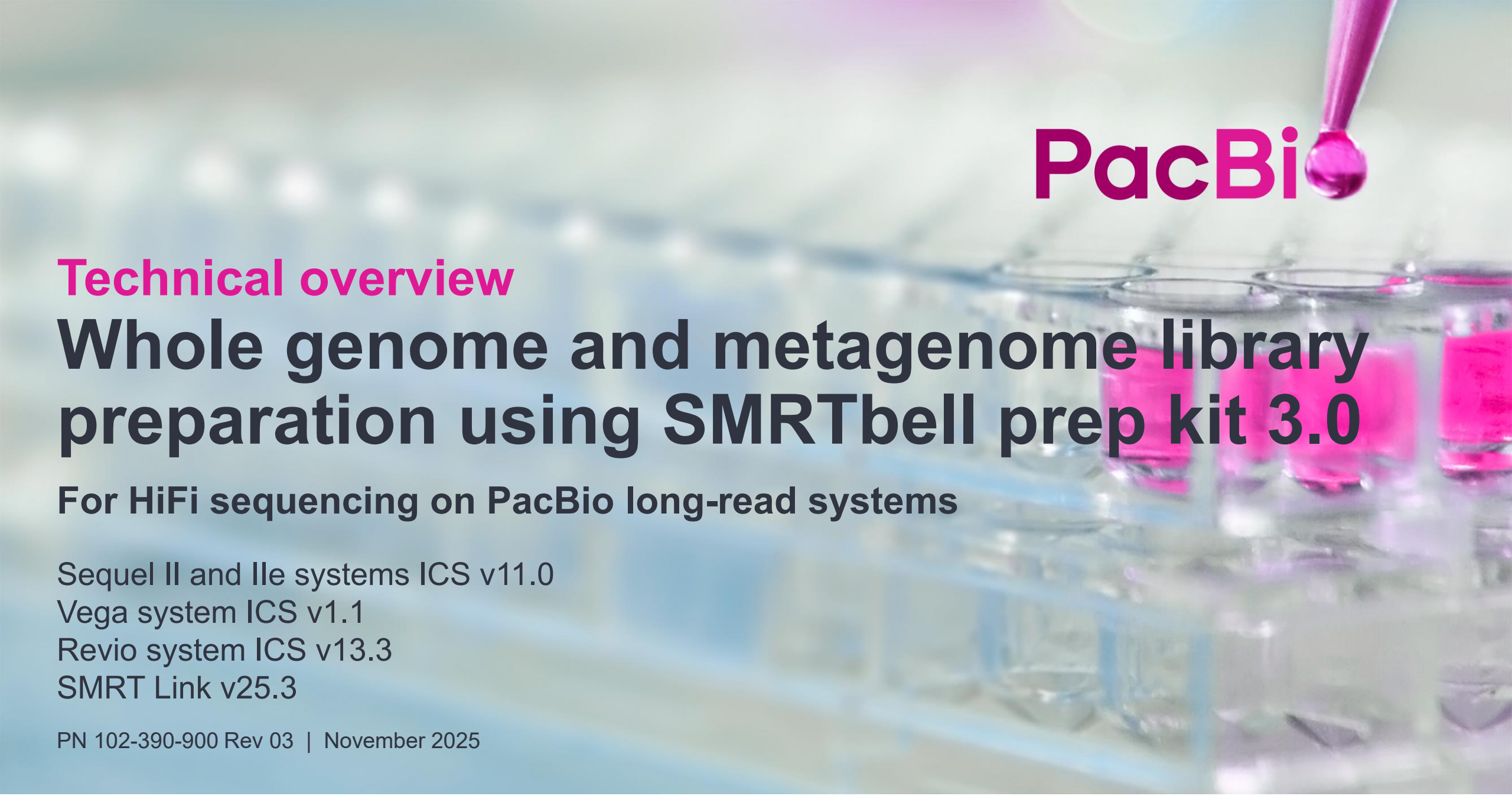


Technical overview

Whole genome and metagenome library preparation using SMRTbell prep kit 3.0

For HiFi sequencing on PacBio long-read systems

Sequel II and IIe systems ICS v11.0
Vega system ICS v1.1
Revio system ICS v13.3
SMRT Link v25.3

