Kinnex 8-fold prep kit (103-920-900)

Procedure & checklist – Preparing Kinnex libraries using Kinnex 12-fold prep kit (103-921-000)

2.1 cDNA synthesis reagent preparation

✓	Step	Instructions
	2.1.1	Quick-spin the Iso-Seq RT enzyme mix in the microcentrifuge to collect liquid, then place on ice.
		Thaw the following components at room temperature, briefly vortex to mix, then quick-spin to collect liquid and place on ice.

Tube color	Reagent
Orange	Iso-Seq RT primer mix (103-104-000)

- After thawing specified reagents **on ice**, perform a quick spin to collect liquid, then **place on ice**

2.2 RT Primer annealing for first-strand synthesis

✓	Step	Instructions
		For each 300 ng RNA sample (≥43 ng/µL) to be processed, prepare Reaction Mix 1 on ice by adding the following components to each tube in the PCR strip tube.
	2.2.1	**Reaction Mix 1 (RM1):**

✓	Tube color	Components	Volume
		Total RNA (300 ng)	<7 µL

- Set up primer annealing reaction mix **on ice** and then transfer to thermal cycler for incubation

2.3 Reverse transcription and template switching

✓	Step	Instructions
		For each RNA sample, prepare Reaction Mix 2 on ice by adding the following components in the order and volume listed below. Adjust component volumes for the number of samples being prepared, plus 10% overage.
	2.3.1	Reaction Mix 2 (RM2):

✓	Tube color	Components	Volume
	Purple	Iso-Seq RT buffer (vortex briefly before use)	5 µL

- Set up reverse transcription reaction mix **on ice** and then transfer to thermal cycler for incubation

2.4 1.3X SMRTbell bead cleanup

✓	Step	Instructions
	2.4.1	For each sample, **add 29 µL of elution buffer** to the 21 µL reverse transcription and template switching reaction (Section 2.3) for a total volume of 50 µL.
	2.4.2	Add **65 µL** (1.3X v/v) of resuspended, room-temperature **SMRTbell cleanup beads**.
	2.4.3	Mix beads by pipetting 10 times or until evenly distributed.

- Perform **1.3X** SMRTbell bead cleanup

PacBio

cDNA amplification + cleanup

In this step, first-strand cDNA products are PCR-amplified and barcoded using barcoded Iso-Seq primers

Procedure & checklist – Preparing Kinnex compatible cDNA using Iso-Seq express 2.0 kit (102-396-000)

1 Input RNA quality control

↓ Safe stop

2 cDNA synthesis + cleanup

↓

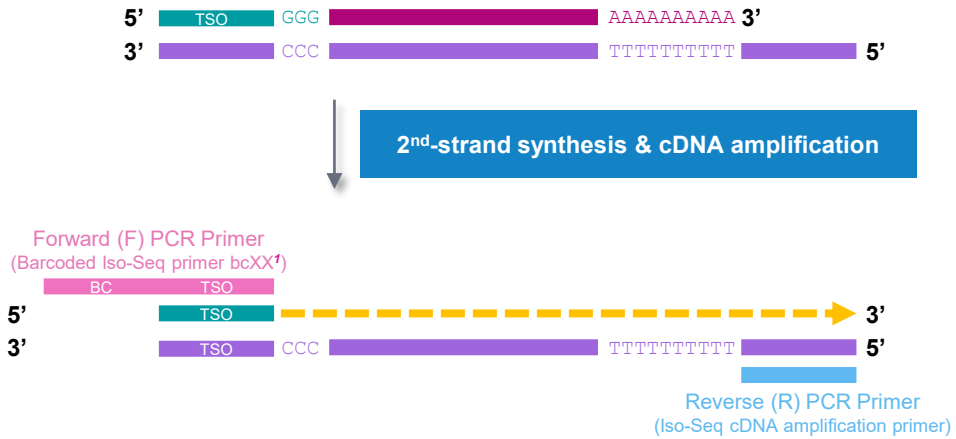
3 cDNA amplification + cleanup

Kinnex library prep

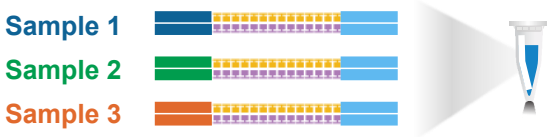
Procedure & checklist – Preparing Kinnex libraries using Kinnex 8-fold prep kit (103-920-900)

Procedure & checklist – Preparing Kinnex libraries using Kinnex 12-fold prep kit (103-921-000)

Barcode up to 12 samples during cDNA amplification step using PacBio barcoded Forward Iso-Seq primers[1]



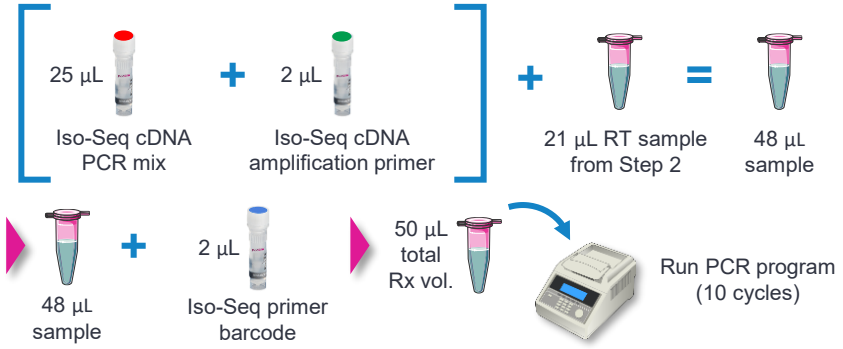
Pool barcoded cDNA (if multiplexing)



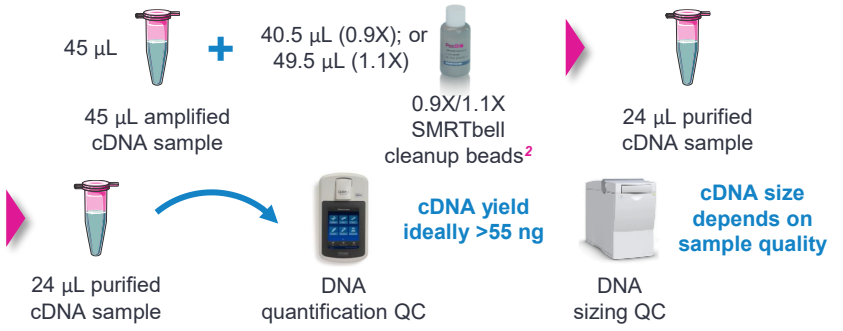
Pooled barcoded full-length cDNA for input into Kinnex library construction

Step 3 procedural summary

3.1 cDNA amplification



3.2 0.9X *or* 1.1X SMRTbell bead cleanup[2]



3.3 Pooling barcoded cDNA (skip if not multiplexing)

Proceed to **Step 4**

[1] Twelve barcoded Iso-Seq primers (Iso-Seq primer bc01–12) are available for cDNA amplification step.
[2] Default recommended SMRTbell bead cleanup ratio is 1.1x followed by library prep using Kinnex 8-fold prep kit, Kinnex 12-fold prep kit or SMRTbell prep kit 3.0. If targeting longer cDNA, 0.9x SMRTbell cleanup beads can be used followed by library prep with Kinnex 8-fold prep kit or SMRTbell prep kit 3.0.

PacBio

cDNA amplification + cleanup

Procedural notes

Procedure & checklist – Preparing Kinnex compatible cDNA using Iso-Seq express 2.0 kit (102-396-000)

1 Input RNA quality control

↓ Safe stop

2 cDNA synthesis + cleanup

↓

3 cDNA amplification + cleanup

Kinnex library prep

Procedure & checklist – Preparing Kinnex libraries using Kinnex 8-fold prep kit (103-920-900)

Procedure & checklist – Preparing Kinnex libraries using Kinnex 12-fold prep kit (103-921-000)

3.1 cDNA amplification

✓	Step	Instructions
	3.1.1	For each sample, prepare Reaction Mix 3 on ice by adding the following components in the order and volume listed below. Adjust component volumes for the number of samples being prepared, plus 10% overage. Pipette mix master mix. **Iso-Seq primer bc01–12 will be added to each sample individually and should not be added to the master mix.**
	3.1.2	On ice, **add 27 µL of RM3** to the 21 µL of the eluted cDNA (from step 2.4.15).
	3.1.3	Add **2 µL of the appropriate Iso-Seq primer barcode** to each sample for a total volume of 50 µL.
	3.1.4	Thoroughly mix by pipetting up and down 10 times and then quick spin to collect all liquid.
	3.1.5	Run the thermal cycler program below. Keep sample on ice until the thermal cycler lid has heated to 105°C.

Reaction Mix 3 (RM3):

✓	Tube color	Components	Volume
	Red	Iso-Seq cDNA PCR mix	25 µL
	Green	Iso-Seq cDNA amplification primer	2 µL
		Total volume	27 µL

✓	Tube color	Component	Volume
	White	Iso-Seq primer bcX	2 µL

Heated lid set at 105°C. Reaction volume set to 50 µL.

PCR program	Kinnex	SPK 3.0
45 seconds at 98°C	1 cycle	1 cycle
10 seconds at 98°C 15 seconds at 60°C 3 minutes at 72°C	10 cycles	12 cycles
5 minutes at 72°C	1 cycle	1 cycle
Hold at 4°C		

SAFE STOPPING POINT -- Store at 4°C or -20°C for long-term storage

- Set up cDNA amplification reaction mix **on ice**
- **Do not add Barcoded Forward Iso-Seq primers to master mix reaction**

- Add desired Barcoded Forward Iso-Seq primer (**select one of bc01-bc12**) to each individual cDNA amplification reaction

- After setting up reactions on ice, add PCR reactions to thermal cycler **after the lid has preheated to 105°C** to avoid digestion of primers by polymerase exonuclease activity

PacBio

cDNA amplification + cleanup (cont.)

Procedural notes

Procedure & checklist – Preparing Kinnex compatible cDNA using Iso-Seq express 2.0 kit (102-396-000)

1 Input RNA quality control

↓ Safe stop

2 cDNA synthesis + cleanup

↓

3 cDNA amplification + cleanup

Kinnex library prep

Procedure & checklist – Preparing Kinnex libraries using Kinnex 8-fold prep kit (103-920-900)

Procedure & checklist – Preparing Kinnex libraries using Kinnex 12-fold prep kit (103-921-000)

- Expected yield of purified cDNA product is **>80 ng**
- Minimum cDNA amount needed to proceed with Kinnex PCR = **25 ng** (**do not proceed with <25 ng**)

3.2 Cleanup of amplified cDNA using 0.9X SMRTbell Cleanup beads

✔	Step	Instructions
	3.2.1	Transfer **45 µL of PCR-amplified cDNA** from Section 3.1 into a clean strip tube . **The correct bead-to-sample ratio is critical at this step.** Refer to the table below for the appropriate bead ratio selection. Add 49.5 µL (1.1x v/v) of resuspended, room-temperature SMRTbell cleanup beads. If targeting longer cDNA, 40.5 µL of SMRTbell cleanup beads (0.9X v/v) can be used.

SMRTbell cleanup bead Ratio	Bead volume (µl)	Recommended library prep kit
1.1X (v/v)	49.5 µl	Kinnex 8-fold Prep kit or Kinnex 12-fold Prep kit or SMRTbell Prep Kit 3.0
0.9X (v/v)	40.5 µl	Kinnex 8-fold Prep kit or SMRTbell Prep Kit 3.0

	3.2.2	Mix beads by pipetting 10 times or until evenly distributed.
	3.2.14	Slowly aspirate the cleared eluate without disturbing the beads. Transfer the entire eluate to a new strip tube. Discard the old strip tube with beads.
	3.2.15	Recommended: Measure concentration and size distribution of each cDNA sample. • Take a 1 µL aliquot from each strip tube. Dilute each aliquot with 4 µL of elution buffer. • Measure DNA concentration with a Qubit fluorometer using the 1x dsDNA HS kit. • Dilute 1:5 dilution further to 1.5 ng/µL based on the Qubit reading if needed. • Run on Agilent Bioanalyzer using High Sensitivity DNA kit; or on Agilent TapeStation using High Sensitivity D5000 ScreenTape Assay. See Figure 2 in the Appendix.
	3.2.16	Refer to table below for the expected cDNA recovery after amplification and the minimum cDNA yield required to proceed with the selected downstream library prep kit.

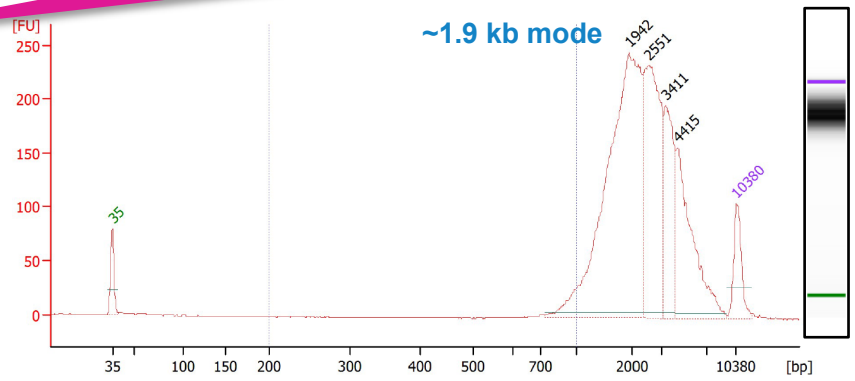
Selected library prep kit	Expected yields per sample (ng)	Minimum yield for library prep (ng)
Kinnex 8-/ 12-fold Prep Kit	> 80ng	> 25ng
SMRTbell Prep Kit 3.0	> 200 ng	> 100ng

SAFE STOPPING POINT -- Store at 4°C or -20°C for long-term storage

- Perform **0.9X or 1.1X** SMRTbell bead cleanup[1]

- **1.1x SMRTbell bead cDNA cleanup + Kinnex 12-fold library prep kit workflow** provides **higher S-read throughput** and **better recovery of <1 kb transcripts** for Kinnex bulk RNA applications
- Can optionally consider using **0.9x SMRTbell bead cDNA cleanup + Kinnex 8-fold library prep kit workflow** for Kinnex bulk RNA applications when retaining the **largest** transcripts is the priority

- Perform DNA concentration QC using Qubit ds DNA HS assay and DNA sizing QC using Bioanalyzer



Example Bioanalyzer DNA sizing QC analysis results for amplified full-length cDNA generated from a universal human RNA reference (UHRR) total RNA sample.

[1] **Note:** Default recommended SMRTbell bead cleanup ratio is 1.1x followed by library prep using Kinnex 8-fold prep kit, Kinnex 12-fold prep kit or SMRTbell prep kit 3.0. If targeting longer cDNA, 0.9x SMRTbell cleanup beads can be used followed by library prep with Kinnex 8-fold prep kit or SMRTbell prep kit 3.0.

cDNA amplification + cleanup (cont.)

Procedural notes

Procedure & checklist – Preparing Kinnex compatible cDNA using Iso-Seq express 2.0 kit (102-396-000)

1 Input RNA quality control

↓ Safe stop

2 cDNA synthesis + cleanup

↓

3 cDNA amplification + cleanup

Kinnex library prep

Procedure & checklist – Preparing Kinnex libraries using Kinnex 8-fold prep kit (103-920-900)

Procedure & checklist – Preparing Kinnex libraries using Kinnex 12-fold prep kit (103-921-000)

3.3 Pooling barcoded cDNA (skip if not multiplexing)

✔	Step	Instructions
	3.3.1	Using the concentration reading from the Qubit fluorometer, pool an **equal mass** of each barcoded cDNA sample. Refer to table below and ensure the total library input is no less than minimum required yield and does not exceed the maximum total multiplex mass for corresponding elution volumn. Store any remaining purified, amplified, barcoded cDNA at 4°C for short-term storage or -20°C for long-term storage.
	3.3.2	Quick-spin the tube strip in a microcentrifuge to collect liquid.
	3.3.3	Proceed to the next step of the protocol: For generating Kinnex™ concatenated libraries, please refer to one of the protocols: • Preparing Kinnex libraries using Kinnex 8-fold prep kit for cDNA with a peak size of 1.5 – 3 kb • Preparing Kinnex libraries using Kinnex 12-fold prep kit for cDNA with a peak size of 1 – 2 kb For generating monomer SMRTbell libraries, please refer to the protocol: • Preparing multiplexed amplicon libraries using SMRTbell prep kit 3.0

Selected library prep kit	Maximum multiplex mass for library prep (ng)
Kinnex 8-/ 12-fold Prep Kit	35ng
SMRTbell Prep Kit 3.0	500ng

PROTOCOL COMPLETE – Store at -20°C for long-term storage

- If multiplexing, perform **equal-mass pooling** of each barcoded cDNA sample after SMRTbell bead cleanup

Comparison of 0.9x vs. 1.1x SMRTbell bead cDNA cleanup for Kinnex bulk RNA sequencing

1.1x SMRTbell bead cDNA cleanup + Kinnex 12-fold library prep workflow provides higher data throughput and better recovery of shorter transcripts for Kinnex bulk RNA applications compared to 0.9x SMRTbell bead cDNA cleanup + Kinnex 8-fold library prep workflow

Example Kinnex bulk RNA workflow

cDNA synthesis and amplification (Iso-Seq express 2.0 kit)

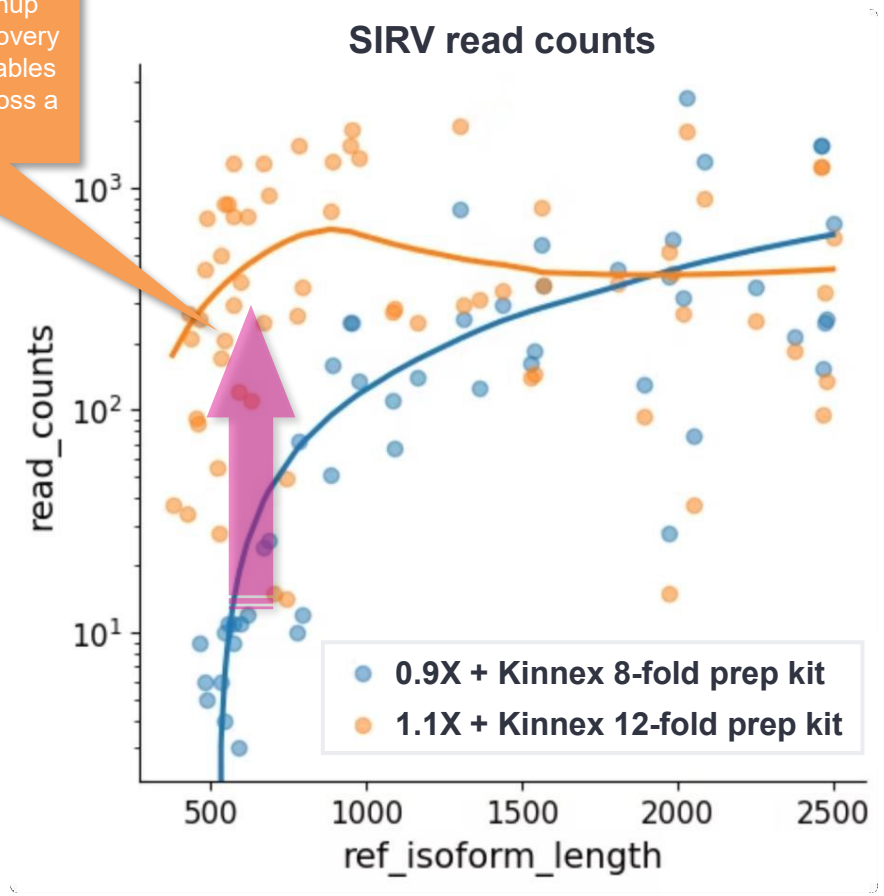
↓

Amplified cDNA cleanup (SMRTbell cleanup beads)

↓

0.9x SMRTbell cleanup beads	1.1x SMRTbell cleanup beads
↓	↓
Kinnex library prep Kinnex 8-fold prep kit	Kinnex library prep Kinnex 12-fold prep kit
↓	↓
SPRQ-Nx	SPRQ-Nx
50 – 70 M S-reads	**70 – 100 M S-reads**
• More effectively retains largest transcripts (>2 kb)	• More effectively recovers shorter transcripts (<1 kb) • Higher overall data throughput

1.1x SMRTbell bead cleanup results in more efficient recovery of <1 kb transcripts and enables improved quantification across a broader size range



Comparison of transcript counts obtained for spike-in RNA variant (SIRV) control samples prepared using 0.9X versus 1.1X SMRTbell bead cleanup procedures during post-cDNA amplification step in *Procedure & checklist – Preparing Kinnex-compatible cDNA using Iso-Seq Express 2.0 kit* (102-396-000). SIRV-Set 2 (Iso Mix E0) (Lexogen Cat. No. 050) contains 69 isoform sequences in equimolar ratios, which originate from 7 genes.

PacBio

Comparison of 0.9x vs. 1.1x SMRTbell bead cDNA cleanup for Kinnex bulk RNA sequencing

1.1x SMRTbell bead cDNA cleanup + Kinnex 12-fold library prep workflow provides higher data throughput and better recovery of shorter transcripts for Kinnex bulk RNA applications compared to 0.9x SMRTbell bead cDNA cleanup + Kinnex 8-fold library prep workflow

Example Kinnex bulk RNA workflow

cDNA synthesis and amplification (Iso-Seq express 2.0 kit)

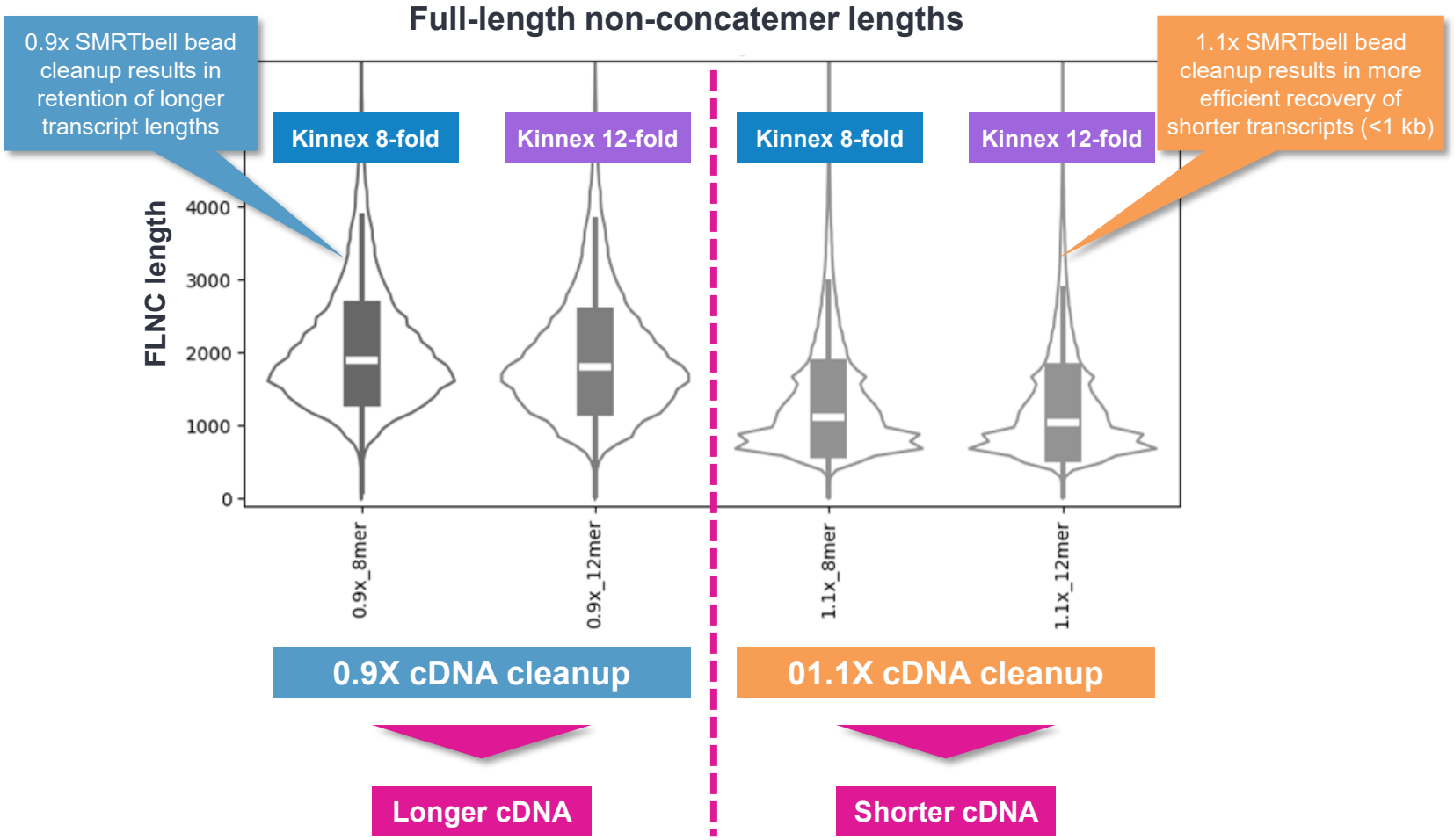
↓

Amplified cDNA cleanup (SMRTbell cleanup beads)

↓

0.9X SMRTbell cleanup beads	1.1X SMRTbell cleanup beads
↓	↓
Kinnex library prep Kinnex 8-fold prep kit	Kinnex library prep Kinnex 12-fold prep kit
↓	↓
SPRQ-Nx	SPRQ-Nx
50 – 70 M S-reads	**70 – 100 M S-reads**
• More effectively retains largest transcripts (>2 kb)	• More effectively recovers shorter transcripts (<1 kb) • Higher overall data throughput

Full-length non-concatemer lengths



- **1.1x SMRTbell bead cDNA cleanup + Kinnex 12-fold prep kit workflow** provides **higher S-read throughput** and **better recovery of <1 kb transcripts** for Kinnex bulk RNA applications
- Can optionally consider using **0.9x SMRTbell bead cDNA cleanup + Kinnex 8-fold prep kit workflow** for Kinnex bulk RNA applications when retaining the **largest** transcripts is the priority

PacBio

Example Kinnex 8-fold bulk RNA library prep inputs & expected yields

Final Kinnex library yield is typically sufficient to support ≥8 SMRT Cell acquisitions on Revio & Vega systems

Kinnex bulk RNA

Procedure & checklist – Preparing Kinnex compatible cDNA using Iso-Seq express 2.0 kit (102-396-000)

Amplicon generation

cDNA synthesis → SMRTbell bead cleanup → cDNA amplification → SMRTbell bead cleanup

IN: 300 ng of total RNA per sample

OUT: ≥100 ng of amplified cDNA per sample

Procedure & checklist – Preparing Kinnex libraries using Kinnex 8-fold prep kit (103-920-900)

Kinnex PCR

Kinnex PCR → SMRTbell bead cleanup

IN: 35 ng of amplified cDNA (single or multiplex)

OUT: 5-9 µg of amplified products after pooling

Procedure & checklist – Preparing Kinnex libraries using Kinnex 8-fold prep kit (103-920-900)

Kinnex array formation

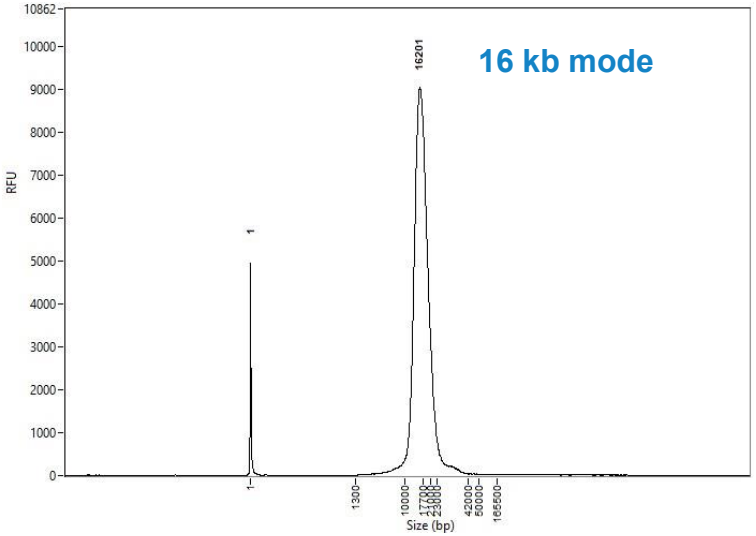
Kinnex digestion / Ligation → Kinnex repair → SMRTbell cleanup → Nuclease treatment → SMRTbell bead cleanup

IN: 5 µg of pooled Kinnex PCR products

OUT: 80% recovery from Kinnex digestion Rx input

IN: 40 µL of purified Kinnex repair reaction products

OUT: 10-30% recovery from Kinnex digestion Rx input



Example Femto pulse DNA sizing QC analysis results for final Kinnex 8-fold bulk RNA library prepared with human universal human reference RNA (UHRR) total RNA sample.

Example Kinnex 8-fold bulk RNA library prep yields

Total RNA input for cDNA synthesis	300 ng
cDNA amplicon input for Kinnex 8-fold array formation	5000 ng
Post-nuclease treatment & final library cleanup yield (%)[1]	1000 ng (20%)

[1] Post-nuclease treatment & final cleanup yields typically ranged from ~10% to ~30% when using UHRR total RNA samples for Kinnex full-length RNA library construction.

Final Kinnex library yield is typically sufficient to run the following number of SMRT Cell acquisitions:

- >8 acquisitions for Revio system using SPRQ-Nx chemistry
- >8 acquisitions for Vega system using SPRQ-Nx chemistry

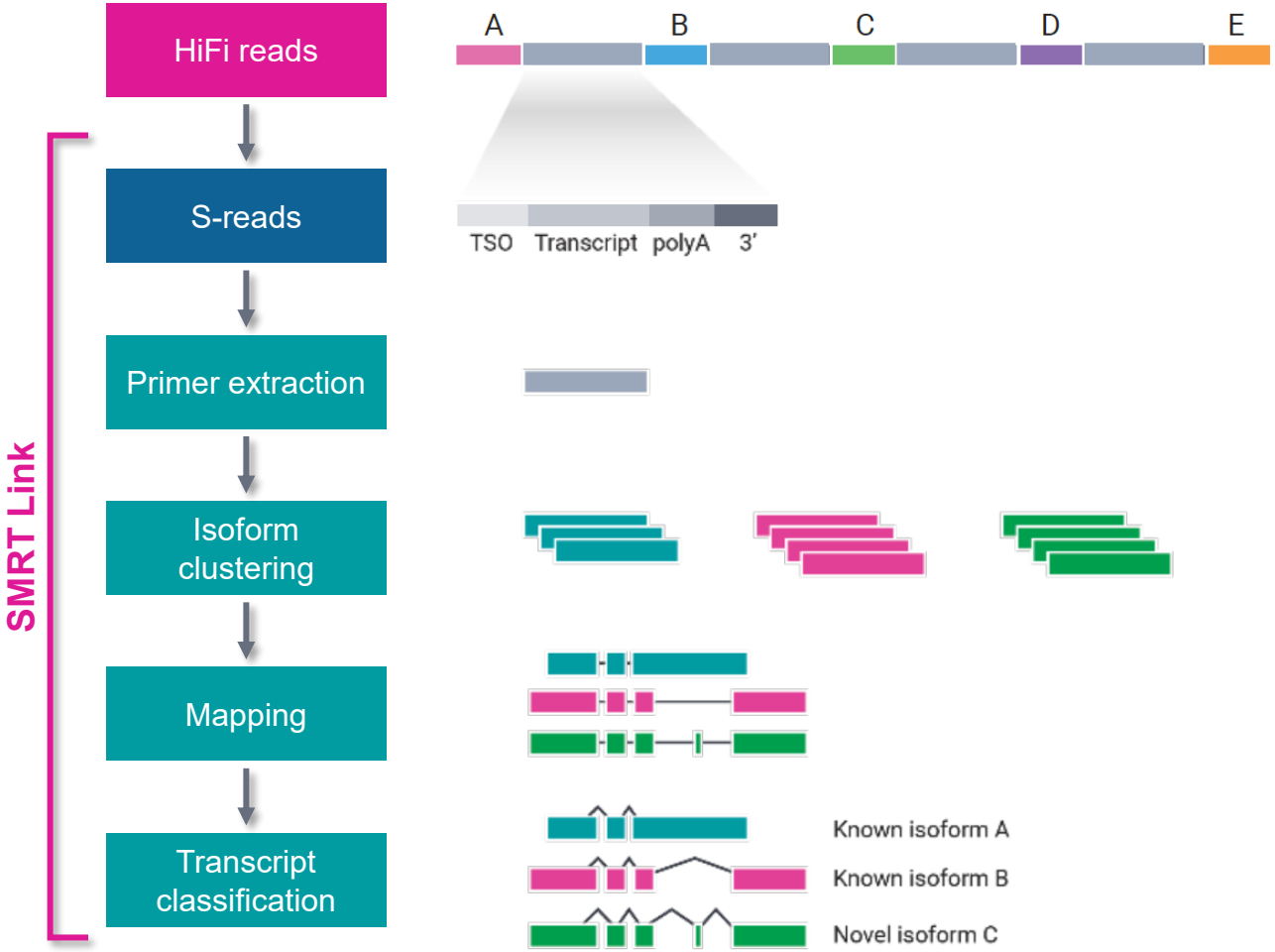
PacBio

Kinnex bulk RNA data analysis workflow overview

Kinnex bulk RNA bioinformatics workflow overview

SMRT Link Read segmentation and Iso-Seq workflow processes HiFi reads generated from Kinnex bulk RNA libraries to produce classified isoforms with read counts that are compatible with tertiary analysis tools

SMRT Link read segmentation and Iso-Seq workflow[1]



Read segmentation

- HiFi reads are segmented into individual segmented reads (S-reads) that represent the original cDNA sequences

Primer extraction

- Primers and polyA tails are removed, but also used to orient the read into 5' → 3' orientation

Isoform clustering

- FLNC reads are clustered by their sequencing similarity to produce isoform consensus sequences
- This step is the last step of Iso-Seq analysis if no genome is provided

Mapping

- If a genome is provided, isoform consensus sequences from the previous step are mapped and further collapsed by their exonic structures to produce isoforms as GFF files for visualization

Transcript classification[2]

- If an annotation (e.g., Gencode) is provided, isoforms are classified against it using pigeon (the PacBio implementation of SQANTI3) to identify known and novel genes/isoforms
- The Iso-Seq workflow can jointly analyze pooled sample reads to produce a unified isoform annotation with per-sample read counts, both raw and normalized as counts per million (CPM)

[1] See ***SMRT Link User Guide*** (Documentation) for detailed descriptions of parameter settings for Read Segmentation and Iso-Seq analysis application. (A video tutorial is also available for viewing.)
[2] Note: SMRT Link only supports transcript classification for human and mouse samples. Non-human/mouse samples will require customized annotation GTF files to be run *via* the command line.

Kinnex bulk RNA bioinformatics workflow recommendations

SMRT Link Read Segmentation and Iso-Seq workflow common considerations and recommendations for analysis of Kinnex bulk RNA data[1]

Analysis recommendations for Iso-Seq data based on reference genome and annotation availability[1]

- With SMRT Link v26.2, the **Read segmentation and Iso-Seq workflow** analysis application supports human and mouse reference genomes and annotations to produce classified isoforms with read counts.
- If working with other organisms, see table below for analysis recommendations

Available reference or annotation	Analysis workflow recommendation
Human or mouse	• Use the Iso-Seq workflow with preloaded human / mouse annotation to get mapped, unique isoforms with classifications and read count information (FASTA, GFF, TXT).
Model organism with good annotation	• Run Iso-Seq workflow with uploaded reference genome to get mapped, unique isoforms (FASTA, GFF) • Generate pigeon-compliant annotation and use the command line for isoform classification with read count information (TXT)
Non-model organism with genome	• Run Iso-Seq workflow with uploaded reference genome to get mapped, unique isoforms (FASTA, GFF)
No genome	• Run Iso-Seq workflow without reference genome to get unique isoforms (FASTA)

[1] See *Application note – Kinnex full-length RNA kit for isoform sequencing* (102-326-591).

Kinnex bulk RNA bioinformatics workflow recommendations (cont.)

SMRT Link Read Segmentation and Iso-Seq workflow common considerations and recommendations for analysis of Kinnex bulk RNA data[1]

Sequencing depth recommendations for Iso-Seq data based on experimental goals and study design

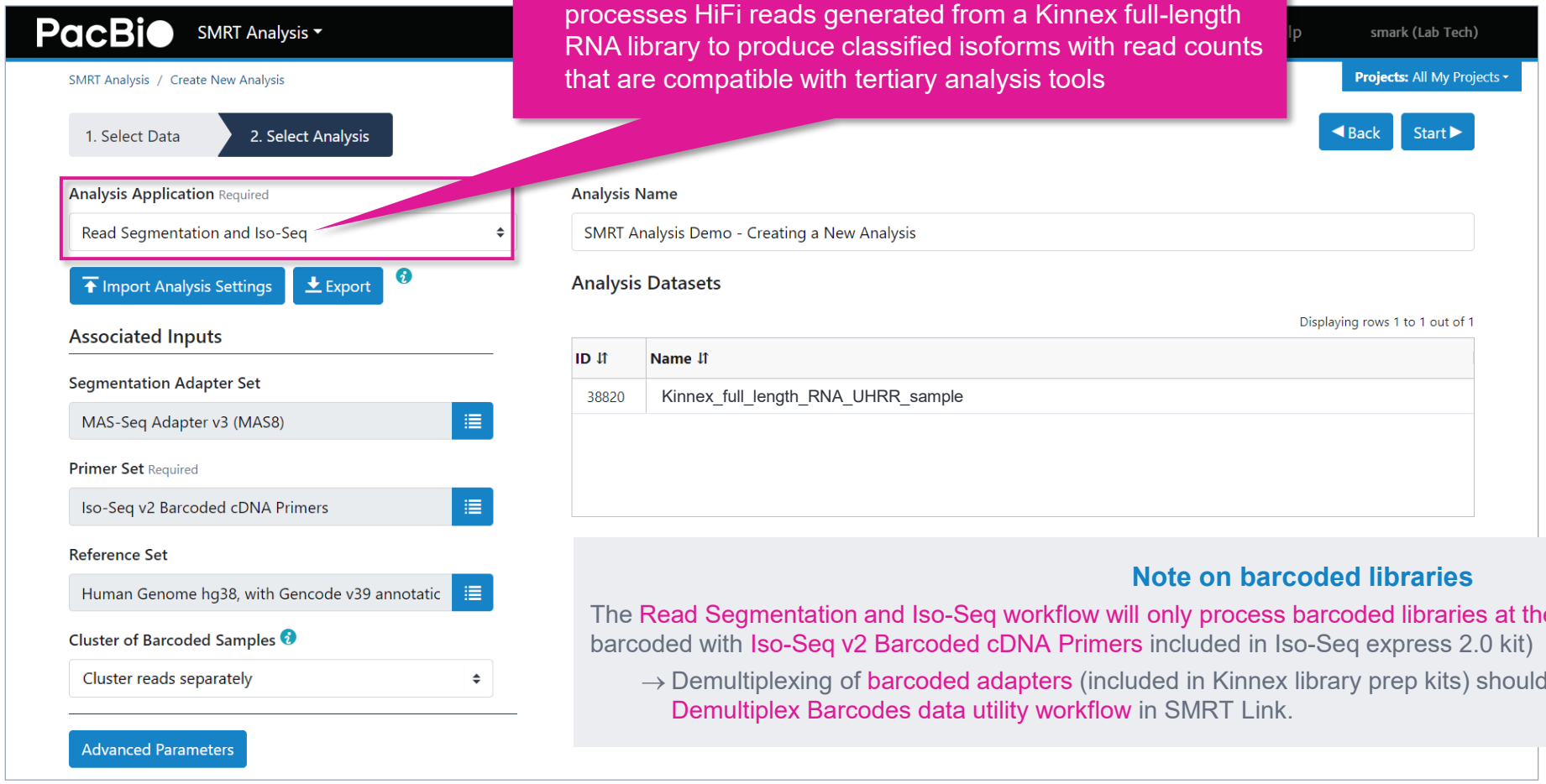
Example application	Human genetics disease studies	Biopharma for identifying highly expressed targets	Plant & animal whole genome annotation
Experimental goal	Isoform discovery and quantification of moderate-to-rare transcripts	Isoform discovery of high expressed transcripts	Comprehensive transcript annotation in a species
Example study design	Disease vs. normal tissues with multiple replicates	Disease cohort with >20+ samples	Plant or animal with multiple tissue types
Target depth of coverage per sample	10 M reads per sample	5 M reads per sample	≤5 M reads per tissue (of same species)
Sample multiplexing[1]	**Vega system + SPRQ-Nx:** Up to 4 samples per Vega acquisition (4-plex)	**Vega system + SPRQ-Nx:** Up to 8 samples per Vega acquisition (8-plex)	
	Revio system + SPRQ-Nx: Up to 6 samples per Revio acquisition (6-plex)	**Revio system + SPRQ-Nx:** Up to 12 samples per Revio acquisition (12-plex)	
SMRT Link data analysis workflows	Read Segmentation and Iso-Seq analysis application with option to “pool reads and cluster together” to get a master isoform classification file with per-sample full-length read counts		

[1] See *Application note – Kinnex full-length RNA kit for isoform sequencing* (102-326-591).

