HiFi sequencing and software v11.0 release: Technical overview for Sequel II and Sequel IIe system users

Sequel II and IIe Systems ICS v11.0 / SMRT Link v11.0

PN 102-399-900 Version 01 (April 2022)

HiFi sequencing and software v11.0 release

Technical overview

A. New consumables for SMRTbell library preparation & sequencing preparation (ABC)

- SMRTbell prep kit 3.0 (SPK 3.0)
- AMPure PB bead size selection kit
- Binding kit 3.1 & cleanup beads
- Binding kit 3.2 & cleanup beads

B. SPK 3.0 WGS sample preparation updates

- SPK 3.0 WGS workflow overview
- SPK 3.0 WGS example performance data

C. SMRT Link Sample Setup updates

- New High-throughput (HT) Sample Setup mode overview
- Sample setup HT mode annealing / binding / cleanup (ABC) and DNA internal control dilution procedure

D. SMRT link run design updates

- GUI updates and new default CCS analysis output changes
- New 5mC detection option
- New heteroduplex detection option
- New AAV application type option
- New on-instrument demultiplexing option

E. SMRT Link Run QC updates

- New instrument status view
- New 5mC-specific report plots

F. SMRT Link SMRT Analysis updates

- Updated GUI nomenclature
- Updated microbial genome analysis application

G. APPENDIX: Technical documentation & applications support resources

- Sequel IIe system documentation
- SMRT Link & other data analysis documentation
- Application technical overviews
- DNA extraction literature
- Sample preparation literature
- Example PacBio data sets

Summary overview of key features, improvements & customer benefits

System v11.0 key feature updates

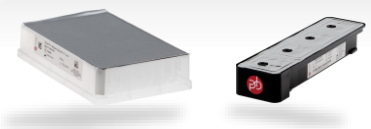
 **ICS v11.0** Sequel II and IIe systems only

 **SMRT Link v11.0** Sequel II and IIe systems only

Workflow step	What's New	Notes
DNA sample prep	 • Human whole blood DNA extraction tech note	• Additional DNA extraction guidance coming
SMRTbell library prep	  • SMRTbell prep kit 3.0 / AMPure PB bead size selection kit • Updated protocols (WGS / amplicon / HiFiViral / Iso-Seq method) and new AAV protocol	• New reagents to accelerate, unify & streamline library prep • TPK 1.0 & TPK 2.0 still available
Sample setup (ABC)	  • Sequel II binding kit 3.1 & 3.2 • Sample Setup HT mode	• Uses Polymerases 2.1/2.2 in a new kit configuration to unify/streamline ABC • Binding kits 2.1/2.2 still available
Run design	  • On-instrument 5mC / heteroduplex detection (HD) / demultiplexing • AAV sequencing mode	• 5mC, HD and demultiplexing capabilities are also available in SMRT Link for Sequel II system
Sequencing	 • SMRT Cell 8M single-use tray • Sequel II sequencing kit 2.0 (1 rxn)	• Functionally the same basic parts as before with updated kit configurations to enable increased run flexibility
Run QC	 • Instrument status monitoring • New 5mC report plots	• Provides real-time ZMW loading performance information during sequencing runs
SMRT Analysis	 • Refreshed GUI emphasizes HiFi data • Microbial genome analysis application	• Deprecates obsoleted subread (CLR) data-based workflows

System v11.0 release key benefits

New products enable simplification & acceleration of sample preparation workflows and consumables ordering



New consumables for SMRTbell library preparation & sequencing preparation

- **New streamlined reagent kit products** for SMRTbell library construction and annealing/binding/cleanup (ABC) of constructed libraries for HiFi sequencing
- Improved consolidation of required core template prep reagents into fewer product bundles reduces complexity and allows for **easier consumables ordering**

Updated SMRTbell library construction workflows to support SPK 3.0

- New whole genome sequencing (WGS) library prep protocol using SPK 3.0 enables **reduced workflow times** and **reduced DNA input** requirements; and **eliminates gel-based size selection requirements** for constructing large/small genome WGS and shotgun metagenomic libraries
- Amplicon, Iso-Seq method, and HiFiViral SARS-CoV-2 library prep protocols are updated to support SPK 3.0 and enable **simpler, more unified sample prep workflows** across different applications
- New adeno-associated virus (AAV) library prep protocol using SPK 3.0 support biopharma and **AAV gene editing vector R&D**

New single-reaction consumables products

- **New Sequel II sequencing kit 2.0 (1 rxn)** and **SMRT Cell 8M single use tray** products
- Enables **improved run schedule flexibility** for lower-throughput customers (no need to wait for 4 samples to accrue) or smaller project sizes

System v11.0 release key benefits (cont.)

New software enables better data, faster – and provides push-button access to the epigenome

Updated software for Sequel II and IIe systems

ICS v11.0

- ICS v11.0 is available for Sequel II and IIe systems only

SMRT Link v11.0

- SMRT Link v11.0 requires Sequel II and IIe systems to be upgraded to ICS v11.0

SMRT Link v11.0 Feature updates

Sample Setup	**New High-Throughput mode** feature provides a more streamlined workflow to efficiently process single samples or multiple samples in parallel using automation
Run Design	**New on-instrument 5mC calling** in CpG motifs. **New heteroduplex detection** option detects and resolves heteroduplex reads **New on-instrument demultiplexing** option **New adeno-associated virus (AAV) application** type option
Run QC	**New Instrument Status view** in Run QC provides real-time ZMW loading performance information about PacBio instruments that are connected to SMRT Link
SMRT Analysis	**New Microbial Genome Analysis application** in SMRT Analysis for *de novo* assembly and base modification detection analysis of microbial genomes using HiFi reads
PacBio SMRT® Link	**General usability and user experience improvements** to SMRT Link graphical user interface to emphasize HiFi sequencing, QC and analysis and deprecate obsoleted subread (CLR) data-based workflows

New consumables for SMRTbell library prep and sequencing prep (ABC)

New consumables for SMRTbell library preparation

New products enable simplification & acceleration of sample preparation workflows and consumables ordering

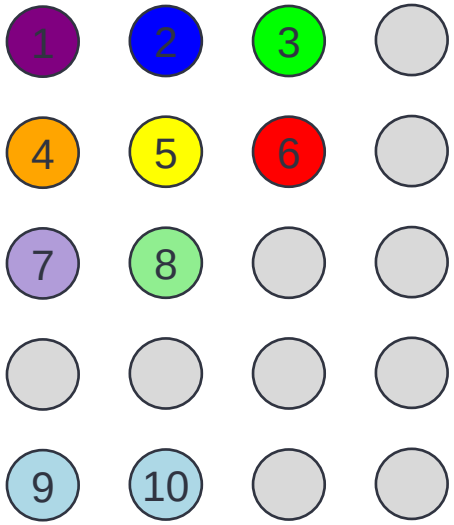
New consumables for SMRTbell library preparation					
New consumables for library construction			**New consumables for size selection**		
SMRTbell prep kit 3.0 Contains sufficient reagents to perform 24 SMRTbell library preparation reactions		102-182-700	**AMPure PB beads size selection kit** Contains sufficient reagents to perform 48 bead-based size selection reactions		102-182-500
	**SMRTbell prep kit 3.0** *Includes all core reagents needed for SMRTbell library construction*			**AMPure PB beads [5 mL]** *Used for performing bead-based library size selection of WGS SMRTbell libraries*	
	**SMRTbell cleanup beads [10 mL]** *Used for routine DNA purification during SMRTbell library construction*			**Elution buffer [50 mL]** *Used for preparing diluted AMPure PB beads for size selection*	
	**Low TE buffer [10 mL]** *Used for shearing genomic DNA samples for WGS SMRTbell library construction*				

Note: Individual kit components are not sold separately. Complete kits can be purchased using the part numbers shown here – refer to your regional price list for details.

SMRTbell prep kit 3.0 configuration

- SPK 3.0 (102-182-700) contains all reagents needed to perform 24 SMRTbell library preparation reactions

1. Repair Buffer
2. End Repair Mix
3. DNA Repair Mix
4. SMRTbell Adapter
5. Ligation Mix
6. Ligation Enhancer
7. Nuclease Buffer
8. Nuclease Mix
9. Elution Buffer
10. Elution Buffer



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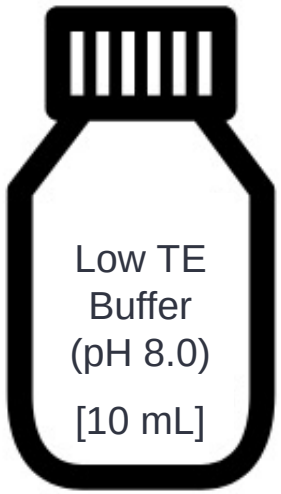

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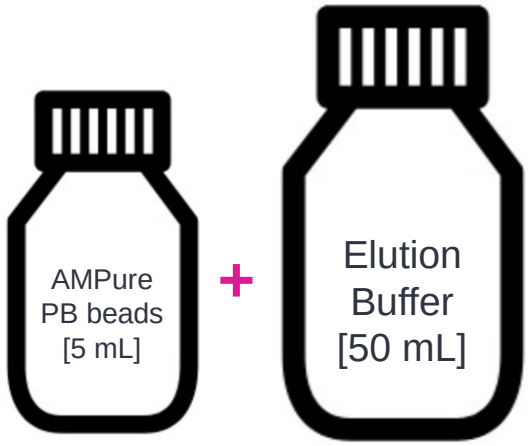
Note: Primer for annealing is now included in Sequel II Binding Kit 3.1/3.2 to streamline ABC workflows.

AMPure PB beads size selection kit

- Bundle of AMPure PB beads and Elution buffer
- Depletes library fragments <5 kb
- Faster, more scalable, and lower capital equipment costs than automated gel-based size selection tools



Recommended

	Size-selection Beads	Gel Cassettes
Size Cutoff	Fixed (>5 kb)	✓ Adjustable
Hands-on Time	✓ Low	✓ Low
Run Time	✓ Minutes	Hours
Automation	✓ Standard liquid handler	Separate instrument
Cost	✓ Low	High
DNA Recovery	✓ High	Low
DNA Input to Library Prep per SMRT® Cell	✓ 1 µg	1.7 µg

Note: Gel size selection is still supported (adds 2 or more hours of run time and increases DNA input requirements). Refer to Technical Note: Alternative Size Selection Methods for SMRTbell prep kit 3.0 (102-326-503), which provides detailed guidance for size selection of WGS libraries using automated gel-based DNA size selection tools.

New consumables for sequencing preparation (ABC)

New products enable simplification & acceleration of sequencing preparation workflows and consumables ordering

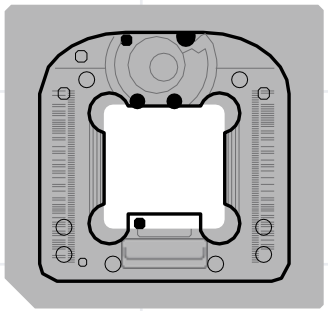
New consumables for sequencing preparation

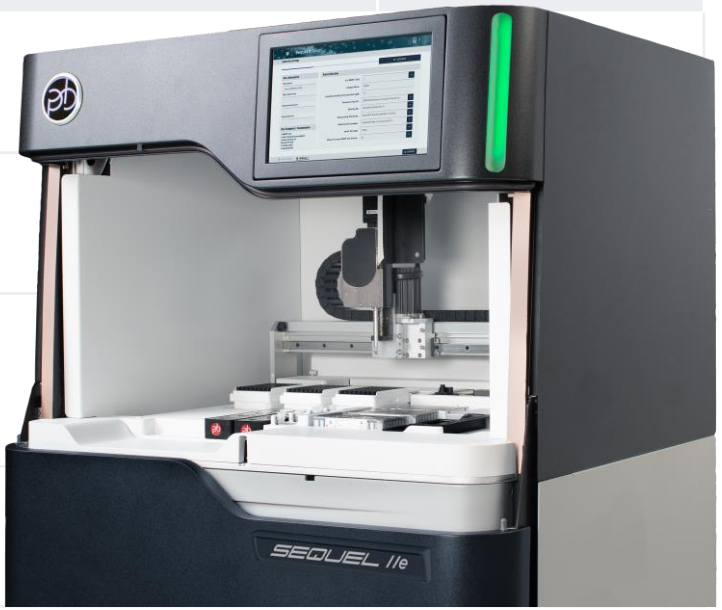
New binding kit consumables

Binding kit 3.1 and cleanup beads Contains sufficient reagents to perform 24 binding reactions with Polymerase 3.1 for samples with inserts <3 kb		102-333-400
	**Sequel II binding kit 3.1 and DNA internal control 3.1** *Include Sequencing primer 3.1, Polymerase 2.1 and DNA internal control 3.1*	
	**SMRTbell cleanup beads [10 mL]** *Used for SMRTbell template cleanup after polymerase binding step*	
Binding kit 3.2 and cleanup beads Contains sufficient reagents to perform 24 binding reactions with Polymerase 3.2 for samples with inserts ≥3 kb		102-333-300
	**Sequel II binding Kit 3.2 and DNA internal control 3.2** *Include Sequencing primer 3.2, Polymerase 2.2 and DNA internal control 3.2*	
	**SMRTbell cleanup beads [10 mL]** *Used for SMRTbell template cleanup after polymerase binding step*	

New SMRT Cell and sequencing kit consumables

	**SMRT Cell 8M single use tray** Contains 1 SMRT Cell to be used with the Sequel II and IIe Systems.	102-182-500
	**Sequel II sequencing kit 2.0 (1 rxn)** Contains sufficient reagents to support sequencing on one SMRT Cell 8M	102-194-400





Summary comparison of new System v11.0 release consumables versus previous consumables

New System v11.0 consumables provide an enhanced user experience with simplified, unified, scalable workflows

New v11.0 product	Number of reactions	Part number	Replaces*
SMRTbell prep kit 3.0	24	102-182-700	• Express TPK 2.0 • Enzyme cleanup kit 2.0 • AMPure PB beads/ProNex
Binding kit 3.1 and cleanup beads	24 (up to 96 SMRT Cells 8M)	102-333-400	• Binding kit 2.1 and internal control 1.0 • AMPure PB beads/ProNex beads
Binding kit 3.2 and cleanup beads	24 (up to 96 SMRT Cells 8M)	102-333-300	• Binding kit 2.2 and internal control 1.0 • AMPure PB beads/ProNex beads • Sequencing primer v5
AMPure PB beads size-selection kit	48	102-182-500	• Gel-based size-selection
Sequel II sequencing kit 2.0 (1 rxn)	1	102-194-400	• N/A
SMRT Cell 8M single-use tray	1	102-281-700	• N/A

* Older consumable products listed in this table will continue to be available for purchase until further notice.

SPK 3.0 WGS sample preparation updates

SPK 3.0 WGS sample preparation workflow overview

Comparison of SPK 3.0 to previous SMRTbell library prep Kits

SMRTbell prep kit 3.0 enables simpler, more unified sample prep workflows across different applications

We recommend using **SPK 3.0** for all supported applications

	SMRTbell prep kit 3.0 *(NEW)*	SMRTbell express template prep kit 2.0
Part Numbers	1. SMRTbell prep kit 3.0	1. SMRTbell express TPK 2.0 2. Enzyme cleanup mix 2.0 3. AMPure PB beads
Samples	24	18
Time, gDNA to SMRTbell library	4.5 hours	8 hours
Input DNA / 30-fold human	3 µg	5 µg
Applications*	WGS, shotgun metagenomics, amplicons, Iso-Seq method, viral sequencing	WGS Amplicons Iso-Seq
Kit cost	$1,800	$1,779
Sample cost	$75	$99

New whole genome sequencing (WGS) library prep protocol using SPK 3.0 enables **reduced workflow times**, uses **reduced DNA input** requirements and **eliminates gel-based size selection** requirements

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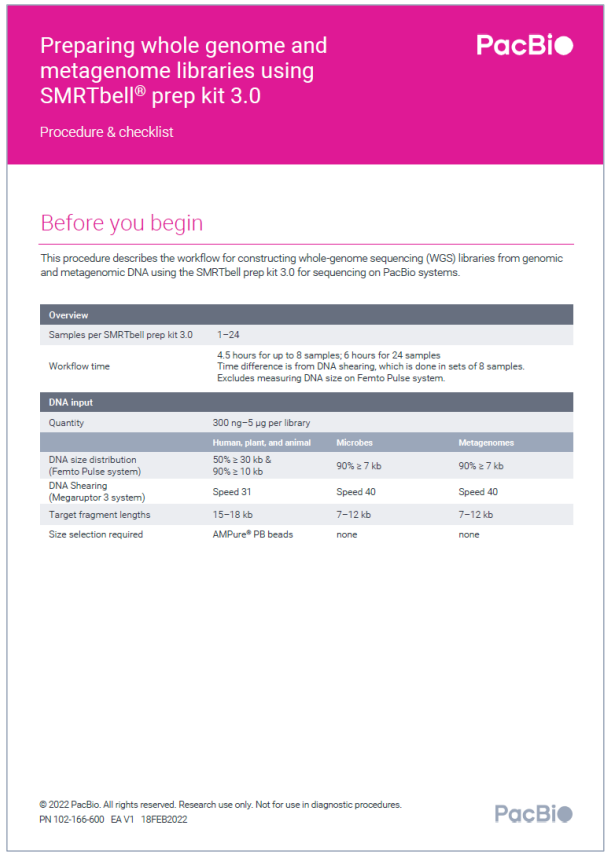
* Future protocol updates for single-cell Iso-Seq and ultra-low DNA input will support SPK 3.0.

New HiFi library preparation protocol using SMRTbell prep kit 3.0 for whole genome and metagenomic shotgun sequencing applications

Procedure & checklist – Preparing whole genome and metagenome libraries using SMRTbell prep kit 3.0 (102-166-600) describes a method for constructing SMRTbell libraries that are suitable for generating HiFi reads on the Sequel II and IIe Systems for WGS and metagenomic shotgun sequencing applications.

Procedure Highlights

- Uses SMRTbell prep kit 3.0 (102-182-70) and supports high-throughput processing using 300 ng – 5 μg of input genomic DNA amounts
  - We recommend starting with **≥1 μg of input DNA per SMRT Cell 8M** (or ~3 μg for up to a 3 Gb WGS sample to enable running 3 SMRT Cells 8M)
- Multiplexing of samples can be performed using SMRTbell barcoded adapter plate 3.0 (102-009-200)
- Recommend shearing high-quality gDNA using a Megaruptor 3 System (Diagenode)
  - 15 kb – 18 kb target insert size for large (plant / animal / human) genomes
  - 7 kb – 12 kb target insert size for small (microbial) genomes
  - 7 kb – 12 kb target insert size for shotgun metagenomic samples
- 4.5-hour workflow time to process up to 8 samples from shearing to size selection (6 hours for 24 samples)
  - Time difference is from DNA shearing, which can be performed in sets of 8 samples.
  - Excludes time needed for DNA sizing QC analysis using a Femto Pulse system.
- WGS SMRTbell libraries can be size-selected using AMPure PB beads without the need for third-party equipment



Preparing whole genome and metagenome libraries using SMRTbell® prep kit 3.0

Procedure & checklist

PacBio

Before you begin

This procedure describes the workflow for constructing whole-genome sequencing (WGS) libraries from genomic and metagenomic DNA using the SMRTbell prep kit 3.0 for sequencing on PacBio systems.

Overview			
Samples per SMRTbell prep kit 3.0	1–24		
Workflow time	4.5 hours for up to 8 samples; 6 hours for 24 samples Time difference is from DNA shearing, which is done in sets of 8 samples. Excludes measuring DNA size on Femto Pulse system.		
DNA input			
Quantity	300 ng–5 μg per library		
	Human, plant, and animal	**Microbes**	**Metagenomes**
DNA size distribution (Femto Pulse system)	50% ≥ 30 kb & 90% ≥ 10 kb	90% ≥ 7 kb	90% ≥ 7 kb
DNA Shearing (Megaruptor 3 system)	Speed 31	Speed 40	Speed 40
Target fragment lengths	15–18 kb	7–12 kb	7–12 kb
Size selection required	AMPure® PB beads	none	none

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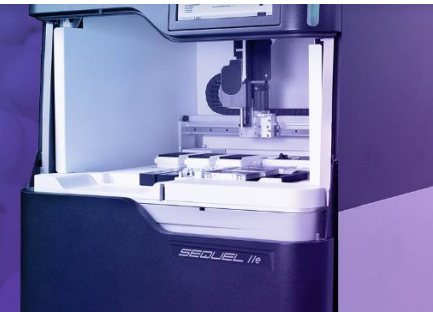
PacBio Documentation (102-166-600)

APPLICATIONS

WHOLE GENOME SEQUENCING

De Novo assembly & variant detection
Microbial assembly
Shotgun metagenomics



SPK 3.0 WGS workflow enables faster library construction times with lower DNA input amounts compared to SMRTbell express TPK 2.0

SPK 3.0 WGS protocol reduces walk-away times and consolidates several enzymatic repair reactions & A-tailing into a single reaction

Lower DNA input
(3 µg instead of 5 µg)

SMRTbell express TPK 2.0

5 µg DNA input for 3 Gb genome

	Walk-away time
DNA Shearing	1.5 hrs (2 cycles)
Remove Single Stranded Overhangs	15 min
DNA Damage Repair	30 min
DNA End Repair/A-tailing	40 min
Adapter Ligation	1 hr
1X AMPure PB Bead Cleanup	30 min
Nuclease Treatment	30 min
1X AMPure PB Bead Cleanup	30 min
Size Selection	2-4 hrs
1X AMPure PB Bead Cleanup	30 min

~8 hrs*

SMRTbell prep kit 3.0

3 µg DNA input for 3 Gb genome

	Walk-away time
DNA Shearing	45 min (1 cycle)
1X SMRTbell Bead Cleanup	20 min
Repair and A-tailing	35 min
Adapter Ligation	30 min
1X SMRTbell Bead Cleanup	20 min
Nuclease Treatment	15 min
AMPure PB Beads Size Selection	30 min

~3 hrs 15 min*

Only **one shearing cycle** needed instead of two

Only **one Repair & A-tailing Rx** performed instead of multiple Rx

Faster adapter ligation Rx
(30 min instead of 1 hr)

Faster nuclease Rx
(15 min instead of 30 min)

Faster size selection by using AMPure PB beads instead of a gel-based system

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* **Excludes** hands-on time and additional time required to perform DNA sizing QC using Femto Pulse and DNA concentration QC

New SMRTbell prep kit 3.0 consolidates all required core template prep reagents into a single orderable part number

SMRTbell prep kit 3.0 WGS workflow *(NEW)*

SMRTbell prep kit 3.0 (102-182-700) *(NEW)*

Included with SPK 3.0 :

- ☑ **Library construction reagents (24 Rx)**
  - Repair buffer, End repair mix, DNA repair mix
  - SMRTbell adapter
  - Ligation mix, Ligation enhancer
  - Other buffers
- ☑ **SMRTbell cleanup beads**
- ☑ **Nuclease mix**

Shear DNA (1 cycle)
↓
1.0X SMRTbell bead cleanup
↓
DNA repair / A-tailing
↓
Adapter ligation
↓
1.0X SMRTbell bead cleanup
↓
Nuclease treatment
↓
Size selection (AMPure PB beads)**

~3 hrs 15 min*

** Note: If performing size selection, use AMPure PB bead size selection kit (102-182-500)

SMRTbell express TPK 2.0 WGS workflow

SMRTbell express TPK 2.0 (100-938-900)

Included with SE TPK 2.0:

- ☑ **Library construction reagents (18 Rx)**
  - DNA prep buffer, NAD, DNA prep additive, DNA prep enzyme, DNA damage repair mix v2, End prep mix
  - SMRTbell overhang adapter v3
  - Ligation mix, Ligation additive, Ligation enhancer
  - Other buffers
- ☒ **AMPure PB beads** (separate purchase required)
- ☒ **SMRTbell enzyme cleanup kit 2.0** (separate purchase required)

Sheared DNA (2 cycles)
↓
Remove ssDNA overhangs
↓
DNA damage repair
↓
DNA end repair / A-tailing
↓
Adapter ligation
↓
1.0X AMPure PB bead cleanup
↓
Nuclease treatment
↓
1.0X AMPure PB bead cleanup
↓
Size selection (PippinHT System)

~8 hrs*

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* **Excludes** hands-on time and additional time required to perform DNA sizing QC using Femto Pulse and DNA concentration QC

New/updated protocols using SPK 3.0 & Binding Kit 3.1/3.2

All WGS applications except ultra-low DNA input now use the **same** protocol

Protocol consolidation with SPK 3.0 unifies sample prep workflows across different application use cases

Main application	Application subtype / Supported use case	Template prep kit(s)	Binding kit	Procedure & checklist for SMRTbell library prep*	
Whole Genome Sequencing	**Large genome WGS, microbial genome WGS, low DNA input & shotgun metagenomics**	**SPK 3.0**	**Binding Kit 3.2**	*Preparing whole genome and metagenome libraries using SMRTbell prep kit 3.0* [102-166-600]	1
	Ultra-Low DNA Input Sequencing[1]	SMRTbell express TPK 2.0	**Binding Kit 3.2**	*Preparing HiFi SMRTbell libraries from ultra-low DNA input* [101-987-800]	
Viral Sequencing	**HiFiViral SARS-CoV-2**	**SPK 3.0**	**Binding Kit 3.1**	*PacBio HiFiViral high-throughput multiplexing for full-viral genome sequencing of SARS-CoV-2* [102-396-100]	2
	AAV Sequencing	**SPK 3.0**	**Binding Kit 3.1**	*Preparing multiplexed AAV SMRTbell libraries using SMRTbell prep kit 3.0* [102-126-400]	3
RNA Sequencing	**Iso-Seq Method (Bulk)**	**SPK 3.0**	**Binding Kit 3.1** **Binding Kit 3.2**	*Preparing Iso-seq libraries using SMRTbell prep kit 3.0* [102-396-000]	4
	Single-Cell Iso-Seq Method[1]	SMRTbell express TPK 2.0	**Binding Kit 3.1** **Binding Kit 3.2**	*Preparing single-cell Iso-seq libraries using SMRTbell express TPK 2.0* [101-892-000]	
Metagenomics	Full-length 16S Sequencing[2]	**SPK 3.0**	**Binding Kit 3.1**	*Preparing multiplexed amplicon libraries using SMRTbell prep kit 3.0* [102-359-000] (using recommended PacBio barcoded 16S primer sequences)	
Targeted Sequencing	**Amplicon Sequencing** (Barcoded adapters or barcoded gene-specific primers)	**SPK 3.0**	**Binding Kit 3.1** **Binding Kit 3.2**	*Preparing multiplexed amplicon libraries using SMRTbell prep kit 3.0* [102-359-000]	5
	Amplicon Sequencing (Barcoded M13 primers)	**SPK 3.0**	**Binding Kit 3.1** **Binding Kit 3.2**	*Preparing multiplexed amplicon libraries using PacBio barcoded M13 primers and SMRTbell prep kit 3.0* [101-921-300]	6

*** Note: SMRTbell express TPK 2.0 protocols are still supported and available:**

- Preparing HiFi SMRTbell Libraries using SMRTbell Express Template Prep Kit 2.0 [101-853-100]
- Preparing Multiplexed Microbial Libraries Using SMRTbell Express Template Prep Kit 2.0 [101-696-100]
- Preparing HiFi Libraries from Low DNA Input Using SMRTbell Express Template Prep Kit 2.0 [101-730-400]
- Preparing 10 kb Library Using SMRTbell Express Template Prep Kit 2.0 for Metagenomics Shotgun Sequencing [101-800-800]
- Preparing SMRTbell Libraries using PacBio Barcoded Universal Primers for Multiplex SMRT Sequencing [101-791-800]

[1] Ultra-low DNA input and Single-cell Iso-Seq method protocols will be updated to support SPK 3.0 in the future

[2] Can use multiplexed amplicon SPK 3.0 protocol 102-359-000 to prepare **16S** and **HLA** SMRTbell libraries

PacBio

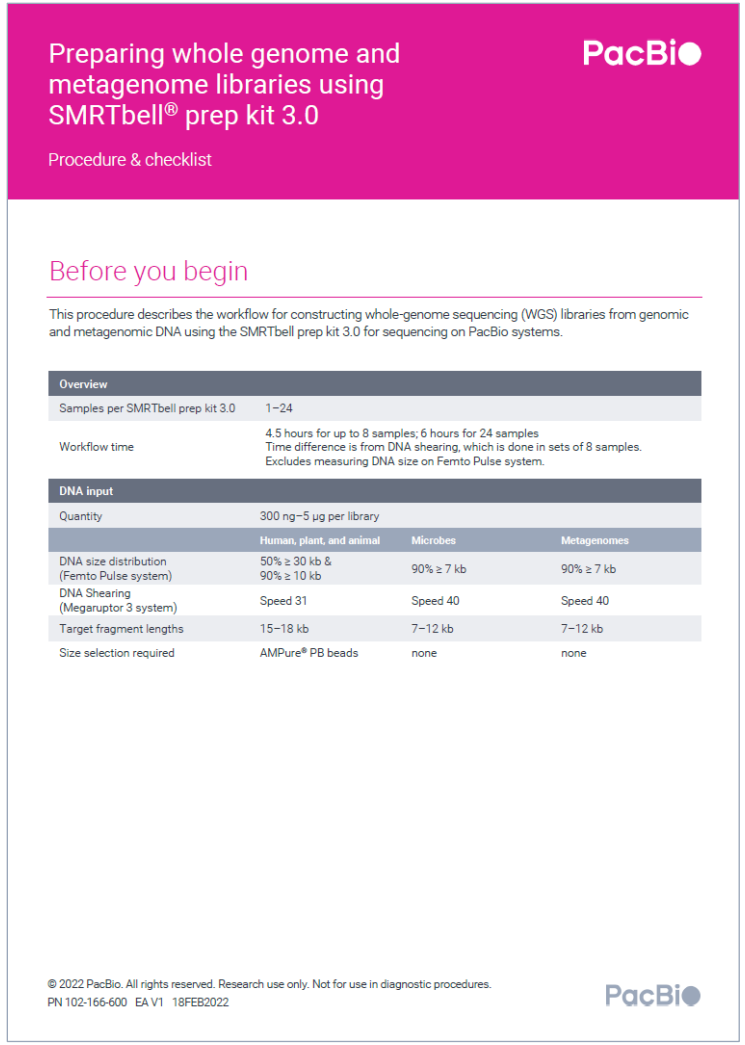
SPK 3.0 WGS sample preparation workflow details

Procedure & checklist – Preparing whole genome and metagenome libraries using SMRTbell prep kit 3.0

Procedure & checklist 102-166-600 describes a method for constructing SMRTbell libraries using SMRTbell prep kit 3.0 that are suitable for generating high-accuracy long reads on the Sequel II and IIe systems for whole genome sequencing (*de novo* assembly, variant detection, microbial multiplexing) and shotgun metagenomic sequencing

Protocol Contents

1. Recommendations for gDNA quantification and sizing QC.
2. Recommendations for shearing gDNA to the desired target mode size using the Megaruptor 3 system (Diagenode).
3. Enzymatic steps for preparation of a WGS SMRTbell library using SMRTbell prep Kit 3.0 (102-182-700). (Instructions for preparing multiplexed samples using SMRTbell barcoded adapter plate 3.0 (102-009-200) are also provided.)
4. Instructions for size-selection of WGS SMRTbell libraries using AMPure PB bead size selection. (Size selection is not required for microbial WGS and metagenomic shotgun libraries where retention of shorter fragments is desired.)
5. Guidance for pooling barcoded WGS SMRTbell libraries for multiplexed sequencing on a single SMRT Cell.



