HiFi Sequencing and Software v10.1 Release:
Technical Overview for Sequel II System & Sequel IIe System Users

Sequel II and IIe Systems ICS v10.1 / SMRT Link v10.1

PN 102-094-100 Version 01 (April 2021)

HiFi Sequencing and Software v10.1 Release: Technical Overview for Sequel II & IIe Systems Users

A. Sequel II and IIe Systems v10.1 Release Overview

Summary Overview of Key Features & Improvements
Sequel II and IIe System Instrument Control Software v10.1 Updates
Sequel II and IIe System Consumables and Sample Preparation Workflow Updates
SMRT Link Sample Setup, Run Design & Run QC Updates
Example HiFi Library Sequencing Performance Data

B. SMRT Link v10.1 Release Overview

Summary Overview of Key Features & Improvements
CCS Analysis Application Features & Reports
SMRT Link v10.1 Cloud
SMRT Link Applications Updates
SMRT Link General Usability Improvements
SMRT Link Fixed & Known Issues

C. Sequel II and IIe Systems Applications Support Resources

HIFI SEQUENCING AND SOFTWARE V10.1 RELEASE: KEY FEATURES & IMPROVEMENTS

- New consumables enable **improved HiFi data quality**
- Updated HiFi sample prep protocol for WGS applications enables **reduced DNA input requirements and higher sample throughput / yr**
- Updated Sequel II and IIe System Instrument Control Software v10.1 enables **on-instrument sequencing workflow improvements**
- Updated SMRT Link v10.1 software features **new analysis applications** and **improves Sample Setup & Run Design ease of use**

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New Consumables

- SMRTbell Enzyme Clean Up Kit 2.0 (101-932-600) *(NEW)*
- Sequencing Primer v5 (102-067-400) *(NEW)*
- Polymerase Binding Kit 2.2 (101-894-200) *(NEW)*

Updated HiFi Sample Prep Protocol for *De Novo* Assembly and Variant Detection

- Enables reduced minimum input gDNA (≥5 µg) for running multiple SMRT Cells
- Supports high-throughput sample processing and automation

Sequel II and IIe System Instrument Control Software v10.1

- Updated on-instrument robotic workflow for improved fluidic handling
- Supports identification of new barcoded overhang adapters
- Supports new Adaptive Loading (AL) feature

SMRT Link v10.1

- Support for new consumables
- Application-specific Sample Setup and Run Design*
- New Adaptive Loading feature in Run Design for WGS applications
- New SARS-CoV-2 analysis application for COVID-19 surveillance
- Usability and user experience improvements

* Feature first introduced with SMRT Link v10.0 limited release

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Sequel II and IIe Systems v10.1 Release Overview

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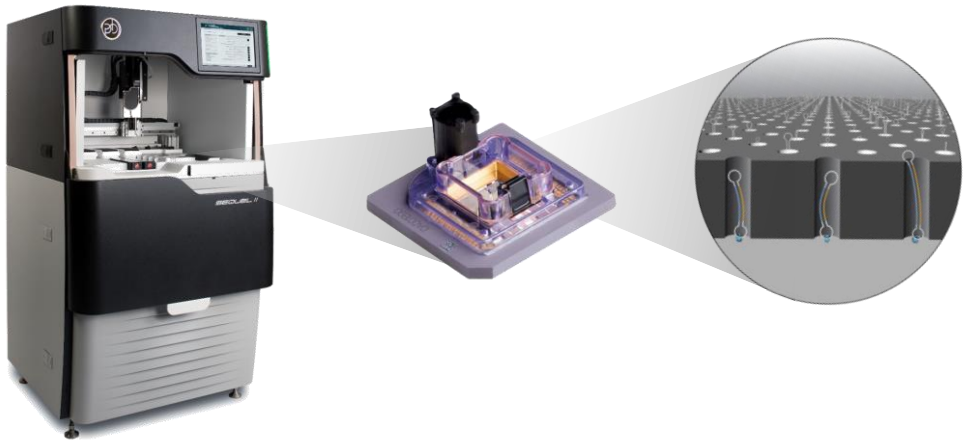
Sequel II and IIe System Instrument Control Software v10.1 Updates

SEQUEL II AND IIE SYSTEM INSTRUMENT CONTROL SOFTWARE V10.1 UPDATES

Updated Sequel II and IIe System ICS v10.1 enables several sequencing workflow improvements

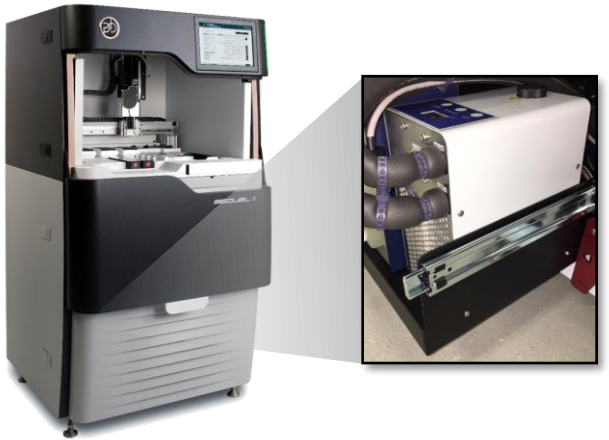
- Updated on-instrument robotic workflow enables improved fluidic handling during reagent/sample aspiration & dispense steps to minimize the impact of drying in low-humidity environments and results in **more uniform loading across the SMRT Cell 8M surface area**
- Enables new **Adaptive Loading (AL)** feature to monitor kinetics of immobilization of polymerase complexes to ZWWs leading to **reduced loading variability and reduced risk of sample overloading conditions**
- Enables support for new barcoded overhang adapters leading to **more streamlined analysis of multiplexed samples**. (**Note:** New redesigned barcoded overhang adapter sequences will become available in a future protocol release.)



SEQUEL II AND IIE SYSTEM INSTRUMENT CONTROL SOFTWARE V10.1 UPDATES (CONT.)

Updated Sequel II and IIe System ICS v10.1 enables several sequencing workflow improvements

- Enables improved environmental systems control leading to **increased instrument reliability**
  - **Note:** Reagent chiller **no longer cools the work deck when the instrument is idle** and no sequencing kit is detected by the NFC reader
  - NFC reader will scan for the presence of unused reagents in the sequencing plate upon instrument power up, upon door closure, and upon end of a sequencing run
  - A sample plate should not be loaded on the work deck without a sequencing plate present since there is no NFC tag on sample plates.



Improved environmental systems control helps extend the service life of the reagent chiller.

Sequel II and IIe System Consumables and Sample Preparation Workflow Updates

NEW SEQUEL II AND IIE SYSTEM REAGENT KIT PRODUCT DESCRIPTIONS

Sequel II Binding Kit 2.2 and DNA Internal Control 1.0 (102-089-000)

- Faster Sequel II Polymerase 2.2 resulting in more subread passes and improved HiFi data quality
- No change to spike-in DNA Internal Control

SMRTbell Enzyme Clean Up Kit 2.0 (101-932-600)

- Improved formulation enables more efficient removal of damaged/incomplete SMRTbell template constructs from final library samples
- For use with HiFi WGS *de novo* assembly and variant detection applications

Sequencing Primer v5 (102-067-400)

- Sequencing Primer v5 is recommended for use with Sequel II Binding Kit 2.2 and enables more processive sequencing of SMRTbell DNA templates leading to improved HiFi data quality

NEW SEQUEL II AND IIE SYSTEM CONSUMABLES PRODUCTS AND REAGENT KIT CONTENTS

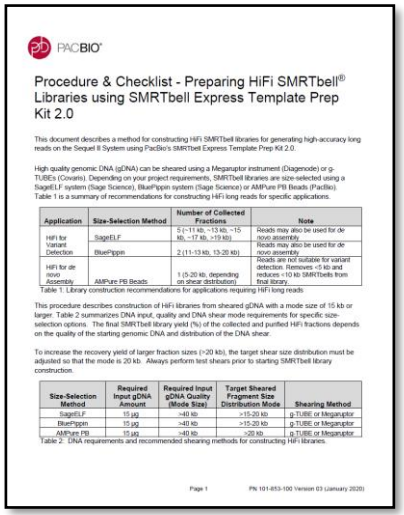
Reagent Kit or Component	Part Number	Quantity	No. of Reactions Supported
SMRTbell Enzyme Clean Up Kit 2.0 (NEW)	**101-932-600**		**18**
SMRTbell Enzyme Clean Up Mix		1 Tube	
SMRTbell Enzyme Clean Up Buffer		1 Tube	
Sequencing Primer v5 (NEW)	**102-067-400**		**10**
Sequencing Primer v5		1 Tube	
Sequel II Binding Kit 2.2 and Internal Control 1.0 (NEW)	**102-089-000**		**24**
Sequel II DNA Polymerase 2.2		1 Tube	
Adaptive loading Buffer		6 Tubes	
Sequel Binding Buffer		2 Tubes	
Sequel dNTP		1 Tube	
Sequel Complex Dilution Buffer		2 Tubes	
Nuclease-Free Water		1 Tube	
DNA Internal Control 1.0		1 Tube	

NEW SEQUEL II AND IIE SYSTEM CONSUMABLES PRODUCTS AND REAGENT KIT CONTENTS

Reagent Kit or Component	Part Number	Quantity	No. of Reactions Supported
HiFi Express Template Prep Kit 2.0 Bundle (UPDATED)	**102-088-900**		**18**
SMRTbell Express Template Prep Kit 2.0		1 Each	
SMRTbell Enzyme Clean Up Kit 2.0 (NEW)		1 Each	
Sequencing Primer v5 (NEW)		1 Tube	
Sequel II HiFi Bundle-18 2.0 (UPDATED)	**102-104-700**		**18**
SMRTbell Express Template Prep Kit 2.0		1 Each	
SMRTbell Enzyme Clean Up Kit 2.0 (NEW)		1 Each	
Sequencing Primer v5 (NEW)		1 Each	
Sequel II Binding Kit 2.2 and Internal Control 1.0 (NEW)		1 Each	
AMPure PB, 5 mLs		1 Tube	

UPDATED HIFI LIBRARY PREPARATION PROTOCOL FOR WGS *DE NOVO* ASSEMBLY & VARIANT DETECTION APPLICATIONS

- Procedure & Checklist – Preparing HiFi SMRTbell Libraries using SMRTbell Express Template Prep Kit 2.0 (PN 101-853-100) protocol document has been updated and describes a method for constructing SMRTbell libraries (~15 kb - 20 kb) that are suitable for generating highly accurate long reads on the Sequel II and IIe Systems for WGS ***de novo*** **assembly** and **variant detection applications**
- Updated workflow supports high-throughput processing using reduced input genomic DNA amounts (5 µg per 3 Gb sample genome size)
- Recommend shearing high-quality gDNA using a Megaruptor 3 System (Diagenode)
- Depending on project requirements, SMRTbell libraries can be size-selected using a PippinHT System (Sage Science), SageELF System (Sage Science), or BluePippin System (Sage Science)



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Procedure & Checklist - Preparing HiFi SMRTbell® Libraries using SMRTbell Express Template Prep Kit 2.0

This document describes a method for constructing HiFi SMRTbell libraries for generating high-accuracy long reads on the Sequel II System using PacBio's SMRTbell Express Template Prep Kit 2.0.

High quality genomic DNA (gDNA) can be sheared using a Megaruptor instrument (Diagenode) or g-TUBEs (Covaris). Depending on your project requirements, SMRTbell libraries are size-selected using a SageELF system (Sage Science), BluePippin system (Sage Science) or AMPure PB Beads (PacBio). Table 1 is a summary of recommendations for constructing HiFi long reads for specific applications.

Application	Size-Selection Method	Number of Collected Fractions	Note
HiFi for Variant Detection	SageELF	5 (~11 kb, ~13 kb, ~15 kb, ~17 kb, >19 kb)	Reads may also be used for de novo assembly.
	BluePippin	2 (11-13 kb, 13-20 kb)	Reads may also be used for de novo assembly.
HiFi for de novo Assembly	AMPure PB Beads	1 (5-20 kb, depending on shear distribution)	Reads are not suitable for variant detection. Removes <5 kb and reduces <10 kb SMRTbells from final library.

Table 1: Library construction recommendations for applications requiring HiFi long reads

This procedure describes construction of HiFi libraries from sheared gDNA with a mode size of 15 kb or larger. Table 2 summarizes DNA input, quality and DNA shear mode requirements for specific size-selection options. The final SMRTbell library yield (%) of the collected and purified HiFi fractions depends on the quality of the starting genomic DNA and distribution of the DNA shear.

To increase the recovery yield of larger fraction sizes (>20 kb), the target shear size distribution must be adjusted so that the mode is 20 kb. Always perform test shears prior to starting SMRTbell library construction.

Size-Selection Method	Required Input gDNA Amount	Required Input gDNA Quality (Mode Size)	Target Sheared Fragment Size Distribution Mode	Shearing Method
SageELF	15 µg	>40 kb	>15-20 kb	g-TUBE or Megaruptor
BluePippin	15 µg	>40 kb	>15-20 kb	g-TUBE or Megaruptor
AMPure PB	15 µg	>40 kb	>20 kb	g-TUBE or Megaruptor

Table 2: DNA requirements and recommended shearing methods for constructing HiFi libraries.

Page 1 PN 101-853-100 Version 03 (January 2020)

https://www.pacb.com/support/documentation/

APPLICATIONS

WHOLE GENOME SEQUENCING

***De Novo* Assembly**

Variant Detection

HIFI LIBRARY PREPARATION & SEQUENCING WORKFLOW IS EFFICIENT AND SCALABLE

Updated HiFi sample preparation workflow provides improved SMRTbell library construction yields and supports high-throughput processing using reduced input genomic DNA amounts

Genomic DNA QC & Shearing

- ≥5 µg of input gDNA for a 3 Gb sample genome size
- Shear up to 8 samples in parallel to ~15 kb - 20 kb target fragment size with the Megaruptor 3 System

SMRTbell Library Construction (5 hrs)

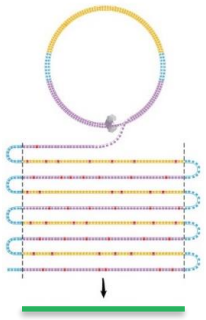
- Process up to 16 samples in parallel manually or up to 96 samples in parallel using automation
- Manual workflow supports construction of ≥32 HiFi libraries per week

SMRTbell Library Size Selection (2 hrs)

- Process up to 20 samples in parallel with the PippinHT System (2 hour run time)
- Higher recovery efficiency with PippinHT provides sufficient SMRTbell library material to run up to ~4 or more SMRT Cells 8M per 5 µg of starting input gDNA

Sequencing Preparation (Sequel II and IIe Systems)

- Anneal Sequencing Primer v5 (1 hr), bind Sequel II Polymerase 2.2 (1 hr) and perform complex cleanup using AMPure PB beads



HiFi Read

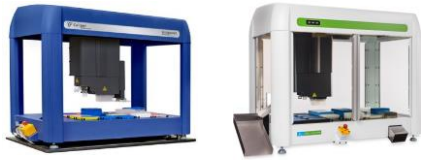
PacBio HiFi reads achieve >99.9% accuracy

HiFi Data Analysis

- For variant detection, can use DeepVariant for small variants <20 bp and SMRT Link Structural Variant Calling for larger variants >20 bp
- For *de novo* assembly, can use SMRT Link Genome Assembly or other third-party software

HIFI LIBRARY CONSTRUCTION WORKFLOW TIMING OVERVIEW

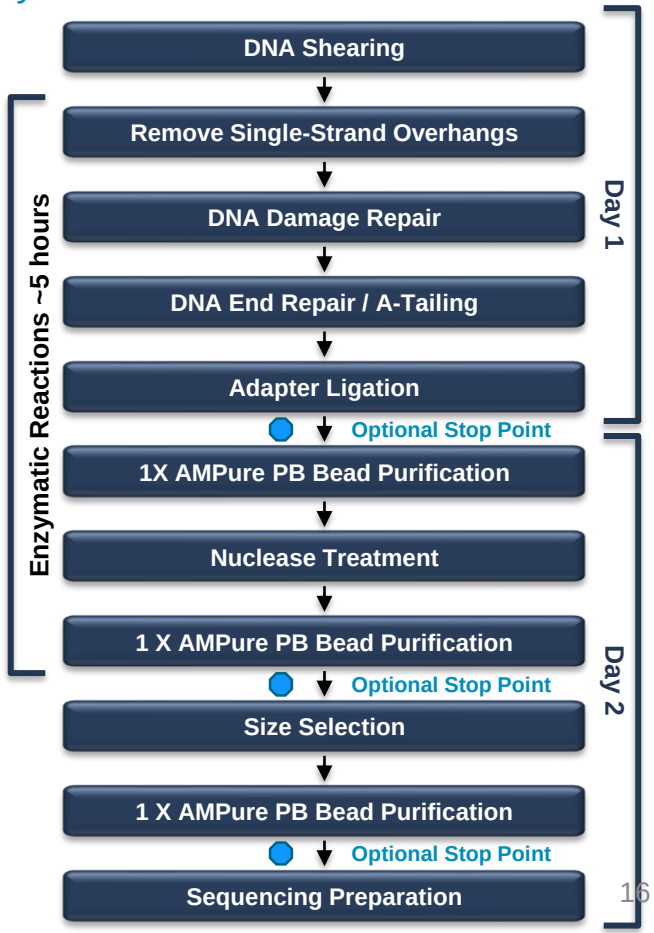
High-throughput library construction and sequencing preparation in 2 days

PROCESS UP TO 16 SAMPLES IN PARALLEL MANUALLY USING 0.2-ML TUBE STRIPS*	PROCESS UP TO 96 SAMPLES IN PARALLEL USING AUTOMATION*
	

* Reagents can be prepared as Master Mixes

STEP	HANDS-ON (MIN)	WALK-AWAY (HRS)
Remove SS to A-Tailing	15	1.4
Adapter Ligation**	5	1.0
AMPure PB Bead Purification	5	0.5
Nuclease Treatment	5	0.5
AMPure PB Bead Purification	5	0.5
Size-selection (PippinHT System)	10	2.0
AMPure PB Bead Purification	5	0.5
Total	**50**	**6.4**

** Adapter Ligation reaction can be performed for 1 hour or left incubating overnight



THE MEGARUPTOR 3 SYSTEM IS RECOMMENDED FOR HIFI SMRTBELL LIBRARY CONSTRUCTION

- Megaruptor 3 System (Diagenode) is generally recommended for shearing*
  - Allows up to 8 samples to be sheared in parallel for high-throughput applications
  - Achieving the same size distribution across multiple samples provides more consistent sequencing performance
- To maximize HiFi yield per SMRT Cell, PacBio recommends fragmenting the gDNA to a size distribution mode between **15 kb – 18 kb** for human whole genome sequencing
- **Note:** Libraries with a size distribution mode larger than 20 kb are ***not*** recommended for HiFi sequencing.
- Recommended library insert size distributions to use for different WGS applications are summarized below and in Table 4 in the procedure.

Application	Recommended Library Insert Size (Mode)
Human Variant Detection	15 kb - 18 kb
Human De Novo Assembly	15 kb - 18 kb
Plant / Animal De Novo Assembly	15 kb - 20 kb

Megaruptor 3 System

*** Note:** The g-TUBE (Covaris) device generates a broader DNA fragment size-distribution compared to the Megaruptor 3 system. As a result, HiFi read quality and overall HiFi data yield may be reduced due to the residual presence of very large DNA fragments generated by g-TUBEs. For additional guidance, see ***Technical Overview: HiFi Library Preparation Using SMRTbell Express TPK 2.0 for De Novo Assembly and Variant Detection*** (PN 101-855-400) or contact PacBio Technical Support or your local Field Applications Scientist.

THE MEGARUPTOR 3 SYSTEM IS RECOMMENDED FOR HIFI SMRTBELL LIBRARY CONSTRUCTION (CONT.)

To use the Megaruptor 3 System, **perform *two* cycles of DNA shearing in the *same* hydropore-syringe device**

- Eliminates very large DNA fragments that may not generate sequencing data that meet HiFi read quality requirements
- Example recommended Megaruptor 3 System software settings to achieve a DNA fragment size distribution mode of ~15 kb - 18 kb (recommended for human whole genome sequencing applications):

gDNA TARGET SHEAR SIZE MODE	MEGARUPTOR 3 SYSTEM SHEARING CYCLE	MEGARUPTOR 3 SYSTEM SPEED SETTING*	RUN TIME PER SHEARING CYCLE
15 – 18 kb	Cycle 1	Speed Setting 31	40 min
	Cycle 2	Speed Setting 32	40 min

- **IMPORTANT:** Genomic DNA must be in **QIAGEN Buffer EB or PacBio Elution Buffer (EB)** or an equivalent low-salt buffer (i.e., 10 mM Tris-Cl, pH 8.5 - 9.0) for shearing

Because the response of individual gDNA samples may differ, **optimization of shearing conditions** is recommended to achieve the desired fragment distribution

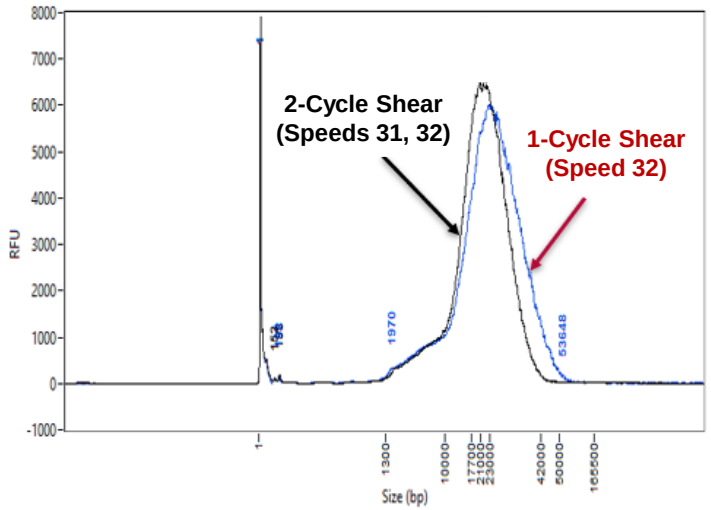
- To minimize sample loss,** the recovered volume (~53 μL) of sheared DNA is used to go **directly** into the first enzymatic reaction in SMRTbell library construction (i.e., no intermediate AMPure PB bead purification step is performed)

*** Note:** The shearing instructions described in this HiFi sample prep procedure are not compatible with the Megaruptor or Megaruptor 2 systems from Diagenode. If using a Megaruptor or Megaruptor 2 system, shearing optimization is necessary before proceeding with this procedure. For additional guidance, see ***Technical Overview: HiFi Library Preparation Using SMRTbell Express TPK 2.0 for De Novo Assembly and Variant Detection*** (PN 101-855-400) or contact PacBio Technical Support or your local Field Applications Scientist.

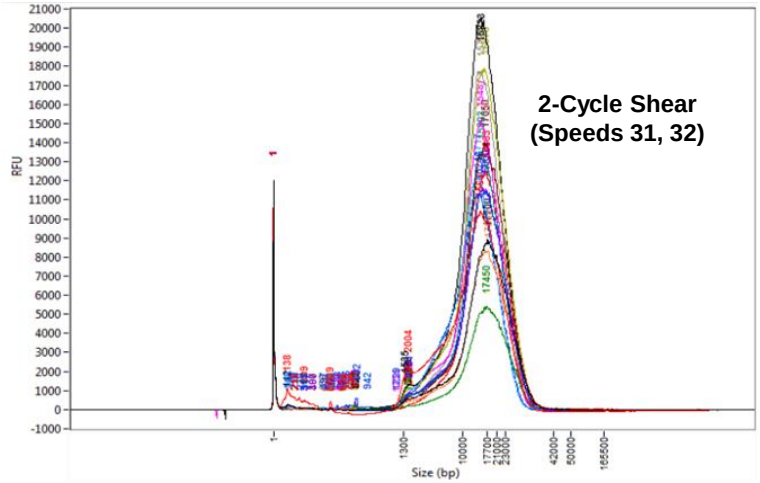
** Losses are mostly due to dead volume in the Megaruptor 3 System [i.e., 5 – 7 μL (<500 ng)]

2-CYCLE SHEARING METHOD USING THE MEGARUPTOR 3 SYSTEM IS RECOMMENDED FOR HIFI SMRTBELL LIBRARY CONSTRUCTION

By performing a 2-cycle shear, the resulting DNA fragment size distribution is tighter and more consistent across multiple samples



Femto Pulse DNA sizing QC analyses of the same human gDNA sample sheared with 1 cycle of shearing versus 2 cycles of shearing using a Megaruptor 3 System. The fragment size distribution is tighter after performing 2 cycles of shearing compared to performing 1 cycle of shearing.