SMRT Link Read Segmentation and Iso-Seq analysis application setup

Specify **Read Segmentation and Iso-Seq** analysis application in SMRT Link[1]

- **Read Segmentation and Iso-Seq** analysis application processes HiFi reads generated from a Kinnex full-length RNA library to produce classified isoforms with read counts that are compatible with tertiary analysis tools



Note on barcoded libraries

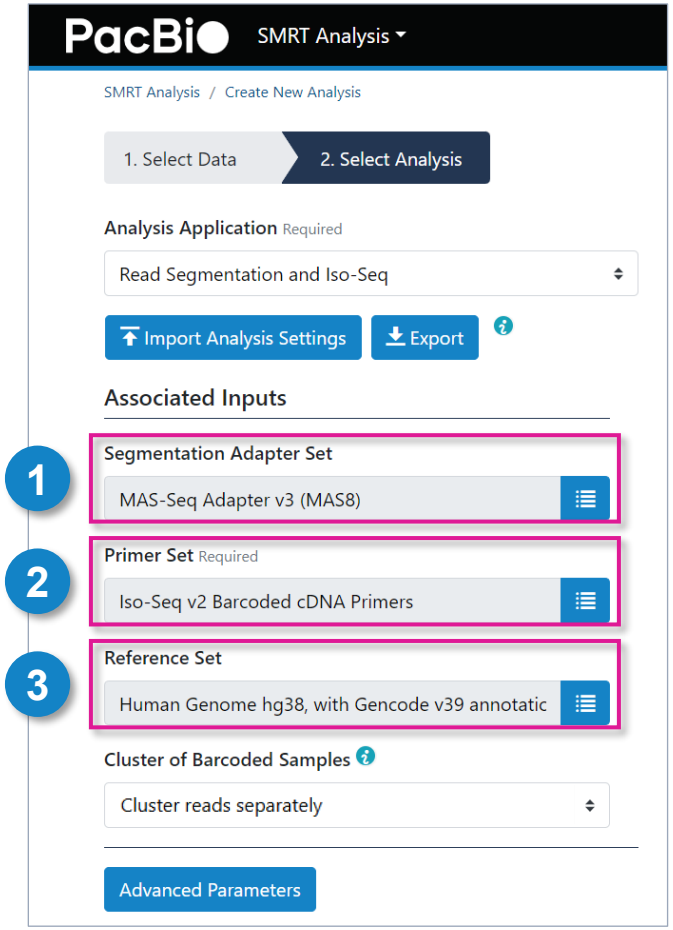
The Read Segmentation and Iso-Seq workflow will only process barcoded libraries at the cDNA level (such as bulk cDNA libraries barcoded with Iso-Seq v2 Barcoded cDNA Primers included in Iso-Seq express 2.0 kit)

→ Demultiplexing of barcoded adapters (included in Kinnex library prep kits) should first be performed by running the Demultiplex Barcodes data utility workflow in SMRT Link.

[1] See *SMRT Link User Guide* (Documentation) for detailed descriptions of parameter settings for Read Segmentation and Iso-Seq analysis application.

SMRT Link Read Segmentation and Iso-Seq analysis application setup (cont.)

Specify **Read Segmentation and Single-Cell Iso-Seq** analysis application required associated inputs[1]



1. Segmentation Adapter Set (Default = MAS-Seq Adapter v3 (MAS8))

- For Kinnex 8-fold libraries, specify Segmentation Adapter Set = MAS-Seq Adapter v3 (MAS8)
- Specify a FASTA file, provided by PacBio, containing segmentation adapters. If you need a custom segmentation adapter set, click Advanced Parameters and use a custom FASTA file formatted as described in the SMRT Link User Guide documentation.

2. Primer Set (Required) (Default = Iso-Seq v2 Barcoded cDNA Primers)

- Specify a primer sequence file in FASTA format to identify cDNA primers for removal. The primer sequence includes the 5' and 3' cDNA primers
- Primer IDs must be specified using the suffix `_5p` to indicate 5' cDNA primers and the suffix `_3p` to indicate 3' cDNA primers. The 3' cDNA primer should not include the `T`s and is written in reverse complement. (See the SMRT Link User Guide for example Iso-Seq v2 Barcoded cDNA Primer IDs and sequences)
- Each primer sequence must be unique

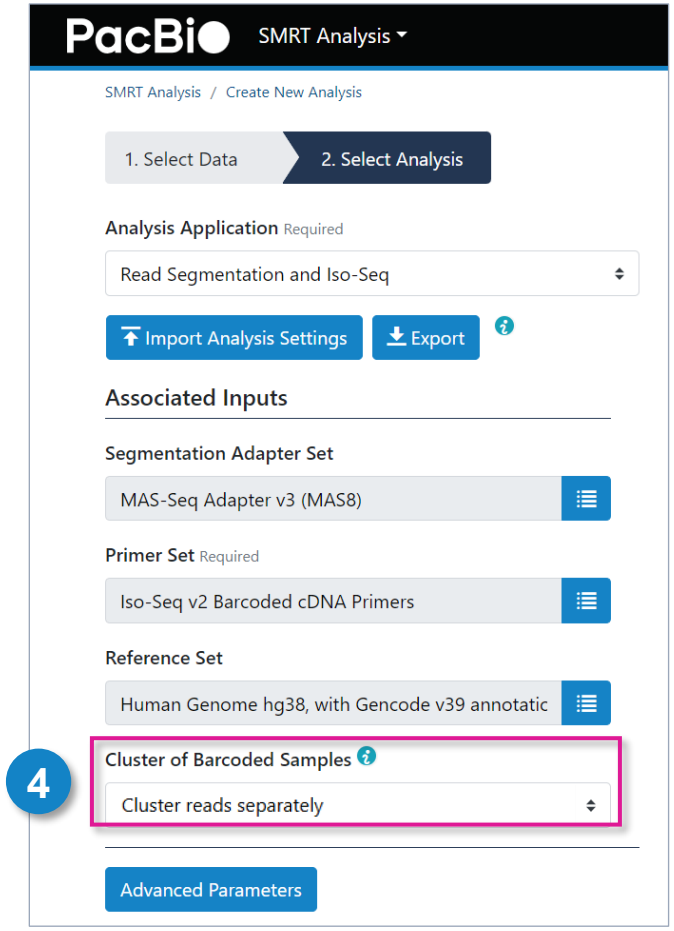
3. Reference Set (Required)

- Specify one of two default reference genome and annotation sets to align high quality isoforms to, and to collapse isoforms mapped to the same genomic loci. The default sets are `Human_hg38_Gencode_v39` and `Mouse_mm39_Gencode_vM28` annotations
- Alternatively, choose other reference genomes (but not with annotations) that were custom-uploaded to SMRT Link
- The Reference Set can be left blank. If blank, the workflow will stop after the isoform clustering step (`isoseq cluster`)

[1] See ***SMRT Link User Guide*** (Documentation) for detailed descriptions of parameter settings for Read Segmentation and Iso-Seq analysis application.

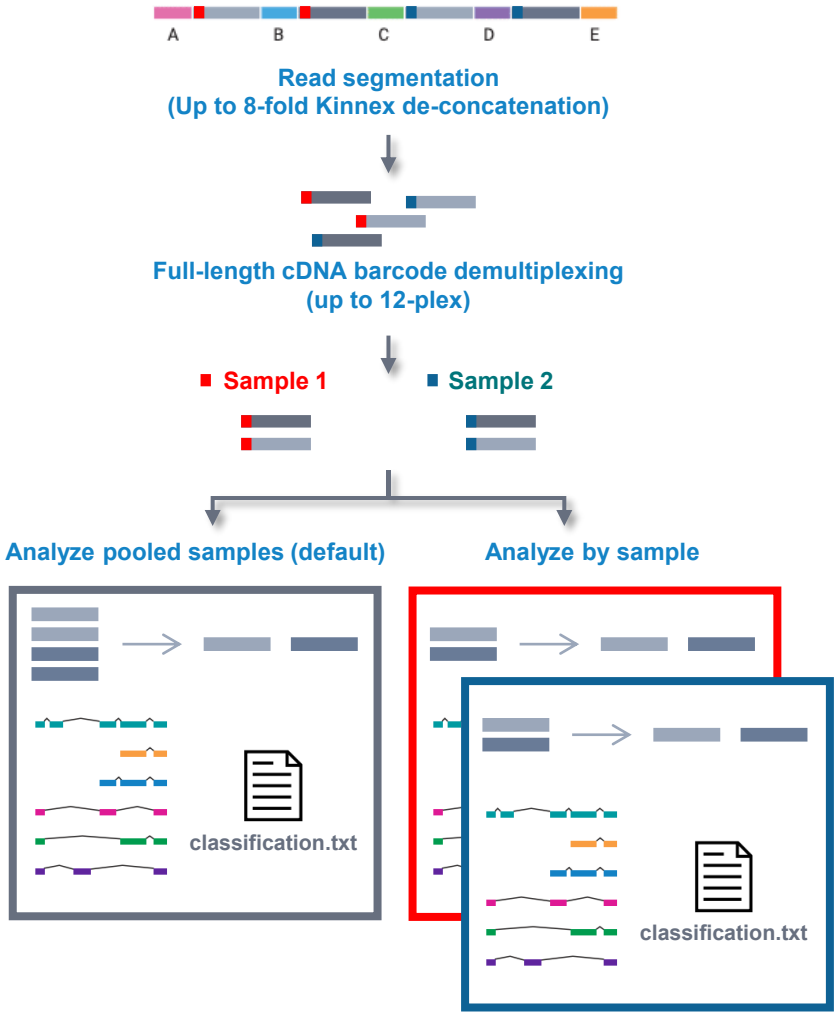
SMRT Link Read Segmentation and Iso-Seq analysis application setup (cont.)

Specify **Read Segmentation and Single-Cell Iso-Seq** analysis application required associated inputs[1]



4. Cluster of Barcoded Samples

- This option specifies barcoded samples that were barcoded at the cDNA level, where the (barcoded) cDNA primers are specified in the Primer Set option. This option does **not** address libraries that were barcoded using barcoded adapters
- Specify whether all FLNC reads will be pooled for clustering, then demultiplexed based on pooled result. **Note:** This setting does **not** apply to non-barcoded samples
- Specify **Pool reads and cluster together** if barcoded samples are from the **same** species, but different tissues, or samples of the same genes but different individuals. The samples are clustered with **all** barcodes pooled
- Specify **Cluster reads separately** if barcoded samples are from different species. The samples are clustered **separately** by barcode
- In either case, the samples on the results page are automatically named `BioSample_1` through `BioSample_N`



[1] See *SMRT Link User Guide* (Documentation) for detailed descriptions of parameter settings for Read Segmentation and Iso-Seq analysis application.

Example SMRT Link Read Segmentation data utility processing results[1] for Kinnex 8-fold bulk RNA library prepared with human UHRR sample

SMRT Link Read Segmentation data utility job report – Summary Metrics and Segmentation Statistics

Summary Metrics

Value	Analysis Metric
10,404,037	Reads
81,012,128	Segmented reads (S-reads)
1,758.004	Mean length of S-reads
94.55 %	Percent of reads with full arrays
7.79	Mean array size (concatenation factor)

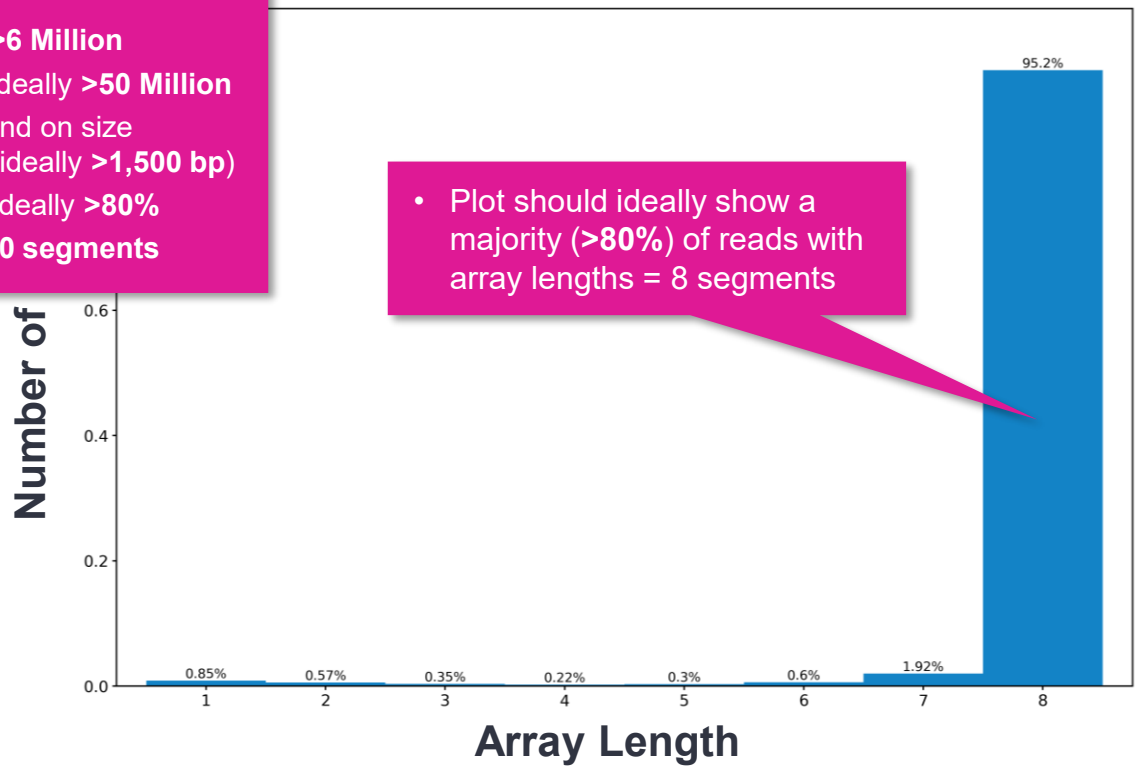
Example Revio system + SPRQ data shown.

For Kinnex 8-fold bulk RNA library run on Revio system:

- Yield of HiFi reads is ideally **>6 Million**
- Yield of segmented reads is ideally **>50 Million**
- Mean S-read length will depend on size distribution of cDNA sample (ideally **>1,500 bp**)
- % of reads with full arrays is ideally **>80%**
- Mean array size is ideally **>7.0 segments**

- **Reads:** Number of input arrayed HiFi reads
- **Segmented reads (S-reads):** Number of generated S-reads
- **Mean length of S-reads:** Mean read length of generated S-reads
- **Percent of reads with full arrays:** Percentage of input HiFi reads containing all adapter sequences in the order listed in the segmentation adapter FASTA file
- **Mean array size:** Mean number of fragments (or S-reads) found in input reads

Segmentation Statistics



- Plot should ideally show a majority (**>80%**) of reads with array lengths = 8 segments

Histogram distribution of the number of S-reads per HiFi read. (Example Revio system + SPRQ data shown.)

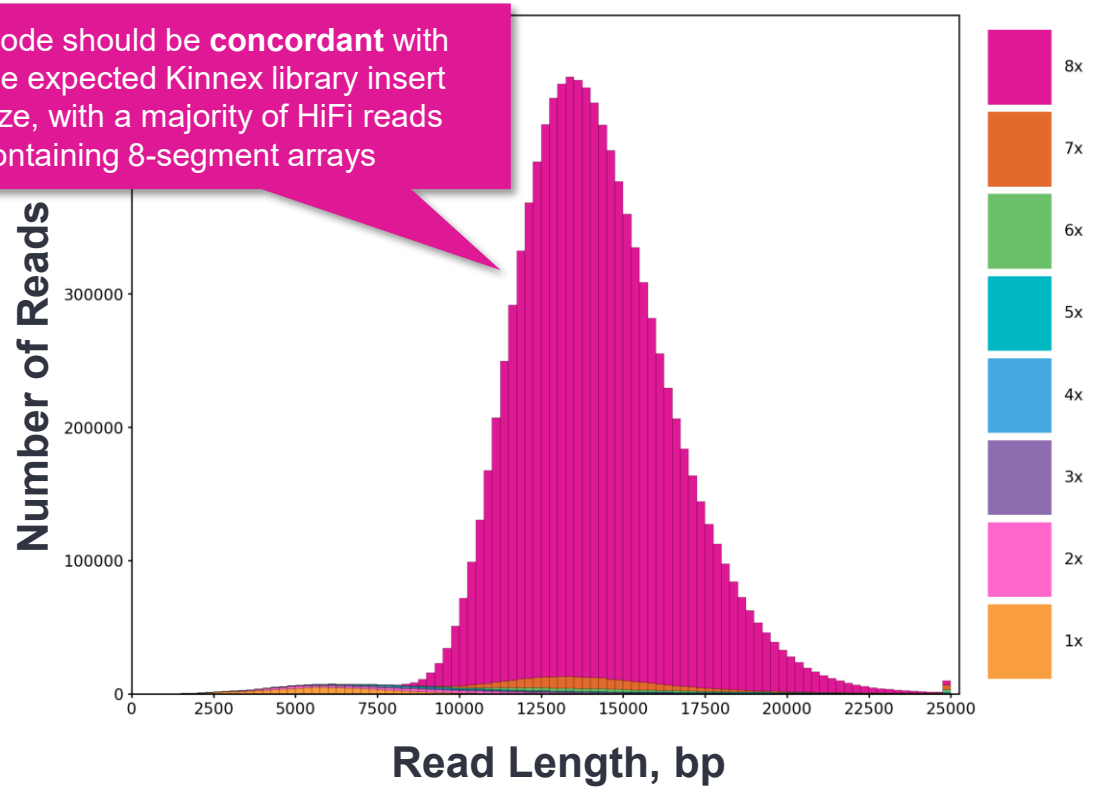
[1] HiFi read lengths, reads/data per SMRT Cell and other sequencing performance results can vary depending on DNA sample quality, insert size, *P1* loading performance & movie time. Note: Refer to ***SMRT Link v13.1 Kinnex single-cell troubleshooting guide*** (103-516-100) for example performance metrics typically achievable with Kinnex libraries under optimal *P1* loading conditions. For Revio system, we recommend aiming for ~60 – 80% *P1* loading for Kinnex full-length RNA libraries.

Example SMRT Link Read Segmentation data utility processing results[1] for Kinnex 8-fold bulk RNA library prepared with human UHRR sample (cont.)

SMRT Link Read Segmentation data utility job report – Length of Reads and S-read Length Distribution

Length of Reads

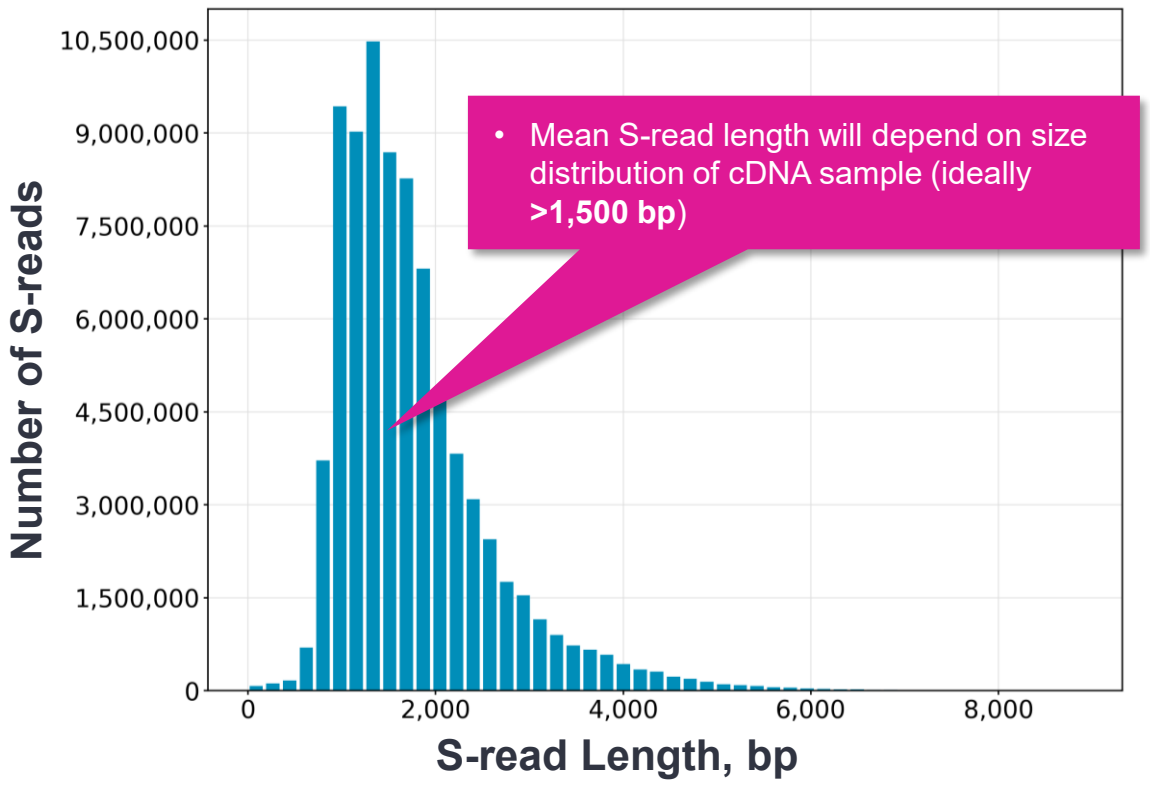
- Mode should be **concordant** with the expected Kinnex library insert size, with a majority of HiFi reads containing 8-segment arrays



Histogram distribution of the number of HiFi reads by read length, in base pairs. (Example Revio system + SPRQ data shown.)

S-read Length Distribution

- Mean S-read length will depend on size distribution of cDNA sample (ideally **>1,500 bp**)



Histogram distribution of the number of S-reads by HiFi read length, in base pairs. (Example Revio system + SPRQ data shown.)

[1] HiFi read lengths, reads/data per SMRT Cell and other sequencing performance results can vary depending on DNA sample quality, insert size, *P1* loading performance & movie time. Note: Refer to ***SMRT Link v13.1 Kinnex single-cell troubleshooting guide*** (103-516-100) for example performance metrics typically achievable with Kinnex libraries under optimal *P1* loading conditions. For Revio system, we recommend aiming for ~60 – 80% *P1* loading for Kinnex bulk RNA libraries.

Example SMRT Link Iso-Seq analysis results for Kinnex 8-fold bulk RNA library prepared with human UHRR sample

SMRT Link Iso-Seq analysis job report – Read Classification statistics

Summary Metrics

Value	Analysis Metric
81,012,128	Reads
78,815,334	Reads with 5' and 3' Primers
78,699,872	Non-Concatamer Reads with 5' and 3' Primers
78,639,000	Non-Concatamer Reads with 5' and 3' Primers and Poly-A Tail (FLNC Reads)
1,643	Mean Length of FLNC Reads
6	Unique Primers
13,135,889	Mean Reads per Primer
13,959,398	Max. Reads per Primer
12,391,746	Min. Reads per Primer
2,196,794	Reads without Primers
96.85%	Percent Bases in Reads with Primers
97.28%	Percent Reads with Primers

- **Reads:** Total number of CCS reads
- **Reads with 5’ and 3’ Primers:** Number of CCS reads with 5’ and 3’ cDNA primers detected
- **Non-Concatemer Reads with 5’ and 3’ Primers:** Number of nonconcatemer CCS reads with 5’ and 3’ primers detected
- **Non-Concatemer Reads with 5’ and 3’ Primers and Poly-A Tail (FLNC Reads):** Number of non-concatemer CCS reads with 5’ and 3’ primers and polyA tails detected. This is usually the number for full-length, nonconcatemer (FLNC) reads, unless polyA tails are not present in the sample
- **Mean Length of FLNC Reads:** Mean length of the non-concatemer CCS reads with 5' and 3' primers and polyA tails detected
- **Unique Primers:** Number of unique primers in the sequence
- **Mean Reads per Primer:** Mean number of CCS reads per primer
- **Max. Reads per Primer:** Maximum number of CCS reads per primer
- **Min. Reads per Primer:** Minimum number of CCS reads per primer
- **Reads without Primers:** Number of CCS reads without a primer
- **Percent Bases in Reads with Primers:** Percentage of bases in CCS reads in the sequence data that contain primers
- **Percent Reads with Primers:** Percentage of CCS reads in the sequence data that contain primers

Example SMRT Link Iso-Seq analysis results for Kinnex 8-fold bulk RNA library prepared with human UHRR sample (cont.)

SMRT Link Iso-Seq analysis job report – Read Classification statistics

Primer Data

Bio Sample Name	Primer Name	CCS Reads	Mean Primer Quality	Reads with 5' and 3' Primers	Non-Concatamer Reads with 5' and 3' Primers
BioSample_1	IsoSeqX_bc01_5p--IsoSeqX_3p	13,800,150	99.7	13,800,150	13,787,052
BioSample_2	IsoSeqX_bc02_5p--IsoSeqX_3p	13,959,398	99.7	13,959,398	13,937,187
BioSample_3	IsoSeqX_bc03_5p--IsoSeqX_3p	12,728,468	99.7	12,728,468	12,707,044
BioSample_4	IsoSeqX_bc04_5p--IsoSeqX_3p	12,404,920	99.7	12,404,920	12,386,009
BioSample_5	IsoSeqX_bc05_5p--IsoSeqX_3p	12,391,746	99.7	12,391,746	12,372,742
BioSample_6	IsoSeqX_bc06_5p--IsoSeqX_3p	13,530,652	99.7	13,530,652	13,509,838
UNASSIGNED	No Primer	2,196,794	0.0	0	0

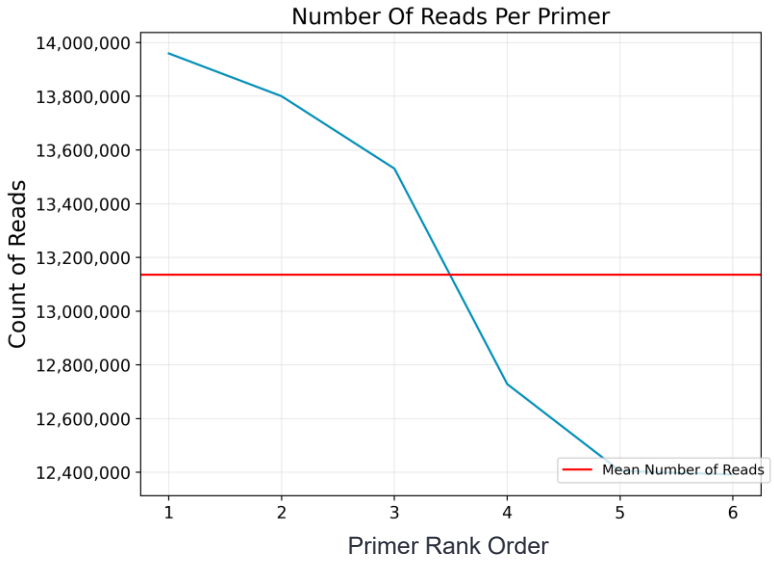
- **Bio Sample Name:** Name of the biological sample associated with the primer
- **Primer Name:** A string containing the pair of primer indices associated with this biological sample
- **CCS Reads:** Number of CCS reads associated with the primer
- **Mean Primer Quality:** Mean primer quality associated with the primer
- **Reads with 5’ and 3’ Primers:** Number of CCS reads with 5’ and 3’ cDNA primers detected
- **Non-Concatemer Reads with 5’ and 3’ Primers:** Number of non-concatemer CCS reads with 5’ and 3’ primers detected
- **Non-Concatemer Reads with 5’ and 3’ Primers and Poly-A Tail:** Number of non-concatemer CCS reads with 5’ and 3’ primers and polyA tails detected. This is usually the number for full-length, non-concatemer (FLNC) reads, unless polyA tails are not present in the sample.

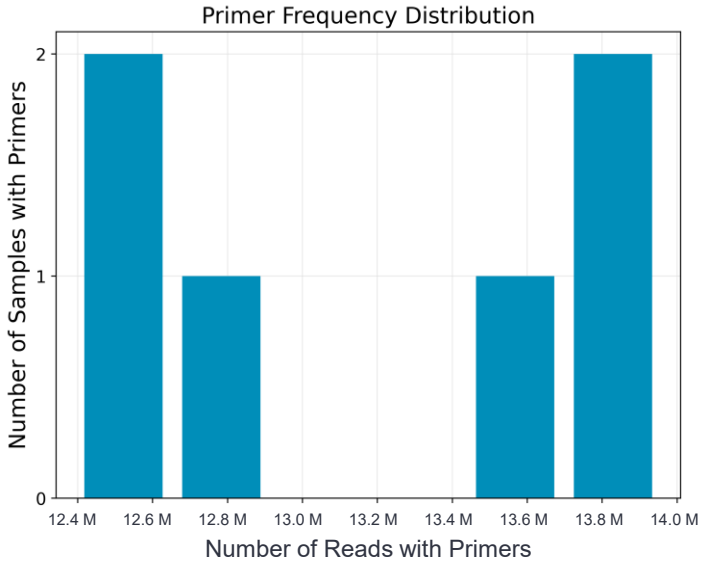
Example Revio system + SPRQ data shown.

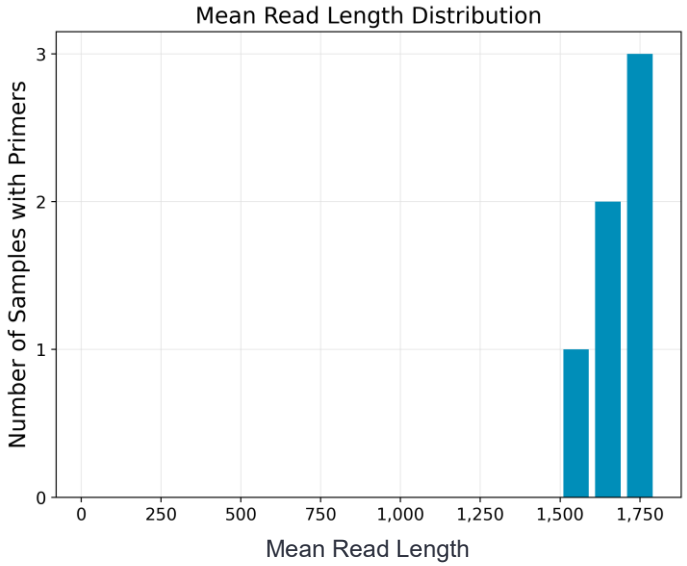
Example SMRT Link Iso-Seq analysis results for Kinnex 8-fold bulk RNA library prepared with human UHRR sample (cont.)

SMRT Link Iso-Seq analysis job report – Read Classification statistics

Primer Read Statistics







Number Of Reads Per Primer: Maps the number of reads per primer, sorted by primer ranking

Primer Frequency Distribution: Maps the number of samples with primers by the number of reads with primers

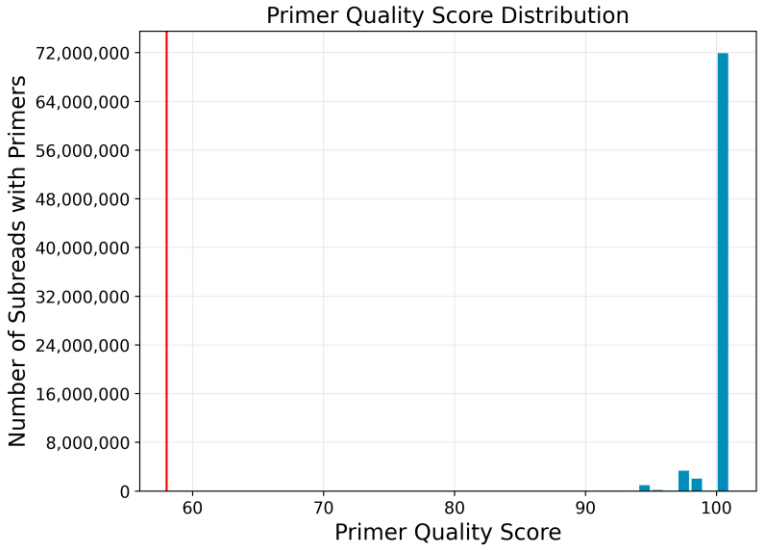
Mean Read Length Distribution: Maps the read mean length against the number of samples with primers

Example Revio system + SPRQ data shown.

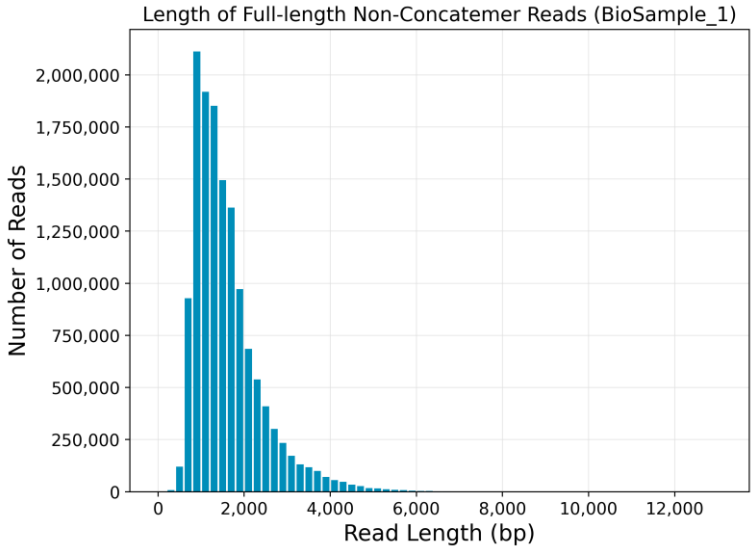
Example SMRT Link Iso-Seq analysis results for Kinnex 8-fold bulk RNA library prepared with human UHRR sample (cont.)

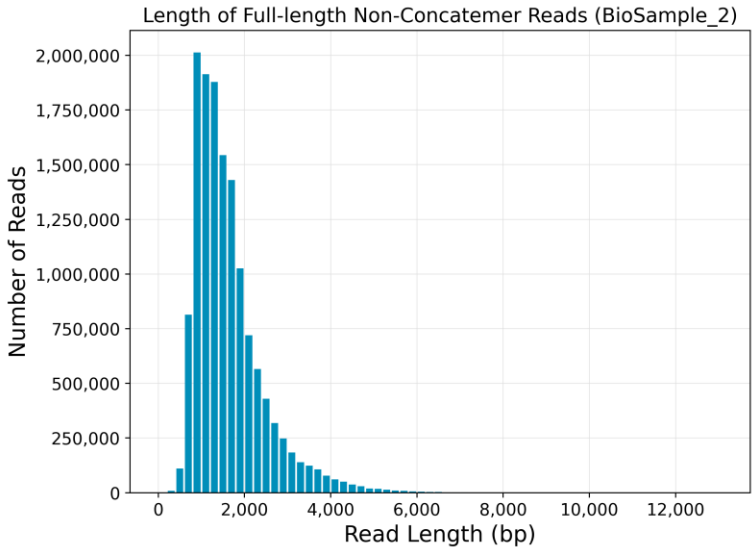
SMRT Link Iso-Seq analysis job report – Read Classification statistics

Primer Quality Scores



Length of Full-length Non-Concatemer Reads





Primer Quality Score Distribution: Histogram of primer scores

Length of Full-Length Non-Concatemer Reads: Per-sample histograms of the read length distribution of non-concatemer CCS reads with 5' and 3' primers and polyA tails detected

Example Revio system + SPRQ data shown.

Example SMRT Link Iso-Seq analysis results for Kinnex 8-fold bulk RNA library prepared with human UHRR sample (cont.)

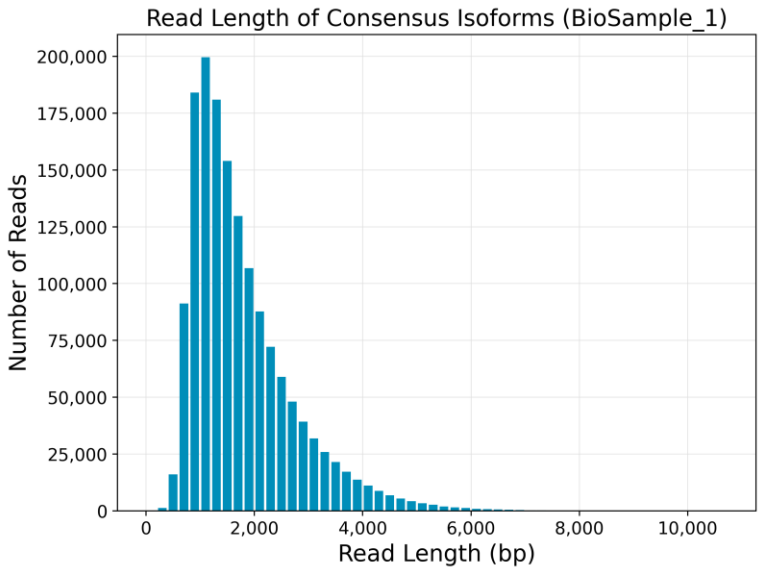
SMRT Link Iso-Seq analysis job report – Transcript Clustering statistics

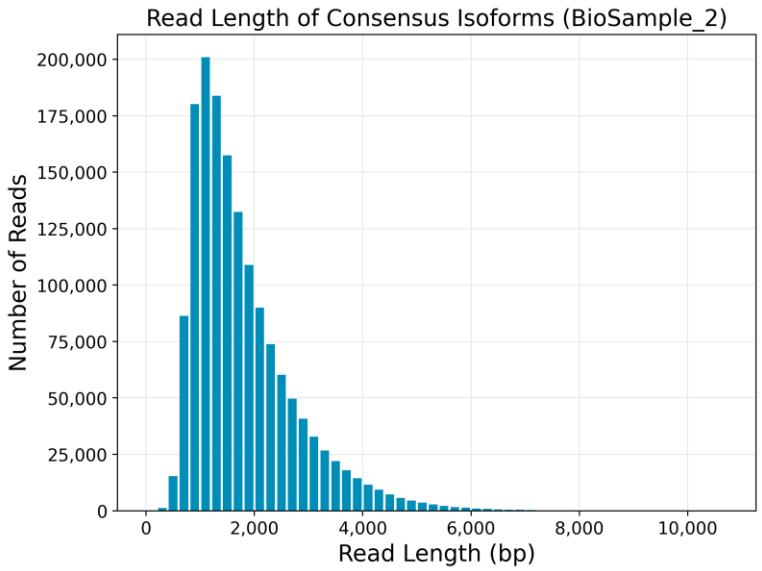
Summary Metrics

Sample Name	Number of High-Quality Isoforms
BioSample_1	1,531,163
BioSample_2	1,549,686
BioSample_3	1,447,494
BioSample_4	1,449,777
BioSample_5	1,442,229
BioSample_6	1,521,973
All Samples	2,930,150

- **Sample Name:** Sample name for which the following metrics apply
- **Number of High-Quality Isoforms:** Number of consensus isoforms that have an estimated accuracy above the specified threshold

Length of Consensus Isoforms





- **Length of Consensus Isoforms:** Per-sample histograms of the consensus isoform lengths and the distribution of isoforms exceeding a read length cutoff. Also includes a single histogram plot for all samples.

Example SMRT Link Iso-Seq analysis results for Kinnex 8-fold bulk RNA library prepared with human UHRR sample (cont.)