Preparing whole genome and metagenome libraries using SMRTbell® prep kit 3.0

PacBio

Procedure & checklist

Before you begin

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DNA input			
Quantity	300 ng–5 µg per library		
	Human, plant, and animal	**Microbes**	**Metagenomes**
DNA size distribution (Femto Pulse system)	50% ≥ 30 kb & 90% ≥ 10 kb	90% ≥ 7 kb	90% ≥ 7 kb
DNA Shearing (Megaruptor 3 system)	Speed 31	Speed 40	Speed 40
Target fragment lengths	15–18 kb	7–12 kb	7–12 kb
Size selection required	AMPure® PB beads	none	none

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PacBio

PacBio Documentation (102-166-600)

SPK 3.0 WGS library prep and sequencing workflow timing overview

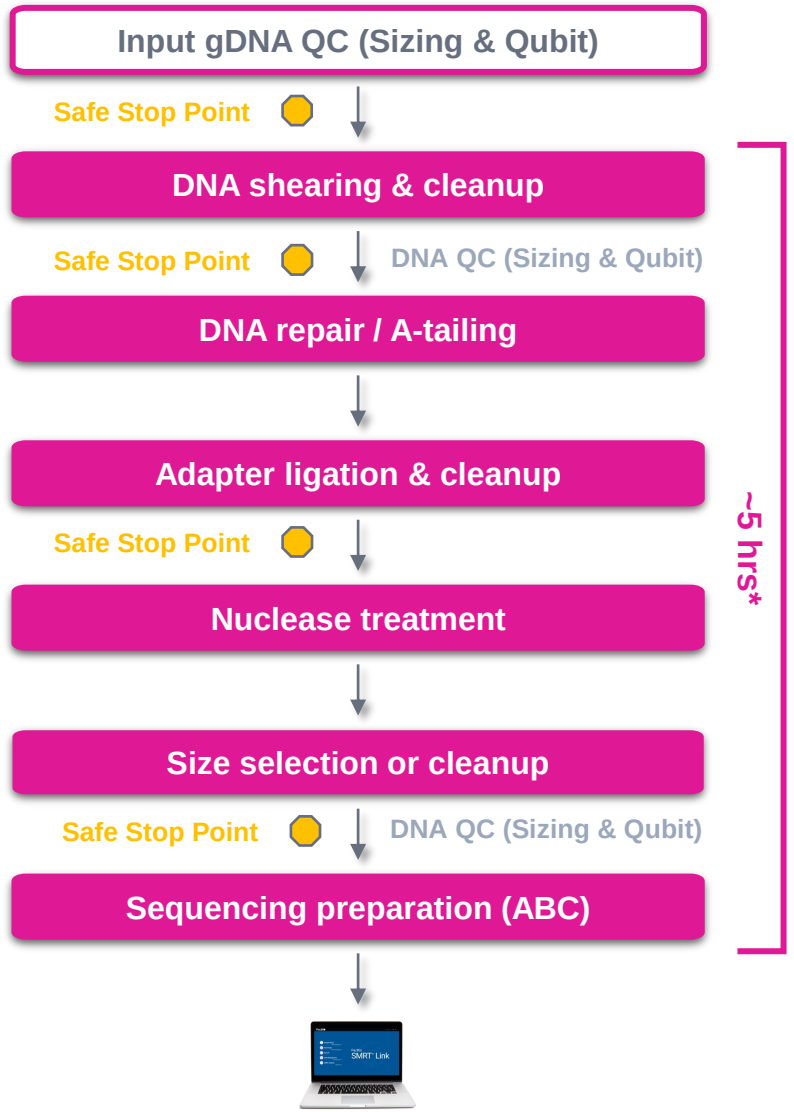
Go from DNA shearing to sequencing preparation in 1 day with SPK 3.0

Workflow step		Hands-on (min)	Walk-away (min)
DNA shearing*	DNA shearing (1 Megaruptor 3 cycle)	5	45
	1.0X SMRTbell bead cleanup	5	20
SMRTbell library construction*	DNA repair / a-tailing	5	35
	Adapter ligation (barcoded or non-barcoded adapter)	5	30
	1.0X SMRTbell bead cleanup	5	20
	Nuclease treatment	5	15
	AMPure PB bead size selection	10	30
Total		**40 min**	**3.3 hrs**

Workflow step		Hands-on (min)	Walk-away (min)
Sequencing preparation (ABC)	Primer annealing (Sequel II primer 3.2)	5	15
	Polymerase binding (Sequel II binding kit 3.2)	5	15
	Complex cleanup (1.2X SMRTbell cleanup beads)	5	20
Total		**15 min**	**0.83 hrs**

SPK 3.0 WGS Workflow

(102-166-600)



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* **Excludes** additional time required to perform DNA sizing QC using Femto Pulse and DNA concentration QC

Input genomic DNA QC recommendations for WGS library construction using SMRTbell prep kit 3.0

- WGS library construction using SMRTbell prep kit 3.0 requires **high-quality, high-molecular weight genomic DNA.***
- Prior to library preparation, evaluate the **quantity** and **size distribution** of the input gDNA to determine whether it is suitable for the protocol.
- For each input gDNA sample:
  - ❑ Measure concentration and total mass of DNA with a **Qubit High Sensitivity dsDNA Assay** system (Thermo Fisher Scientific)
  - ❑ Measure DNA size distribution with a **Femto Pulse** system (Agilent)
  - ❑ Proceed with SMRTbell library construction if the **gDNA sample quality** is acceptable (see Table below)

Sample type	Input DNA metric	Requirement	Notes
All	**Per Library**	300 ng – 5 µg	• Starting with low DNA input amounts approaching ~300 ng may in some cases produce insufficient amounts of SMRTbell library to load at concentrations that optimize sequencing data yield. • For multiplexing applications, generally aim to use ≥300 ng of DNA input per sample, with a total mass ≥1 µg across all samples
All	**Per SMRT Cell 8M**	≥1 µg	• Start with ≥1 µg of total input DNA per SMRT Cell 8M (for a single sample or across multiple samples when pooling) to enable generation of sufficient library to load at concentrations that optimize sequencing data yield.
Large genome (Animal/plant/human)	**Longer than 30 kb**	≥50%	• Required to achieve target fragment lengths after DNA shearing. • For large genome samples, the Femto Pulse Genome Quality Number (GQN) at 30 kb should be ≥5.0. (Not applicable to microbial and metagenomic samples)
Large genome (Animal/plant/human)	**Longer than 10 kb**	≥90%	• Required for effective AMPure PB bead size selection. • For large genome samples, the GQN at 10 kb should be ≥9.0.
Small genome (microbial/metagenomic)	**Longer than 7 kb**	≥90%	• For microbial and metagenomic samples, the input DNA should be at least as large as the recommended insert lengths of 7–12 kb with a GQN at 7 kb ≥9.0. • Any degradation present should be due to shearing from the extraction process (e.g., bead beating) and not from poor sample handling or storage, or biochemical processes

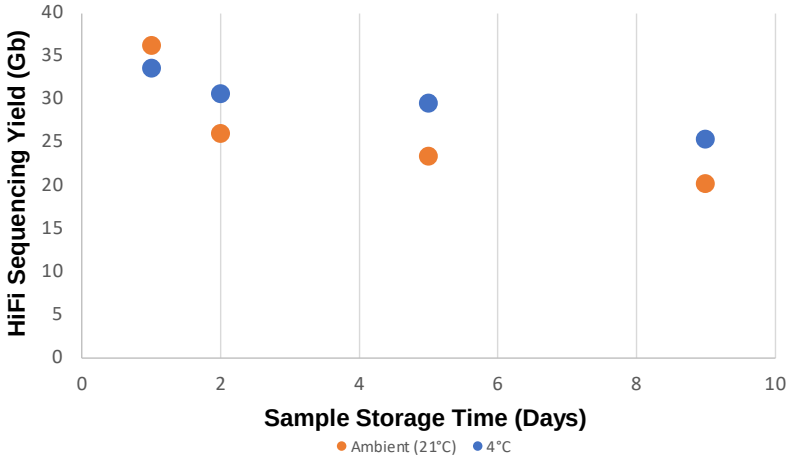
* See Technical note: Sample preparation for PacBio HiFi sequencing from human whole blood (102-326-500)

NEW DNA extraction techical note: Sample preparation for PacBio HiFi sequencing from human whole blood (102-326-500)

Provides best practices for handling human whole blood samples* to generate optimal sequencing performance

Technical note 102-326-500 discusses the effect of anticoagulant, sample storage time, storage conditions, and white blood cell count on the sequencing performance of DNA extracted using the Nanobind CBB Big DNA Kit (NB-900-001-01)

Nanobind CBB Big DNA Kit (NB-900-001-01) for isolating HMW DNA from cells, bacteria, & blood.



For optimal HiFi yield and read length performance, store human whole blood samples for **fewer than 2 days** at 4°C.

Stage	Variable	Best practice for PacBio HiFi sequencing
Before DNA extraction	Sample type	Human whole blood
	Anticoagulant	Potassium EDTA (K_2EDTA)
	Sample storage temperature	4 ± 3°C
	Sample storage time	≤ 2 days from collection to extraction
DNA extraction	Volume of whole blood	200 µL
	White blood cell (WBC) count	≥ 4 × 10^6 cells/mL for ≥ 3 µg of DNA
	DNA extraction method	Nanobind CBB Big DNA kit
After DNA extraction	DNA storage	Rest 1 day at ambient temperature, then store at 4 ± 3°C
	DNA size distribution	• 90% of DNA ≥ 10 kb (genomic quality number at 10 kb ≥ 9.0) • 50% of DNA ≥ 30 kb (genomic quality number at 30 kb ≥ 5.0)
	UV absorbance	• A260/280 nm ≥ 1.7 • A260/230 nm ≥ 1.5

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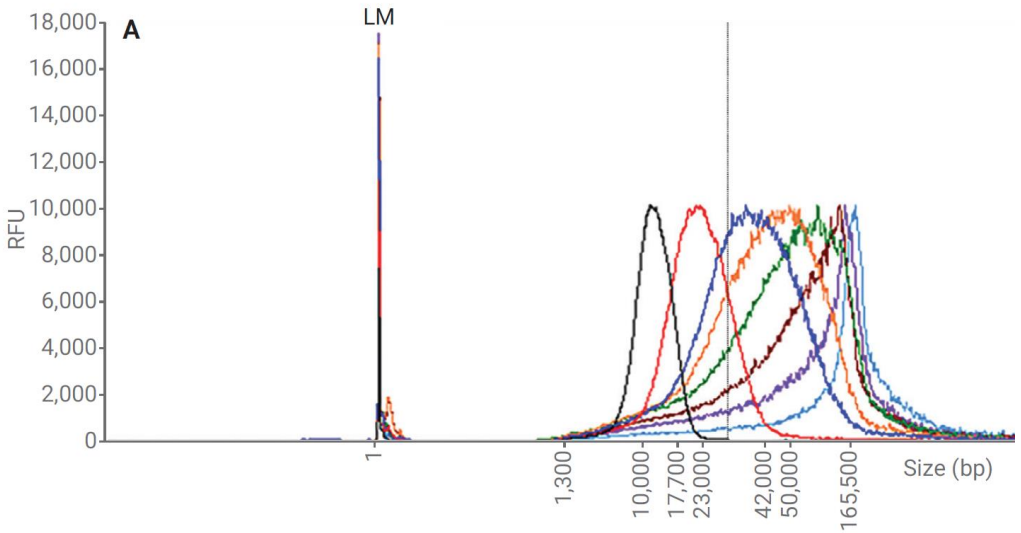
* For general DNA extraction recommendations see Technical note: Preparing DNA for PacBio HiFi sequencing – Extraction and quality control (102-193-651)

The Femto Pulse System is recommended for DNA sizing QC of genomic DNA for WGS applications

- Femto Pulse System (Agilent) is highly recommended for DNA sizing QC of input genomic DNA and SMRTbell libraries
  - Enables sizing of gDNA samples ranging from 1,300 bp to 165 kb
  - Requires <1 ng of sample DNA
  - Can analyze up to 12 samples in <1.5 hours
- The Femto Pulse system can be used in place of traditional pulse-field gel electrophoresis (PFGE) to quickly assess the initial integrity of genomic DNA, evaluate shears, determine appropriate size-selection thresholds, and conduct final QC before preparing libraries for SMRT Sequencing

Femto Pulse outputs quality metrics such as the **Genomic Quality Number (GQN)*** to quickly score the integrity of HMW gDNA

Ave. Smear Size (bp)	GQN Set at 30 kb
12,147	0
23,339	1.5
45,304	6.4
57,789	7.1
73,267	7.8
94,045	7.8
109,968	8.2
164,292	8.8



Femto Pulse System

Femto Pulse system offers a simplified QC workflow to generate SMRTbell libraries for WGS sequencing in **reduced time, and conserves sample** by using femtogram ranges of input DNA

* See Agilent Application Note – Quality Metrics for Nucleic Acids with the Agilent Fragment Analyzer and Femto Pulse Systems (5994-0521EN)

The Megaruptor 3 system is recommended for shearing genomic DNA For WGS applications

- Megaruptor 3 system (Diagenode) is highly recommended for DNA shearing*
  - Up to 8 samples can be sheared in parallel in ~45 minutes for high-throughput applications
  - Achieving the same size distribution across multiple samples provides more consistent sequencing performance
- Recommended library insert size distributions and Megaruptor 3 shear speed settings to use for different WGS applications are summarized on Page 7 in the procedure
  - Bring input gDNA to a final volume of **100 – 130 µL** with **Low TE buffer** [10 mM Tris-HCl (pH 8.0) + 0.1 mM EDTA] to target a DNA concentration of **3 – 39 ng/µL (ideal: 30 ng/µL)**
  - Perform shearing (**1 cycle**) using the conditions described in the table below

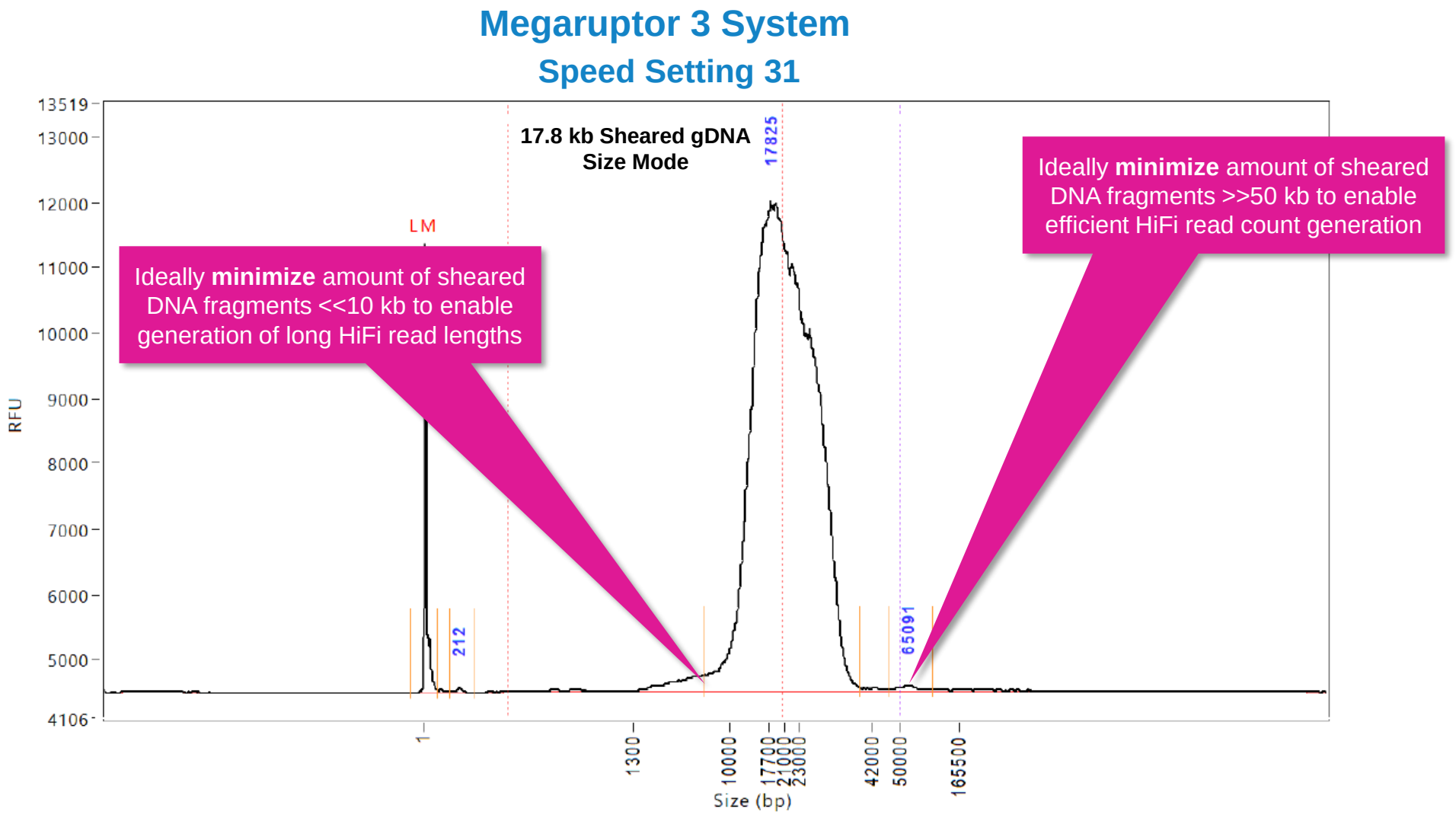
Megaruptor 3 System

Application	Recommended library Insert size (mode)	Recommended Megaruptor 3 shear speed setting
Animal / plant / human WGS	15 kb - 18 kb	31
Microbial WGS or shotgun metagenomics	7 kb - 12 kb	40

Because the response of individual gDNA samples can differ, **optimization of shearing conditions** may be needed to achieve the desired fragment distribution

*** 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 Note: Covaris g-TUBE DNA Shearing for SMRTbell prep kit 3.0 (102-326-501) or contact PacBio Technical Support or your local Field Applications Scientist.

Example Megaruptor 3 shearing results for a human genomic DNA sample

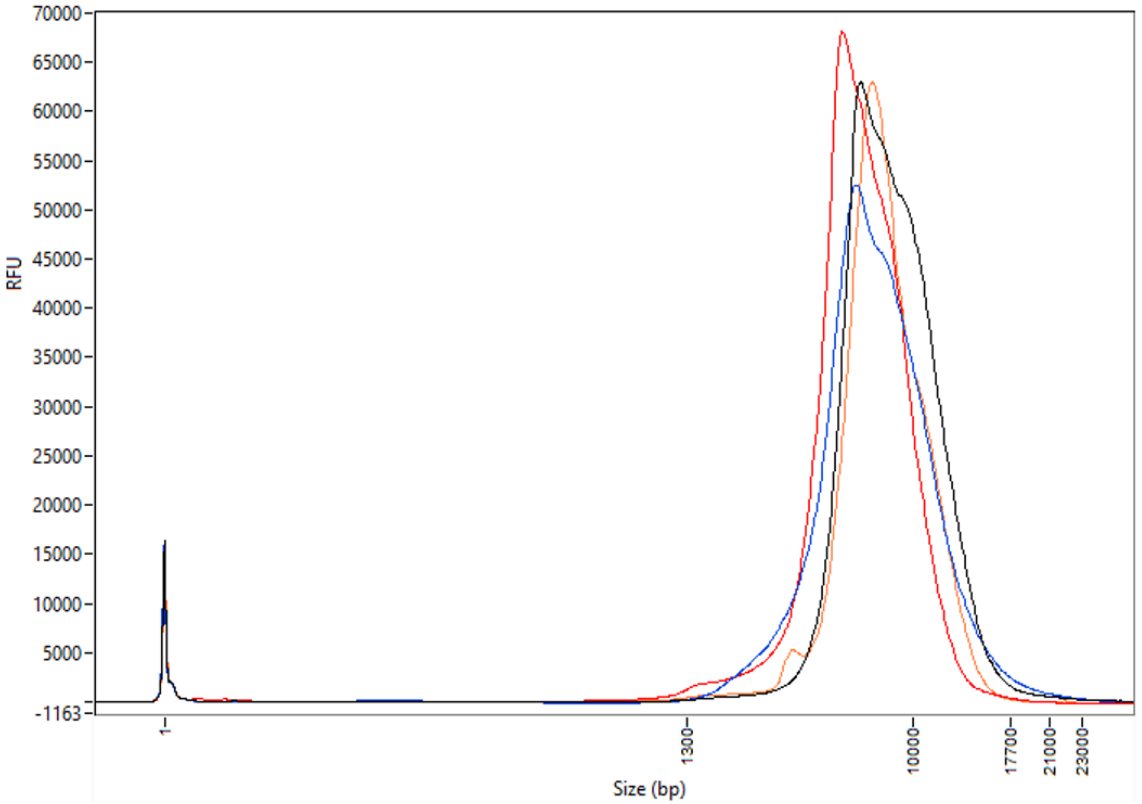


Femto Pulse DNA sizing QC analysis of a human gDNA sample sheared using a Megaruptor 3 with speed setting 31 (1-cycle shear). The fragment size distribution mode is 17.8 kb.

Example Megaruptor 3 shearing results for microbial genomic DNA samples

Megaruptor 3 System

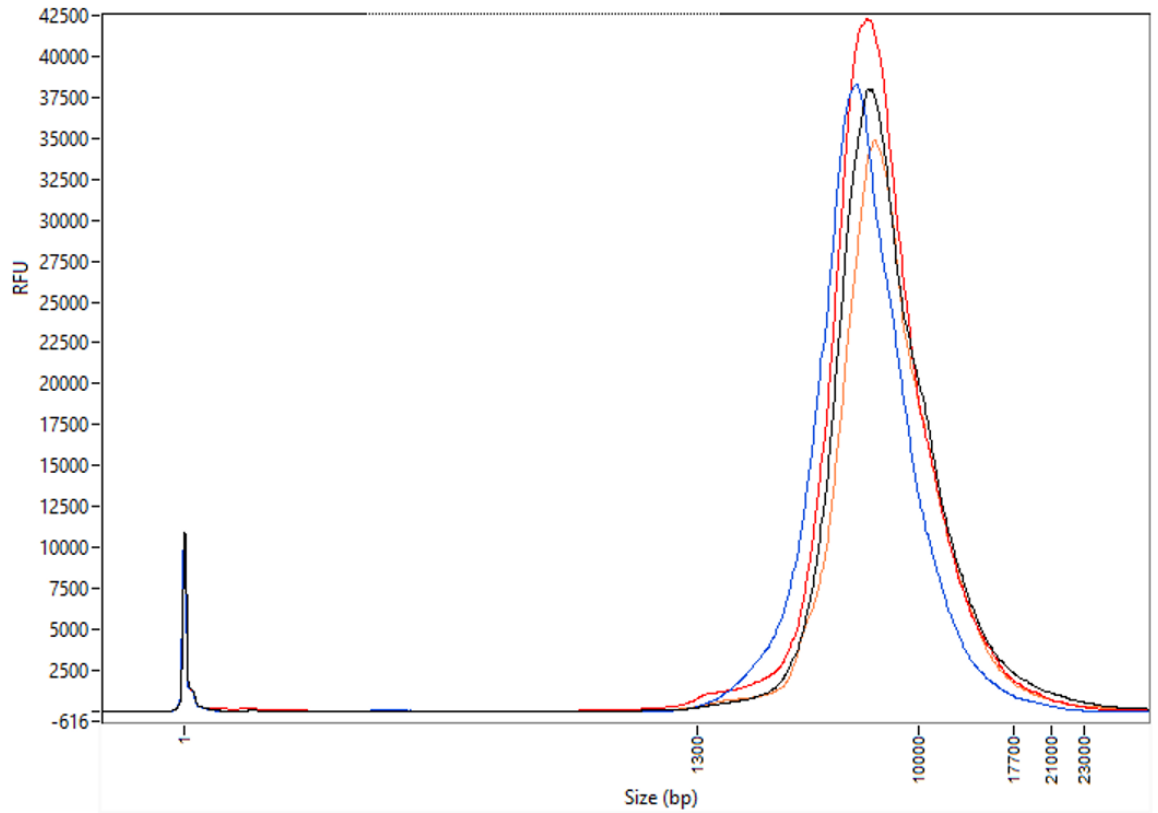
Speed Setting 40



Femto Pulse DNA sizing QC analyses of four different microbial gDNA samples sheared using a Megaruptor 3 System with speed setting 40 (1-cycle shear). The mean sheared DNA fragment size for all samples is ~7 kb – 10 kb.

g-TUBE

3287 x *g* [7000 RPM with Eppendorf MiniSpin Plus)



Femto Pulse DNA sizing QC analyses of four different microbial gDNA samples sheared using g-TUBES with a centrifugation speed of 3287 x *g*. The mean sheared DNA fragment size for all samples is ~7 kb – 10 kb.

SMRTbell barcoded adapter plate 3.0 is recommended for barcoding WGS samples

For Sequel II and IIe Systems, SMRTbell barcoded adapter plate 3.0 (102-009-200) is available for multiplexing up to 96 microbes per SMRT Cell 8M.

- Contains 96 barcoded adapters to support multiplexed SMRTbell library construction for up to 96 samples using SPK 3.0
- Can be used for Microbial Assembly and any other WGS or amplicon sequencing application that employs barcoded overhang adapters
- Each barcoded adapter contains a 5 bp padding sequence for more uniform ligation performance across different barcode sequences
- Each well on the plate contains a barcoded adapter with a unique 10-base pair PacBio barcode sequence
- Each barcoded adapter is present in only one well and supports a single reaction
- SMRT Link comes pre-installed with the following barcode set FASTA file containing SMRTbell barcoded adapter plate 3.0 barcode sequences*:
  `SMRTbell Barcoded Adapter Plate 3.0 (bc2001-bc2096)`

	1	2	3	4	5	6	7	8	9	10	11	12
A	BC2001	BC2009	BC2017	BC2025	BC2033	BC2041	BC2049	BC2057	BC2065	BC2073	BC2081	BC2089
B	BC2002	BC2010	BC2018	BC2026	BC2034	BC2042	BC2050	BC2058	BC2066	BC2074	BC2082	BC2090
C	BC2003	BC2011	BC2019	BC2027	BC2035	BC2043	BC2051	BC2059	BC2067	BC2075	BC2083	BC2091
D	BC2004	BC2012	BC2020	BC2028	BC2036	BC2044	BC2052	BC2060	BC2068	BC2076	BC2084	BC2092
E	BC2005	BC2013	BC2021	BC2029	BC2037	BC2045	BC2053	BC2061	BC2069	BC2077	BC2085	BC2093
F	BC2006	BC2014	BC2022	BC2030	BC2038	BC2046	BC2054	BC2062	BC2070	BC2078	BC2086	BC2094
G	BC2007	BC2015	BC2023	BC2031	BC2039	BC2047	BC2055	BC2063	BC2071	BC2079	BC2087	BC2095
H	BC2008	BC2016	BC2024	BC2032	BC2040	BC2048	BC2056	BC2064	BC2072	BC2080	BC2088	BC2096

Figure illustration of mapping between a specific well location and a unique PacBio barcode sequence on a 96-well plate in the SMRTbell barcoded adapter plate (102-009-200)

Reagent kit quantities support a **single use** of each of the 96 barcoded adapters in the plate for SMRTbell library preparations.

Plate Layout (Excel) [Link]

Barcode Sequences (FASTA) [Link]

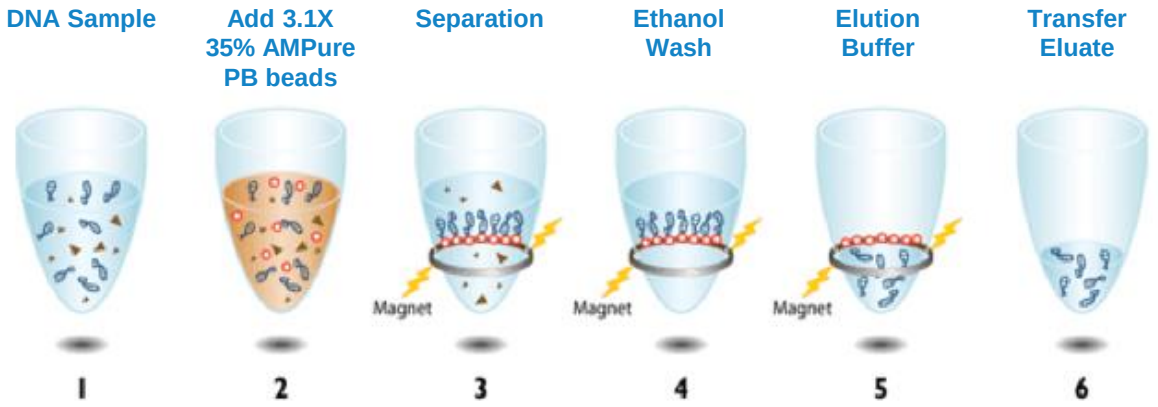
Product insert: SMRTbell barcoded adapter plate 3.0 (96 barcodes, 96 samples) [Link]

* If your SMRT Link version does not have the required barcode set FASTA file for a specific analysis application, please visit PacBio's Multiplexing website to download the appropriate FASTA file or contact PacBio Technical Support.