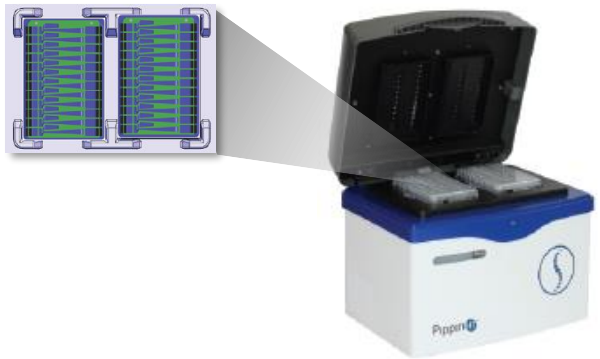


Femto Pulse DNA sizing QC analysis overlay of 15 human gDNA samples sheared with 2 cycles of shearing using a Megaruptor 3 System. The size distribution mode of the samples after 2 cycles of shearing is ~18 kb.

SIZE-SELECTION WITH THE PippinHT SYSTEM IS RECOMMENDED FOR HIFI SMRTBELL LIBRARY CONSTRUCTION

- PippinHT System (Sage Science) is recommended for size selection
- PippinHT System enables faster run times and higher throughput compared to the SageELF and BluePippin Systems
  - Can process up to 20 samples per instrument run
  - 2-hour run time
- SMRTbell templates <10 kb are removed during PippinHT size selection
- PippinHT Cassette Definition File and Run Protocol Setup
  - ***"6-10kb High Pass Marker 75E"***
  - Using the "Range" selection mode, enter a desired "Start" value of 10000 and a "End" value of 50000.
  - Be sure to assign a marker lane
- PippinHT System shows efficient post-size selection recovery yields (approx. ≥35% – 50%), which enables reduction of the input gDNA amount required for HiFi SMRTbell library construction*

PippinHT System



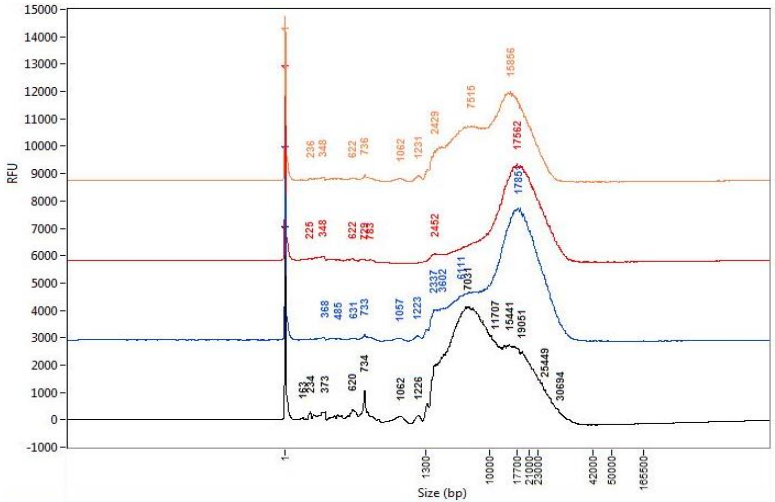
With high-quality samples, higher recovery efficiency with PippinHT size selection can provide sufficient SMRTbell library material to run **up to ~4 or more SMRT Cells 8M per 5 µg of starting input gDNA**

* **Note:** If using BluePippin or SageELF size-selection, library recovery yields may be lower with this HiFi sample prep procedure. For additional guidance, see ***Technical Overview: HiFi Library Preparation Using SMRTbell Express TPK 2.0 for De Novo Assembly and Variant Detection*** (PN 101-855-400) or contact PacBio Technical Support or your local Field Applications Scientist.

SIZE-SELECTION WITH THE PippinHT SYSTEM IS RECOMMENDED FOR HIFI SMRTBELL LIBRARY CONSTRUCTION (CONT.)

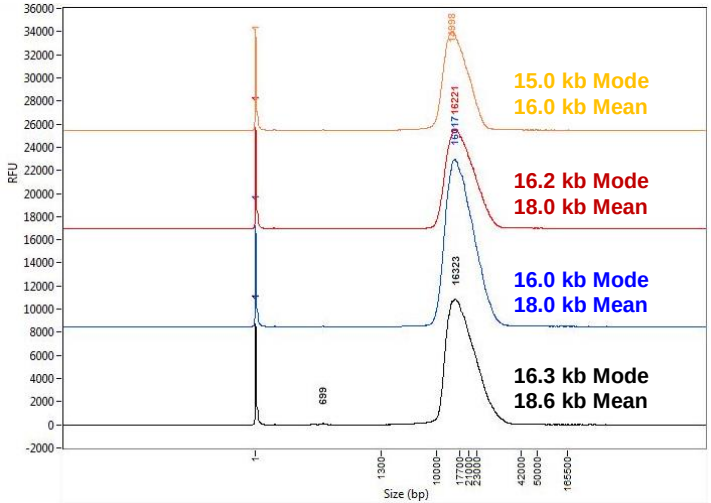
PippinHT size selection is fast (2-hr run time) and efficient (can process up to 20 samples per instrument run)

Before PippinHT Size Selection



Femto Pulse DNA sizing QC analyses of the several human SMRTbell library samples before size selection. The size distribution mode ranges from ~7 kb to ~18 kb for the different samples.

After PippinHT Size Selection



Femto Pulse DNA sizing QC analyses of the several human SMRTbell library samples after PippinHT size selection using a 10-kb lower cutoff setting. The size distribution mode of the size-selected samples is similar (~18 kb).

HIFI SAMPLE PREPARATION WORKFLOW IS AMENABLE TO AUTOMATION

HiFi SMRTbell library construction can be automated using the Sciclone Liquid Handling Workstation (Perkin-Elmer)

- Enables greater reproducibility compared with manual-only methods
- Can process up to 96 samples at a time

Input Requirements:

- Recommend using >6 μg of input sheared gDNA due to liquid dead volumes

Automated Workflow Steps:

- Enzymatic reactions
- AMPure PB bead purifications

Output:

- SMRTbell libraries ready for downstream size-selection

https://www.perkinelmer.com/category/sciclone-g3-liquid-handling-workstations

COMPARISON OF HIFI SAMPLE PREPARATION WORKFLOW CHANGES FOR *DE NOVO* ASSEMBLY AND VARIANT DETECTION APPLICATIONS

SAMPLE PREP WORKFLOW STEP	*OLD* HIFI PROCEDURE & CHECKLIST (VERSION 3, JAN. 2020)	*NEW* HIFI PROCEDURE & CHECKLIST (VERSION 4, APR. 2021)
LIBRARY CONSTRUCTION		
Input gDNA Amount	~15 µg	≥5 µg
DNA Shearing	Megaruptor 1/2/3 System; or g-TUBEs	Megaruptor 3 System (2-cycle shearing)
Post Shearing AMPure PB Bead Purification	Yes	No
Post-ligation Heat Kill	Yes	No
Buffer Exchange prior to Nuclease cleanup (AMPure PB beads)	No	Yes
SMRTbell Enzyme Clean up Kit Version (Reaction Time)	1.0 (60 min)	2.0 (30 min)
Size-Selection Options*	SageELF System; or BluePippin System; or AMPure PB Bead Size Selection	PippinHT System; or SageELF System; or BluePippin System

** AMPure PB bead size selection is under development for the new HiFi sample preparation Procedure & Checklist (PN 101-853-100, Version 4) and specific guidance will be provided in a future protocol update.*

COMPARISON OF HIFI SAMPLE PREPARATION WORKFLOW CHANGES FOR *DE NOVO* ASSEMBLY AND VARIANT DETECTION APPLICATIONS (CONT.)

SAMPLE PREP WORKFLOW STEP	***OLD*** HIFI PROCEDURE & CHECKLIST (VERSION 3, JAN. 2020)	***NEW*** HIFI PROCEDURE & CHECKLIST (VERSION 4, APR. 2021)
SMRT LINK SAMPLE SETUP		
Primer Annealing	Sequencing Primer v2	Sequencing Primer v5
Polymerase binding	Sequel II Polymerase 2.0 Binding Time = 4 h	Sequel II Polymerase 2.2 Binding Time = 1 h
Complex Cleanup	Dilute Bound Complex Volume by 3.33-fold and purify sample using 1.2X AMPure PB beads	If Bound Complex Volume is <100 µL, bring up to 100 µL with Complex Dilution Buffer and purify sample using 1.2X AMPure PB beads.
SMRT LINK RUN DESIGN SETUP		
Pre-Extension Time	2 h (<20 kb) or 4 h (≥20 kb)	0 h (**No** Pre-extension)
Adaptive Loading (AL)	OFF	ON

ENABLING PRODUCTION-SCALE THROUGHPUTS FOR HUMAN WHOLE GENOME SEQUENCING FOR RARE AND INHERITED DISEASE RESEARCH



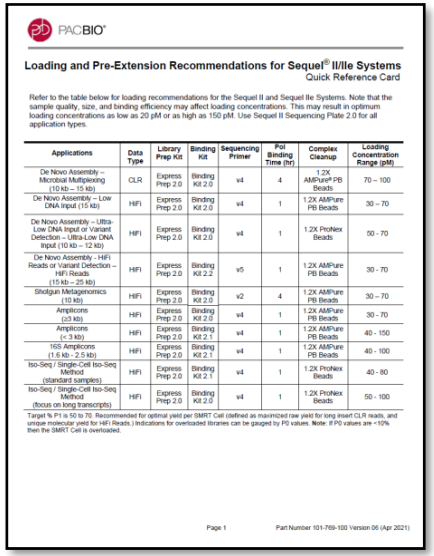
		STRUCTURAL VARIANTS (SVs)	VARIANT DETECTION (SNVs, INDELs, SVs)	VARIANT DETECTION (SNVs, INDELs, SVs)
Number of SMRT Cells 8M/sample		**1**	**2**	**3**
Sequencing time per sample (hrs)		30	60	90
Coverage per human genome sample		~10-fold	~20-fold	~30-fold
Variant Detection Performance (% Accuracy, F1)	SNV	99.0%	99.8%	99.9%
	Indel	92.6%	96.9%	97.9%
	SV	92.6%	95.7%	95.8%
Annual Sample Throughput	1 Sequel IIe System	256	128	84
	6 Sequel IIe Systems	1,536	768	504
	12 Sequel IIe Systems	3,072	1,536	1,008

*This efficient HiFi sample preparation workflow, developed in collaboration with **Children's Mercy Kansas City**, provides a scalable solution for sequencing 100s to 1000s of whole human genomes per year on the Sequel II and IIe Systems.*

SMRT Link Sample Setup, Run Design & Run QC Updates

SMRT LINK V10.1 SAMPLE SETUP & RUN DESIGN RECOMMENDATIONS FOR SPECIFIC APPLICATIONS

Generally follow SMRT Link Sample Setup & Run Design instructions using the recommendations provided in the **Quick Reference Card – Loading and Pre-Extension Time Recommendations for the Sequel II and IIe Systems** unless specified otherwise in the relevant Procedure & Checklist



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Loading and Pre-Extension Recommendations for Sequel® II/IIe Systems
Quick Reference Card

Refer to the table below for loading recommendations for the Sequel II and Sequel IIe Systems. Note that the sample quality, size, and binding efficiency may affect loading concentrations. This may result in optimum loading concentrations as low as 20 pM or as high as 150 pM. Use Sequel II Sequencing Plate 2.0 for all application types.

Applications	Data Type	Library Prep Kit	Binding Kit	Sequencing Primer	Pol Binding Time (hr)	Complex Cleanup	Loading Concentration Range (pM)
De Novo Assembly – Microbial Multiplexing (10 kb – 15 kb)	CLR	Express Prep 2.0	Binding Kit 2.0	v4	4	1.2X AMPureP PB Beads	70 – 100
De Novo Assembly – Low DNA Input (15 kb)	HiFi	Express Prep 2.0	Binding Kit 2.0	v4	1	1.2X AMPure PB Beads	30 – 70
De Novo Assembly – Ultra-Low DNA Input or Variant Detection – Ultra-Low DNA Input (10 kb – 12 kb)	HiFi	Express Prep 2.0	Binding Kit 2.0	v4	1	1.2X ProNex Beads	50 - 70
De Novo Assembly - HiFi Reads or Variant Detection – HiFi Reads (15 kb – 25 kb)	HiFi	Express Prep 2.0	Binding Kit 2.2	v5	1	1.2X AMPure PB Beads	30 - 70
Shotgun Metagenomics (10 kb)	HiFi	Express Prep 2.0	Binding Kit 2.0	v2	4	1.2X AMPure PB Beads	30 – 70
Amplicons (≥3 kb)	HiFi	Express Prep 2.0	Binding Kit 2.0	v4	1	1.2X AMPure PB Beads	30 - 70
Amplicons (< 3 kb)	HiFi	Express Prep 2.0	Binding Kit 2.1	v4	1	1.2X AMPure PB Beads	40 - 150
16S Amplicons (1.6 kb - 2.5 kb)	HiFi	Express Prep 2.0	Binding Kit 2.1	v4	1	1.2X AMPure PB Beads	40 - 100
Iso-Seq / Single-Cell Iso-Seq Method (standard samples)	HiFi	Express Prep 2.0	Binding Kit 2.1	v4	1	1.2X ProNex Beads	40 - 80
Iso-Seq / Single-Cell Iso-Seq Method (focus on long transcripts)	HiFi	Express Prep 2.0	Binding Kit 2.0	v4	1	1.2X ProNex Beads	50 - 100

Target % P1 is 50 to 70. Recommended for optimal yield per SMRT Cell (defined as maximized raw yield for long insert CLR reads, and unique molecular yield for HiFi Reads.) Indications for overloaded libraries can be gauged by P0 values. Note: If P0 values are <10% then the SMRT Cell is overloaded.

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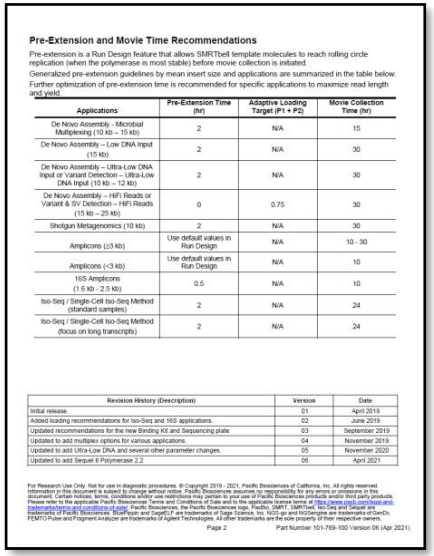


Pre-Extension and Movie Time Recommendations

Pre-extension is a Run Design feature that allows SMRTbell template molecules to reach rolling circle replication (when the polymerase is most stable) before movie collection is initiated.

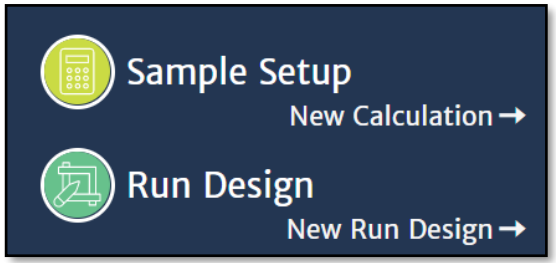
Generalized pre-extension guidelines by mean insert size and applications are summarized in the table below. Further optimization of pre-extension time is recommended for specific applications to maximize read length and yield.

Applications	Pre-Extension Time (hr)	Adaptive Loading Target (P1 + P2)	Movie Collection Time (hr)
De Novo Assembly - Microbial Multiplexing (10 kb – 15 kb)	2	N/A	15
De Novo Assembly – Low DNA Input (15 kb)	2	N/A	30
De Novo Assembly – Ultra-Low DNA Input or Variant Detection – Ultra-Low DNA Input (10 kb – 12 kb)	2	N/A	30
De Novo Assembly – HiFi Reads or Variant & SV Detection – HiFi Reads (15 kb – 25 kb)	0	0.75	30
Shotgun Metagenomics (10 kb)	2	N/A	30
Amplicons (≥3 kb)	Use default values in Run Design	N/A	10 - 30
Amplicons (<3 kb)	Use default values in Run Design	N/A	10
16S Amplicons (1.6 kb - 2.5 kb)	0.5	N/A	10
Iso-Seq / Single-Cell Iso-Seq Method (standard samples)	2	N/A	24
Iso-Seq / Single-Cell Iso-Seq Method (focus on long transcripts)	2	N/A	24

Revision History (Description)	Version	Date
Initial release	01	April 2019
Added loading recommendations for Iso-Seq and 16S applications.	02	June 2019
Updated recommendations for the new Binding Kit and Sequencing plate.	03	September 2019
Updated to add multiplex options for various applications.	04	November 2019
Updated to add Ultra-Low DNA and several other parameter changes.	05	November 2020
Updated to add Sequel II Polymerase 2.2	06	April 2021

Page 2 Part Number 101-769-100 Version 06 (Apr 2021)

In SMRT Link v10.1, most Sample Setup and Run Design parameter fields are auto-filled with the recommended settings for each application type.



https://www.pacb.com/support/documentation/

UPDATED SAMPLE SETUP WORKFLOW: SPECIFYING APPLICATION TYPE

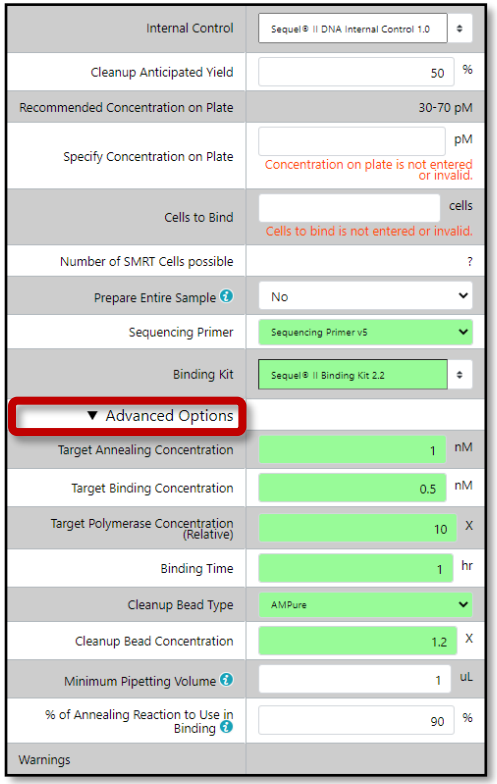
Sample Setup auto-populates application-specific information for selected fields

- The user starts by first entering the **Sample Name** and then selecting an **Application Type**
- Once an application is selected, default values are auto-populated for various fields and highlighted in **green**

UPDATED SAMPLE SETUP WORKFLOW: SPECIFYING APPLICATION TYPE (CONT.)

Auto-populated Sample Setup fields are highlighted in green color

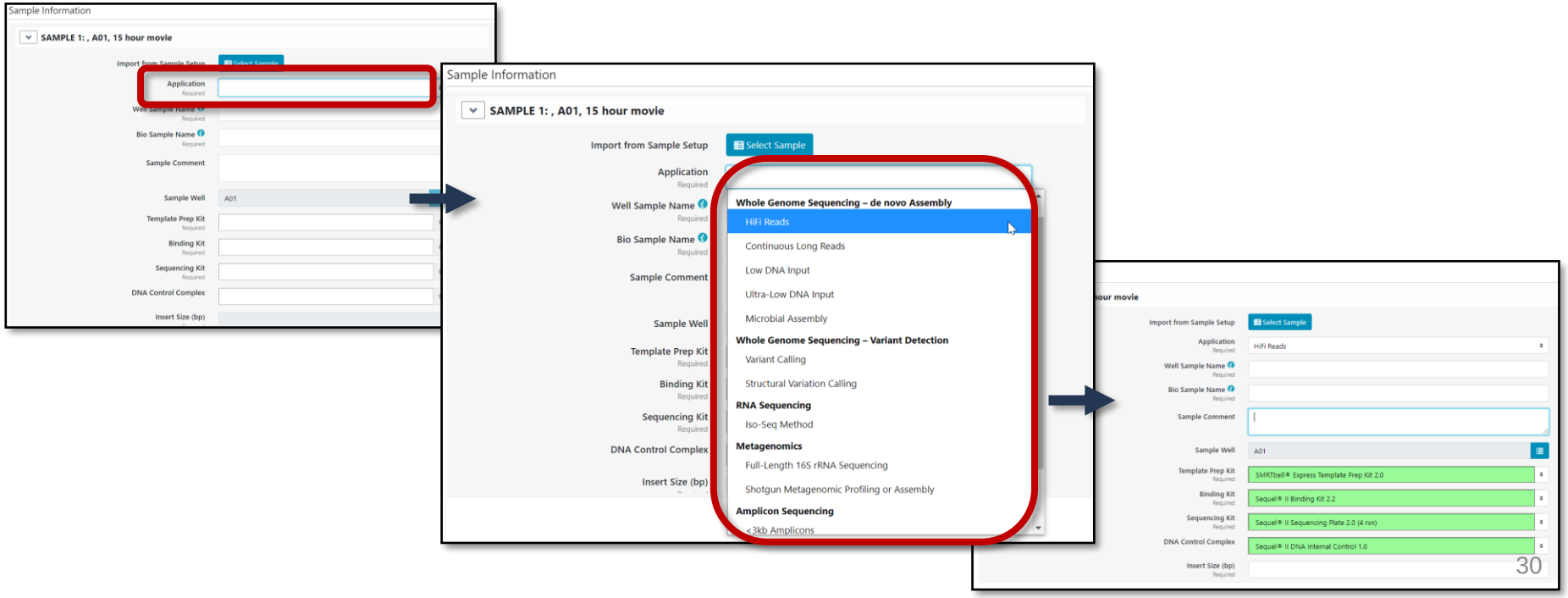
- The following fields are auto-populated and highlighted in **green**:
  - Sequencing Primer
  - Binding Kit
- Note: The following auto-populated fields are located in **Advanced Options**:
  - Target Annealing Concentration
  - Target Binding Concentration
  - Target Polymerase Concentration (Relative)
  - Binding Time
  - Cleanup Bead Type
  - Cleanup Bead Concentration
- If any auto-populated entry is manually changed to a different value, then the field will be highlighted in **yellow** color



UPDATED RUN DESIGN WORKFLOW: SPECIFYING APPLICATION TYPE

Run Design auto-populates application-specific information for selected fields

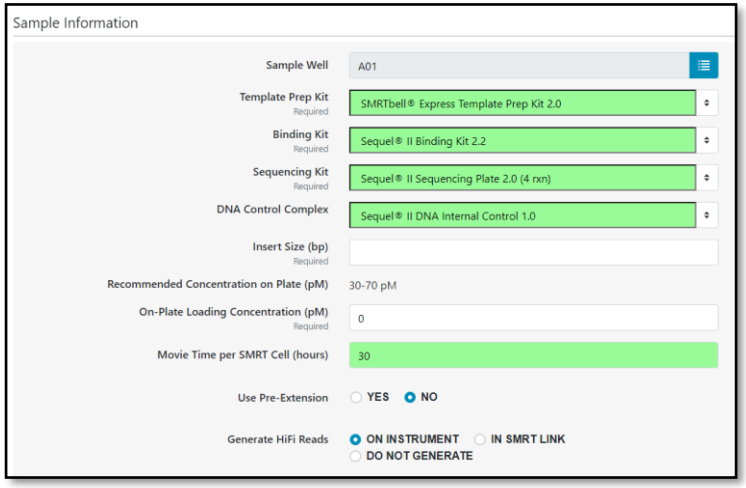
- In not importing sample information from Sample Setup, the user can start by first selecting an **Application Type**
- Once an application is selected, default values are auto-populated for various fields and highlighted in **green**



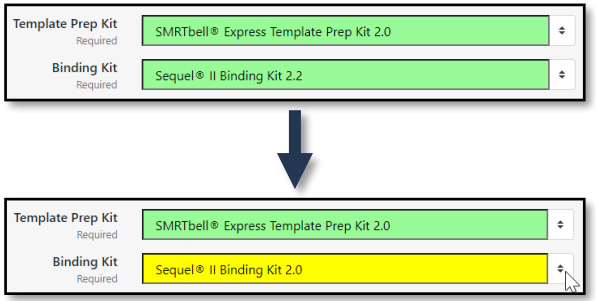
UPDATED RUN DESIGN WORKFLOW: SPECIFYING APPLICATION TYPE (CONT.)