SMRT Link Iso-Seq analysis job report – Transcript Mapping and Classification statistics

Summary Metrics (All samples)

Sample Name	Genes	Genes, filtered	Known Genes, filtered	Isoforms	Isoforms, filtered	Known isoforms, filtered	Novel isoforms > 1TPM, filtered
BioSample_1	403,607	25,116	21,839	829,922	236,783	64,884	8,731
BioSample_2	405,157	25,172	21,978	840,868	240,486	65,292	8,933
BioSample_3	375,913	24,601	21,629	778,487	223,422	64,022	7,682
BioSample_4	369,570	24,459	21,607	781,424	231,051	63,712	9,386
BioSample_5	367,898	24,382	21,574	778,483	230,618	63,540	9,473
BioSample_6	393,111	24,862	21,827	827,709	241,390	64,789	9,234

- **Sample Name:** Sample name for which the following metrics apply
- **Total unique genes:** The total number of unique genes across all cells.
- **Total unique genes, filtered:** The total number of unique genes, after filtering out reads based on the SQANTI transcript filtering criteria.
- **Total unique isoforms:** The total number of unique isoforms across all cells
- **Total unique isoforms, filtered:** The total number of unique isoforms across all cells, after filtering out reads based on the SQANTI transcript filtering criteria.

Example SMRT Link Iso-Seq analysis results for Kinnex 8-fold bulk RNA library prepared with human UHRR sample (cont.)

SMRT Link Iso-Seq analysis job report – Transcript Mapping and Classification statistics

Transcript Classification, filtered (All samples)[1]

Category	Count	CAGE Detected	CAGE Detected, (%)	polyA Detected	polyA Detected, (%)
FSM	202364	91165	45.05%	108319	53.52%
ISM	314609	40144	12.75%	191810	60.96%
NIC	183274	114701	62.58%	96830	52.83%
NNC	152653	92299	60.46%	82649	54.14%
Antisense	3074	599	19.48%	1890	61.48%
Fusion	5204	2937	56.43%	2921	56.12%
More junctions	135	76	56.29%	82	60.74%
Genic intron	0	0	0.00%	0	0.00%
Genic genomic	2059	802	38.95%	1177	57.16%
Intergenic	6606	509	7.70%	5297	80.18%

- **Category:** Transcript classification[2] assigned by the classification and filtering tool `pigeon`, based on the SQANTI3 software
- **Count:** The number of transcripts, after filtering out reads based on the SQANTI filtering criteria, in a specific classification
- **CAGE Detected:** The number of transcripts where the transcription start site falls within 50 bp of an annotated CAGE (Cap Analysis of Gene Expression) peak site
- **CAGE Detected, (%):** The percentage of transcripts where the transcription start site falls within 50 bp of an annotated CAGE peak site
- **polyA Motif Detected:** The number of transcripts where a known polyA motif is detected upstream of the transcription end site
- **polyA Motif Detected, (%):** The percentage of transcripts where a known polyA motif is detected upstream of the transcription end site

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[1] Example Revio system + SPRQ data shown. **Note:** Unfiltered transcript classification data are also displayed in the Iso-Seq analysis job report.
[2] Refer to the ***SMRT Link User Guide*** (Documentation) for descriptions of transcript classification categories (e.g., FSM – Full splice match, ISM – Incomplete splice match, etc.).

Example SMRT Link Iso-Seq analysis results for Kinnex 8-fold bulk RNA library prepared with human UHRR sample (cont.)

SMRT Link Iso-Seq analysis job report – Transcript Mapping and Classification statistics

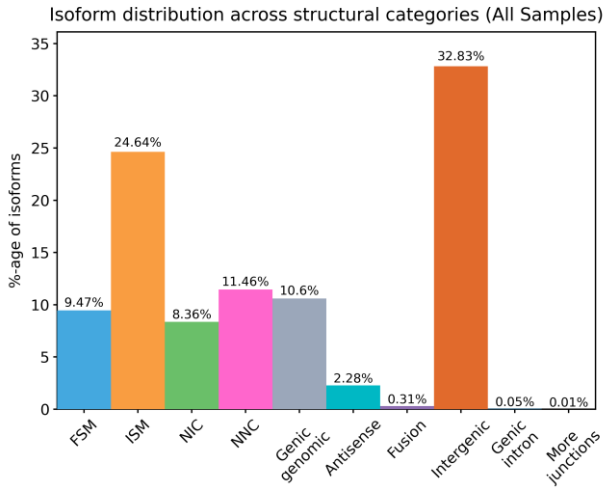
Transcript Classification Plots

Transcript Classification Plots, Filtered

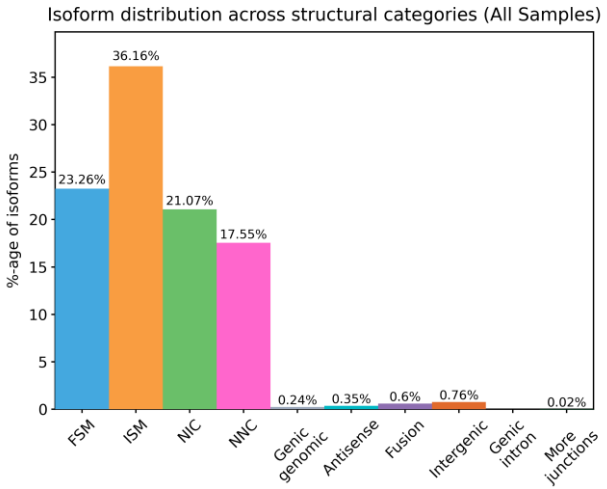
Isoform distributions across structural categories:

- Distribution of the % of isoforms by structural categories

Example Revio system + SPRQ data shown.



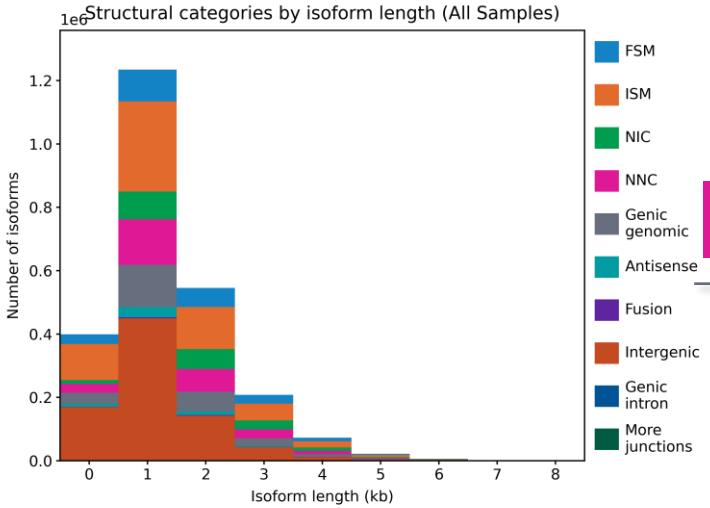
Filter out reads based on the SQANTI3 transcript filtering criteria



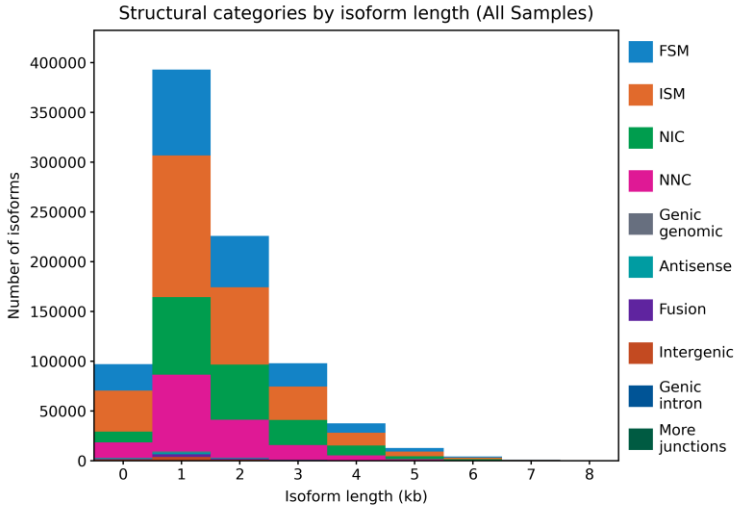
Structural categories by isoform lengths:

- Histogram display of the number of isoforms by their length in kb and their structural category

Example Revio system + SPRQ data shown.



Filter out reads based on the SQANTI3 transcript filtering criteria



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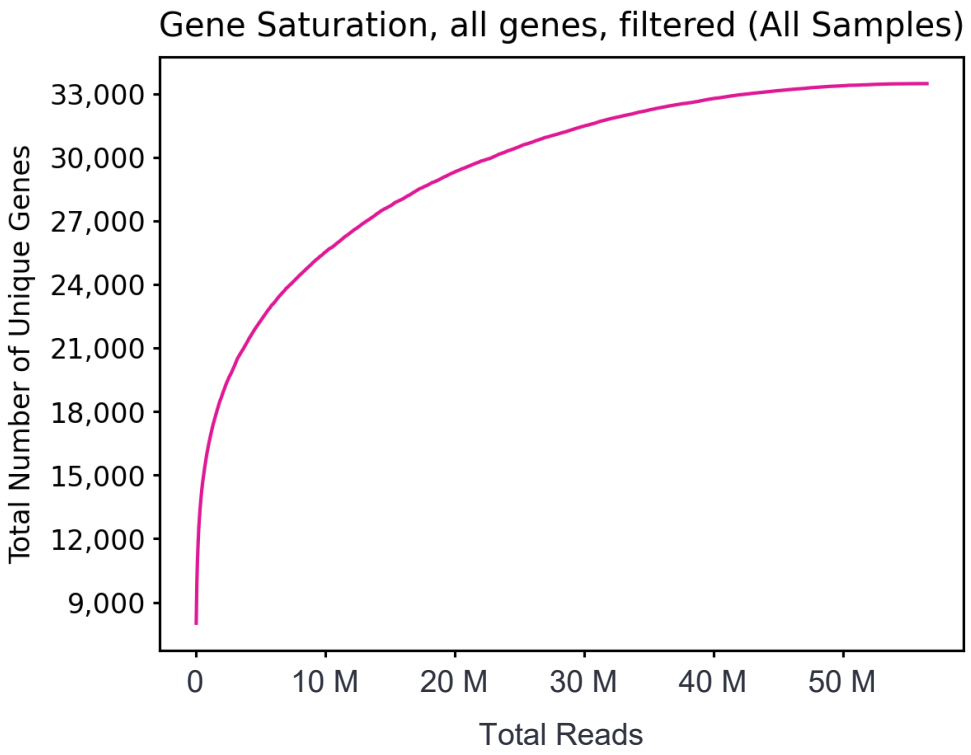
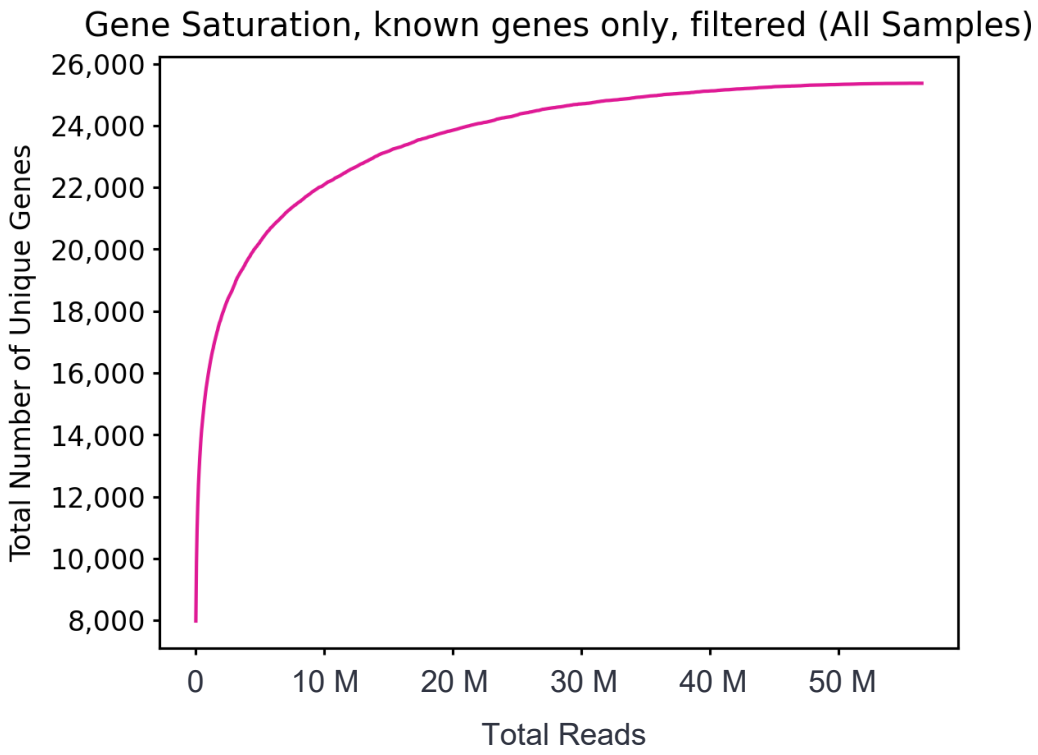
Example SMRT Link Iso-Seq analysis results for Kinnex 8-fold bulk RNA library prepared with human UHRR sample (cont.)

SMRT Link Iso-Seq analysis job report – Transcript Mapping and Classification statistics

Gene Saturation





Gene Saturation, all genes, filtered: Saturation plot showing the level of gene saturation for all genes, after filtering out reads based on the SQANTI transcript filtering criteria

Gene Saturation, known genes only, filtered: Saturation plot showing the level of gene saturation, for unique known genes only (genes annotated in the reference annotation) per cell, after filtering out reads based on the SQANTI transcript filtering criteria

Example Revio system + SPRQ data shown.

Example SMRT Link Iso-Seq analysis results for Kinnex 8-fold bulk RNA library prepared with human UHRR sample (cont.)

File Downloads tab

Edit Output File Name Prefix **Example:**analysis-Bio Sample 4-2110

File	Size	Type
Non-passing reads, unaligned	4 GB	bam
Report read_segmentation	3 KB	JsonReport
SMRT Link Log	13 KB	log
Segmented Reads, passing, unaligned	65 GB	bam
UHRR_verification_DL_bc4 (demux1) (Bio Sample 4) Segmented Reads	20 KB	ConsensusReadSet
Unique mapped transcripts, GFF (All Samples)	895 MB	gff
Unique mapped transcripts, classification TXT (All Samples)	728 MB	txt
Unique mapped transcripts, filtered, GFF (All Samples)	599 MB	gff
Unique mapped transcripts, filtered, classification TXT (All Samples)	307 MB	txt
Unique mapped transcripts, filtered, junctions TXT (All Samples)	624 MB	txt
Unique mapped transcripts, junctions TXT (All Samples)	771 MB	txt

Files shown in the File Downloads tab are available on the analysis results page. Additional files are also available on the SMRT Link server in the analysis output directory.

Refer to SMRT Link user guide for descriptions of downloadable output files

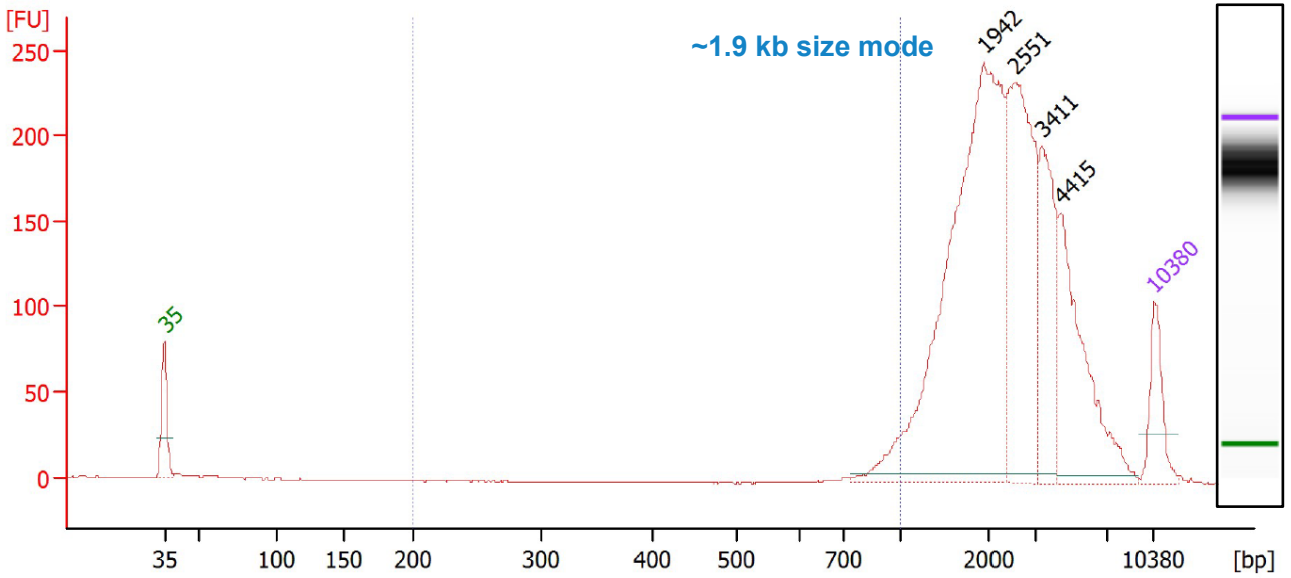
- These files are useful for **visualizing** isoform structures in Integrative Genomics Viewer (IGV) / UCSC genome browser and enable understanding of why an isoform is novel/known, etc.
  - GFF file containing unique mapped transcripts after filtering
  - Text file containing unique mapped transcript classifications against annotations, after filtering
  - Text file containing information about unique mapped transcript junctions, after filtering

Kinnex bulk RNA example sequencing performance data

Example Kinnex 8-fold bulk RNA library preparation QC results

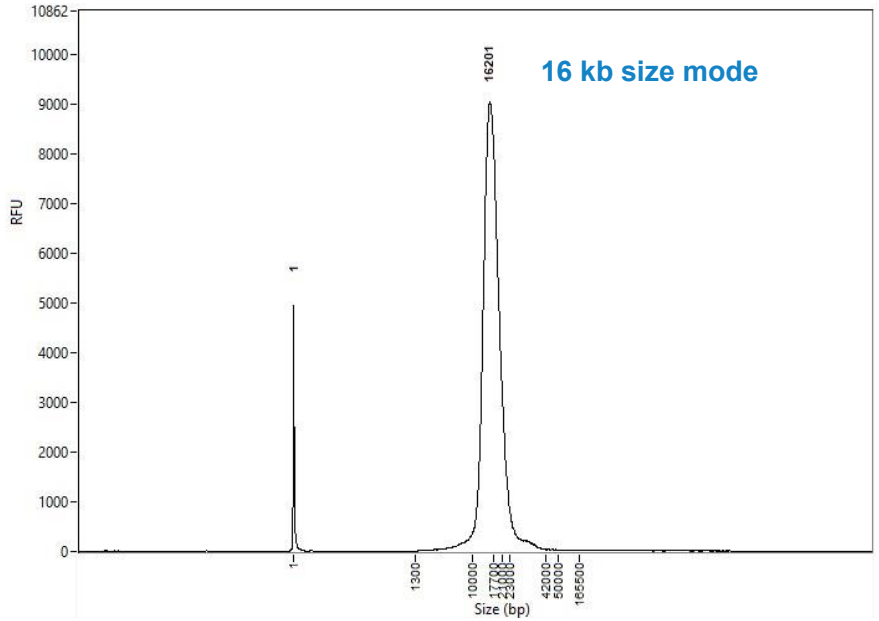
Kinnex 8-fold bulk RNA library prepared from universal human reference RNA (UHRR)

Amplified full-length cDNA QC



Example Bioanalyzer DNA sizing QC analysis results for amplified full-length cDNA generated from a universal human RNA reference (UHRR) total RNA sample.

Final Kinnex full-length RNA library QC



Example Femto Pulse DNA sizing QC analysis results for final Kinnex full-length RNA library.

Final Kinnex library yield is typically sufficient to run the following number of SMRT Cell acquisitions:

- >8 acquisitions for Revio system using SPRQ-Nx chemistry
- >8 acquisitions for Vega system using SPRQ-Nx chemistry

Total RNA input for cDNA synthesis	300 ng
cDNA input for Kinnex array formation	5000 ng
Post-nuclease treatment & final library cleanup yield (%)[1]	1000 ng (20%)

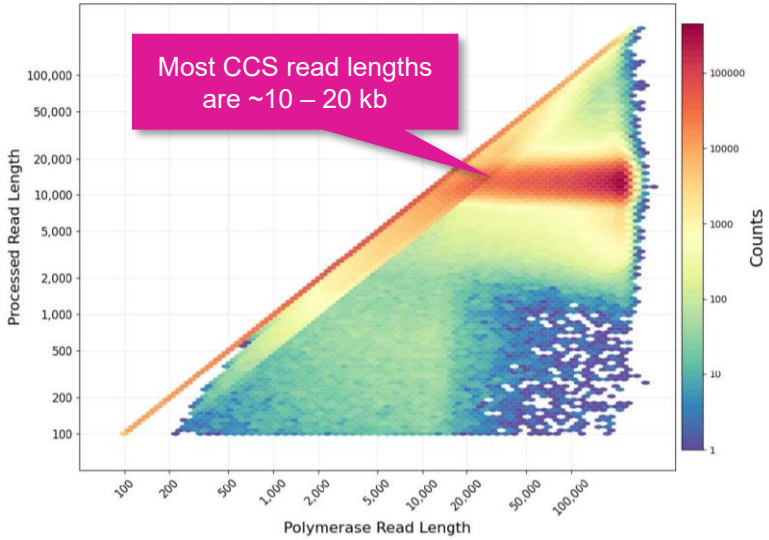
[1] Post-nuclease treatment & final cleanup yields typically ranged from ~10% to ~30% when using UHRR total RNA samples for Kinnex full-length RNA library construction.

PacBio

Example Kinnex 8-fold bulk RNA sequencing results [Revio system[1]]

Kinnex 8-fold bulk RNA library prepared from universal human reference RNA (UHRR)

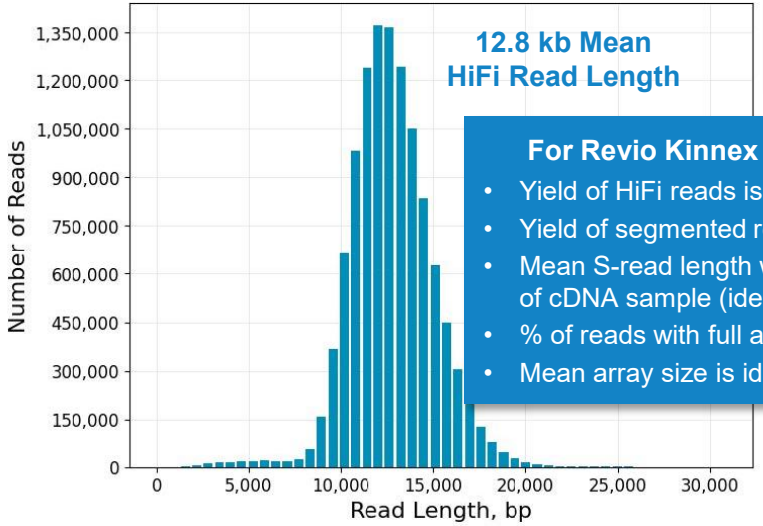
Raw Data Report



Raw Base Yield	1,969 Gb
Mean Polymerase Read Length	108.1 kb
P1	72%

Example sequencing metrics for a Universal Human Reference RNA (UHRR) Kinnex full-length RNA library sample run on a Revio system with Revio SPRQ polymerase kit / 160 pM on-plate loading concentration (OPLC) / 24-hrs movie time.

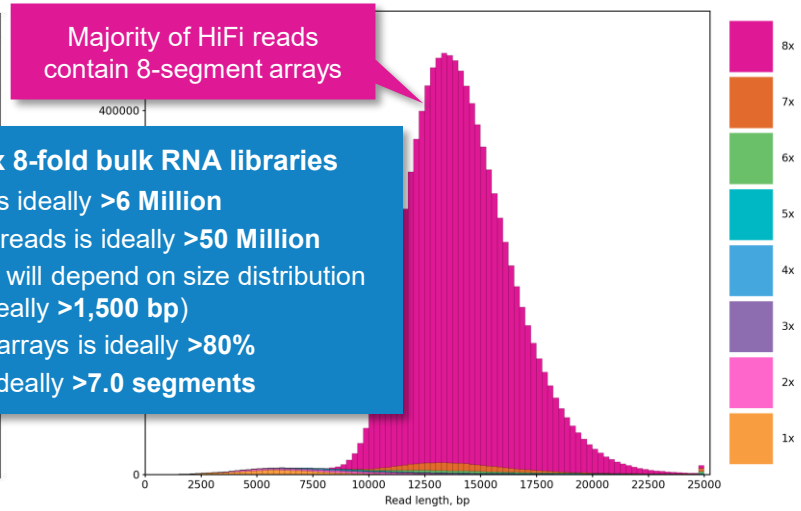
HiFi Read Length



HiFi Reads	11.4 M
HiFi Base Yield	145.1 Gb
Mean HiFi Read Length	12.8 kb
Median HiFi Read Quality	Q36
HiFi Read Mean # of Passes	12

For UHRR Kinnex full-length RNA libraries, per-Revio SMRT Cell acquisition HiFi read counts were typically >6 Million depending on the final library insert size and *P1* loading performance.

Read Segmentation Metrics



For Revio Kinnex 8-fold bulk RNA libraries

- Yield of HiFi reads is ideally **>6 Million**
- Yield of segmented reads is ideally **>50 Million**
- Mean S-read length will depend on size distribution of cDNA sample (ideally **>1,500 bp**)
- % of reads with full arrays is ideally **>80%**
- Mean array size is ideally **>7.0 segments**

Input HiFi Reads	11.4 M
Segmented reads (S-reads)	89.2 M
Mean length of S-reads	1.7 kb
Percent of reads with full arrays	94.5%
Mean array size (concentration factor)	7.8

For Kinnex 8-fold bulk RNA libraries, per-Revio SMRT Cell acquisition segmentation read counts were typically >50 Million.

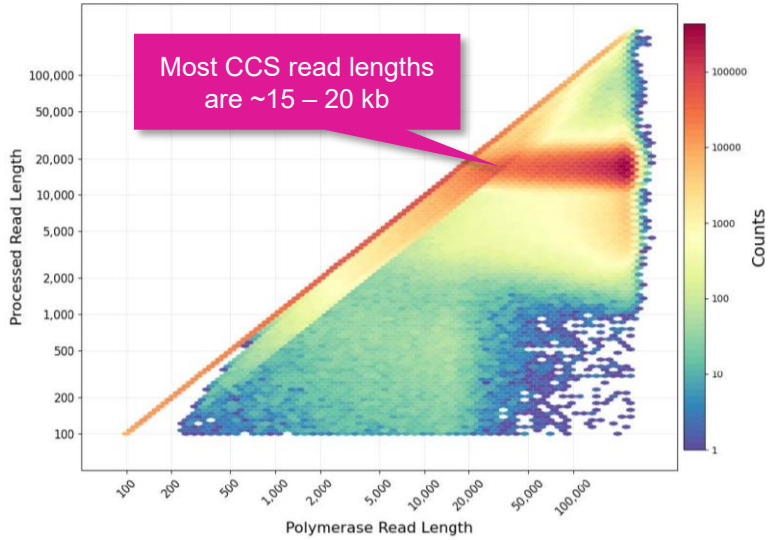
PacBio

[1] HiFi read lengths, reads/data per SMRT Cell and other sequencing performance results can vary depending on DNA sample quality, insert size, sample loading performance & movie time. Note: Shorter library insert sizes (<15 kb), lower DNA quality samples, and suboptimal sample loading performance may result in reduced HiFi data yields per SMRT Cell.

Example Kinnex 12-fold bulk RNA sequencing results [Revio system[1]]

Kinnex 12-fold bulk RNA library prepared from universal human reference RNA (UHRR)

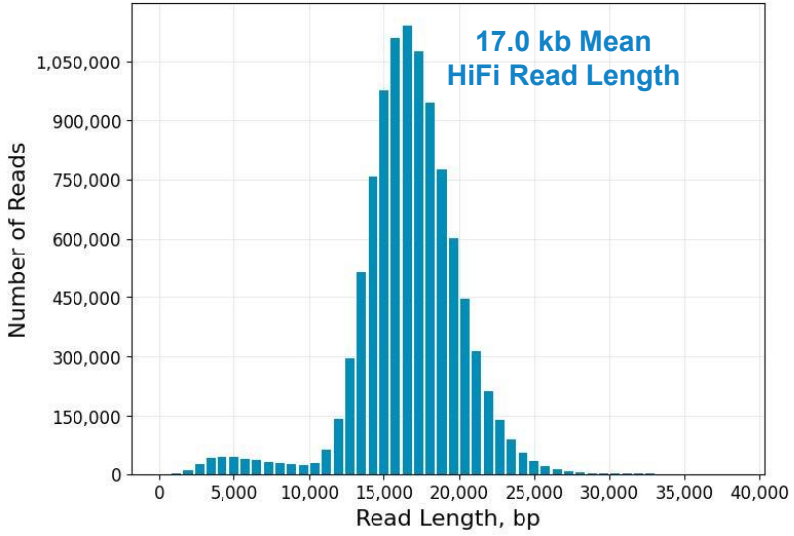
Raw Data Report



Raw Base Yield	1,870 Gb
Mean Polymerase Read Length	106.1 kb
P1	70%

Example sequencing metrics for a Universal Human Reference RNA (UHRR) Kinnex full-length RNA library sample run on a Revio system with Revio SPRQ polymerase kit / 160 pM on-plate loading concentration (OPLC) / 24-hrs movie time.

HiFi Read Length & Read Segmentation Metrics



For Revio Kinnex 12-fold bulk RNA libraries

- Yield of HiFi reads is ideally **>6 Million**
- Yield of segmented reads is ideally **>70 Million**
- Mean S-read length will depend on size distribution of cDNA sample (ideally **>1,500 bp**)
- % of reads with full arrays is ideally **>80%**
- Mean array size is ideally **>11.0 segments**

HiFi Reads	9.5 M
HiFi Base Yield	161.1Gb
Mean HiFi Read Length	17.0 kb
Median HiFi Read Quality	Q33
HiFi Read Mean # of Passes	10

For UHRR Kinnex full-length RNA libraries, per-Revio SMRT Cell acquisition HiFi read counts were typically >6 Million depending on the final library insert size and *P1* loading performance.

Input HiFi Reads	9.5 M
Segmented reads (S-reads)	110.6 M
Mean length of S-reads	1.4 kb
Percent of reads with full arrays	94.8%
Mean array size (concentration factor)	11.7

For Kinnex 12-fold bulk RNA libraries, per-Revio SMRT Cell acquisition segmentation read counts were typically >70 Million.

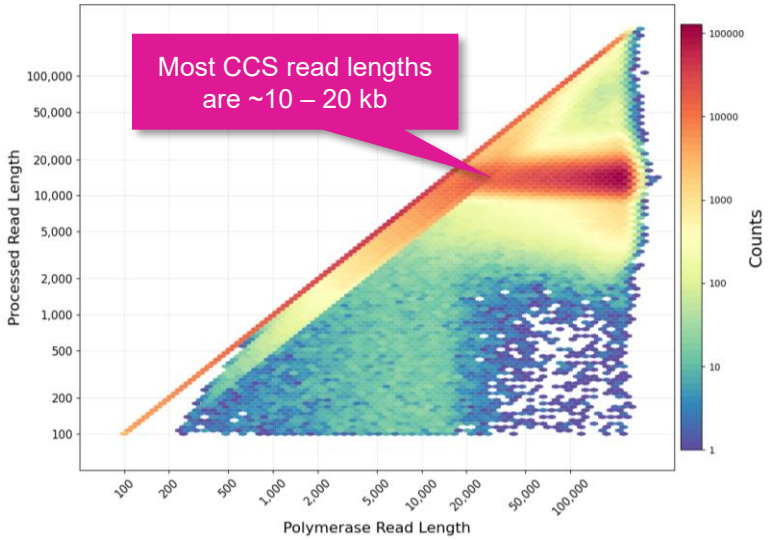
PacBio

[1] HiFi read lengths, reads/data per SMRT Cell and other sequencing performance results can vary depending on DNA sample quality, insert size, sample loading performance & movie time. Note: Shorter library insert sizes (<15 kb), lower DNA quality samples, and suboptimal sample loading performance may result in reduced HiFi data yields per SMRT Cell.

Example Kinnex 8-fold bulk RNA sequencing results [Vega system[1]]

Kinnex 8-fold bulk RNA library prepared from universal human reference RNA (UHRR)

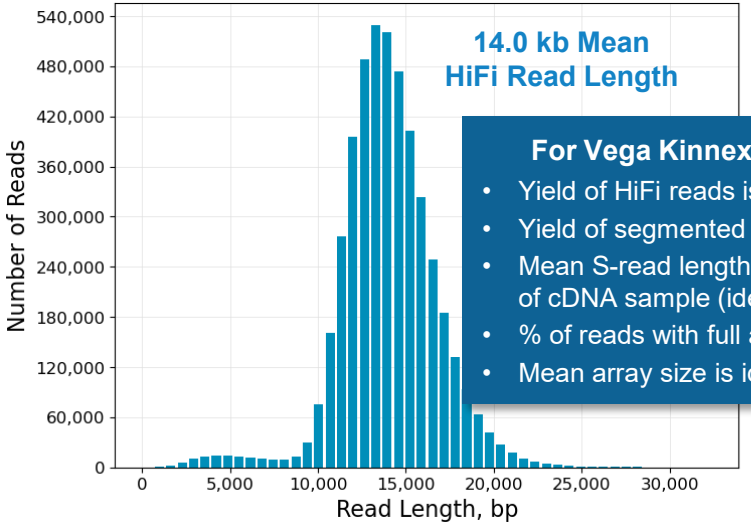
Raw Data Report



Mean Polymerase Read Length	95.3 kb
Loading level	63%

Example sequencing metrics for a Universal Human Reference RNA (UHRR) Kinnex full-length RNA library sample run on a Vega system with Vega SPRQ polymerase kit / 130 pM on-plate loading concentration (OPLC) / 24-hrs movie time.

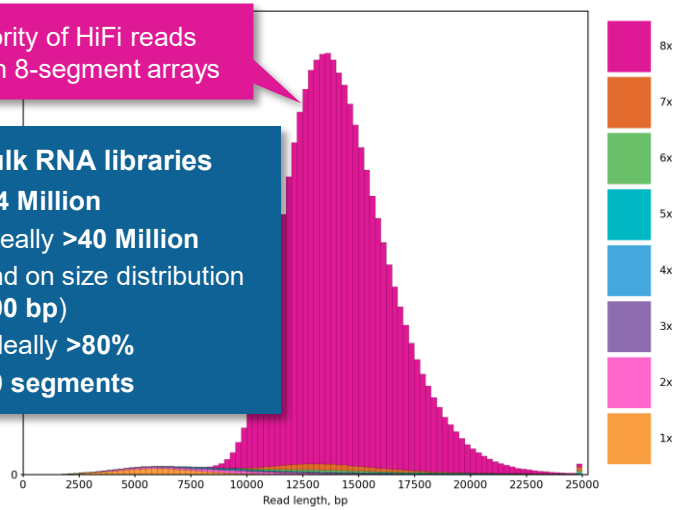
HiFi Read Length



HiFi Reads	6.5 M
HiFi Base Yield	99.9 Gb
Mean HiFi Read Length	14.0
Median HiFi Read Quality	Q34
HiFi Read Mean # of Passes	11

For UHRR Kinnex full-length RNA libraries, per-Vega SMRT Cell acquisition HiFi read counts were typically >4 Million depending on the final library insert size and sample loading performance.

Read Segmentation Metrics



For Vega Kinnex 8-fold bulk RNA libraries

- Yield of HiFi reads is ideally **>4 Million**
- Yield of segmented reads is ideally **>40 Million**
- Mean S-read length will depend on size distribution of cDNA sample (ideally **>1,500 bp**)
- % of reads with full arrays is ideally **>80%**
- Mean array size is ideally **>7.0 segments**

Input HiFi Reads	6.5 M
Segmented reads (S-reads)	47.9 M
Mean length of S-reads	1.7 kb
Percent of reads with full arrays	94.1%
Mean array size (concentration factor)	7.7

For Kinnex 8-fold bulk RNA libraries, per-Vega SMRT Cell acquisition segmentation read counts were typically >40 Million.

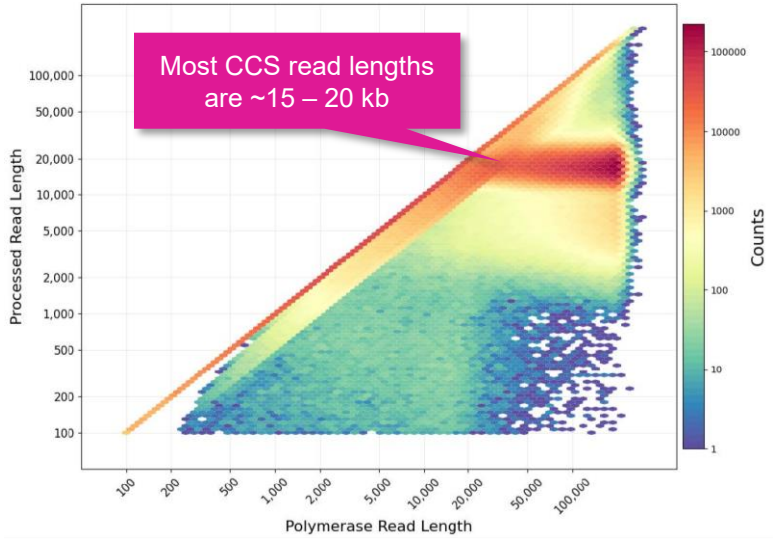
PacBio

[1] HiFi read lengths, reads/data per SMRT Cell and other sequencing performance results can vary depending on DNA sample quality, insert size, sample loading performance & movie time. Note: Shorter library insert sizes (<15 kb), lower DNA quality samples, and suboptimal sample loading performance may result in reduced HiFi data yields per SMRT Cell.

Example Kinnex 12-fold bulk RNA sequencing results [Vega system[1]]

Kinnex 12-fold bulk RNA library prepared from universal human reference RNA (UHRR)

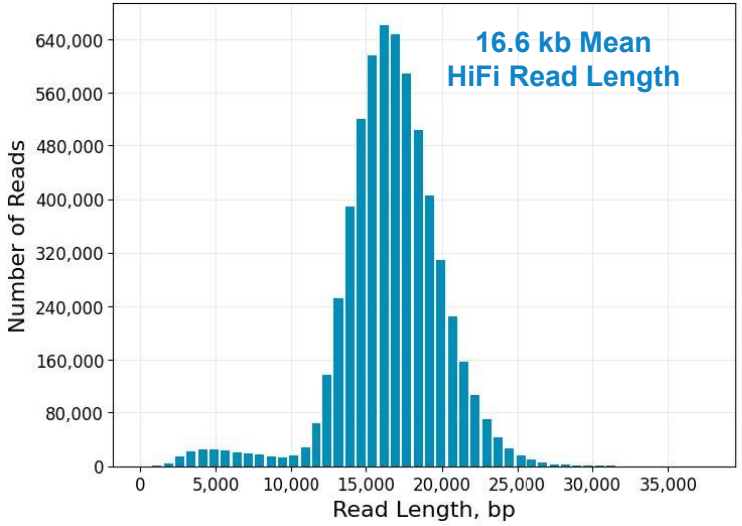
Raw Data Report



Mean Polymerase Read Length	88.5 kb
Loading level	83%

Example sequencing metrics for a Universal Human Reference RNA (UHRR) Kinnex full-length RNA library sample run on a Vega system with Vega SPRQ polymerase kit / 130 pM on-plate loading concentration (OPLC) / 24-hrs movie time.