Size-selection of SMRTbell libraries with AMPure PB beads is recommended for WGS applications

- AMPure PB beads are used as the default size selection method* to remove short DNA fragments (<5 kb) and enrich for the long fragments when preparing SMRTbell libraries for whole genome sequencing
- AMPure PB bead size selection of SMRTbell templates is performed follows:
  - Prepare a 35% dilution (v/v) of the AMPure PB bead stock by adding 1.75 mL of resuspended AMPure PB beads to 3.25 mL of Elution Buffer (EB). [35% AMPure PB beads solution can be stored at 4°C for 30 days.]
  - Add 3.1X v/v of resuspended, room-temperature 35% AMPure PB beads solution to each sample tube and incubate for 20 min at RT to allow beads to bind to DNA
  - Place sample tubes on a magnetic rack to immobilize AMPure PB beads; wash samples with 80% ethanol 2X; then elute samples in 15 µL of EB for 5 min at RT

AMPure PB beads

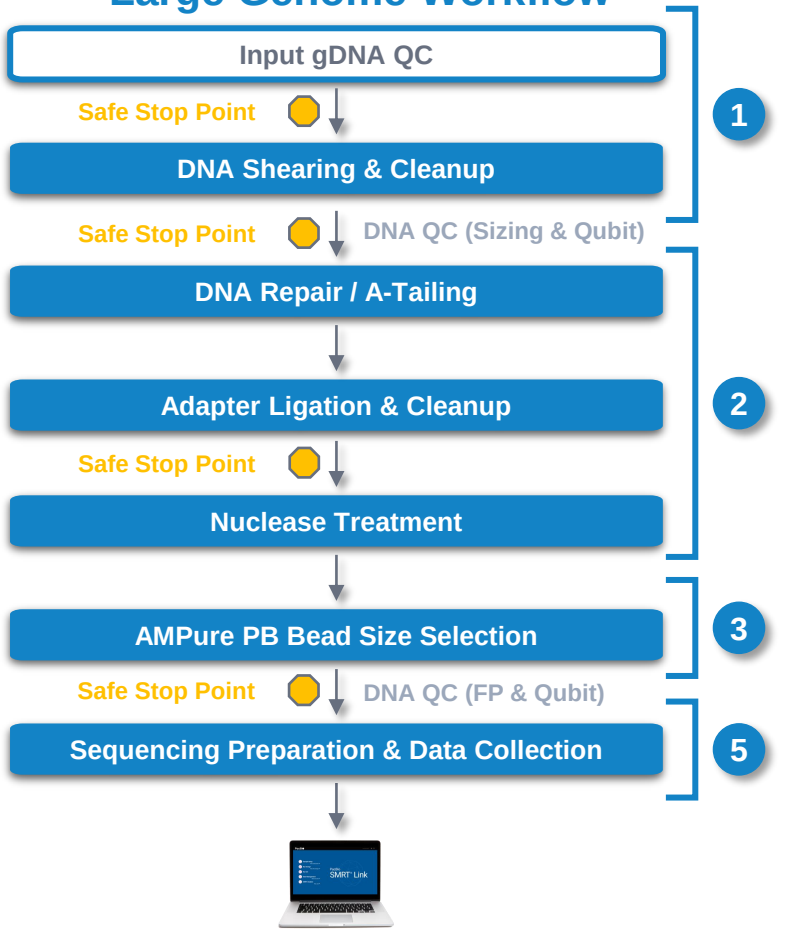


With high-quality WGS samples, AMPure PB bead size selection can recover sufficient SMRTbell library material to run **up to ~3 or more SMRT Cells 8M per 3 µg of starting input gDNA**

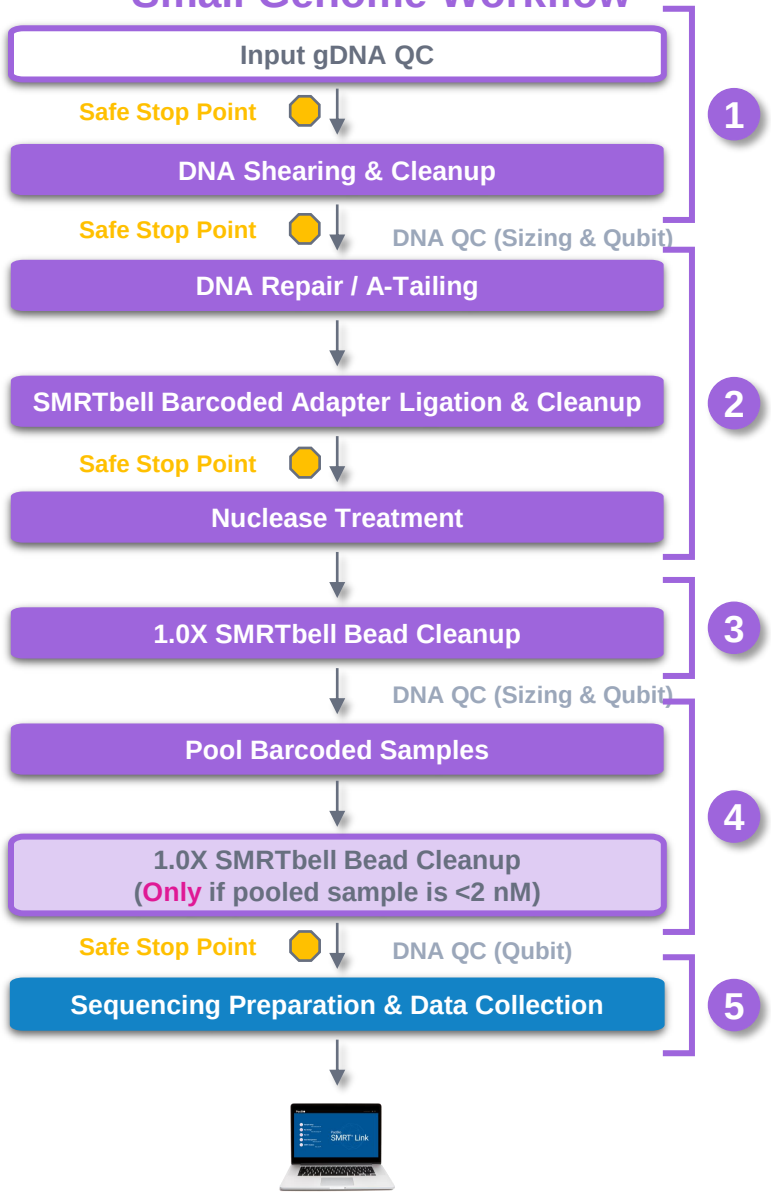
* **Note:** Although size-selection beads have many advantages, automated DNA size selection systems that utilize gel cassettes offer more flexibility in defining a size cutoff. Three automated DNA size selection tools that may **optionally** be employed for performing size selection on SMRTbell libraries for HiFi WGS applications include the PippinHT, BluePippin and SageELF systems from Sage Science. Note that use of these tools requires higher input DNA amounts (≥1.5 µg/SMRT Cell 8M). For more information, refer to Technical Note: Alternative size selection methods for SMRTbell prep kit 3.0 (TN103-110921), which provides detailed guidance for size selection of WGS libraries using automated DNA size selection tools or contact PacBio Technical Support or your local Field Applications Scientist.

Summary comparison of SPK 3.0 library sample preparation for large genome *vs*. small genome (microbial / shotgun metagenomic) WGS applications

Large Genome Workflow



Small Genome Workflow



Step	Action or metric	Large genome (Single sample)	Small genome (Multiplexed samples)
1	Input DNA quality	• 50% ≥ 30 kb • & 90% ≥ 10 kb	• 90% ≥ 7 kb
	Input DNA amount*	• Use ≥1 µg of total input DNA per SMRT Cell 8M	• Use ≥300 ng of input DNA per sample, with a total mass ≥1 µg across all samples
	Megaruptor system shearing	• Use speed setting 31	• Use Speed setting 40
2	Adapter ligation	• Use standard (non-barcoded) adapter included with SPK 3.0	• Use Barcoded adapter plate 3.0
3	Size selection	• Use AMPure PB bead size selection	• AMPure PB bead size selection is optional; otherwise perform a standard 1X cleanup with SMRTbell cleanup beads
4	Pooling	• N/A	• Perform equal mass pooling with barcoded samples
5	Sequencing prep & data collection	• 30 hr movie time	• 15 hr movie time

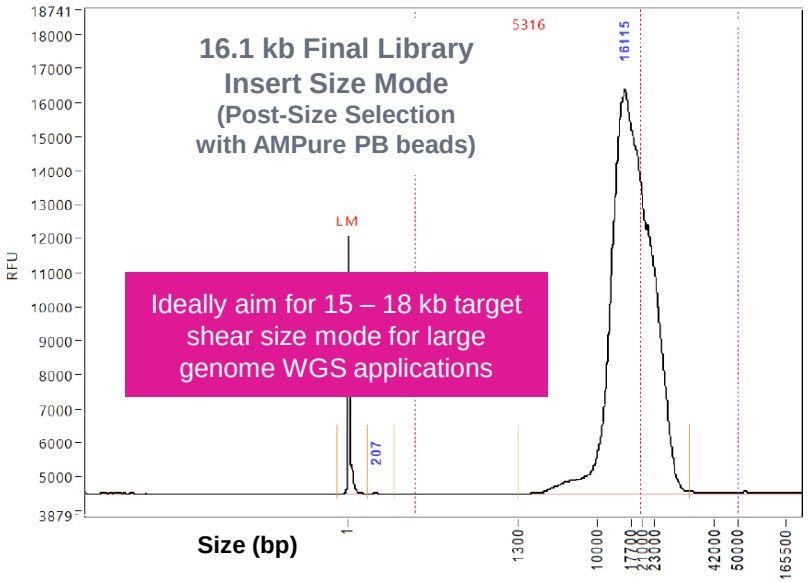
* Increase DNA input amounts to ≥1.5 µg per SMRT Cell 8M when using a gel-based automated size selection system.

SPK 3.0 WGS example performance data

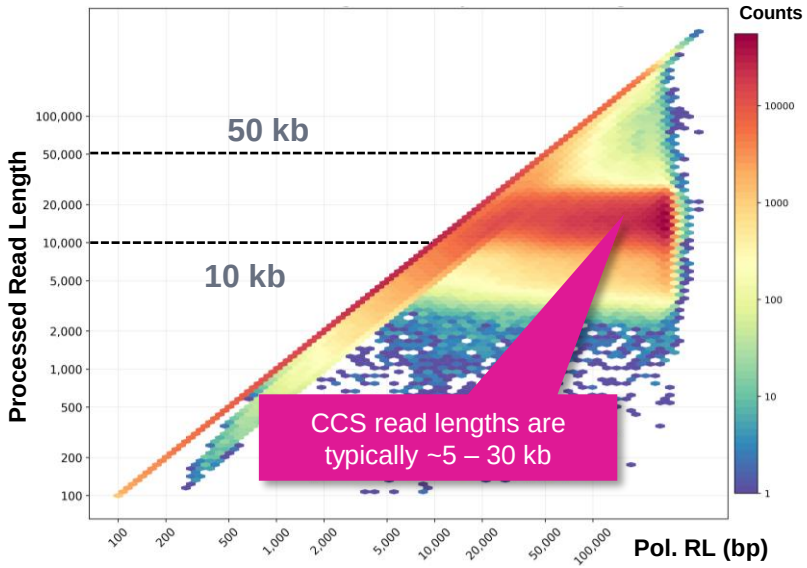
Example library QC and sequencing performance for human WGS libraries prepared with SMRTbell prep kit 3.0

SMRTbell library QC and primary sequencing metrics

Size-Selected Library QC

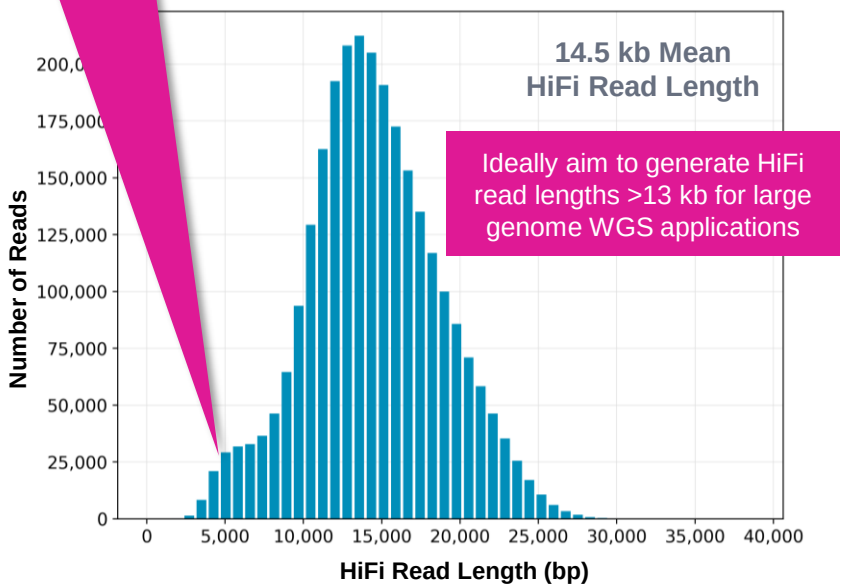


Raw Data Report



A secondary left hand peak may also be visible depending on DNA sample quality

CCS Analysis Report



Input gDNA for Megaruptor 3 shearing	3000 ng
Post-shearing recovery (%)*	2620 ng (87%)
Final yield of AMPure PB bead Size-selected library (%)**	1070 ng (36%)

* Post-shearing recoveries typically ranged from ~70% to >95% when using input human DNA samples (1 μg to 5 μg)

** Final post-size selected library yields typically ranged from ~25% to ~50% when using input human DNA samples (1 μg to 5 μg)

Raw Base Yield	617.65 Gb
Mean Polymerase Read Length	102.8 kb
P0	23.4%
P1	75.0%
P2	1.6%

Example sequencing metrics for a human WGS sample run with Binding Kit 3.2 (Polymerase 2.2) / 85 pM on-plate concentration / 30-h movie time / 2-h Pre-Extension Time / Adaptive Loading Target = 0.85

HiFi Reads	2.7 M
HiFi Base Yield	39.2 Gb
Mean HiFi Read Length	14,490 bp
Median HiFi Read Quality	Q34
HiFi Read Mean # of Passes	12

For SPK 3.0 human WGS libraries, per-SMRT Cell HiFi base yields typically ranged from ~28 to 39 Gb.

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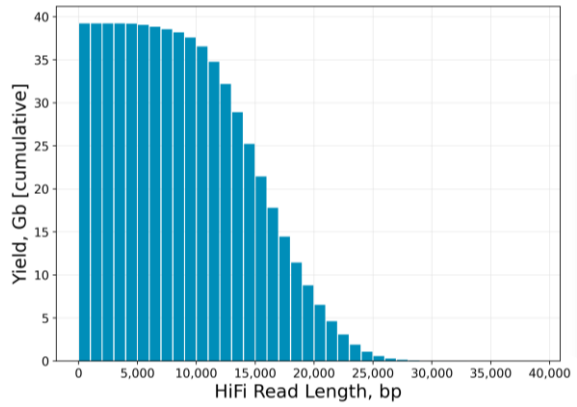
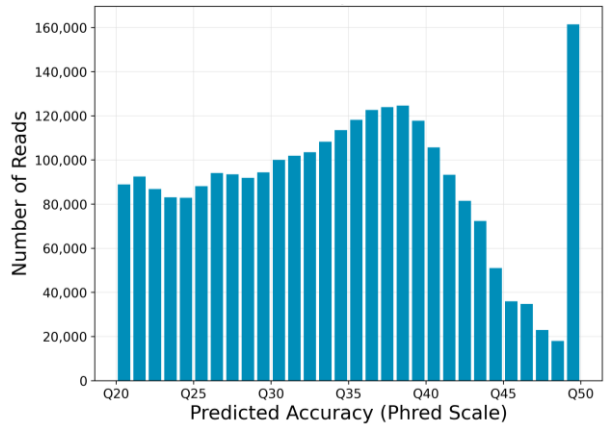
Example sequencing performance for human WGS libraries prepared with SMRTbell prep kit 3.0 (cont.)

Primary sequencing metrics (Cont.)

Yield by HiFi Read Length



Read Quality Distribution



HiFi Read Length Summary

Read Length (bp)	Reads	Reads (%)	Yield (bp)	Yield (%)
≥ 0	2,707,732	100	39,236,168,651	100
≥ 5,000	2,664,322	98	39,051,919,399	100
≥ 10,000	2,353,137	87	36,541,368,326	93
≥ 15,000	1,164,272	43	21,435,305,025	55
≥ 20,000	294,460	11	6,522,779,501	17
≥ 25,000	21,062	1	559,040,421	1
≥ 30,000	1,012	0	35,294,569	0
≥ 35,000	388	0	15,240,023	0
≥ 40,000	129	0	5,578,841	0

HiFi Read Quality Summary

Read Quality (Phred)	Reads	Reads (%)	Yield (bp)	Yield (%)
≥ Q20	2,707,732	100	39,236,168,651	100
≥ Q30	1,811,377	67	25,413,473,886	65
≥ Q40	679,582	25	8,150,599,400	21
≥ Q50	146,257	5	1,355,549,531	3

Example *de novo* assembly performance for human WGS libraries prepared with SMRTbell prep kit 3.0

HiFi WGS data sets generated with SPK 3.0 provide highly contiguous and highly accurate assemblies

HG002 Library ID	Contig_N50_Mbp
64009e_s10_cov30	35.4
64012e_s10_cov30	36.5
64015e_s10_cov30	36.7
64438e_s10_cov30	34.1
64441e_s10_cov30	33.6

HG002 Library ID	deNovo_asm_QV
64009e_s10_cov30	48.3
64012e_s10_cov30	48.3
64015e_s10_cov30	48.2
64438e_s10_cov30	48.3
64441e_s10_cov30	48.2

- Data were generated from five different human HG002 WGS libraries run on five different Sequel IIe systems
- Data were subsampled to 30-fold coverage and assembled using SMRT Link Genome Assembly analysis application

Example variant detection performance for human WGS libraries prepared with SMRTbell prep kit 3.0

HiFi WGS data sets generated with SPK 3.0 provide highly accurate variant calls

HG002 Library ID	INDEL.F1_Score
64009e_s10_cov30	0.995
64012e_s10_cov30	0.994
64015e_s10_cov30	0.994
64438e_s10_cov30	0.993
64441e_s10_cov30	0.994

HG002 Library ID	SNP.F1_Score
64009e_s10_cov30	0.999
64012e_s10_cov30	0.999
64015e_s10_cov30	0.999
64438e_s10_cov30	0.999
64441e_s10_cov30	0.999

- Data were generated from five different human HG002 WGS libraries run on five different Sequel IIe systems
- Data were subsampled to 30-fold coverage and analyzed with DeepVariant

F1 score = Harmonic mean of precision and recall

SMRT Link Sample Setup updates

New High-Throughput Sample Setup mode overview

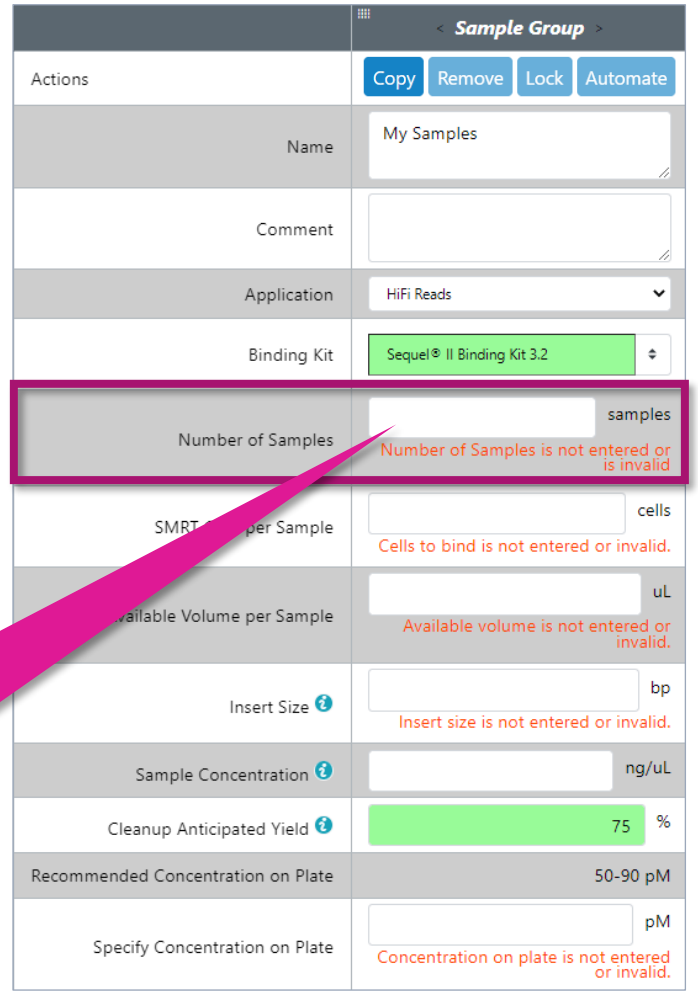
Specifying multiple samples in one protocol instance

New High-Throughput mode feature provides a more streamlined workflow to efficiently process single samples or multiple samples in parallel using automation

- The user can specify multiple samples to all be processed at once
  - A *single* protocol will be generated for *all* of these samples
  - This greatly simplifies batch processing and automation
  - All samples in a batch should have approximately the same concentration and insert size*

    → This generally requires that the user does a concentration normalization step at the end of library prep if working with multiple samples

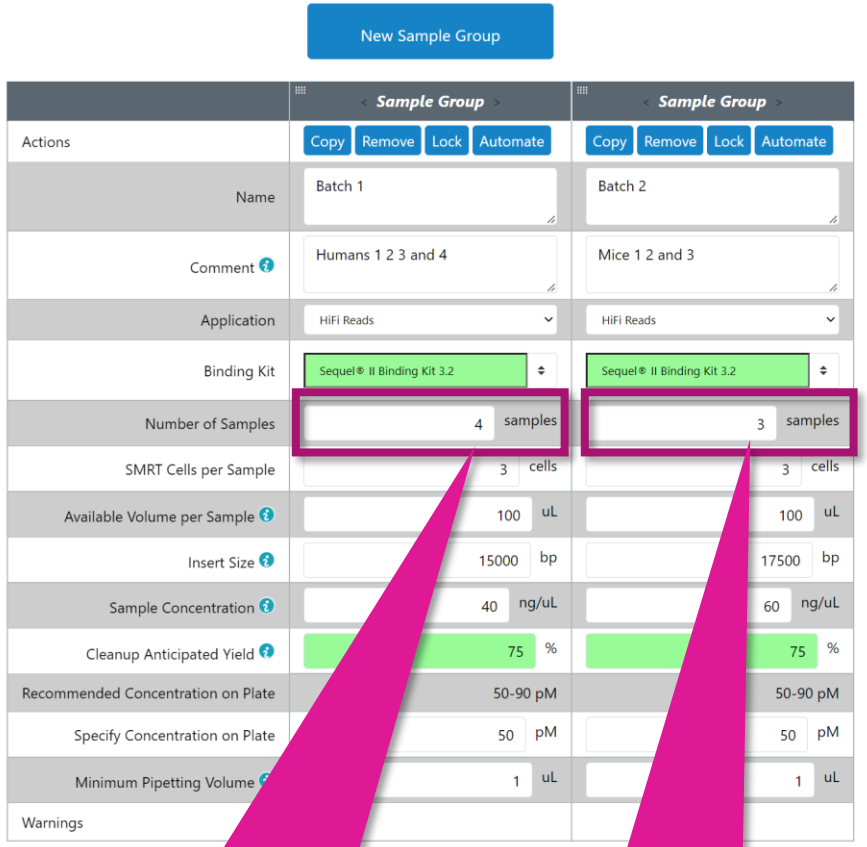
* **All samples in a group should have substantially equivalent library properties** (i.e., insert sizes and concentrations within +/- 15% of the specified values.)



The user can enter any number of samples (including 1)

Parallel display of multiple batches at sample information entry step

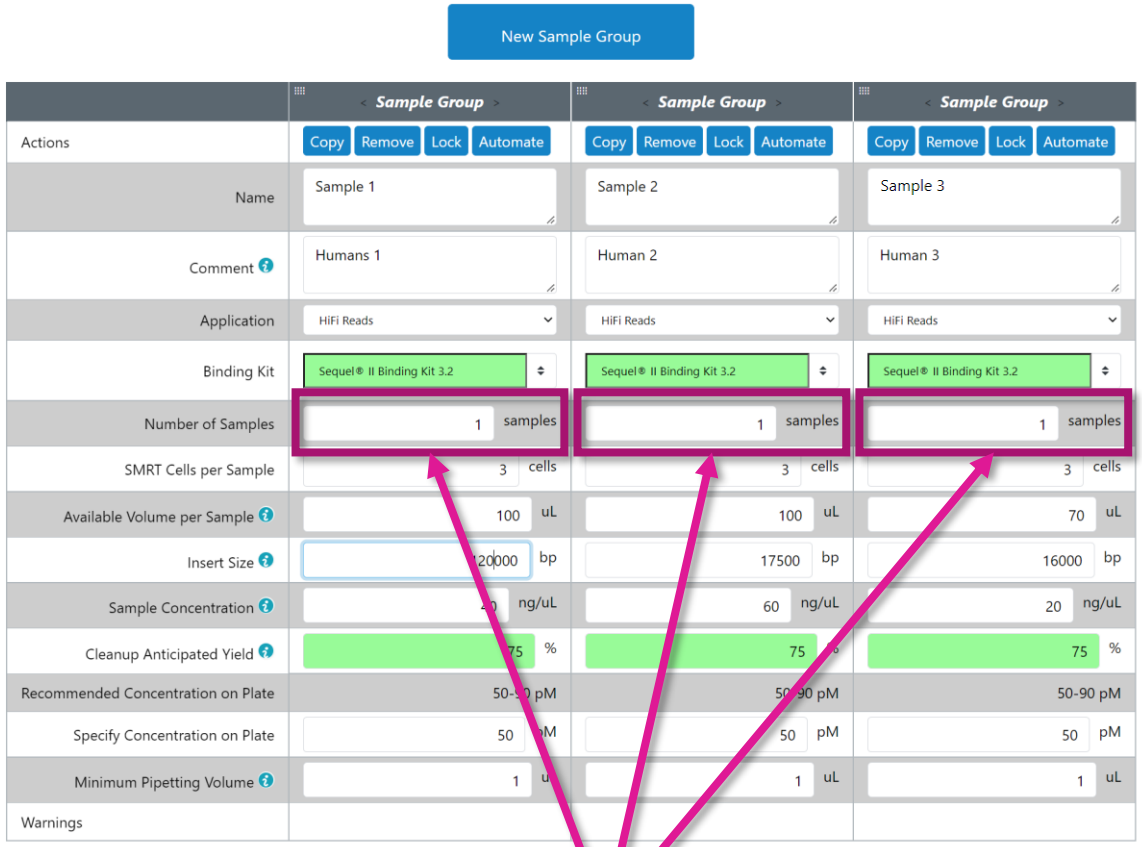
Example: Multiple samples per batch



All 4 samples in 'Batch 1' group have **equivalent** library properties and are processed with the **same** ABC protocol

All 3 samples in 'Batch 2' group have **equivalent** library properties and are processed with the **same** ABC protocol

Example: Single samples (Each "batch" = 1 sample)



Sample 1, Sample 2 and Sample 3 have **non-equivalent** library properties and are processed with three **different** ABC protocols

New Sequel II binding kit component details

Sequel II binding kit 3.1 and cleanup beads (102-333-400)

- Sequel II Primer 3.1 (pre-diluted AND pre-conditioned)
- Sequel II Polymerase 2.1
- SMRTbell Cleanup Beads for complex cleanup
- DNA Internal Control 3.1 (defined 2 kb template bound to Polymerase 2.1)
- Supports ≥24 binding reactions, and up to 4 SMRT Cells 8M per binding reaction (96 cells total), depending on use case, sample size and concentration
- Note: Sequel II Binding Kit 3.1 is recommended for inserts <3 kb

Sequel II Primer 3.1
= Sequencing Primer v4*

DNA Internal Control 3.1
= DNA Internal Control 1.0

Sequel II binding kit 3.2 and cleanup beads (102-333-300)

- Sequel II Primer 3.2 (pre-diluted AND pre-conditioned)
- Sequel II Polymerase 2.2
- SMRTbell Cleanup Beads for complex cleanup
- DNA Internal Control 3.2 (defined 11 kb template bound to Polymerase 2.2)
- Supports ≥24 binding reactions, and up to 4 SMRT Cells 8M per binding reaction (96 cells total), depending on use case, sample size and concentration
- Note: Sequel II Binding Kit 3.2 is recommended for inserts >3 kb

Sequel II Primer 3.2
= Sequencing Primer v5*

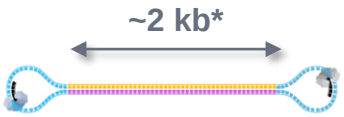
* Users should **NOT** use legacy sequencing primer products (Primer v4, v5) in place of the pre-diluted and pre-conditioned sequencing primers (Primer 3.1/3.2) included with Sequel II binding kits 3.1 and 3.2.

Sequel II DNA internal control complex 3.1 and 3.2 descriptions

Sequel II DNA control complexes are fixed SMRTbell templates pre-bound to a polymerase and are used as a spike-in sequencing control

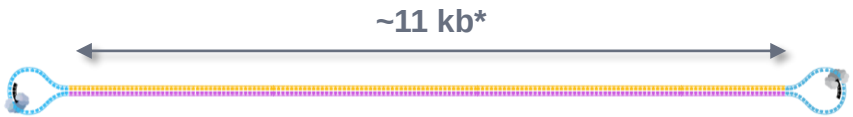
Sequel II DNA Internal Control 3.1

DNA Control 3.1 is the **same** as DNA Control 1.0



- Included with **Sequel II Binding Kit 3.1**
- Synthetic 2 kb construct bound to Sequel II Polymerase 2.1
- Sequence available in NCBI GenBank (Accession MG495226)

Sequel II DNA Internal Control 3.2



- Included with **Sequel II Binding Kit 3.2**
- Synthetic 11 kb construct bound to Sequel II Polymerase 2.2

- DNA internal control complex is added to the sample at the end of Sample Setup and is intended to serve as an ideal (well-behaved) sample for obtaining good sequencing results on the Sequel IIe System
  - Therefore, poor sequencing results obtained with the control can indicate potential issues with the system, reagents, or consumables
- DNA internal control templates are synthetic constructs with a known sequence and therefore can be easily separated from the sample data
- Note: PacBio **requires** the use of a DNA Internal Control to be eligible for reimbursement requests arising from sequencing run failures

PacBio * Not to scale

Example Sequel II DNA internal control 3.2 performance

Sequel II DNA Internal Control 3.2 polymerase read length performance more closely matches large insert WGS sample library performance compared to Control 1.0

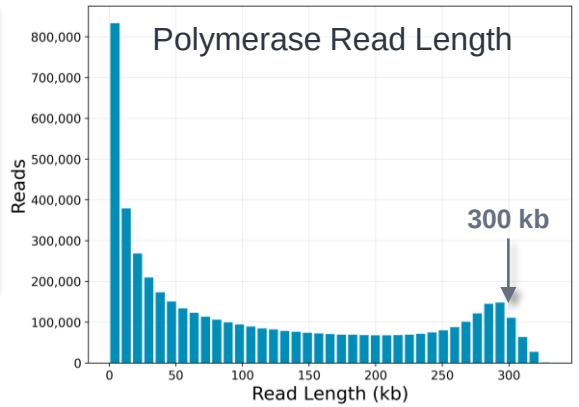
Sample

Control 1.0

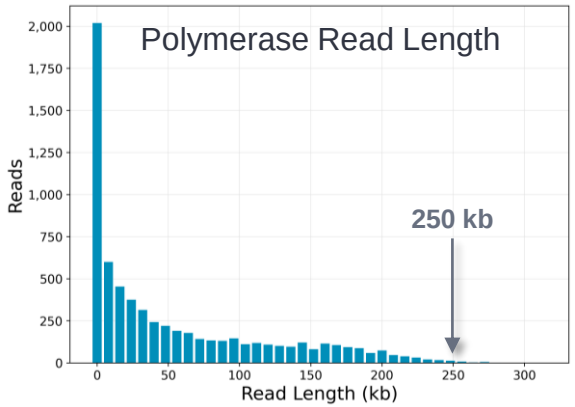
	Sample Information	Run Settings	Reads	Control			
			Polymerase Read Length			Concordance	
Well	Name	Movie Time (hrs)	Mean	Poly RL Mean (bp)	Total Reads	Mean	Mode
A01	WGS Sample 1 Pol 2.2	30	113986	56757	6610	0.86	0.89
B01	WGS Sample 2 Pol 2.2	30	108072	55405	6886	0.86	0.89
C01	WGS Sample 3 Pol 2.2	30	111207	52599	4522	0.86	0.89
D01	WGS Sample 4 Pol 2.2	30	110184	55776	6854	0.86	0.89

Sequel II DNA Internal Control 1.0 polymerase read length is typically **>40 kb** for 30-h movies with WGS samples bound to Polymerase 2.2

Sample



Control 1.0



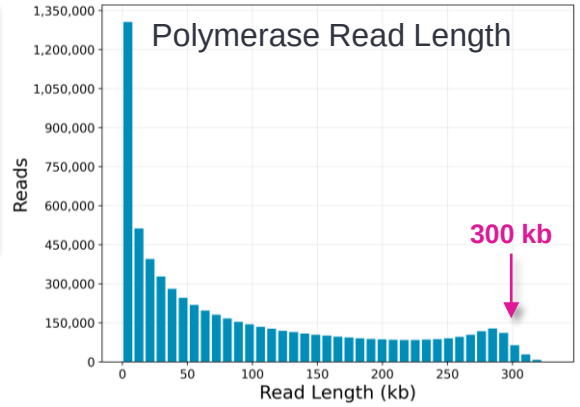
Sample

Control 3.2

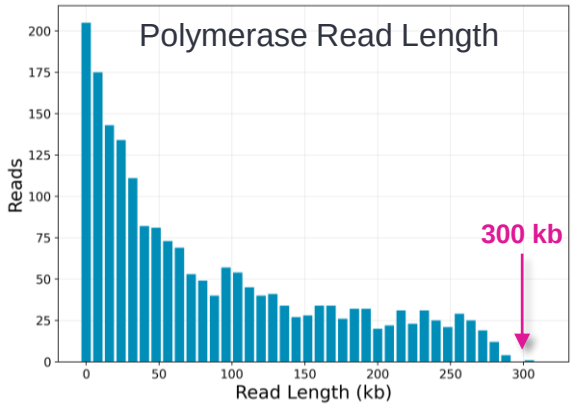
	Sample Information	Run Settings	Reads	Control			
			Polymerase Read Length			Concordance	
Well	Name	Movie Time (hrs)	Mean	Poly RL Mean (bp)	Total Reads	Mean	Mode
A01	WGS Sample 1 Pol 2.2	30	93898	89237	1963	0.88	0.91
B01	WGS Sample 2 Pol 2.2	30	102673	91526	1926	0.89	0.91
C01	WGS Sample 3 Pol 2.2	30	107079	99541	1855	0.89	0.91

Sequel II DNA Internal Control 3.2 polymerase read length is typically **>80 kb** for 30-h movies with WGS samples bound to Polymerase 2.2

Sample



Control 3.2



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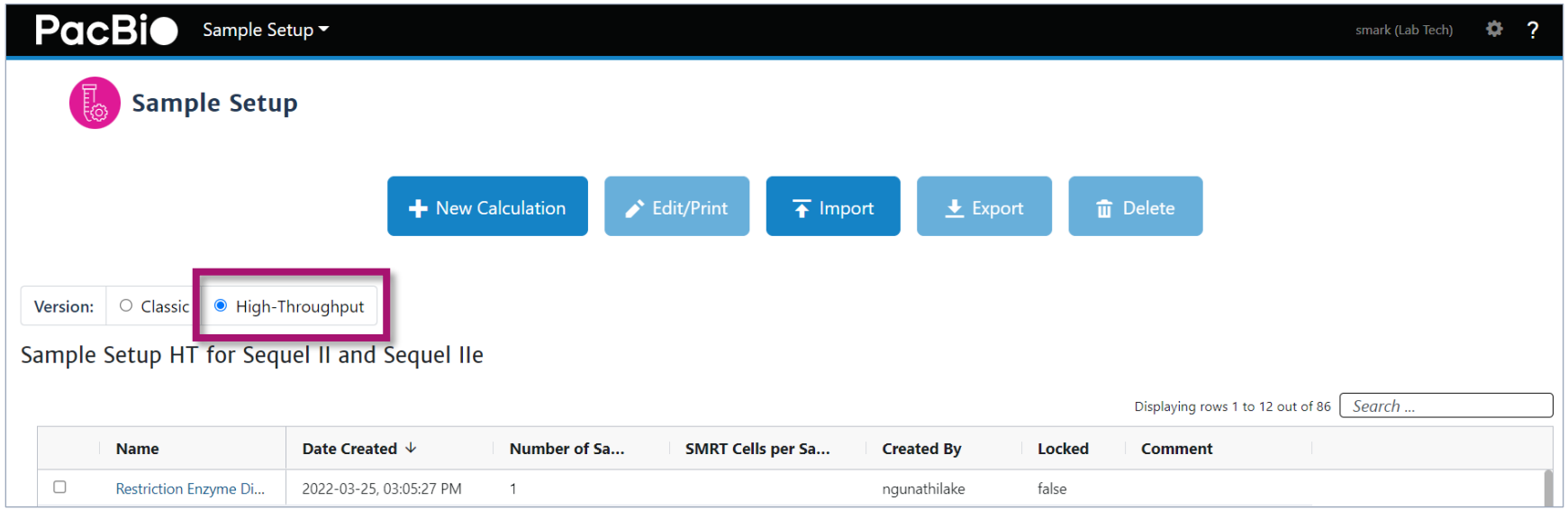
Note: No changes to existing troubleshooting guidance or credit reimbursement policy for Sequel II systems at this time.