Auto-populated fields are highlighted in green color

- The following fields are auto-populated and highlighted in **green**:
  - Template Prep Kit
  - Binding Kit
  - Sequencing Kit
  - DNA Control Complex
  - Movie Time Per SMRT Cell
  - Pre-Extension Time (If applicable)



- If any auto-populated entry is manually changed to a different value, then the field will be highlighted in **yellow** color



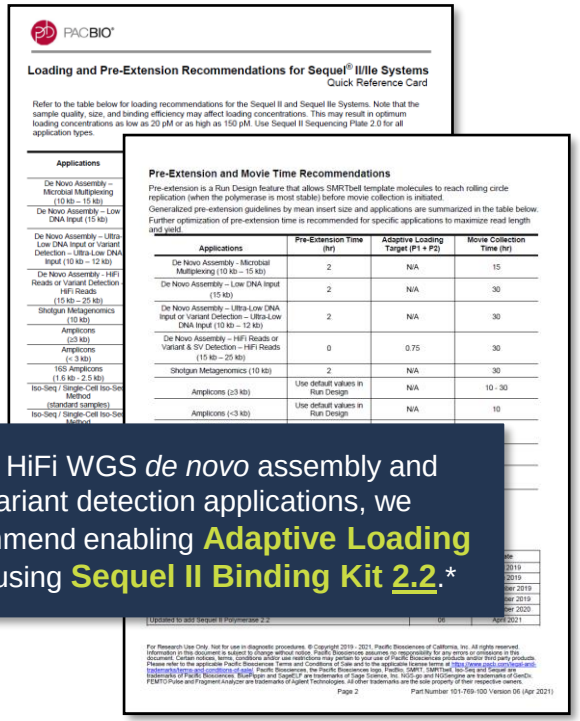
SAMPLE SETUP RECOMMENDATIONS FOR HIFI *DE NOVO* ASSEMBLY AND VARIANT DETECTION APPLICATIONS (SEQUEL II AND IIe SYSTEM V10.1 RELEASE)

- Follow **SMRT Link Sample Setup** instructions using the recommendations provided in the Quick Reference Card – Loading and Pre-Extension Time Recommendations for the Sequel II/IIe Systems for sequencing HiFi library samples for *De Novo* Assembly or Variant Detection applications

  → For **SMRT Link v10.1** (or higher): Select **'WGS – De Novo Assembly / HiFi Reads' or 'WGS – Variant Detection / Variant Calling'** from the **Application** field drop-down menu in the SMRT Link Sample Setup and SMRT Link Run Design user interface

Applications	Data Type	Library Prep Kit	Binding Kit	Sequencing Primer	Pol Binding Time (hr)	Complex Cleanup	Loading Concentration Range (pM)
De Novo Assembly - HiFi Reads or Variant Detection – HiFi Reads (15 – 25 kb)	HiFi	Express Prep 2.0	Binding Kit 2.2	v5	1	1.2X AMPure PB Beads	30 - 70

Applications	Pre-Extension Time (hr)	Adaptive Loading Target (P1 + P2)	Movie Collection Time (hr)
De Novo Assembly – HiFi Reads or Variant & SV Detection – HiFi Reads (15 kb – 25 kb)	0	0.75	30



For HiFi WGS *de novo* assembly and variant detection applications, we recommend enabling **Adaptive Loading** and using **Sequel II Binding Kit 2.2**.*

https://www.pacb.com/support/documentation/

** Recommendations for using Sequel II Binding Kit 2.2 with other application use cases aside from WGS de novo assembly and variant detection will be provided in future protocol documentation releases.*

NEW ADAPTIVE LOADING FEATURE FOR SEQUEL II AND IIe SYSTEMS

Adaptive Loading reduces sample overloading, allowing users to load higher with confidence

- Adaptive loading technology actively monitors polymerase complex binding to the bottom of ZMWs during the sample immobilization step.
- Detection of these active polymerase complexes allows the system to terminate the immobilization step when the desired loading target has been achieved.

  → This approach can help reduce sample overloading and run-to-run yield variability

Adaptive Loading (AL) uses active monitoring of polymerase binding to the bottom of the ZMW during loading to reduce variability and the risk of overloading with high-concentration samples

OVERVIEW OF SMRT LINK V10.1 SAMPLE SETUP AND RUN DESIGN WORKFLOW TO ENABLE ADAPTIVE LOADING

Adaptive Loading is **automatically** enabled by default in SMRT Link v10.1 Sample Setup and Run Design for sequencing applications using **Sequel II Binding Kit 2.2**

Sample Setup
New Calculation →

1

1. Binding Kit Version Selection

- Specify **Sequel II Binding Kit 2.2***

2. Complex Cleanup Step

- Use **Adaptive Loading (AL) Buffer** in place of Complex Dilution Buffer (CDB) to elute purified polymerase-bound sample from AMPure PB beads

3. Final Loading Dilution Step

- Use **AL Buffer** in place of CDB to dilute the purified polymerase-bounds sample to the final on-plate loading concentration

Run Design
New Run Design →

2

Advanced Options

- Specify **Use Adaptive Loading = YES**
- Specify **Loading Target (P1 + P2)**
- Specify **Maximum Loading Time (hours)**

3

Load Sample Plate

- **Start** sequencing run

** In SMRT Link v10.1 Sample Setup, the Adaptive Loading sample setup procedure is **only** enabled by selecting **Sequel II Binding Kit 2.2**. Selection of other Sequel II Binding Kit versions will not enable the Adaptive Loading sample setup procedure.*

SMRT LINK V10.1 SAMPLE SETUP WORKFLOW TO ENABLE ADAPTIVE LOADING

Sample Setup
New Calculation →

Binding Kit Version Selection

- Specify **Sequel II Binding Kit 2.2***

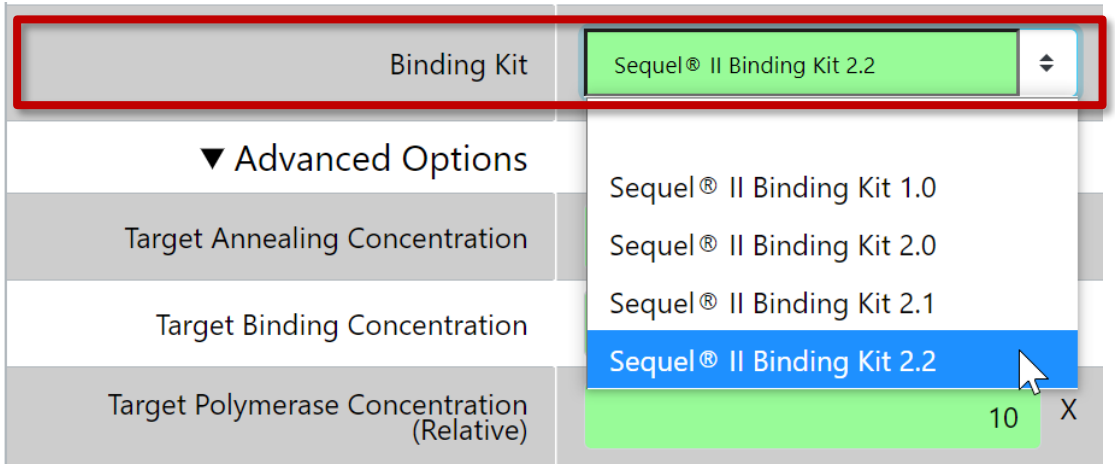
Complex Cleanup Step

- Use **Adaptive Loading (AL) Buffer** in place of Complex Dilution Buffer (CDB) to elute purified polymerase-bound sample from AMPure PB beads

Final Loading Dilution Step

- Use **AL Buffer** in place of CDB to dilute the purified polymerase-bounds sample to the final on-plate loading concentration

1. Binding Kit Version Selection



** In SMRT Link v10.1 Sample Setup, the Adaptive Loading sample setup procedure is **only** enabled by selecting **Sequel II Binding Kit 2.2**. Selection of other Sequel II Binding Kit versions will not enable the Adaptive Loading sample setup procedure.*

SMRT LINK V10.1 SAMPLE SETUP WORKFLOW TO ENABLE ADAPTIVE LOADING (CONT.)

Binding Kit Version Selection

- Specify **Sequel II Binding Kit 2.2**

Complex Cleanup Step

- Use **Adaptive Loading (AL) Buffer** in place of Complex Dilution Buffer (CDB) to elute purified polymerase-bound sample from AMPure PB beads

Final Loading Dilution Step

- Use **AL Buffer** in place of CDB to dilute the purified polymerase-bounds sample to the final on-plate loading concentration

2. Complex Cleanup Step

4. Add AMPure PB beads and gently pipette-mix. Pipette-mixing with wide orifice pipette tips is recommended.

	HiFi WGS De Novo Sample	✓	Notes
Volume of AMPure PB beads (uL)	120.0 uL		

5. To bind the polymerase-bound complexes to AMPure PB beads, incubate the mixture on the benchtop for 5 minutes. Note: Longer incubation times have not been tested and may have a negative impact on polymerase-template complex stability due to high salt concentration.
6. Place the tube in a magnetic bead rack until the beads collect to the side of the tube and the solution appears clear. Discard the supernatant. DO NOT wash the collected bead pellet with ethanol.
7. Immediately resuspend the beads in room temperature Adaptive Loading Buffer and pipette-mix. Pipette-mixing with wide orifice pipette tips is recommended.

	HiFi WGS De Novo Sample	✓	Notes
Volume of Adaptive Loading Buffer (uL)	50.0 uL		

SMRT LINK V10.1 SAMPLE SETUP WORKFLOW TO ENABLE ADAPTIVE LOADING (CONT.)

Binding Kit Version Selection

- Specify **Sequel II Binding Kit 2.2**

Complex Cleanup Step

- Use **Adaptive Loading (AL) Buffer** in place of Complex Dilution Buffer (CDB) to elute purified polymerase-bound sample from AMPure PB beads

Final Loading Dilution Step

- Use **AL Buffer** in place of CDB to dilute the purified polymerase-bounds sample to the final on-plate loading concentration

3. Final Loading Dilution Step*

Final Loading Dilution

Reagent	HiFi WGS De Novo Sample	✓
Adaptive Loading Buffer	61.9 uL	
Prepared sample	49.3 uL	
Diluted Internal Control (Dilution 2)	3.8 uL	
DTT	0.0 uL	
Sequel Additive	0.0 uL	
Total Volume	115.0 uL	

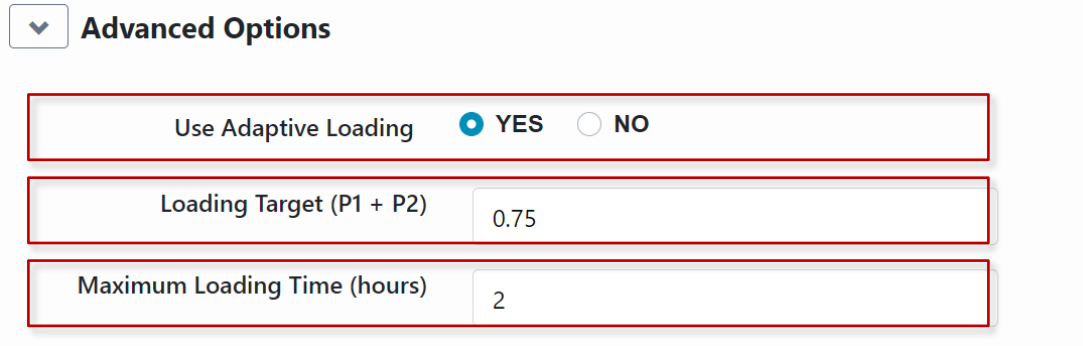
** In SMRT Link v10.1 Sample Setup, **no** DTT or Sequel Additive is added during the Final Loading Dilution step for sample complexes prepared with Sequel II Binding Kit 2.2.*

SMRT LINK V10.1 RUN DESIGN WORKFLOW TO ENABLE ADAPTIVE LOADING

Advanced Options

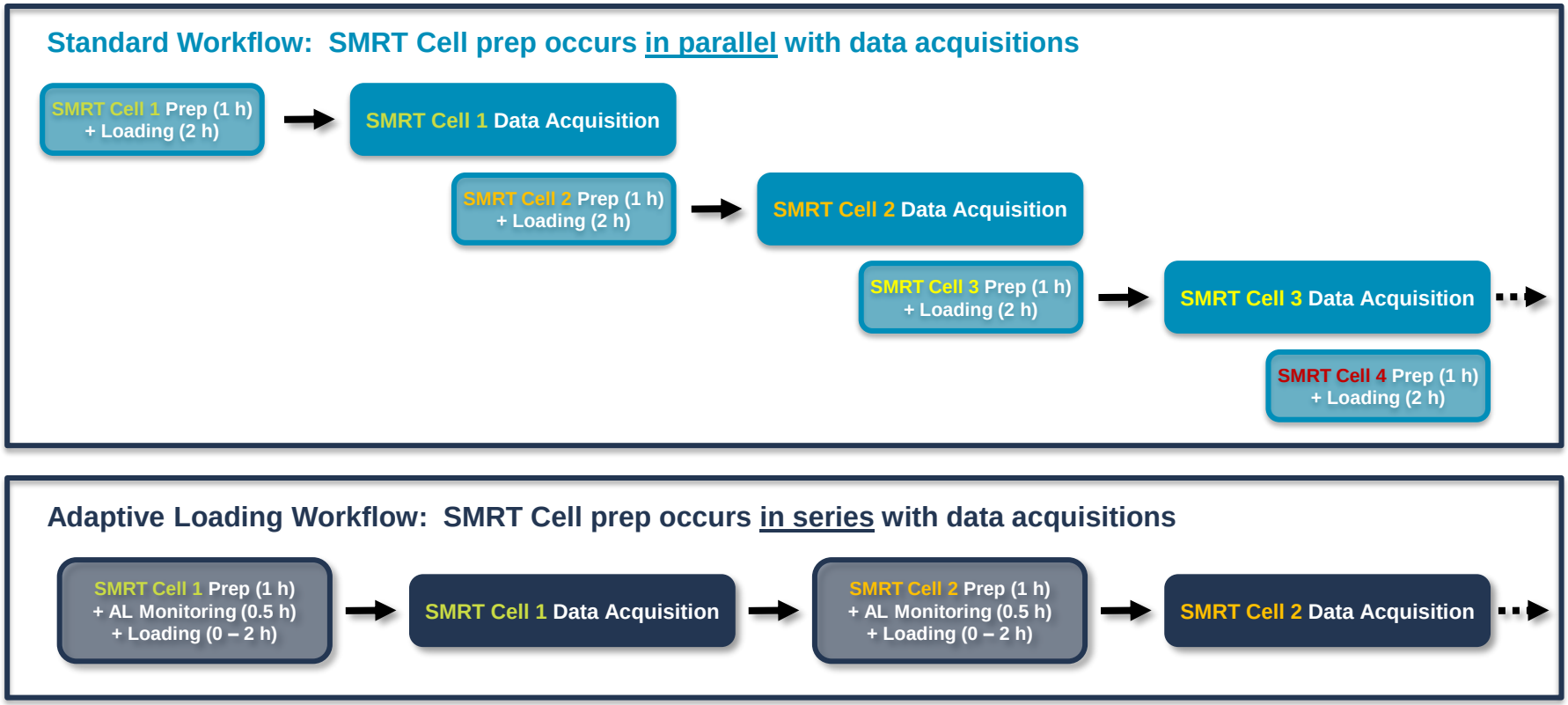
- Specify **Use Adaptive Loading = YES**
- Specify **Loading Target (P1 + P2)**
- Specify **Maximum Loading Time (hours)**

Run Design Advanced Options



For HiFi WGS *de novo* assembly and variant detection applications using Sequel II Binding Kit 2.2, we highly recommend using the Adaptive Loading **default values of 0.75 for the Loading Target and 2 hours for Maximum Loading Time.**

ADAPTIVE LOADING REQUIRES SERIALIZATION OF THE SEQUEL II/IIe SYSTEM INSTRUMENT ROBOTIC WORKFLOW

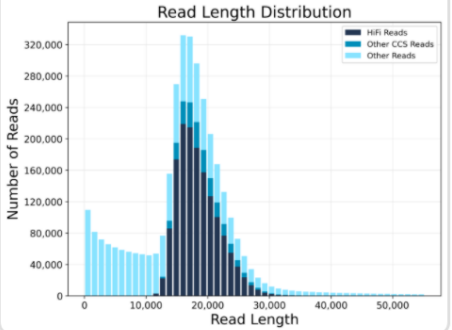


- With the Adaptive Loading feature enabled, instrument run times will be **longer** compared to non-AL runs depending on the actual duration of the AL monitoring + immobilization (loading) time period (up to ~2.5 hours) per SMRT Cell 8M.

NEW RUN QC VISUALIZATION PLOTS IN SMRT LINK V10.1

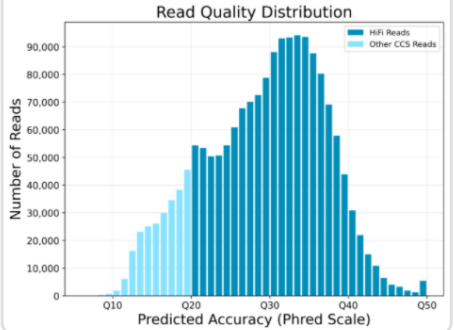
The following plots below are generated for any run where CCS processing is enabled on-instrument (Sequel IIe System) or through CCS Pre-Analysis (Sequel or Sequel II Systems)

HiFi Read Length Distribution



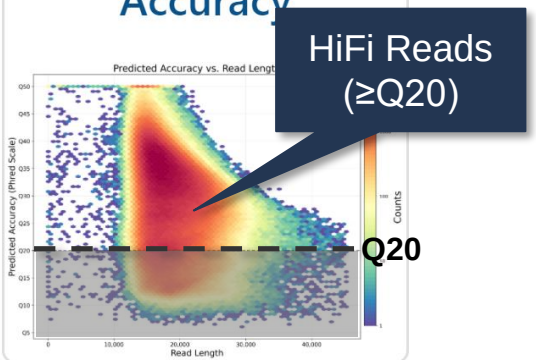
Displays a histogram distribution of HiFi Reads (QV ≥20), other CCS Reads (three or more passes, but QV <20), and other reads, by read length.

Read Quality Distribution



Displays a histogram distribution of HiFi Reads (QV ≥20) and other CCS Reads by read quality.

Read Length vs Predicted Accuracy

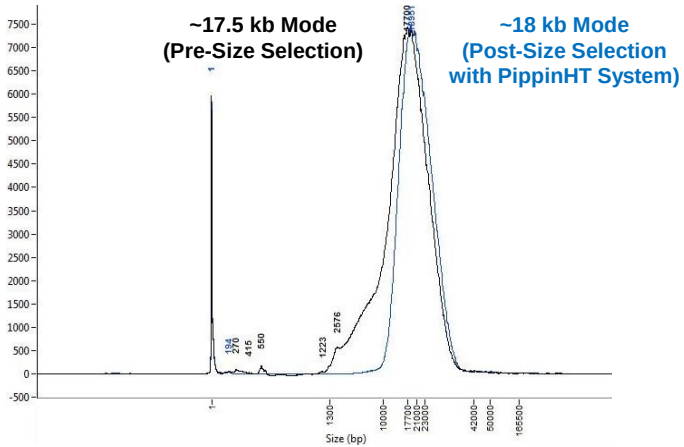


Displays a heat map of CCS Read lengths and predicted accuracies. The boundary between HiFi Reads and other CCS Reads is shown as a dashed line at QV 20.

Example HiFi Library Sequencing Performance Data

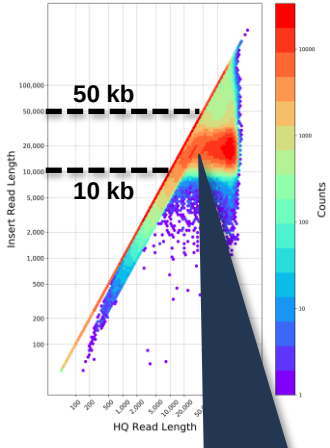
EXAMPLE SEQUENCING PERFORMANCE OF A 18-KB HUMAN HIFI LIBRARY FOR WGS VARIANT DETECTION APPLICATIONS

Size-Selected HiFi Library QC



Input gDNA for Megaruptor 3 Shearing	5 µg
Post-Library Construction Recovery (%) [Pre-Nuclease Treatment]	3540 ng (71%)
Post-Library Construction Recovery (%) [Post-Nuclease Treatment]	1368 ng (27%)
Post-PippinHT Size Selection Recovery (%)	640 ng (13%)

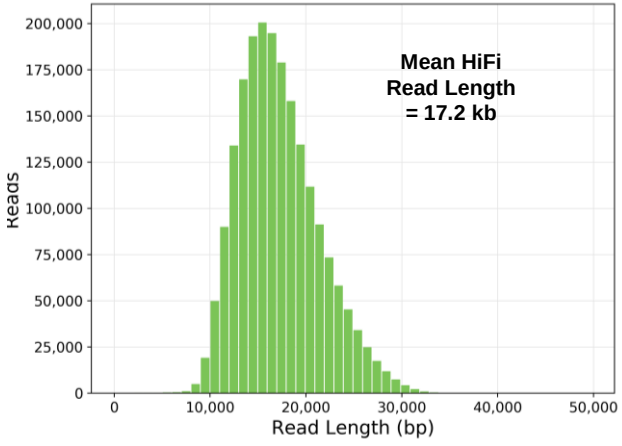
Insert Read Length Density Plot



IRLD plot shows most HiFi read lengths are ~10 – 30 kb*

* 40 pM on-plate loading concentration generated P1 = 70% using a 30-hour movie collection time (Sequel IIe System)

HiFi Read Length Distribution

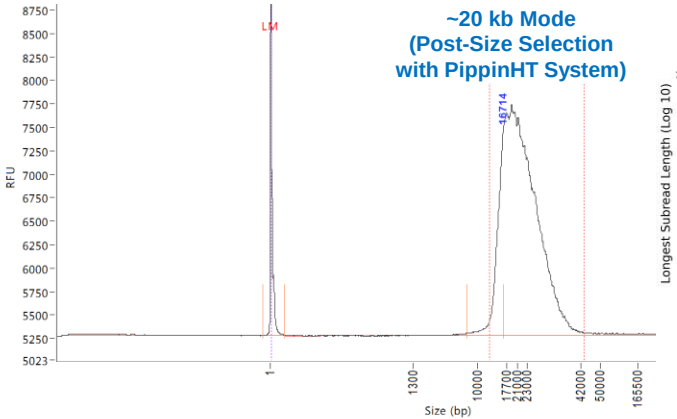


HiFi Reads	2.0 M
HiFi Base Yield**	34.7 Gb
Mean HiFi Read Length	17,212 bp
Median HiFi Read Quality	Q30

** For this human library data set, typical HiFi base yields were ~25 Gb – 35 Gb per SMRT Cell 8M.

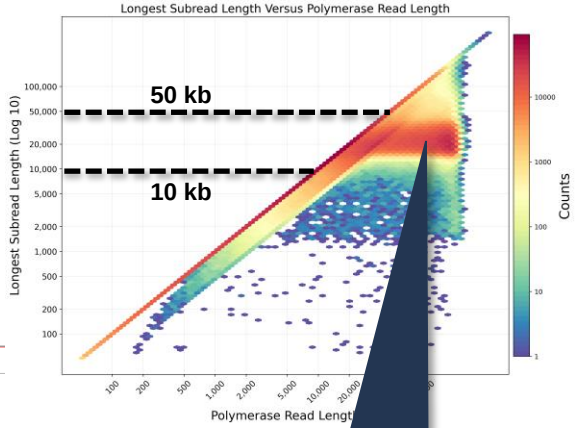
EXAMPLE SEQUENCING PERFORMANCE OF A 20-KB PLANT HIFI LIBRARY FOR WGS *DE NOVO* ASSEMBLY APPLICATIONS

Size-Selected HiFi Library QC



Input gDNA for Megaruptor 3 Shearing	5 µg
Post-Library Construction Recovery (%) [Post-Nuclease Treatment]	1900 ng (38%)
Post-PippinHT Size Selection Recovery (%)	850 ng (17%)

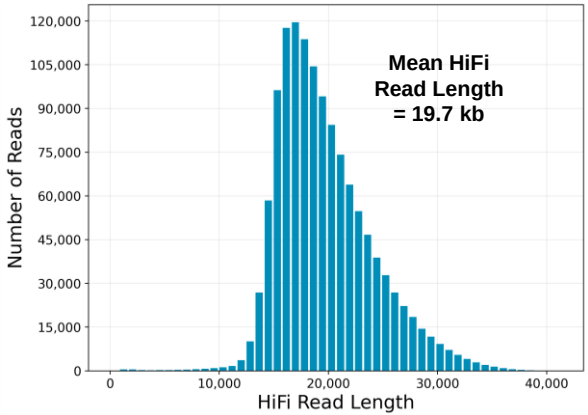
Insert Read Length Density Plot



IRLD plot shows most HiFi read lengths are ~10 – 30 kb*

* 60 pM on-plate loading concentration generated P1 = 85% [Adaptive Loading Target (P1 + P2) = 0.85] using a 30-hour movie collection time (Sequel IIe System)

HiFi Read Length Distribution



HiFi Reads	1.3 M
HiFi Base Yield	25.1 Gb
Mean HiFi Read Length	19,696 bp
Median HiFi Read Quality	Q28

SEQUEL II DNA INTERNAL CONTROL PERFORMANCE UPDATE

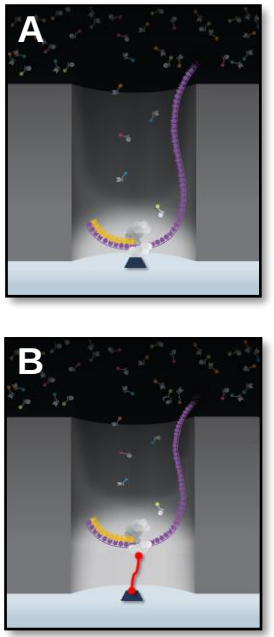
Sequel II DNA Internal Control Complex 1.0 is included with Sequel II Binding Kit 2.0 / 2.1 / 2.2

- Sequel II Binding Kits 2.0, 2.1 and 2.2 include Sequel II DNA Internal Control Complex pre-bound with **Sequel II Polymerase 1.0**
- Sequel II Polymerase 2.2 is bound (tethered) slightly higher above the surface of the ZMW compared to Sequel II Polymerases 2.0 and 2.1
- A higher laser power setting is required to illuminate the ZMW when sequencing samples bound to Sequel II Polymerase 2.2

→ **Sequel II DNA Internal Control Complex 1.0 shows reduced mean read length performance when used with samples bound to Sequel II Polymerase 2.2** compared to samples bound with Sequel II Polymerase 2.0 or 2.1

Polymerase Version Bound to Sample	Estimated DNA Internal Control Complex 1.0 Mean Polymerase Read Length (30-h Movie)
Sequel II Polymerase 2.2	~30 kb
Sequel II Polymerase 2.0 **Sequel II Polymerase 2.1**	~50 kb

The higher laser power setting required for sequencing samples bound to Sequel II Polymerase 2.2 results in increased photodamage to the Sequel II DNA internal Control Complex 1.0 and hence shorter control polymerase read lengths



Comparison of Sequel II Polymerase 2.0-DNA Template complex (**A**) vs. Sequel II Polymerase 2.2-DNA Template complex (**B**) immobilized to a ZMW.