PN 102-390-900 Rev 03 | November 2025

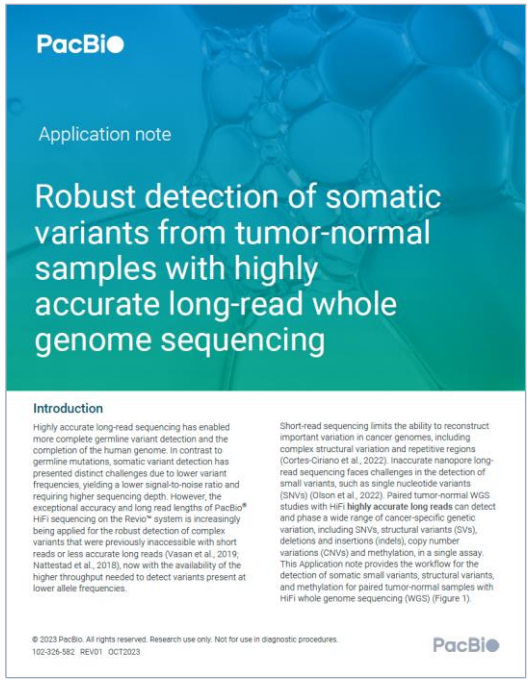
Technical overview

Whole genome and metagenome library preparation using SMRTbell prep kit 3.0

1. WGS library preparation & sequencing workflow overview
2. WGS library preparation workflow details
3. WGS library sequencing preparation workflow details
4. WGS data analysis recommendations for *de novo* assembly, variant detection and shotgun metagenomics applications
5. WGS library example sequencing performance data
6. Technical documentation & applications support resources
7. APPENDIX 1 – Genomic DNA isolation & QC recommendations for PacBio WGS sample preparation
8. APPENDIX 2 – Sequencing preparation workflow overview for Sequel II/IIe systems
9. APPENDIX 3 – PacBio compatible WGS library preparation workflows
    i. seqWell LongPlex WGS workflow overview
    ii. EpiCypher Fiber-seq assay workflow overview

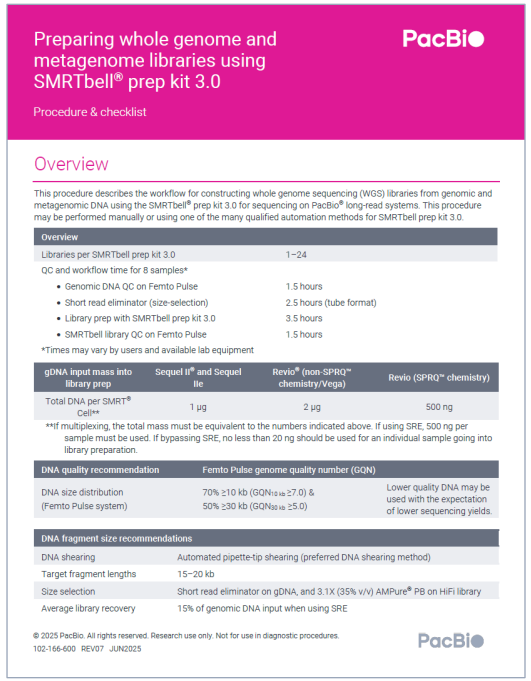
Whole genome and metagenome library preparation using SMRTbell prep kit 3.0: Getting started