Creating a new sample setup calculation worksheet from the SMRT Link v11.0 Sample Setup homepage

High-Throughput mode is the new default mode to create or edit calculations to define reaction conditions for primer annealing, polymerase binding, complex cleanup and sequencing.



High-Throughput mode provides a simplified, streamlined workflow to efficiently process either one sample or multiple samples with similar library properties (such as mean insert size and DNA concentration) in parallel. You can also export the calculated values to a CSV file for laboratory automation.

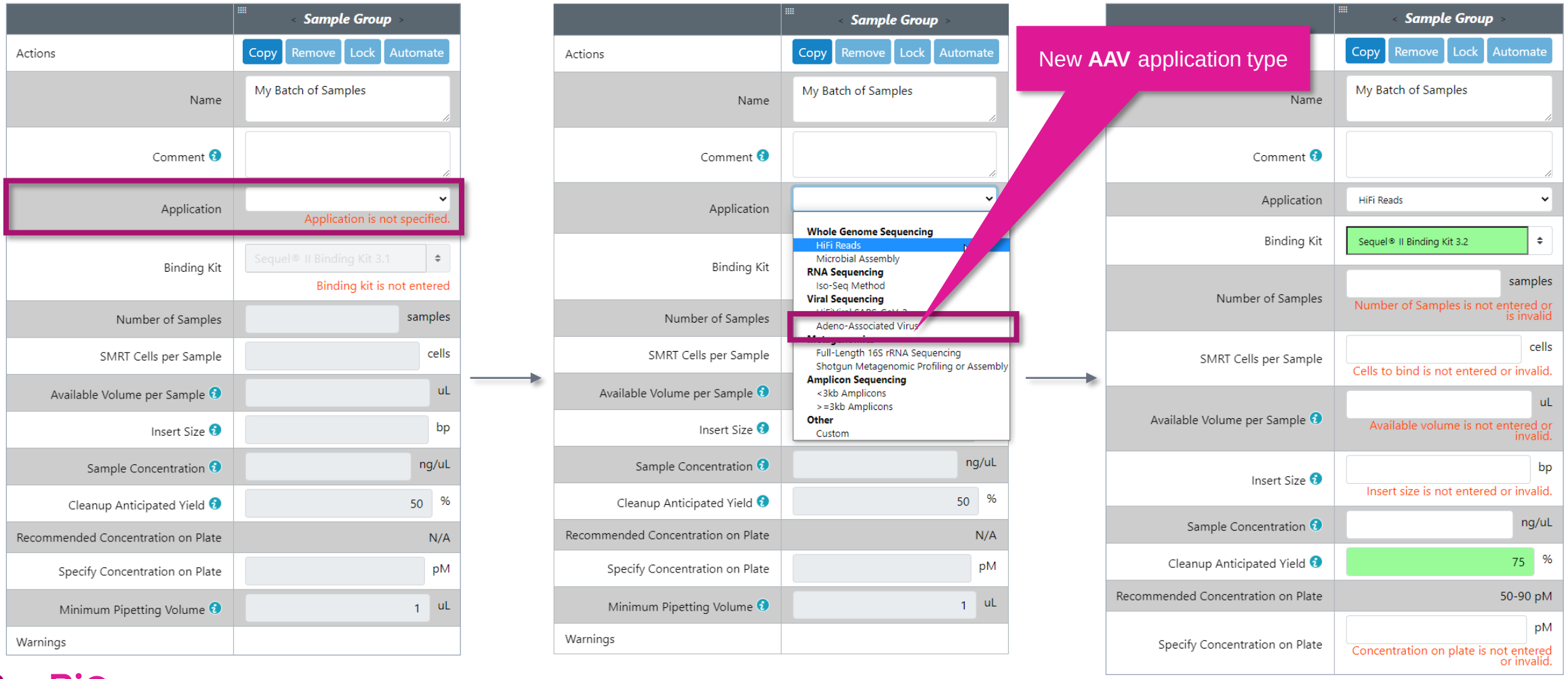
- HT mode is designed to support higher throughput operation by **simplifying and unifying protocol steps** across multiple samples
- HT mode is designed to be used in an identical manner across **all** applications
- HT mode **only** supports the new Binding Kits 3.1 and 3.2
- HT mode is designed to be automation-compatible
- **Classic mode* does not support Sequel II Binding Kit 3.1/3.2**

* **Note:** Sample Setup Classic mode is provided for **legacy support purposes only** and is **not recommended for general use**.

High-Throughput Sample Setup mode application type specification

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

- Select an **Application Type**
- Once an application is selected, default values are auto-populated for various fields and highlighted in **green**

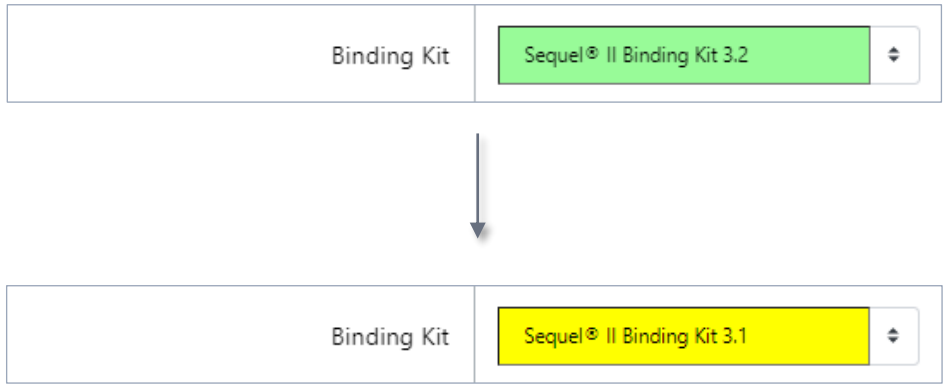


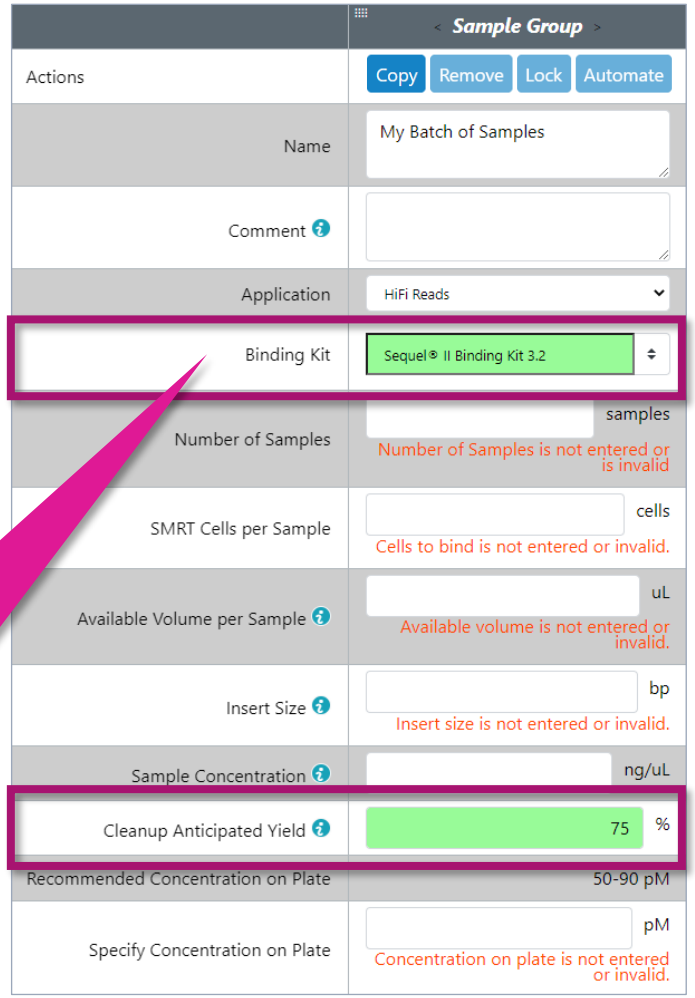
High-Throughput Sample Setup mode application type specification (cont.)

Auto-populated fields are highlighted in green color

- The following fields are auto-populated and highlighted in **green**:
  - Binding Kit
  - Cleanup Anticipated Yield

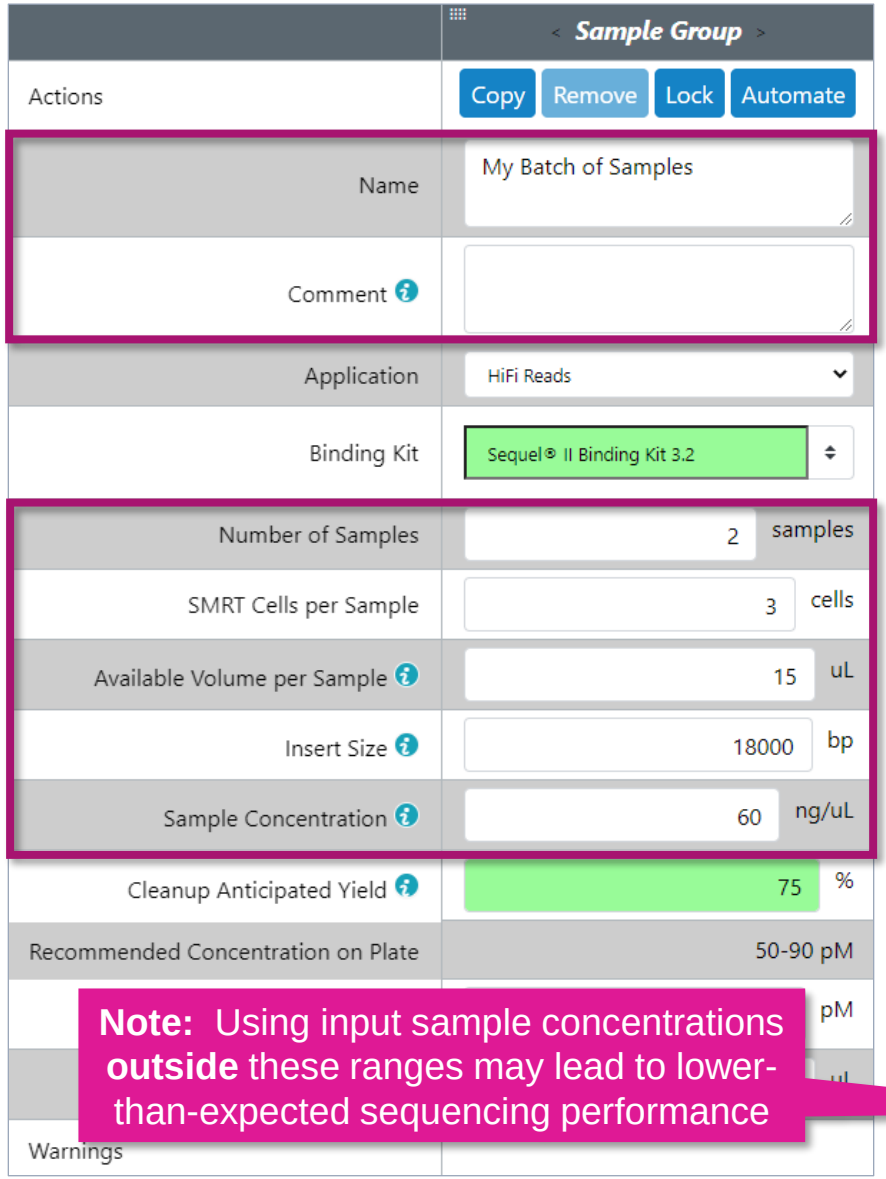
If any auto-populated entry is manually changed to a different value, then the field will be highlighted in **yellow** color





Sequencing primer version does not need to be specified. Use the sequencing primer **included** with the Sequel II Binding Kit.

Entering sample library information



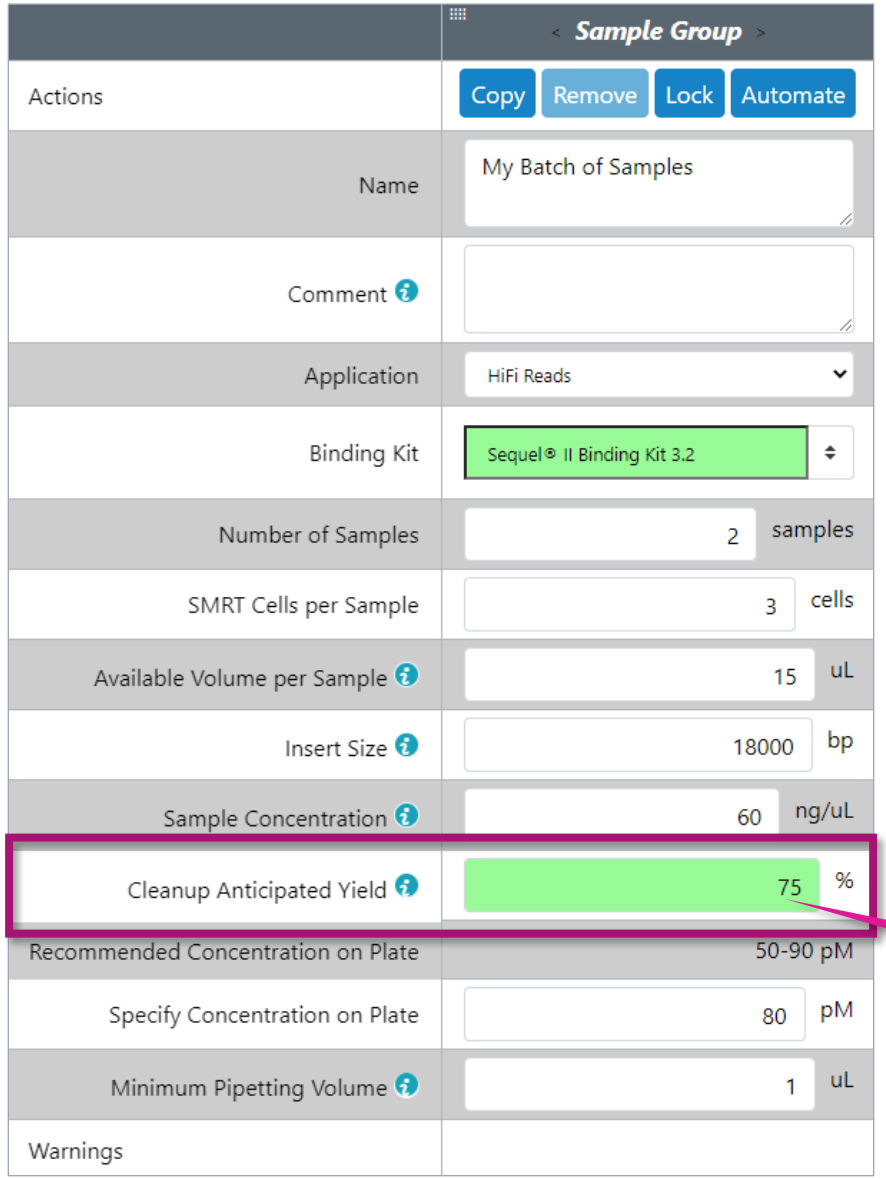
Note: Using input sample concentrations **outside** these ranges may lead to lower-than-expected sequencing performance

- Enter the following information for each group of samples:
  - **Name**
    - Enter a sample group name (if processing multiple similar samples in a group) or a sample name (if processing a single sample)
  - **Comment**
    - You can enter information meaningful to you like a batch identifier for a LIMS and/or information for each sample such as: "1: Sample ID1234", "2: Sample ID5678", etc.
  - **Number of Samples**
    - All samples in a group should have insert sizes and concentrations within +/- 15%*
  - **SMRT Cells per Sample**
  - **Available Volume per Sample (µL)**
    - Please enter the volume of the least abundant sample in this batch, to ensure adequate volume is available for all samples
  - **Sample Insert Size (bp)**
    - Enter the mean insert size based on a DNA sizing QC (smear) analysis of the final library. All samples in a batch should be within +/- 15%* of the entered Insert Size value
  - **Sample Concentration (ng/µl)**
    - All samples in a batch should be within +/- 15%* of the entered Sample Concentration value. The acceptable range of input concentrations depends on insert size as shown in the table at right:

Insert size	Concentration range
≥10 kb	20 – 60 ng/µL
3 kb – 9999 bp	6 – 20 ng/µL
1.5 kb – 2999 bp	3 – 10 ng/µL
500 bp – 1499 bp	1 – 3 ng/µL

* **Note:** If your samples have insert sizes or concentrations that are different by more than +/- 15%, we recommend setting them up as separate batches.

Entering sample clean-up information



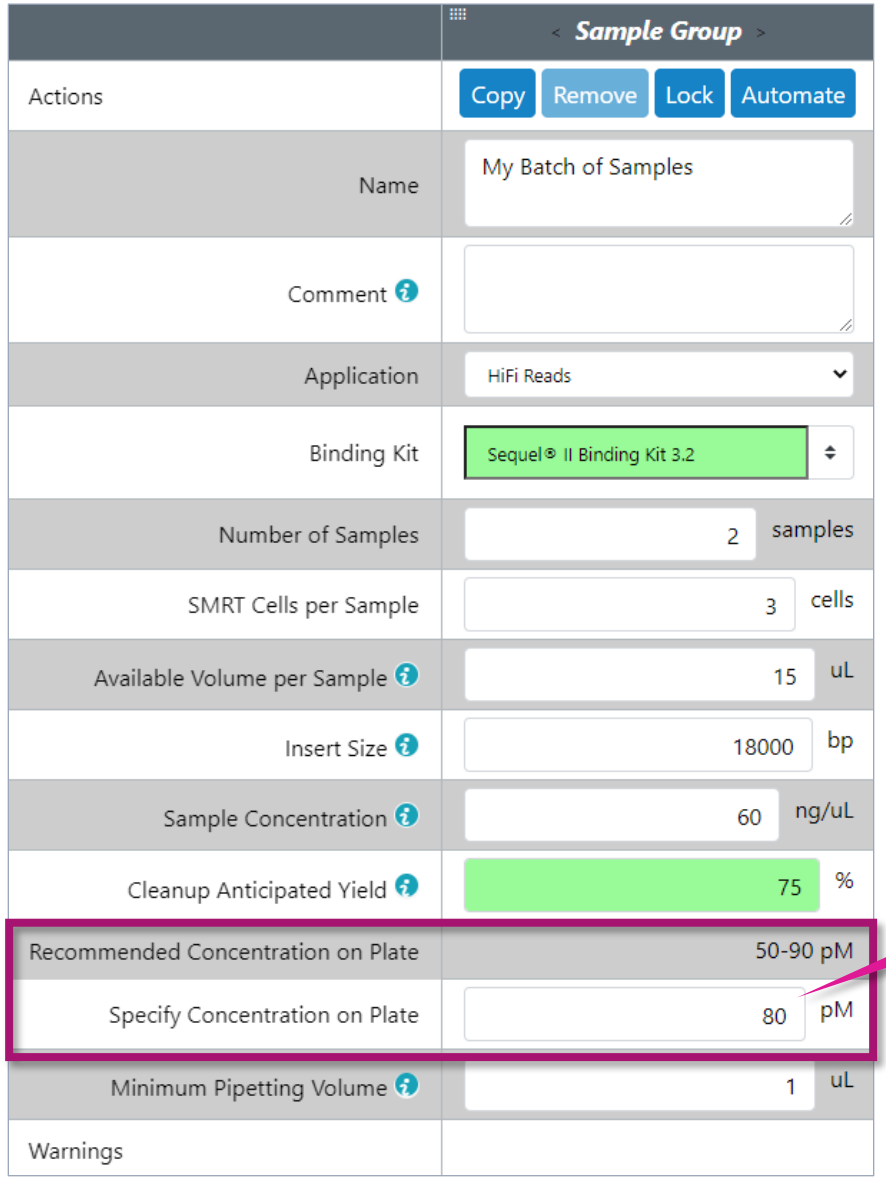
Sample Setup entry fields

- **Cleanup Anticipated Yield** field is **auto-filled with** default values based on the specified Binding Kit
  - 60% for Sequel II binding kit 3.1
  - 75% for Sequel II binding kit 3.2
- Sample cleanup is performed using **SMRTbell cleanup beads** included with the binding kit

Cleanup of the bound complex allows removal of excess polymerase and sequencing primers, resulting in **higher-quality data**

Can **adjust Cleanup Anticipated Yield** based on previous experience.

Sample loading concentration specification



Sample Setup entry fields

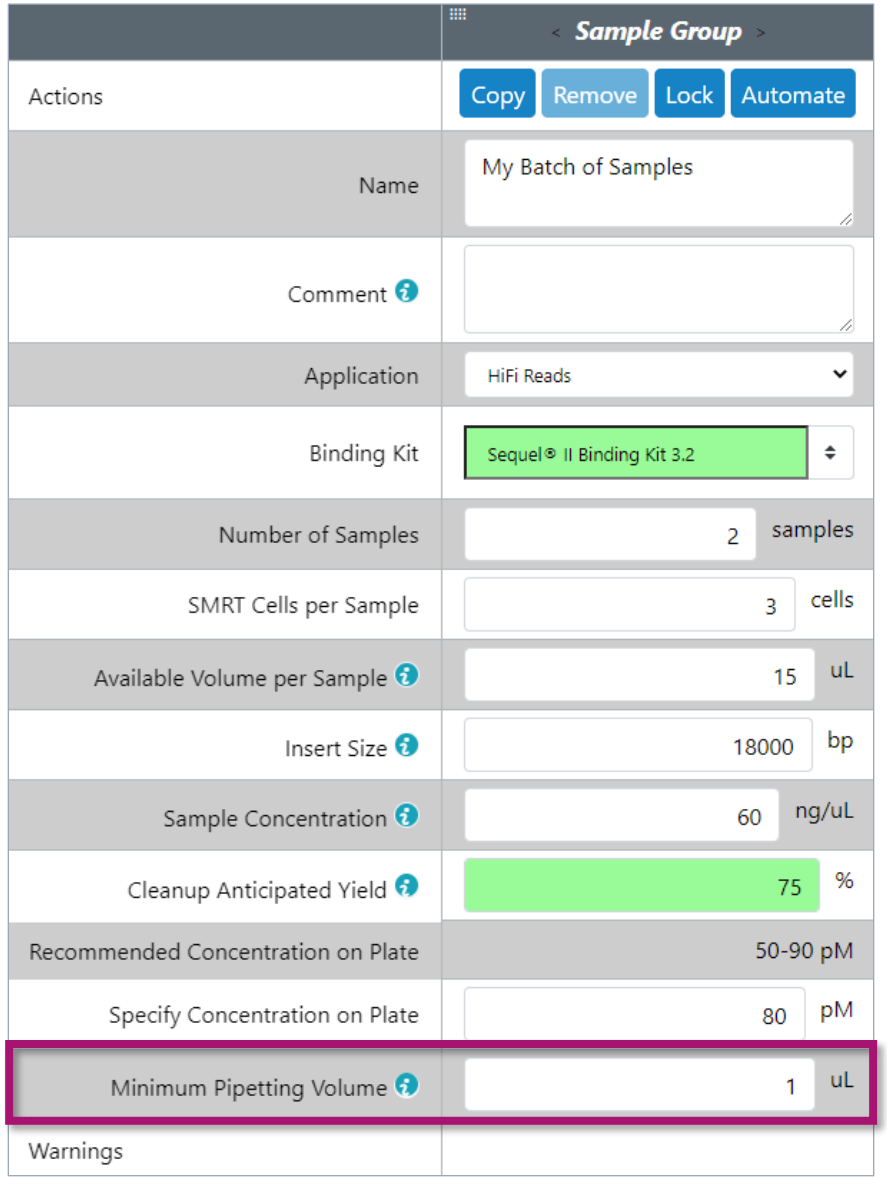
- Specify **Concentration On-Plate (OPLC)* (pM)**

See Quick reference card – loading and pre-extension recommendations for Sequel II and IIe systems (101-769-100) for any specific **changes** to SMRT Link Sample Setup recommendations

Can **adjust OPLC** based on previous experience with a particular sample type.

* OPLC = On-Plate Loading Concentration

Minimum pipetting volume field is no longer hidden in Advanced Options



Sample Setup entry fields

- Specify the **Minimum Pipetting Volume**
- This allows you to set a lower limit on pipetting volumes to use in certain protocol steps, such as sample annealing and binding
- We recommend setting this to 1 μL, though in some cases, for example if sample availability is very limited, it may be appropriate to set a value below 1 μL
- Some protocol steps include fixed values of 1 μL that will not be affected by this setting

Note: Sample Setup High-Throughput mode **does not contain an Advanced Options section**

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* OPLC = On-Plate Loading Concentration

Sample information summary table

Summary table provides complete sample information for review after completion of all required field entries

Sample Group Information

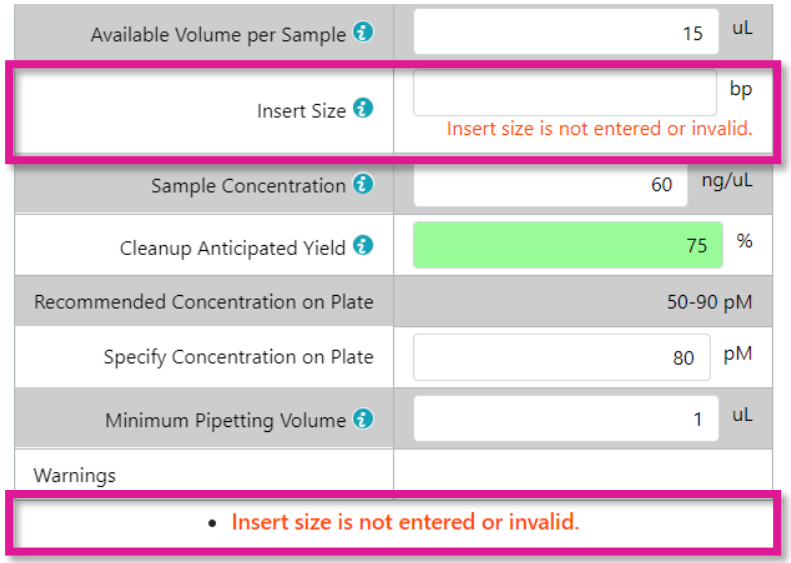
	Sample Group
Name	My Batch of Samples
Comment	
Number of Samples	2
SMRT Cells Per Sample	3
Sample Volume to Use	8.0 uL
Sample Concentration	60 ng/uL 5.13 nM
Insert Size	18000 bp
Binding Kit	Sequel® II Binding Kit 3.2
Cleanup Anticipated Yield	75 %
Concentration On Plate	80 pM
Application	HiFi Reads
Minimum Pipetting Volume	1 uL
Warnings	

- For HT mode, we **no longer prioritize primer:template or polymerase:template ratios**
  - For HT mode, primer concentration is locked at 20 nM and the polymerase concentration is locked at 15 nM
  - This is more consistent with the biochemistry and allows more efficient use of reagents
- When following the recommended input sample concentration guidance for different insert sizes:
  - Template concentration can range from ~1 nM to 5 nM in the annealing reaction and from ~0.5 nM to 2.5 nM in the binding reaction

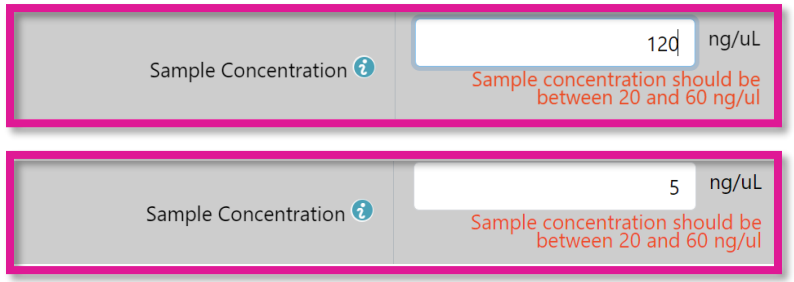
Sample Setup HT summary only shows the **starting** sample template concentration and does not display the template concentration in the annealing or binding reactions

Sample setup warnings

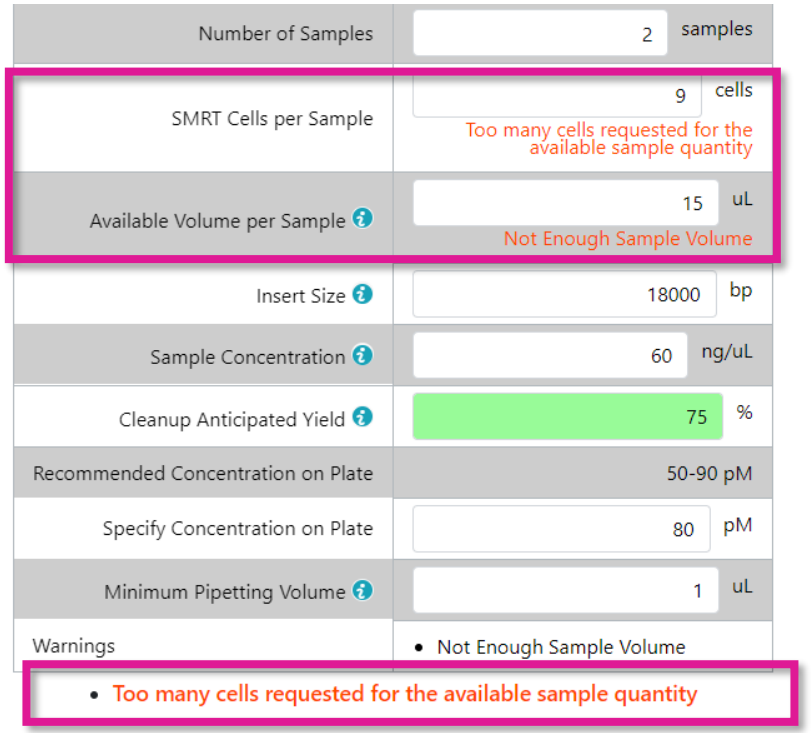
Sample Setup flags any incomplete / invalid entries and other sample issues