HiFi Read Length & Read Segmentation Metrics



For Vega Kinnex 8-fold bulk RNA libraries

- Yield of HiFi reads is ideally **>4 Million**
- Yield of segmented reads is ideally **>50 Million**
- Mean S-read length will depend on size distribution of cDNA sample (ideally **>1,500 bp**)
- % of reads with full arrays is ideally **>80%**
- Mean array size is ideally **>7.0 segments**

HiFi Reads	6.0 M
HiFi Base Yield	99.9 Gb
Mean HiFi Read Length	16.6
Median HiFi Read Quality	Q32
HiFi Read Mean # of Passes	8

For UHRR Kinnex full-length RNA libraries, per-Vega SMRT Cell acquisition HiFi read counts were typically >4 Million depending on the final library insert size and sample loading performance.

Input HiFi Reads	6.0 M
Segmented reads (S-reads)	69.1 M
Mean length of S-reads	1.4 kb
Percent of reads with full arrays	93.2%
Mean array size (concentration factor)	11.5

For Kinnex 12-fold bulk RNA libraries, per-Vega SMRT Cell acquisition segmentation read counts were typically >50 Million.

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[1] HiFi read lengths, reads/data per SMRT Cell and other sequencing performance results can vary depending on DNA sample quality, insert size, sample loading performance & movie time. Note: Shorter library insert sizes (<15 kb), lower DNA quality samples, and suboptimal sample loading performance may result in reduced HiFi data yields per SMRT Cell.

Technical resources for Kinnex bulk RNA library preparation, sequencing & data analysis

Technical resources for Kinnex bulk RNA library preparation, sequencing & data analysis

RNA sample preparation resources

- **Note:** The products below have not been tested or validated by PacBio but are listed here as examples of third-party kits used by other PacBio customers for isolating total RNA for PacBio RNA sequencing (Iso-Seq) applications

Kit type	Product name
mRNA isolation	Ambion Poly(A) Purist MAG Kit [Link]
Total RNA isolation	Qiagen RNeasy Plus Kits [Link]
	Sigma Spectrum Plant Total RNA Kit [Link]
	iNtRON Easy Spin Total RNA [Link]
	TRIzol Reagent can be used to isolate total RNA from tissues or cells, including lipid-rich and difficult samples [Link]
RNA stabilization & storage	RNALater is an aqueous, nontoxic tissue storage reagent that rapidly permeates tissues to stabilize and protect cellular RNA [Link]

Technical resources for Kinnex bulk RNA library preparation, sequencing & data analysis

RNA sample preparation resources (cont.)

Some important considerations to bear in mind when isolating total RNA for Iso-Seq analysis include the following:

- RNA sample has not been exposed to high temperatures (e.g.: >65°C for 1 hour can cause a detectable decrease in sequence quality) or pH extremes (<6 or >9).
- RNA sample has an OD260/OD280 ratio ~2.0.
- RNA sample has an OD260/OD230 ratio ≥2.0.
- RNA sample has a RIN number ≥7.0 (ideally recommend ≥8.0).
- RNA sample has not been exposed to intercalating fluorescent dyes or ultraviolet radiation. SYBR dyes are not RNA damaging, but do avoid ethidium bromide.
- RNA sample does not contain denaturants (e.g., guanidinium salts or phenol) or detergents (e.g., SDS or Triton-X100).
- RNA sample does not contain carryover contamination from the original organism / tissue (e.g., heme, humic acid, polyphenols, etc.).
- Only use RNase-free water supplied in the reagent kit or other suppliers.
- Make aliquots of the RNA sample and TSO to avoid excessive freeze-thaw cycles.
- Thaw RNA samples and TSO on ice before use – DO NOT leave on the benchtop.
- Avoid excessive pipetting and vortexing when working with RNA.
- **Note:** RNA samples should only be shipped with dry ice.

Technical resources for Kinnex bulk RNA library preparation, sequencing & data analysis (cont.)

Kinnex library preparation literature & other resources

- Application note – Kinnex full-length RNA kit for isoform sequencing (102-326-591)
- Procedure & checklist – Preparing Kinnex libraries using Kinnex 8-fold prep kit (103-920-900)
- Procedure & checklist – Preparing Kinnex libraries using Kinnex 12-fold prep kit (103-921-000)
- Technical overview – Kinnex library preparation using Kinnex prep kits (103-911-600)
- Technical overview – Kinnex library preparation using Kinnex 8-fold prep kit (103-916-000)
- Technical overview – Kinnex library preparation using Kinnex 12-fold prep kit (103-916-100)
- Whitepaper – Bulk and single-cell isoform sequencing for human disease research (102-326-576)

Data analysis resources

- Application note – Bioinformatics tools for full length isoform sequencing (102-326-593)
- SMRT Link Kinnex full-length RNA troubleshooting guide (103-552-100)
- SMRT Link Kinnex single-cell troubleshooting guide (103-516-100)
- SMRT Link MAS-Seq troubleshooting guide (102-994-400)
- SMRT Link software installation guide [Link]
- SMRT Link user guide [Link]
- SMRT Tools reference guide [Link]
- Video tutorial – Read Segmentation and Iso-Seq workflow in SMRT Link [Link]

Publications

- Wissel, D. et al. (2026) A systematic benchmark of high-accuracy PacBio long-read RNA sequencing for transcript-level quantification. Genome Biology [Link]
- Al'Khafaji, A.M. et al. (2024) High-throughput RNA isoform sequencing using programmable cDNA concatenation. Nature biotechnology [Link]
- Pardo-Palacios, F. et al. (2024) Systematic assessment of long-read RNA-seq methods for transcript identification and quantification. Nature Biotech [Link]
- Pardo-Palacios, F., et al., (2024) SQANTI3: curation of long-read transcriptomes for accurate identification of known and novel isoforms. Nature Methods [Link]
- Schertzer, M.D. et al. (2023) Cas13d-mediated isoform-specific RNA knockdown with a unified computational and experimental toolbox. Nature comm [Link]

Technical resources for Kinnex bulk RNA library preparation, sequencing & data analysis (cont.)

Webinars

- PacBio webinar (2025) – Detection and quantification of transcript isoforms using high-depth Kinnex full-length RNA sequencing [Link]
- PacBio video (2024) – Kinnex explained – how concatenating smaller amplicons increases throughput for PacBio HiFi sequencing [Link]
- PacBio PRISM webinar (2024) – Let's stick together – exploring PacBio Kinnex kits [Link]
- PacBio Iso-Seq social club webinar (2022) – TappAS for isoform differential expression analysis [Link]
- PacBio Iso-Seq social club webinar (2022) – Single-cell Iso-Seq applications in cancer and neurological disorders [Link]

Example PacBio data sets

Application	Dataset	Data type	PacBio system
Kinnex bulk RNA sequencing	Homo sapiens – Universal human reference RNA (UHRR) [Link]	HiFi long read	Vega system
	Homo sapiens – Universal human reference RNA (UHRR) [Link]	HiFi long read	Revio system – SPRQ chemistry
	Homo sapiens – Universal human reference RNA (UHRR) [Link]	HiFi long read	Sequel II & Revio systems
	Homo sapiens – HG002 [Link]	HiFi long read	Revio system
	Homo sapiens – Heart [Link]	HiFi long read	Revio system
	Homo sapiens – Cerebellum [Link]	HiFi long read	Revio system
	Homo sapiens – Brain [Link]	HiFi long read	Revio system

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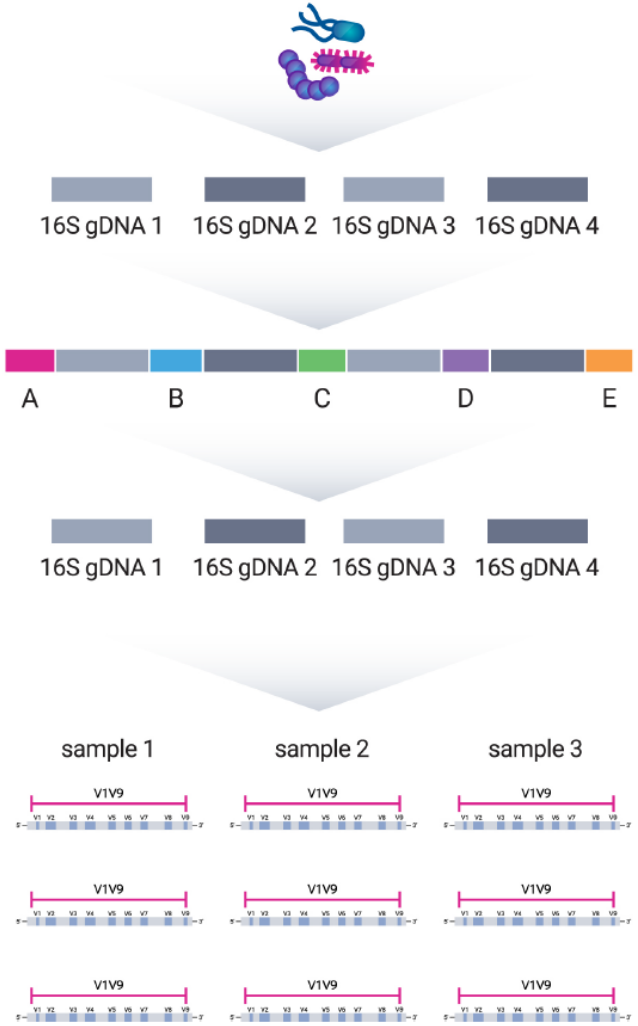
APPENDIX 2: Example Kinnex 16S rRNA workflow

Kinnex 16S rRNA library preparation workflow overview
Kinnex 16S rRNA data analysis workflow overview
Kinnex 16S rRNA example sequencing performance data
Technical resources for Kinnex 16S rRNA library preparation, sequencing & data analysis

Kinnex 16s rRNA library preparation workflow overview

Kinnex 16S rRNA method overview

Use Kinnex full-length RNA kit to perform high-accuracy, full-length isoform sequencing with PacBio long-read systems



16S amplicon generation

Procedure & checklist – Preparing Kinnex-compatible 16S rRNA amplicons (103-885-200)

Kinnex library preparation

Procedure & checklist – Preparing Kinnex libraries using Kinnex 8-fold prep kit (103-238-700); ***OR***

Procedure & checklist – Preparing Kinnex libraries using Kinnex 12-fold prep kit (103-238-800)

PacBio sequencing

Read segmentation + Amplicon demultiplexing

SMRT Link user guide (103-891-900)

- Generate 16S amplicons (up to 384-plex) using user-supplied barcoded 16S PCR primers (obtained from third-party oligo synthesis provider)
- 1-day Kinnex library preparation using Kinnex 8-fold prep kit or Kinnex 12-fold prep kit
- SMRT Link Run Design support for 'Kinnex 16S rRNA' application type option with auto-analysis (read segmentation only)
- Demultiplex 16S amplicon barcodes in SMRT Link to generate per-sample read BAM files
- Analyze per-sample BAM files using PacBio GitHub tools or other custom 16S analysis pipeline

Kinnex 16S rRNA workflow key steps

Full-length 16S gene amplification

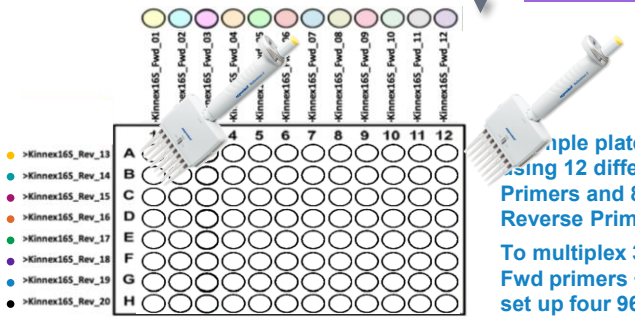
Procedure & checklist – Preparing Kinnex-compatible 16S rRNA amplicons
(103-885-200)

Bacterial gDNA fragments isolated from metagenomic samples

Recommended gDNA input for full-length 16S PCR amplification = 1-2 ng

PCR amplification of full-length 16S genes[1]

Can barcode up to 384 samples during 16S gene amplification step using PacBio barcoded Fwd and Rev primers[1]



ple plate layout for 96-plex PCR design ing 12 different 16S Barcoded Forward Primers and 8 different 16S Barcoded Reverse Primers.

To multiplex 384 samples, use 12 barcoded Fwd primers + 32 barcoded Rev primers and set up four 96-well PCR reaction plates.

Forward (F) PCR primer
(Kinnex16S_Fwd_ XX[1])
Fwd BC

Reverse (R) PCR primer
(Kinnex16S_Rev_ XX[1])
Rev BC

Dual-barcoded full-length 16S amplicon product

Contains Kinnex PCR handle 1

Equal-volume pooling & cleanup

Contains Kinnex PCR handle 2

Pooled barcoded full-length 16S amplicons for input into Kinnex library construction

[1] 12 different 16S barcoded Forward PCR primers + 32 different 16S barcoded Reverse PCR primers are available for 16S gene amplification step to multiplex up to 384 samples.

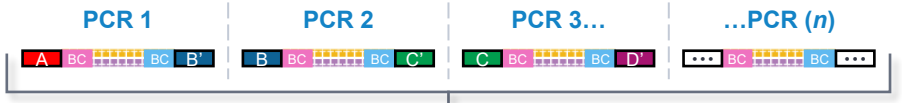
Kinnex library prep, sequencing & analysis

Procedure & checklist – Preparing Kinnex libraries using Kinnex 8-fold prep kit
(103-238-700); ***OR***

Procedure & checklist – Preparing Kinnex libraries using Kinnex 12-fold prep kit
(103-238-800)

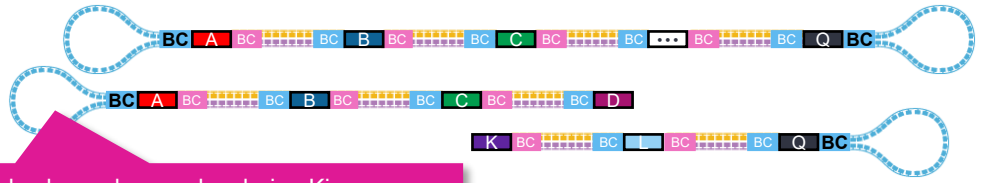
Amplified 16S products containing a different PacBio barcode at each end (Fwd BC ≠ Rev BC)

Kinnex PCR (parallel PCR Rxs to append Kinnex segmentation adapters)



Pooling & Kinnex array formation

Barcoded Kinnex terminal adapter
(bcM0001-bcM0004)[2]



Can also barcode samples during Kinnex array formation using up to 4 different barcoded Kinnex terminal SMRTbell adapters[2]

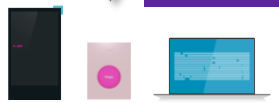
Kinnex array repair & nuclease Tx

Anneal Kinnex sequencing primer / Bind polymerase / Cleanup (ABC)

Purified polymerase-bound Kinnex library

Long-read sequencing & data analysis

Revio or Vega system



SMRT Link read segmentation & 3rd-party metagenomics 2° analysis tools

[2] Kinnex adapter barcode sequences can be downloaded from SMRT Link Data Management module.

Kinnex 16S rRNA experimental design considerations

Kinnex 16S rRNA application use case recommendations for PacBio systems

	Vega system + SPRQ-Nx chemistry		Revio system + SPRQ-Nx chemistry	
Experimental goal	Determine the microbial diversity (phylogeny and taxonomy) of bacteria in a metagenomic sample			
Sample multiplexing[1]	Up to 1,536 samples per Vega SMRT Cell acquisition		Up to 1,536 samples per Revio SMRT Cell acquisition	
Expected coverage per sample[2]	**Kinnex 8-fold library**	Up to 50 M reads divided by *N* samples	**Kinnex 8-fold library**	Up to 70 M reads divided by *N* samples
	Example coverage for 1,536-plex	Up to ~30k reads per sample	**Example coverage for 1,536-plex**	Up to ~45k reads per sample
	Kinnex 12-fold library	Up to 60 M reads divided by *N* samples	**Kinnex 12-fold library**	Up to 100 M reads divided by *N* samples
	Example coverage for 1,536-plex	Up to ~39k reads per sample	**Example coverage for 1,536-plex**	Up to ~65k reads per sample
16S amplicon generation protocol	Procedure & checklist – Preparing Kinnex-compatible 16S rRNA amplicons (103-885-200) [Min. input requirement: Total genomic DNA of 1–2 ng per metagenomic sample]			
Kinnex library prep protocol	Procedure & checklist – Preparing Kinnex libraries using the Kinnex 8-fold prep kit (103-920-900); or Procedure & checklist – Preparing Kinnex libraries using the Kinnex 12-fold prep kit (103-921-000)			
16S amplicon DNA input into Kinnex library prep workflow	35 ng of purified pooled 16S amplicon DNA			
SMRT Link data analysis workflows	Read Segmentation			
Community data analysis tools	Analyze full-length 16S data with DADA2, QIIME 2, microbiome helper, OneCodex, EZBiome, seqSight, or PacBio GitHub pipeline (pb-16S-nf)			

[1] Kinnex prep kits can support up to 1,536-plex sample multiplexing through the combined use of 12 different 16S barcoded Forward PCR primers + 32 different 16S barcoded Reverse PCR primers (supplied by user) and 4 different barcoded Kinnex terminal SMRTbell adapters (included in Kinnex concatenation kit 2.0) during Kinnex 16s rRNA library construction.
[2] For most 16S analysis applications, typically aim for ~30-50 K reads/sample.

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General best practices recommendations for preparing Kinnex 16S rRNA libraries (cont.)

Preparation of barcoded 16S gene-specific forward and reverse primers

- Resuspend stock oligos with a target concentration of 100 µM in 10 mM Tris-HCl pH 8.0–8.5 (Elution buffer) or low TE (10 mM Tris-HCl with 0.1 mM EDTA)
- To prepare oligo working solutions, dilute each primer individually to 2.5 µM in 10 mM Tris-HCl pH 8.0–8.5 (elution buffer) or low TE
  - E.g., add 5 µL of 100 µM primer stock to 195 µL of 10 mM Tris-HCl pH 8.0–8.5 buffer. This volume of diluted oligo is sufficient for running more than 50 PCR reactions
- Always mix primer stocks well before preparing dilutions. Prior to use, verify that the concentration of each diluted oligo solution is 2.5 µM by directly measuring the OD260 value using a Nanodrop system
- Aliquot the diluted oligos in 96-well plates in the format shown in the plate maps below

Plate map for preparing barcoded 16S gene-specific forward primers

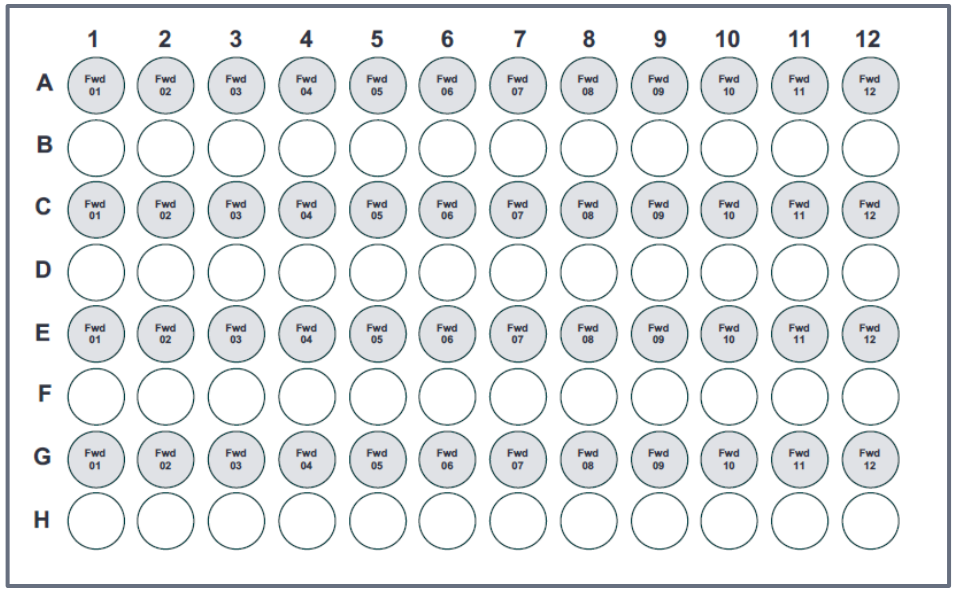
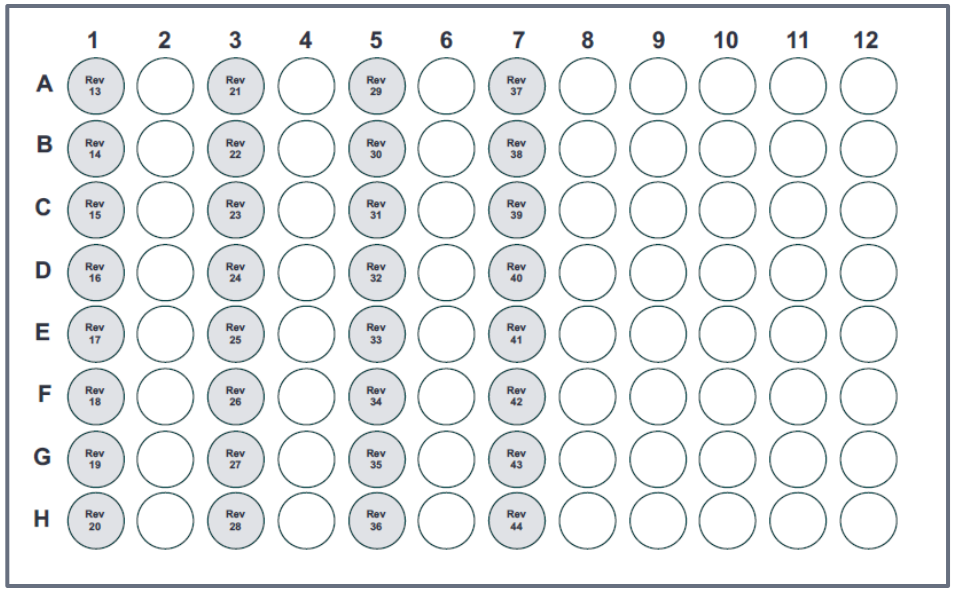


Plate map for preparing barcoded 16S gene-specific reverse primers



General best practices recommendations for preparing Kinnex 16S rRNA libraries (cont.)

DNA input and reagent & sample handling

Required DNA input amount

- Recommended input bacterial genomic DNA amount per sample is 1–2 ng
- Expected 16S amplicon size is approximately 1,500 bases and typical 16S PCR product yields are 50–300 ng per sample

DNA QC & handling recommendations for bacterial gDNA isolated from metagenomic samples

- For best results, characterize bacterial gDNA samples thoroughly and normalize gDNA concentrations before use
- Bring gDNA samples to room temperature and mix well by pipetting to ensure sample homogeneity, then measure gDNA concentration using Qubit dsDNA HS assay reagents
- Assess sample purity using a Nanodrop system → OD260/280 should be between 1.8 and 2.0 for purified double-stranded DNA
- To ensure pipetting accuracy, plan to deliver 1–2 ng of gDNA to each individual PCR reaction in a constant 5 µL volume
  - Normalize sample gDNA concentration to 0.2–0.4 ng/µL in 10 mM Tris-HCl pH 8.0–8.5 (elution buffer) prior to setting up PCR reactions
  - **Note:** Nuclease-free water and Elution buffer (EB) from PacBio can be used in place of 10mM Tris-HCl pH 8.0–8.5 for gDNA normalization
- Based on prior PacBio experience, QIAgen Powerfecal Pro kit extracts DNA of sufficient quality for this workflow

Reagent and sample handling

- Thaw PCR Ready Mix on ice and mix well before use
- Note: All PCR reactions described in this procedure must be set up and kept on ice until read to load onto thermal cycler instrument
  - High proofreading activity of the enzyme in the PCR Ready Mix will rapidly degrade primers at room-temperature

16S full-length PCR

Perform PCR amplification of 16S gene with barcoded forward and reverse primers

Procedure & checklist – Preparing Kinnex-compatible 16S rRNA amplicons (103-885-200)

1 16S full-length PCR

↓ Safe stop

2 Pooling + cleanup

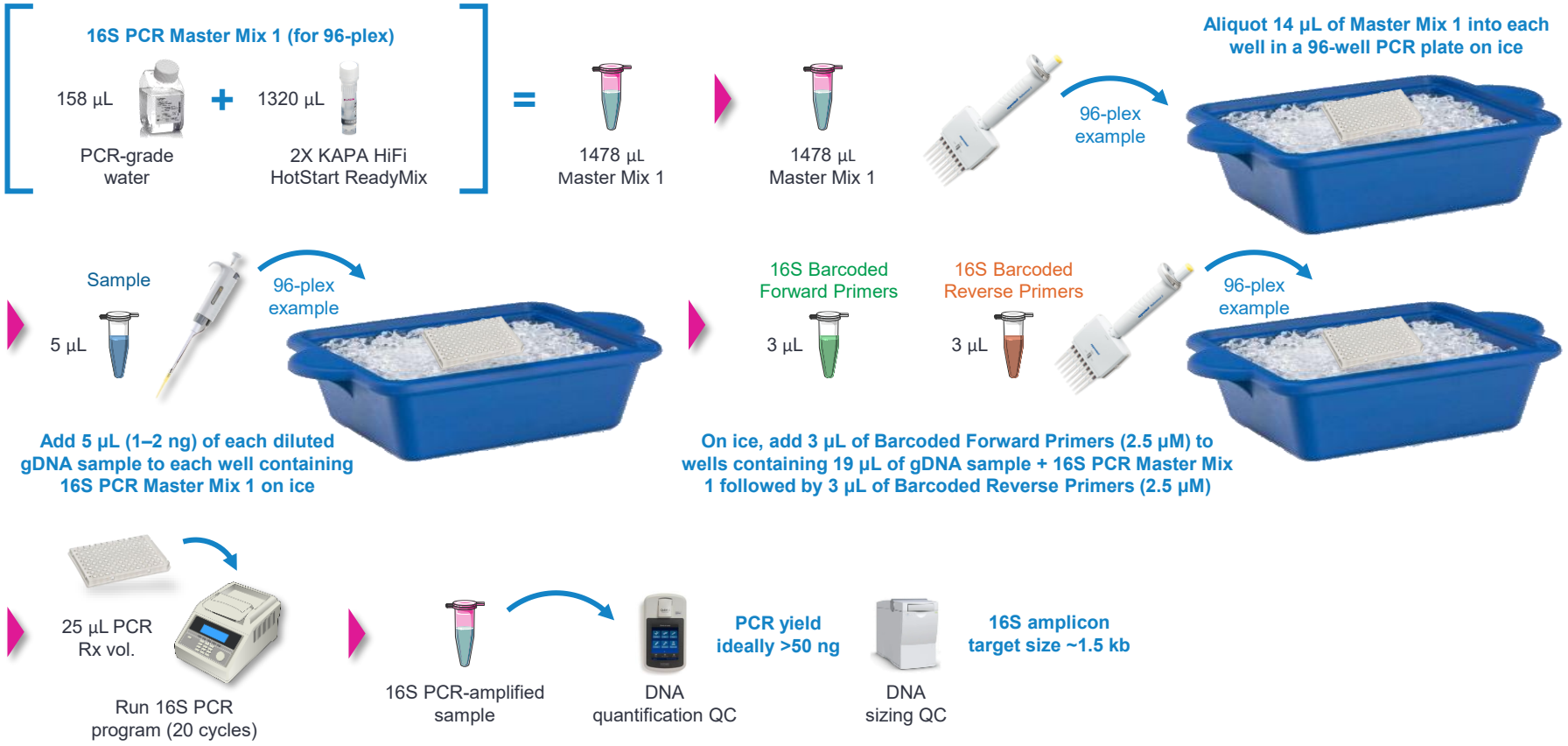
Kinnex library prep

Procedure & checklist – Preparing Kinnex libraries using Kinnex 8-fold prep kit (103-920-900)

Procedure & checklist – Preparing Kinnex libraries using Kinnex 12-fold prep kit (103-921-000)

Step 1 procedural summary

1. PCR amplification of 16S gene with barcoded primers



Proceed to **Step 2**

16S full-length PCR (cont.)

Perform PCR amplification of 16S gene with barcoded forward and reverse primers

Procedure & checklist – Preparing Kinnex-compatible 16S rRNA amplicons (103-885-200)

1 16S full-length PCR

↓ Safe stop

2 Pooling + cleanup

Kinnex library prep

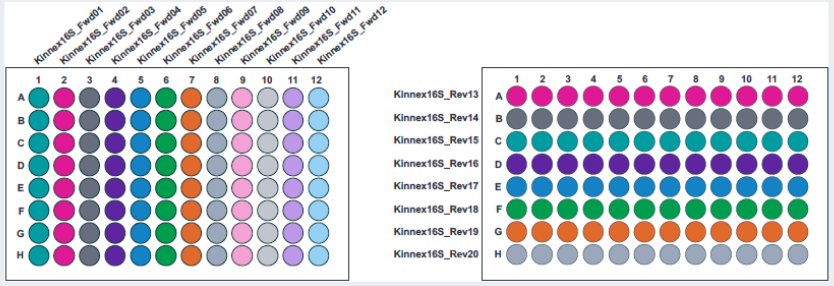
Procedure & checklist – Preparing Kinnex libraries using Kinnex 8-fold prep kit (103-920-900)

Procedure & checklist – Preparing Kinnex libraries using Kinnex 12-fold prep kit (103-921-000)

1. PCR amplification of 16S gene with barcoded primers

✔ Step	Instructions
	Thaw the PCR Ready Mix, briefly vortex to mix, and place on ice. Note that all PCR reactions must be set up on ice; the high proofreading activity of the enzyme will result in rapid primer degradation at room temperature.
1.1	**Thaw plates containing the diluted forward and reverse primers.** Briefly spin the plate to ensure that the entire volume is at the bottom of each well.
1.2	Transfer 14 µL of the prepared 16S PCR Master Mix 1 into a 96-well PCR plate for each 96-plex. For a 96-plex experiment design, use one 96-well plate. For a 384-plex experiment design, use four 96-well plates. Add 5 µL (1ng) of each diluted gDNA sample to each well containing 16S PCR Master Mix 1 on ice.
1.3	The figures below illustrate an example plate layout for setting up a 96-plex PCR design using twelve different 16S Barcoded Forward Primers and eight different 16S Barcoded Reverse Primers. Please refer to Appendix section A2 - 384 barcodes layout for all of the plate designs.

16S PCR Master Mix 1 components	1 sample	N	For 96-plex	For 192-plex	For 384-plex
PCR-grade Water	1.5 µL	1.5 x N x 1.1	158 µL	317 µL	634 µL
2X KAPA HiFi HotStart ReadyMix	12.5 µL	12.5 x N x 1.1	1320 µL	2640 µL	5280 µL
Total	14 µL	14 x N x 1.1	1478 µL	2957 µL	5914 µL



- Prepare PCR Master Mix[1] on ice for processing up to 384 metagenomic DNA samples (include 10% overage)
- Ensure all reagents are thawed and mixed prior to use

- **IMPORTANT: All PCR reactions described in this procedure must be set up and kept on ice** until read to load onto thermal cycler instrument
  - High proofreading activity of the enzyme in the PCR Ready Mix will rapidly degrade primers at room temperature

[1] Kapa PCR mix (HiFi HotStart ReadyMix) and 16S primer oligos are user-supplied and must be purchased from a third-party vendor.

16S full-length PCR (cont.)

Perform PCR amplification of 16S gene with barcoded forward and reverse primers

Procedure & checklist – Preparing Kinnex-compatible 16S rRNA amplicons (103-885-200)

1 16S full-length PCR

↓ Safe stop

2 Pooling + cleanup

Kinnex library prep

Procedure & checklist – Preparing Kinnex libraries using Kinnex 8-fold prep kit (103-920-900)

Procedure & checklist – Preparing Kinnex libraries using Kinnex 12-fold prep kit (103-921-000)

1. PCR amplification of 16S gene with barcoded primers (cont.)

✔ Step	Instructions
1.4	On ice, add 3 µL of the **Barcoded Forward Primers (2.5 µM)** to wells containing 19 µL of gDNA and 16S PCR Master Mix followed by 3 µL of the **Barcoded Reverse Primers (2.5 µM)**. The final concentration of the barcoded forward and reverse primers in each well is 0.3 µM. The final reaction volume in each well is 25 µL. Mix well by pipetting. Seal the plates to prevent evaporation during PCR. Briefly spin the plate in a refrigerated centrifuge (4°C) to ensure that the entire sample volume is at the bottom of each well.
1.5	Set up and run a thermal cycler with the program shown below. **Keep the 96-well PCR plates on ice until the lid has heated to 105°C.** The duration of PCR is a 1 hour. Heated lid set at 105°C. Reaction volume set to 25 µL
1.6	Spot-check amplification results with Agilent Bioanalyzer or TapeStation systems. The expected target amplicon size is ~1,500 bp, and the amount of amplicon material generated from each sample should be comparable as assessed by analyzing the relative intensity of the ~1,500 bp PCR product. See Figure 2 in the Appendix.
1.7	Proceed to pooling and SMRTbell cleanup in the next step.
	SAFE STOPPING POINT -- Store at 4°C or -20°C for long-term storage

Step	Temperature	Duration	Cycle
Initial Denaturation	95 °C	3 min	1
Denaturation	98 °C	20 s	20
Annealing	57 °C	30 s	
Extension	72 °C	75 s	
Final Extension	72 °C	5 min	1
Hold	4 °C	Hold	

- **IMPORTANT:** Pre-heat thermal cycler until lid temperature reaches 105˚C before loading the 96-well PCR plate.
  - Keep the 96-well PCR plates on ice until the lid is pre-heated.

- Expected 16S amplicon size is approx. 1,500 bp and typical 16S PCR product yields are ~50–300 ng per sample

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16S PCR amplicon pooling & SMRTbell cleanup

Pool barcoded 16S PCR amplicons and perform cleanup using 1.1X SMRTbell cleanup beads

Procedure & checklist – Preparing Kinnex-compatible 16S rRNA amplicons (103-885-200)

1 16S full-length PCR

↓ Safe stop

2 Pooling + cleanup

Kinnex library prep

Procedure & checklist – Preparing Kinnex libraries using Kinnex 8-fold prep kit (103-920-900)

Procedure & checklist – Preparing Kinnex libraries using Kinnex 12-fold prep kit (103-921-000)

Step 2 procedural summary

2.1 Pooling of barcoded 16S PCR amplicons

For <96-plex

16S PCR 1 (20 µL) + 16S PCR 2 (20 µL) + 16S PCR 3 (20 µL) + ... + 16S PCR (*N* < 96) (20 µL) → **Pooled 16S PCR reactions** (*N* x 20 µL total pooled vol.)

For 96-plex

16S PCR 1 (10 µL) + 16S PCR 2 (10 µL) + 16S PCR 3 (10 µL) + ... + 16S PCR 96 (10 µL) → **Pooled 16S PCR reactions** (960 µL total pooled vol.)

For 192-plex

16S PCR 1 (5 µL) + 16S PCR 2 (5 µL) + 16S PCR 3 (5 µL) + ... + 16S PCR 192 (5 µL) → **Pooled 16S PCR reactions** (960 µL total pooled vol.)

A total mass of 35 ng is required for Kinnex PCR step [Step 3]

2.2. Cleanup of pooled 16S PCR amplicon using 1.1X SMRTbell cleanup beads

960 µL Pooled 16S PCR amplicons [96-plex example] + 1,056 µL 1.1X SMRTbell cleanup beads → 100 µL purified 16S PCR amplicon pool

Proceed to **Step 3**

PacBio

16S PCR amplicon pooling & SMRTbell cleanup

Pool barcoded 16S PCR amplicons and perform cleanup using 1.1X SMRTbell cleanup beads

Procedure & checklist – Preparing Kinnex-compatible 16S rRNA amplicons (103-885-200)

1 16S full-length PCR

↓ Safe stop

2 Pooling + cleanup

Kinnex library prep

Procedure & checklist – Preparing Kinnex libraries using Kinnex 8-fold prep kit (103-920-900)

Procedure & checklist – Preparing Kinnex libraries using Kinnex 12-fold prep kit (103-921-000)

2.1 Pooling of barcoded 16S PCR amplicons

✔	Step	Instructions
	2.1.1	If PCR products are of the expected size and comparable quantity, pool equal volumes of each PCR reaction in a clean DNA LoBind microcentrifuge tube according to the recommendations below: • For a 96-plex experiment design, we recommend pooling **10 µL** from each PCR reaction. • For a 192-plex or higher-plex experiment design, we recommend pooling **5 µL** from each PCR reaction. If doing less than 96-plex, pool **20uL from each PCR reaction into subsequent steps. A total mass of 35ng is required for the Kinnex PCR step.** Store unused PCR reactions at -20°C for future use.
	2.1.2	Proceed to SMRTbell cleanup in the next step.

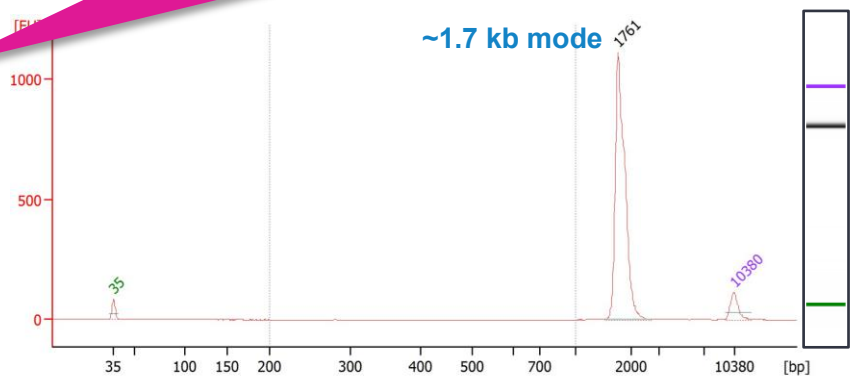
- **For 96-plex:** Pool 10 µL from each PCR reaction
- **For 192-plex or higher:** Pool 5 µL from each PCR reaction
- **For <96-plex:** Pool 20 µL from each PCR reaction into subsequent steps
- Typical total yield from each 25 µL PCR reaction is **~50-300 ng**
- Ensure there is **at least 35 ng** of pooled amplicon DNA to proceed to the Kinnex PCR step

2.2 Cleanup of pooled 16S PCR amplicon using 1.1X SMRTbell cleanup beads

✔	Step	Instructions
	2.2.1	Add **1.1X** v/v (volume over volume) of resuspended, room-temperature SMRTbell cleanup beads to the tube of pooled 16S amplicons. Note: use a 5 mL LoBind tube if the volume is more than 2mL.
	2.2.2	Pipette-mix the beads until evenly distributed.
	2.2.3	Quick-spin the tube in a microcentrifuge to collect liquid.
	2.2.14	Slowly aspirate the cleared eluate without disturbing the beads. Transfer the eluate to a **single new 1.5mL LoBind tube or tube strip**. Discard the old tube with beads.
	2.2.15	Measure DNA concentration with a Qubit fluorometer using the 1x dsDNA HS kit using **1 µL** aliquot from the LoBind tube. Typical total yield from each 25 µL PCR reaction is ~50–300 ng.

PROTOCOL COMPLETE – Store at 4°C or -20°C for long-term storage

- Perform 1.1X SMRTbell bead cleanup at room temp.



Example Bioanalyzer DNA sizing QC analysis results for pooled 16S amplicon DNA samples generated from mock microbial community genomic DNA (ATCC MSA-1003 20 Strain Staggered Mix).