PACIFIC BIOSCIENCES®

SMRT Link v10.1 Overview

Subhead should be no longer than 1 line

SMRT LINK V10.1 SOFTWARE RELEASE: KEY FEATURES & IMPROVEMENTS

- Supports complete **SMRT Link analysis workflow on Amazon Cloud** (SMRT Link Cloud)
- Features several **new and improved analysis applications** for HiFi de novo assembly, SV calling, multiplexed Iso-Seq analysis and SARS-CoV-2 full-viral genome sequencing
- Reduces HPC requirements to enable **lower-cost data analysis & storage configurations**
- Provides a **simplified user experience** for run setup and includes **usability improvements** to support high-throughput sequencing

SMRT LINK V10.1 SOFTWARE RELEASE: KEY FEATURES & IMPROVEMENTS

- Supports complete **SMRT Link analysis workflow on Amazon Cloud** (SMRT Link Cloud)
- Features several **new and improved analysis applications** for HiFi de novo assembly, SV calling, multiplexed Iso-Seq analysis and SARS-CoV-2 full-viral genome sequencing
- Reduces HPC requirements to enable **lower-cost data analysis & storage configurations**
- Provides a **simplified user experience** for run setup and includes **usability improvements** to support high-throughput sequencing

SMRT Link Cloud

Supports Complete SMRT Link Workflow on Amazon Cloud*

- Flexible data analysis optimized for speed or cost based on user preferences
- No need for internal HPC

SMRT Analysis

BIOCONDA

Features New and Improved Analysis Applications

- New Genome Assembly application for HiFi data*
- New SARS-CoV-2 application for COVID-19 surveillance
- Updated Iso-Seq application for improved multiplexed sample analysis
- Updated SV Calling application for improved precision
- Enhanced alignment concordance in mapping applications
- Bioconda: New HiFi Amplicon Analysis application*
- Bioconda: New Single-cell Iso-Seq application*

Welcome to SMRT® Link

Provides Simplified User Experience & Usability Improvements

- New application-centric Sample Setup and Run Design*
- New HiFi sequencing metrics and data visualizations in Run QC*
- Reduced HPC requirements to enable lower-cost configurations*
- New Sample Setup import feature to support for high-throughput production environments

* Feature first introduced with SMRT Link v10.0 limited release

SEQUEL IIe SYSTEM ENABLES REDUCED COMPUTE REQUIREMENTS FOR SMRT LINK INSTALLATIONS

Head Node	
Cores	**32**
RAM	**64 GB**
Local Storage	**1 TB SSD/Flash storage**
db_datadir (Local Storage)	**250 GB**
Compute Nodes	
Cores (Total)	**64** ← Previously 384 (Sequel II System)
Minimum RAM per slot (1 slot = 1 core)	**>4 GB**
Local Storage	**100 GB**
Shared Data Storage	
Sequencing Data	**20 TB** [a] ← Previously >100 TB (Sequel II System)
Analysis Data	**40 TB** [a]
Network	
10 GbE strongly recommended, 1GbE required [b]	

[a] Storage is calculated for one Sequel IIe System, assuming 100 human genomes per year at 30-fold HiFi coverage, *de novo* assembly

[b] Connection between the Head Node and Sequel IIe System

Reduced HPC requirements for the Sequel IIe System enable **lower-cost compute configurations** compared to the Sequel II System

- Approx. 5-fold lower HPC costs: $20K (Sequel IIe System) *vs*. $100K (Sequel II System)
- Note: For a single Sequel IIe System deployment, a **Single System Compute** configuration is available – contact PacBio Technical Support or your local Bioinformatics FAS for details. (Supports ONE Sequel IIe system ONLY. Not suggested for sites with multiple instruments.)

CCS Analysis Application Features & Reports

CCS ANALYSIS ALGORITHM AND DATA OUTPUTS

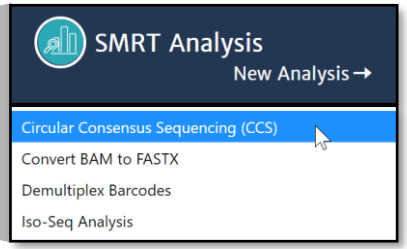
CCS analysis and output are unified for Sequel IIe System and SMRT Link

- CCS algorithm and output files (metrics, reports) are the ***same*** for Sequel IIe System and SMRT Link

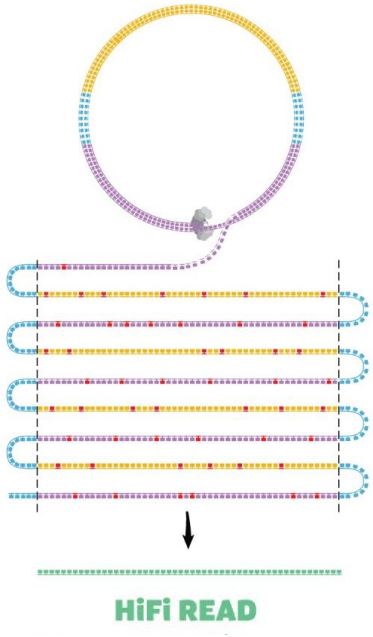
Sequel IIe System

On-Instrument CCS

SMRT Link



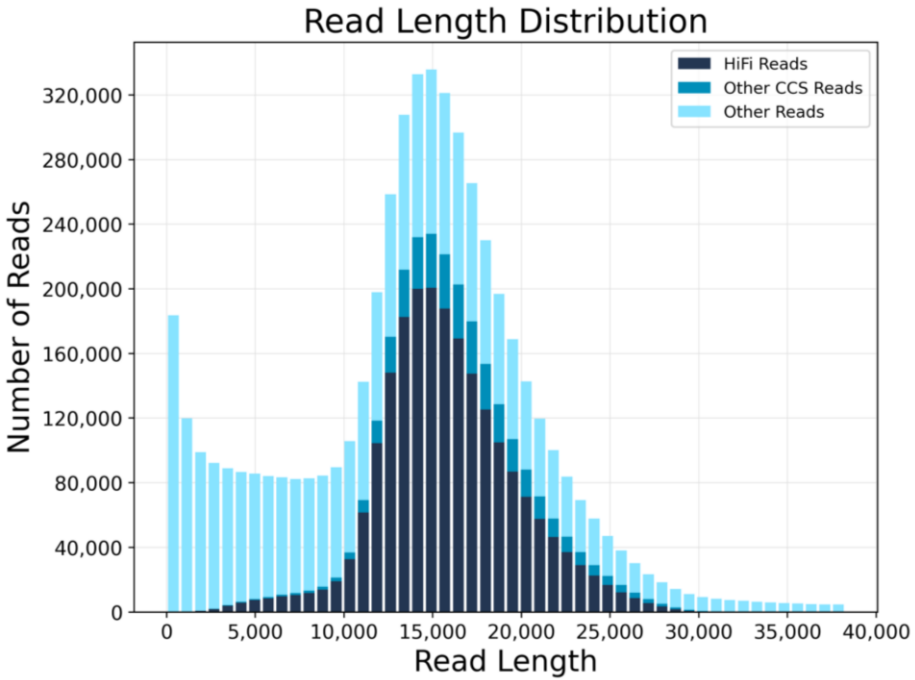
CCS Analysis Application



HiFi reads achieve ≥99.9% accuracy

CCS ANALYSIS `reads.bam` DATA FILE FORMAT

- Sequel IIe System on-instrument CCS (OICCS)* and SMRT Link CCS Analysis application outputs a **`reads.bam`** file containing **<u>one</u> read per productive ZMW**.
- This format is **far more compact** than subread data.
- There are three classes of reads in this `reads.bam` file:
  - **HiFi Reads:** CCS reads with QV ≥20
  - **Other CCS Reads:** CCS reads with lower quality (<Q20)
  - **Other Reads:** Single-pass reads or reads not meeting minimum CCS requirements
- For OICCS, when the `reads.bam` file is imported into SMRT Link, a filtered file containing **only** HiFi reads is automatically generated

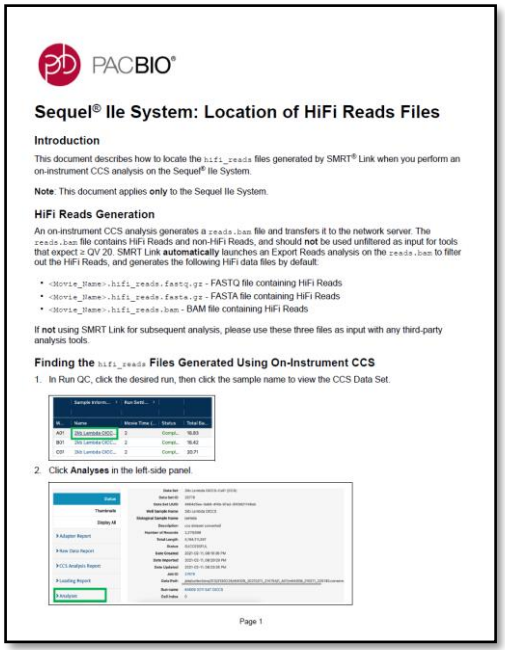


* ***Note:*** *Users can optionally specify in SMRT Link Run Design to include polymerase kinetics information (for secondary epigenetics analysis) in the `reads.bam` file produced through either on-instrument CCS or SMRT Link – however, BAM file size is 5X larger if kinetics information is included.*

HIFI DATA-SPECIFIC FILES ARE AUTOMATICALLY GENERATED IN SMRT LINK WHEN CCS ANALYSIS IS PERFORMED

HiFi Data File Generation With On-instrument CCS Analysis (Sequel IIe System ICS v10.1+)

- An on-instrument CCS analysis generates a `reads.bam` file and transfers it to the network server.
- The `reads.bam` file contains HiFi Reads *and* non-HiFi Reads, and should not be used unfiltered as input for third-party tools that expect ≥QV20 sequencing data .
- SMRT Link automatically launches an **Export Reads** analysis on the `reads.bam` to filter out the HiFi Reads, and generates the following three HiFi data files by default*:
  - **`<Movie_Name>.hifi_reads.fastq.gz`** → FASTQ file with HiFi reads
  - **`<Movie_Name>.hifi_reads.fasta.gz`** → FASTA file with HiFi reads
  - **`hifi_reads.bam`** → BAM file with HiFi reads
- Refer to ***Sequel IIe System: Location of HiFi Reads Files*** (PN 102-110-200) to locate the `hifi_reads` files generated by SMRT Link when you perform an on-instrument CCS analysis on the Sequel IIe System.



PACBIO®

Sequel® IIe System: Location of HiFi Reads Files

Introduction

This document describes how to locate the `hifi_reads` files generated by SMRT® Link when you perform an on-instrument CCS analysis on the Sequel® IIe System.

Note: This document applies **only** to the Sequel IIe System.

HiFi Reads Generation

An on-instrument CCS analysis generates a `reads.bam` file and transfers it to the network server. The `reads.bam` file contains HiFi Reads and non-HiFi Reads, and should **not** be used unfiltered as input for tools that expect ≥ QV 20. SMRT Link **automatically** launches an Export Reads analysis on the `reads.bam` to filter out the HiFi Reads, and generates the following HiFi data files by default:

- `<Movie_Name>.hifi_reads.fastq.gz` - FASTQ file containing HiFi Reads
- `<Movie_Name>.hifi_reads.fasta.gz` - FASTA file containing HiFi Reads
- `<Movie_Name>.hifi_reads.bam` - BAM file containing HiFi Reads

If **not** using SMRT Link for subsequent analysis, please use these three files as input with any third-party analysis tools.

Finding the `hifi_reads` Files Generated Using On-Instrument CCS

1. In Run QC, click the desired run, then click the sample name to view the CCS Data Set.

2. Click **Analyses** in the left-side panel.

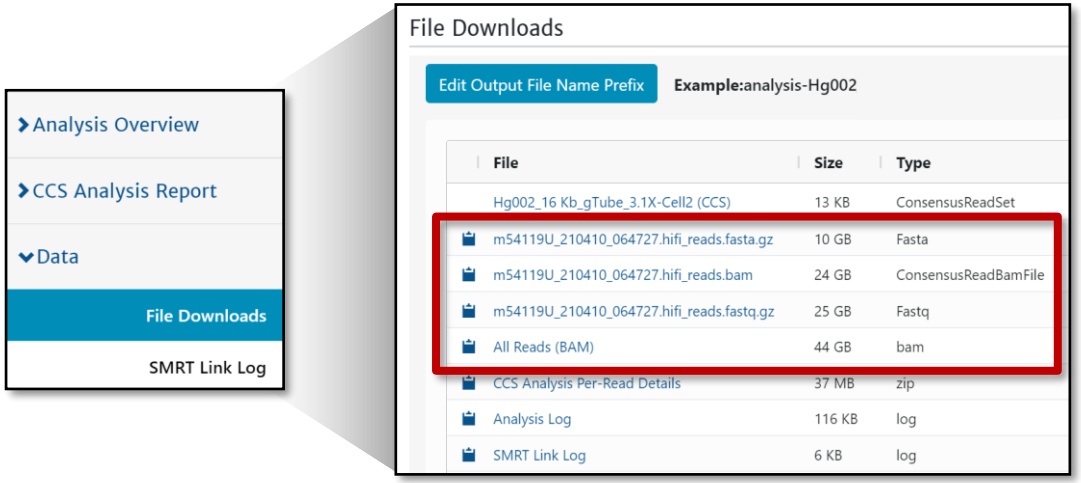
Page 1

** If not using SMRT Link for subsequent analysis, please use these three files as input with any third-party analysis tools*

HIFI DATA-SPECIFIC FILES ARE AUTOMATICALLY GENERATED IN SMRT LINK WHEN CCS ANALYSIS IS PERFORMED (CONT.)

HiFi Data File Generation With SMRT Link CCS Analysis Application (SMRT Link v10.1+)

- The Circular Consensus Sequencing (CCS) analysis application in SMRT Link generates one `reads.bam` file (labeled **'All Reads (BAM)'** in the **File Downloads** tab) plus the following three HiFi data-specific files below by default*:
  - **`<Movie_Name>.hifi_reads.fastq.gz`**
    - → FASTQ file with HiFi reads
  - **`<Movie_Name>.hifi_reads.fasta.gz`**
    - → FASTA file with HiFi reads
  - **`hifi_reads.BAM`**
    - → BAM file with HiFi reads
- To download the above files in SMRT Link, go to the **File Downloads** tab for the CCS analysis job



SMRT LINK ANALYSIS APPLICATIONS AND DATA FILTERING

CCS-based analysis applications require a `reads.bam` file as input and use built-in default read quality filter settings

- All SMRT Link CCS-based applications* use **`reads.bam`** dataset as input
  - **Built-in default filtering** applied prior to analysis execution for each application
  - All applications except Iso-Seq analysis use default HiFi reads (Q20 or higher)
  - Iso-Seq application uses reads with Q10 or higher

The following analysis parameters are **deprecated** as they no longer need to be specified by the user:

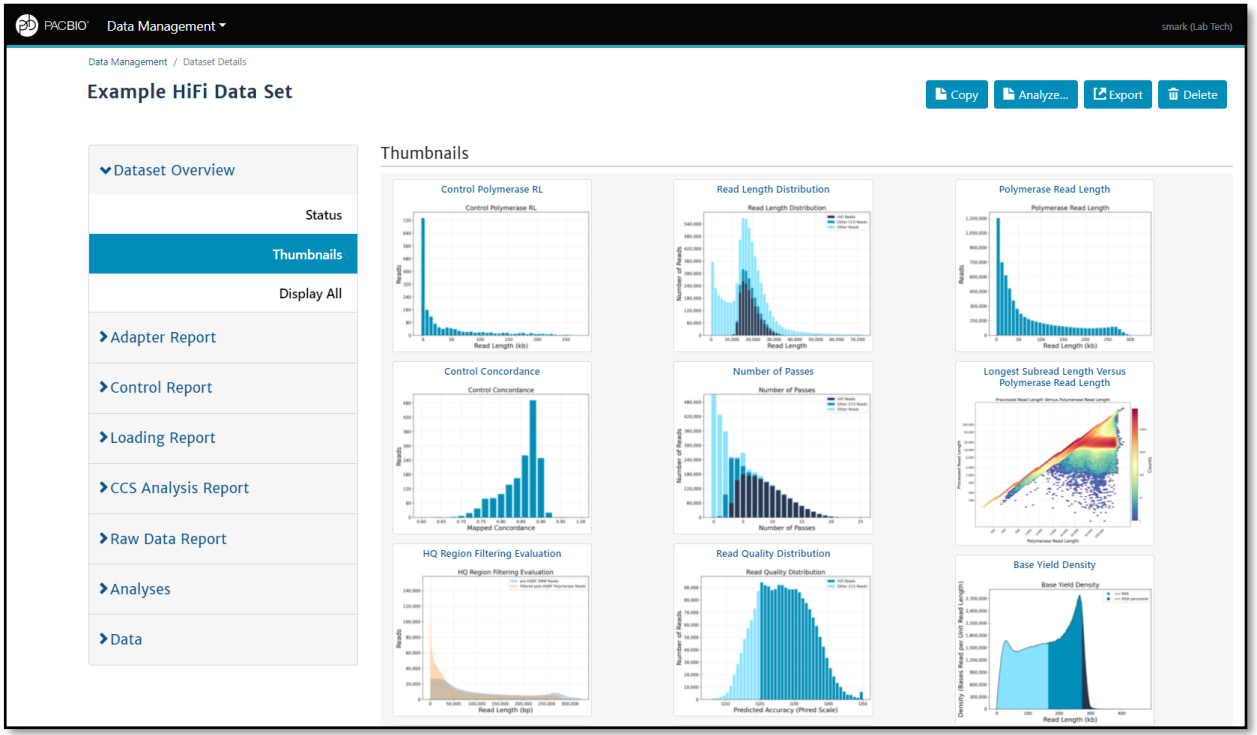
- Minimum Number of Passes
- Minimum Predicted Accuracy

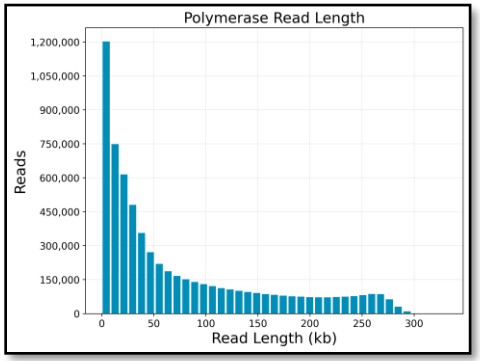
- Custom/non-HiFi data filtering:
  - Use **SMRT Link "Export Reads"** application to specify a custom QV value in Advanced Parameters to create FASTX and/or BAM files containing reads with a specified minimum CCS Predicted Accuracy
  - Use **SMRT Link Data Management** to create a Data Set with custom read quality filtering

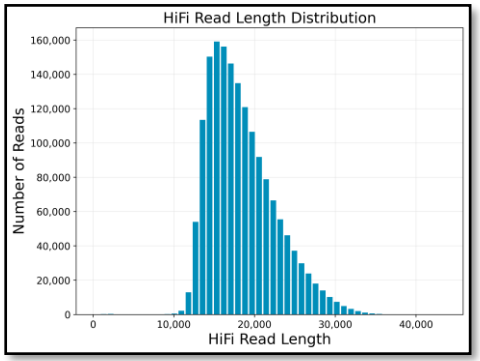
* Note: Microbial Assembly, Base Modification Analysis and HGAP4 Assembly remain CLR-based analysis applications

SMRT LINK DATA MANAGEMENT DATA SET REPORTS

Data Set Overview tab displays summary information for all reads *and* HiFi reads

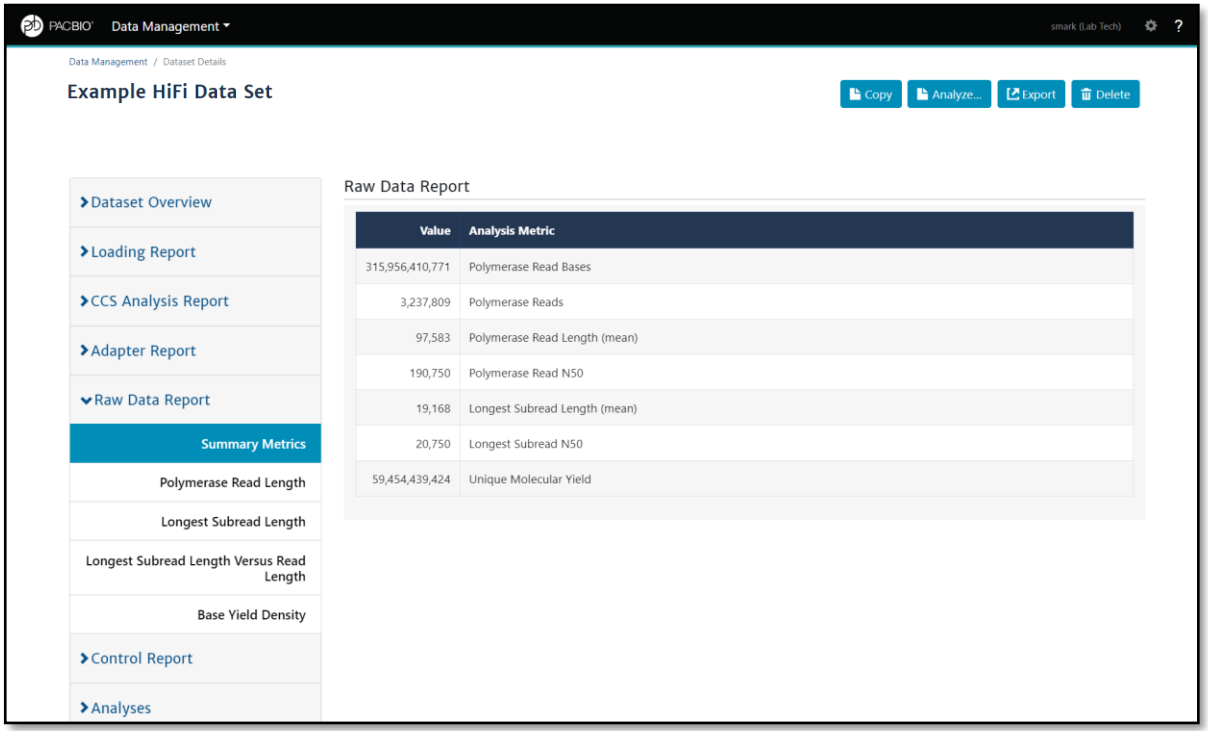






SMRT LINK DATA MANAGEMENT DATA SET REPORTS (CONT.)