- If any field is **missing** an entry or contains an invalid entry, then **no** calculations will be performed
  - Enter a valid value to enable calculations to be performed



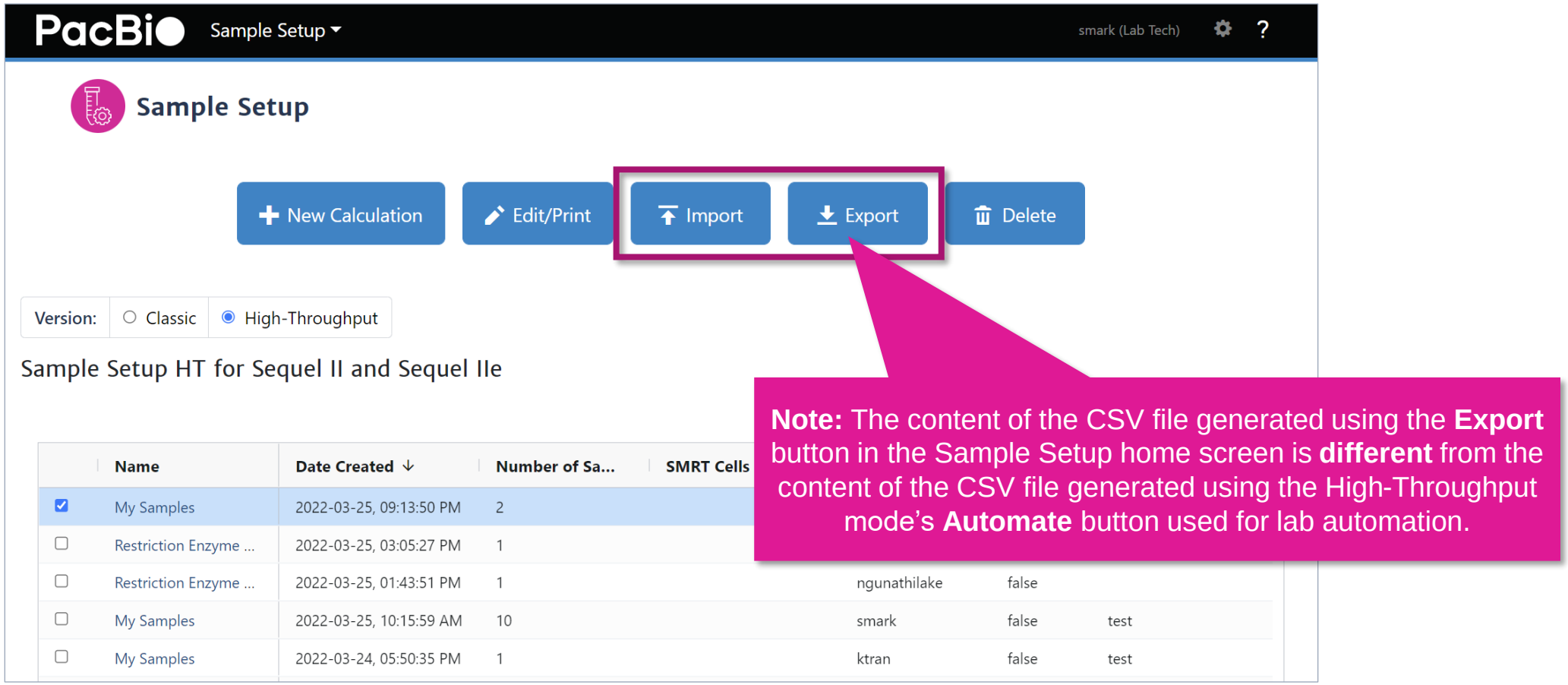
- If the sample concentration is **outside** the recommended range, calculations will still be performed **only** if there is a sufficient amount of sample to proceed with annealing and binding for the number of SMRT Cells specified
  - However, annealing and binding reaction conditions may not be optimal and generate lower than expected sequencing performance



- If there is an **insufficient** amount of sample to proceed with annealing and binding for the number of SMRT Cells specified, then **no** calculations will be performed
  - Can try reducing the on-plate loading concentration or the number of SMRT Cells to enable calculations to be performed

Importing and exporting sample setup worksheets

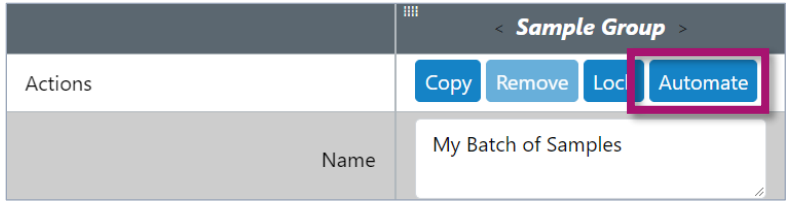
Sample Setup supports importing and exporting calculations in CSV format

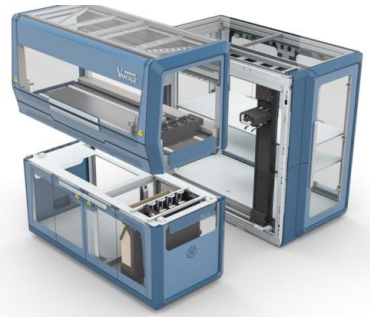


- To **import** a new calculation, first find (or create) a calculation **similar** to that you wish to import, then **export** it in CSV format. You can then customize the exported CSV file as needed, then **import** the modified CSV file

Exporting a sample setup worksheet for lab automation

From the sample setup worksheet, click **Automate** to export the calculated values to a CSV file for lab automation





Final Loading Dilution

For each of your 10 samples, combine the following **and protect from light**:

Component	High-Throughput Mode Demo	✓	
Prepared Sample	150.0 uL		
Sequel II Loading Buffer 3.2	200.0 uL		
Diluted Internal Control (Dilution 3)	10.0 uL		
Total Volume	360.0 uL		

Load 115 uL of sample per well and/or store at 4C for up to 24 hours before use.(*)

The CSV file generated by the **Automate** button in High-Throughput mode includes all the fields that display in the Sample Setup page, with the volumes listed in each table easily accessible for liquid handling automation purposes.

Row	Variable Name	Example Value
1	Export Version	4
2	Instructions Version	SMRT Link: 11.0.0.145013; Chemistry Bundle: 11.0.0.143406; Params: 11.0.0
3	Sampl	
4	Reque	
5	Annea	
6	Annea	
7	Annea	
8	Annea	
9	Annea	
10	Polym	
11	Sequel II Polymerase Dilution Buffer Volume	171.5
12	Binding Annealed Sample Volume	16
13	Binding Diluted Polymerase Volume	16
14	Binding Incubation Temperature (C)	25
15	Binding Incubation Time (minutes)	15
16	ICD Buffer Name	ABC Buffer
17	ICD1 Buffer Volume	19
18	ICD1 Internal Control Stock Volume	1
19	ICD2 Buffer Volume	19
20	ICD2 Diluted Internal Control (ICD1) Volume	1
21	ICD3 Buffer Volume	95
22	ICD3 Dil	
23	Cleanup	
24	Cleanup	
25	Cleanup	
26	Cleanup	
27	Cleanup	
28	Cleanup	
29	Cleanup S2 Dilution Total Volume	100
30	Cleanup S3 Bead Solution Volume	120
31	Cleanup S5 Elution Volume	150
32	Final Loading Prepared Sample Volume	150
33	Final Loading Dilution Buffer Volume	200
34	Final Loading Diluted Internal Control (ICD3) Volume	10
35	Final Loading Volume (microliter)	115

Note: If needed, HT automation scripts will instruct the robot to distribute the final loading dilution volume across the required number of plate wells to prevent liquid overflow.*

* If manually preparing libraries using 0.2 mL strip tubes, you may need to use a larger tube that can accommodate the final loading dilution volume.

SMRT Link Sample Setup ABC (annealing / binding / cleanup) recommendations

Follow SMRT Link Sample Setup instructions for using the recommended reagent kits for each application*

Main application	Application subtype / Supported use case	Template prep kit(s)	Annealing[1]	Binding[2,3]	Complex cleanup
Whole genome sequencing	Large genome WGS, microbial genome WGS, low DNA input & shotgun metagenomics	SPK 3.0	Use sequencing primer included with binding kit	Sequel II Binding Kit 3.2	SMRTbell cleanup beads
	Ultra-low DNA input sequencing	SMRTbell express TPK 2.0	Use sequencing primer included with binding kit	Sequel II Binding Kit 3.2	SMRTbell cleanup beads
Viral sequencing	HiFiViral SARS-CoV-2	SPK 3.0	Use sequencing primer included with binding kit	Sequel II Binding Kit 3.1	SMRTbell cleanup beads
	AAV sequencing	SPK 3.0	Use sequencing primer included with binding kit	Sequel II Binding Kit 3.1	SMRTbell cleanup beads
RNA sequencing	Iso-Seq method (bulk)[2]	SPK 3.0	Use sequencing primer included with binding kit	Sequel II Binding Kit 3.1 Sequel II Binding Kit 3.2	SMRTbell cleanup beads
	Single-cell Iso-Seq method[2]	SMRTbell express TPK 2.0	Use sequencing primer included with binding kit	Sequel II Binding Kit 3.1 Sequel II Binding Kit 3.2	SMRTbell cleanup beads
Metagenomics	Full-length 16S sequencing	SPK 3.0	Use sequencing primer included with binding kit	Sequel II Binding Kit 3.1	SMRTbell cleanup beads
Targeted sequencing	Amplicon sequencing (barcoded adapters or barcoded gene-specific primers)[3]	SPK 3.0	Use sequencing primer included with binding kit	Sequel II Binding Kit 3.1 Sequel II Binding Kit 3.2	SMRTbell cleanup beads
	Amplicon sequencing (barcoded M13 primers)[3]	SPK 3.0	Use sequencing primer included with binding kit	Sequel II Binding Kit 3.1 Sequel II Binding Kit 3.2	SMRTbell cleanup beads

1 Sequencing primer 3.1 is included with Sequel II binding kit 3.1; Sequencing primer 3.2 is included with Sequel II binding kit 3.2.

2 For standard Iso-Seq Method (bulk) and standard Single-Cell Iso-Seq samples, use Sequel II binding kit 3.1. For Iso-Seq Method (bulk) and Single-Cell Iso-Seq samples with a focus on long transcripts (≥3 kb), use Sequel II binding kit 3.2. (**Note:** The default binding kit specified for the Iso-Seq application type in SMRT Link Run Design is Binding kit 3.1.)

3 For amplicons <3 kb, use Sequel II binding kit 3.1. For amplicons ≥3 kb, use Sequel II binding kit 3.2.

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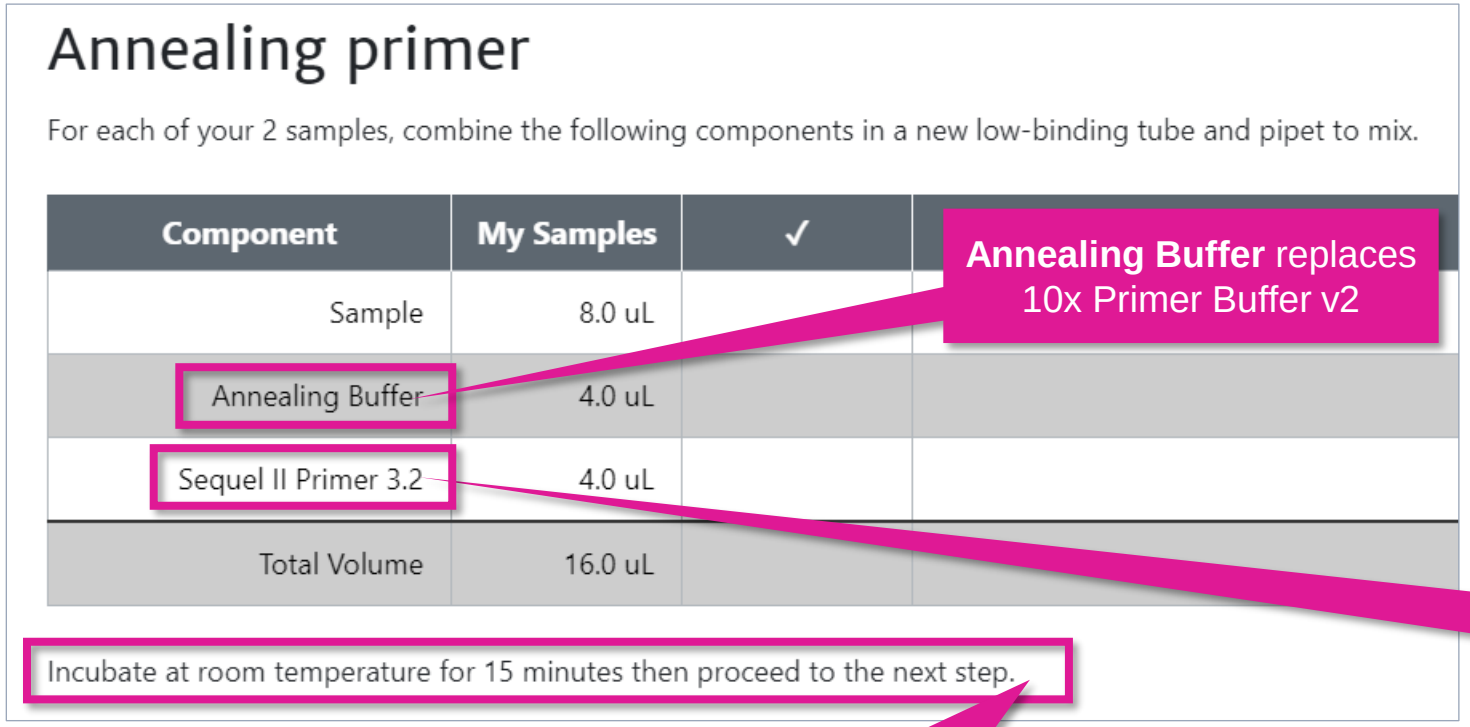
* Refer to Quick reference card – Loading and pre-extension time recommendations for the Sequel II and IIe systems (101-769-100) for updates to recommended ABC workflow parameters for specific applications.

Sample Setup High-Throughput mode annealing / binding / cleanup (ABC) and DNA internal control dilution procedure

Annealing procedure

Sequencing primer annealing reaction volumes are automatically calculated in SMRT Link Sample Setup



Annealing Buffer replaces 10x Primer Buffer v2

- Sample Setup calculates annealing and binding reaction volumes for each sample after all the entries have been made
- For primer annealing reactions, always add 1 part buffer and 1 part primer to 2 parts of sample
- Anneal samples to sequencing primer by incubating at room temperature (15 - 25°C) for 15 min
- After annealing the primer to each sample, proceed to polymerase binding

Use the pre-diluted and pre-conditioned sequencing primer **included** with the Sequel II Binding Kit.* **No primer conditioning step is needed**.

Classic Mode still states: *"Incubate at room temperature for 15 minutes then transfer to a 4C location for immediate use, or store at -20C for long-term use.(*)"*

- Sequel II Binding Kit 3.1 **includes** Sequel II Primer 3.1 (= v4 Primer)
- Sequel II Binding Kit 3.2 **includes** Sequel II Primer 3.2 (= v5 Primer)

* **Note:** Users should **NOT** use legacy sequencing primer products (Primer v4, v5) in place of the pre-diluted and pre-conditioned sequencing primers (Primer 3.1/3.2) included with Sequel II binding kits 3.1 and 3.2.

Binding procedure

Polymerase binding reaction volumes are automatically calculated in SMRT Link Sample Setup

Polymerase Dilution

Combine the following components in a single low-bind tube and pipet to mix. The prepared volume of diluted polymerase is sufficient to process all specified samples.

Component	My Samples	✓
Polymerase Stock	1.0 uL	
Sequel® II Polymerase Dilution Buffer	49.0 uL	
Total Volume	50.0 uL	

Sequel II Polymerase Dilution Buffer replaces Sequel Binding Buffer

Total volume supports **all** samples

Diluted Polymerase must be used immediately, any remaining should be discarded.

- All samples require an initial **polymerase dilution step** (i.e., a 50-fold dilution of the polymerase stock tube)
- Diluted polymerase must be used immediately, any remaining should be discarded.
- For binding reactions, always add 1 part diluted polymerase to 1 part of annealed sample
- Bind polymerase to annealed samples by incubating at room temperature* for 15 min
- After binding the polymerase to each sample, proceed to complex cleanup

Use the **same** tube from annealing step

Binding

For each of your 2 samples, add Diluted Polymerase and finger tap or pipet to mix.

Component	My Samples	✓
Annealed Sample	16.0 uL	
Diluted Polymerase	16.0 uL	
Total Volume	32.0 uL	

Incubate at room temperature for 15 minutes. Bound complex can be stored at 4C for 4 weeks.(*)

Updated storage time for bound complexes to **4 weeks** (instead of 7 days)

We recommend performing a ***fresh*** **binding reaction** for each sample whenever possible

* Room Temperature = 15°C - 25°C

Cleanup procedure

Complex cleanup reaction volumes are automatically calculated in SMRT Link Sample Setup

Purification of Polymerase Bound SMRTbell® Complexes

1. Equilibrate the Clean-up Beads and Sequel II Loading Buffer 3.2 to room temperature.

2. Please add the following buffer volumes to each sample in each batch, as indicated:

	Demo	✓	Notes
# of Samples in Batch	**2**		
Binding Reaction	32.0 uL		
ABC Buffer	68.0 uL		
Total Volume	100.0 uL		

3. Add the indicated volume of Clean-up Beads to each sample in each batch and gently pipette-mix. Incubate on the benchtop for 10 minutes.

	Demo	✓
# of Samples in Batch	**2**	
Clean-up Beads	120.0 uL	

4. Place each tube or tube strip 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.

5. Immediately resuspend the beads in the indicated volumes of room temperature Sequel II Loading Buffer 3.2 and pipette-mix:

	Demo	✓
# of Samples in Batch	**2**	
Sequel II Loading Buffer 3.2	150.0 uL	

Sequel II Loading Buffer 3.1 replaces Complex Dilution Buffer (CDB)

Sequel II Loading Buffer 3.2 replaces Adaptive Loading Buffer (ALB)

ABC Buffer replaces Complex Dilution Buffer (CDB)

Use **1.2X SMRTbell Clean-up Beads included** with the Sequel II Binding Kit

1.2X vol.

- **Note:** To streamline high-throughput workflows, DNA quantification of pre-cleanup and post-cleanup complexes is **no longer required***
- As a result, the actual sample OPLC in the final dilution step **may vary** from the specified target OPLC
- If needed, users can adjust the Cleanup Anticipated Yield to improve concordance of target OPLC vs. actual OPLC values

- After performing complex cleanup, proceed to prepare the DNA Internal Control

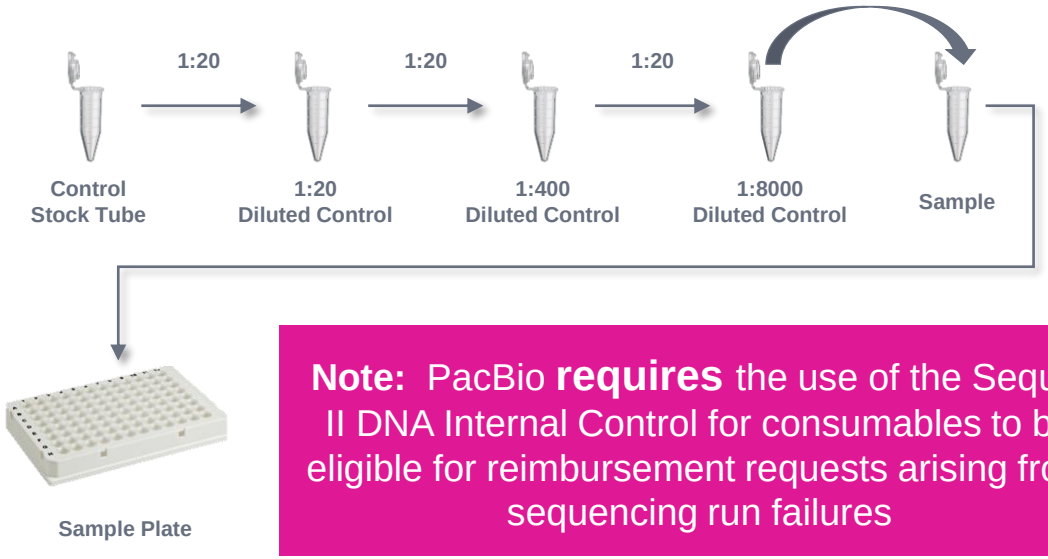
PacBio

*** Optional:** If desired, you may quantify sample recovery efficiency using a Qubit instrument, but this is not required.

DNA internal control dilution procedure

Sample Setup provides instructions for diluting the DNA Internal Control stock before adding to the final solution for loading into the sample plate well

- Addition of (Spike-In) DNA Internal Control for each sample is **highly recommended**
  - Can help distinguish instrument-related issues from sample library quality issues and enables quicker troubleshooting
- Perform **three** sequential 1:20 dilution steps using ABC Buffer to prepare the DNA Internal Control working solution
- Add 3 µL (per SMRT Cell 8M) of serially diluted DNA Internal Control to final on-plate sample dilution



Note: PacBio **requires** the use of the Sequel II DNA Internal Control for consumables to be eligible for reimbursement requests arising from sequencing run failures

Internal Control Dilution

Perform three sequential dilution steps using ABC Buffer. Use a new low-binding tube for each dilution step.

1. First Dilution

Reagent	Internal Control		
ABC Buffer	19.0 uL		
Sequel II DNA Internal Control Complex 3.2	1.0 uL		

Mix well by flicking tube by hand and pulse spin to collect contents and keep on ice.

2. Second Dilution

Reagent	Internal Control		
ABC Buffer	19.0 uL		
Diluted Internal Control (Dilution 1)	1.0 uL		

Mix well by flicking tube by hand and pulse spin to collect contents and keep on ice.

3. Third Dilution

Reagent	Internal Control	✓	
ABC Buffer	19.0 uL		
Diluted Internal Control (Dilution 2)	1.0 uL		

Mix well by flicking tube by hand and pulse spin to collect contents and keep on ice.

Use the DNA internal control **included** with the Sequel II Binding Kit

Three small-volume dilution steps are performed for better compatibility with automation systems

Final loading dilution procedure

Follow Sample Setup instructions to add diluted DNA Internal Control and Loading Buffer to your sample

Final Loading Dilution

For each of your 2 samples, combine the following **and protect from light**:

Component	My Samples	✓	
Prepared Sample	100.0 uL		
Sequel II Loading Buffer 3.2	134.0 uL		
Diluted Internal Control (Dilution 3)	6.0 uL		
Total Volume	240.0 uL		

Load 115 uL of sample per well and/or store at 4C for up to 24 hours before use.(*)

(*) Sequencing performance after storage is dependent on DNA sample quality/type and cannot be guaranteed.

Use the Sequel II Loading Buffer **included** with the Sequel II Binding Kit

Parallel display of multiple batches at annealing and binding steps

Annealing primer

For each sample in each batch, combine the following components in a new low-binding tube (one tube per sample) and pipet to mix.

	Sample 1	Sample 2	My Batch of Samples	✓	
# of Samples in Batch	**1**	**1**	**1**		
Sample	5.0 uL	5.0 uL	12.0 uL		
Annealing Buffer	2.5 uL	2.5 uL	6.0 uL		
Sequel II Primer 3.2	2.5 uL	2.5 uL	6.0 uL		
Total Volume	10.0 uL	10.0 uL	24.0 uL		

Incubate at room temperature for 15 minutes then proceed to the next step.

Total volume of diluted polymerase master mix prepared is sufficient to process **all** samples in **all** batches

Polymerase Dilution

This dilution produces one **Diluted Polymerase Master Mix** with enough volume for all samples in all batches.

Combine the following components in a single low-bind tube and pipet to mix.

Reagent	Diluted Polymerase	✓	
Polymerase Stock	1.0 uL		
Sequel® II Polymerase Dilution Buffer	49.0 uL		
Total Volume	50.0 uL		

Diluted Polymerase must be used immediately, any remaining should be discarded.

Binding

For each sample in each batch, add Diluted Polymerase and finger tap or pipet to mix.

	Sample 1	Sample 2	My Batch of Samples	✓	
# of Samples in Batch	**1**	**1**	**1**		
Annealed Sample	10.0 uL	10.0 uL	24.0 uL		
Diluted Polymerase	10.0 uL	10.0 uL	24.0 uL		
Total Volume	20.0 uL	20.0 uL	48.0 uL		

Incubate at room temperature for 15 minutes. Bound complex can be stored at 4C for 4 weeks.(*)

Parallel display of multiple batches at cleanup step

Purification of Polymerase Bound SMRTbell® Complexes

Cleanup instructions are displayed in table form for **easier viewing**

1. Equilibrate the Clean-up Beads and Sequel II Loading Buffer 3.2 to room temperature.

2. Please add the following buffer volumes to each sample in each batch, as indicated:

	Humans 1 2 and 3	Humans 4 and 5	Human 6	✓	
# of Samples in Batch	**3**	**2**	**1**		
Binding Reaction	20.0 uL	20.0 uL	48.0 uL		
ABC Buffer	80.0 uL	80.0 uL	52.0 uL		
Total Volume	100.0 uL	100.0 uL	100.0 uL		

3. Add the indicated volume of Clean-up Beads to each sample in each batch and gently pipette-mix. Incubate on the benchtop for 10 minutes.

	Humans 1 2 and 3	Humans 4 and 5	Human 6	✓	
# of Samples in Batch	**3**	**2**	**1**		
Clean-up Beads	120.0 uL	120.0 uL	120.0 uL		

4. Place each tube or tube strip 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.

5. Immediately resuspend the beads in the indicated volumes of room temperature Sequel II Loading Buffer 3.2 and pipette-mix:

	Humans 1 2 and 3	Humans 4 and 5	Human 6	✓	
# of Samples in Batch	**3**	**2**	**1**		
Sequel II Loading Buffer 3.2	150.0 uL	150.0 uL	150.0 uL		

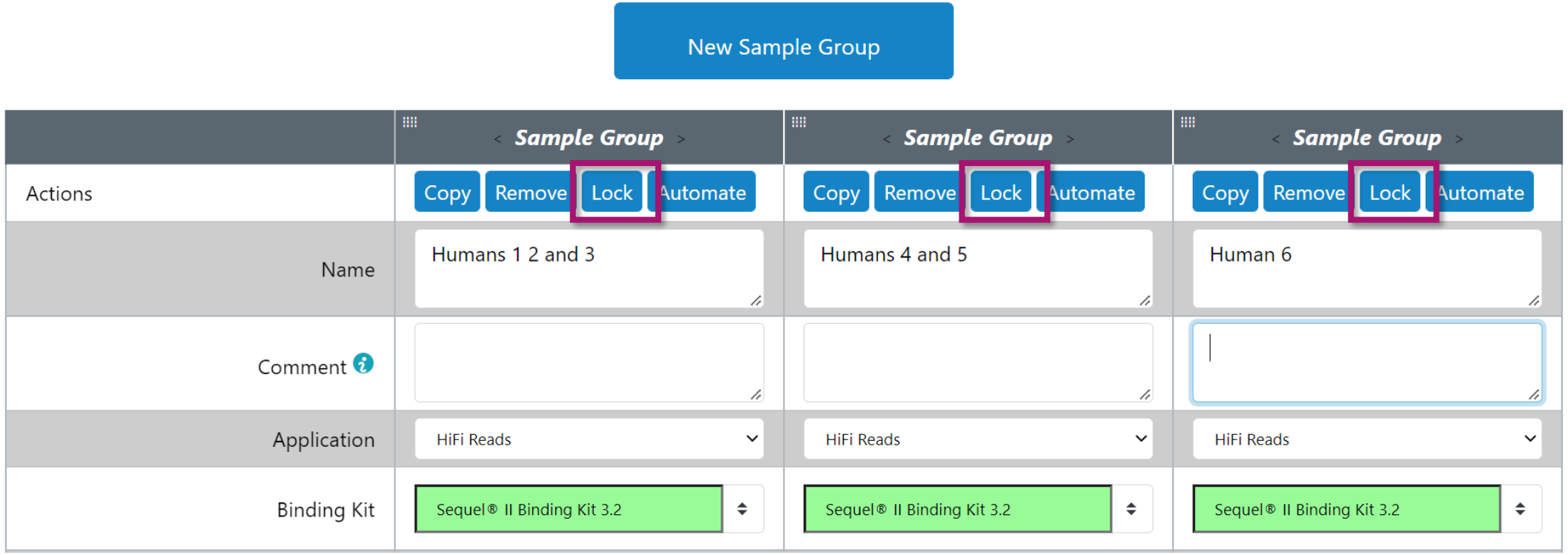
6. To elute the polymerase-bound complexes, incubate the samples on the benchtop for at least 5 minutes at room temperature.

7. Place each tube or tube strip in a magnetic bead rack until the beads collect to the side of the tube and the solution appears clear.

8. Transfer eluates to new low-binding tubes or tube strips. Place on ice **and protect from light**.

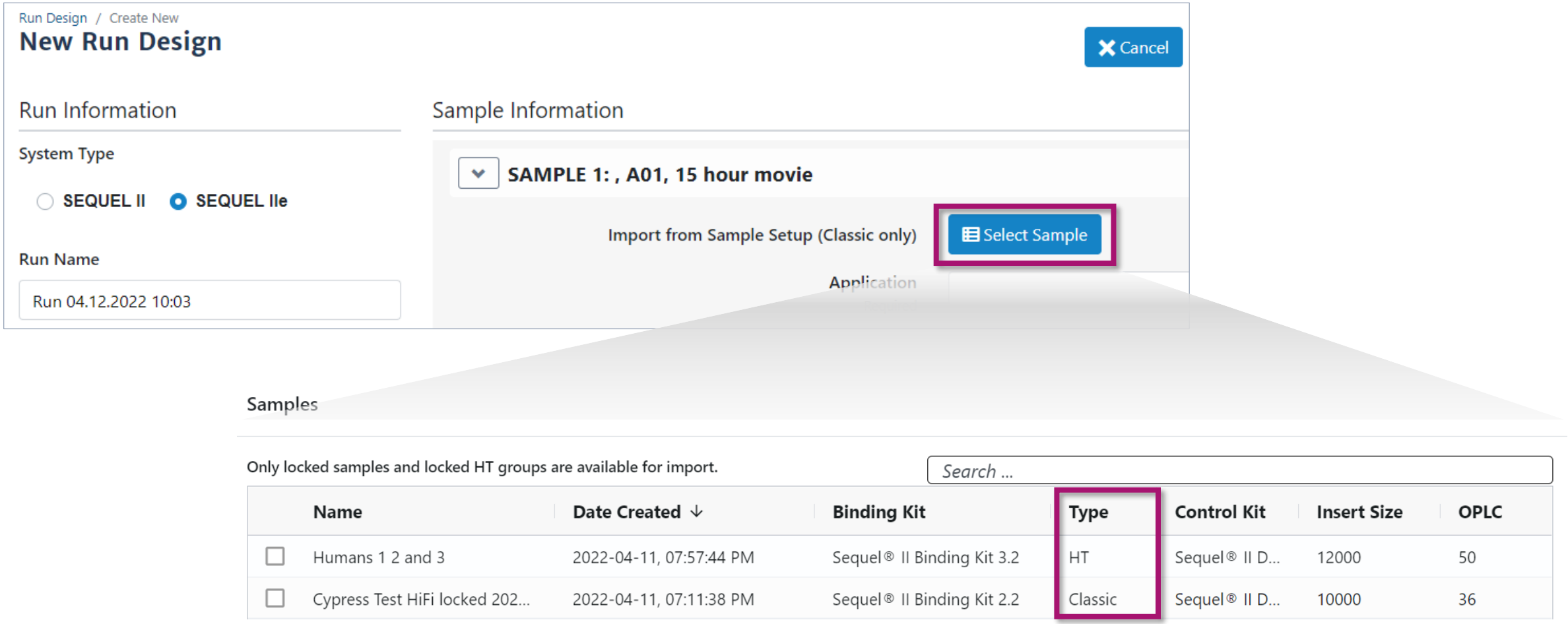
Optional: If desired, you may quantify sample recovery efficiency using a Qubit instrument, but this is not required.