- Minimum amount of pooled 16S amplicon DNA needed to proceed with Kinnex PCR = **35 ng**

PacBio

Comparison of Kinnex 8-fold prep kit versus 12-fold prep kit for Kinnex 16S rRNA sequencing

Kinnex 8-fold prep kit provides higher data quality that is suitable for downstream 16S metagenomics analysis tools

Example Kinnex 16S rRNA workflow

16S rRNA amplification (Kinnex-compatible 16S PCR primers)

↓

Amplified 16S DNA cleanup (1.1x SMRTbell cleanup beads)

↓

Kinnex library prep Kinnex 8-fold prep kit

↓

SPRQ-Nx

50 – 70 M S-reads

↓

- More reads pass quality filtering using DADA2 despite lower raw S-read throughput with 8-fold kit
- **Kinnex 8-fold prep kit is generally recommended for 16S applications**

Kinnex library prep Kinnex 12-fold prep kit

↓

SPRQ-Nx

70 – 100 M S-reads

↓

- Fewer reads pass quality filtering using DADA2 despite higher raw S-read throughput with 12-fold kit

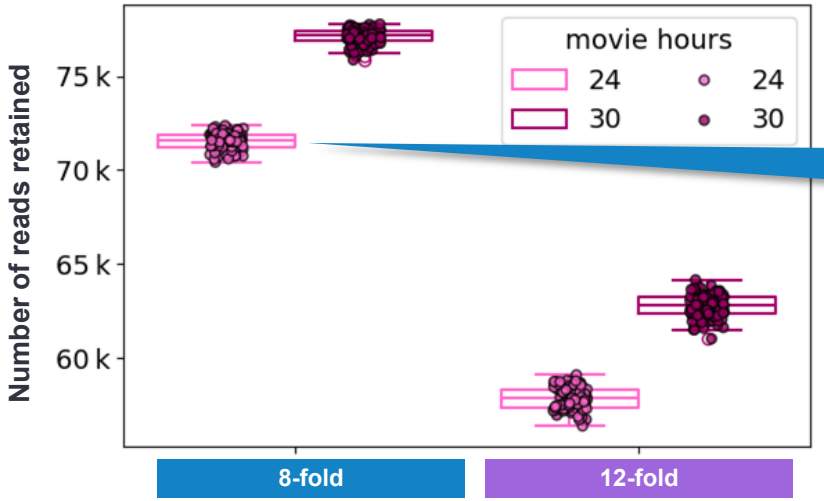
Example HiFi sequencing metrics for 16S rRNA libraries (384-plex) prepared with Kinnex 8-fold prep kit *vs.* Kinnex 12-fold prep kit

Sample	Movie time (hrs)	P1 (%)	HiFi Yield (Gb)	HiFi reads	Read quality (median)	Q30+ BQ (%)	S-reads per acquisition
16S_12mer	24	69.14	147.9	7,860,184	Q34	93.9%	93,421,796
16S_8mer	24	74.96	120.4	9,541,465	Q37	95.4%	75,949,260
16S_12mer	30	65.52	151.2	8,028,963	Q35	94.4%	95,454,790
16S_8mer	30	70.03	123.8	9,811,370	Q40	96.4%	78,112,438

Using Kinnex 12-fold prep kit generates higher raw S-read counts but lower median read quality

Using Kinnex 8-fold prep kit generates lower raw S-read counts but **higher median read quality**

Number of reads retained per sample after DADA2 quality filtering[1] (100k downsample per sample)



16S analysis tools such as DADA2 discard lower-quality reads, **so Kinnex 8-fold kit leaves more usable reads per sample**

For downstream 16S analysis using DADA2,[1] Kinnex 8-fold library prep workflow retains a higher proportion of useful reads after quality filtering compared to Kinnex 12-fold prep workflow

Comparison of number of reads retained after DADA2 quality filtering. Kinnex 16S rRNA libraries (384-plex) were prepared with Kinnex 8-fold prep kit or Kinnex 12-fold prep kit and run on a Revio system with SPRQ-Nx chemistry using 24 or 30 movie acquisition times.

[1] Refer to HiFi-16S-workflow documentation available on PacBio GitHub for details about quality filtering steps when processing HiFi full-length 16S data into high- =quality amplicon sequence variants (ASVs) using QIIME 2 and DADA2.

Example Kinnex 12-fold 16S rRNA library prep inputs & expected yields

Final Kinnex library yield is typically sufficient to support ≥8 SMRT Cell acquisitions on Revio & Vega systems

Kinnex 16S r RNA

Amplicon generation

Procedure & checklist – Preparing Kinnex-compatible 16S rRNA amplicons (103-885-200)

16S gene PCR amplification → SMRTbell bead cleanup → Pool barcoded 16S amplicons → SMRTbell bead cleanup

IN: 1-2 ng of bacterial gDNA per sample

OUT: 50-300 ng of PCR products per sample

Kinnex PCR

Procedure & checklist – Preparing Kinnex libraries using Kinnex 12-fold prep kit (103-921-000)

Kinnex PCR → SMRTbell bead cleanup

IN: 35 ng of pooled 16S amplicons

OUT: 5-9 µg of amplified products after pooling

Kinnex array formation

Procedure & checklist – Preparing Kinnex libraries using Kinnex 12-fold prep kit (103-921-000)

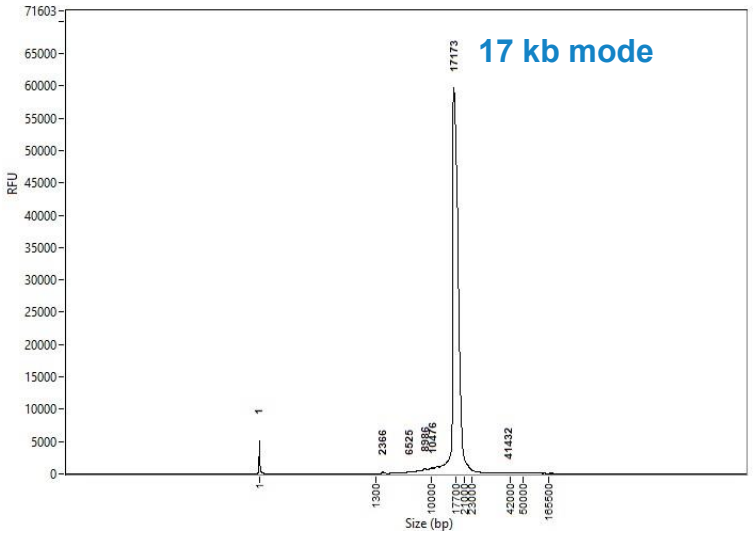
Kinnex digestion / Ligation → Kinnex repair → SMRTbell cleanup → Nuclease treatment → SMRTbell bead cleanup

IN: 5 µg of pooled Kinnex PCR products

OUT: 80% recovery from Kinnex digestion Rx input

IN: 40 µL of purified Kinnex repair reaction products

OUT: 10-20% recovery from Kinnex digestion Rx input



Example Femto Pulse DNA sizing QC analysis results for final Kinnex 12-fold library prepared with pooled 16S amplicon DNA samples generated from mock microbial community genomic DNA (ATCC MSA-1003 20 Strain Staggered Mix).

Example Kinnex 12-fold 16S rRNA library prep yields

gDNA input for 16S PCR	1.1 ng
16S amplicon DNA input for Kinnex 12-fold array formation	5000 ng
Post-nuclease treatment & final library cleanup yield (%)[1]	750 ng (15%)

[1] Post-nuclease treatment & final cleanup yields typically ranged from ~10% to ~20% when using 16S amplicon DNA samples generated from mock microbial community genomic DNA (ATCC MSA-1003 20 Strain Staggered Mix).

Final Kinnex library yield is typically sufficient to run the following number of SMRT Cell acquisitions:

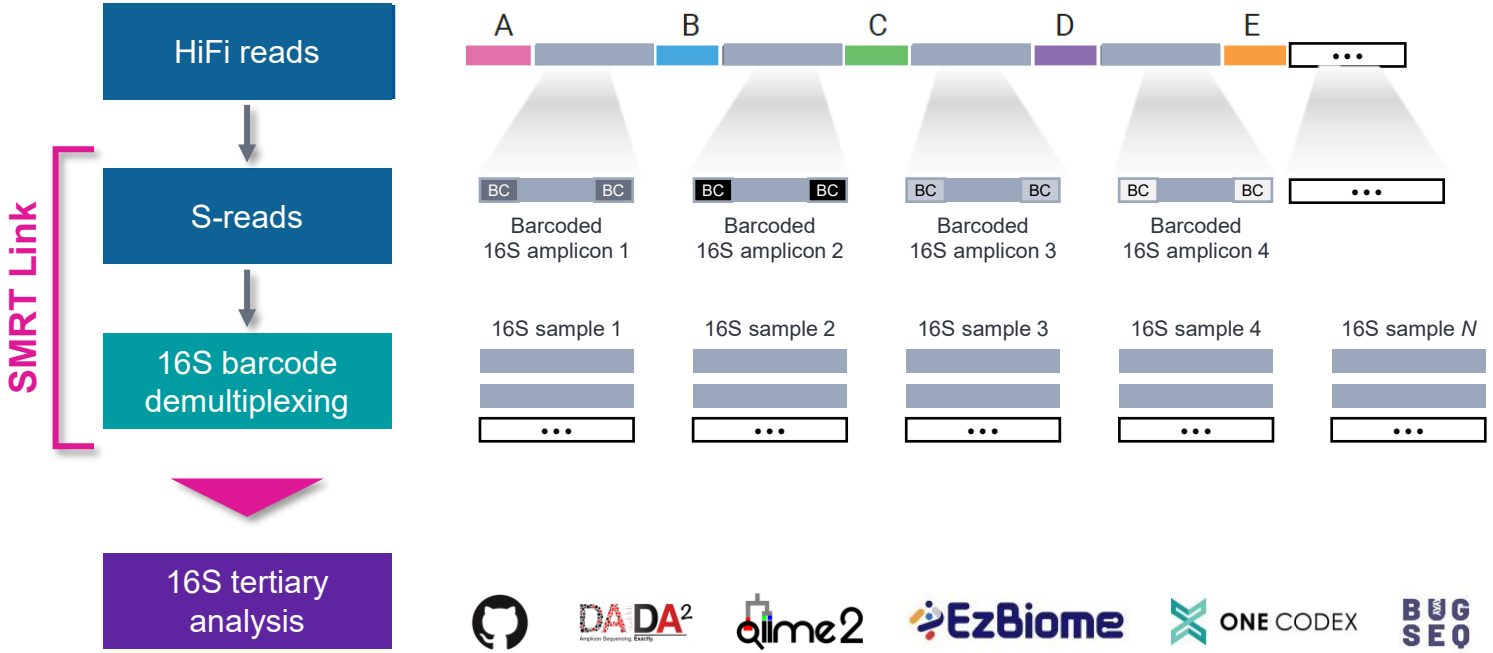
- >8 acquisitions for Revio system using SPRQ-Nx chemistry
- >8 acquisitions for Vega system using SPRQ-Nx chemistry

Kinnex 16s rRNA data analysis workflow overview

Kinnex 16S rRNA bioinformatics workflow overview

SMRT Link Read Segmentation processes HiFi reads generated from Kinnex 16S rRNA libraries to produce individual segmented reads (S-reads) that are compatible with tertiary analysis tools

SMRT Link read segmentation and barcode demultiplexing workflow[1]



Read segmentation

- Use SMRT Link Read Segmentation data utility to split arrayed Kinnex library HiFi reads into individual segmented reads (S-reads) that represent the original barcoded 16S amplicon sequences

Demultiplex barcodes

- Use SMRT Link Demultiplex barcodes data utility to separate sequence reads by barcode

Tertiary analysis

- Use Github or other third-party tools (e.g., PacBio GitHub, *DADA2*, *QIIME2*, *microbiomehelper*, *One Codex*, *EZBiome*, *BugSeq*)

Example Kinnex 16S rRNA data set

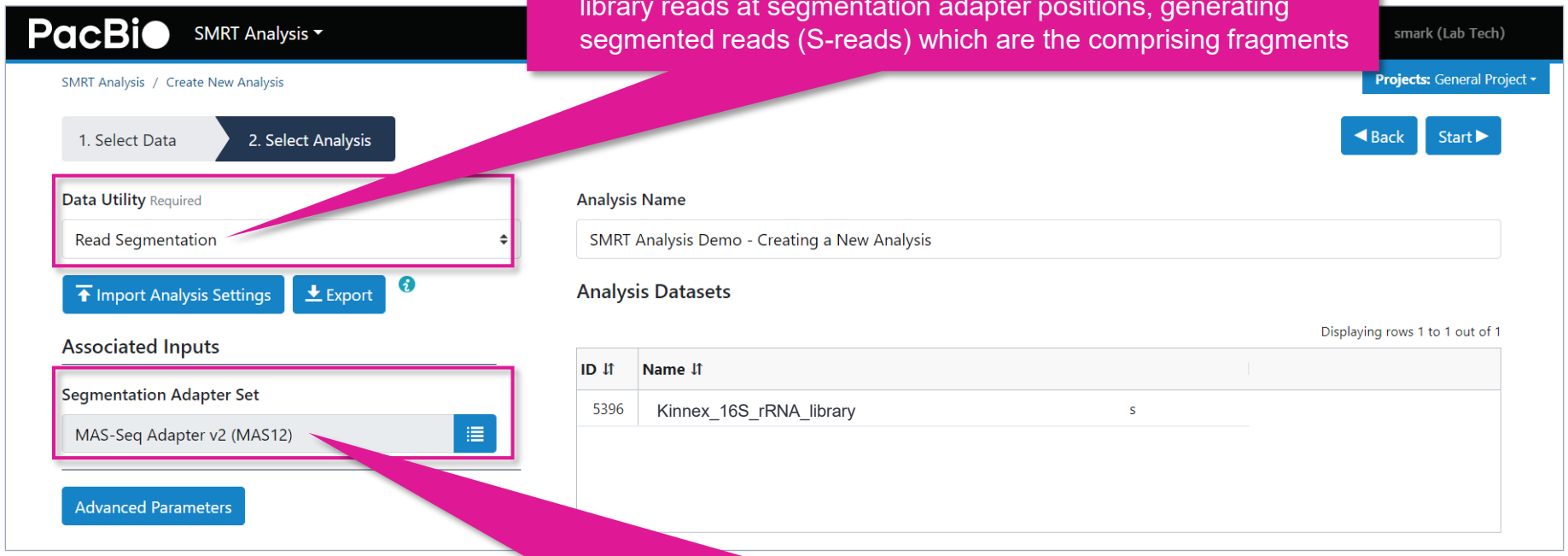
- ZymoBIOMICS Fecal Reference with TruMatrix Technology (human) [Link]
- Mixture: ZymoBIOMICS Gut Microbiome Standard, ZymoBIOMICS Fecal Reference with TruMatrix™ Technology, ATCC 20 Strain Even Mix Genomic Material, ATCC 20 Strain Staggered Mix Genomic Material [Link]

[1] See ***SMRT Link User Guide*** (Documentation) for detailed descriptions of parameter settings for Read Segmentation and Demultiplex Barcodes data utilities.

SMRT Link Read Segmentation setup

Specify **Read Segmentation** data utility in SMRT Link[1]

- **Read Segmentation** data utility splits arrayed Kinnex HiFi library reads at segmentation adapter positions, generating segmented reads (S-reads) which are the comprising fragments



- For Kinnex 12-fold libraries, specify Segmentation Adapter Set = **MAS-Seq Adapter v2 (MAS12)**
- Specify a FASTA file, provided by PacBio, containing segmentation adapters. If you need a custom segmentation adapter set, click Advanced Parameters and use a custom FASTA file formatted as described in the SMRT Link User Guide

[1] See ***SMRT Link User Guide*** (103-008-800) for detailed descriptions of parameter settings for Read Segmentation data utility.

Example SMRT Link Read Segmentation data utility processing results[1] for Kinnex 12-fold 16S rRNA library prepared from mock microbial community gDNA

SMRT Link Read Segmentation data utility job report – Summary Metrics and Segmentation Statistics

Summary Metrics

Value	Analysis Metric
7,713,616	Reads
91,255,042	Segmented reads (S-reads)
1,558	Mean length of S-reads
96.16 %	Percent of reads with full arrays
11.83	Mean array size (concatenation factor)

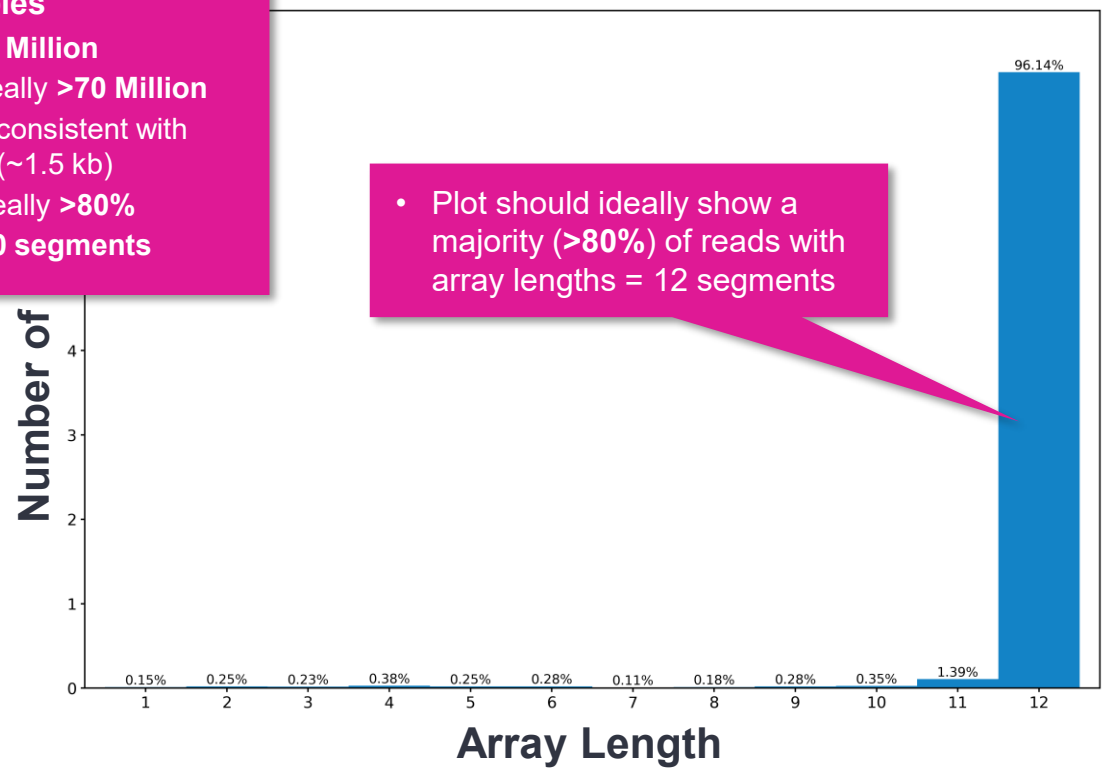
Example Revio system + SPRQ data shown.

For Revio system Kinnex 12-fold 16S rRNA samples

- Yield of HiFi reads is ideally **>6 Million**
- Yield of segmented reads is ideally **>70 Million**
- Mean S-read length should be consistent with expected 16S amplicon length (~1.5 kb)
- % of reads with full arrays is ideally **>80%**
- Mean array size is ideally **>11.0 segments**

- **Reads:** Number of input arrayed HiFi reads
- **Segmented reads (S-reads):** Number of generated S-reads
- **Mean length of S-reads:** Mean read length of generated S-reads
- **Percent of reads with full arrays:** Percentage of input HiFi reads containing all adapter sequences in the order listed in the segmentation adapter FASTA file
- **Mean array size:** Mean number of fragments (or S-reads) found in input reads

Segmentation Statistics



- Plot should ideally show a majority (**>80%**) of reads with array lengths = 12 segments

Histogram distribution of the number of S-reads per HiFi read for Kinnex 12-fold 16S rRNA library. (Example Revio system + SPRQ data shown.)

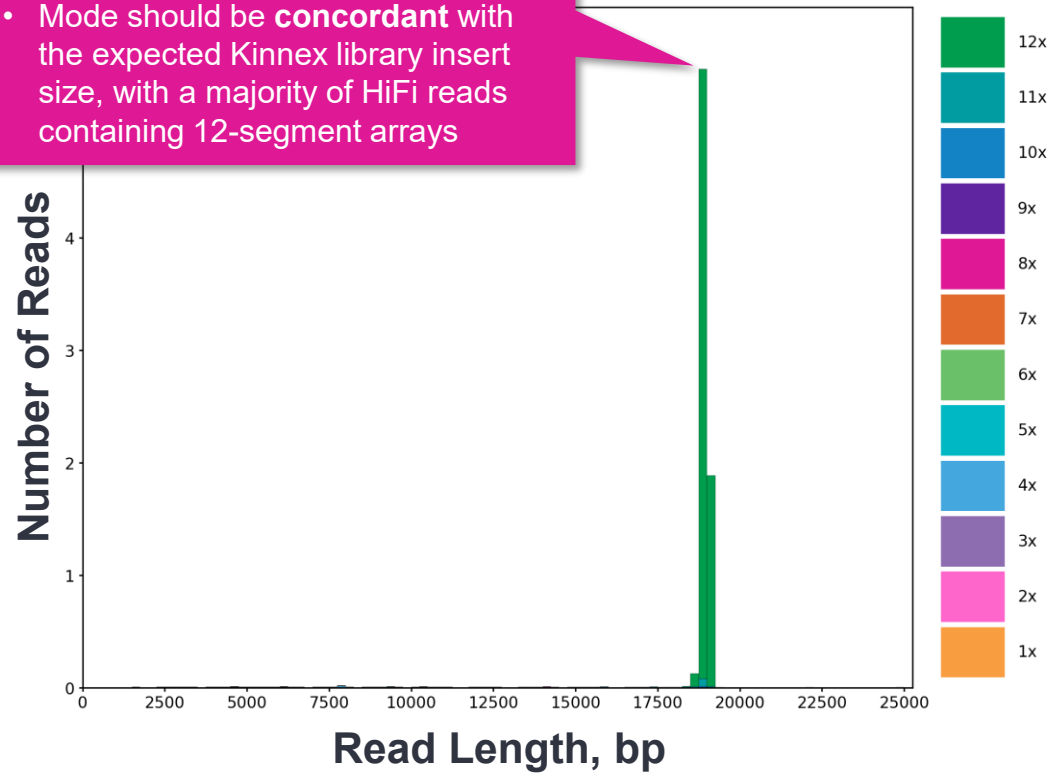
[1] HiFi read lengths, reads/data per SMRT Cell and other sequencing performance results can vary depending on DNA sample quality, insert size, *P1* loading performance & movie time. For Revio system, we recommend aiming for ~60 – 75% *P1* loading for Kinnex 16S rRNA libraries.

Example SMRT Link Read Segmentation data utility processing results[1] for Kinnex 12-fold 16S rRNA library prepared from mock microbial community gDNA (cont.)

SMRT Link Read Segmentation data utility job report – Length of Reads and S-read Length Distribution

Length of Reads

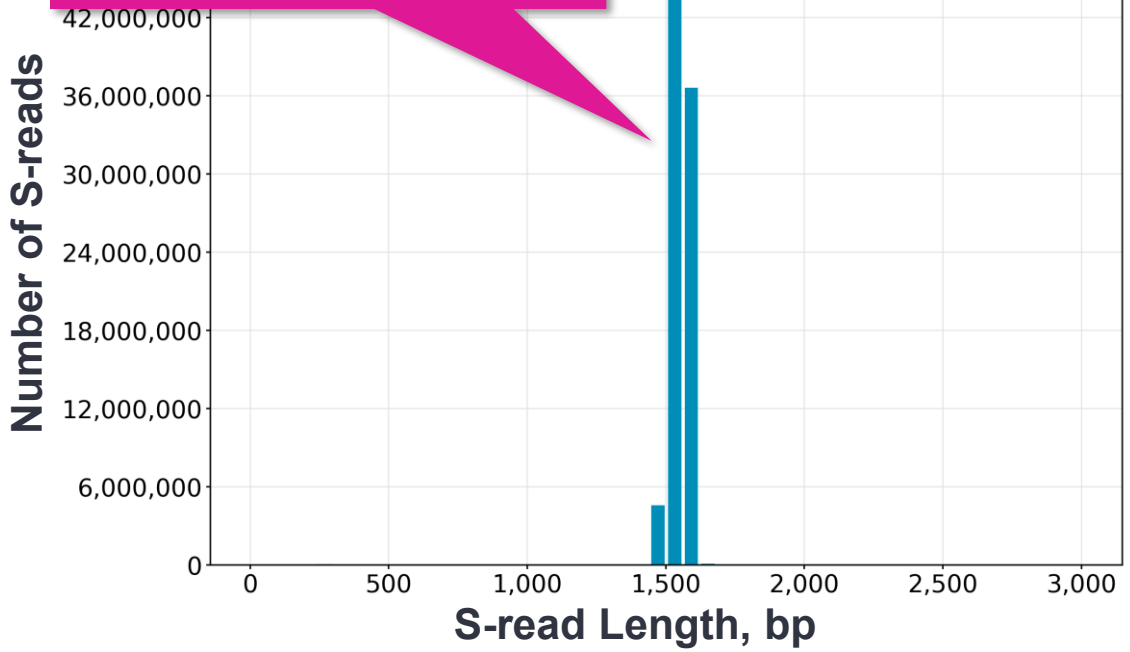
- Mode should be **concordant** with the expected Kinnex library insert size, with a majority of HiFi reads containing 12-segment arrays



Histogram distribution of the number of HiFi reads by read length, in base pairs for Kinnex 12-fold 16S rRNA. (Example Revio system + SPRQ data shown.)

S-read Length Distribution

- Mean S-read length should correspond to expected 16S amplicon insert size (**~1,500 bp**)



Histogram distribution of the number of S-reads by HiFi read length, in base pairs for Kinnex 12-fold 16S rRNA library. (Example Revio system + SPRQ data shown.)

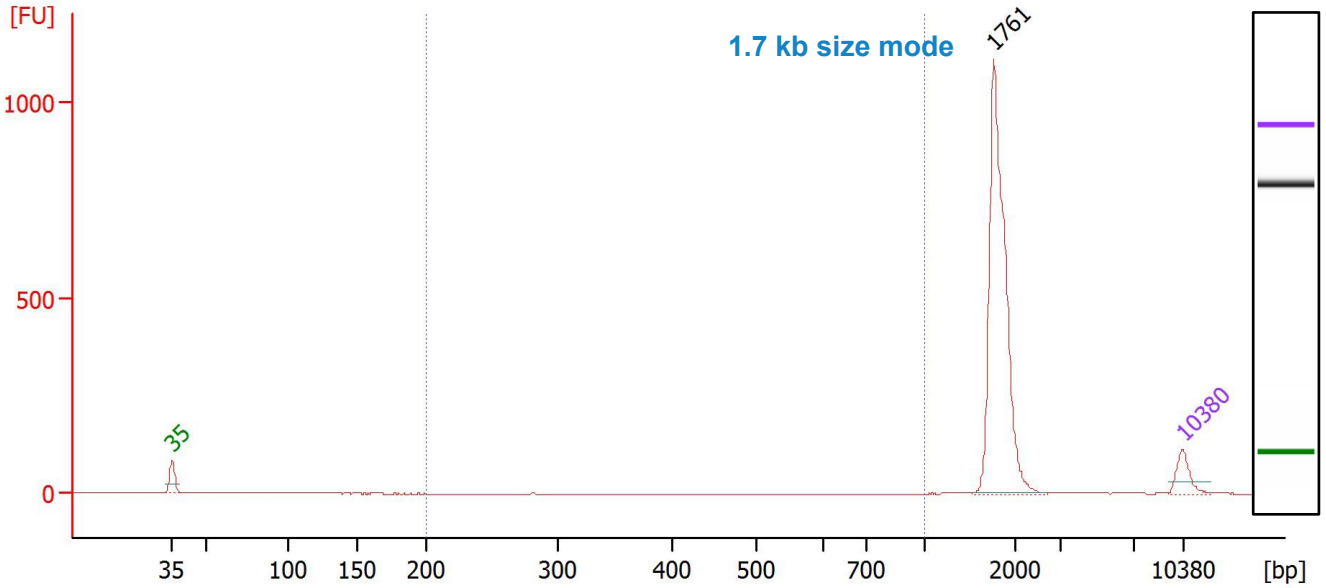
[1] HiFi read lengths, reads/data per SMRT Cell and other sequencing performance results can vary depending on DNA sample quality, insert size, *P1* loading performance & movie time. For Revio system, we recommend aiming for ~60 – 75% *P1* loading for Kinnex 16S rRNA libraries.

Kinnex 16s rRNA example sequencing performance data

Example Kinnex 12-fold bulk 16S rRNA library preparation QC results

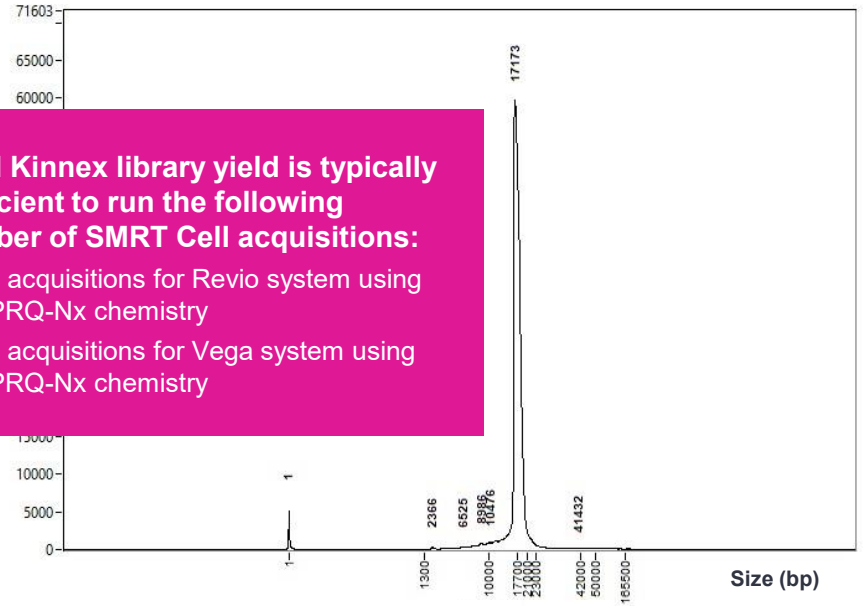
Kinnex 12-fold 16S rRNA library prepared from mock microbial community genomic DNA

16S amplicon DNA QC



Example Bioanalyzer DNA sizing QC analysis results for pooled 16S amplicon DNA samples generated from mock microbial community genomic DNA (ATCC MSA-1003 20 Strain Staggered Mix).

Final Kinnex 16S rRNA library QC



Final Kinnex library yield is typically sufficient to run the following number of SMRT Cell acquisitions:

- >8 acquisitions for Revio system using SPRQ-Nx chemistry
- >8 acquisitions for Vega system using SPRQ-Nx chemistry

Example Femto Pulse DNA sizing QC analysis results for final Kinnex 16S rRNA library.

Example sample multiplexing design for 1,536-plex Kinnex 16S rRNA library

384-plex 16S PCR x 4-plex Kinnex adapter barcoding → Total sample multiplex level = **1,536-plex**

gDNA input for 16S PCR	1.1 ng
16S amplicon DNA input Kinnex PCR products for Kinnex array formation	5000 ng
Post-nuclease treatment & final library cleanup yield (%)[1]	750 ng (15%)

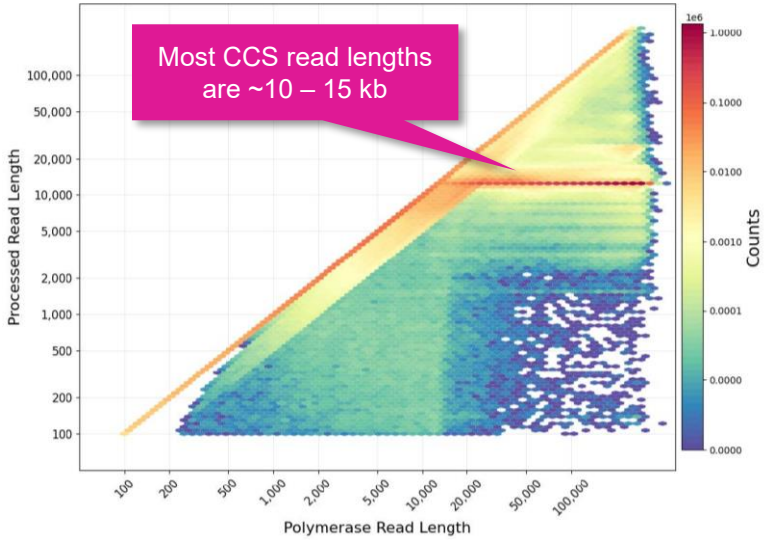
[1] Post-nuclease treatment & final cleanup yields typically ranged from ~10% to ~20% when using mock microbial community genomic DNA for Kinnex full-length 16S rRNA library construction.

PacBio

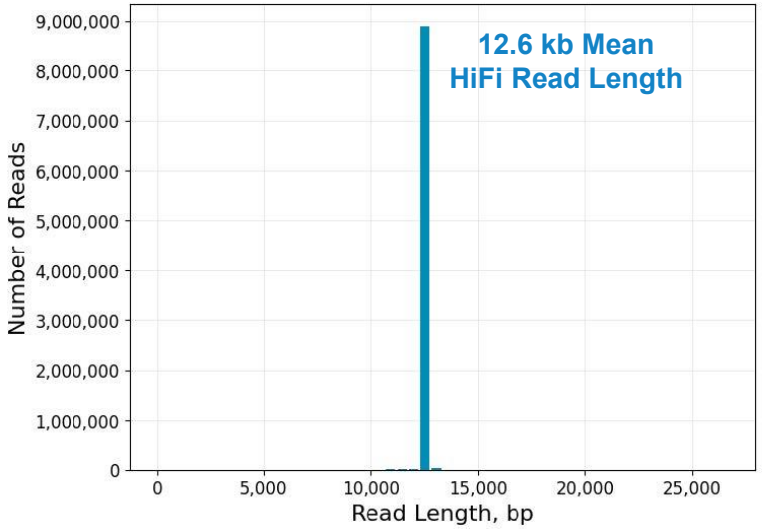
Example Kinnex 8-fold 16S rRNA sequencing results
[Revio system[1]]

Kinnex 8-fold 16S rRNA library prepared from mock microbial community gDNA sample mixture (384-plex)

Raw Data Report



HiFi Read Length & Read Segmentation Metrics



For Revio Kinnex 8-fold 16S rRNA libraries

- Yield of HiFi reads is ideally **>6 Million**
- Yield of segmented reads is ideally **>50 Million**
- Mean S-read length should be consistent with expected 16S amplicon length (~1.5 kb)
- % of reads with full arrays is ideally **>80%**
- Mean array size is ideally **>7.0 segments**

Raw Base Yield	1,837 Gb
Mean Polymerase Read Length	103.0 kb
P1	71%

Example sequencing metrics for a Kinnex 16S rRNA library sample run on a Revio system with Revio SPRQ polymerase kit / 130 pM on-plate loading concentration (OPLC) / 24-hrs movie time.

HiFi Reads	10.0 M
HiFi Base Yield	126.1 Gb
Mean HiFi Read Length	12.6 kb
Median HiFi Read Quality	Q39
HiFi Read Mean # of Passes	13

For Kinnex 16S rRNA libraries, per-Revio SMRT Cell acquisition HiFi read counts were typically >6 Million depending on the final library insert size and *P1* loading performance.

Input HiFi Reads	10.0 M
Segmented reads (S-reads)	79.5 M
Mean length of S-reads	1.6 kb
Percent of reads with full arrays	97.9%
Mean array size (concentration factor)	7.97

For Kinnex 8-fold 16S rRNA libraries, per-Revio SMRT Cell segmentation read counts were typically >50 Million.

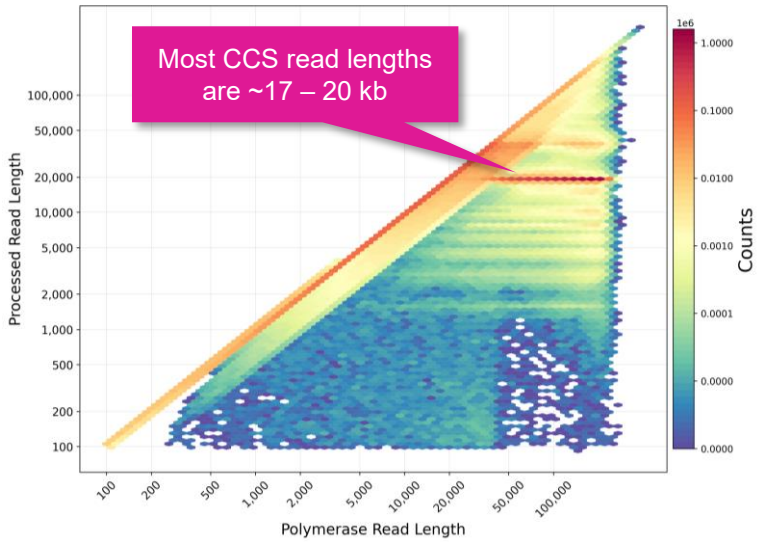
PacBio

[1] HiFi read lengths, reads/data per SMRT Cell and other sequencing performance results can vary depending on DNA sample quality, insert size, sample loading performance & movie time. Note: Shorter library insert sizes (<15 kb), lower DNA quality samples, and suboptimal sample loading performance may result in reduced HiFi data yields per SMRT Cell.

Example Kinnex 12-fold 16S rRNA sequencing results
[Revio system[1]]

Kinnex 12-fold 16S rRNA library prepared from mock microbial community gDNA sample mixture (384-plex)

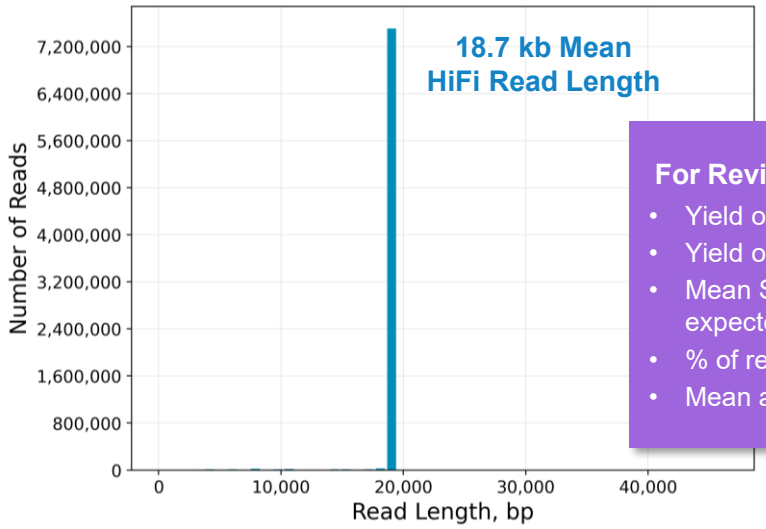
Raw Data Report



Raw Base Yield	1,400 Gb
Mean Polymerase Read Length	80.5
P1	69%

Example sequencing metrics for a Kinnex 16S rRNA library sample run on a Revio system with Revio SPRQ polymerase kit / 130 pM on-plate loading concentration (OPLC) / 24-hrs movie time.

HiFi Read Length

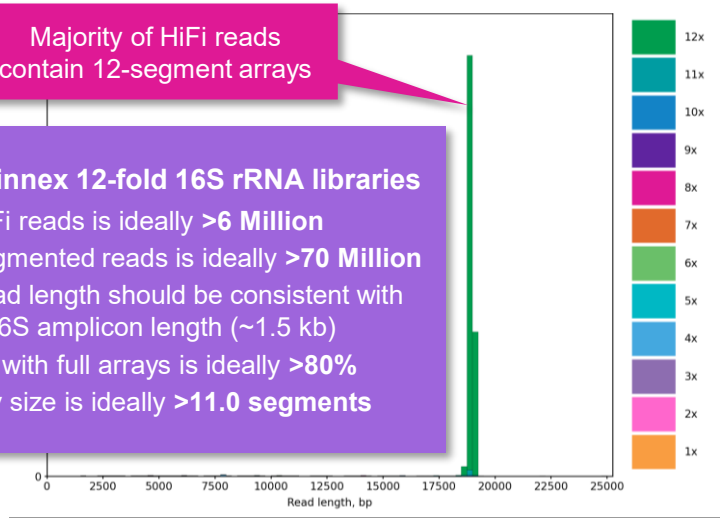


HiFi Reads	7.7 M
HiFi Base Yield	144.5 Gb
Mean HiFi Read Length	18.7 kb
Median HiFi Read Quality	Q34
HiFi Read Mean # of Passes	7

For Kinnex 16S rRNA libraries, per-Revio SMRT Cell acquisition HiFi read counts were typically >6 Million depending on the final library insert size and *P1* loading performance.