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



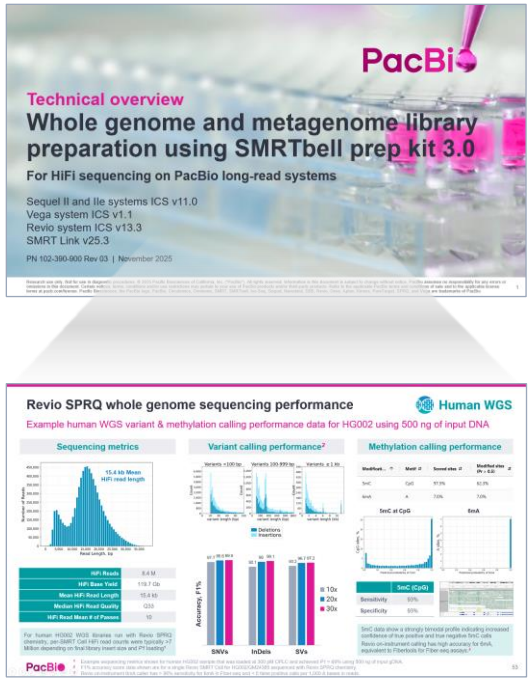
Application notes and Application briefs (Literature)

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



Procedure & Checklist – Preparing whole genome and metagenome libraires using SMRTbell prep kit 3.0 (102-166-600)

Technical documentation containing sample library construction and sequencing preparation protocol details.



Technical overview: Whole genome and metagenome library preparation using SMRTbell prep kit 3.0 (102-390-900)

Technical overview presentations describe sample preparation details for constructing HiFi libraries for specific applications. Example sequencing performance data for a given application are also summarized.

Genomic DNA QC & shearing

Extract HMW DNA using Nanobind kits

≥500 ng input gDNA per Revio SMRT Cell with SPRQ chemistry

Shear DNA to 15 – 20 kb for WGS

Library construction (SMRTbell prep kit 3.0)

Multiplex WGS samples using SMRTbell adapter index plate 96A/B/C/D

Sequencing (Sequel II/IIe, Vega & Revio systems)

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

Data analysis (SMRT Link or third-party tools)

Genome assembly

Variant detection

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

WGS library preparation & sequencing workflow overview

Whole genome and metagenome library preparation procedure description

Procedure & checklist – Preparing whole genome and metagenome libraries using SMRTbell prep kit 3.0 (102-166-600) 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 long-read systems. This procedure may be performed manually or using one of the many qualified automation methods for SMRTbell prep kit 3.0.

Overview	
Libraries per SMRTbell prep kit 3.0	1–24
QC and workflow time for 8 samples*	
• Genomic DNA QC on Femto Pulse	1.5 hours
• Short read eliminator (size-selection)	2.5 hours (tube format)
• Library prep with SMRTbell prep kit 3.0	3.5 hours
• SMRTbell library QC on Femto Pulse	1.5 hours

*Times may vary by user and available lab equipment

gDNA input mass into library prep	Sequel II® and Sequel IIe	Revio® (non-SPRQ™ chemistry/Vega)	Revio (SPRQ™ chemistry)
Total DNA per SMRT® Cell**	1 µg	2 µg	500 ng

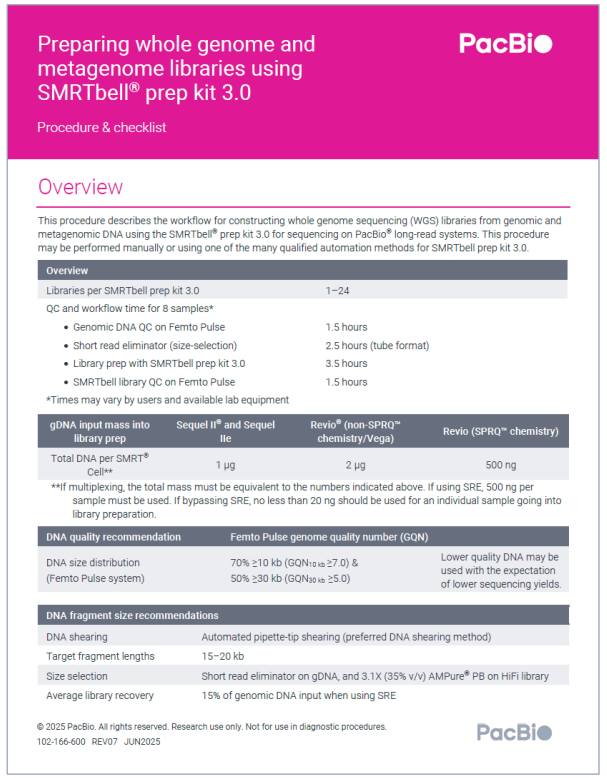
**If multiplexing, the total mass must be equivalent to the numbers indicated above. If using SRE, 500 ng per sample must be used. If bypassing SRE, no less than 20 ng should be used for an individual sample into library preparation.