Raw Data Report tab displays summary metrics for all reads

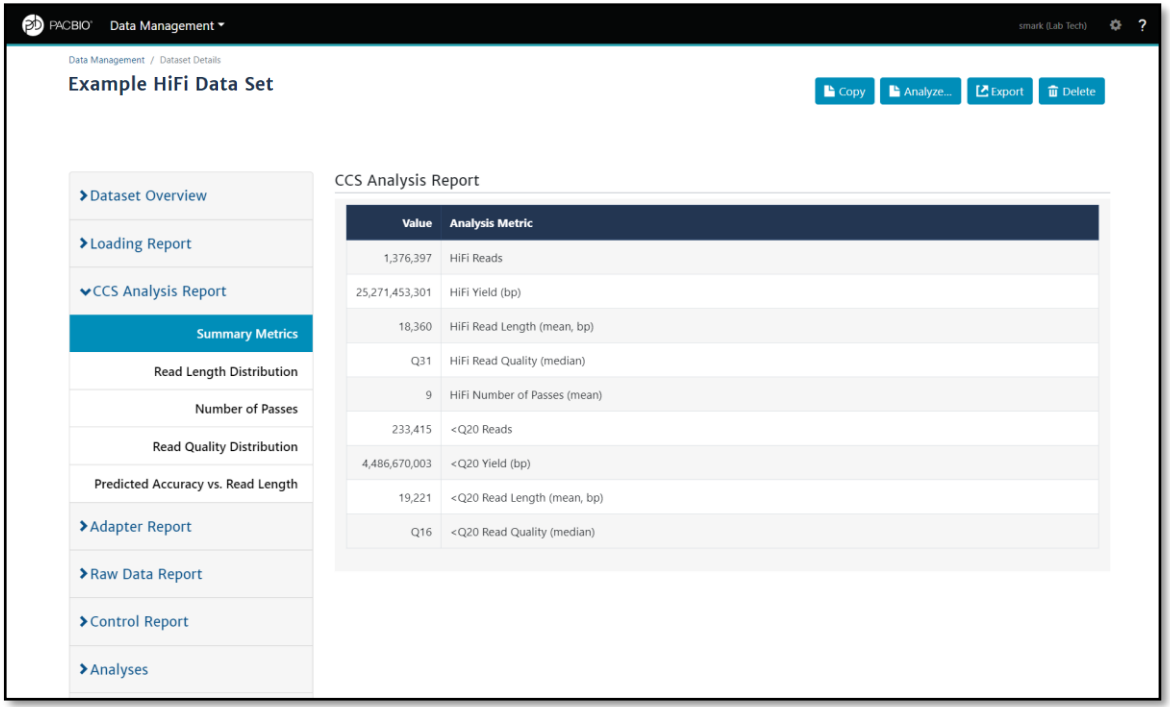


Raw Data Report

Value	Analysis Metric
315,956,410,771	Polymerase Read Bases
3,237,809	Polymerase Reads
97,583	Polymerase Read Length (mean)
190,750	Polymerase Read N50
19,168	Longest Subread Length (mean)
20,750	Longest Subread N50
59,454,439,424	Unique Molecular Yield

SMRT LINK DATA MANAGEMENT DATA SET REPORTS (CONT.)

CCS Analysis Report tab includes summary metrics for HiFi Reads (≥Q20) and Other CCS Reads (<Q20)



CCS Analysis Report

Value	Analysis Metric
1,376,397	HiFi Reads
25,271,453,301	HiFi Yield (bp)
18,360	HiFi Read Length (mean, bp)
Q31	HiFi Read Quality (median)
9	HiFi Number of Passes (mean)
233,415	<Q20 Reads
4,486,670,003	<Q20 Yield (bp)
19,221	<Q20 Read Length (mean, bp)
Q16	<Q20 Read Quality (median)

SMRT Link v10.1 Cloud

SMRT LINK CLOUD INTEGRATION

A cloud-based end-to-end analysis workflow enabled on Amazon Web Services

- Complete SMRT Link v10.1 functionality is now available on the cloud
- Cloud-agnostic solution – Amazon Web Services (AWS) support is being offered first
- Enabled for ***all*** Sequel Systems – Sequel, Sequel II and Sequel IIe Systems
- SMRT Link Cloud Advantages:
  - No dependency on having internal compute hardware infrastructure
  - Flexible data analysis options to optimize for speed or cost based on user preferences
  - Ability to easily share data with collaborators

DATA STREAMING TO THE CLOUD

SMRT Link v10.1 enables data streaming to AWS

- Seamless automated data streaming from the sequencing instrument to AWS
  - Once the Sequel / Sequel II / Sequel IIe System, SMRT Link and local network storage are configured, automated data streaming is enabled through Amazon tools and utilities included with SMRT Link
- Fail-safe data streaming is enabled through use of a local fail-safe network storage server
  - The sequencing data for each run is transferred to a local network storage server and then streamed to AWS
  - Network storage server requirements (provided by customer)
    - Disk space requirements are based on a typical run for Sequel II/IIe Systems
    - Disk space is managed by the customer – availability, data back up, etc.

DATA STREAMING TO THE CLOUD (CONT.)

SMRT Link provides a solution for streaming data from the network server to AWS

Sequel Instrument

Generate Sequencing Data

Network Server

Transfer Data to a Network Server

Amazon Web Services

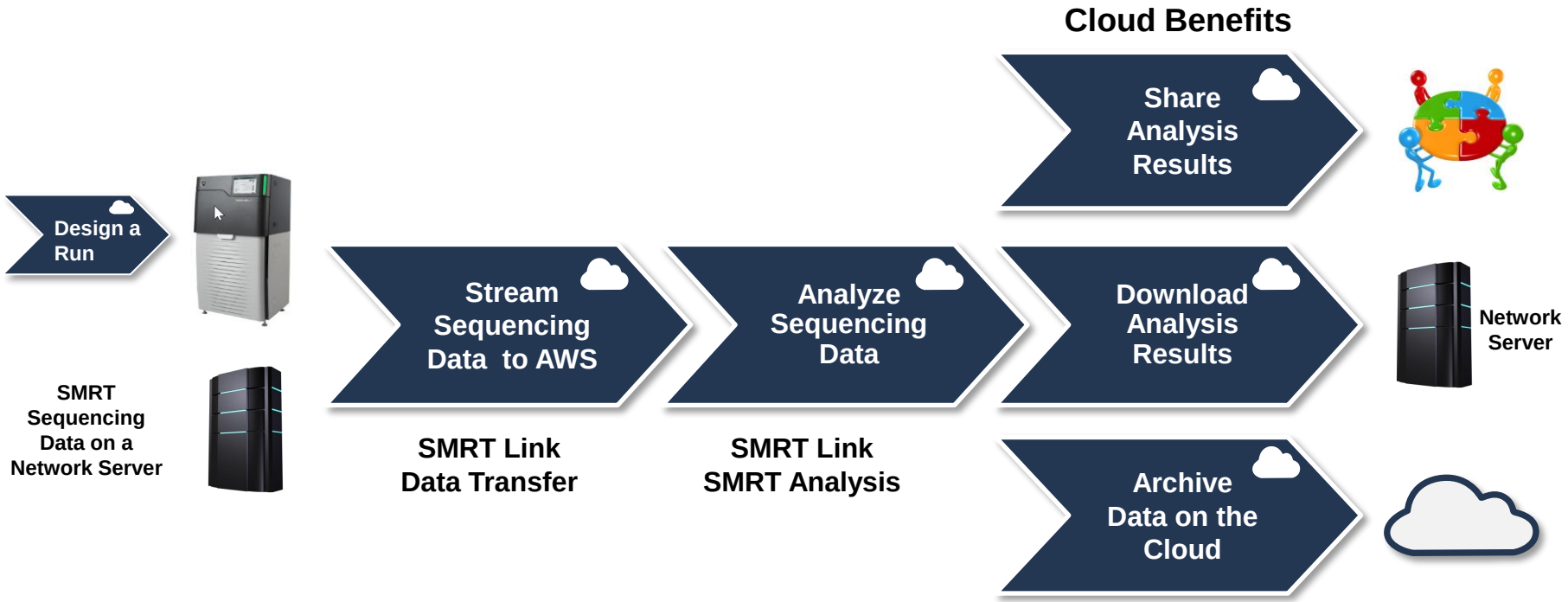
Stream data to AWS*

* Optionally, a customer-preferred transfer mechanism can be used

See SMRT Link Cloud Reference Guide (v10.1) (PN 101-985-900)

SMRT LINK CLOUD WORKFLOW

Versatile post-analysis options



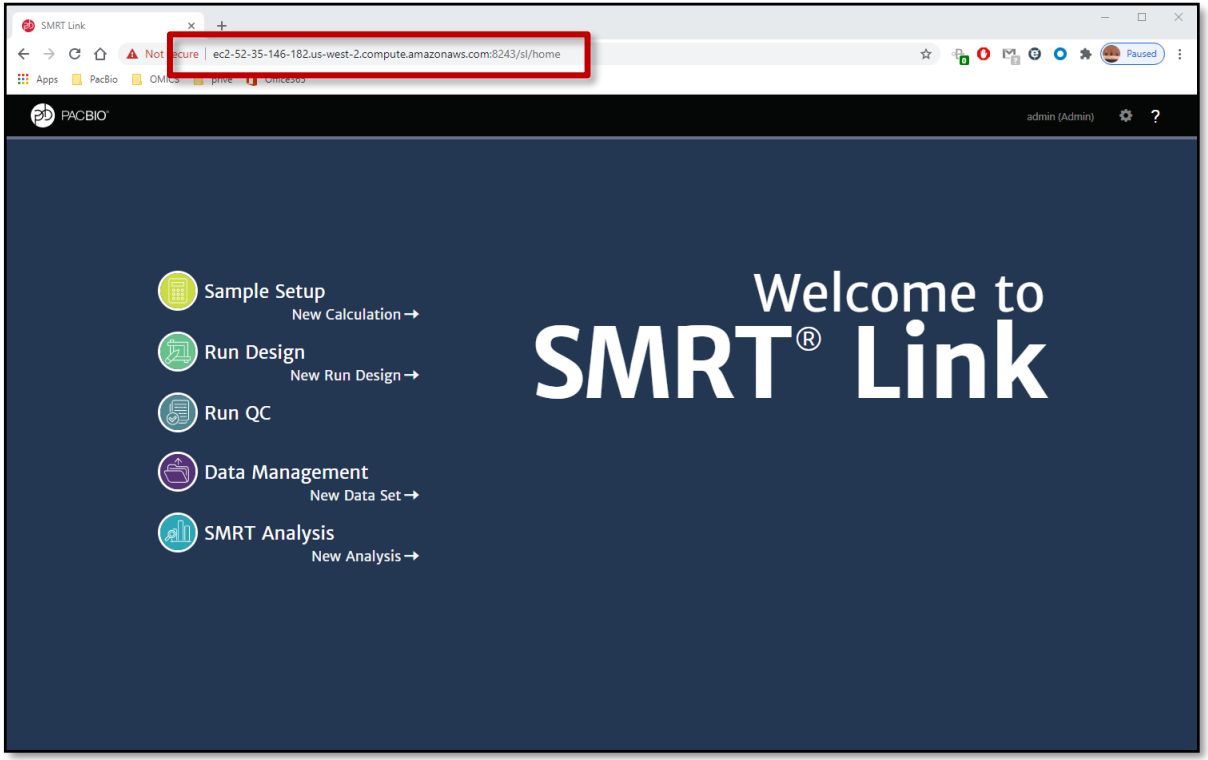
SMRT LINK CLOUD DATA SAFETY AND SECURITY

Compliance and safety in place

- Data safety and security mechanisms
  - Utilizing existing AWS and SMRT Link safety features
- Compliance with data localization requirements
  - Data localization provided by AWS data locality
- For information regarding specific security questions and concerns, see the **SMRT Link Cloud Reference Guide (v10.1)** (PN 102-043-900)

SMRT LINK CLOUD USAGE

Same functionality and usage as a local SMRT Link instance



- All SMRT Link features available
  - SMRT Link access URL points to the Cloud instance
- All available User Documents are applicable for SMRT Link Cloud

SMRT LINK CLOUD – TRANSFER SERVER REQUIREMENTS

AWS DATASYNC VM SPECIFICATIONS (MINIMUM REQUIREMENTS)
Virtual processors – **Four** (4) virtual processors assigned to the VM **Disk space** – **80 GB** of disk space for installation of VM image and system data **RAM** – Depending on your configuration, one of the following: • **32 GB of RAM** assigned to the VM, for tasks to transfer EC2 instance types with up to 20 million files. • **64 GB of RAM** assigned to the VM, for tasks to transfer more than 20 million files

STORAGE REQUIREMENTS	
Sequel IIe System	**1.5 – 2 TB (no kinetics info)**, **9 – 12 TB (with kinetics info)**
Sequel II System	**12 TB**
Sequel System	**1.5 – 2 TB**

NETWORK
10 GBE highly recommended, 1 GBE required **Time for data transfer to AWS is highly dependent on network speed and load**

COST SAVINGS: HUMAN TRIO ANALYSIS (20-FOLD HIFI COVERAGE) WITH CLOUD COMPUTE

	Sequel II System	Sequel IIe System	Sequel IIe System Savings
Instrument data transfer (data type)	6,000 GB (Subreads)	320 GB (HiFi Reads)	**95%**
HiFi read generation	12,000 CPU-hours	-	100%
Application compute	2,000 CPU-hours	2,000 CPU-hours	-
Total compute	14,000 CPU-hours	2,000 CPU-hours	**85%**
Total data storage	6,000 GB	320 GB	**95%**
CPU cost (AWS)*	$602	$85	$517
Annual data storage cost (AWS)**	$1,671	$85	$1,586
Compute cost (AWS)* **	$2,273	$170	**$2,103**

* m5a.12xlarge (48 vCPUs, 192 GiB RAM, $2.064/hr), https://aws.amazon.com/ec2/pricing/on-demand/

** Assumes 1 trio for 1 year at $0.023 per month, https://aws.amazon.com/s3/pricing/

*** All prices are listed in USD and costs may vary by region.

SMRT Link Applications Updates

ANALYSIS APPLICATION UPDATES

Genome Assembly – *De Novo* Assembly Using HiFi Reads (SMRT Link v10.0 Release) ***[NEW]***

- Generate highly accurate polished contiguous assemblies and fully phased haplotigs
- Fast and easy to use

SARS-CoV-2 Analysis (SMRT Link v10.1 Release) ***[NEW]***

- Analyze multiplexed SARS-CoV-2 viral amplicon samples to identify variants and call a single consensus sequence per sample using HiFi reads

Iso-Seq Analysis (SMRT Link v10.1 Release) ***[UPDATED]***

- Features improved graphical and tabular report outputs for analysis of multiplexed Iso-Seq samples

Single-Cell Iso-Seq Analysis (Bioconda Release) ***[NEW]***

- Supports analysis of Unique Molecular Identifier (UMI) sequence tags in single-cell Iso-Seq samples

Amplicon Analysis – HiFi Reads (Bioconda Release) ***[NEW]***

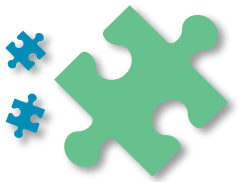
- Clustering and allele detection using HiFi reads

GENOME ASSEMBLY ANALYSIS APPLICATION *[NEW]*

SMRT Link Genome Assembly analysis application uses HiFi reads for improved *de novo* assemblies

Contiguity

- Resolve Repetitive Regions
- High Contig N50



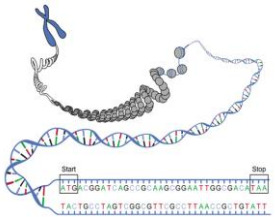
Correctness

- Base QV
- Phasing accuracy

```
AGTTTCGATAGA
AGTT-CGAAAGA
```

Completeness

- Gene Space
- Repetitive Regions



Compute

- CPU / Wall Time
- RAM
- Disk Storage

GENOME ASSEMBLY ANALYSIS APPLICATION ALGORITHM

Powered by IPA (Improved and Phased Assembly) algorithm

- Fast and efficient assembly – 5.5 hours* for a human assembly with 20-fold HiFi read coverage
- High contiguity
- Fully phased haplotigs
- High per-base quality of polished assemblies
- Easy to use

* Compute environment:
  Head Node - Cores: 32, RAM: 64 GB, 1 TB local tmp, 256 GB local db_datadir
  Compute Nodes – Cores 64, RAM: 4GB per core, 1 TB local tmp, 256 GB local db_datadir

GENOME ASSEMBLY ANALYSIS APPLICATION WORKFLOW

1. Convert inputs to compressed database format for fast and easy retrieval
2. Overlap reads to form read piles using local alignment initialization and extension
3. Separate overlapped reads by phase using de Bruijn graph
4. Remove chimeras and repeats to improve contiguity and reduce missasembly
5. Build a string graph with primary and associate contigs, assign reads to contigs based on phased overlaps
6. Polish using phased-aware assignment-based mapping
7. Purge duplicates

SARS-CoV-2 ANALYSIS APPLICATION *[NEW]*

Use Case

- Analysis support for HiFiViral for SARS-CoV-2 Workflow (See ***Procedure & Checklist – Multiplexing 1.2 kb Amplicons for Full-Viral Genome Sequencing*** [PN 102-075-000 (High-Throughput) / PN 102-082-500 (Low-Throughput)]
- For each sample, identifies a single SARS-CoV-2 species and consensus sequence
- Input Data: HiFi sequencing data for multiplexed SARS-CoV-2 amplicon samples*
    - Amplicon sizes supported: From a few hundred bases to kilobases, tiled across the entire 30 kb SARS-CoV-2 viral genome
    - Sample multiplexing level supported: 10- to 1000-plex

Analysis output per sample

- Amplicon coverage (CSV)
- Variant calls (VCF)
- Consensus sequence (FASTA)
- Aligned reads (BAM)

* SARS-CoV-2 analysis application does not support non-amplicon SARS-CoV-2 data (capture-based data, WGS or transcriptome) and non-SARS-CoV-2 viral data.

SARS-CoV-2 ANALYSIS APPLICATION WORKFLOW

Use the SARS-CoV-2 analysis application in SMRT Link to analyze multiplexed viral surveillance samples for SARS-CoV-2

HiFi Reads

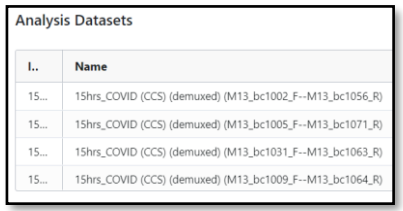
↓

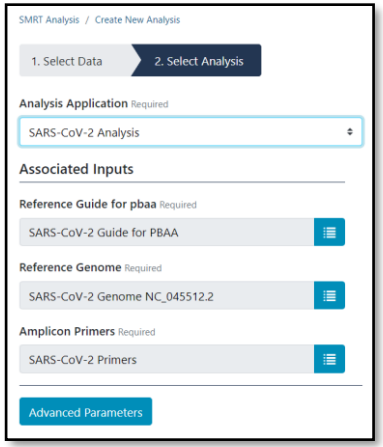
Demultiplex Barcodes

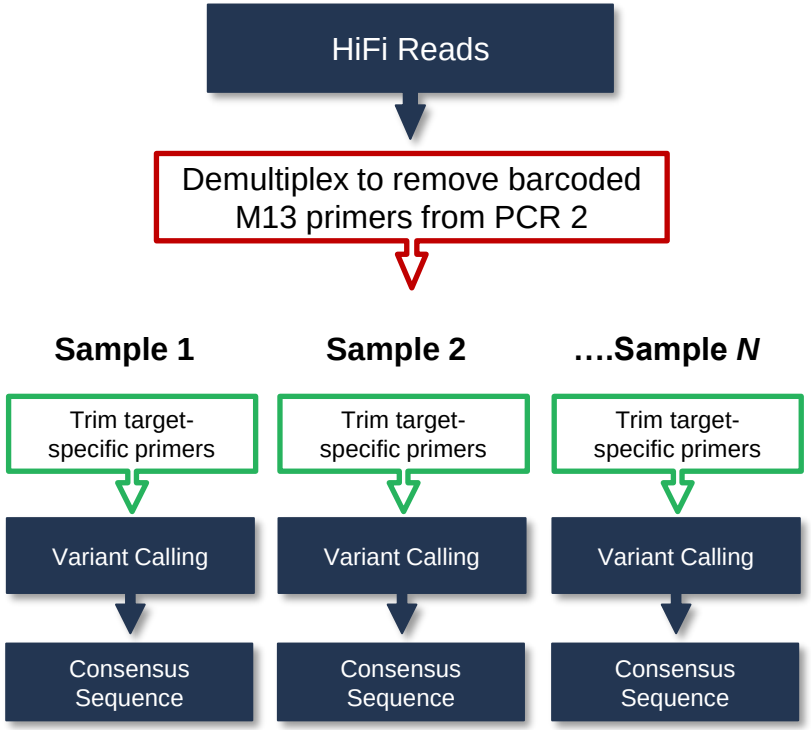
↓

SARS-CoV-2 Analysis Pipeline

(Select all samples to analyze)



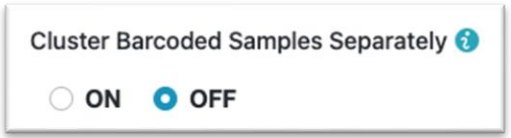




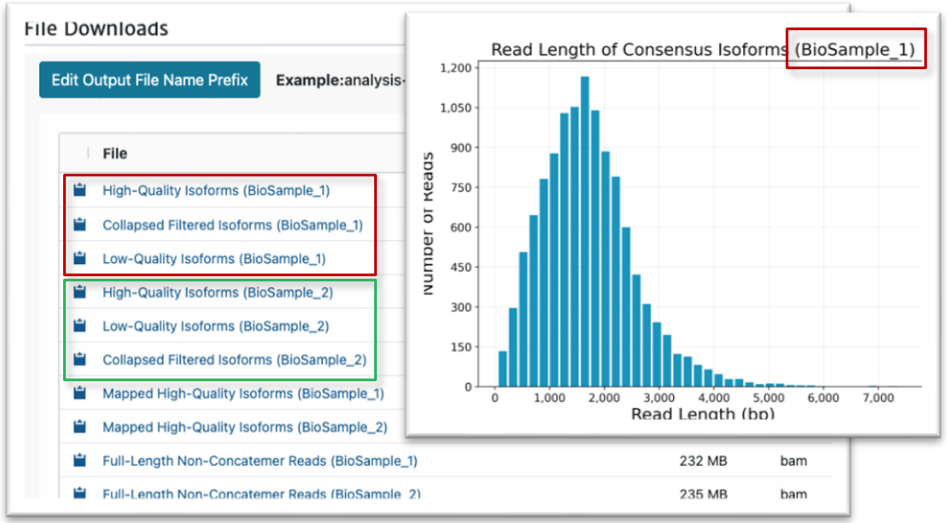
IMPROVED ISO-SEQ ANALYSIS APPLICATION FOR MULTIPLEXED SAMPLES

In SMRT Link v10.1, **use the Iso-Seq Analysis application to analyze multiplexed Iso-Seq samples*** – do not run Demultiplex Barcodes first

- For multiplexed datasets, Iso-Seq Analysis reports and graphs now include isoforms **per barcode** in addition to the total number of isoforms across all barcodes
- Per-barcode summary metrics, plots, and file downloads are generated
- Iso-Seq Analysis now supports both separate and joint clustering of barcoded samples



Sample Name	Number of High-Quality Isoforms	Number of Low-Quality Isoforms
BioSample_1	11,246	288
BioSample_2	10,914	289



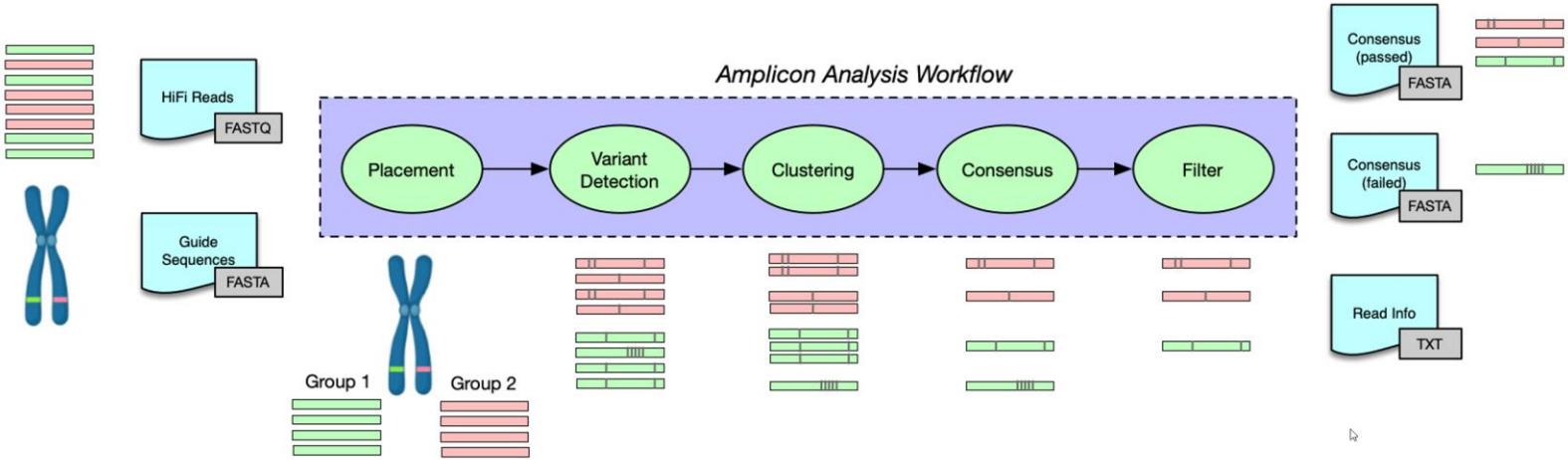
* In Iso-Seq Analysis, select primer set IsoSeq_Primers_12_Barcodes_v1 or another custom set containing multiple barcodes Do not use the default of IsoSeqPrimers_v2

PACBIO AMPLICON ANALYSIS APPLICATION (*pbAA)* [BIOCONDA RELEASE]

PacBio Amplicon Analysis Application for HiFi reads

- **Functionality:**
  - A reference-guided application for clustering and generation of high-quality phased consensus sequences using HiFi data
- **Benefits:**
  - Accurate - base level resolution
  - Sensitive – no missed alleles, favoring false positive over false negatives
  - Fast – results in less than five minutes for samples with high read depth (>500-fold)
    - Optimized performance with low computational complexity
  - Flexible – general amplicon analysis application with tunable parameters
  - **Bonus feature**: Visualization sub-tool for coloring aligned reads by cluster (helpful for interpreting and troubleshooting analysis results)

PACBIO AMPLICON ANALYSIS APPLICATION (*pbAA)* WORKFLOW AND OUTPUT



Analysis Workflow

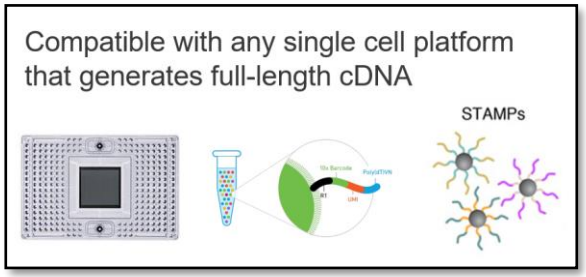
1. Generate guide information
2. Assign reads to a loci
3. Detect variants
4. Cluster using a custom model
5. Generate consensus
6. Filter results

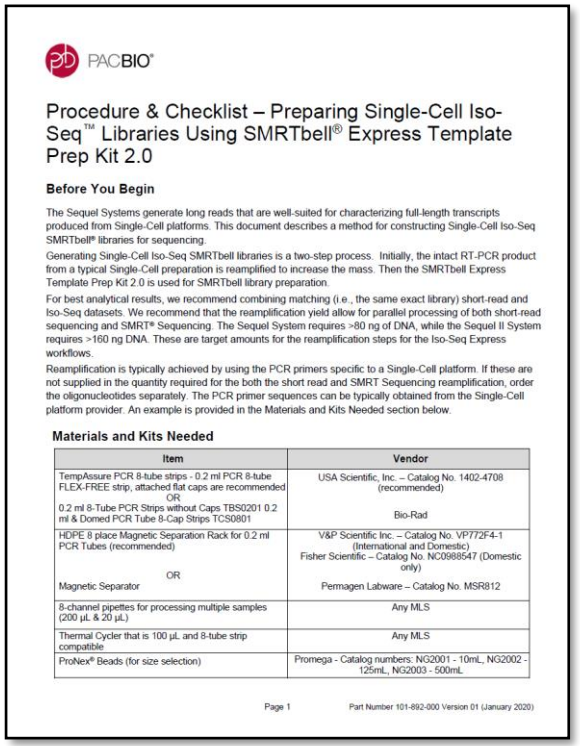
Output

- Two cluster consensus files – reads passing and failing filtering
- Reads information file – details on read classification

SINGLE-CELL ISO-SEQ METHOD SUMMARY

- Procedure & Checklist – Preparing Single-Cell Iso-Seq Libraries Using SMRTbell Express Template Prep Kit 2.0 protocol (PN 101-892-000) provides detailed workflow guidance
- Uses standard Iso-Seq Express library preparation & sequencing workflow
- Generating matching short-read data from the same library sample is recommended
- Characterize alternative splicing with up to 3 Million full-length transcript reads generated per SMRT Cell 8M
  - Each FL transcript read contains single-cell barcode and UMI information





PACBIO

Procedure & Checklist – Preparing Single-Cell Iso-Seq™ Libraries Using SMRTbell® Express Template Prep Kit 2.0

Before You Begin

The Sequel Systems generate long reads that are well-suited for characterizing full-length transcripts produced from Single-Cell platforms. This document describes a method for constructing Single-Cell Iso-Seq SMRTbell® libraries for sequencing.