Read Segmentation Metrics

Majority of HiFi reads contain 12-segment arrays



For Revio Kinnex 12-fold 16S rRNA libraries

- Yield of HiFi reads is ideally **>6 Million**
- Yield of segmented reads is ideally **>70 Million**
- Mean S-read length should be consistent with expected 16S amplicon length (~1.5 kb)
- % of reads with full arrays is ideally **>80%**
- Mean array size is ideally **>11.0 segments**

Input HiFi Reads	7.7 M
Segmented reads (S-reads)	91.2 M
Mean length of S-reads	1.6 kb
Percent of reads with full arrays	96.1%
Mean array size (concentration factor)	11.8

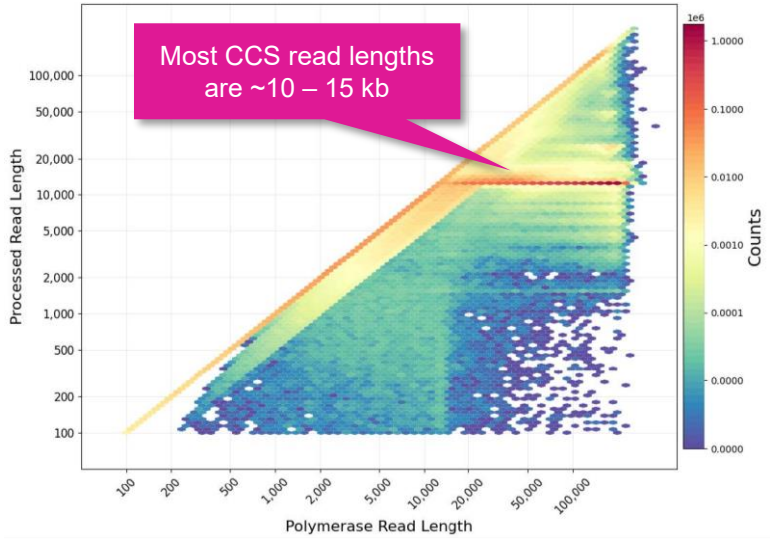
For Kinnex 12-fold 16S rRNA libraries, per-Revio SMRT Cell segmentation read counts were typically >70 Million.

[1] HiFi read lengths, reads/data per SMRT Cell and other sequencing performance results can vary depending on DNA sample quality, insert size, sample loading performance & movie time. Note: Shorter library insert sizes (<15 kb), lower DNA quality samples, and suboptimal sample loading performance may result in reduced HiFi data yields per SMRT Cell.

Example Kinnex 8-fold 16S rRNA sequencing results [Vega system[1]]

Kinnex 8-fold 16S rRNA library prepared from mock microbial community gDNA sample mixture (384-plex)

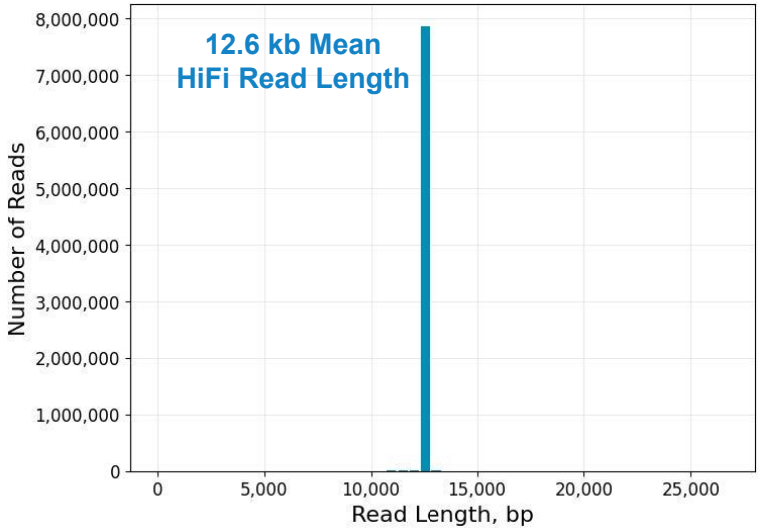
Raw Data Report



Mean Polymerase Read Length	101.0 kb
Loading level	73%

Example sequencing metrics for a Kinnex 16S rRNA library sample run on a Vega system with Vega SPRQ polymerase kit / 80 pM on-plate loading concentration (OPLC) / 24-hrs movie time.

HiFi Read Length & Read Segmentation Metrics



For Vega Kinnex 8-fold 16S rRNA libraries

- Yield of HiFi reads is ideally **>4 Million**
- Yield of segmented reads is ideally **>40 Million**
- Mean S-read length should be consistent with expected 16S amplicon length (~1.5 kb)
- % of reads with full arrays is ideally **>80%**
- Mean array size is ideally **>7.0 segments**

HiFi Reads	7.9 M
HiFi Base Yield	99.9 Gb
Mean HiFi Read Length	12.6 kb
Median HiFi Read Quality	Q40
HiFi Read Mean # of Passes	10

For Kinnex 16S rRNA libraries, per-Vega SMRT Cell acquisition HiFi read counts were typically >4 Million depending on the final library insert size and sample loading performance.

Input HiFi Reads	7.9 M
Segmented reads (S-reads)	63.0 M
Mean length of S-reads	1.6 kb
Percent of reads with full arrays	98.2%
Mean array size (concentration factor)	7.97

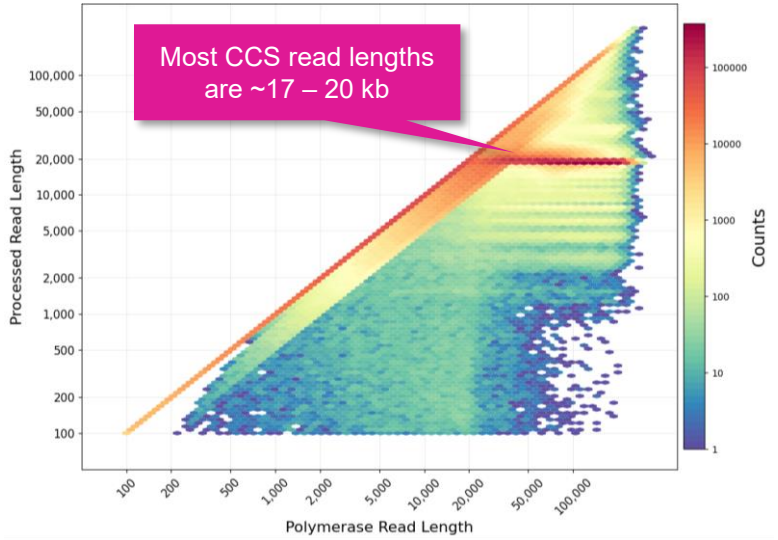
For Kinnex 8-fold 16S rRNA libraries, per-Vega SMRT Cell acquisition segmentation read counts were typically >40 Million.

[1] HiFi read lengths, reads/data per SMRT Cell and other sequencing performance results can vary depending on DNA sample quality, insert size, sample loading performance & movie time. Note: Shorter library insert sizes (<15 kb), lower DNA quality samples, and suboptimal sample loading performance may result in reduced HiFi data yields per SMRT Cell.

Example Kinnex 12-fold 16S rRNA sequencing results [Vega system[1]]

Kinnex 12-fold 16S rRNA library prepared from mock microbial community gDNA sample mixture (384-plex)

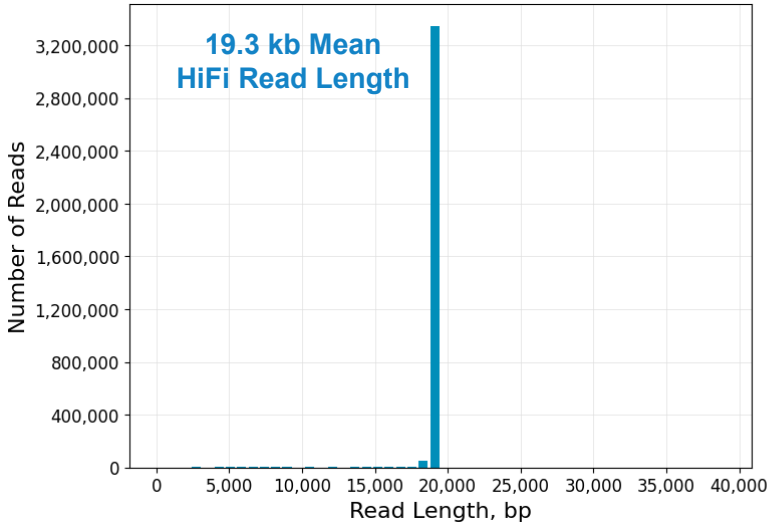
Raw Data Report



Mean Polymerase Read Length	64.1 kb
Loading level	67%

Example sequencing metrics for a Kinnex 16S rRNA library sample run on a Vega system with Vega SPRQ polymerase kit / 80 pM on-plate loading concentration (OPLC) / 24-hrs movie time.

HiFi Read Length & Read Segmentation Metrics



For Vega Kinnex 12-fold 16S rRNA libraries

- Yield of HiFi reads is ideally **>4 Million**
- Yield of segmented reads is ideally **>50 Million**
- Mean S-read length should be consistent with expected 16S amplicon length (~1.5 kb)
- % of reads with full arrays is ideally **>80%**
- Mean array size is ideally **>11.0 segments**

HiFi Reads	5.2
HiFi Base Yield	99.9 Gb
Mean HiFi Read Length	19.3 kb
Median HiFi Read Quality	Q34
HiFi Read Mean # of Passes	6

For Kinnex 16S rRNA libraries, per-Vega SMRT Cell acquisition HiFi read counts were typically >4 Million depending on the final library insert size and sample loading performance.

Input HiFi Reads	5.2 M
Segmented reads (S-reads)	60.6 M
Mean length of S-reads	1.6 kb
Percent of reads with full arrays	95.8%
Mean array size (concentration factor)	11.8

For Kinnex 12-fold 16S rRNA libraries, per-Vega SMRT Cell acquisition segmentation read counts were typically >50 Million.

PacBio

[1] HiFi read lengths, reads/data per SMRT Cell and other sequencing performance results can vary depending on DNA sample quality, insert size, sample loading performance & movie time. Note: Shorter library insert sizes (<15 kb), lower DNA quality samples, and suboptimal sample loading performance may result in reduced HiFi data yields per SMRT Cell.

Improving sequencing performance of “difficult” 16S samples

Performing AMPure PB bead size-selection on Kinnex 16S rRNA libraries can help improve sample loading of challenging metagenomic samples

Sample Name	*P1* % [Revio system]	Gb Yield	Mean Length	Mean QV
16S_Kinnex_library_SOP	26	46	18,813 bp	Q29
16S_Kinnex_library_3.1X AMPure	**80**	**87**	**18,851 bp**	**Q28**

Some bacterial 16S samples may have carry-over contaminants present leading to low sample (*P1*) loading on PacBio long-read systems

→ Using AMPure size-selection (3.1X 35% AMPure PB beads) can help mitigate this issue

[1] Example data shown were obtained for a Kinnex 16S rRNA library run on a Revio system with Revio v1 chemistry.

Technical resources for Kinnex 16s rRNA library preparation, sequencing & data analysis

Technical resources for Kinnex 16S rRNA library preparation, sequencing & data analysis

Metagenomic DNA sample preparation resources

- Based on prior PacBio experience, the PowerFecal Pro DNA Kit (QIAGEN) extracts DNA of sufficient quality for the Kinnex full-length 16S rRNA library preparation workflow.

Kinnex library preparation literature & other resources

- Application note – Kinnex 16S rRNA kit for full-length 16S sequencing (102-326-601)
- Procedure & checklist – Preparing Kinnex libraries using Kinnex 8-fold prep kit (103-920-900)
- Procedure & checklist – Preparing Kinnex libraries using Kinnex 12-fold prep kit (103-921-000)
- Technical overview – Kinnex library preparation using Kinnex prep kits (103-911-600)
- Technical overview – Kinnex library preparation using Kinnex 8-fold prep kit (103-916-000)
- Technical overview – Kinnex library preparation using Kinnex 12-fold prep kit (103-916-100)
- Whitepaper – Bulk and single-cell isoform sequencing for human disease research (102-326-576)

Data analysis resources

- SMRT Link Kinnex full-length RNA troubleshooting guide (103-552-100)
- SMRT Link Kinnex single-cell troubleshooting guide (103-516-100)
- SMRT Link MAS-Seq troubleshooting guide (102-994-400)
- SMRT Link software installation guide [Link]
- SMRT Link user guide [Link]
- SMRT Tools reference guide [Link]
- Video tutorial – Read Segmentation and Iso-Seq workflow in SMRT Link [Link]

Technical resources for Kinnex full-length 16S rRNA library preparation, sequencing & data analysis

Posters & publications

- Anderson, M.A.J. et al. (2025) Unlocking River Biofilm Microbial Diversity: A Comparative Analysis of Sequencing Technologies. Molecular Ecology Resources [Link]
- PacBio PAG poster (2025) – High-resolution microbiome species profiling at scale with the Kinnex kit for full- length 16S rRNA sequencing [Link]
- PacBio ASM Microbe poster (2024) - Microbiome species profiling at scale with the Kinnex kit for full-length 16S rRNA sequencing [Link]
- PacBio PAG poster (2024) – Increasing the throughput of full-length 16S sequencing with Kinnex kits [Link]
- Al'Khafaji, A.M. et al. (2024) High-throughput RNA isoform sequencing using programmable cDNA concatenation. Nature biotechnology [Link]
- Notario, E. et al. (2023) Amplicon-based microbiome profiling: from second- to third-generation sequencing for higher taxonomic resolution. Genes [Link]

Webinars

- PacBio webinar (2025) – Unlocking human microbiome clarity with PacBio HiFi sequencing [Link]
- PacBio video (2024) – Kinnex explained – how concatenating smaller amplicons increases throughput for PacBio HiFi sequencing [Link]
- PacBio PRISM webinar (2024) – Let's stick together – exploring PacBio Kinnex kits [Link]
- PacBio webinar (2023) – Increasing the throughput of full-length 16s and RNA-Seq with Kinnex kits [Link]
- PacBio webinar (2023) – Creating a reliable microbiome testing service for companion animal health using PacBio sequencing [Link]

Example PacBio data sets

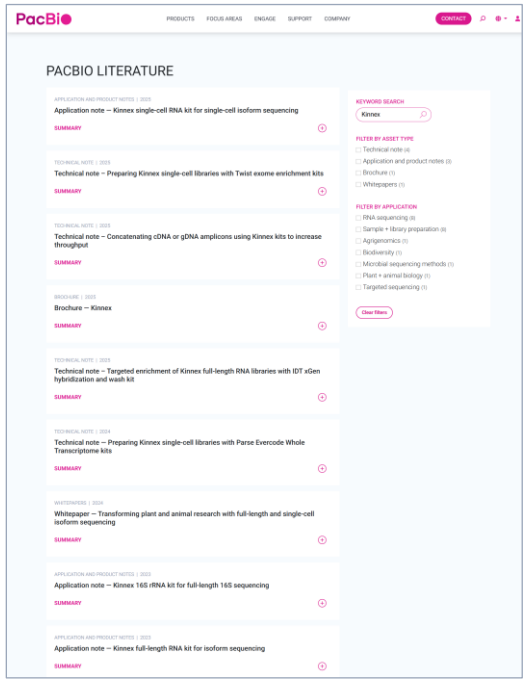
Application	Dataset	Data type	PacBio system
Kinnex 16S rRNA sequencing	ZymoBIOMICS Fecal Reference with TruMatrix Technology (human) [Link]	HiFi long read	Sequel II & Revio systems
	Mixture: ZymoBIOMICS Gut Microbiome Standard, ZymoBIOMICS Fecal Reference with TruMatrix™ Technology, ATCC 20 Strain Even Mix Genomic Material, ATCC 20 Strain Staggered Mix Genomic Material [Link]	HiFi long read	Sequel II & Revio systems

APPENDIX 3: PacBio compatible Kinnex library preparation workflows

Preparing Kinnex single-cell libraries with ArgenTag kits

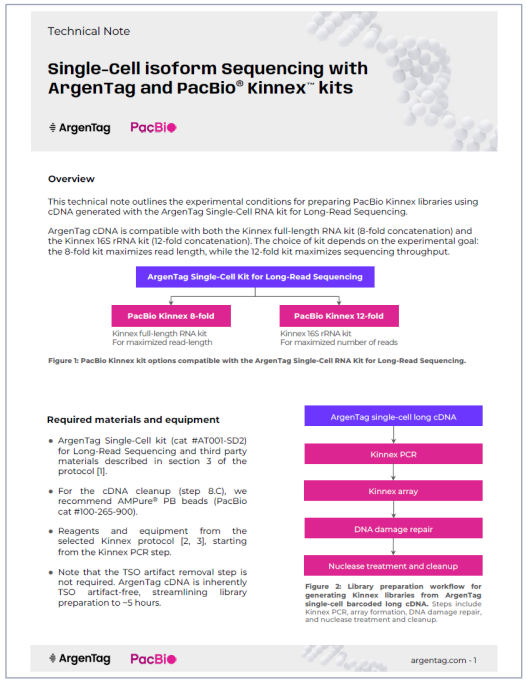
Preparing Kinnex single-cell libraries with ArgenTag kits: Getting started

Application-specific educational literature → **Application-specific protocol documentation** → **Application-specific technical overviews**



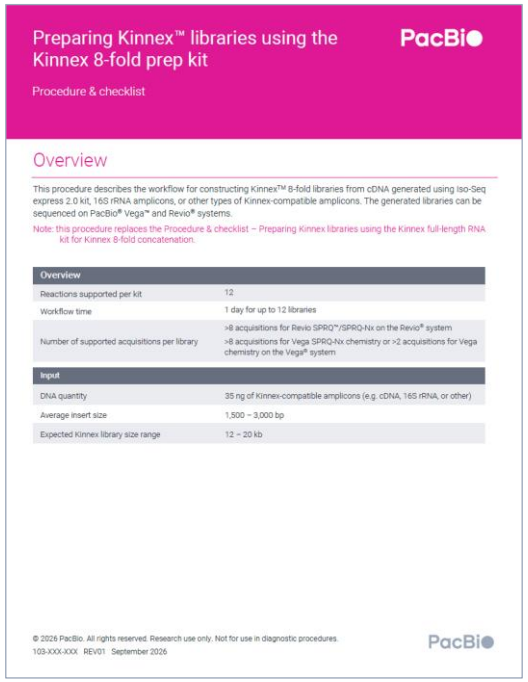
PacBio literature website [Link]

Application-specific brochures, informational guides and other product literature containing best practices recommendations for library preparation and data analysis workflows.



Technical note – Single-Cell isoform Sequencing with ArgenTag and PacBio Kinnex kits [ArgenTag]

Technical documentation describing the workflow for preparing PacBio Kinnex single-cell libraries with single-cell cDNA generated using ArgenTag Single-Cell RNA kit for Long-Read Sequencing.



Procedure & checklist – Preparing Kinnex libraries using Kinnex 8-fold prep kit (103-920-900) **or Kinnex 12-fold prep kit** (103-921-000) [PacBio]

Technical documentation containing PacBio SMRTbell library construction details.

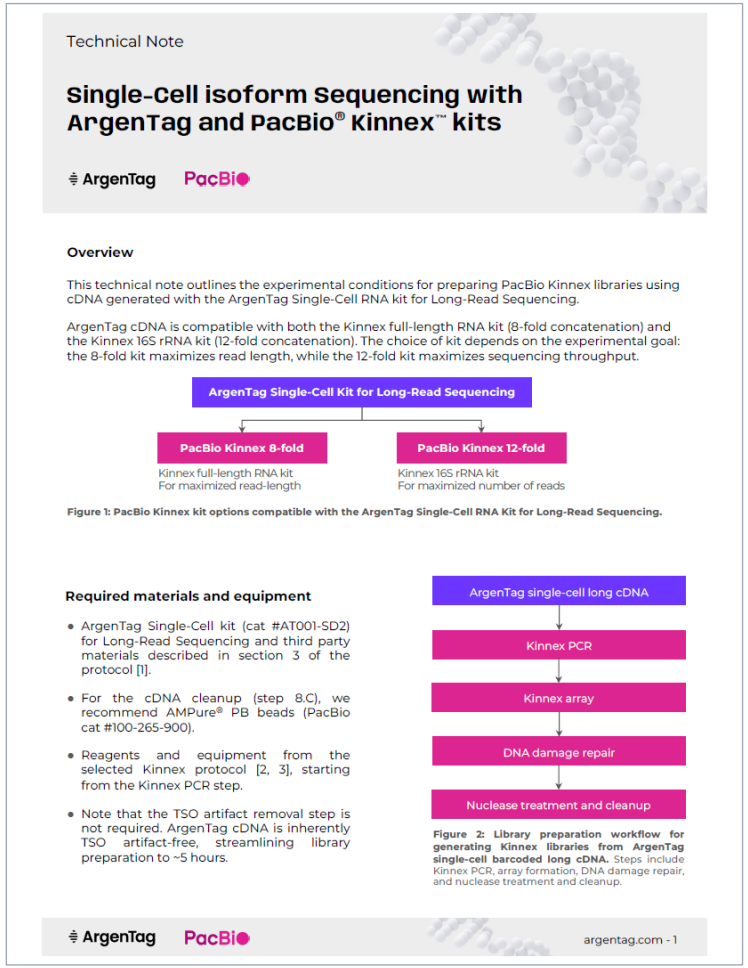
Technical overview – Kinnex library preparation using Kinnex 8-fold prep kit (103-916-000) **or Kinnex 12-fold prep kit** (103-916-100) [PacBio]

Technical overview presentations describe sample preparation details for constructing HiFi libraries for specific applications. Example sequencing performance data for a given application are also summarized.

PacBio

Technical reference for preparing Kinnex single-cell libraries with ArgenTag Single-Cell RNA kit for Long-Read Sequencing

Technical note – Single-Cell isoform Sequencing with ArgenTag and PacBio Kinnex kits (ArgenTag) describes the workflow for preparing PacBio Kinnex single-cell libraries with single-cell cDNA generated using ArgenTag Single-Cell RNA kit for Long-Read Sequencing



Technical Note

Single-Cell isoform Sequencing with ArgenTag and PacBio® Kinnex™ kits

ArgenTag PacBio

Overview

This technical note outlines the experimental conditions for preparing PacBio Kinnex libraries using cDNA generated with the ArgenTag Single-Cell RNA kit for Long-Read Sequencing.

ArgenTag cDNA is compatible with both the Kinnex full-length RNA kit (8-fold concatenation) and the Kinnex 16S rRNA kit (12-fold concatenation). The choice of kit depends on the experimental goal: the 8-fold kit maximizes read length, while the 12-fold kit maximizes sequencing throughput.

ArgenTag Single-Cell Kit for Long-Read Sequencing

PacBio Kinnex 8-fold — Kinnex full-length RNA kit, For maximized read-length

PacBio Kinnex 12-fold — Kinnex 16S rRNA kit, For maximized number of reads

Figure 1: PacBio Kinnex kit options compatible with the ArgenTag Single-Cell RNA Kit for Long-Read Sequencing.

Required materials and equipment

- ArgenTag Single-Cell kit (cat #AT001-SD2) for Long-Read Sequencing and third party materials described in section 3 of the protocol [1].
- For the cDNA cleanup (step 8.C), we recommend AMPure® PB beads (PacBio cat #100-265-900).
- Reagents and equipment from the selected Kinnex protocol [2, 3], starting from the Kinnex PCR step.
- Note that the TSO artifact removal step is not required. ArgenTag cDNA is inherently TSO artifact-free, streamlining library preparation to ~5 hours.

ArgenTag single-cell long cDNA → Kinnex PCR → Kinnex array → DNA damage repair → Nuclease treatment and cleanup

Figure 2: Library preparation workflow for generating Kinnex libraries from ArgenTag single-cell barcoded long cDNA. Steps include Kinnex PCR, array formation, DNA damage repair, and nuclease treatment and cleanup.

ArgenTag PacBio argentag.com - 1

ArgenTag Technical note

Single-Cell isoform Sequencing with ArgenTag and PacBio Kinnex kits (ArgenTag)

PacBio Procedure & checklist

Preparing Kinnex libraries using Kinnex 8-fold prep kit (103-920-900)

Or

Preparing Kinnex libraries using Kinnex 12-fold prep kit (103-921-000)

Overview

- ArgenTag Technical note (ArgenTag) outlines the experimental conditions for preparing PacBio Kinnex libraries using cDNA generated with the ArgenTag Single-Cell RNA kit for Long-Read Sequencing
- ArgenTag cDNA is compatible with both the PacBio Kinnex 8-fold prep kit and the Kinnex 12-fold prep kit
- The choice of Kinnex prep kit depends on the experimental goal:
  - **Kinnex 8-fold kit** maximizes **read length**, while the **Kinnex 12-fold kit** maximizes **sequencing throughput**

[1] Learn more about how Parse technology uniquely labels cells without needing to isolate individual cells at Parse Biosciences website.

Workflow overview for preparing Kinnex single-cell libraries with ArgenTag Single-Cell RNA kit for Long-Read Sequencing

Follow ArgenTag ArgenTag Single-Cell RNA kit procedure[1] to generate single-cell cDNA samples suitable for Kinnex library prep and HiFi sequencing on PacBio long-read systems

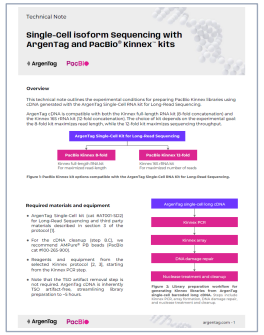
1. ArgenTag single-cell cDNA generation
2. Kinnex PCR
3. Kinnex array formation
4. DNA damage repair
5. Nuclease treatment + cleanup

ArgenTag single-cell cDNA generation procedure overview[1]

Technical note – Single-Cell isoform Sequencing with ArgenTag and PacBio Kinnex kits

[ArgenTag]

ArgenTag



ArgenTag single-cell cDNA generation procedure key steps

- Single-cell cDNA material first needs to be generated as described in the ArgenTag protocol[1] ***Procedure & checklist – ArgenTag Single-Cell kit for Long-Read Sequencing handbook***

Procedure & checklist – ArgenTag Single-Cell kit for Long-Read Sequencing handbook [ArgenTag]

- Use the generated cDNA as input into the PacBio Kinnex 8-fold prep protocol (103-920-900) or Kinnex 12-fold prep protocol (103-921-000) starting at **Step 3: Kinnex PCR**.

ArgenTag single-cell cDNA generation workflow[1] (Step 1)

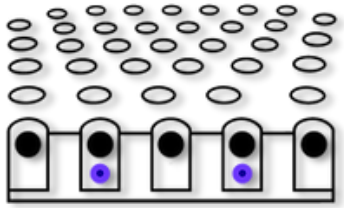
1: Load the ArgenTag chip

Pipette your cell suspension onto the ArgenTag microwell chip. Cells will settle into individual microwells by gravity.

5 min

2: Capture and barcode

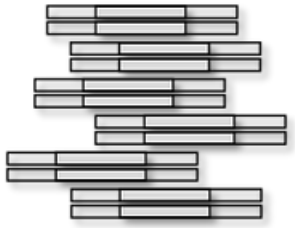
Add the barcoded magnetic beads to pair with the cells in microwells. Lyse the cells in place and recover the beads with the poly-A mRNA.



15 min

3: Reverse transcribe and amplify

Perform the reverse transcription with the magnetic beads, and amplify to generate full-length, barcoded cDNA.



30 min

PacBio

[1] For single-cell cDNA generation procedure details, refer to ***Procedure & checklist – ArgenTag Single-Cell kit for Long-Read Sequencing handbook*** available from the ArgenTag website.

Workflow overview for preparing Kinnex single-cell libraries with Parse Evercode Whole Transcriptome kits (cont.)

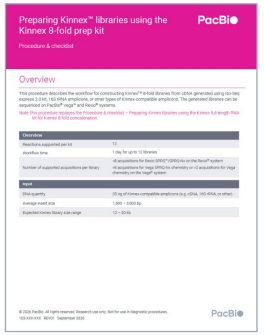
Parse single-cell cDNA samples may be used for Kinnex library construction by following PacBio *Procedure & checklist – Preparing Kinnex libraries using Kinnex single-cell RNA kit* (103-254-300)

1. ArgenTag single-cell cDNA generation
2. Kinnex PCR
3. Kinnex array formation
4. DNA damage repair
5. Nuclease treatment + cleanup

PacBio Kinnex library construction procedure[1]

Procedure & checklist – Preparing Kinnex libraries using Kinnex 8-fold prep kit (103-920-900) or Kinnex 12-fold prep kit (103-921-000)

PacBio



Kinnex single-cell library prep procedure key steps

- Following cDNA QC per the ArgenTag user manual: Ideal cDNA profile should have an average peak above 1,200 bp with fragments in the 1,000–5,000 bp range exceeding 30% of total molecules
- Proceed directly to Kinnex PCR step using 35 ng input for 8-fold concatenation using Kinnex 8-fold prep kit (103-920-900) or 12-fold concatenation using Kinnex 12-fold prep kit (103-921-000)
- Library preparation then continues according to the respective PacBio Kinnex protocol
- Following sample setup ABC, prepare libraries for sequencing using SMRT Link Run Design using same parameters as standard Kinnex libraries

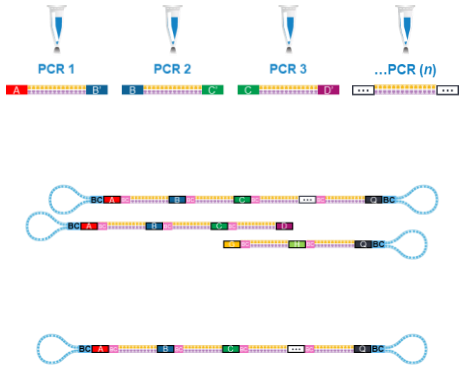
1. ArgenTag single-cell cDNA generation

→ See **ArgenTag Technical note**

Technical note – Single-Cell isoform Sequencing with ArgenTag and PacBio Kinnex kits (ArgenTag)

Kinnex single-cell library construction workflow (Steps 2 – 5)

2 Kinnex PCR
↓ Safe stop
3 Kinnex array
↓ Safe stop
4 DNA repair
↓ Safe stop
5 Nuclease treatment and cleanup

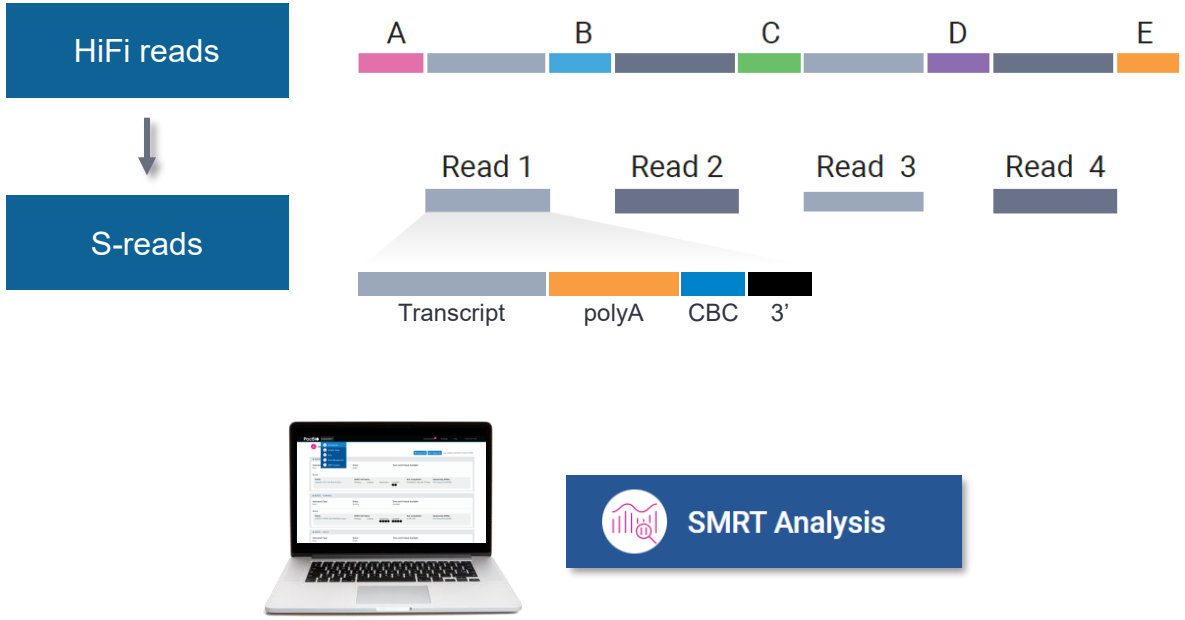


SMRT Link informatics and secondary analysis recommendations for Kinnex single-cell libraries generated with ArgenTag Single-Cell RNA kit

Read Segmentation can be run in SMRT Link (or skera in the command line) to generate segmented reads

- Read Segmentation can be run in SMRT Link (or skera in the command line) to generate segmented reads (S-reads)
  - The S-reads will contain the original ArgenTag single-cell cDNA barcode information
- S-reads can be demultiplexed and analyzed using methods described in ***Technical note – Single-Cell isoform Sequencing with ArgenTag and PacBio Kinnex kits*** [ArgenTag][1]

SMRT Link read segmentation workflow



[1] Refer to ArgenTag website or ***Technical note – Single-Cell isoform Sequencing with ArgenTag and PacBio Kinnex kits*** [ArgenTag] and ***ArgenTag single-cell read demultiplexing pipeline*** website [ArgenTag GitHub] for secondary analysis recommendations using command line and community tools.

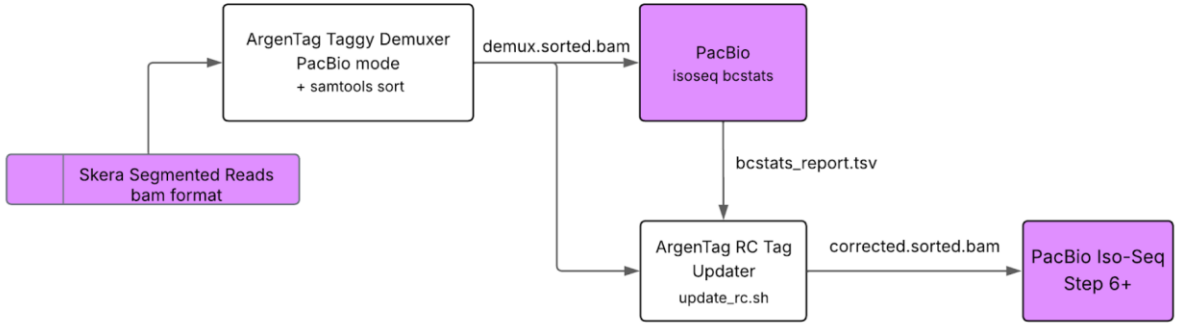
SMRT Link informatics and secondary analysis recommendations for Kinnex single-cell libraries generated with ArgenTag Single-Cell RNA kit (cont.)

Segmented reads (S-reads) can be demultiplexed and analyzed using ArgenTag + PacBio tools

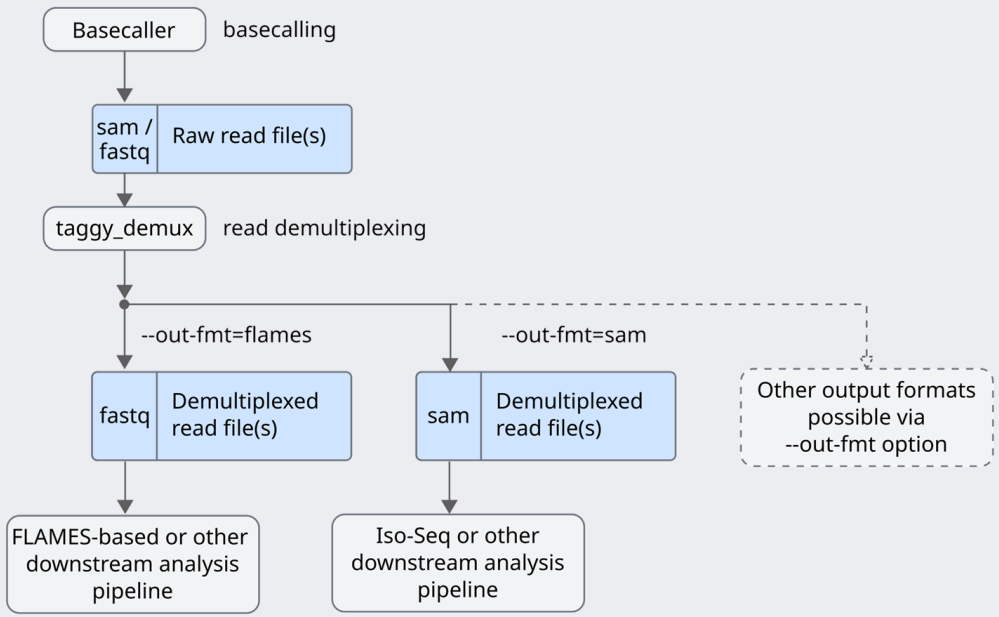
- S-reads can be demultiplexed and analyzed using the bioinformatics pipeline described below[1]:

AT taggy demux + update_rc.sh + PBIso-Seq pipeline description

- **Input:** S-reads (.bam)
- **Steps:** AT Taggy Demuxer, + update_rc.sh +, PB Iso-Seq workflow (step 6+), Pigeon.
- **Output:** GTF, gene and isoform classification, gene- and isoform-level count matrix, isoform classification.



ArgenTag single-cell read demultiplexing workflow

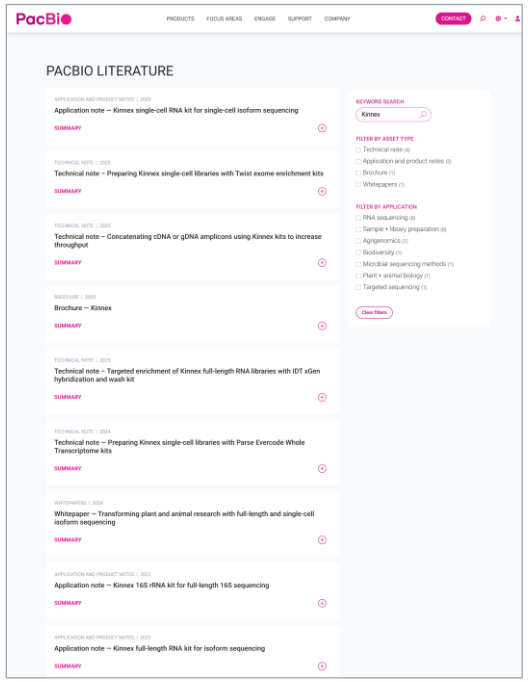


[1] Refer to ArgenTag website or ***Technical note – Single-Cell isoform Sequencing with ArgenTag and PacBio Kinnex kits*** [ArgenTag] and ***ArgenTag single-cell read demultiplexing pipeline*** website [ArgenTag GitHub] for secondary analysis recommendations using command line and community tools.

Preparing Kinnex single-cell libraries with Parse Evercode Whole Transcriptome kits

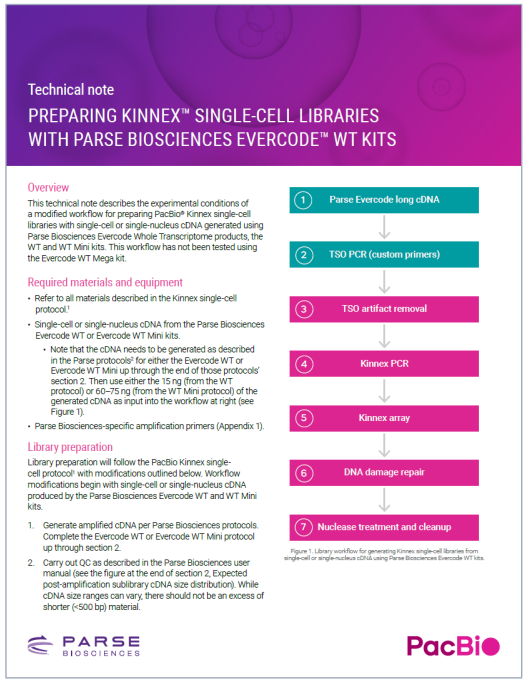
Preparing Kinnex single-cell libraries with Parse Evercode Whole Transcriptome kits: Getting started

Application-specific educational literature → **Application-specific protocol documentation** → **Application-specific technical overviews**



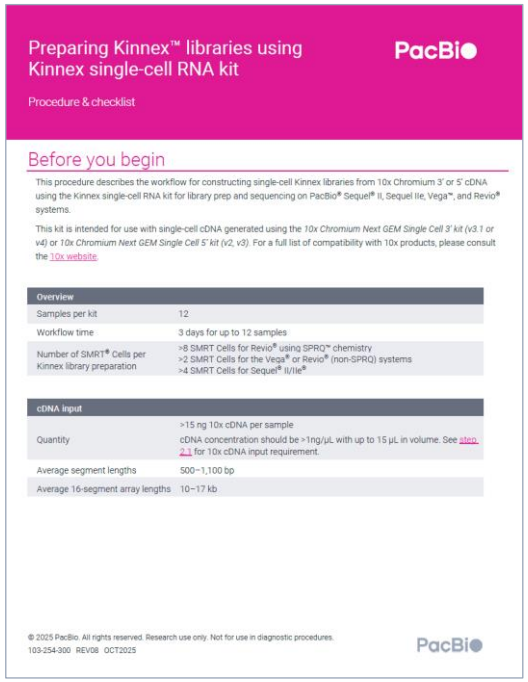
PacBio literature website [Link]

Application-specific brochures, informational guides and other product literature containing best practices recommendations for library preparation and data analysis workflows.