DNA quality recommendation	Femto Pulse genome quality number (GQN)	
DNA size distribution (Femto Pulse system)	70% ≥10 kb ($GQN_{10\ kb}$ ≥7.0) & 50% ≥30 kb ($GQN_{30\ kb}$ ≥5.0)	Lower quality DNA may be used with the expectation of lower sequencing yields.

DNA fragment size recommendations	
DNA shearing	Automated pipette-tip shearing (preferred DNA shearing method)
Target fragment lengths	15–20 kb
Size selection	Short read eliminator on gDNA, and 3.1X (35% v/v) AMPure® PB on HiFi library
Average library recovery	15% of genomic DNA input when using SRE

SMRTbell prep kit 3.0 (102-182-700)

SMRTbell template (15 – 20 kb) containing SMRTbell adapters[2]



PacBio Documentation (102-166-600)

Note: For multiplexed **microbial WGS applications**, we recommend using the HiFi plex prep kit 96 (PN 103-381-200) and following *Procedure & checklist – Preparing multiplexed whole genome and amplicon libraries using the HiFi plex prep kit 96* (103-418-800)[1]

[1] For multiplexed microbial WGS applications where ≥50 ng of input gDNA per sample is available, we recommend using the HiFi plex prep kit 96 (PN 103-381-200) and following ***Procedure & checklist – Preparing multiplexed whole genome and amplicon libraries using the HiFi plex prep kit 96*** (103-418-800). Also see ***Technical overview – HiFi library preparation using HiFi prep kits for high-throughput sequencing on PacBio long-read systems*** (103-424-600) for general data analysis recommendations for microbial WGS applications.

[2] To enable sample multiplexing, SMRTbell adapter index plate 96A (102-009-200) (or adapter index plate 96B/C/D) must be purchased separately from SMRTbell prep kit 3.0 bundle (102-182-700).

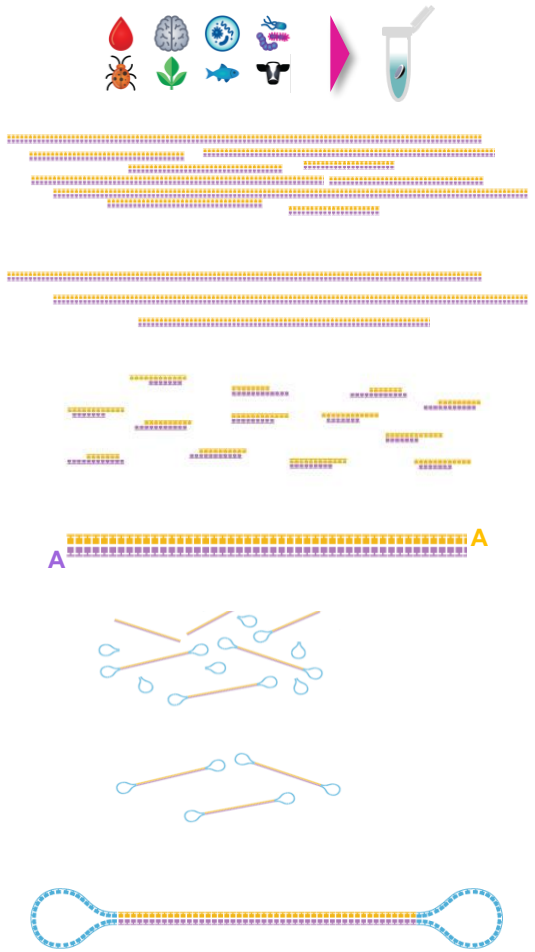
PacBio

SMRTbell prep kit 3.0 (SPK 3.0) whole genome and metagenome library preparation workflow overview