Generating Single-Cell Iso-Seq SMRTbell libraries is a two-step process. Initially, the intact RT-PCR product from a typical Single-Cell preparation is reamplified to increase the mass. Then the SMRTbell Express Template Prep Kit 2.0 is used for SMRTbell library preparation.

For best analytical results, we recommend combining matching (i.e., the same exact library) short-read and Iso-Seq datasets. We recommend that the reamplification yield allow for parallel processing of both short-read sequencing and SMRT® Sequencing. The Sequel System requires >80 ng of DNA, while the Sequel II System requires >160 ng DNA. These are target amounts for the reamplification steps for the Iso-Seq Express workflows.

Reamplification is typically achieved by using the PCR primers specific to a Single-Cell platform. If these are not supplied in the quantity required for the both the short read and SMRT Sequencing reamplification, order the oligonucleotides separately. The PCR primer sequences can be typically obtained from the Single-Cell platform provider. An example is provided in the Materials and Kits Needed section below.

Materials and Kits Needed

Item	Vendor
TempAssure PCR 8-tube strips - 0.2 ml PCR 8-tube FLEX-FREE strip, attached flat caps are recommended OR 0.2 ml 8-Tube PCR Strips without Caps TBS0201 0.2 ml & Domed PCR Tube 8-Cap Strips TCS0801	USA Scientific, Inc. – Catalog No. 1402-4708 (recommended) Bio-Rad
HDPE 8 place Magnetic Separation Rack for 0.2 ml PCR Tubes (recommended) OR Magnetic Separator	V&P Scientific Inc. – Catalog No. VP772F4-1 (International and Domestic) Fisher Scientific – Catalog No. NC0988547 (Domestic only) Permagen Labware – Catalog No. MSR812
8-channel pipettes for processing multiple samples (200 µL & 20 µL)	Any MLS
Thermal Cycler that is 100 µL and 8-tube strip compatible	Any MLS
ProNex® Beads (for size selection)	Promega - Catalog numbers: NG2001 - 10mL, NG2002 - 125mL, NG2003 - 500mL

Page 1 Part Number 101-892-000 Version 01 (January 2020)

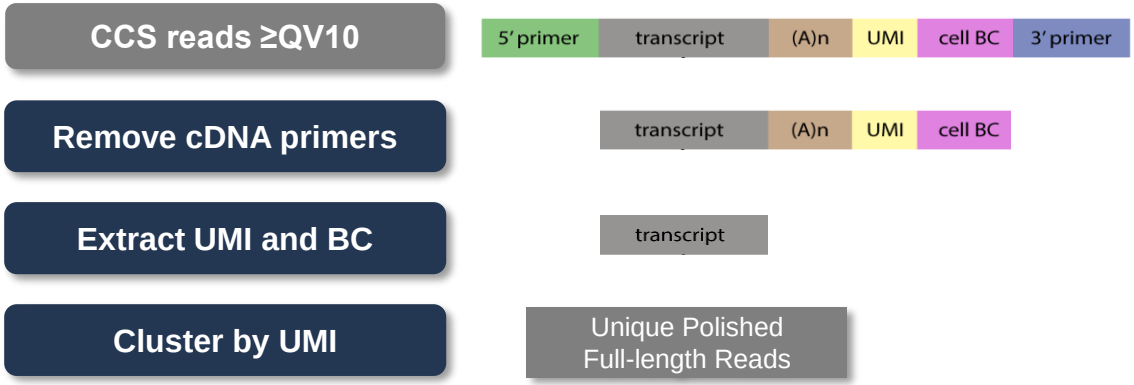
https://www.pacb.com/support/documentation/

SINGLE-CELL ISO-SEQ ANALYSIS WORKFLOW [BIOCONDA RELEASE]

Supports analysis of Unique Molecular Identifier (UMI) sequence tags in single-cell Iso-Seq samples

Analysis Workflow

1. Remove cDNA 5’ and 3’ primers
2. Extract and trim UMI and cell BC
3. Remove polyA tails and concatemers
4. Cluster by UMI using QV-guided approach



OTHER ANALYSIS APPLICATION UPDATES

Improved Structural Variant Calling Analysis Application

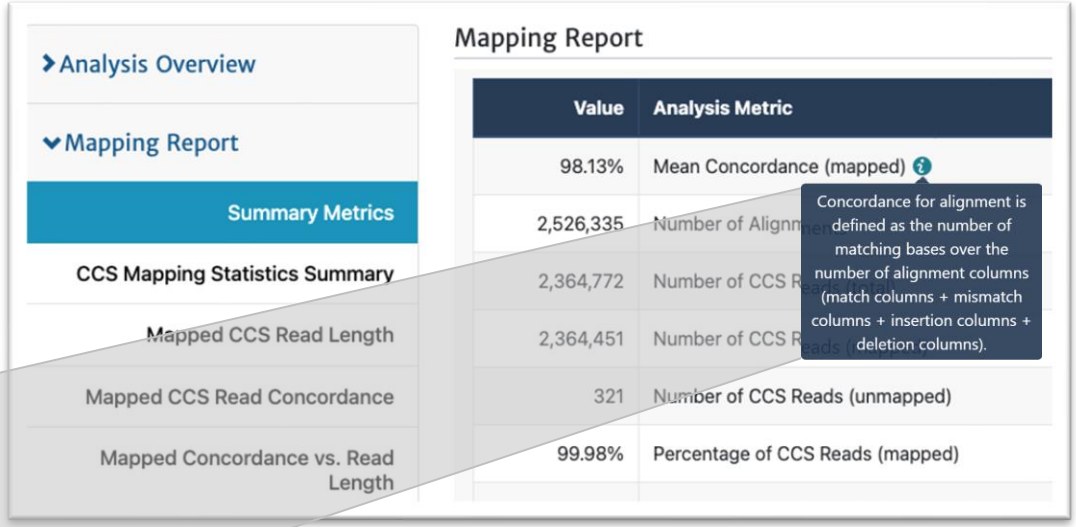
- Improved precision:
  - Breakend (BND) calls - filtering of short and low-identity alignments
- Added SVLEN annotation for inversion variants

Improvement to Mapping Applications

- Enhanced alignment concordance
  - Industry-standard BLAST-style alignment identity

IMPROVED CALCULATION OF ALIGNMENT CONCORDANCE IN MAPPING APPLICATIONS

- Alignment concordance is now reported as industry-standard BLAST-style alignment identity (matches/alignment columns)
  - In earlier versions we used a non-standard calculation for concordance



Concordance for alignment is defined as the **number of matching bases over the number of alignment columns** (match columns + mismatch columns + insertion columns + deletion columns).

DATA TYPE	MEAN MAPPED CONCORDANCE Δ (OLD VS. NEW CALCULATION)
HiFi Data	0.1 – 0.2% Lower
CLR Data	0.5% Higher

SMRT Link General Usability Improvements

SMRT LINK USER INTERFACE AND USABILITY IMPROVEMENTS

More Streamlined Application-centric Sample Setup and Run Design

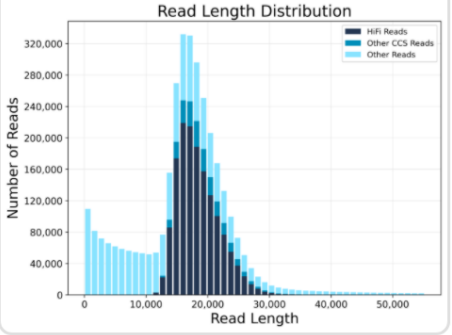
- Default protocol and run settings are auto-filled for each selected application type

SMRT LINK USER INTERFACE AND USABILITY IMPROVEMENTS

New HiFi Metrics and Data Visualization Reports

- Available in Run QC when On-Instrument CCS is enabled for the Sequel IIe System

HiFi Read Length Distribution

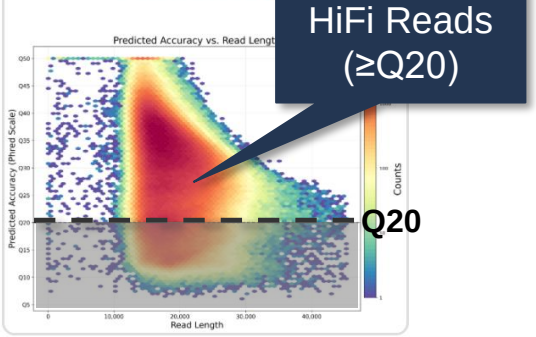


Displays a histogram distribution of HiFi Reads (QV ≥20), other CCS Reads (three or more passes, but QV <20), and other reads, by read length.

Read Quality Distribution

Displays a histogram distribution of HiFi Reads (QV ≥20) and other CCS Reads by read quality.

Read Length vs Predicted Accuracy



Displays a heat map of CCS Read lengths and predicted accuracies. The boundary between HiFi Reads and other CCS Reads is shown as a dashed line at QV 20.

SMRT LINK USER INTERFACE AND USABILITY IMPROVEMENTS

Improved Sample Setup Support for High-Throughput Sequencing

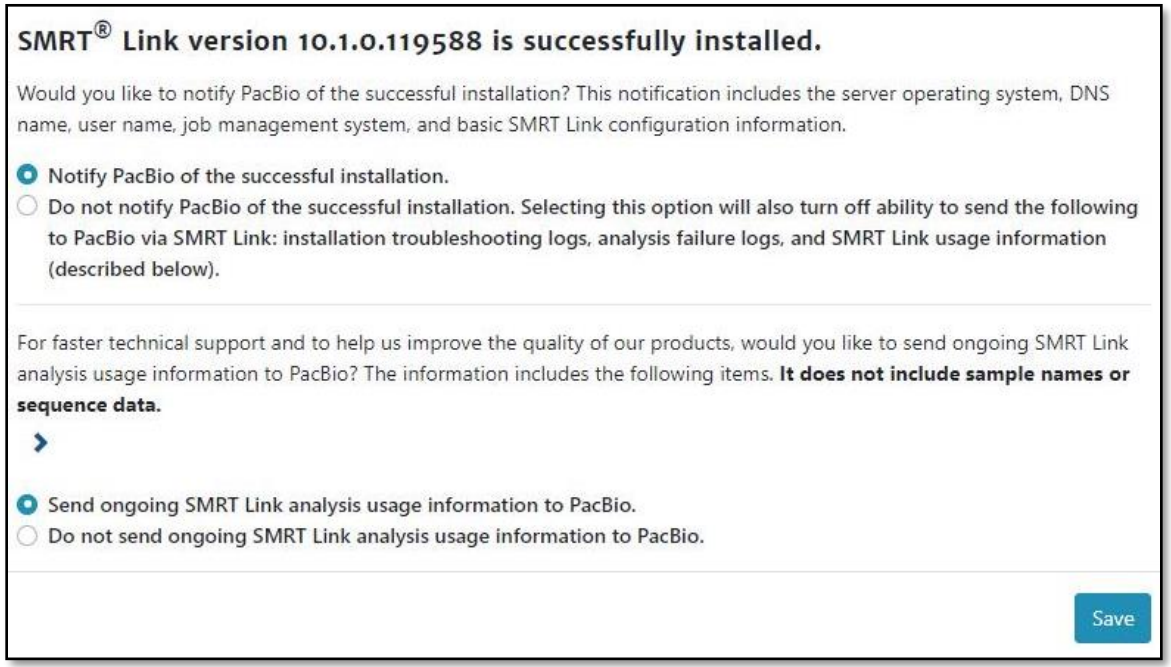
- Sample Setup features enhanced support for high-throughput production environments through new Sample Setup sheet *.CSV import function

Sample Name	System Name	Application	Available Starting Sample Volume (uL)	Starting Sample Concentration (ng/uL)	Insert Size (bp)
Sample 1 Demo	Sequel IIe	HiFi Reads	10	100	18000

Field Name	Required	Description
Sample Name	**Yes**	Enter alphanumeric characters, spaces, hyphens, underscores, colons, or periods **only**.
System Name	**Yes**	**Must** be `Sequel`, `Sequel II`, or `Sequel IIe`.
Application	**Yes**	Enter one of the following values: • `HiFi Reads` • `Continuous Long Reads` • `Low DNA Input` • `Ultra-Low DNA Input` • `Microbial Assembly` • `Variant Calling` • `Structural Variation Calling` • `HiFiViral SARS-CoV-2` • `Iso-Seq Method` • `Full-Length 16S rRNA Sequencing` • `Shotgun Metagenomic Profiling or Assembly` • `<3kb Amplicons` • `>=3kb Amplicons` • `Custom`
Available Starting Sample Volume (uL)	**Yes**	Enter a positive integer. Units are in microliters.
Starting Sample Concentration (ng/uL)	**Yes**	Enter a positive integer. Units are in nanograms per microliter.
Insert Size (bp)	**Yes**	Enter a positive integer. Units are in base pairs.

SMRT LINK USER INTERFACE AND USABILITY IMPROVEMENTS

We recommend notifying PacBio of your successful SMRT Link v10.1 installation and sending ongoing SMRT Link analysis usage information to PacBio in order to expedite case troubleshooting and to help us continually improve our products



SMRT Link Fixed & Known Issues

SMRT LINK V10.1 FIXED ISSUES HIGHLIGHTS

See the latest SMRT Link Release Notes for an updated list of fixed issues

- SV calling: Joint SV calling on demultiplexed Data Sets from the same cell/collection – demultiplexed Data Sets are now analyzed separately, and the Bio Sample name is used.
- Sample Setup: Columns in the edit/print view can now be drag-and-dropped.
- Copying to the clipboard now works as expected.
- Exporting large analysis directories now works correctly and does not fail.
- Absolute file paths are now included in the `subreadset.xml` file.
- Login for local SMRT Link WSO2 users is now enabled.
- The outputs analysis directory now includes symbolic links to the BAM files.
- BAM files consolidation for microbial assemblies now works correctly.
- Using the hyphen character "-" in barcode and Bio Sample names no longer causes the Demultiplex Barcodes application to fail.
- HGAP4 analysis no longer fails if the Genome Size is set to more than 2.0 GB.

SMRT LINK V10.1 KNOWN ISSUES HIGHLIGHTS

See the latest SMRT Link Release Notes for an updated list of known issues

- Run Design: When opening a saved Run Design, you will sometimes be asked to save changes when no changes were made.
- Run Design: The Import from Sample Setup feature does not distinguish between Sample Setup designs created for Sequel II and for Sequel IIe, instead showing both.
- A cached URL containing the string /welcome at the end of the SMRT Link URL (Example: https://URL/sl/welcome) in the browser's history causes an error when accessing SMRT Link.
- Motif detection is designed for microbial genomes and has not been tested on non-microbial genomes; it may run out of memory on large genomes.
- When copying an analysis using the Demultiplex Barcodes application (using Copy from an Analysis Results page or Copy From on the New Analysis page), the input of sample names for each barcode is not preserved from the copied analysis. In the second step of the New Analysis wizard, users must either re-enter the sample names using the Interactive Barcode Selector and Sample Name Editor, or re- upload a Barcoded Sample File. The Start button is not enabled until users do so.
- When creating a user, ensure that the new user profile has the Username attribute populated with the account/login name. This is required for the user search in the configuration and project pages to find local users. (See SMRT Link Software Installation (v10.1) for details.)
- When using Bio Sample Names with a PacBio analysis application, you can enter names that include spaces. Please avoid using spaces in Bio Sample Names as spaces may lead to third-party compatibility issues.

PACIFIC BIOSCIENCES®

Sequel II and IIe Systems Applications Support Resources

Subhead should be no longer than 1 line

OVERVIEW – SEQUEL SYSTEMS APPLICATION OPTIONS AND SEQUENCING RECOMMENDATIONS

This document provides high-level application workflow guidance and links to protocols for preparing samples for sequencing on the Sequel Systems and analysis.

Whole Genome Sequencing

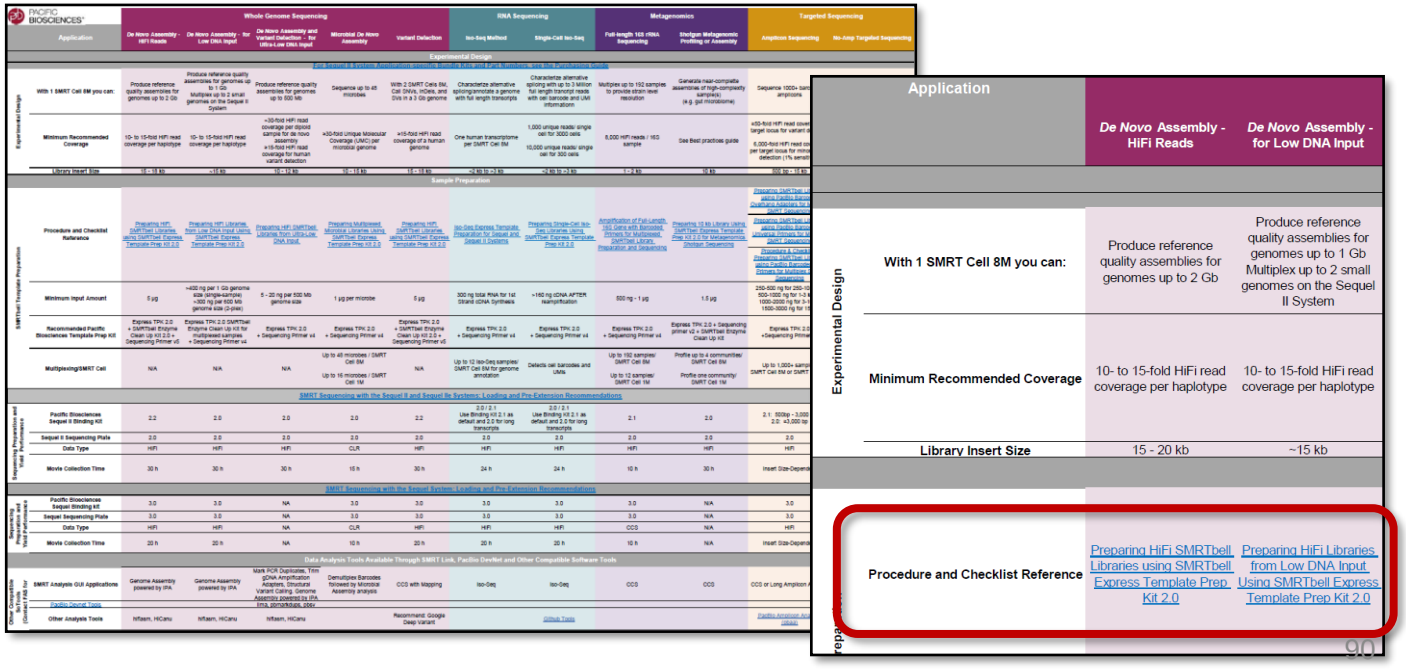
Variant Detection

RNA Sequencing

Targeted Sequencing

Complex Populations

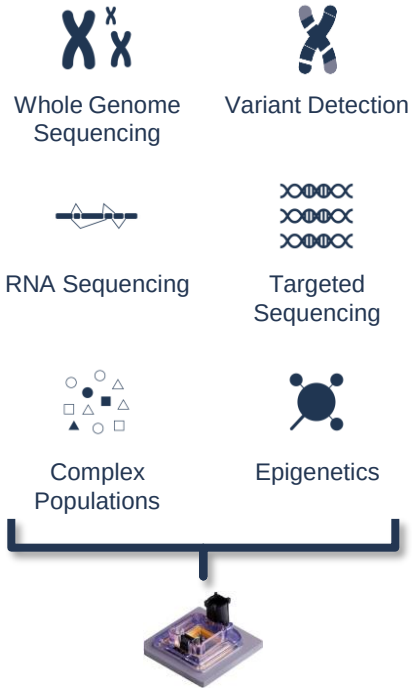
Epigenetics



https://www.pacb.com/wp-content/uploads/Overview-Sequel-Systems-Application-Options-and-Sequencing-Recommendations.pdf

WHAT CAN YOU DO WITH ONE SMRT CELL 8M?

With PacBio Single Molecule, Real-Time Sequencing on the Sequel II and IIe Systems you can characterize whole genomes and transcriptomes with just one SMRT Cell 8M.