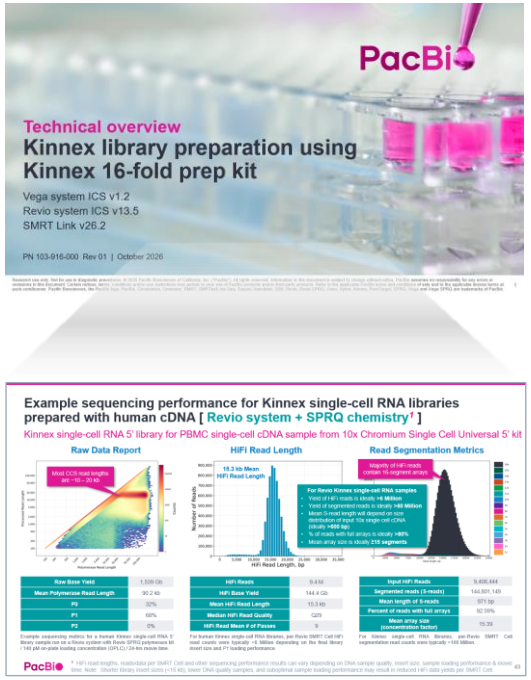
Technical note – Preparing Kinnex single-cell libraries with Parse Evercode Whole Transcriptome kits (102-326-610) [PacBio]

Technical documentation describing a modified workflow for preparing PacBio Kinnex single-cell libraries with single-cell or single-nucleus cDNA generated using Parse Biosciences Evercode Whole Transcriptome products.



Procedure & checklist – Preparing Kinnex libraries using Kinnex 16-fold prep kit (103-885-300) [PacBio]

Technical documentation containing PacBio SMRTbell library construction details.



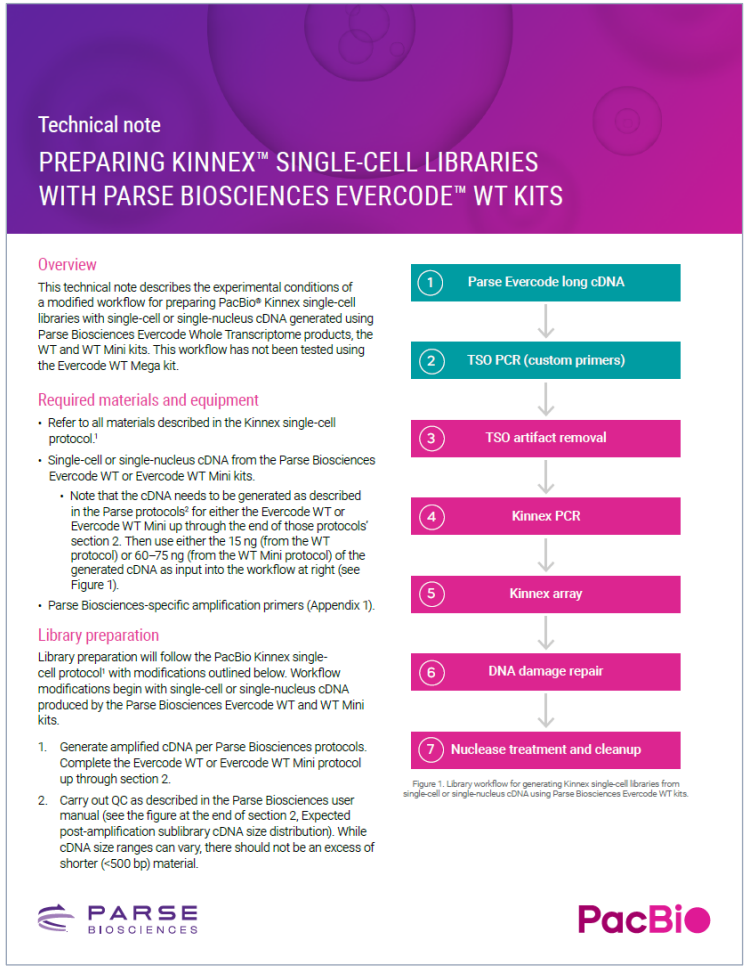
Technical overview – Kinnex library preparation using Kinnex 16-fold prep kit (103-916-200)

Technical overview presentations describe sample preparation details for constructing HiFi libraries for specific applications. Example sequencing performance data for a given application are also summarized.

PacBio

Technical reference for preparing Kinnex single-cell libraries with Parse Evercode Whole Transcriptome kits

Technical note – Preparing Kinnex single-cell libraries with Parse Evercode Whole Transcriptome kits (102-326-610) describes a modified workflow for preparing PacBio Kinnex single-cell libraries with single-cell or single-nucleus cDNA generated using Parse Biosciences Evercode Whole Transcriptome products



Technical note

PREPARING KINNEX™ SINGLE-CELL LIBRARIES WITH PARSE BIOSCIENCES EVERCODE™ WT KITS

Overview

This technical note describes the experimental conditions of a modified workflow for preparing PacBio® Kinnex single-cell libraries with single-cell or single-nucleus cDNA generated using Parse Biosciences Evercode Whole Transcriptome products, the WT and WT Mini kits. This workflow has not been tested using the Evercode WT Mega kit.

Required materials and equipment

- Refer to all materials described in the Kinnex single-cell protocol.[1]
- Single-cell or single-nucleus cDNA from the Parse Biosciences Evercode WT or Evercode WT Mini kits.
  - Note that the cDNA needs to be generated as described in the Parse protocols[2] for either the Evercode WT or Evercode WT Mini up through the end of those protocols' section 2. Then use either the 15 ng (from the WT protocol) or 60–75 ng (from the WT Mini protocol) of the generated cDNA as input into the workflow at right (see Figure 1).
- Parse Biosciences-specific amplification primers (Appendix 1).

Library preparation

Library preparation will follow the PacBio Kinnex single-cell protocol[1] with modifications outlined below. Workflow modifications begin with single-cell or single-nucleus cDNA produced by the Parse Biosciences Evercode WT and WT Mini kits.

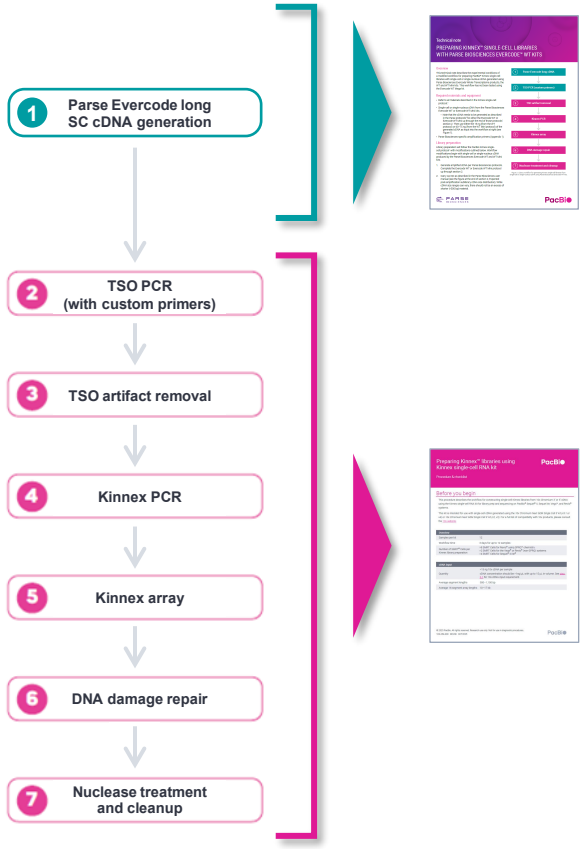
1. Generate amplified cDNA per Parse Biosciences protocols. Complete the Evercode WT or Evercode WT Mini protocol up through section 2.
2. Carry out QC as described in the Parse Biosciences user manual (see the figure at the end of section 2, Expected post-amplification sublibrary cDNA size distribution). While cDNA size ranges can vary, there should not be an excess of shorter (<500 bp) material.

1 Parse Evercode long cDNA
2 TSO PCR (custom primers)
3 TSO artifact removal
4 Kinnex PCR
5 Kinnex array
6 DNA damage repair
7 Nuclease treatment and cleanup

Figure 1. Library workflow for generating Kinnex single-cell libraries from single-cell or single-nucleus cDNA using Parse Biosciences Evercode WT kits.

PARSE BIOSCIENCES

PacBio



PacBio Technical note

Preparing Kinnex single-cell libraries with Parse Evercode Whole Transcriptome kits (102-326-610)

[Select to use either single-cell or single-nucleus cDNA from Parse Biosciences Evercode WT or Evercode WT Mini kits]

PacBio Procedure & checklist

Preparing Kinnex libraries using Kinnex 16-fold prep kit (103-885-300)

Overview

- Parse whole transcriptome kits use split-pool combinatorial barcoding to enable scale-up of single cell projects to millions of cells or nuclei[1]
- This Kinnex library prep workflow has been tested with single-cell or single-nucleus cDNA generated using Parse Biosciences Evercode WT and WT Mini kit whole transcriptome products
- This workflow has not been tested using the Evercode WT Mega kit

[1] Learn more about how Parse technology uniquely labels cells without needing to isolate individual cells at Parse Biosciences website.

Workflow overview for preparing Kinnex single-cell libraries with Parse Evercode Whole Transcriptome kits

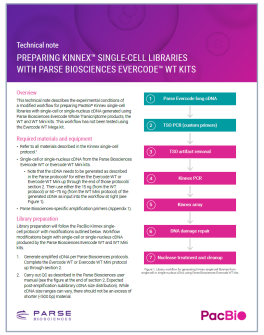
Follow Parse Bioscience whole transcriptome kit procedure[1] to generate single-cell cDNA samples suitable for Kinnex library prep and HiFi sequencing on PacBio long-read systems

1. Parse Evercode SC cDNA generation
2. TSO PCR (with custom primers)
3. TSO artifact removal
4. Kinnex PCR
5. Kinnex array formation
6. DNA damage repair
7. Nuclease treatment and cleanup

Parse single-cell cDNA generation procedure overview[1]

Technical note – Preparing Kinnex single-cell libraries with Parse Evercode Whole Transcriptome kits (102-326-610)

PacBio



Parse single-cell cDNA generation procedure key steps

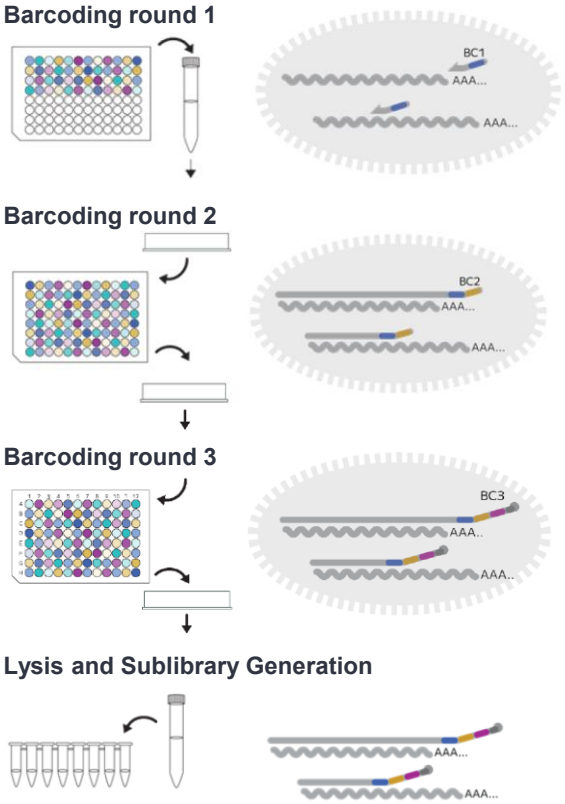
- Single-cell cDNA material first needs to be generated as described in the Parse protocols[1] for either the **Evercode WT or Evercode WT Mini kit** up through the end of those protocols' **Section 2**.

Evercode WT User Guides
UMWT3300 or UMWT3100

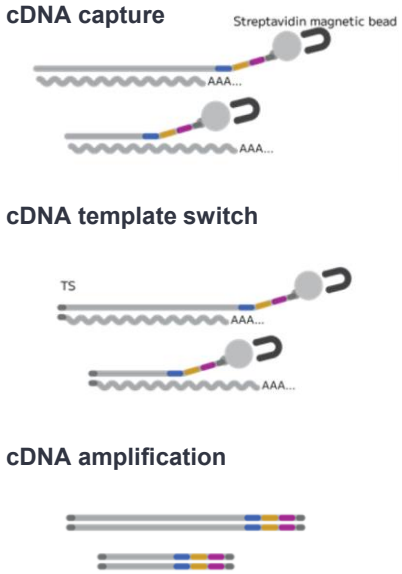
- Use either 15 ng (from the WT protocol) or 60–75 ng (from the WT Mini protocol) of the generated cDNA as input into the **PacBio Kinnex 16-fold prep kit protocol** (103-885-300) starting at **Step 2: TSO PCR (with custom primers[2])**.

Parse Evercode single-cell cDNA generation workflow[1] (Step 1)

Section 1: In situ cell / nuclei barcoding



Section 2: cDNA capture and amplification



PacBio

[1] For single-cell cDNA generation procedure details, refer to **Evercode WT User Guides** (UMWT3300 or UMWT3100) available from the Parse Biosciences website.
[2] Perform TSO PCR (Step 2) with Parse Biosciences-specific amplification primers (see Appendix 1 in **Parse Technical note** 102-326-610).

Workflow overview for preparing Kinnex single-cell libraries with Parse Evercode Whole Transcriptome kits (cont.)

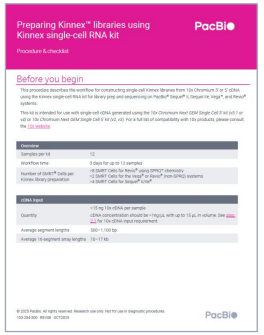
Parse single-cell cDNA samples may be used for Kinnex library construction by following PacBio *Procedure & checklist – Preparing Kinnex libraries using the Kinnex 16-fold prep kit* (103-885-300)

1. Parse Evercode SC cDNA generation
2. TSO PCR (with custom primers)
3. TSO artifact removal
4. Kinnex PCR
5. Kinnex array formation
6. DNA damage repair
7. Nuclease treatment and cleanup

PacBio Kinnex library construction procedure[1]

Procedure & checklist – Preparing Kinnex libraries using Kinnex 16-fold prep kit (103-885-300)

PacBio



Kinnex SC library prep procedure key steps

- Follow **PacBio Kinnex 16-fold prep kit protocol** (103-885-300) (**Steps 2 – 7**) with modifications:
  - Generate amplified single-cell cDNA per Parse Biosciences protocols. Complete the**Evercode WT or Evercode WT Mini protocol** up through **Section 2**.
  - Carry out cDNA quantification and sizing QC as described in the Parse Biosciences user manual.
  - Begin **Kinnex 16-fold prep kit protocol from step 2 (TSO PCR)** with the following modification: Follow step 2.1 and use 5 µL of **Parse Biosciences-specific amplification primers** as described in Appendix 1 instead of Kinnex 5' or 3' capture primer mixes.[1]
  - After running TSO PCR program, proceed with the subsequent Kinnex workflow from cleanup with 1.5X SMRTbell cleanup beads in the TSO PCR step through the rest of the protocol.[2]

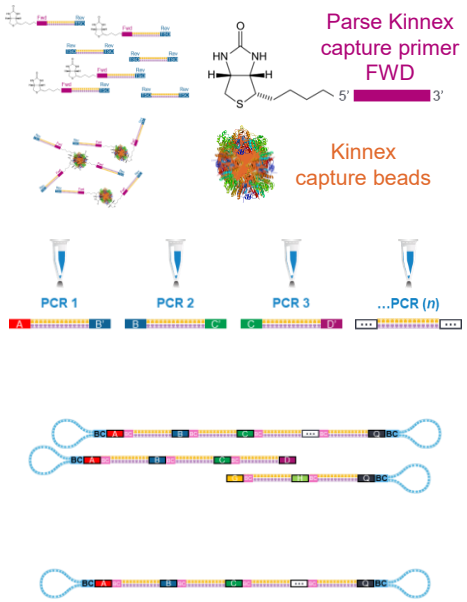
1. Parse Evercodesingle-cell cDNA generation

→ See **PacBio Technical note**

Preparing Kinnex single-cell libraries with Parse Evercode Whole Transcriptome kits (102-326-610)

Kinnex single-cell library construction workflow (Steps 2 – 7)

2 TSO PCR
↓
3 TSO artifact removal
↓
4 Kinnex PCR
↓
5 Kinnex array
↓
6 DNA repair
↓
7 Nuclease treatment and cleanup



[1] This replacement is critical to adapt the Parse library termini to function with the Kinnex concatenation workflow.
[2] Subsequent QC and library quantification can be carried out as described in the Kinnex 16-fold pre kit protocol (step 5.21 in 103-885-300).

TSO PCR amplification primer oligo recommendations for preparing Kinnex single-cell libraries with Parse Evercode Whole Transcriptome kits

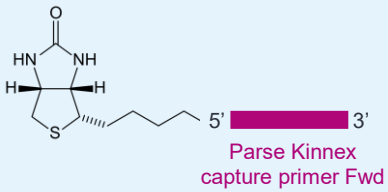
Order custom TSO PCR amplification primers as described below from your preferred oligo vendor

Parse Biosciences-specific primers required for TSO PCR amplification step

- Order custom primers from your preferred oligo vendor
- An example of IDT code is shown at right
- Primers should be HPLC-purified
- Order these primers and reconstitute in TE buffer (10 mM Tris HCl pH 8, 0.1 mM EDTA).
- Use 10 µM solutions of each of these primers in place of the yellow-capped Kinnex 3’ capture primer mix or the red-capped Kinnex 5’ capture primer mix in the Kinnex 16-fold prep kit (103-836-900)

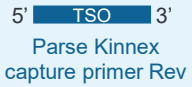
Parse Kinnex forward capture primer

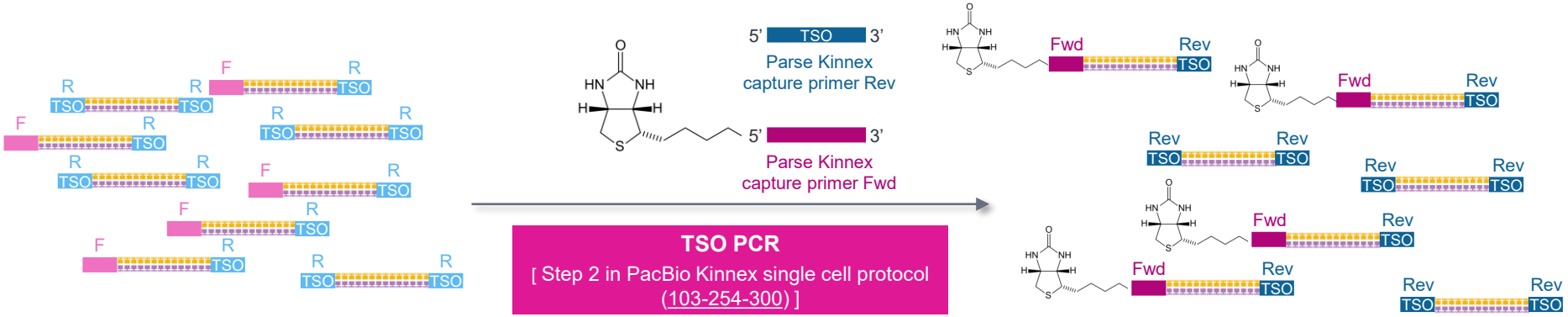
/5Biosg/AA/ideoxyU/CTACACGACGCTCTT
CCGATCTCAGACGTGTGCTCTTCCGATC



Parse Kinnex reverse capture primer

AAGCAGTGGTATCAACGCAGAG





TSO PCR step in Kinnex single-cell RNA procedure uses a modified PCR primer (Parse Kinnex capture primer Fwd) to incorporate a biotin tag into desired cDNA products with the correct structure.[1]

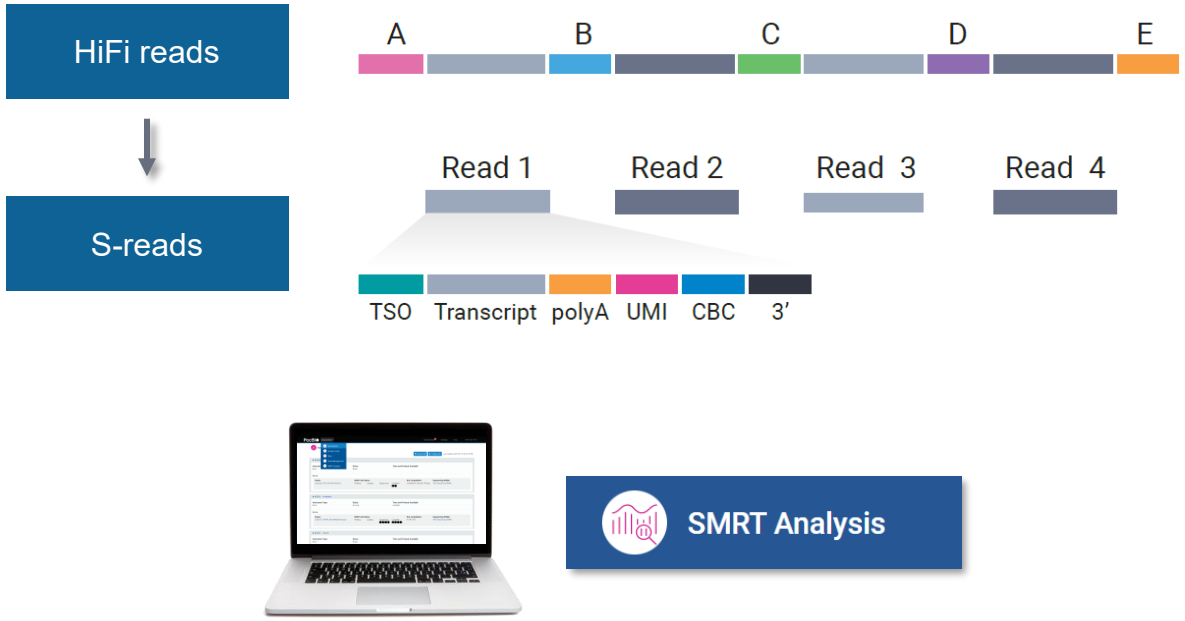
[1] The subsequent TSO artifact removal step (Step 3) in the **Kinnex 16-fold prep kit protocol** (103-885-300) uses streptavidin-coated Kinnex capture beads to pull down and enrich for biotin-tagged cDNA products with the correct structure.

SMRT Link informatics and secondary analysis recommendations for Kinnex single-cell libraries generated with Parse Evercode Whole Transcriptome kits

Read Segmentation can be run in SMRT Link (or skera in the command line) to generate segmented reads

- Note: The SMRT Link single-cell Iso-Seq workflow does not currently support analysis of Parse Biosciences data.
- Instead, Read Segmentation can be run in SMRT Link (or skera in the command line) to generate segmented reads (S-reads).
- The S-reads will contain the original single-cell or single-nucleus cDNA-containing barcode information. Refer to Rebboah et al. (2021) (section Preprocessing of LR-Split-seq data)[1] and Parse Biosciences long-read data processing guide[2] (login required) for secondary analysis recommendations using command line and community tools.

SMRT Link read segmentation workflow

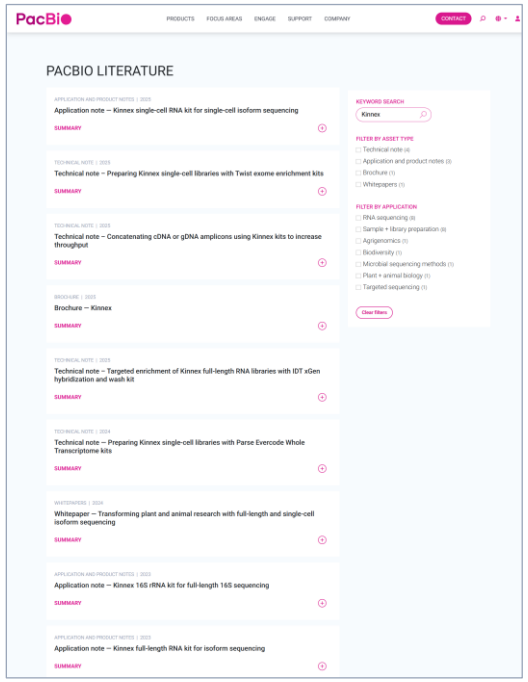


[1] Rebboah, E., et al. (2021). Mapping and modeling the genomic basis of differential RNA isoform expression at single-cell resolution with LR-Split-seq. Genome biology, 22, 1-28 [Link].
[2] Workflow-for-Processing-Long-Read-Data-with-the-Parse-Biosciences-Pipeline. Parse Biosciences long-read data processing guide.

Preparing Kinnex single-cell libraries with Twist exome enrichment kits

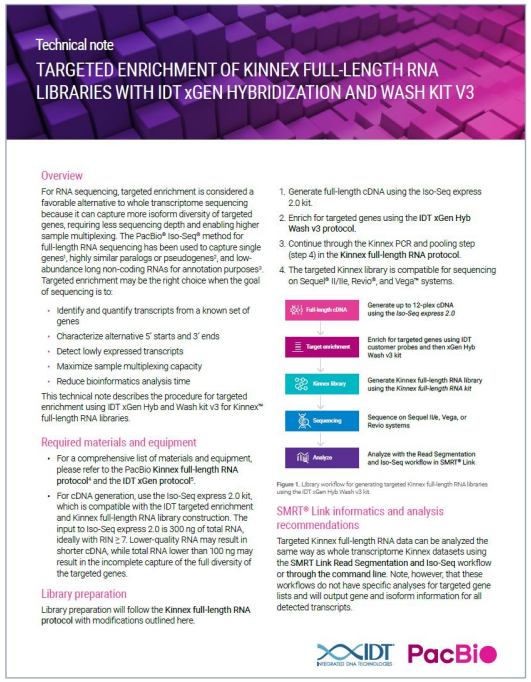
Targeted enrichment of Kinnex full-length RNA libraries with IDT xGen hybridization and wash kit v3: Getting started

Application-specific educational literature → **Application-specific protocol documentation** → **Application-specific technical overviews**



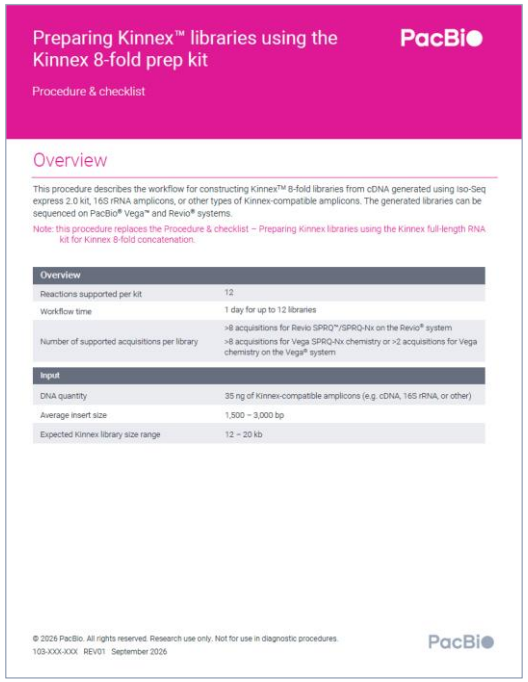
PacBio literature website [Link]

Application-specific brochures, informational guides and other product literature containing best practices recommendations for library preparation and data analysis workflows.



Technical note – Targeted enrichment of Kinnex full-length RNA libraries with IDT xGen hybridization and wash kit v3 (102-326-632) [PacBio]

Technical documentation describing the procedure for targeted enrichment using IDT xGen Hyb and Wash kit v3 for preparing Kinnex full-length RNA libraries for PacBio sequencing.



Procedure & checklist – Preparing Kinnex libraries using Kinnex 8-fold prep kit (103-920-900) [PacBio]

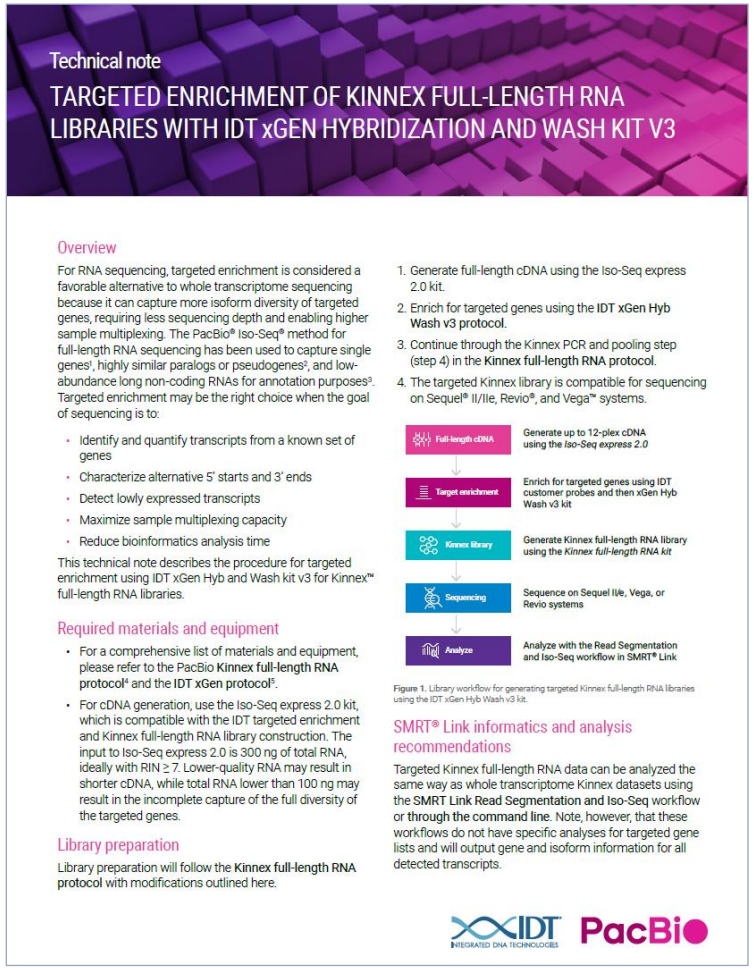
Technical documentation containing PacBio SMRTbell library construction details.

Technical overview – Kinnex library preparation using Kinnex 8-fold prep kit (103-916-000) **or Kinnex 12-fold prep kit** (103-916-100) [PacBio]

Technical overview presentations describe sample preparation details for constructing HiFi libraries for specific applications. Example sequencing performance data for a given application are also summarized.

Technical reference for targeted enrichment of Kinnex full-length RNA libraries with IDT xGen hybridization and wash kit v3

Technical note – Targeted enrichment of Kinnex full-length RNA libraries with IDT xGen hybridization and wash kit (102-326-632) describes the procedure for targeted enrichment using IDT xGen Hyb and Wash kit v3 for Kinnex full-length RNA libraries



Technical note

TARGETED ENRICHMENT OF KINNEX FULL-LENGTH RNA LIBRARIES WITH IDT xGEN HYBRIDIZATION AND WASH KIT V3

Overview

For RNA sequencing, targeted enrichment is considered a favorable alternative to whole transcriptome sequencing because it can capture more isoform diversity of targeted genes, requiring less sequencing depth and enabling higher sample multiplexing. The PacBio® Iso-Seq® method for full-length RNA sequencing has been used to capture single genes[1], highly similar paralogs or pseudogenes[2], and low-abundance long non-coding RNAs for annotation purposes[3]. Targeted enrichment may be the right choice when the goal of sequencing is to:

- Identify and quantify transcripts from a known set of genes
- Characterize alternative 5' starts and 3' ends
- Detect lowly expressed transcripts
- Maximize sample multiplexing capacity
- Reduce bioinformatics analysis time

This technical note describes the procedure for targeted enrichment using IDT xGen Hyb and Wash kit v3 for Kinnex™ full-length RNA libraries.

Required materials and equipment

- For a comprehensive list of materials and equipment, please refer to the PacBio **Kinnex full-length RNA protocol**[4] and the **IDT xGen protocol**[5].
- For cDNA generation, use the Iso-Seq express 2.0 kit, which is compatible with the IDT targeted enrichment and Kinnex full-length RNA library construction. The input to Iso-Seq express 2.0 is 300 ng of total RNA, ideally with RIN ≥ 7. Lower-quality RNA may result in shorter cDNA, while total RNA lower than 100 ng may result in the incomplete capture of the full diversity of the targeted genes.

Library preparation

Library preparation will follow the **Kinnex full-length RNA protocol** with modifications outlined here.

1. Generate full-length cDNA using the Iso-Seq express 2.0 kit.
2. Enrich for targeted genes using the **IDT xGen Hyb Wash v3 protocol**.
3. Continue through the Kinnex PCR and pooling step (step 4) in the **Kinnex full-length RNA protocol**.
4. The targeted Kinnex library is compatible for sequencing on Sequel® II/IIe, Revio®, and Vega™ systems.

Full-length cDNA — Generate up to 12-plex cDNA using the *Iso-Seq express 2.0*

Target enrichment — Enrich for targeted genes using IDT customer probes and then xGen Hyb Wash v3 kit

Kinnex library — Generate Kinnex full-length RNA library using the *Kinnex full-length RNA kit*

Sequencing — Sequence on Sequel IIe, Vega, or Revio systems

Analyze — Analyze with the Read Segmentation and Iso-Seq workflow in SMRT® Link

Figure 1. Library workflow for generating targeted Kinnex full-length RNA libraries using the IDT xGen Hyb Wash v3 kit.

SMRT® Link informatics and analysis recommendations

Targeted Kinnex full-length RNA data can be analyzed the same way as whole transcriptome Kinnex datasets using the **SMRT Link Read Segmentation and Iso-Seq** workflow or **through the command line**. Note, however, that these workflows do not have specific analyses for targeted gene lists and will output gene and isoform information for all detected transcripts.

IDT INTEGRATED DNA TECHNOLOGIES PacBio

1. Input RNA quality control
2. cDNA synthesis + cleanup
3. cDNA amplification + cleanup
4. Target enrichment with IDT xGen kit
5. Kinnex PCR
6. Kinnex array formation + cleanup
7. Nuclease treatment + cleanup
8. Annealing, binding and cleanup (ABC)

PacBio Procedure & checklist

Preparing Kinnex compatible cDNA using Iso-Seq express 2.0 kit (102-396-000)

PacBio Technical note

Targeted enrichment of Kinnex full-length RNA libraries with IDT xGen hybridization and wash kit v3 (102-326-632)[1]

PacBio Procedure & checklist

Preparing Kinnex libraries using Kinnex 8-fold prep kit (103-920-900)

Overview

- For RNA sequencing, **targeted enrichment** may be the right choice when the goal of sequencing is to:
  - Identify and quantify transcripts from a known set of genes
  - Characterize alternative 5' starts and 3' ends
  - Detect lowly expressed transcripts
  - Maximize sample multiplexing capacity
  - Reduce bioinformatics analysis time
- ***Technical note – Targeted enrichment of Kinnex full-length RNA libraries with IDT xGen hybridization and wash kit v3*** (102-326-632) describes the procedure for targeted enrichment using IDT xGen Hyb and Wash kit v3 for Kinnex full-length RNA libraries

[1] For step-by-step IDT hybridization capture workflow details, refer to the **IDT xGen Hybridization and Wash Kit v3 (EAP) for PacBio Targeted Kinnex protocol** (RUO24-3081_001).

Workflow overview for targeted enrichment of Kinnex full-length RNA libraries with IDT xGen hybridization and wash kit v3

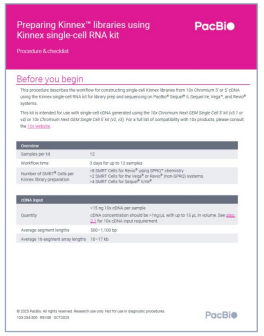
Follow PacBio Kinnex library prep protocol and IDT target enrichment procedure to generate full-length RNA hybrid capture libraries suitable for HiFi sequencing on PacBio long-read systems

1. Input RNA QC
2. cDNA synthesis + cleanup
3. cDNA amplification + cleanup
4. Target enrichment with IDT xGen kit
5. Kinnex PCR
6. Kinnex array formation + cleanup
7. Nuclease treatment + cleanup

PacBio Kinnex library construction procedure[1]

Procedure & checklist – Preparing Kinnex libraries using the Kinnex 8-fold prep kit (103-920-900)

PacBio



Kinnex full-length RNA library prep procedure key steps

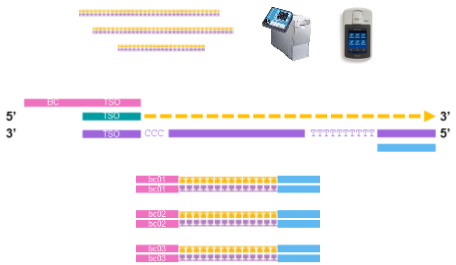
- For cDNA generation, use the Iso-Seq express 2.0 kit with 300 ng of total RNA input, ideally with RIN ≥ 7.[1]
- Library preparation will generally follow the **Kinnex 8-fold prep kit protocol** with the following additional steps:
  - Generate full-length cDNA using the Iso-Seq express 2.0 kit by following **Steps 1-3** in the Iso-Seq cDNA synthesis protocol (102-396-000).
  - Enrich for targeted genes using the **IDT xGen Hyb Wash v3 protocol** (RUO24-3081_001; also see **IDT xGen Technical note** (102-326-632)).
  - Continue through the **Kinnex PCR step (Step 2)** in the Kinnex 8-fold prep kit (103-920-900) protocol and complete the remainder of the library construction workflow

Bulk Iso-Seq cDNA amplicon generation workflow

1 Input RNA quality control

2 cDNA synthesis + cleanup

3 cDNA amplification + cleanup



4. Target enrichment with IDT xGen kit

→ See **PacBio Technical note**

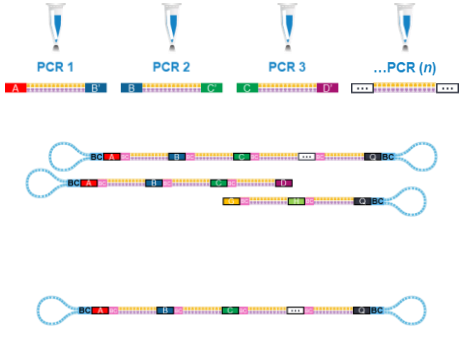
Targeted enrichment of Kinnex full-length RNA libraries with IDT xGen hybridization and wash kit v3 (102-326-632)

Kinnex bulk RNA library construction workflow

5 Kinnex PCR

6 Kinnex array formation + cleanup

7 Nuclease treatment + cleanup



[1] Lower-quality RNA may result in shorter cDNA, while total RNA input amounts lower than 100 ng may result in the incomplete capture of the full diversity of the targeted genes.

Workflow overview for targeted enrichment of Kinnex full-length RNA libraries with IDT xGen hybridization and wash kit v3 (cont.)