Sample Batches are now Lockable



Lock is required before samples can be imported into Run Design module and also sends a finalized version of the instructions to the server for use in Data Set reports.

Sample Setup HT batches can now be imported into Run Design



Limitation: Import is still performed at the level of single collections (individual SMRT Cells) in Run Design.

→ The user will need to import the same Sample Setup HT batch worksheet for all pertinent samples and define appropriate sample names (and other run design parameters if needed) for each SMRT Cell to be run

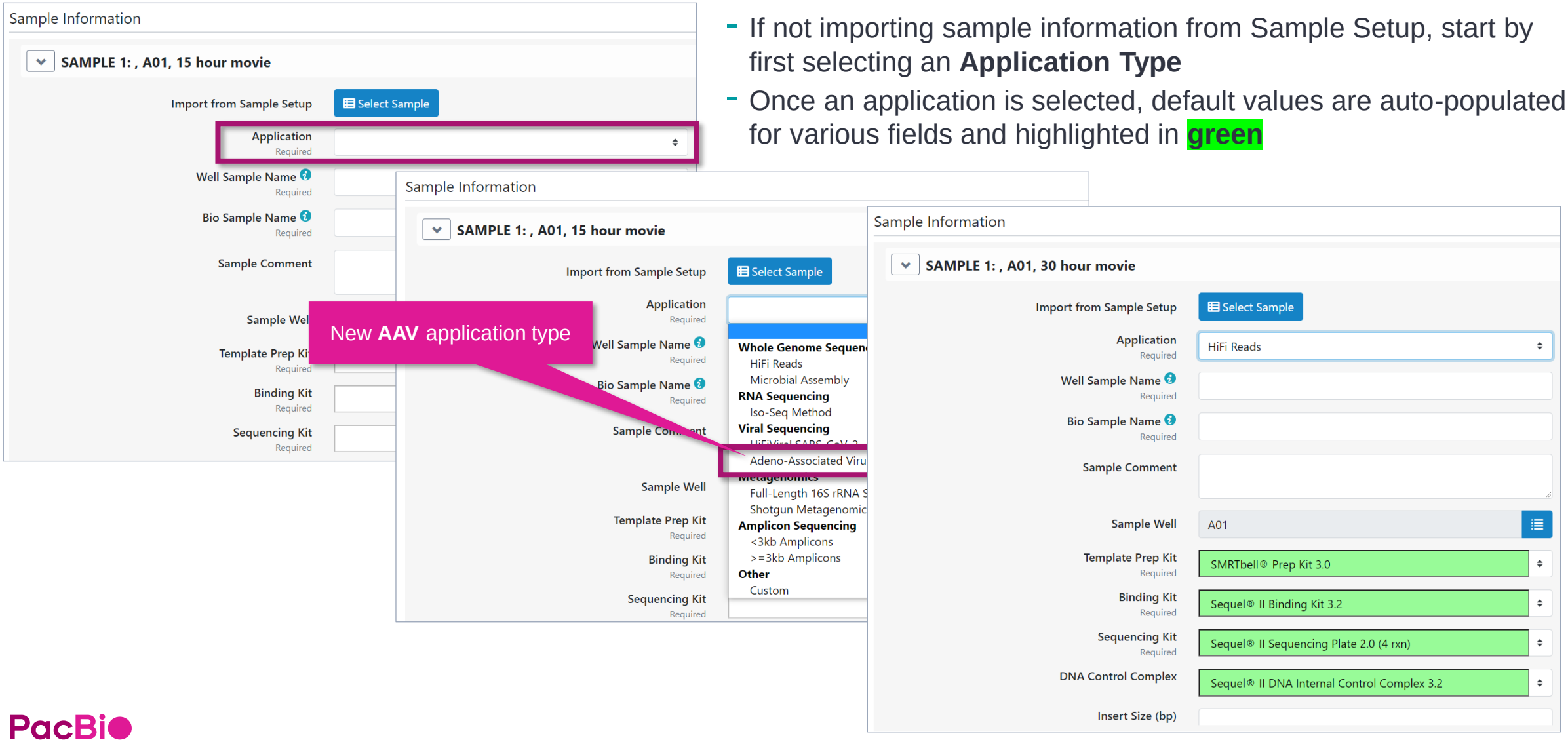
SMRT Link Run Design updates

GUI and new default CCS analysis output changes

Application type specification

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

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

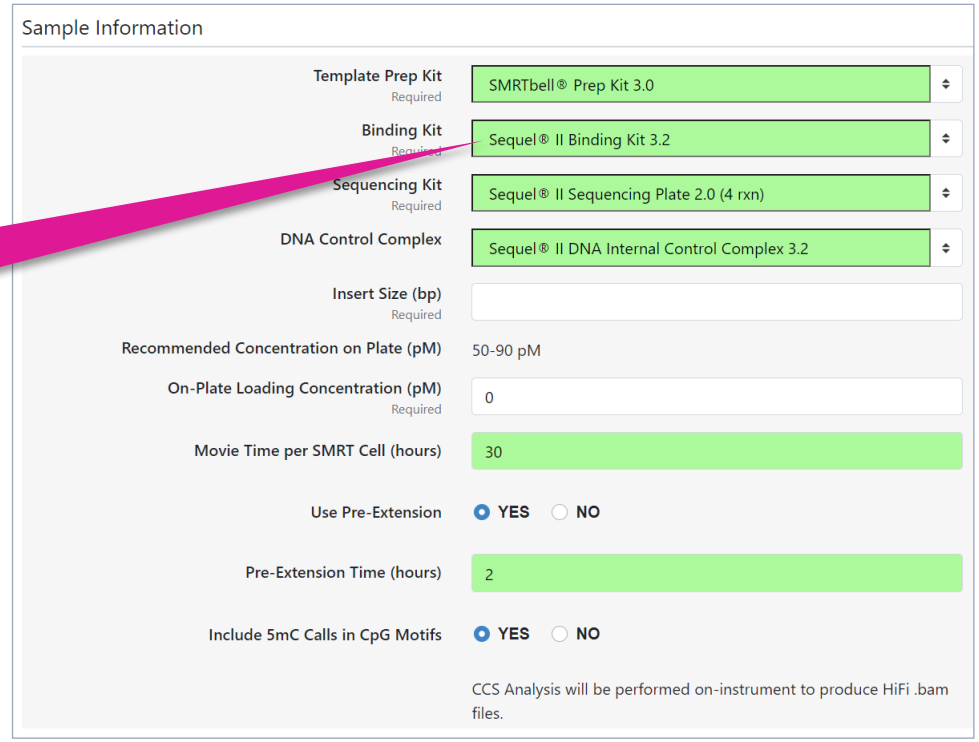


Application type specification (cont.)

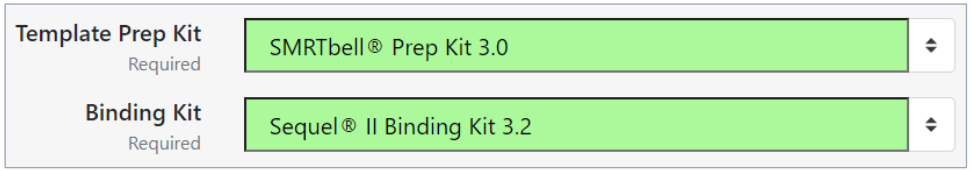
Auto-populated fields are highlighted in green color

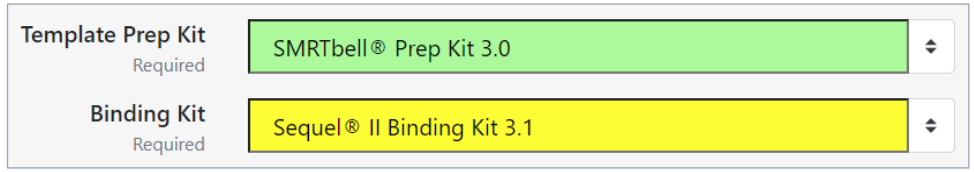
- The following fields are auto-populated and highlighted in **green**:
  - Template Prep Kit
  - Binding Kit
  - Sequencing Kit
  - DNA Control Complex
  - Movie Time Per SMRT Cell
  - Pre-Extension Time

Adaptive Loading is automatically enabled when Sequel II binding kit 2.2 or 3.2 is specified as the default binding kit to use for an application



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





SMRT Link Run Design setup recommendations

Follow SMRT Link Run Design instructions for using the recommended run conditions for each application

Main application	Application subtype / Supported use case	Template prep kit(s)	Pre-extension time	Movie collection time
Whole genome sequencing	**Large genome WGS, Low DNA input WGS & shotgun metagenomics**	SPK 3.0	2 hrs	30 hrs
	Microbial WGS	SPK 3.0	2 hrs	15 hrs
	Ultra-low DNA input sequencing	SMRTbell express TPK 2.0	2 hrs	30 hrs
Viral sequencing	**HiFiViral SARS-CoV-2**	SPK 3.0	0 hrs	8 hrs
	AAV sequencing	SPK 3.0	2 hrs	24 hrs
RNA sequencing	**Iso-Seq method (bulk)**	SPK 3.0	2 hrs	24 hrs
	Single-cell Iso-Seq method	SMRTbell express TPK 2.0	2 hrs	24 hrs
Metagenomics	**Full-length 16S sequencing**	SPK 3.0	0.5 hrs	10 hrs
Targeted sequencing	**Amplicon sequencing** (barcoded adapters or barcoded gene-specific primers)	SPK 3.0	Use default value in Run Design for the specified insert size	10 hrs (<3 kb) 30 hrs (≥3 kb)
	Amplicon sequencing (barcoded M13 primers)	SPK 3.0	Use default value in Run Design for the specified insert size	10 hrs (<3 kb) 30 hrs (≥3 kb)

- Refer to Quick reference card – Loading and pre-extension time recommendations for the Sequel II and IIe systems (101-769-100) for **updates** to recommended run setup parameters for specific applications

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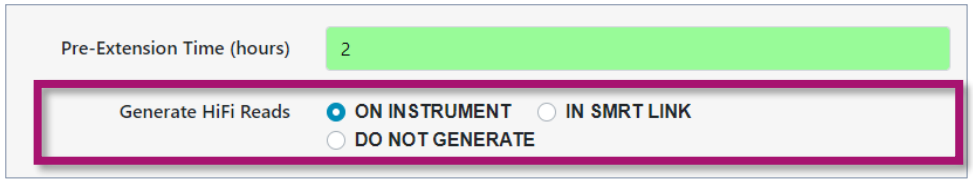
HiFi read generation is automatically enabled in SMRT Link v11.0

IMPORTANT! By default, all newly created Sequel II and IIe system run designs will specify to **automatically perform CCS analysis** and output **only** HiFi reads

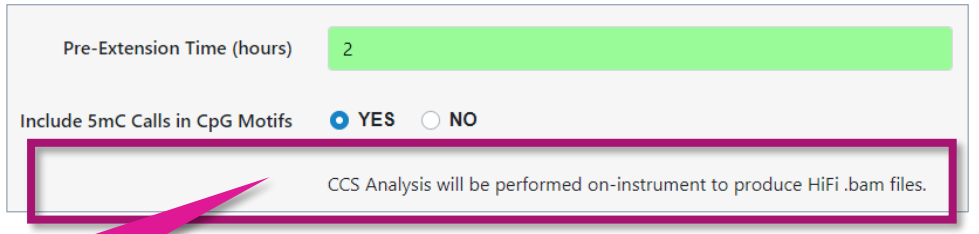
Sequel IIe system

- With Sequel IIe system run designs, the on-instrument CCS (OICCS) analysis workflow is **automatically** enabled and outputs a `hifi_reads.bam` file that contains **only HiFi (≥QV 20 CCS) reads**.*
  - In the Run Design **Advanced Options** section, users can specify whether to include low quality reads (non-HiFi reads) in the CCS analysis output (i.e., generate the full `reads.bam` file to support legacy CLR experiments). Note that specifying YES for this option **disables** automated on-instrument barcode demultiplexing, 5mC detection and heteroduplex detection, if applicable.

SMRT Link v10.2 Run Design



SMRT Link v11.0 Run Design



'On-Instrument / In SMRT Link / Do Not Generate' control is now **hidden** and locked to defaults

* Note for **Sequel IIe system** users: To access subread-level (CLR) data, run designs must be created using the *.CSV import feature. In this case, both HiFi **and** subread data are saved and transferred to the SMRT Link storage server. (The `scraps.bam` file, however, is not available.) See the SMRT Link User Guide (102-278-200) for details.

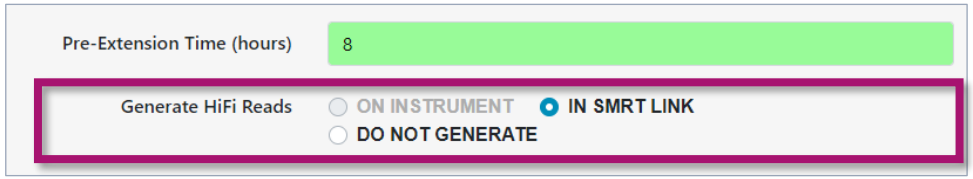
HiFi read generation is automatically enabled in SMRT Link v11.0 (cont.)

IMPORTANT! By default, all newly created Sequel II and IIe system run designs will specify to **automatically perform CCS analysis** and output **only** HiFi reads

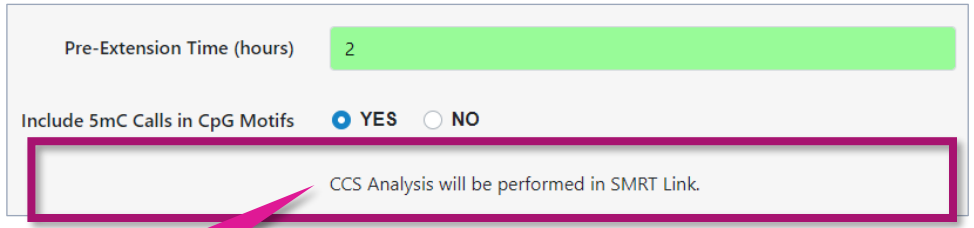
Sequel II system

- With Sequel II system run designs, CCS analysis in SMRT Link is **automatically** enabled and outputs a `hifi_reads.bam` file that contains **only HiFi (≥QV 20 CCS) reads**.
  - In the Run Design **Advanced Options** section, users can specify whether to include low quality reads (non-HiFi reads) in the CCS analysis output (i.e., generate the full `reads.bam` file to support legacy CLR experiments). Note that specifying YES for this option **disables** automated (in SMRT Link) barcode demultiplexing, 5mC detection and heteroduplex detection, if applicable

SMRT Link v10.2 Run Design



SMRT Link v11.0 Run Design

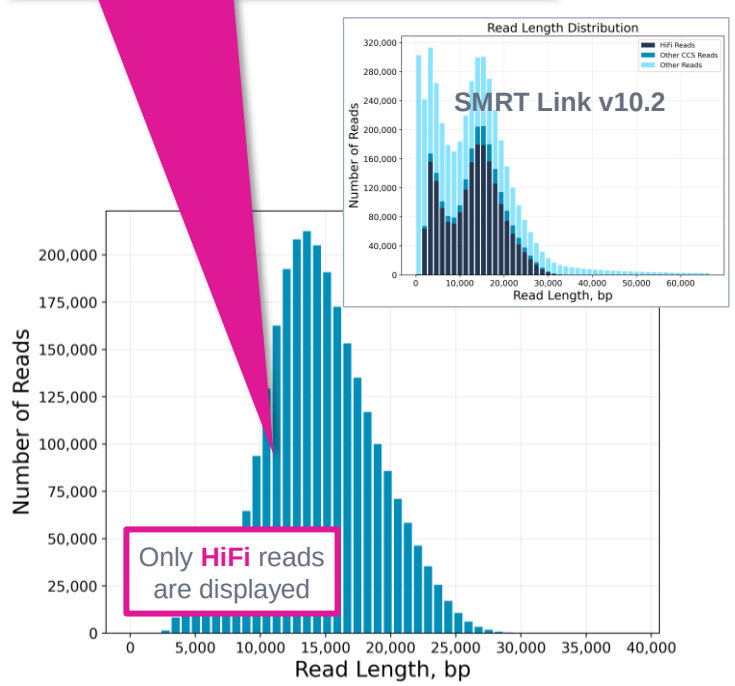


'In SMRT Link / Do Not Generate' control is now **hidden** and locked to defaults

HiFi read generation is automatically enabled in SMRT Link v11.0 (cont.)

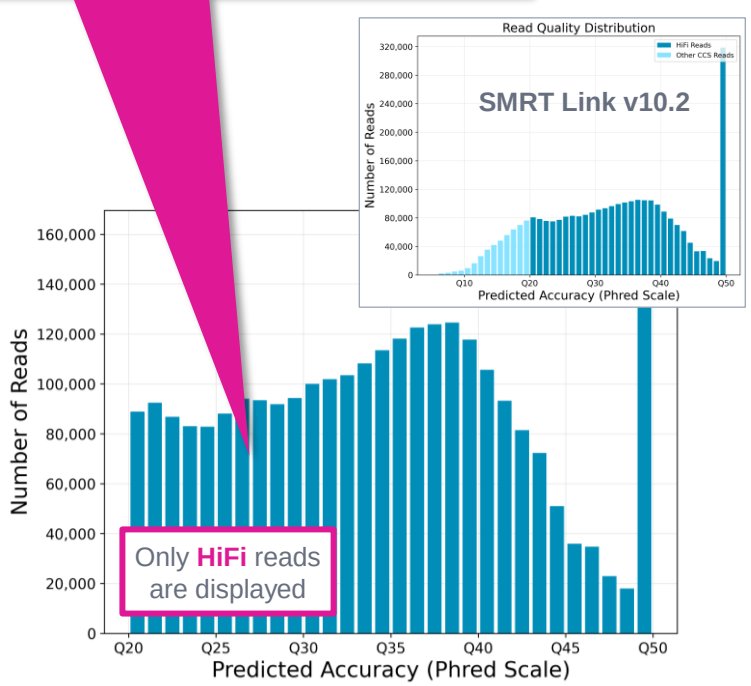
- The following **HiFi (QV ≥20) data-specific** reports are automatically generated in SMRT Link Run QC:
  - Read Length Distribution: Displays a read length histogram distribution for HiFi reads
  - Read Quality Distribution: Displays a read quality histogram distribution for HiFi reads
  - Read Length vs Predicted Accuracy: Displays a heat map of HiFi read lengths and predicted accuracies.

No longer displays 'Other CCS Reads' (<QV 20) or 'Other Reads'



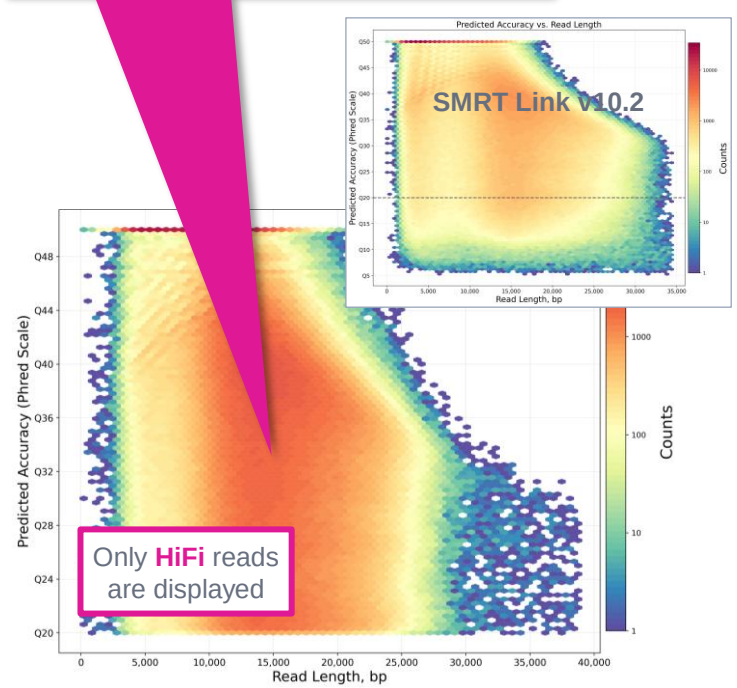
Read Length Distribution
SMRT Link v11.0 Run QC

No longer displays 'Other CCS Reads' (<QV 20)



Raw Quality Distribution
SMRT Link v11.0 Run QC

No longer displays 'Other CCS Reads' (<QV 20)



Predicted Accuracy vs. Read Length
SMRT Link v11.0 Run QC

File and directory structure output by Sequel IIe system ICS v11.0

Example default file and directory structure output by the Sequel IIe system for each SMRT Cell transferred to network storage

```
<your_specified_output_directory>/r64012e_211206ee_183753/1_A01/
|--m64012e_211206_183753.baz2bam_1.log
|--m64012e_211206_183753.ccs.log
|--m64012e_211206_183753.ccs_reports.json
|--m64012e_211206_183753.ccs_reports.txt
|--m64012e_211206_183753.consensusreadset.xml
|--m64012e_211206_183753.hifi_reads.bam
|--m64012e_211206_183753.hifi_reads.bam.pbi
|--m64012e_211206_183753.sts.xml
|--m64012e_211206_183753.zmw_metrics.json.gz
|--m64012e_211206_183753.transferdone
```

In ICS v11.0, **default** output files include `*.hifi_reads.bam` and `*.hifi_reads.bam.pbi` **instead of** `*.reads.bam` and `*.reads.bam.pbi`

(**Note:** *Unless* low quality reads are optionally included in CCS output)

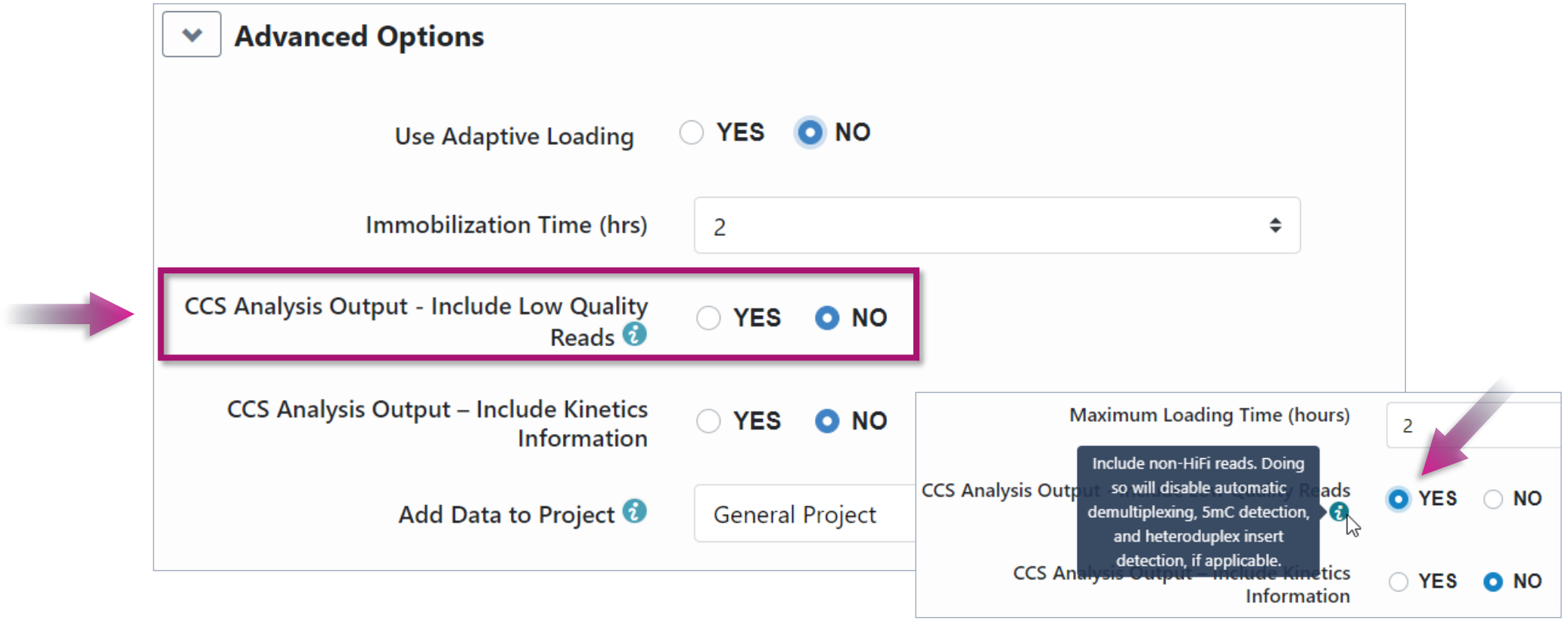
If 5mC CpG Detection is performed, the following additional files are output:

```
|-- m64012e_211206_183753.5mc_report.json
|-- m64012e_211206_183753.primrose.log
```

Advanced option: Include low quality reads in CCS analysis output

Optional: Click **Advanced Options** and specify, for this Run Design **only**, whether to include **low quality reads** (non-HiFi reads) in the CCS analysis output.

- **Default setting = NO** for all application types
- Note that specifying YES for this option **disables** automatic on-instrument barcode demultiplexing, 5mC detection, and heteroduplex insert detection on Sequel IIe systems since lower-quality data are **not** compatible with these automated workflows*



* Note for **Sequel II System** users: Specifying YES for this option disables automatic barcode demultiplexing in SMRT Link, 5mC detection in SMRT Link, and heteroduplex insert detection in SMRT Link since lower-quality data are not compatible with these automated workflows*

Advanced option: Include low quality reads in CCS analysis output (cont.)

Note: Specification in Run Design to include low quality reads (non-HiFi reads) in the CCS analysis output affects on-instrument and in-SMRT Link data processing options

System	Data processing feature	Include low quality reads in CCS analysis output?	
		NO (Default)	YES
Sequel IIe system	CCS analysis	• `hifi_reads.bam` generated on-instrument	• `reads.bam` generated on-instrument
	Heteroduplex detection	• Automated on-instrument: available • In SMRT Link: **unavailable**	• On-instrument: **unavailable** • In SMRT Link: **unavailable**
	5mC detection	• Automated on-instrument: available • Automated in SMRT Link: available*	• On-instrument: **unavailable** • Manual in SMRT Link: available*
	Barcode demultiplexing	• Automated on-instrument: available • Automated in SMRT Link: available	• On-instrument **unavailable** • Manual in SMRT Link: available
Sequel II system	CCS analysis	• `hifi_reads.bam` generated in SMRT Link	• `reads.bam` generated in SMRT Link
	Heteroduplex detection	• Automated in SMRT Link: available	• Manual in SMRT Link: available (`subreads.bam` input only)
	5mC detection	• Automated in SMRT Link: available	• Manual in SMRT Link: available* (`subreads.bam` or `reads.bam` input)
	Barcode demultiplexing	• Automated in SMRT Link: available	• Manual in SMRT Link: available (`reads.bam` input only)

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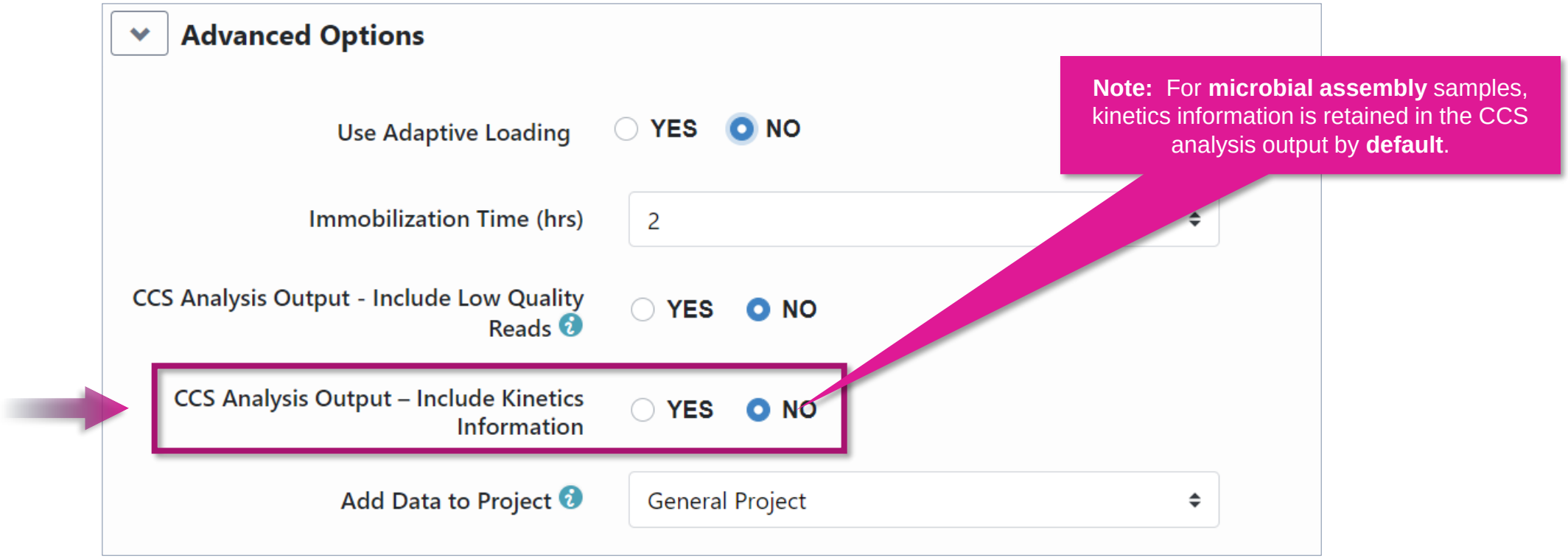
* Kinetics information must be retained in the CCS analysis output

Advanced option: Include kinetics information in CCS analysis output

Optional: Click **Advanced Options** and specify, for this Run Design **only**, whether to include kinetics information (used for epigenetics analysis) in the CCS Analysis output.

- **Default setting = NO** for all application types **except** microbial assembly*
- Adjusting this setting in Run Design **overwrites** any previous global setting specified in the SMRT Link configuration home page.
- **Note:** Adding kinetics information can increase the amount of storage used by the output BAM files by up to **5 times**.



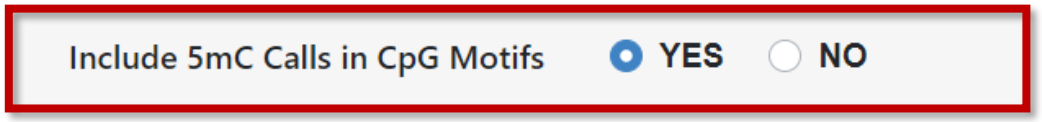
* **Note:** If automated 5mC detection is specified, users have the option of retaining or not retaining kinetics information in the CCS analysis output.