SMRT Cell 8M

SMRT Sequencing Applications		Number of SMRT Cells 8M*	PacBio Consumable Estimated Costs (US List Price)†
WHOLE GENOME SEQUENCING	***De Novo* Assembly:** Produce reference-quality assemblies for genomes up to 2 Gb	1	$1300/sample
	Microbial *De Novo* Assembly: Generate reference-quality assemblies for up to 48 microbial isolates	1	$70/sample
	Variant Detection: Call single nucleotide, indel, and structural variants in a ~3 Gb genome	2	$2600/sample
	Structural Variant Detection: Call structural variants for up to 2 samples with ~3 Gb genomes	1	$670/sample
RNA SEQUENCING	**Whole Transcriptome:** Characterize alternative splicing with full-length transcripts	1	$1300/sample
	Genome Annotation: Sequence full-length transcripts and multiplex up to 8 tissues	1	$185/tissue
TARGETED SEQUENCING	**Amplicon Sequencing:** Detect variation in specific regions by multiplexing 1000 samples (1-10 kb)	1	$1-2/sample
	No-Amp Sequencing: Enrich hard-to-amplify targets and multiplex up to 48 samples	1	$82-118/sample
COMPLEX POPULATIONS	**Full-length 16S:** Gain strain-level resolution by multiplexing up to 192 samples	1	$7.50/sample
	Metagenomic Functional Profiling: Examine up to 3 low-complexity samples with multiplexing	1	$450/sample
	Shotgun Metagenomic Assembly: Generate near-complete assemblies of high-complexity samples (e.g. gut microbiome)	1	$1300/sample

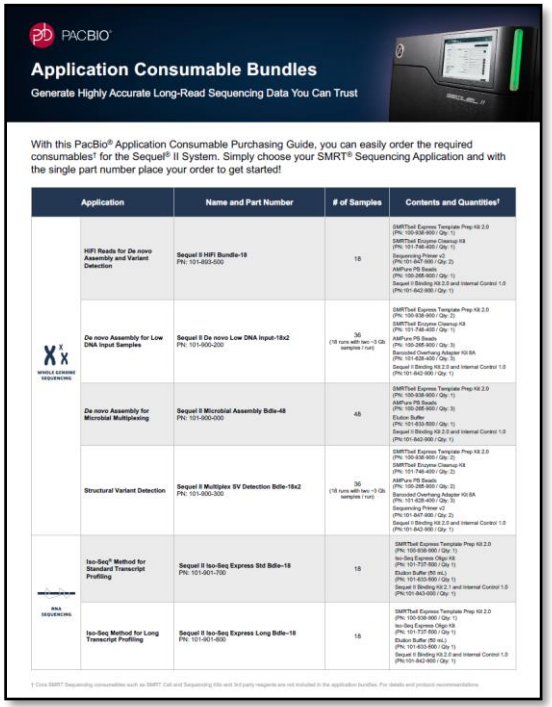
**Study design, sample type, and level of multiplexing may affect the number of SMRT Cells 8M required. †All prices are listed in USD and cost may vary by region. Pricing includes library and sequencing reagents run on your Sequel II System and does not include instrument amortization or other reagents.*

Application Brochure: What Can You Do with One SMRT Cell?

APPLICATION CONSUMABLE BUNDLES & PURCHASING GUIDE

Purchasing Guide brochure enables users to easily order required consumables needed to run a specific type of application on the Sequel II and IIe Systems.

- Customers can use a **single part number** to order a consumables bundle containing PacBio-branded reagents needed for SMRTbell library construction, primer annealing & polymerase binding
- **Exclusions:**
  - Core PacBio-branded SMRT Sequencing consumables (SMRT Cells, Sequencing Kits & SMRT Oil), plastics and other 3rd-party reagents are not included in the application bundles
  - For Barcoded Adapter bundles that support >16-plex, PacBio recommends customers purchase barcoded adapters directly from a third-party oligo synthesis company.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Application Consumable Bundles

Generate Highly Accurate Long-Read Sequencing Data You Can Trust

With this PacBio® Application Consumable Purchasing Guide, you can easily order the required consumables† for the Sequel® II System. Simply choose your SMRT® Sequencing Application and with the single part number place your order to get started!

Application		Name and Part Number	# of Samples	Contents and Quantities‡
WHOLE GENOME SEQUENCING	HiFi Reads for *De novo* Assembly and Variant Detection	Sequel II HiFi Bundle-1B PN: 101-893-500	1B	SMRTbell Express Template Prep Kit 2.0 (PN: 100-938-900 / Qty: 1) SMRTbell Enzyme Cleanup Kit (PN: 101-746-400 / Qty: 1) Sequencing Primer v2 (PN:101-847-900 / Qty: 2) AMPure PB Beads (PN: 100-265-900 / Qty: 1) Sequel II Binding Kit 2.0 and Internal Control 1.0 (PN:101-842-900 / Qty: 1)
	De novo Assembly for Low DNA Input Samples	Sequel II De novo Low DNA Input-18x2 PN: 101-900-200	36 (18 runs with two ~3 Gb samples / run)	SMRTbell Express Template Prep Kit 2.0 (PN: 100-938-900 / Qty: 2) SMRTbell Enzyme Cleanup Kit (PN: 101-746-400 / Qty: 1) AMPure PB Beads (PN: 100-265-900 / Qty: 3) Barcoded Overhang Adapter Kit 8A (PN: 101-628-400 / Qty: 3) Sequel II Binding Kit 2.0 and Internal Control 1.0 (PN:101-842-900 / Qty: 1)
	De novo Assembly for Microbial Multiplexing	Sequel II Microbial Assembly 8dbc-48 PN: 101-900-000	48	SMRTbell Express Template Prep Kit 2.0 (PN: 100-938-900 / Qty: 1) AMPure PB Beads (PN: 100-265-900 / Qty: 3) Elution Buffer (PN: 101-633-500 / Qty: 1) Sequel II Binding Kit 2.0 and Internal Control 1.0 (PN:101-842-900 / Qty: 1)
	Structural Variant Detection	Sequel II Multiplex 9V Detection 8dbc-18x2 PN: 101-900-300	36 (18 runs with two ~3 Gb samples / run)	SMRTbell Express Template Prep Kit 2.0 (PN: 100-938-900 / Qty: 2) SMRTbell Enzyme Cleanup Kit (PN: 101-746-400 / Qty: 2) AMPure PB Beads (PN: 100-265-900 / Qty: 2) Barcoded Overhang Adapter Kit 8A (PN: 101-628-400 / Qty: 3) Sequencing Primer v2 (PN:101-847-900 / Qty: 2) Sequel II Binding Kit 2.0 and Internal Control 1.0 (PN:101-842-900 / Qty: 1)
RNA SEQUENCING	Iso-Seq® Method for Standard Transcript Profiling	Sequel II Iso-Seq Express 9td 8dbc-1B PN: 101-901-700	1B	SMRTbell Express Template Prep Kit 2.0 (PN: 100-938-900 / Qty: 1) Iso-Seq Express Oligo Kit (PN: 101-737-500 / Qty: 1) Elution Buffer (50 mL) (PN: 101-633-900 / Qty: 1) Sequel II Binding Kit 2.1 and Internal Control 1.0 (PN:101-843-000 / Qty: 1)
	Iso-Seq Method for Long Transcript Profiling	Sequel II Iso-Seq Express Long 8dbc-1B PN: 101-901-800	1B	SMRTbell Express Template Prep Kit 2.0 (PN: 100-938-900 / Qty: 1) Iso-Seq Express Oligo Kit (PN: 101-737-500 / Qty: 1) Elution Buffer (50 mL) (PN: 101-633-900 / Qty: 1) Sequel II Binding Kit 2.0 and Internal Control 1.0 (PN:101-842-900 / Qty: 1)

† Core SMRT Sequencing consumables such as SMRT Cell and Sequencing Kits and 3rd party reagents are not included in the application bundles. For details and product recommendations

https://www.pacb.com/wp-content/uploads/Application-Consumable-Bundle-Purchasing-Guide.pdf

CORE PACBIO REAGENTS & CONSUMABLES REQUIRED FOR SMRTBELL EXPRESS LIBRARY CONSTRUCTION

SMRTbell Express Template Prep Kit 2.0

- Remove Single-Strand Overhangs
- ↓ DNA Damage Repair
- ↓ DNA End Repair / A-Tailing
- ↓ Adapter Ligation

SMRTbell Enzyme Cleanup Kit 2.0

- ↓ Nuclease Treatment*

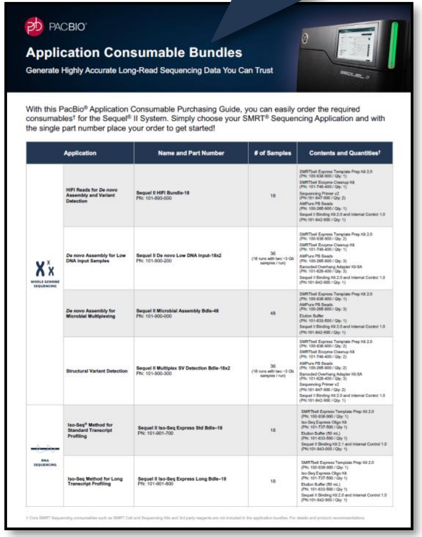
AMPure PB Beads

- ↓ Purify SMRTbell Templates**

* A Nuclease Treatment step is included in some protocols to remove non-intact SMRTbell templates

** Some protocols may specify the use of ProNex Beads (instead of AMPure PB Beads) for SMRTbell purification

PacBio **Purchasing Guide** brochure enables users to easily order required consumables needed to prepare a SMRTbell library to run a specific type of application on the Sequel II/IIe System.***



PACBIO

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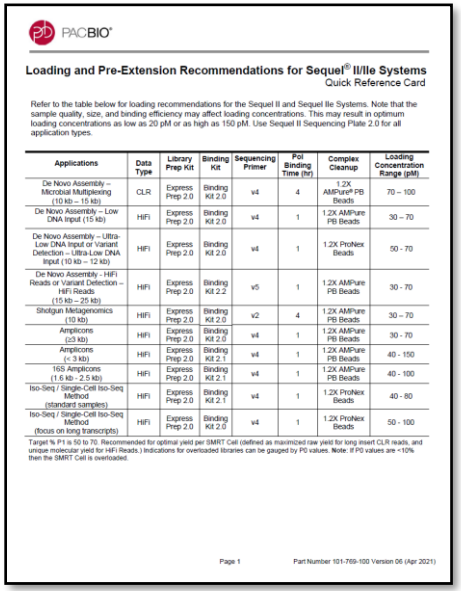
Application		Name and Part Number	# of Samples	Contents and Quantities†
WHOLE GENOME SEQUENCING	HiFi Reads for *De novo* Assembly and Variant Detection	Sequel II HiFi Bundle-18 PN: 101-893-500	18	SMRTbell Express Template Prep Kit 2.0 (PN: 100-938-900 / Qty: 1) SMRTbell Enzyme Cleanup Kit (PN: 101-746-400 / Qty: 1) Sequencing Primer v2 (PN:101-847-900 / Qty: 2) AMPure PB Beads (PN: 100-265-900 / Qty: 1) Sequel II Binding Kit 2.0 and Internal Control 1.0 (PN:101-842-900 / Qty: 1)
	De novo Assembly for Low DNA Input Samples	Sequel II De novo Low DNA Input-18x2 PN: 101-900-200	36 (18 runs with two ~3 Gb samples / run)	SMRTbell Express Template Prep Kit 2.0 (PN: 100-938-900 / Qty: 2) SMRTbell Enzyme Cleanup Kit (PN: 101-746-400 / Qty: 1) AMPure PB Beads (PN: 100-265-900 / Qty: 3) Barcoded Overhang Adapter Kit 8A (PN: 101-628-400 / Qty: 3) Sequel II Binding Kit 2.0 and Internal Control 1.0 (PN:101-842-900 / Qty: 1)
	De novo Assembly for Microbial Multiplexing	Sequel II Microbial Assembly Bdle-48 PN: 101-900-000	48	SMRTbell Express Template Prep Kit 2.0 (PN: 100-938-900 / Qty: 1) AMPure PB Beads (PN: 100-265-900 / Qty: 3) Elution Buffer (PN: 101-633-500 / Qty: 1) Sequel II Binding Kit 2.0 and Internal Control 1.0 (PN:101-842-900 / Qty: 1)
	Structural Variant Detection	Sequel II Multiplex SV Detection Bdle-18x2 PN: 101-900-300	36 (18 runs with two ~3 Gb samples / run)	SMRTbell Express Template Prep Kit 2.0 (PN: 100-938-900 / Qty: 2) SMRTbell Enzyme Cleanup Kit (PN: 101-746-400 / Qty: 2) AMPure PB Beads (PN: 100-265-900 / Qty: 2) Barcoded Overhang Adapter Kit 8A (PN: 101-628-400 / Qty: 3) Sequencing Primer v2 (PN:101-847-900 / Qty: 2) Sequel II Binding Kit 2.0 and Internal Control 1.0 (PN:101-842-900 / Qty: 1)
RNA SEQUENCING	Iso-Seq® Method for Standard Transcript Profiling	Sequel II Iso-Seq Express Std Bdle-18 PN: 101-901-700	18	SMRTbell Express Template Prep Kit 2.0 (PN: 100-938-900 / Qty: 1) Iso-Seq Express Oligo Kit (PN: 101-737-500 / Qty 1) Elution Buffer (50 mL) (PN: 101-633-500 / Qty 1) Sequel II Binding Kit 2.1 and Internal Control 1.0 (PN:101-843-000 / Qty: 1)
	Iso-Seq Method for Long Transcript Profiling	Sequel II Iso-Seq Express Long Bdle-18 PN: 101-901-800	18	SMRTbell Express Template Prep Kit 2.0 (PN: 100-938-900 / Qty: 1) Iso-Seq Express Oligo Kit (PN: 101-737-500 / Qty 1) Elution Buffer (50 mL) (PN: 101-633-500 / Qty 1) Sequel II Binding Kit 2.0 and Internal Control 1.0 (PN:101-842-900 / Qty: 1)

*** Core PacBio-branded SMRT Sequencing consumables (SMRT Cells, Sequencing Kits & SMRT Oil), plastics and other 3rd-party reagents are not included in the application bundles

SEQUEL IIe SYSTEM QUICK REFERENCE CARD – DIFFUSION LOADING AND PRE-EXTENSION RECOMMENDATIONS

Follow **SMRT Link Sample Setup & Run Design** instructions using the recommendations provided in the Quick Reference Card – Loading and Pre-Extension Time Recommendations for the Sequel II/IIe System unless specified otherwise in the relevant Procedure & Checklist



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Loading and Pre-Extension Recommendations for Sequel® II/IIe Systems
Quick Reference Card

Refer to the table below for loading recommendations for the Sequel II and Sequel IIe Systems. Note that the sample quality, size, and binding efficiency may affect loading concentrations. This may result in optimum loading concentrations as low as 20 pM or as high as 150 pM. Use Sequel II Sequencing Plate 2.0 for all application types.

Applications	Data Type	Library Prep Kit	Binding Kit	Sequencing Primer	Pol Binding Time (hr)	Complex Cleanup	Loading Concentration Range (pM)
De Novo Assembly – Microbial Multiplexing (10 kb – 15 kb)	CLR	Express Prep 2.0	Binding Kit 2.0	v4	4	1.2X AMPure® PB Beads	70 – 100
De Novo Assembly – Low DNA Input (15 kb)	HiFi	Express Prep 2.0	Binding Kit 2.0	v4	1	1.2X AMPure PB Beads	30 – 70
De Novo Assembly – Ultra-Low DNA Input or Variant Detection – Ultra-Low DNA Input (10 kb – 12 kb)	HiFi	Express Prep 2.0	Binding Kit 2.0	v4	1	1.2X ProNex Beads	50 - 70
De Novo Assembly - HiFi Reads or Variant Detection – HiFi Reads (15 kb – 25 kb)	HiFi	Express Prep 2.0	Binding Kit 2.2	v5	1	1.2X AMPure PB Beads	30 - 70
Shotgun Metagenomics (10 kb)	HiFi	Express Prep 2.0	Binding Kit 2.0	v2	4	1.2X AMPure PB Beads	30 – 70
Amplicons (≥3 kb)	HiFi	Express Prep 2.0	Binding Kit 2.0	v4	1	1.2X AMPure PB Beads	30 - 70
Amplicons (< 3 kb)	HiFi	Express Prep 2.0	Binding Kit 2.1	v4	1	1.2X AMPure PB Beads	40 - 150
16S Amplicons (1.6 kb - 2.5 kb)	HiFi	Express Prep 2.0	Binding Kit 2.1	v4	1	1.2X AMPure PB Beads	40 - 100
Iso-Seq / Single-Cell Iso-Seq Method (standard samples)	HiFi	Express Prep 2.0	Binding Kit 2.1	v4	1	1.2X ProNex Beads	40 - 80
Iso-Seq / Single-Cell Iso-Seq Method (focus on long transcripts)	HiFi	Express Prep 2.0	Binding Kit 2.0	v4	1	1.2X ProNex Beads	50 - 100

Target % P1 is 50 to 70. Recommended for optimal yield per SMRT Cell (defined as maximized raw yield for long insert CLR reads, and unique molecular yield for HiFi Reads.) Indications for overloaded libraries can be gauged by P0 values. **Note:** If P0 values are <10% then the SMRT Cell is overloaded.

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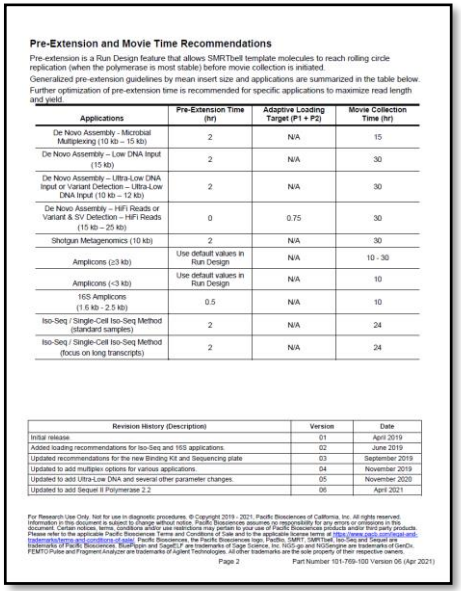

Pre-Extension and Movie Time Recommendations

Pre-extension is a Run Design feature that allows SMRTbell template molecules to reach rolling circle replication (when the polymerase is most stable) before movie collection is initiated.
Generalized pre-extension guidelines by mean insert size and applications are summarized in the table below. Further optimization of pre-extension time is recommended for specific applications to maximize read length and yield.

Applications	Pre-Extension Time (hr)	Adaptive Loading Target (P1 + P2)	Movie Collection Time (hr)
De Novo Assembly - Microbial Multiplexing (10 kb – 15 kb)	2	N/A	15
De Novo Assembly – Low DNA Input (15 kb)	2	N/A	30
De Novo Assembly – Ultra-Low DNA Input or Variant Detection – Ultra-Low DNA Input (10 kb – 12 kb)	2	N/A	30
De Novo Assembly - HiFi Reads or Variant & SV Detection – HiFi Reads (15 kb – 25 kb)	0	0.75	30
Shotgun Metagenomics (10 kb)	2	N/A	30
Amplicons (≥3 kb)	Use default values in Run Design	N/A	10 - 30
Amplicons (<3 kb)	Use default values in Run Design	N/A	10
16S Amplicons (1.6 kb - 2.5 kb)	0.5	N/A	10
Iso-Seq / Single-Cell Iso-Seq Method (standard samples)	2	N/A	24
Iso-Seq / Single-Cell Iso-Seq Method (focus on long transcripts)	2	N/A	24

Revision History (Description)	Version	Date
Initial release.	01	April 2019
Added loading recommendations for Iso-Seq and 16S applications.	02	June 2019
Updated recommendations for the new Binding Kit and Sequencing plate	03	September 2019
Updated to add multiplex options for various applications.	04	November 2019
Updated to add Ultra-Low DNA and several other parameter changes.	05	November 2020
Updated to add Sequel II Polymerase 2.2	06	April 2021

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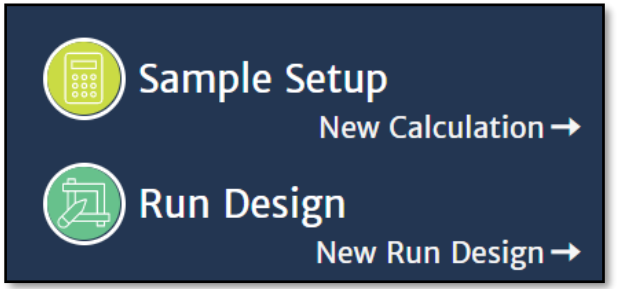
In SMRT Link v10.1, most Sample Setup and Run Design parameter fields are auto-filled with the recommended settings for each application type.



https://www.pacb.com/support/documentation/

TECHNICAL DOCUMENTATION & SOFTWARE DOWNLOAD RESOURCES

Sequel IIe System Documentation

- Sequel II and Sequel IIe Systems Operations Guide (PN 101-774-700)
- Sequel II/IIe System v10.1 Release Notes (PN 102-041-700)
- Sequel IIe System: Location of HiFi Reads Files (PN 102-110-200)
- Quick Reference Card – Loading and Pre-Extension Recommendations for the Sequel II/IIe Systems (PN 101-769-100)
- Pacific Biosciences Glossary of Terms (PN 000-710-267)

SMRT Link Documentation

- SMRT Link v10.1 Software Download Site: https://www.pacb.com/support/software-downloads/
- SMRT Link v10.1 Software Installation Instructions (PN 102-036-900)
- SMRT Link v10.1 Release Notes (PN 102-040-000)
- SMRT Link v10.1 User Guide (PN 102-037-000)
- SMRT Link Cloud Reference Guide (v10.1) (PN 102-043-900)
- SMRT Link Web Services API Use Cases (v10.1) (PN 102-040-300)

https://www.pacb.com/support/documentation/

TECHNICAL DOCUMENTATION & SOFTWARE DOWNLOAD RESOURCES (CONT.)

Sample Library Preparation Documentation

- Overview – Sequel Systems Application Options and Sequencing Recommendations (PN 101-851-300)
- Procedure & Checklist – Using AMPure PB Beads for Size-Selection (PN 101-854-900)
- Whole Genome Sequencing Applications
  - *De Novo* Assembly – HiFi Reads
    - Procedure & Checklist – Preparing HiFi SMRTbell Libraries using SMRTbell Express Template Prep Kit 2.0 (PN 101-853-100)
  - *De Novo* Assembly – Low DNA Input
    - Procedure & Checklist - Preparing SMRTbell Libraries Using Express Template Prep Kit 2.0 With Low DNA Input (PN 101-730-400)
  - *De Novo* Assembly – Ultra-Low DNA Input
    - Procedure & Checklist – Preparing HiFi SMRTbell Libraries from Ultra-Low DNA Input (PN 101-987-800)
  - Microbial *De Novo* Assembly
    - Procedure & Checklist – Preparing Multiplexed Microbial Libraries Using SMRTbell Express Template Prep Kit 2.0 (PN 101-696-100)
  - Variant Detection
    - Procedure & Checklist – Preparing HiFi SMRTbell Libraries using SMRTbell Express Template Prep Kit 2.0 (PN 101-853-100)

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TECHNICAL DOCUMENTATION & SOFTWARE DOWNLOAD RESOURCES (CONT.)

Sample Library Preparation Documentation (Cont.)

- RNA Sequencing Applications
  - Iso-Seq Method
    - Procedure & Checklist – Iso-Seq Express Template Preparation for Sequel and Sequel II Systems (PN 101-763-800)
    - Procedure & Checklist – Preparing Single-Cell Iso-Seq Libraries Using SMRTbell Express Template Prep Kit 2.0 (PN 101-892-000)
- Metagenomics Applications
  - Full-length 16S Sequencing
    - Procedure & Checklist – Amplification of Full-Length 16S Gene with Barcoded Primers for Multiplexed SMRTbell Library Preparation and Sequencing (PN 101-599-700)
  - Metagenomics Shotgun Sequencing
    - Procedure & Checklist – Preparing 10 kb Library Using SMRTbell Express Template Prep Kit 2.0 for Metagenomics Shotgun Sequencing (PN 101-800-800)
- Targeted Sequencing Applications
  - Amplicon Sequencing
    - Procedure & Checklist – Preparing SMRTbell Libraries using PacBio Barcoded Overhang Adapters for Multiplexing Amplicons (PN 101-791-700)
    - Procedure & Checklist – Preparing SMRTbell Libraries using PacBio Barcoded Universal Primers for Multiplex SMRT Sequencing (PN 101-791-800)
    - Procedure & Checklist – Preparing SMRTbell Libraries using PacBio Barcoded M13 Primers for Multiplex SMRT Sequencing (PN 101-921-300)
  - No-Amp Targeted Sequencing
    - Procedure & Checklist – No-Amp Targeted Sequencing Utilizing the CRISPR-Cas9 System (PN 101-801-500)

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TECHNICAL DOCUMENTATION & SOFTWARE DOWNLOAD RESOURCES (CONT.)

Applications Best Practices Guides

- Whole Genome Sequencing Applications
  - Application Brief: Whole genome sequencing for de novo assembly – Best Practices (PN BP102-121219)
  - Application Brief: Variant detection using whole genome sequencing with HiFi reads – Best Practices (PN BP106-092419)
  - Application Brief: Microbial whole genome sequencing – Best Practices (PN BP101-013020)
- RNA Sequencing Applications
  - Application Brief: Long-read RNA sequencing – Best Practices (PN BP103-062619)
  - Application Brief: Single-cell RNA sequencing with HiFi reads - Best Practices (PN BP109-102020)
- Metagenomics Applications
  - Application Brief: Metagenomic sequencing with HiFi reads – Best Practices (PN BP108-030220)
- Targeted Sequencing Applications
  - Application Brief: Targeted sequencing for amplicons – Best Practices (PN BP105-071919)
  - Application Brief: No-Amp targeted sequencing – Best Practices (PN BP107-092319)

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TECHNICAL DOCUMENTATION & SOFTWARE DOWNLOAD RESOURCES (CONT.)