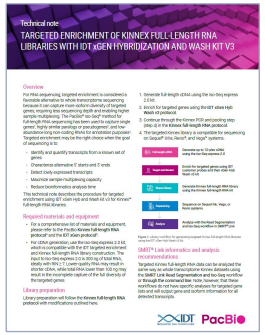
Follow PacBio Kinnex library prep protocol and IDT target enrichment procedure to generate full-length RNA hybrid capture libraries suitable for HiFi sequencing on PacBio long-read systems

1. Input RNA QC
2. cDNA synthesis + cleanup
3. cDNA amplification + cleanup
4. **Target enrichment with IDT xGen kit**
5. Kinnex PCR
6. Kinnex array formation + cleanup
7. Nuclease treatment + cleanup

IDT xGen hybrid capture procedure overview[1]

Technical note – Targeted enrichment of Kinnex full-length RNA libraries with IDT xGen hybridization and wash kit v3 (102-326-632)

PacBio

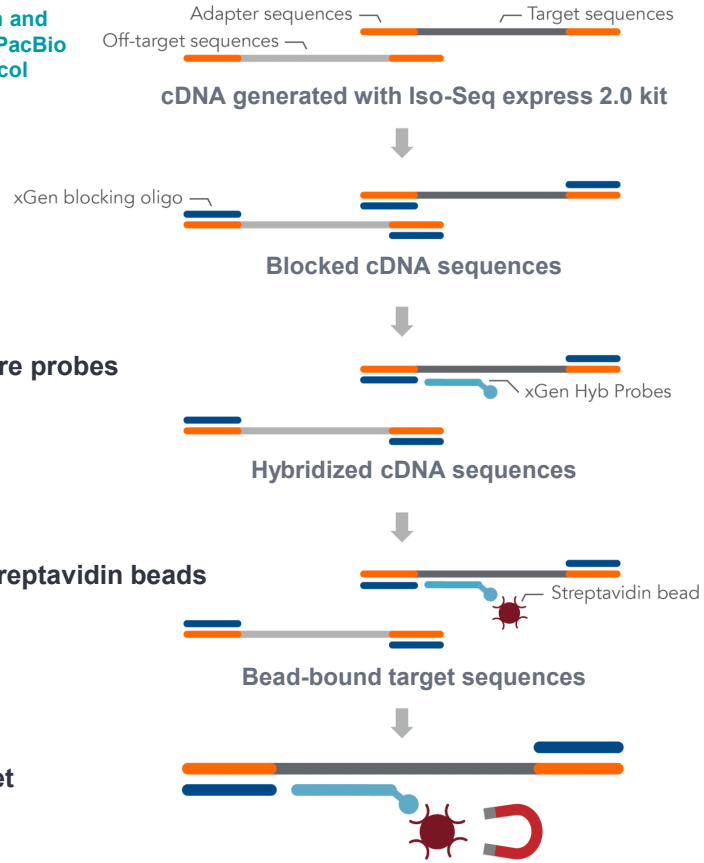

Technical note
TARGETED ENRICHMENT OF KINNEX FULL-LENGTH RNA LIBRARIES WITH IDT xGEN HYBRIDIZATION AND WASH KIT V3

IDT xGEN hybrid capture procedure key steps[1]

- Add Blocker Master Mix to each cDNA sample (500 ng[2]) and dry down
- Add Hybridization Master Mix to each sample well and incubate for desired hybridization time.[3]
- Prepare hybridization wash buffer and capture beads within 2 hours before use
- Add (streptavidin) capture beads to sample (30 min)
- Perform 3 rounds of washing with wash buffers
- Perform post-capture PCR reaction + cleanup
- Perform DNA quantification QC using Qubit assay and DNA sizing QC using Agilent TapeStation or other equivalent system

IDT xGEN hybrid capture workflow

IDT xGen Hybridization and Wash Kit v3 (EAP) for PacBio Targeted Kinnex protocol (RUO24-3081_001).

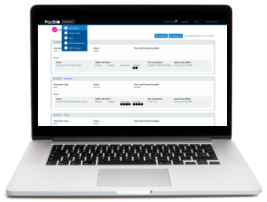


[1] For hybridization capture workflow details, refer to the **IDT xGen Hybridization and Wash Kit v3 (EAP) for PacBio Targeted Kinnex protocol** (RUO24-3081_001).
[2] 100 ng to 6 µg cDNA inputs can be used for short hybridizations (1-2 hrs). Generally, we recommend not to exceed 2.5 µg total input for 1-hr hybridization time for very large panels (>30 Mb).
[3] This procedure supports short hybridization times of only 1 hr as well as the standard 4- or 16-hr incubations. We recommend users empirically test the shorter hybridization time for their workflows.

SMRT Link informatics and secondary analysis recommendations for targeted Kinnex full-length RNA data

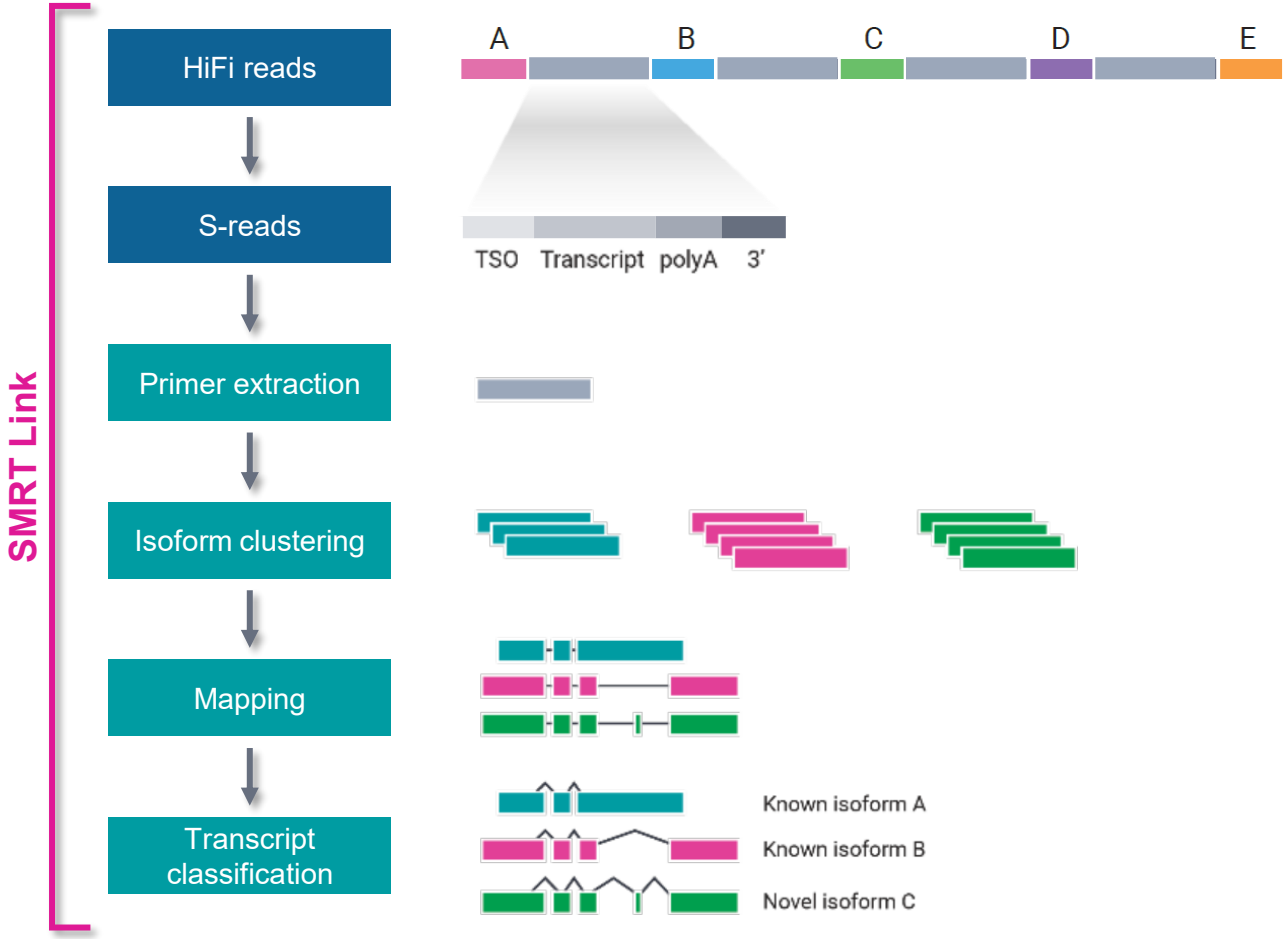
Use SMRT Link Read Segmentation and Iso-Seq application to perform isoform-classification analysis to identify novel genes & isoforms with abundance information

- Targeted Kinnex full-length RNA data can be analyzed the same way as whole transcriptome Kinnex datasets using the SMRT Link Read Segmentation and Iso-Seq workflow or through the command line.
- Note, however, that these workflows do not have specific analyses for targeted gene lists and will output gene and isoform information for all detected transcripts.



SMRT Analysis

SMRT Link read segmentation and Iso-Seq workflow[1]

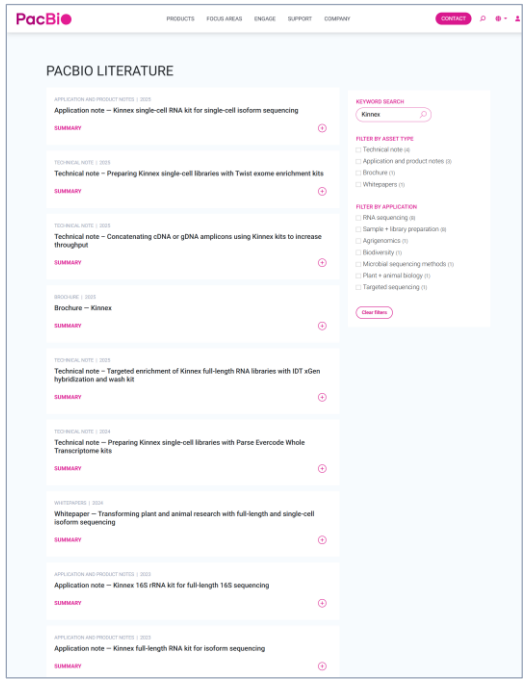


[1] See *SMRT Link User Guide* (Documentation) for detailed descriptions of parameter settings for Read Segmentation and Iso-Seq analysis application.

Targeted enrichment of Kinnex full-length RNA libraries with IDT xGen hybridization capture kits

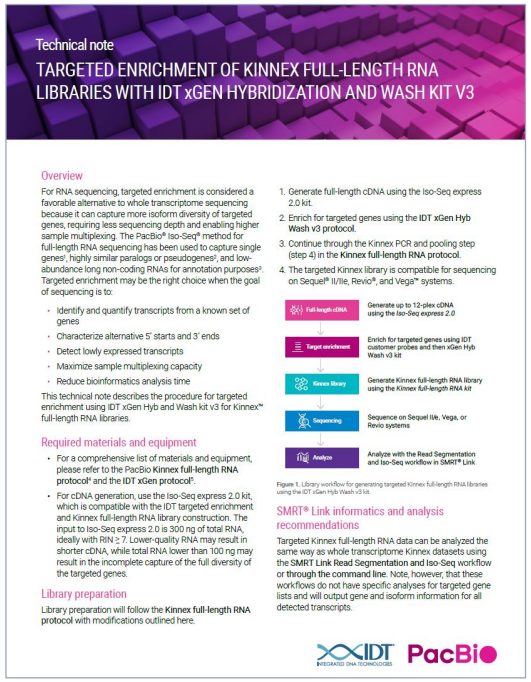
Targeted enrichment of Kinnex full-length RNA libraries with IDT xGen hybridization and wash kit v3: Getting started

Application-specific educational literature → **Application-specific protocol documentation** → **Application-specific technical overviews**



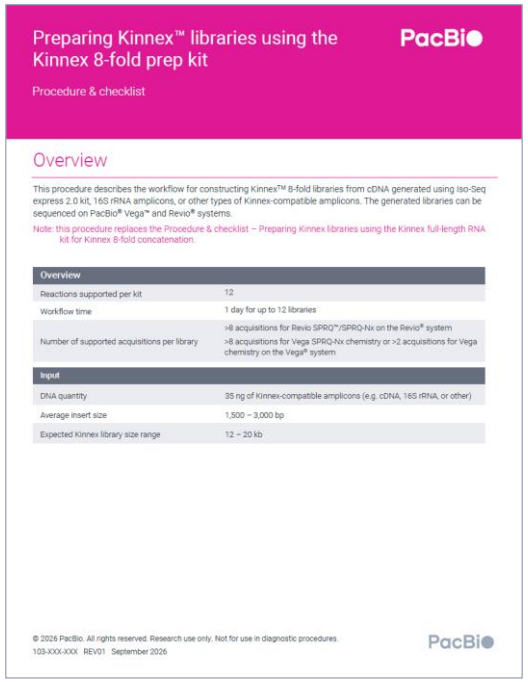
PacBio literature website [Link]

Application-specific brochures, informational guides and other product literature containing best practices recommendations for library preparation and data analysis workflows.



Technical note – Targeted enrichment of Kinnex full-length RNA libraries with IDT xGen hybridization and wash kit v3 (102-326-632) [PacBio]

Technical documentation describing the procedure for targeted enrichment using IDT xGen Hyb and Wash kit v3 for preparing Kinnex full-length RNA libraries for PacBio sequencing.



Procedure & checklist – Preparing Kinnex libraries using Kinnex 8-fold prep kit (103-920-900) **or Kinnex 12-fold prep kit** (103-921-000) [PacBio]

Technical documentation containing PacBio SMRTbell library construction details.

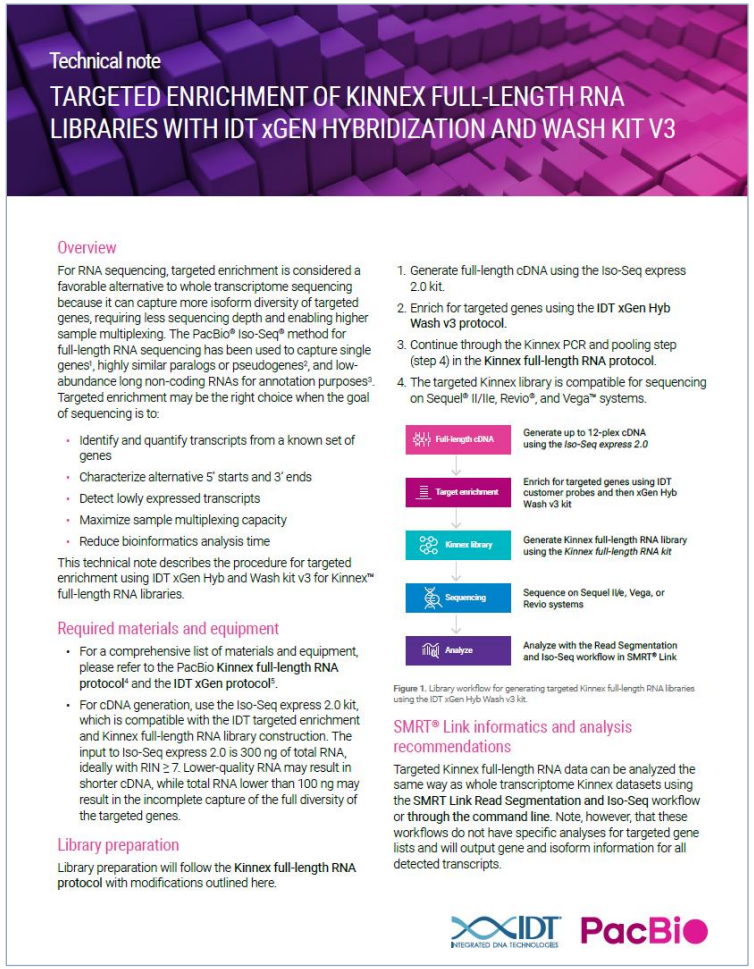
Technical overview – Kinnex library preparation using Kinnex 8-fold prep kit (103-916-000) **or Kinnex 12-fold prep kit** (103-916-100) [PacBio]

Technical overview presentations describe sample preparation details for constructing HiFi libraries for specific applications. Example sequencing performance data for a given application are also summarized.

PacBio

Technical reference for targeted enrichment of Kinnex full-length RNA libraries with IDT xGen hybridization and wash kit v3

Technical note – Targeted enrichment of Kinnex full-length RNA libraries with IDT xGen hybridization and wash kit (102-326-632) describes the procedure for targeted enrichment using IDT xGen Hyb and Wash kit v3 for Kinnex full-length RNA libraries



Technical note

TARGETED ENRICHMENT OF KINNEX FULL-LENGTH RNA LIBRARIES WITH IDT xGEN HYBRIDIZATION AND WASH KIT V3

Overview

For RNA sequencing, targeted enrichment is considered a favorable alternative to whole transcriptome sequencing because it can capture more isoform diversity of targeted genes, requiring less sequencing depth and enabling higher sample multiplexing. The PacBio® Iso-Seq® method for full-length RNA sequencing has been used to capture single genes[1], highly similar paralogs or pseudogenes[2], and low-abundance long non-coding RNAs for annotation purposes[3]. Targeted enrichment may be the right choice when the goal of sequencing is to:

- Identify and quantify transcripts from a known set of genes
- Characterize alternative 5' starts and 3' ends
- Detect lowly expressed transcripts
- Maximize sample multiplexing capacity
- Reduce bioinformatics analysis time

This technical note describes the procedure for targeted enrichment using IDT xGen Hyb and Wash kit v3 for Kinnex™ full-length RNA libraries.

Required materials and equipment

- For a comprehensive list of materials and equipment, please refer to the PacBio **Kinnex full-length RNA protocol**[4] and the **IDT xGen protocol**[5].
- For cDNA generation, use the Iso-Seq express 2.0 kit, which is compatible with the IDT targeted enrichment and Kinnex full-length RNA library construction. The input to Iso-Seq express 2.0 is 300 ng of total RNA, ideally with RIN ≥ 7. Lower-quality RNA may result in shorter cDNA, while total RNA lower than 100 ng may result in the incomplete capture of the full diversity of the targeted genes.

Library preparation

Library preparation will follow the **Kinnex full-length RNA protocol** with modifications outlined here.

1. Generate full-length cDNA using the Iso-Seq express 2.0 kit.
2. Enrich for targeted genes using the **IDT xGen Hyb Wash v3 protocol**.
3. Continue through the Kinnex PCR and pooling step (step 4) in the **Kinnex full-length RNA protocol**.
4. The targeted Kinnex library is compatible for sequencing on Sequel® II/IIe, Revio®, and Vega™ systems.

Full-length cDNA — Generate up to 12-plex cDNA using the *Iso-Seq express 2.0*

Target enrichment — Enrich for targeted genes using IDT customer probes and then xGen Hyb Wash v3 kit

Kinnex library — Generate Kinnex full-length RNA library using the *Kinnex full-length RNA kit*

Sequencing — Sequence on Sequel IIe, Vega, or Revio systems

Analyze — Analyze with the Read Segmentation and Iso-Seq workflow in SMRT® Link

Figure 1. Library workflow for generating targeted Kinnex full-length RNA libraries using the IDT xGen Hyb Wash v3 kit.

SMRT® Link informatics and analysis recommendations

Targeted Kinnex full-length RNA data can be analyzed the same way as whole transcriptome Kinnex datasets using the **SMRT Link Read Segmentation and Iso-Seq** workflow or **through the command line**. Note, however, that these workflows do not have specific analyses for targeted gene lists and will output gene and isoform information for all detected transcripts.

IDT INTEGRATED DNA TECHNOLOGIES PacBio

1. Input RNA quality control
2. cDNA synthesis + cleanup
3. cDNA amplification + cleanup
4. Target enrichment with IDT xGen kit
5. Kinnex PCR
6. Kinnex array formation + cleanup
7. Nuclease treatment + cleanup
8. Annealing, binding and cleanup (ABC)

PacBio Procedure & checklist

Preparing Kinnex compatible cDNA using Iso-Seq express 2.0 kit (102-396-000)

PacBio Technical note

Targeted enrichment of Kinnex full-length RNA libraries with IDT xGen hybridization and wash kit v3 (102-326-632)[1]

PacBio Procedure & checklist

Preparing Kinnex libraries using Kinnex 8-fold prep kit (103-920-900)

Or

Preparing Kinnex libraries using Kinnex 12-fold prep kit (103-921-000)

Overview

- For RNA sequencing, **targeted enrichment** may be the right choice when the goal of sequencing is to:
  - Identify and quantify transcripts from a known set of genes
  - Characterize alternative 5' starts and 3' ends
  - Detect lowly expressed transcripts
  - Maximize sample multiplexing capacity
  - Reduce bioinformatics analysis time
- ***Technical note – Targeted enrichment of Kinnex full-length RNA libraries with IDT xGen hybridization and wash kit v3*** (102-326-632) describes the procedure for targeted enrichment using IDT xGen Hyb and Wash kit v3 for Kinnex full-length RNA libraries

[1] For step-by-step IDT hybridization capture workflow details, refer to the **IDT xGen Hybridization and Wash Kit v3 (EAP) for PacBio Targeted Kinnex protocol** (RUO24-3081_001).

Workflow overview for targeted enrichment of Kinnex full-length RNA libraries with IDT xGen hybridization and wash kit v3

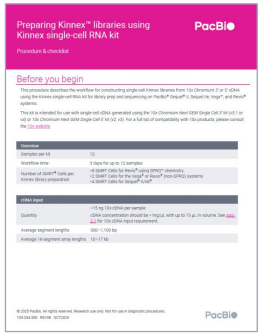
Follow PacBio Kinnex library prep protocol and IDT target enrichment procedure to generate full-length RNA hybrid capture libraries suitable for HiFi sequencing on PacBio long-read systems

1. Input RNA QC
2. cDNA synthesis + cleanup
3. cDNA amplification + cleanup
4. Target enrichment with IDT xGen kit
5. Kinnex PCR
6. Kinnex array formation + cleanup
7. Nuclease treatment + cleanup

PacBio Kinnex library construction procedure[1]

Procedure & checklist – Preparing Kinnex libraries using the Kinnex 8-fold (103-920-900)/12-fold prep kit (103-921-000)

PacBio


Preparing Kinnex™ libraries using Kinnex single-cell RNA kit

PacBio

Procedure & checklist

Before you begin

Kinnex full-length RNA library prep procedure key steps

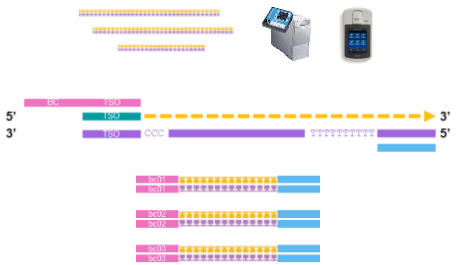
- For cDNA generation, use the Iso-Seq express 2.0 kit with 300 ng of total RNA input, ideally with RIN ≥ 7.[1]
- Library preparation will generally follow the **Kinnex 8-fold/12-fold prep kit protocol** with the following additional steps:
  - Generate full-length cDNA using the Iso-Seq express 2.0 kit by following **Steps 1-3** in the Iso-Seq cDNA synthesis protocol (102-396-000).
  - Enrich for targeted genes using the **IDT xGen Hyb Wash v3 protocol** (RUO24-3081_001; also see **IDT xGen Technical note** (102-326-632)).
  - Continue through the **Kinnex PCR step (Step 2)** in the Kinnex 8-fold (103-920-900) /12-fold prep kit (103-921-000) protocol and complete the remainder of the library construction workflow

Bulk Iso-Seq cDNA amplicon generation workflow

1 Input RNA quality control

2 cDNA synthesis + cleanup

3 cDNA amplification + cleanup



4. Target enrichment with IDT xGen kit

→ See **PacBio Technical note**

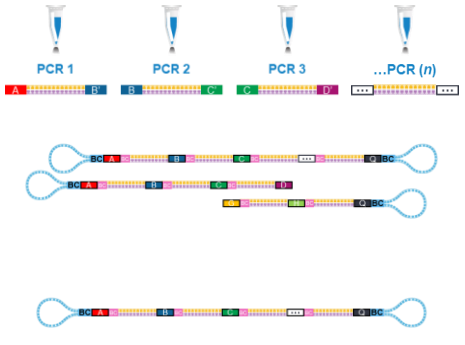
Targeted enrichment of Kinnex full-length RNA libraries with IDT xGen hybridization and wash kit v3 (102-326-632)

Kinnex bulk RNA library construction workflow

5 Kinnex PCR

6 Kinnex array formation + cleanup

7 Nuclease treatment + cleanup



[1] Lower-quality RNA may result in shorter cDNA, while total RNA input amounts lower than 100 ng may result in the incomplete capture of the full diversity of the targeted genes.

Workflow overview for targeted enrichment of Kinnex full-length RNA libraries with IDT xGen hybridization and wash kit v3 (cont.)

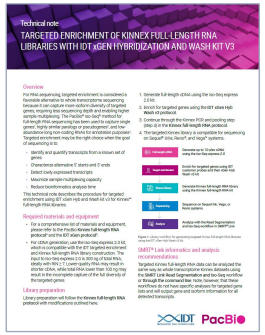
Follow PacBio Kinnex library prep protocol and IDT target enrichment procedure to generate full-length RNA hybrid capture libraries suitable for HiFi sequencing on PacBio long-read systems

1. Input RNA QC
2. cDNA synthesis + cleanup
3. cDNA amplification + cleanup
4. **Target enrichment with IDT xGen kit**
5. Kinnex PCR
6. Kinnex array formation + cleanup
7. Nuclease treatment + cleanup

IDT xGen hybrid capture procedure overview[1]

Technical note – Targeted enrichment of Kinnex full-length RNA libraries with IDT xGen hybridization and wash kit v3 (102-326-632)

PacBio

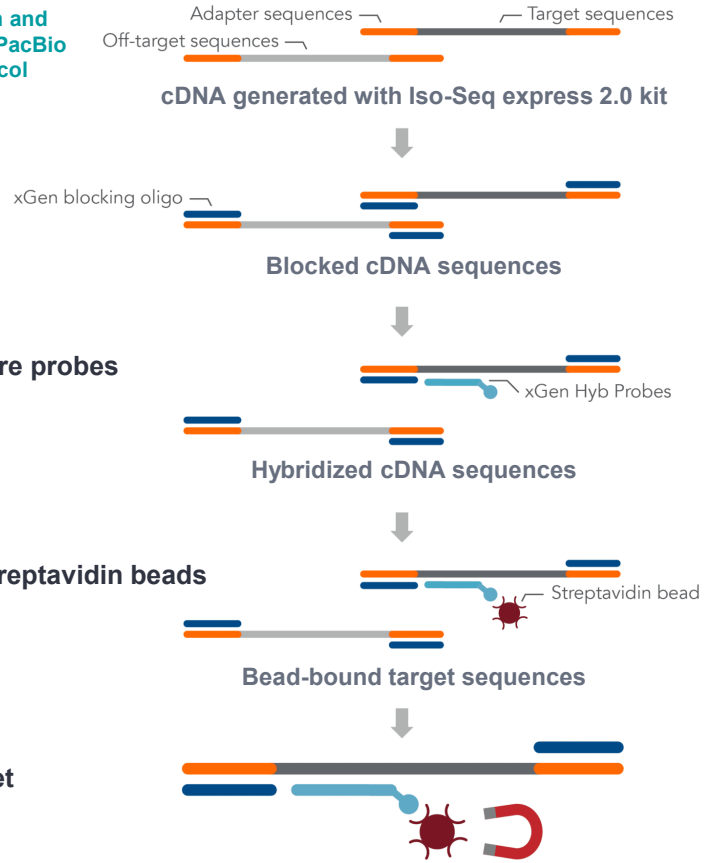

Technical note
TARGETED ENRICHMENT OF KINNEX FULL-LENGTH RNA LIBRARIES WITH IDT xGEN HYBRIDIZATION AND WASH KIT V3

IDT xGEN hybrid capture procedure key steps[1]

- Add Blocker Master Mix to each cDNA sample (500 ng[2]) and dry down
- Add Hybridization Master Mix to each sample well and incubate for desired hybridization time.[3]
- Prepare hybridization wash buffer and capture beads within 2 hours before use
- Add (streptavidin) capture beads to sample (30 min)
- Perform 3 rounds of washing with wash buffers
- Perform post-capture PCR reaction + cleanup
- Perform DNA quantification QC using Qubit assay and DNA sizing QC using Agilent TapeStation or other equivalent system

IDT xGEN hybrid capture workflow

IDT xGen Hybridization and Wash Kit v3 (EAP) for PacBio Targeted Kinnex protocol (RUO24-3081_001).

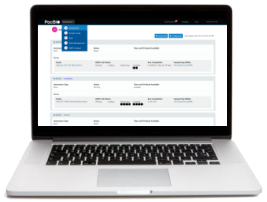


[1] For hybridization capture workflow details, refer to the **IDT xGen Hybridization and Wash Kit v3 (EAP) for PacBio Targeted Kinnex protocol** (RUO24-3081_001).
[2] 100 ng to 6 µg cDNA inputs can be used for short hybridizations (1-2 hrs). Generally, we recommend not to exceed 2.5 µg total input for 1-hr hybridization time for very large panels (>30 Mb).
[3] This procedure supports short hybridization times of only 1 hr as well as the standard 4- or 16-hr incubations. We recommend users empirically test the shorter hybridization time for their workflows.

SMRT Link informatics and secondary analysis recommendations for targeted Kinnex full-length RNA data

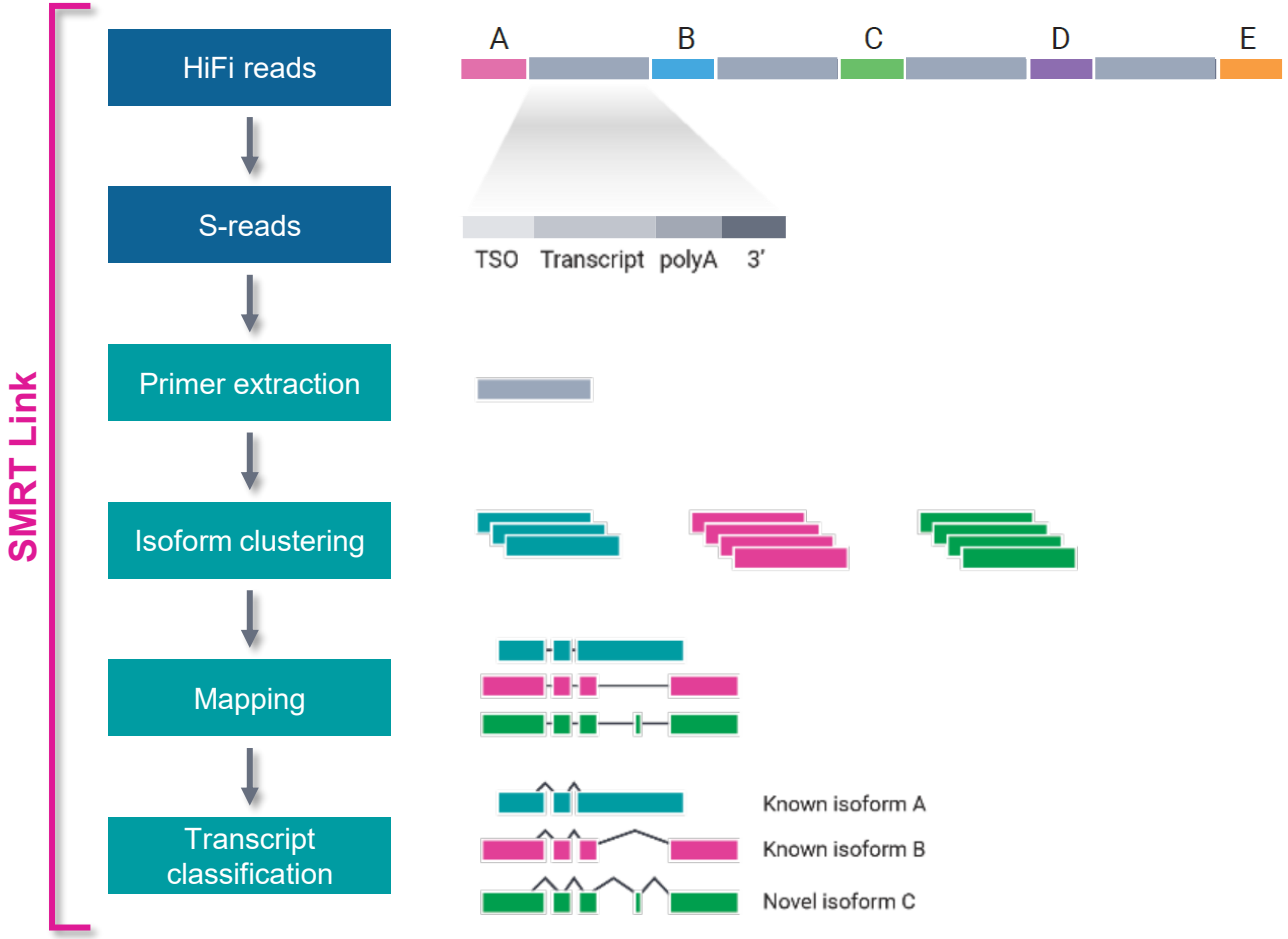
Use SMRT Link Read Segmentation and Iso-Seq application to perform isoform-classification analysis to identify novel genes & isoforms with abundance information

- Targeted Kinnex full-length RNA data can be analyzed the same way as whole transcriptome Kinnex datasets using the SMRT Link Read Segmentation and Iso-Seq workflow or through the command line.
- Note, however, that these workflows do not have specific analyses for targeted gene lists and will output gene and isoform information for all detected transcripts.



SMRT Analysis

SMRT Link read segmentation and Iso-Seq workflow[1]



[1] See *SMRT Link User Guide* (Documentation) for detailed descriptions of parameter settings for Read Segmentation and Iso-Seq analysis application.

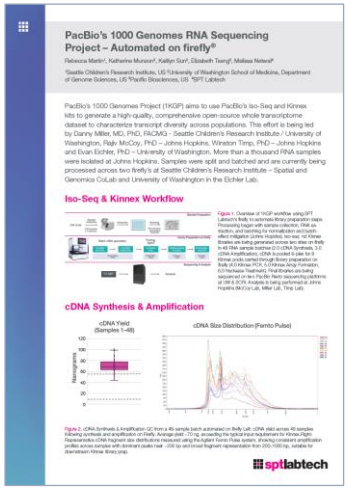
Kinnex library prep automation system options

Automated Kinnex full-length RNA library preparation with the SPT Labtech firefly platform

SPT firefly automated Kinnex workflow improves reproducibility and scalability in large-scale transcriptomics studies by reducing manual steps in library preparation[1]

Automated walk-away workflow and processing with the SPT Labtech firefly system enables:

- Reduced hands-on time – Manual pipetting decreased by up to 80%, allowing researchers to focus on data analysis and experimental design
- Improved consistency – User-to-user and day-to-day variation is minimized
- Scalability – Generation of high-quality, full-length transcript data is enabled at higher throughput to support large-scale genomics initiatives


PacBio's 1000 Genomes RNA Sequencing Project – Automated on firefly®

Iso-Seq & Kinnex Workflow

cDNA Synthesis & Amplification

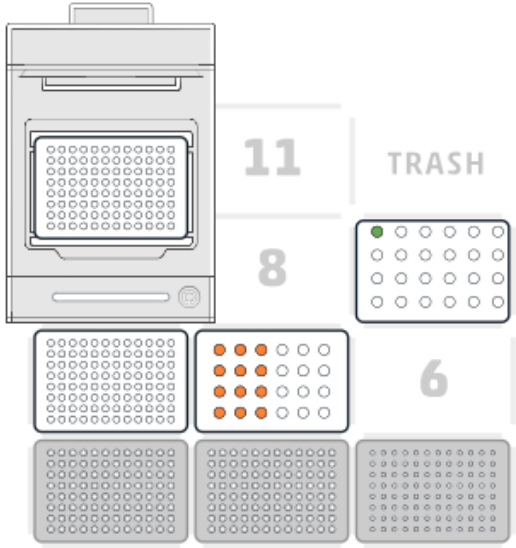
[1] See *PacBio's 1000 Genomes RNA Sequencing Project – Automated on firefly* case study (SPT Labtech).

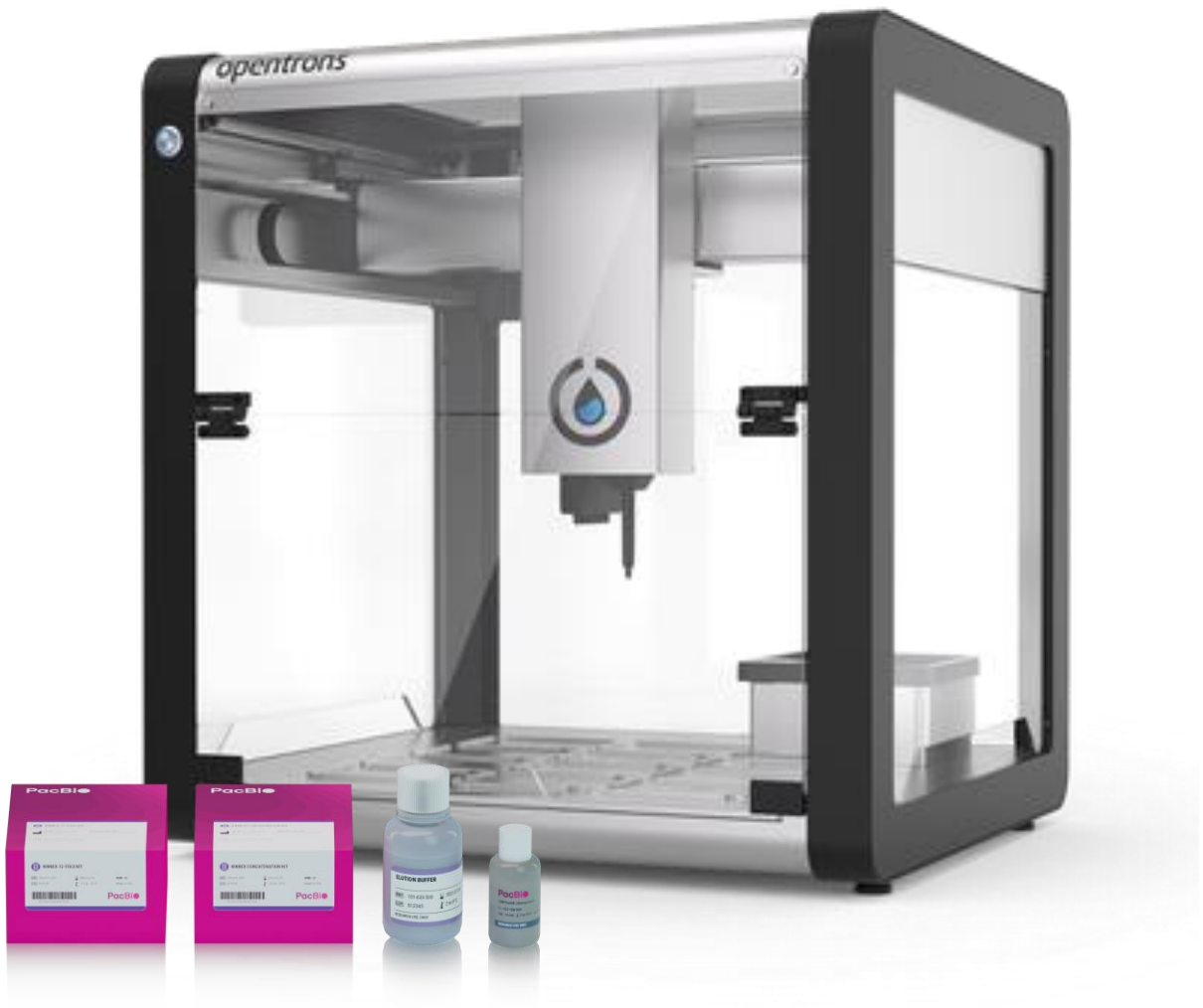
Automated Kinnex library preparation with the Opentrons OT-2 system

Kinnex PCR workflow step for Kinnex 8-fold/12-fold/16-fold prep kits can be automated on the Opentrons OT-2 system[1]

Opentrons OT-2 is a high-precision, open-source liquid handler designed to be easy for scientists to use

- OT-2 has a user-friendly, plug and play graphical interface to design protocols compatible with any ANSI/SBS compliant or automation friendly labware.
- OT-2 base model includes an Opentrons GEN2 Single-Channel Pipette; you can also use up to two pipettes at the same time.





[1] Visit https://library.opentrons.com/p/pacbio_kinnex_pcr to obtain automation protocol for running the Kinnex PCR workflow step on Opentrons OT-2 system.