SMRTbell prep kit 3.0 WGS workflow

Library construction

- DNA sample extraction
- DNA sample QC
- DNA size selection
- DNA shearing
- DNA repair & A-tailing
- Adapter ligation
- Nuclease treatment
- Library cleanup or size selection



Protocol documentation or reference

Guide & overview – Nanobind PanDNA kit (103-394-800)

Refer to third-party user guide documenation

SPK 3.0 protocol reference for manual & automated workflows

Procedure & checklist – Preparing whole genome and metagenome sequencing libraries using SMRTbell prep kit 3.0 (102-166-600)

Recommended equipment & consumables

Nanobind PanDNA kit
(103-260-000 / 24 RXN)

Qubit 4 fluorometer system
Femto Pulse system

Short read eliminator (SRE) kit (102-208-300) for progressively depleting fragments up to 25 kb from genomic DNA samples

Hamilton Microlab Prep system for pipette-based DNA shearing[1]

SMRTbell prep kit 3.0
(102-182-700 / 24 RXN)

WGS library insert size >10 kb:[2]
3.1X (35%) AMPure PB size selection

WGS library insert size <10 kb:
1X SMRTbell bead cleanup

1 Alternatively, can shear DNA using Megaruptor 3 system (Diagenode) or g-TUBEs (Covaris) if Microlab Prep system is unavailable.
2 Can optionally perform gel cassette-based size selection to enrich for SMRTbell library inserts >10 kb.

SMRTbell prep kit 3.0 bundle (102-182-700)

SPK 3.0 bundle supports whole genome and metagenome library preparation workflows[1]

- Contains the necessary reagents for library preparation with SMRTbell adapters
- Kit also includes SMRTbell cleanup beads and low TE buffer
- Indexed (barcoded) adapters and size-selection reagents[1] are sold separately
- Supports 24 SMRTbell libraries per kit
- Compatible with the Vega system, Revio system and Sequel II & IIe systems

SMRTbell prep kit 3.0 bundle components

Component		Description
1		**SMRTbell prep kit 3.0** • Contains core reagents for SMRTbell template construction
2		**Low TE buffer** • For DNA shearing and cleanup
3		**SMRTbell cleanup beads** • For DNA cleanup

SMRTbell prep kit 3.0 bundle configuration

SMRTbell prep kit 3.0 (102-141-700)

#	Component	Part number	Qty	Color	Volume
1	Repair buffer	102-166-000	1	purple	220 µL
2	End repair mix	102-166-100	1	blue	110 µL
3	DNA repair mix	102-167-700	1	green	55 µL
4	SMRTbell adapter	102-167-800	1	orange	125 µL
5	Ligation mix	102-167-200	1	yellow	860 µL
6	Ligation enhancer	102-179-100	1	red	55 µL
7	Nuclease buffer	102-167-900	1	light purple	155 µL
8	Nuclease mix	102-166-200	1	light green	155 µL
9-10	Elution buffer	100-159-800	2	white	1.5 mL

Low TE buffer (102-178-400)

#	Component	Part number	Qty	Color	Volume
1	Low TE buffer (pH 8.0)	102-178-400	1	clear	10 mL

SMRTbell cleanup beads (102-158-300)

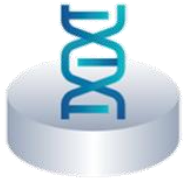
#	Component	Part number	Qty	Color	Volume
1	SMRTbell cleanup beads	102-158-300	1	clear	10 mL

[1] Short read eliminator (SRE) kit (102-208-300) for performing gDNA size selection and AMPure PB beads kit (102-182-500) for performing SMRTbell library size selection must be purchased separately.