Applications Technical Training Documentation

- Whole Genome Sequencing Applications
  - Technical Overview: HiFi Library Preparation Using SMRTbell Express Template Prep Kit 2.0 (PN 101-855-400)
  - Technical Overview: Low DNA Input Library Preparation Using SMRTbell Express Template Prep Kit 2.0 (PN 101-781-000)
  - Technical Overview: Ultra-Low DNA Input Library Preparation Using SMRTbell Express Template Prep Kit 2.0 (101-998-000)
  - Technical Overview: Multiplexed Microbial Library Preparation Using SMRTbell Express Template Prep Kit 2.0 (PN 101-742-600)
- RNA Sequencing Applications
  - Technical Overview: Iso-Seq Express Library Preparation Using SMRTbell Express Template Prep Kit 2.0 (PN 101-814-400)
  - Technical Overview: Single-Cell Iso-Seq Library Preparation Using SMRTbell Express TPK 2.0 (PN 101-925-400)
- Metagenomics Applications
  - Technical Overview: Metagenomics Shotgun Library Preparation Using SMRTbell Express Template Prep Kit 2.0 (PN 101-894-900)
  - Technical Overview: Full-Length 16S Library Preparation Using SMRTbell Express Template Prep Kit 2.0 (PN 101-916-900)
- Targeted Sequencing Applications
  - Technical Overview: Multiplexed Amplicon Library Preparation Using SMRTbell Express Template Prep Kit 2.0 (PN 101-814-300)
  - Technical Overview: No-Amp Targeted Sequencing Library Preparation and Data Analysis Technical Overview (PN 101-840-800)
  - PacBio HiFiViral Workflow Overview: Multiplexed Amplicon Library Preparation for Full-Viral Genome Sequencing of SARS-CoV-2 (PN 102-084-800)

https://www.pacb.com/support/documentation/

TECHNICAL DOCUMENTATION & SOFTWARE DOWNLOAD RESOURCES (CONT.)

Data Analysis Documentation

- Analysis Procedure – Multiplexed Microbial Assembly with SMRT Link v8.0 and SMRTbell Express Template Prep Kit 2.0 (PN 101-855-300)
- Analysis Procedure – No-Amp Data Preparation and Repeat Analysis (PN 101-801-400)
- Brief Primer and Lexicon for PacBio SMRT Sequencing Webpage (v10.0)
- PacBio Bioinformatics File Formats Documentation Webpage (v10.0)
- SMRT Analysis Barcoding Overview (v9.0) (PN 101-923-200)
- SMRT Tools Reference Guide (v10.1) (PN102-037-300)

Sequencing Performance Troubleshooting Documentation

- Guide – Step-by-Step Run Performance Evaluation (For Sequel II and Sequel IIe Systems) (PN 101-993-600)

https://www.pacb.com/support/documentation/

TECHNICAL DOCUMENTATION & SOFTWARE DOWNLOAD RESOURCES (CONT.)

Technical Notes

- Technical Note: Preparing samples for PacBio whole genome sequencing for de novo assembly – Collection and storage (PN TN100-040518)
- Technical Note: Preparing DNA for PacBio HiFi sequencing – Extraction and quality control (PN TN101-081420)

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DNA SAMPLE PREPARATION ONLINE RESOURCE

Literature resource for sample collection and DNA extraction protocol references

The listing below is a collection of publications by the scientific community describing extraction protocols for high-molecular weight DNA followed by PacBio sequencing. When possible, the links point directly to the methods section (or supplementary information).

Animals | Plants | Fungi | Protists

If you have protocols you would like to share, or have questions about DNA extraction for PacBio sequencing, contact ExtractDNA@pacb.com.

Animals

- Invertebrates
  - Panova2016 – DNA extraction protocols for whole-genome sequencing in marine organisms
  - *microinvertebrates*
    - Laumer2020 – protocols for diverse meiofauna species, including *C. elegans* & *Castrella truncata* (SMRT Le… confer… presentation)
  - *arthropods*
    - arachnids
      - Guerrero2019 – The Pacific Biosciences de novo assembled genome dataset from a parthenogenetic New Zealand wild population of the longhorned tick, *Haemaphysalis longicornis* Neumann, 1901
      - Liu2019 – DNA Methylation Patterns in the Social Spider, *Stegodyphus dumicola*
      - Burgess2018 – Draft genome assembly of the sheep scab mite, *Psoroptes ovis*
      - Randall2018 – The draft genome assembly of *Dermatophagoides pteronyssinus* supports identification of novel allergen isoforms in *Dermatophagoides* species

Methods

Sample collection

A female yellowbelly pufferfish (Fig. 2), reared in the fish breeding centre of Fujian Normal University in Fuzhou City of Fujian Province was used for genome sequencing and assembly. Fresh white muscle, eye, skin, gonad, gut, liver, kidney, blood, gall bladder and air bladder tissues were collected and quickly frozen in liquid nitrogen for one hour. White muscle tissues were used for DNA sequencing for genome assembly, while all tissues were used for transcriptome sequencing.

Fig. 2

A picture of the yellowbelly pufferfish used in the genome sequencing and assembly.

DNA and RNA sequencing

Genomic DNA from white muscle tissue was extracted using the

Plants

- Panova2016 – DNA extraction protocols for whole-genome sequencing in marine organisms (algae)

Fungi

- Faure2019 – Long-Read Genome Sequence of the Sugar Beet Rhizosphere Mycoparasite *Pythium oligandrum*
- Nagappan2018 – Improved nucleic acid extraction protocols for *Ganoderma boninense*, *G. miniatocinctum* and *G. tornatu*
- Schwessinger2017 – Extraction of high molecular weight DNA from fungal rust spores for long read sequencing
- Solomon2016 – Robust and effective methodologies for cryopreservation and DNA extraction from anaerobic gut fungi
- Sonnenberg2016 – A detailed analysis of the recombination landscape of the button mushroom *Agaricus bisporus var. bisporus*

www.ExtractDNAforPacBio.com

PacBio does not assume responsibilities/guarantees for these external publications/protocols, but we are happy to help as best as we can to guide / connect. Please contact **ExtractDNA@pacb.com** for more discussions around your particular species & sequencing project!

SEQUEL II AND IIe SYSTEM BEST PRACTICES OVERVIEW GUIDES

Whole Genome Sequencing for *De novo* Assembly

Variant Detection Using Whole Genome Sequencing with HiFi Reads

No-Amp Targeted Sequencing

RNA Sequencing / Single-Cell RNA Sequencing

16S / Metagenomics Shotgun Sequencing of Complex Populations

https://www.pacb.com/smrt-science/smrt-resources/pacbio-literature/

BEST PRACTICES: WHOLE GENOME SEQUENCING FOR *DE NOVO* ASSEMBLY

SMRTbell Template Preparation

- Start with unamplified genomic DNA input (≥5 µg for a ~3-Gb sample genome size) from any sample type (blood, tissue, cell lines)
- Using SMRTbell Express Template Prep Kit 2.0, prepare libraries for HiFi sequencing of up to 16 samples at a time with manual prep, or 96 samples using an automation-friendly workflow
- Enrich for ~15 kb - 20 kb inserts with size selection.

Sequence on the Sequel II or IIe Systems

- With highly accurate long reads (HiFi reads) from the Sequel II or IIe Systems, you can assemble up to a 2 Gb genome in a single SMRT Cell 8M for ~$1,300* or scale up for larger genomes
- Run up to 200 samples (2 Gb) per year, per Sequel II or IIe System
- Sequence to desired coverage depth based on the complexity of the genome sample:
  - Recommend aiming for 10- to 15-fold HiFi read coverage per haplotype for phased *de novo* assembly

Data Analysis Solutions with the PacBio Analytical Portfolio

- Use SMRT Link Genome Assembly (powered by IPA), or open-source tools including HiCanu or hifiasm to assemble and phase the genome
- Example datasets are available at pacb.com/dataset

** Read lengths, reads/data per SMRT Cell 8M and other sequencing performance results vary based on sample quality/type and insert size.*

† Prices, listed in USD, are approximate and may vary by region. Pricing includes library and sequencing reagents run on a Sequel II or IIe System and does not include instrument amortization or other reagents.

BEST PRACTICES: VARIANT DETECTION USING WHOLE GENOME SEQUENCING WITH HIFI READS

SMRTbell Template Preparation

- Start with unamplified genomic DNA input (≥5 µg for a ~3-Gb sample genome size) from any sample type (blood, tissue, cell lines)
- Using SMRTbell Express Template Prep Kit 2.0, prepare libraries for HiFi sequencing of up to 16 samples at a time with manual prep, or 96 samples using an automation-friendly workflow
- Enrich for ~15 kb - 18 kb inserts with size selection. Inserts larger than this range may reduce read and variant calling accuracy

Sequence on the Sequel II or IIe Systems

- With highly accurate long reads (HiFi reads) from the Sequel II or IIe Systems you can comprehensively detect variants in 100s to 1000s of genomes in a year
- Sequence to desired coverage based on study needs:*
  - Aim for ≥15-fold HiFi read coverage of a Human genome for variant detection applications
  - Recommend 2 SMRT Cells 8M to achieve ≥15-fold coverage of a human genome for comprehensive variant detection for $2600†

Data Analysis Solutions with the PacBio Analytical Portfolio

- Detect all variant types – including SNVs, indels, SVs, and CNVs – with the highest precision and recall using SMRT Link Structural Variant Calling analysis application (powered by pbsv) and Google DeepVariant (PacBio model)
  - Use joint calling in pbsv and DeepVariant for multiple samples
- Expand variant calling into previously inaccessible regions of the genome, including repetitive regions and medically relevant genes that are difficult to map
- Phase small variants into phase blocks using WhatsHap and Confirm variant calls visually with IGV and GenomeRibbon

** Read lengths, reads/data per SMRT Cell 8M and other sequencing performance results vary based on sample quality/type and insert size.*

† Prices, listed in USD, are approximate and may vary by region. Pricing includes library and sequencing reagents run on a Sequel II or IIe System and does not include instrument amortization or other reagents.

BEST PRACTICES: RNA SEQUENCING (ISO-SEQ ANALYSIS)

SMRTbell Template Preparation

- Prepare full-length cDNA from 300 ng of total RNA using the NEBNext Single Cell/Low Input cDNA Synthesis & Amplification Module kit
- Use the SMRTbell Express Template Prep Kit 2.0 to prepare libraries in one day
- Multiplex up to 12 samples with barcoding

Sequence on the Sequel II or IIe Systems

- Maximize output and turn-around-time with adjustable sequencing parameters
  - Sequel IIe System: 24 hour movies with 2 hours pre-extension is recommended
- Use the Sequel IIe System to generate up to 4 million* full-length, non-concatemer (FLNC) reads per SMRT Cell 8M
- Scale throughput based on project needs – With a single SMRT Cell 8M you can:
  - Characterize a whole transcriptome
  - Multiplex multiple tissues for genome annotation

Data Analysis Solutions with the PacBio Analytical Portfolio

- Generate highly accurate long reads (HiFi reads), with single-molecule resolution using circular consensus sequencing (CCS) mode
- Use the Iso-Seq analysis in SMRT Link to output high-quality, full-length transcript FASTA sequences, with no assembly required, to characterize transcripts and splice variants
- Run Iso-Seq analysis with or without a reference genome, and annotate the genome using community tools such as SQANTI2, TAMA, and LoReAn

** Read lengths, reads/data per SMRT Cell 8M and other sequencing performance results vary based on sample quality/type and insert size.*

BEST PRACTICES: SINGLE-CELL RNA SEQUENCING (SINGLE-CELL ISO-SEQ ANALYSIS)

SMRTbell Template Preparation

- Enrich for single-cell cDNA using a single-cell sorting platform that generates full-length cDNA*
- Start library preparation with at least 160 ng of input cDNA (post-single-cell platform PCR reaction) for 1-2 SMRT Cells 8M
- Use the SMRTbell Express Template Prep Kit 2.0 to prepare libraries in one day

Sequence on the Sequel II or IIe Systems

- Use HiFi sequencing on the Sequel II or IIe Systems to generate 3 million full-length transcript reads from one SMRT Cell 8M to obtain ~1,000 unique molecules for 3,000 single cells**
- Use a 24-hour movie collection time with a 2-hour pre-extension time
- For human samples, run up to 240 SMRT Cell 8M/year at a cost of ~$1,300/SMRT Cell 8M, excluding single-cell enrichment cost†

Data Analysis Solutions with the PacBio Analytical Portfolio

- Analyze HiFi reads which allow accurate single-cell barcode and UMI identification
- Use the single-cell Iso-Seq analysis tools on GitHub to output high-quality, full-length transcript FASTA sequences per UMI, with no assembly required, to characterize transcript variants for each cell

** Number of usable reads, containing the UMI and cell barcode, vary by single-cell platform. Any platform that generates full-length cDNA is compatible with the single-cell RNA sequencing workflow.*

*** Read lengths, reads/data per SMRT Cell type and other sequencing performance results vary based on single-cell platform, sample quality/type and insert size.*

† Prices, listed in USD, are approximate and may vary by region. Pricing includes library and sequencing reagents run on a Sequel II or IIe System and does not include instrument amortization or other reagents.

BEST PRACTICES: NO-AMP TARGETED SEQUENCING

SMRTbell Template Preparation

- Start with high-quality genomic DNA (~1-20 µg / SMRT Cell)
- Prepare SMRTbell libraries in 2-days with stream-lined protocol
  - Block 5' & 3' ends to prevent off-target ligation
  - Use custom-design guide RNAs to enrich for target regions of interest
  - Multiplex up to 48 samples SMRT Cell 8M using barcoded adapters to analyze 5 or more targeted regions per sample

Sequence on the Sequel II or IIe Systems

- Sequence multiplexed targets and/or samples on the Sequel II or IIe Systems using the latest chemistry*
  - Run up to 48 samples per SMRT Cell 8M at ~$82-118/sample on the Sequel IIe System†

Data Analysis Solutions with the PacBio Analytical Portfolio

- Use command line tools to perform de-multiplexing and circular consensus sequencing (CCS) analysis to generate highly accurate long reads (HiFi reads)
- Output data in FASTQ format for results summary reporting on repeat counts and on-target rates
- Visualize results with IGV and command-line scripts for easy review of repeat count of both alleles, mosaic characterization, identification of interruption sequences and CRISPR / Cas9 off-targets

** Read lengths, reads/data per SMRT Cell 8M and other sequencing performance results vary based on sample quality/type and insert size.*

† Prices, listed in USD, are approximate and may vary by region. Pricing includes library and sequencing reagents run on a Sequel II or IIe System and does not include instrument amortization or other reagents.

BEST PRACTICES: MICROBIAL WHOLE GENOME SEQUENCING

LIBRARY PREP

SMRTbell Template Preparation

- Start with 1.0 µg of high-quality input gDNA per microbial sample and construct a 10 – 15 kb library using SMRTbell Express TPK 2.0
- Reduce costs by multiplexing samples to assemble most bacterial genomes into 5 contigs or fewer, exclusive of plasmids
  - Simplify equimolar pooling with Microbial Multiplexing Calculator
  - Adjust multiplexing depth to balance cost per genome with genome completeness
- Note: Closure of class III complexity genomes with large repeat regions may require 20–30 kb library preparations & size-selection and may not be suitable for multiplexing

SMRT SEQUENCING

Sequence on the Sequel II or IIe Systems

- Maximize output and turn-around-time with adjustable sequencing parameters*
- Multiplex up to 48 isolates per SMRT Cell 8M for $70/sample† with a 15 hour collection time
- Recommend generating ≥30-fold unique molecular coverage (UMC) per microbial genome

DATA ANALYSIS

Data Analysis Solutions with the PacBio Analytical Portfolio

- Use SMRT Link for fully automated demultiplexing, assembly, circularization, and polishing of both chromosomes and plasmids to produce gold standard references
- Achieve high-quality consensus accuracies >99.999%
- Detect and annotate active m6A and m4C Restriction-Modification system motifs with the ‘Base Modification and Motif Analysis’ application in SMRT Analysis [m4C (≥25-fold coverage per strand) / m6A (≥25-fold coverage per strand)]

** Read lengths, reads/data per SMRT Cell 8M and other sequencing performance results vary based on sample quality/type and insert size.*

† Prices, listed in USD, are approximate and may vary by region. Pricing includes library and sequencing reagents run on a Sequel II or IIe System and does not include instrument amortization or other reagents.

BEST PRACTICES: 16S AMPLICON SEQUENCING

SMRTbell Template Preparation

- Perform library construction with SMRTbell Express TPK 2.0
- PacBio full-length 16S protocol available with recommended barcoded 16S primer sequences; OR use third-party Shoreline Biome kits for DNA extraction and PCR amplification
- Multiplex up to 192 16S samples per SMRT Cell 8M

Sequence on the Sequel II or IIe Systems

- Produce HiFi reads with circular consensus sequencing (CCS)
  - Generate up to 2 Million 16S HiFi (Q30) reads per Sequel SMRT Cell 8M* using a 10-hour movie collection time
- Recommend generating ≥8000 HiFi reads per multiplexed 16S sample for analysis

Data Analysis Solutions with the PacBio Analytical Portfolio

- De-multiplex barcodes within SMRT Link GUI or on the command line
- Output data in standard file formats, (BAM and FASTA/Q) for seamless integration with downstream analysis tools
- Analyze 16S HiFi data using third-party analysis tools like Shoreline Biome SB Analyzer or DADA2

** Read lengths, number of reads, data per SMRT Cell 8M, and other sequencing performance results vary based on sample quality/type and insert size, among other factors*

BEST PRACTICES: METAGENOMICS SHOTGUN SEQUENCING

SMRTbell Template Preparation

- Start with recommended amount of input gDNA per sample (1.5 µg) and construct a 10 kb library with SMRTbell Express TPK 2.0
- The size distribution of the starting genomic DNA is critical for shearing and PacBio recommends working with samples where the majority of the input gDNA is greater than 15 kb whenever possible.

Sequence on the Sequel II or IIe Systems

- Produce HiFi (≥Q20) reads with circular consensus sequencing (CCS)
  - Generate up to 2.4 Million 16S HiFi (Q20) reads per Sequel SMRT Cell 8M* using a 30-hour movie collection time
- Target coverage recommendations:
  - 5-fold coverage (~3000 HiFi reads) of least abundant species for profiling intact genes and operons
  - 20-fold coverage (~12,000 HiFi reads) for near-complete genome assemblies

Data Analysis Solutions with the PacBio Analytical Portfolio

- De-multiplex barcodes within SMRT Link GUI or on the command line
- Analyze metagenomic shotgun HiFi reads using third-party analysis tools
  - Perform taxonomic classification and functional gene profiling using QIIME and MEGAN
  - Perform gene prediction and discovery using FragGeneScan and Prodigal
  - Perform metagenomic shotgun assembly directly with HiFi reads using Canu
- Bin contigs and plasmids originating from the same strain by leveraging epigenetic signatures

** Read lengths, number of reads, data per SMRT Cell 8M, and other sequencing performance results vary based on sample quality/type and insert size, among other factors*

PACBIO DOCUMENTATION RESOURCES

DOCUMENTATION

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Asset Type

SDS (36) | Guide & Overview (31) | Package Insert (28) | Procedure & Checklist (24) | Release Notes (12) | Quick Reference Card (5) | Developer Documentation (4) | Experimental Protocol (2) | User Bulletin (1)

Workflow Step

Template Preparation (65) | Sequencing (38) | Analysis (35) | Binding (27) | Sample Preparation (6)

Product Line

Sequel System (115) | Sequel II System (104) | PacBio RS II (24) | Sequel IIe System (23)

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TOGGLE ALL DESCRIPTIONS

Overview – Sequel Systems Application Options and Sequencing Recommendations — *April 23, 2021*

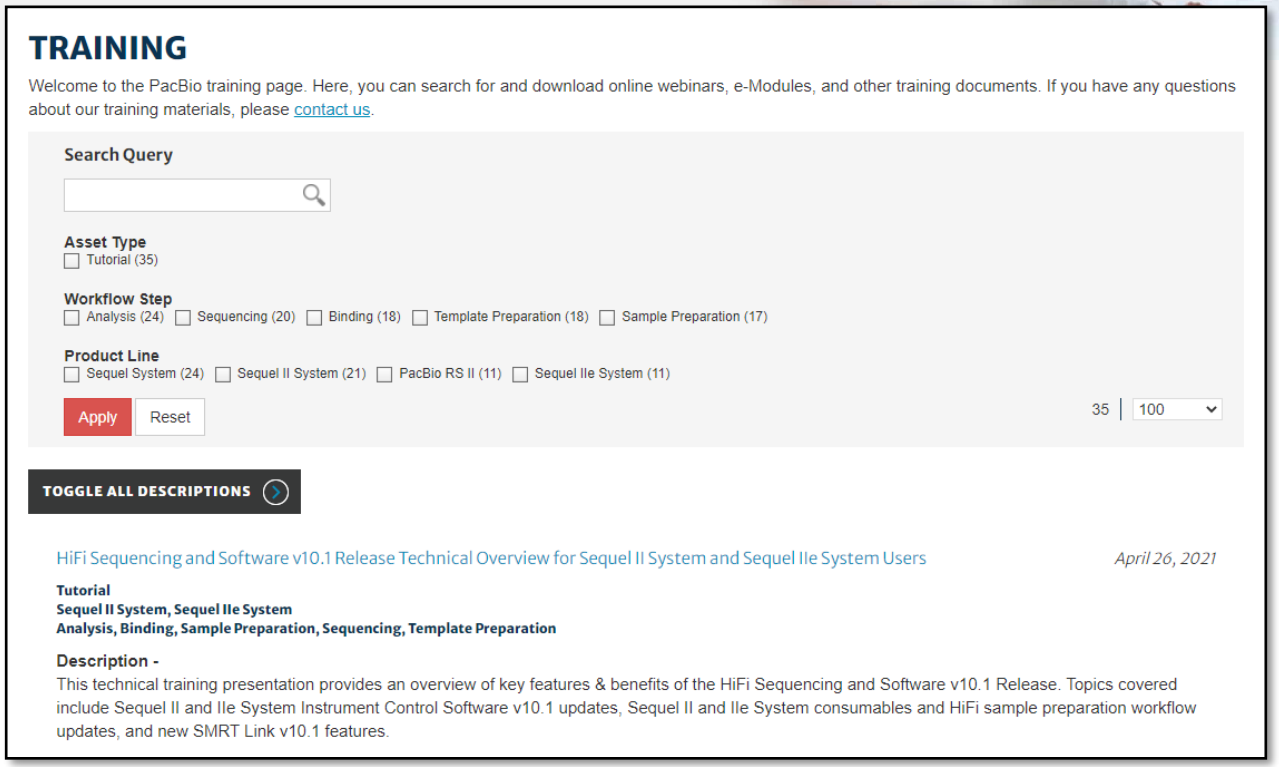
Guide & Overview
Sequel II System, Sequel IIe System, Sequel System
Analysis, Binding, Sequencing, Template Preparation

Description -
This document provides high-level application workflow guidance for preparing sample, sequencing on the Sequel or Sequel II System, and analysis.

- PacBio Documentation page allows you to search for and download the latest guides, protocols, product information, and more.

https://www.pacb.com/support/documentation/

PACBIO TRAINING RESOURCES



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Workflow Step
☐ Analysis (24) ☐ Sequencing (20) ☐ Binding (18) ☐ Template Preparation (18) ☐ Sample Preparation (17)

Product Line
☐ Sequel System (24) ☐ Sequel II System (21) ☐ PacBio RS II (11) ☐ Sequel IIe System (11)

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HiFi Sequencing and Software v10.1 Release Technical Overview for Sequel II System and Sequel IIe System Users — *April 26, 2021*

Tutorial
Sequel II System, Sequel IIe System
Analysis, Binding, Sample Preparation, Sequencing, Template Preparation

Description -
This technical training presentation provides an overview of key features & benefits of the HiFi Sequencing and Software v10.1 Release. Topics covered include Sequel II and IIe System Instrument Control Software v10.1 updates, Sequel II and IIe System consumables and HiFi sample preparation workflow updates, and new SMRT Link v10.1 features.

- PacBio Training page allows you to search for and download video tutorials and other training resources

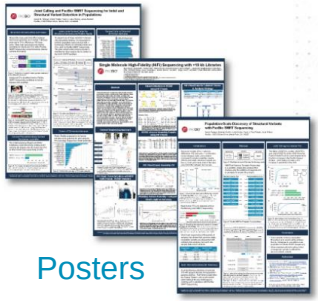
http://www.pacb.com/support/training/

SMRT SEQUENCING RESOURCES

A Foundation for the Future of Genomic Discovery

Scientific Publications

PacBio Literature



Posters

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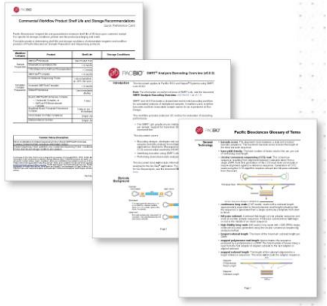
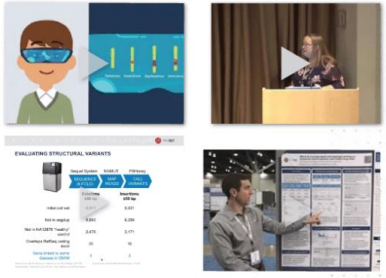


Documentation



Video Gallery

https://www.pacb.com/smrt-science/smrt-resources/

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