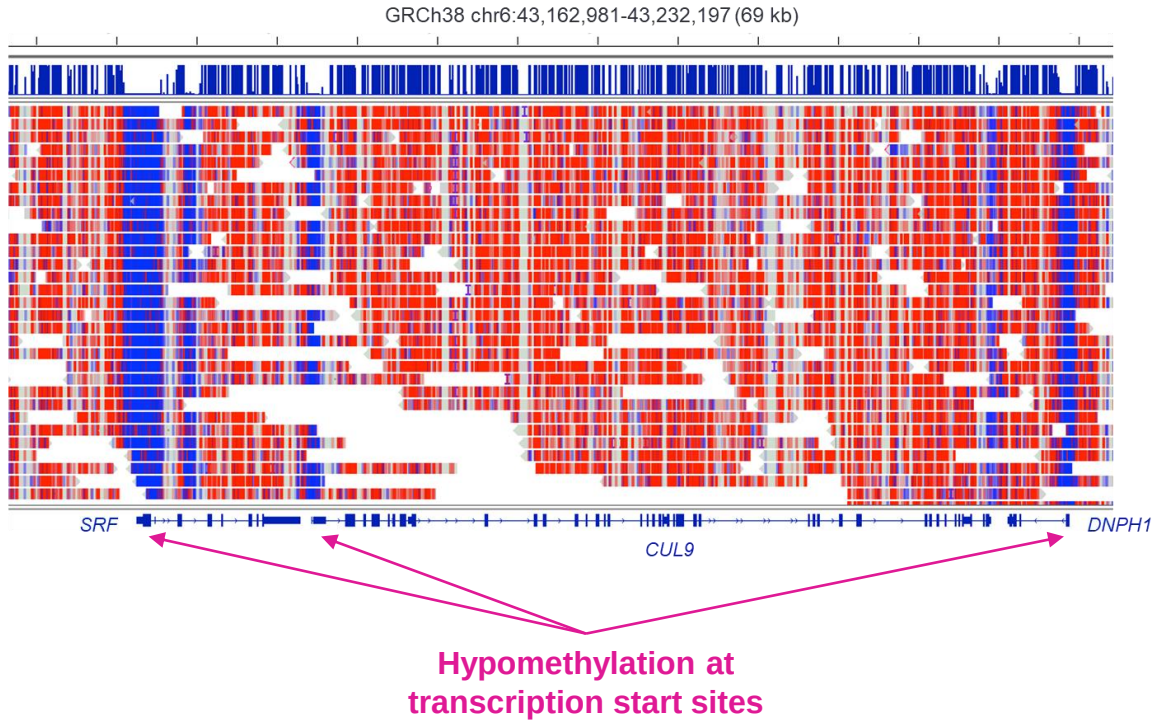
New 5mC detection option

Include 5mC calls in CpG motifs

If selected, kinetic signatures of cytosine bases in CpG motifs will be automatically analyzed to identify the presence of 5mC during on-instrument CCS (Sequel IIe system only) or during CCS analysis in SMRT Link

- **Default setting = YES** when specifying 'HiFi Reads' or 'Custom' application types
- 5mC detection is automatically performed on-instrument with the Sequel IIe system and in SMRT Link with the Sequel II system (data outputs are the **same** for both methods)
- 5mC calls are output in `hifi_reads.bam` as BAM standard MM and ML tags and can be easily visualized in IGV
- Processing and storage requirements are **minimal**:
  - File size increase is ~5%
  - On-instrument processing time for Sequel IIe systems is ~10 minutes
- Kinetics are not retained in the CCS analysis output by default, but they can **optionally** be retained as well.
- 5mC calls require a **CpG context and symmetrical methylation** (i.e., does not detect hemi-methylated sites)
- Though trained on human data, 5mC detection has been demonstrated to work on non-human data (e.g., plants (Maize)).
- 5mC consensus calling and other tools planned for a a future SMRT Link version.
  - For guidance on command line tool options for 5mC analysis, please contact your local PacBio support team or PacBio Technical Support



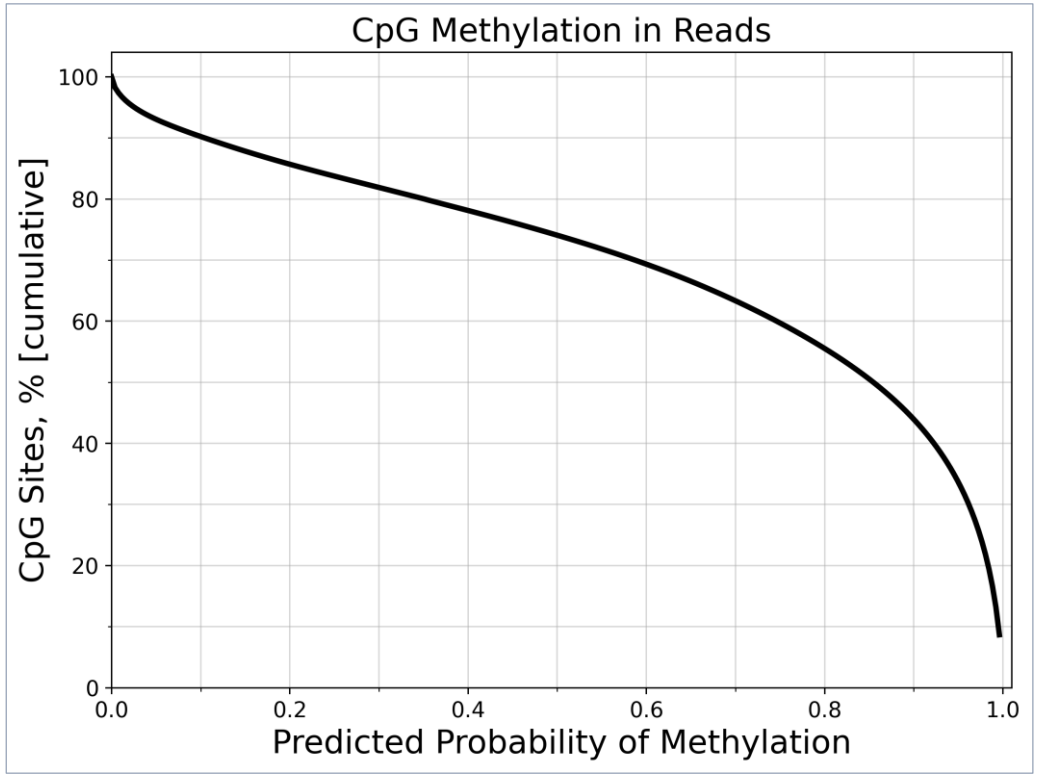


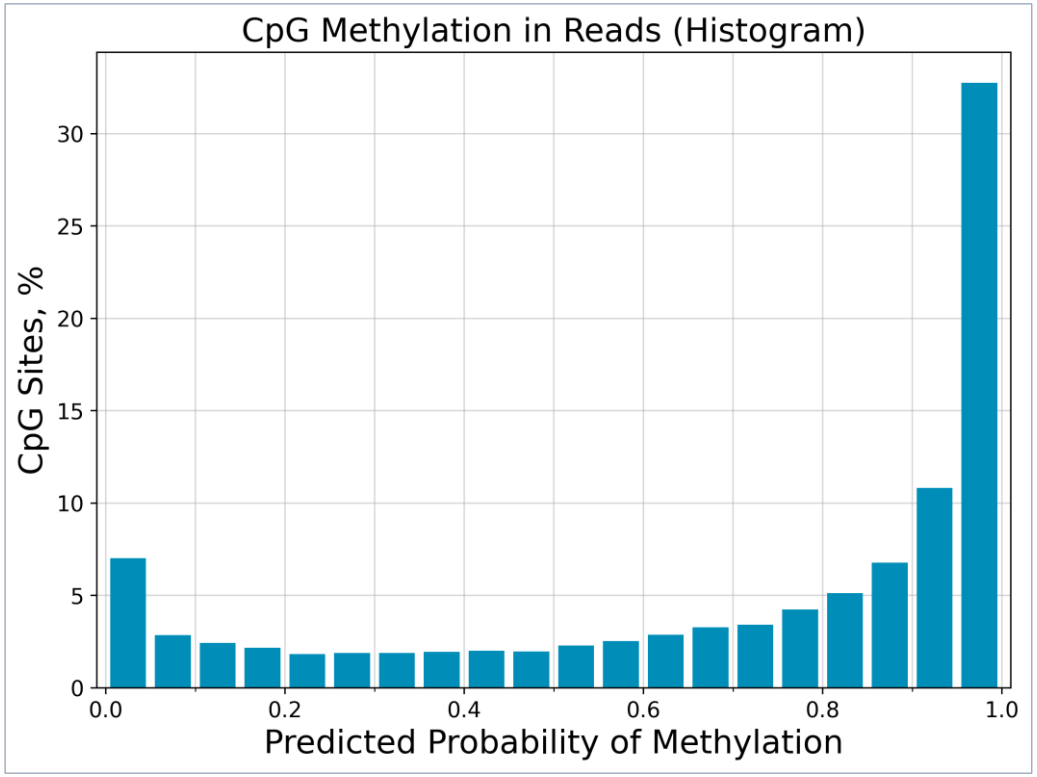
Example IGV plot demonstrating 5mC detection in HiFi reads for a human HG002 sample. Hypomethylation at active transcription start sites can be easily visualized (unpublished data).

Example 5mC data set: https://downloads.pacbcloud.com/public/dataset/HG002-CpG-methylation-202202/

Include 5mC calls in CpG motifs (cont.)

- The **5mC CpG detection** utility generates the following reports:
  - CpG Methylation in Reads: Plots the cumulative percentage of CpG sites in the sample against the predicted probability of methylation. (Report appears in SMRT Link Run QC and Data Management)
  - CpG Methylation in Reads (Histogram): Histogram plot displaying the percentage of CpG sites in the sample versus the predicted probability of methylation (Report appears in Data Management only)

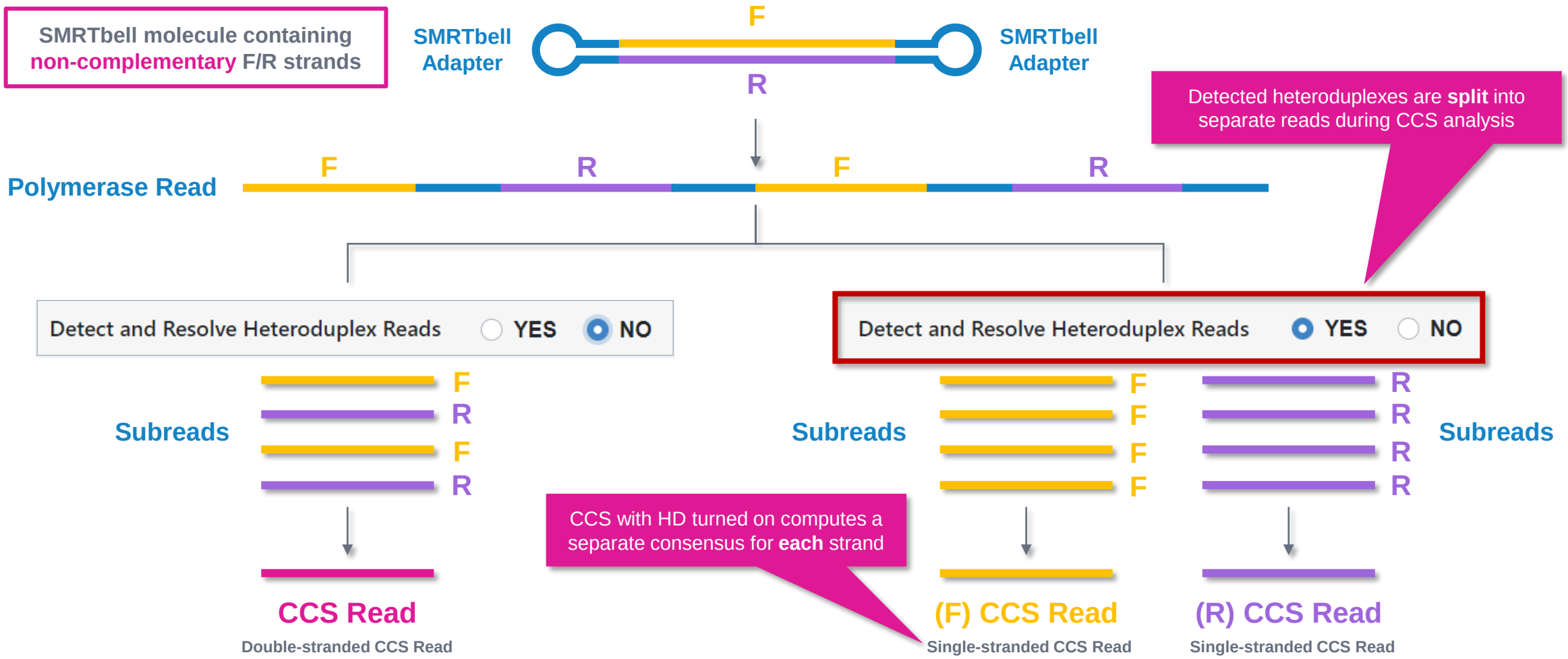




New heteroduplex detection option

Detect and resolve heteroduplex reads

If selected,* any detected heteroduplexes (HD) are separated into separate reads during on-instrument CCS (Sequel IIe system only) or during CCS analysis in SMRT Link

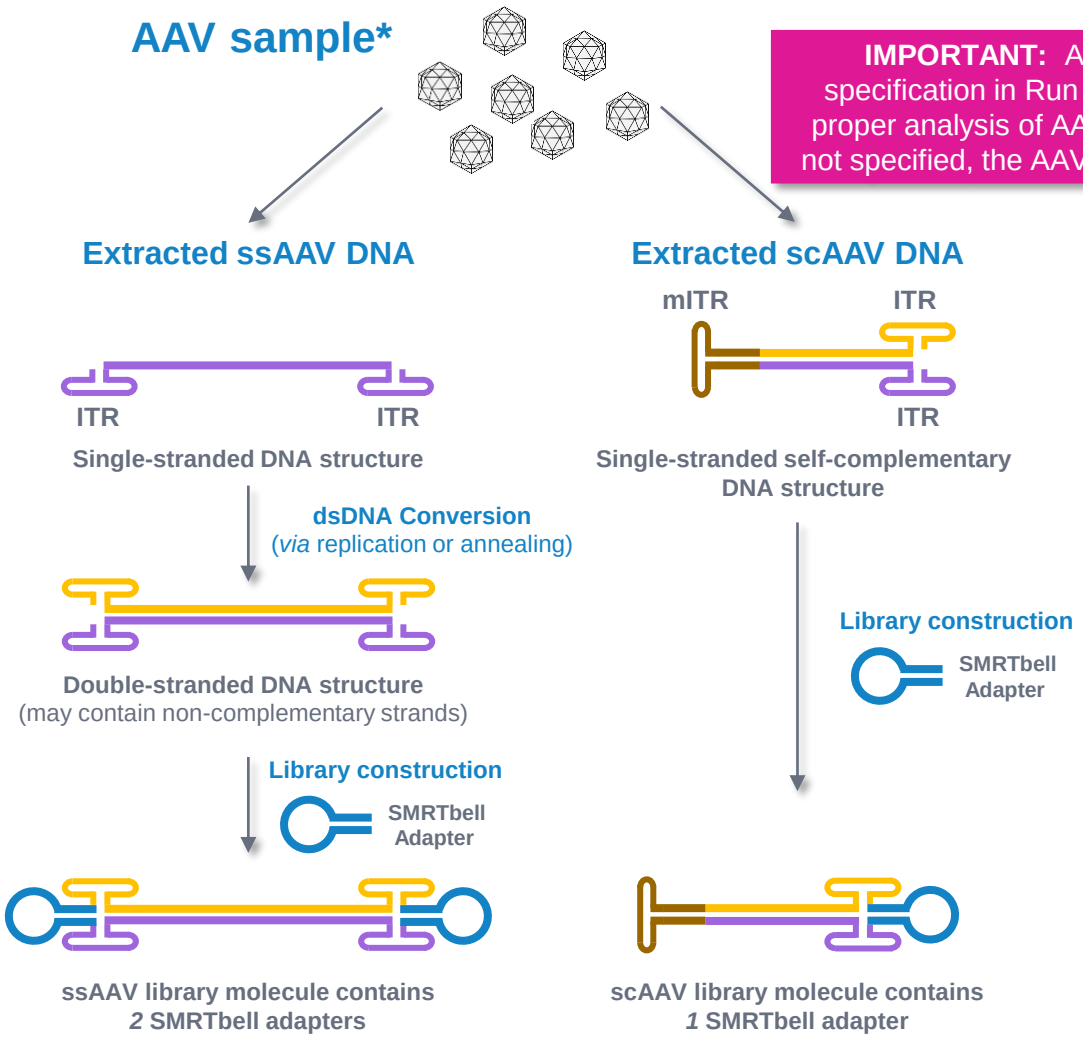


* This optional feature is applicable **only** if application type is set to one of the following: Adeno-Associated Virus (AAV), Full-Length 16S rRNA Sequencing, <3 kb Amplicons, ≥3 kb Amplicons, or Custom

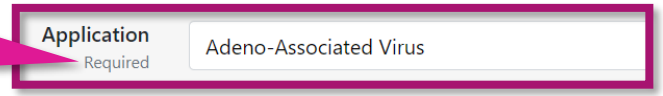
New AAV application type option

Adeno-associated virus (AAV) application type specification

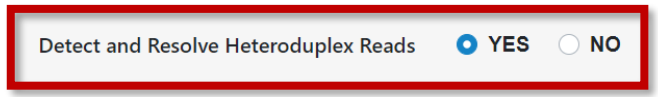
If selected, AAV sequencing mode applies a modified adapter calling algorithm during post-primary analysis and enables automated heteroduplex (HD) read detection and resolution during CCS analysis



IMPORTANT: AAV application type specification in Run Design is **required** for proper analysis of AAV data. If inadvertently not specified, the AAV sample must be re-run.



Modified adapter calling + HD detection are **both** enabled by default when AAV application type is selected in Run Design



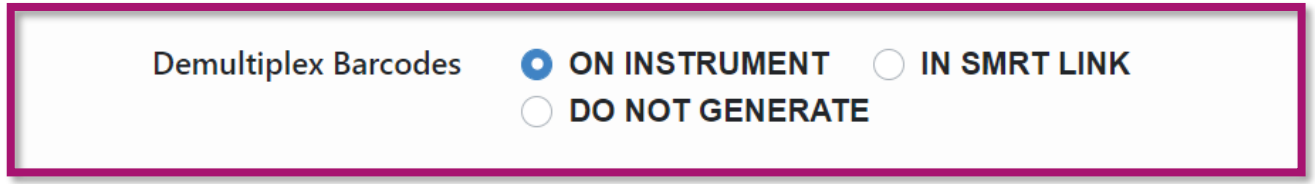
AAV data processing step	Purpose	Sequel IIe system workflow	Sequel II system workflow
Modified adapter calling	• Enables correct annotation of full-pass subreads within library molecules containing only 1 SMRTbell adapter • Required for analysis of scAAV libraries	Automated on-instrument analysis 	Automated on-instrument analysis 
Heteroduplex detection and splitting	• CCS analysis feature to identify a double-stranded sequence comprised of two non-complementary strands within the same library molecule • Separates the forward and reverse strands and generates a consensus read for each strand • Required for analysis of ssAAV libraries	Automated on-instrument analysis 	Automated in-SMRT Link analysis 

*** Note:** Extracted AAV DNA samples typically contain a mixture of ssAAV DNA and scAAV DNA

New on-instrument demultiplexing option

On-instrument barcode demultiplexing (Sequel IIe system only)

SMRT Link v11.0 Run Design enables specification to perform barcode demultiplexing on-instrument



- If on-instrument barcode demultiplexing is performed, the following additional files are output:

```
|--  bc1001--bc1001/m64012e_211206_183753.bc1001--bc1001.consensusreadset.xml
|--  bc1001--bc1001/m64012e_211206_183753.hifi_reads.bc1001--bc1001.bam
|--  bc1001--bc1001/m64012e_211206_183753.hifi_reads.bc1001--bc1001.bam.pbi
|-- m64012e_211206_183753.barcodes.fasta
|-- m64012e_211206_183753.lima.log
|-- m64012e_211206_183753.lima_counts.txt
|-- m64012e_211206_183753.lima_guess.json
|-- m64012e_211206_183753.lima_guess.txt
|-- m64012e_211206_183753.lima_reports.txt
|-- m64012e_211206_183753.lima_summary.txt
|-- m64012e_211206_183753.unbarcoded.consensusreadset.xml
|-- m64012e_211206_183753.unbarcoded.hifi_reads.bam
|-- m64012e_211206_183753.unbarcoded.hifi_reads.bam.pbi
```

Each demultiplexed child data set is output into a subfolder containing the same name as the barcode(s), e.g., 'bc1001-bc1001' for easy identification

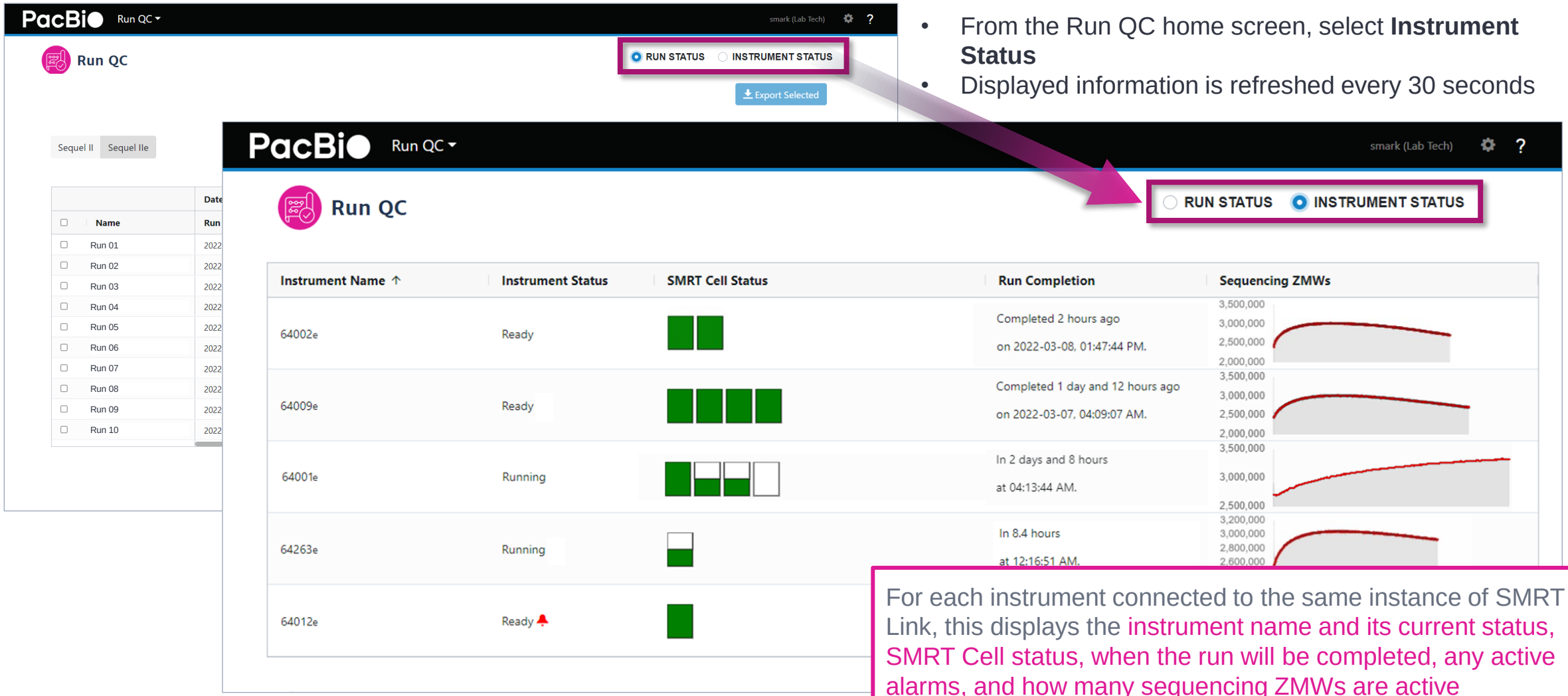
- **Note:** The **un-demultiplexed `hifi_reads.bam` file is not** transferred, it is partitioned into the example file structure shown here.
- Command line tool (`lima`) is available to re-merge demultiplexed files to re-create the original un-demultiplexed `hifi_reads.bam` file if needed

SMRT Link Run QC updates

New Instrument Status view

New Instrument Status view

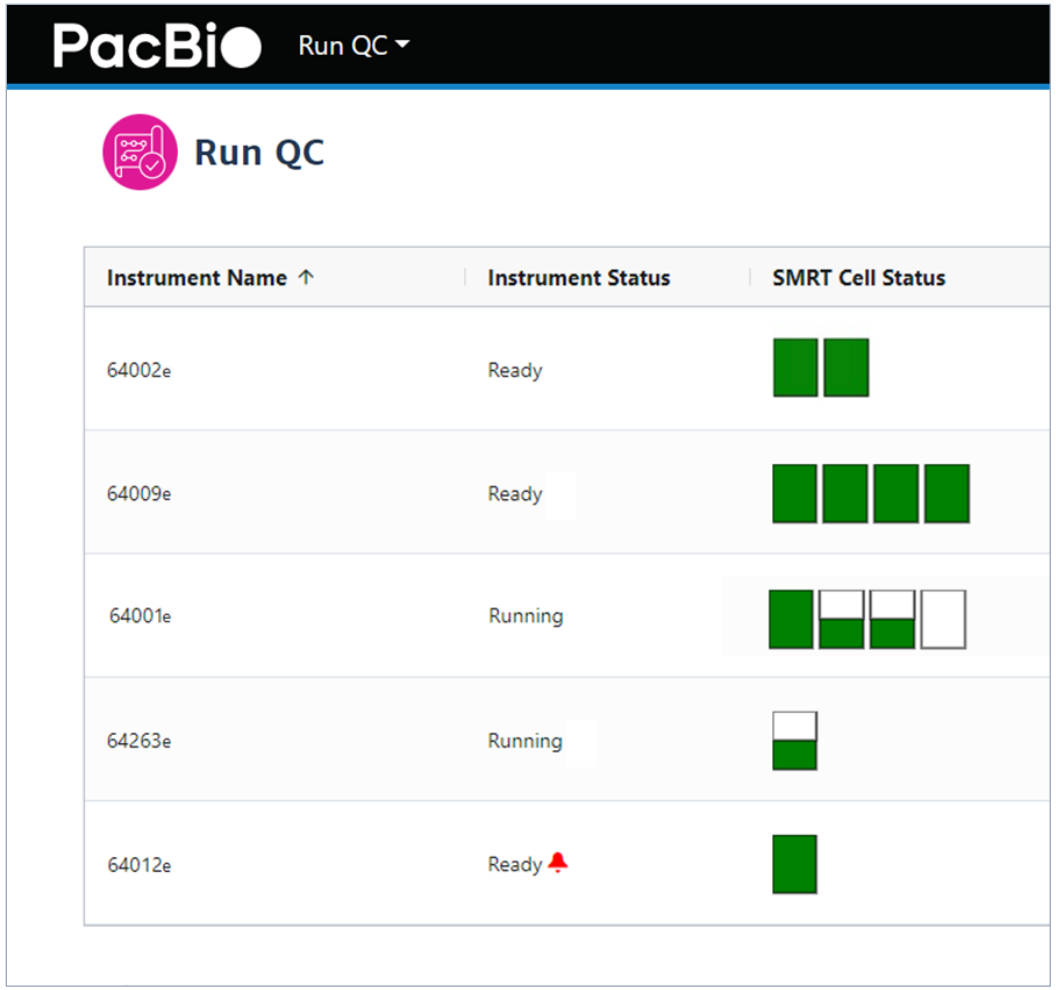
Instrument Status view enables real-monitoring of sequencing ZMW activity across multiple instruments



- From the Run QC home screen, select **Instrument Status**
- Displayed information is refreshed every 30 seconds

For each instrument connected to the same instance of SMRT Link, this displays the instrument name and its current status, SMRT Cell status, when the run will be completed, any active alarms, and how many sequencing ZMWs are active

Instrument Status and SMRT Cell Status



- **Instrument Status**
  - A **red alarm symbol** displays next to the instrument status if any errors or warnings appear during a sequencing run.

- **SMRT Cell Status**
  - If an instrument does not have a SMRT Cell tray loaded, the SMRT Cell Status field will **not** display any icons.

SMRT Cell Status Icons

Fully green: SMRT Cell has completed sequencing

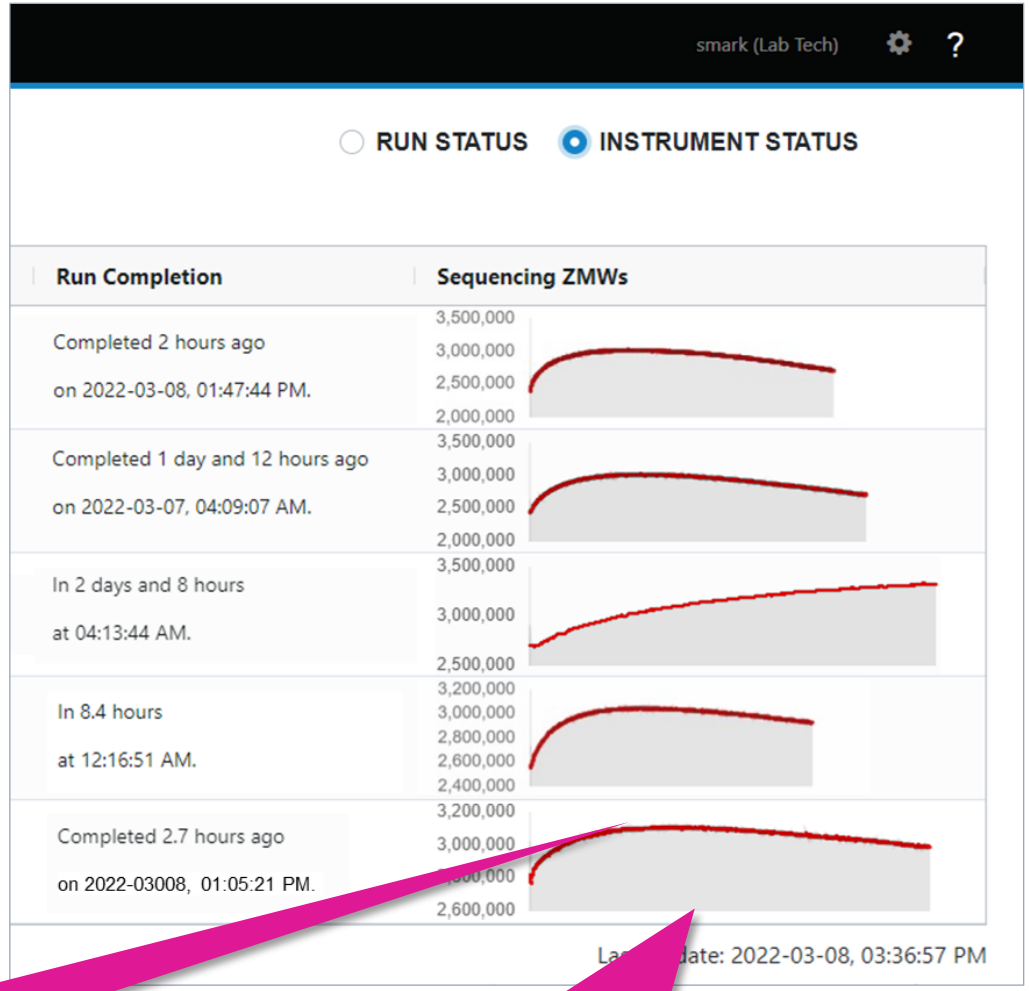
Half Green: SMRT Cell is in cell prep or SMRT Cell is currently sequencing

White: SMRT Cell is in the queue for sequencing, but cell prep has not started

Run Completion and Sequencing ZMWs

- **Run Completion**
  - Displays the estimated time remaining to complete sequencing run or the time elapsed since the sequencing run completed.
  - Also displays the date (in YYYY-MM-DD format) when the last sequencing run was completed
  - Note: If an instrument has been idle for 2 days, then the Run Completion field will be blank

- **Sequencing ZMWs**
  - Displays a plot of how many ZMWs on a SMRT Cell are actively sequencing during a movie collection
    - For sequencing runs using **Binding Kit 2.2 and 3.2**, only the number of actively sequencing **singly-loaded ZMWs (*P1*)** displays
    - For sequencing runs using **Binding Kit 2.1 and 3.1**, the **total number of actively sequencing ZMWs (*P1* + *P2*)** displays
  - Note: Both the Y-axis (Sequencing ZMW count) and X-axis (Elapsed Time) are **auto-scaled** as a sequencing run progresses



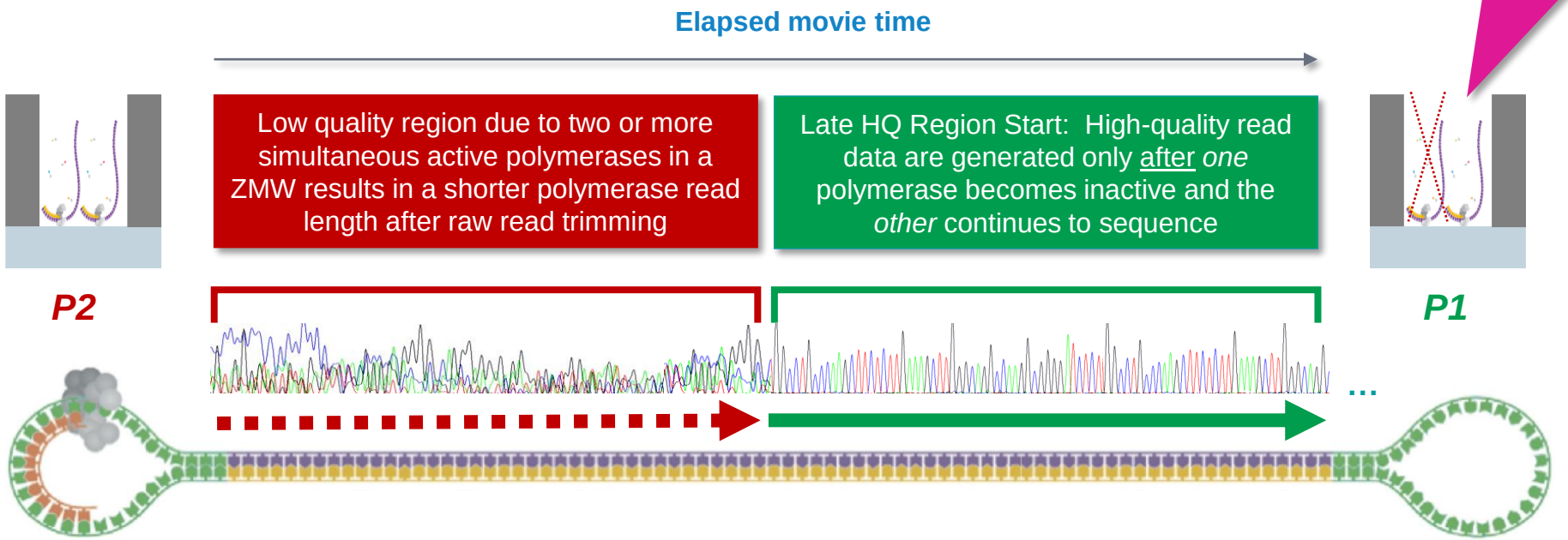
For a SMRT Cell that achieves ≥50% P1 loading and ≥10% P0, the ZMW Sequencing plot should typically display a peak value >2,000,000

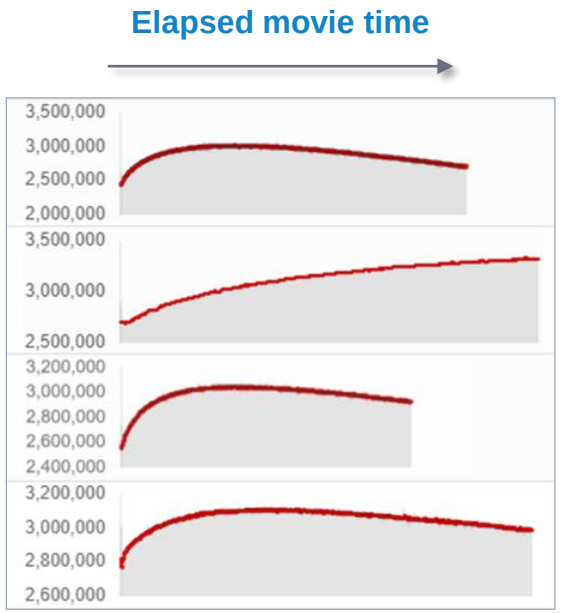
Note: Elapsed Time axis is **auto-scaled** and so the shape of the plots may change as movie collection progresses

Sequencing ZMWs plot characteristics depend on binding kit type specification

- **Sequencing ZMWs (cont.)**
  - For optimally loaded runs (*P1* >50%) conducted with **Binding Kit 2.2 and 3.2**, the peak concurrent Sequencing ZMWs value will typically be **less than** the final %*P1* ZMW yield reported in Run QC at the end of a movie collection. (For poorly loaded runs (*P1* <50%), the two values will typically be more similar.)
  - For optimally loaded runs (*P1* >50%) conducted with **Binding Kit 2.1 and 3.1**, the peak concurrent Sequencing ZMWs value will typically be **higher** than the final %*P1* ZMW yield reported in Run QC. (For poorly loaded runs, the two values will typically be more similar.)