Other recommended kits & consumables for SPK 3.0 WGS library preparation and HiFi sequencing

Ancillary kits must be purchased separately from SMRTbell prep kit 3.0 bundle (102-182-700)

HMW DNA extraction



Nanobind PanDNA kit (103-260-000)

- For HMW DNA extraction from cultured cells, cultured bacteria, whole blood, tissues, insects & plant nuclei[1]

Final WGS library size selection

AMPure PB bead size selection kit (102-182-500)

- Contains paramagnetic beads and elution buffer to selectively remove dsDNA <5 kb

Input gDNA size selection

Short read eliminator (SRE) kit (102-208-300)

- Contains reagents for depleting <10 kb fragments from input gDNA using size-selective precipitation

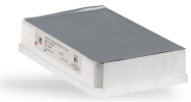
Sample multiplexing

SMRTbell adapter index plate 96A (102-009-200)

- Contains 96 indexed SMRTbell adapters (bc2001–bc2096) in plate format (1 sample per index)

HiFi sequencing

Revio SPRQ polymerase kit (103-520-100)



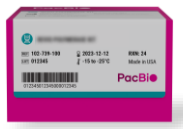
Revio SPRQ sequencing plate (4-rxn: 103-504-900)

Revio SMRT Cell tray (4 cells: 102-202-200)

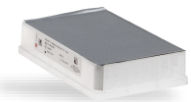
Vega polymerase kit (103-517-600)

Vega sequencing plate (103-274-300)

Vega SMRT Cell tray (103-406-700)



Sequel II binding kit 3.2 (102-333-300)



Sequel II sequencing kit 2.0 (4-rxn: 101-820-200)

Sequel II SMRT Cell 8M tray (4 cells: 101-389-001)

[1] Nanobind CBB kit (102-301-900) may also be used for HMW DNA extraction from cultured mammalian cells, cultured bacteria and whole blood.

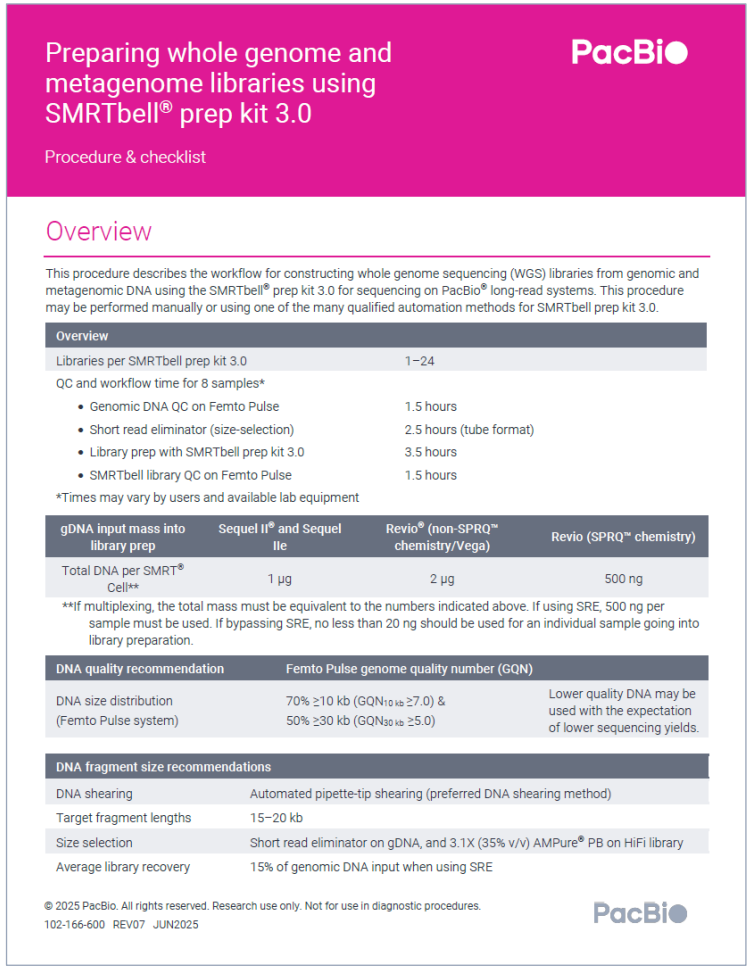
WGS library preparation workflow details

Procedure & checklist – Preparing whole genome libraries using SMRTbell prep kit 3.0 (102-166-600)

Procedure & checklist 102-166-600 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 long-read systems. This procedure may be performed manually or using one of the many qualified automation methods for SMRTbell prep kit 3.0.