Due to terminations, **not all ZMWs are singly-loaded at the same time** – e.g., some ZMWs are singly-loaded only at or near the end of a movie collection, whereas others are singly-loaded only at the beginning (and undergo termination before the end of the movie).





As acquisition progresses, active complexes in ***P2*** ZMWs will terminate at a certain rate and only when one SMRTbell complex per ZMW is left sequencing will a high-quality (HQ) region within the raw read be detected and the ZMW state will turn to ***P1***.

Comparison of Instrument Status view vs. Run Status view

Example instrument status and run status reports for a well-loaded sample achieving >70% *P1* loading

Peak value of Sequencing ZMWs plot for last cell (Sample D01) is ~3,000,000

Run QC

RUN STATUS ● INSTRUMENT STATUS

Instrument Name ↑	Instrument Status	SMRT Cell Status	Run Completion	Sequencing ZMWs
64009e	Ready		Completed 1 day and 12 hours ago on 2022-03-07, 04:09:07 AM.	3,500,000 3,000,000 2,500,000 2,000,000

Run QC

● RUN STATUS ○ INSTRUMENT STATUS

Final *P1* loading for sample D01 is >70% (6,144,000 *P1* ZMWs)

Expand All

	Sample Information	Run Settings			Productivity (%)			Reads								Control
								HiFi Reads				Polymerase Read Length		Longest Subread		
Well	Name	Movie Time (hrs)	Status	Total Bases (Gb)	P0	P1	P2	≥Q20 Reads	Yield	Mean Length	Median QV	Mean	N50	Mean	N50	Poly RL Mean (bp)
A01	WGS Sample 1 Pol 2.2	30	Complete	637.19	25.3	72.5	2.2	2817138	36.53 Gb	12967	Q37	109721	224250	17115	21250	98761
B01	WGS Sample 2 Pol 2.2	30	Complete	531.02	30.5	67.3	2.2	1844770	28.79 Gb	15608	Q32	98521	197750	27164	46250	91774
C01	WGS Sample 3 Pol 2.2	30	Complete	673.07	15.7	81.8	2.5	2802352	45.45 Gb	16218	Q33	102674	206750	19974	22750	94451
D01	WGS Sample 4 Pol 2.2	30	Complete	570.69	19.6	76.8	3.6	2178787	31.12 Gb	14282	Q34	92686	190250	24667	40250	87702

Example Instrument Status report (top) and Run QC report (bottom) for a WGS sample bound with Binding Kit 3.2 and sequenced using a 30-hour movie collection time. The Sequencing ZMWs plot in the Instrument Status report shows that the peak concurrent Sequencing ZMWs value for the final SMRT Cell in the run (Well D01) is approximately 3,000,000 ZMWs, whereas the final %*P1* ZMW yield reported in the corresponding Run QC metrics table for Well D01 is 76.8% (or 6,144,000 *P1* ZMWs).

Comparison of Instrument Status view vs. Run Status view

Example instrument status and run status reports for a poorly-loaded sample achieving <50% *P1* loading

Run QC

RUN STATUS ● INSTRUMENT STATUS

Peak value of Sequencing ZMWs plot for last cell (Sample D01) is ~2,000,000

Instrument Name ↑	Instrument Status	SMRT Cell Status	Run Completion	Sequencing ZMWs
64444e	Ready			2,000,000 1,500,000 1,000,000 500,000

Run QC

● RUN STATUS ○ INSTRUMENT STATUS

Expand All

Final *P1* loading for sample D01 is <30% (2,065,000 *P1* ZMWs)

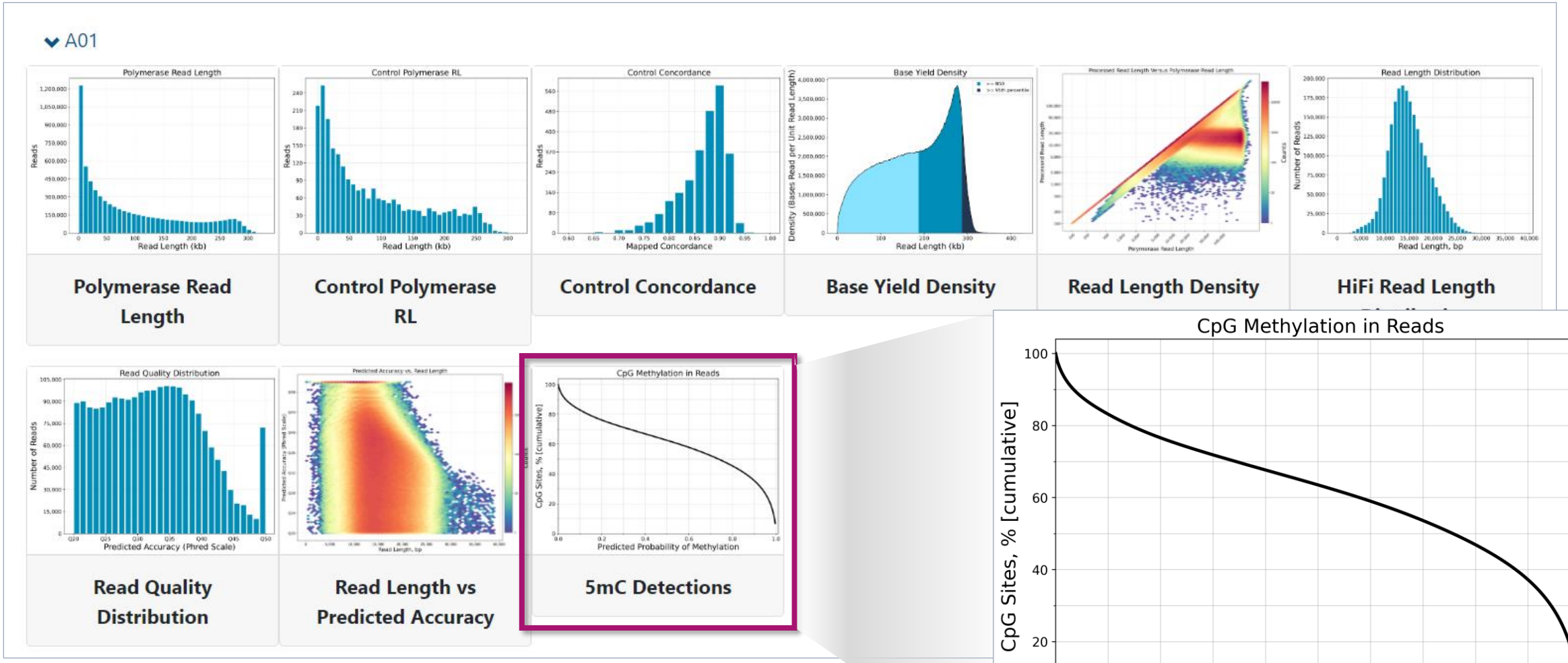
	Sample Information	Run Settings			Productivity (%)			Reads								
								HiFi Reads				Read Length		Longest Subread		
Well	Name	Movie Time (hrs)	Status	Total Bases (Gb)	P0	P1	P2	≥Q20 Reads	Yield	Mean Length	Mean QV	Mean	N50	Mean	N50	Poly RL Mean (bp)
A01	WGS Sample 1 Pol 2.2	30	Complete	605.22	26.6	71.6	1.8	2914608	30.72 Gb		Q39	105...	222250	14637	14750	86336
B01	WGS Sample 2 Pol 2.2	30	Complete	572.02	29.8	68.3	1.9	2535778	26.57 Gb	10479	Q39	104...	214750	20328	42750	87317
C01	WGS Sample 3 Pol 2.2	30	Complete	68.73	84.8	14.7	0.5	575681	1.61 Gb	2797	Q30	60179	229750	10683	67750	75807
D01	WGS Sample 4 Pol 2.2	10	Complete	69.41	73.4	25.8	0.8	1199084	2.65 Gb	2210	Q52	34005	61750	5368	17250	29914

Example Instrument Status report (top) and Run QC report (bottom) for a WGS sample bound with Binding Kit 3.2 and sequenced using a 10-hour movie collection time. The Sequencing ZMWs plot in the Instrument Status report shows that the peak concurrent Sequencing ZMWs value for the final SMRT Cell in the run (Well D01) is approximately 2,000,000 ZMWs, whereas the final %*P1* ZMW yield reported in the corresponding Run QC metrics table for Well D01 is 25.8% (or 2,065,000 *P1* ZMWs).

New 5mC-specific report plots

New Run QC report plot: 5mC Detections

Plots



- **CpG Methylation in Reads:** Plots the cumulative percentage of CpG sites in the sample against the predicted probability of methylation. (Report appears in SMRT Link Run QC and Data Management)

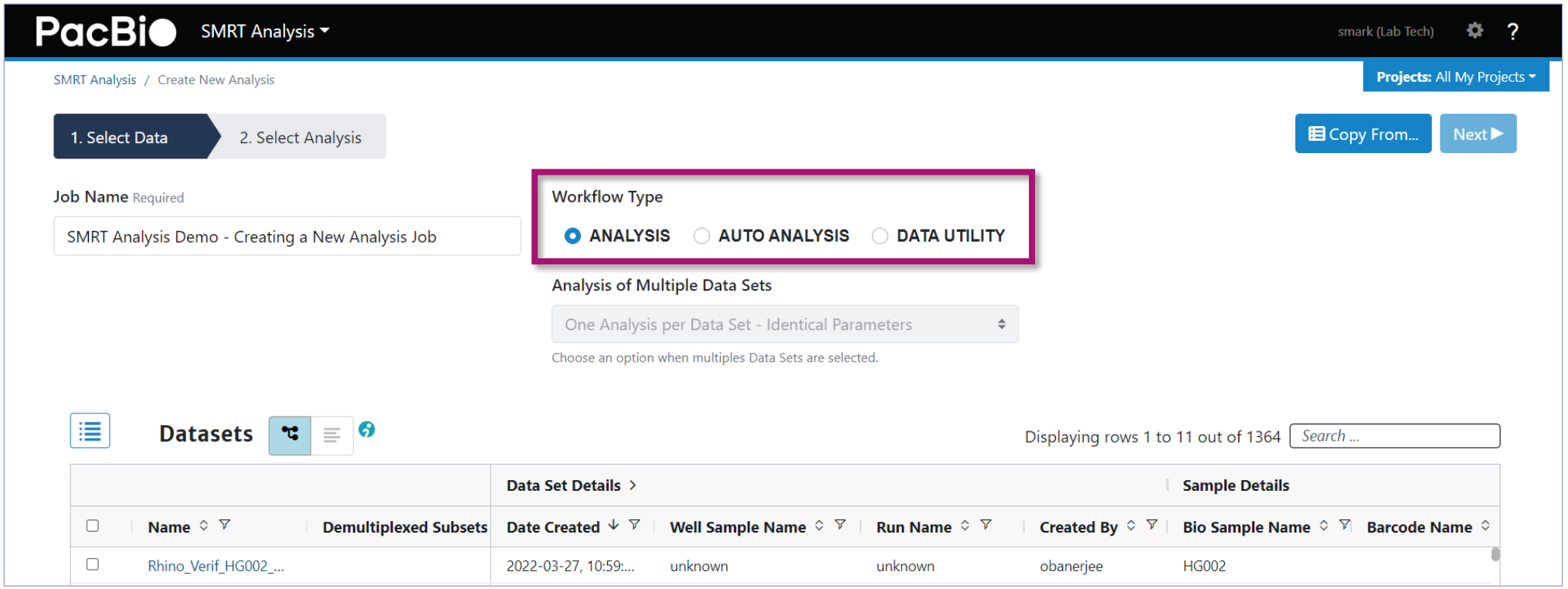
Note: A different CpG Methylation in Reads Histogram plot displaying the percentage of CpG sites in the sample versus the predicted probability of methylation Is available only in Data Management.

SMRT Analysis updates

Updated GUI nomenclature

New conceptual structure in SMRT Analysis v11.0 for specifying workflow type

Data processing workflows are now separated into ‘Analyses’ and ‘Utilities’

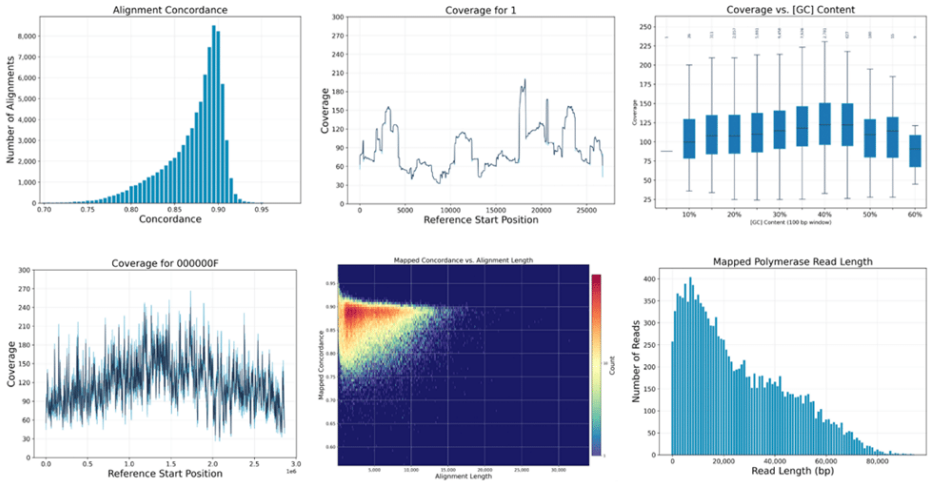


- **Analysis:** An analysis uses applications designed to produce biologically-meaningful results. These analysis applications **only** accept HiFi reads
- **Data Utilities:** Data processing utilities are used as intermediate steps to producing biologically-meaningful results. Some data utilities accept **only** HiFi reads whereas other data utilities accept **only** subreads (formerly known as “Continuous Long Reads” in previous SMRT Link versions)

Analysis Applications

SMRT Link Analysis Applications are designed to produce biologically-meaningful results

SMRT Link analysis applications accept **only** HiFi reads as input.



- **Genome Assembly**
  - Generate de novo assemblies of genomes, using HiFi reads.
- **HiFi Mapping**
  - Align (or map) reads to a user-provided reference sequence.
- **HiFiViral SARS-CoV-2 Analysis**
  - Analyze multiplexed viral surveillance samples for SARS-CoV-2, using HiFi reads.
- **Iso-Seq Analysis**
  - Characterize full-length transcript isoforms, using HiFi reads.
- **Microbial Genome Analysis**
  - Note: This combines and replaces the Microbial Assembly and Base Modification Analysis applications in the previous release.
  - Generate de novo assemblies of small prokaryotic genomes between 1.9-10 Mb and companion plasmids between 2 – 220 kb, and identify methylated bases and associated nucleotide motifs.
  - Optionally include identification of 6mA and 4mC modified bases and associated DNA sequence motifs.
- **Minor Variants Analysis**
  - Identify and phase minor single nucleotide substitution variants in complex populations.
- **Structural Variant Calling**
  - Identify structural variants (Default: ≥20 bp) in a sample or set of samples relative to a reference.

Data Utilities

PacBio Data Utilities are used as intermediate steps to producing biologically-meaningful results

The following data utilities accept **only** HiFi reads as input:

- **5mC CpG Detection**
  - Analyze the kinetic signatures of cytosine bases in CpG motifs to identify the presence of 5mC. (Sequel II only.)
- **Demultiplex Barcodes**
  - Separate reads by barcode.
- **Export Reads**
  - Export HiFi reads that pass filtering criteria as FASTA, FASTQ and BAM files.
  - For barcoded runs, you must first run the Demultiplex Barcodes application to create BAM files before using this application.
- **Mark PCR Duplicates**
  - Remove duplicate reads from a HiFi reads Data Set created using an ultra-low DNA sequencing protocol.
- **Trim Ultra-Low Adapters**
  - Trim PCR Adapters from a HiFi reads Data Set created using an ultra- low DNA sequencing library.

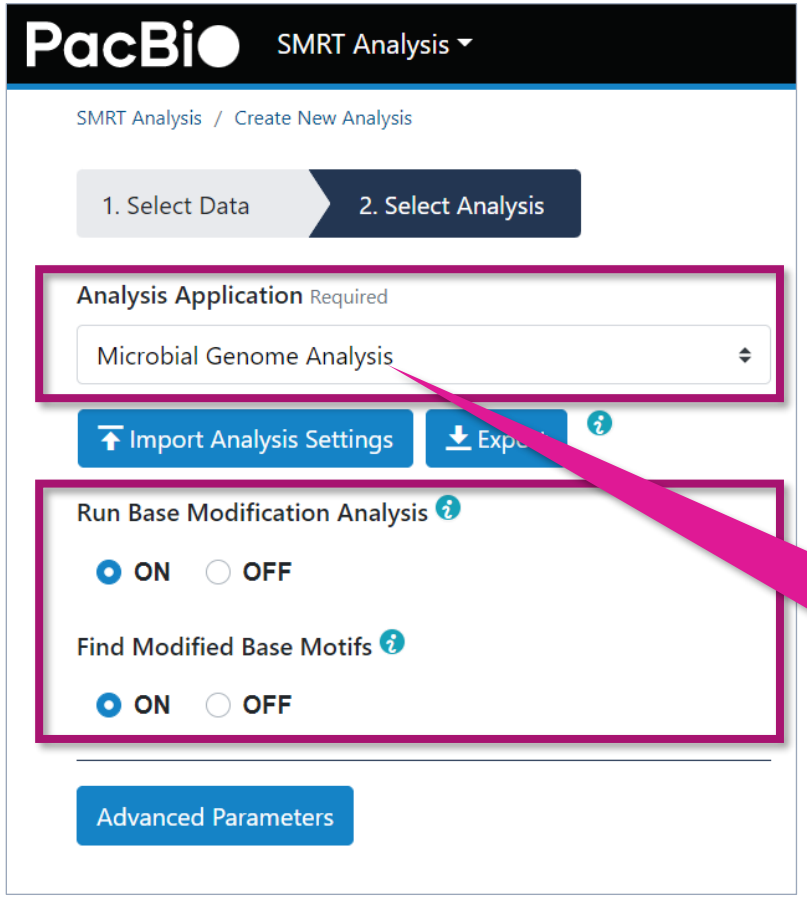
The following data utilities accept **only** subreads as input:

- **Circular Consensus Sequencing (CCS)**
  - Identify consensus sequences for single molecules.

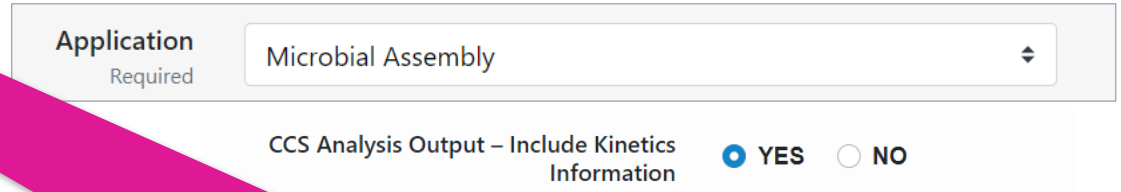
Updated Microbial Genome Analysis application

Updated Microbial Genome Analysis application

Use SMRT Link v11.0 Microbial Genome Analysis application to perform microbial assembly and base modification detection using HiFi reads



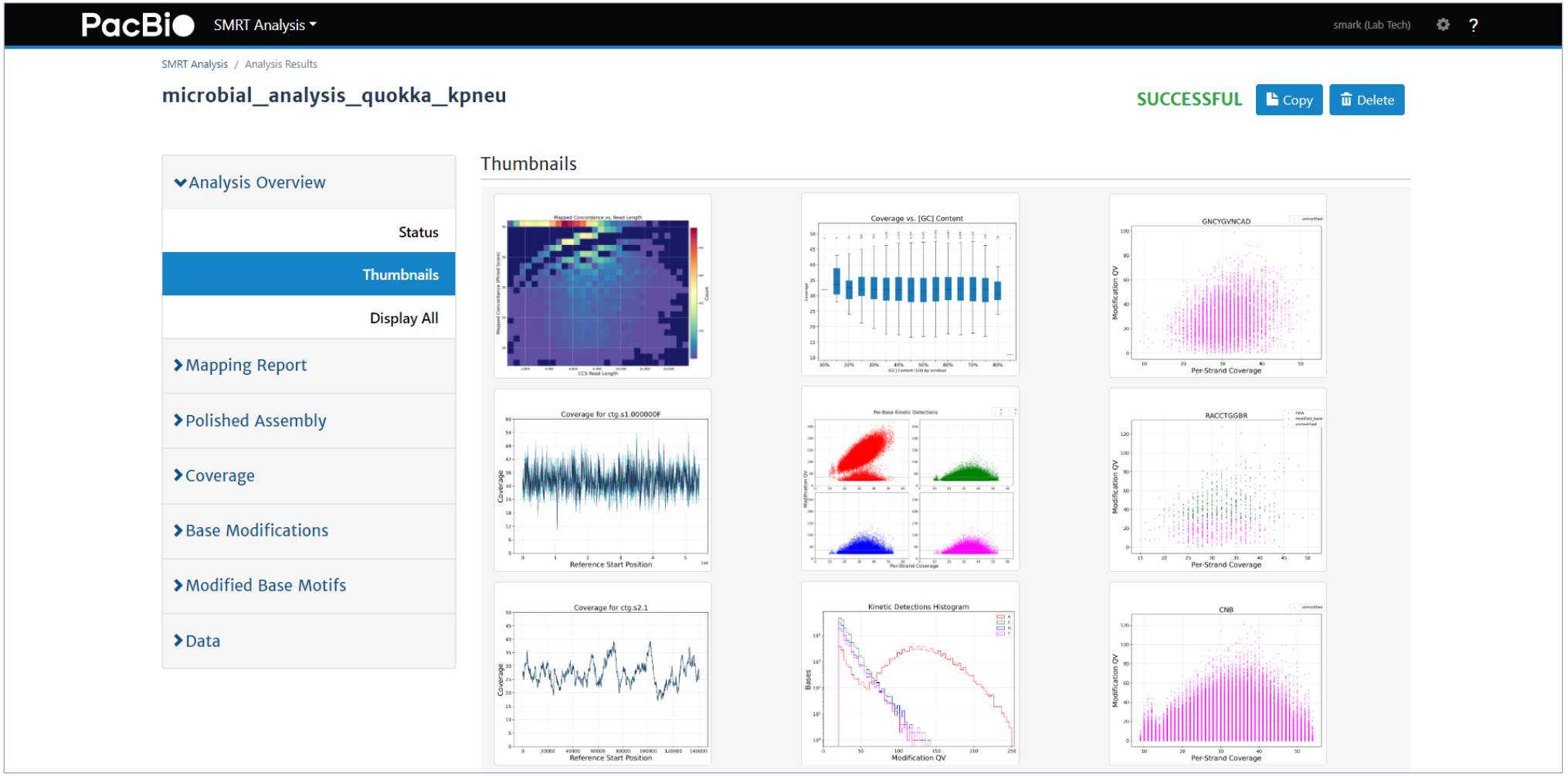
- Generate *de novo* assemblies of small prokaryotic genomes between 1.9-10 Mb and companion plasmids between 2 – 220 kb, and identify methylated bases and associated nucleotide motifs.
- Optionally include identification of 6mA and 4mC modified bases and associated DNA sequence motifs. (This requires kinetic information.)
  - Unlike 5mC calling, microbial base modification detection is performed off-instrument (i.e., in SMRT Link only)
  - This requires a Run Design to specify that kinetic information be retained in the CCS analysis output
    - For the Microbial Assembly application type, Run Design automatically defaults to specifying **YES** for the 'CCS Analysis Output – Include Kinetics Information' field



Note: This combines and **replaces** the Microbial Assembly and Base Modification Analysis applications in the previous SMRT Link release.

Updated Microbial Genome Analysis application (cont.)

View microbial assembly results, detected base modifications and identified modified base motifs in a single analysis job report



APPENDIX: Technical documentation & applications support resources

HiFi sequencing and software v11.0 release technical documentation

Sequel IIe system documentation

- Sequel II and Sequel IIe systems operations guide (101-774-700)
- Sequel II/IIe system v11.0 release notes (102-279-600)
- Sequel II and IIe systems: Data files (102-144-100)
- Sequel IIe system: Location of HiFi reads files (102-110-200)
- Quick reference card – Loading and pre-extension recommendations for the Sequel II and IIe systems (101-769-100)
- Pacific Biosciences glossary of terms (000-710-267)

SMRT Link & other data analysis documentation

- Brief primer and lexicon for PacBio SMRT sequencing webpage (v11.0)
- PacBio bioinformatics file formats documentation webpage (v11.0)
- SMRT Link v11.0 cloud reference guide (102-295-600)
- SMRT Link v11.0 release notes (102-279-500)
- SMRT Link v11.0 software installation guide (102-278-600)
- SMRT Link v11.0 user guide (102-278-200)
- SMRT Link v11.0 web services API use cases (102-298-700)
- SMRT Tools v11.0 reference guide (102-278-500)

HiFi sequencing and software v11.0 release technical documentation (cont.)

Application technical overviews

- HiFi sequencing and software v11.0 release: Technical overview for Sequel II & Sequel IIe system users (102-399-900)
- Technical overview: Adeno-associated virus (AAV) library preparation using SMRTbell prep kit 3.0 (102-390-400)
- Technical overview: Iso-Seq library preparation using SMRTbell prep kit 3.0 (102-393-400)
- Technical overview: Multiplexed amplicon library preparation using SMRTbell prep kit 3.0 (102-395-900)
- Technical overview: Multiplexed SARS-CoV-2 library preparation for full-viral genome sequencing using SMRTbell prep kit 3.0 (102-399-300)
- Technical overview: Whole genome and metagenome library preparation using SMRTbell prep kit 3.0 (102-390-900)

DNA extraction literature

- Circulomics Nanobind Application notes, Kit handbooks, and Protocols
- Technical note: Preparing DNA for PacBio HiFi sequencing – Extraction and quality control (102-193-651)
- Technical note: Preparing samples for PacBio whole genome sequencing for *de novo* assembly – collection and storage (TN100-040518)
- Technical note: Sample preparation for PacBio HiFi sequencing from human whole blood (102-326-500)

HiFi sequencing and software v11.0 release technical documentation (cont.)

Sample preparation literature

- Overview – Sequel systems application options and sequencing recommendations (101-851-300)
- Procedure & checklist – Preparing multiplexed AAV SMRTbell libraries using SMRTbell prep kit 3.0 (102-126-400)
- Procedure & checklist – Preparing multiplexed amplicon libraries using SMRTbell prep kit 3.0 (102-359-000)
- Procedure & checklist – Preparing multiplexed amplicon libraries using PacBio barcoded M13 primers and SMRTbell prep kit 3.0 (101-921-300)
- Procedure & checklist – PacBio HiFiViral high-throughput multiplexing for full-viral genome sequencing of SARS-CoV-2 using SMRTbell prep kit 3.0 (102-396-100)
- Procedure & checklist – Preparing Iso-Seq libraries using SMRTbell prep kit 3.0 (102-396-000)
- Procedure & checklist – Preparing whole genome and metagenome sequencing libraries using SMRTbell prep kit 3.0 (102-166-600)
- Quick reference card – Loading and pre-extension recommendations for the Sequel II and IIe systems (101-769-100)
- Technical note: Alternative size selection methods for SMRTbell prep kit 3.0 (TN103-110921)
- Technical note: Covaris g-TUBE DNA shearing for SMRTbell prep kit 3.0 (102-326-501)

Example PacBio data sets

5mC detection at CpG sites	Dataset	Data type	PacBio system
5mC detection at CpG sites	Human HG002 CpG methylation status	HiFi Reads	Sequel IIe System

www.pacb.com