Procedure & checklist contents

1. Genomic DNA (gDNA) input QC recommendations and general best practices for reagent & sample handling.
2. Sample multiplexing guidance and recommendations.
3. Instructions for performing size selection on input genomic DNA using Short read eliminator kit (SRE)[1].
4. Instructions for automated DNA shearing for WGS using Hamilton robots.[2]
5. Enzymatic workflow steps for SMRTbell library construction using SMRTbell prep kit 3.0.
6. Instructions for performing final cleanup and size selection on SMRTbell library using AMPure PB beads.[3]
7. Workflow steps for sample setup ABC[4] (annealing, binding, and cleanup) to prepare samples for sequencing using Vega polymerase kit or Revio SPRQ polymerase kit.



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

PacBio

Procedure & checklist

Overview

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® long-read systems. This procedure may be performed manually or using one of the many qualified automation methods for SMRTbell prep kit 3.0.

Overview	
Libraries per SMRTbell prep kit 3.0	1–24
QC and workflow time for 8 samples*	
• Genomic DNA QC on Femto Pulse	1.5 hours
• Short read eliminator (size-selection)	2.5 hours (tube format)
• Library prep with SMRTbell prep kit 3.0	3.5 hours
• SMRTbell library QC on Femto Pulse	1.5 hours

*Times may vary by users and available lab equipment

gDNA input mass into library prep	Sequel II® and Sequel IIe	Revio® (non-SPRQ™ chemistry/Vega)	Revio (SPRQ™ chemistry)
Total DNA per SMRT® Cell**	1 µg	2 µg	500 ng

**If multiplexing, the total mass must be equivalent to the numbers indicated above. If using SRE, 500 ng per sample must be used. If bypassing SRE, no less than 20 ng should be used for an individual sample going into library preparation.

DNA quality recommendation	Femto Pulse genome quality number (GQN)	
DNA size distribution (Femto Pulse system)	70% ≥10 kb ($GQN_{10\,kb}$ ≥7.0) & 50% ≥30 kb ($GQN_{30\,kb}$ ≥5.0)	Lower quality DNA may be used with the expectation of lower sequencing yields.

DNA fragment size recommendations	
DNA shearing	Automated pipette-tip shearing (preferred DNA shearing method)
Target fragment lengths	15–20 kb
Size selection	Short read eliminator on gDNA, and 3.1X (35% v/v) AMPure® PB on HiFi library
Average library recovery	15% of genomic DNA input when using SRE

102-166-600 REV07 JUN2025

PacBio

PacBio Documentation (102-166-600)

[1] If performing SRE using a Hamilton Microlab Prep liquid handling instrument, refer to ***Guide and overview – Short Read Eliminator (SRE), DNA shearing, and cleanup for the Hamilton Microlab Prep system*** (103-424-100) for details about third-party consumables requirements.

[2] Refer to the Appendix section of Procedure 102-166-600 for instructions on shearing DNA with the Megaruptor 3 system or Covaris g-TUBE devices.

[3] If the DNA library is <10 kb, can proceed with a 1x SMRTbell bead cleanup instead of performing DNA size selection using diluted AMPure PB beads.

[4] For primer annealing, polymerase binding & complex cleanup (ABC) using Vega chemistry or Revio SPRQ chemistry, follow sample setup instructions in Procedure & checklist – Do not use SMRT Link Sample Setup. For ABC using Revio non-SPRQ chemistry or Sequel II/IIe chemistry, follow SMRT Link Sample Setup software for preparing samples for sequencing.

Key workflow steps for SPK 3.0 WGS & metagenomic sequencing applications

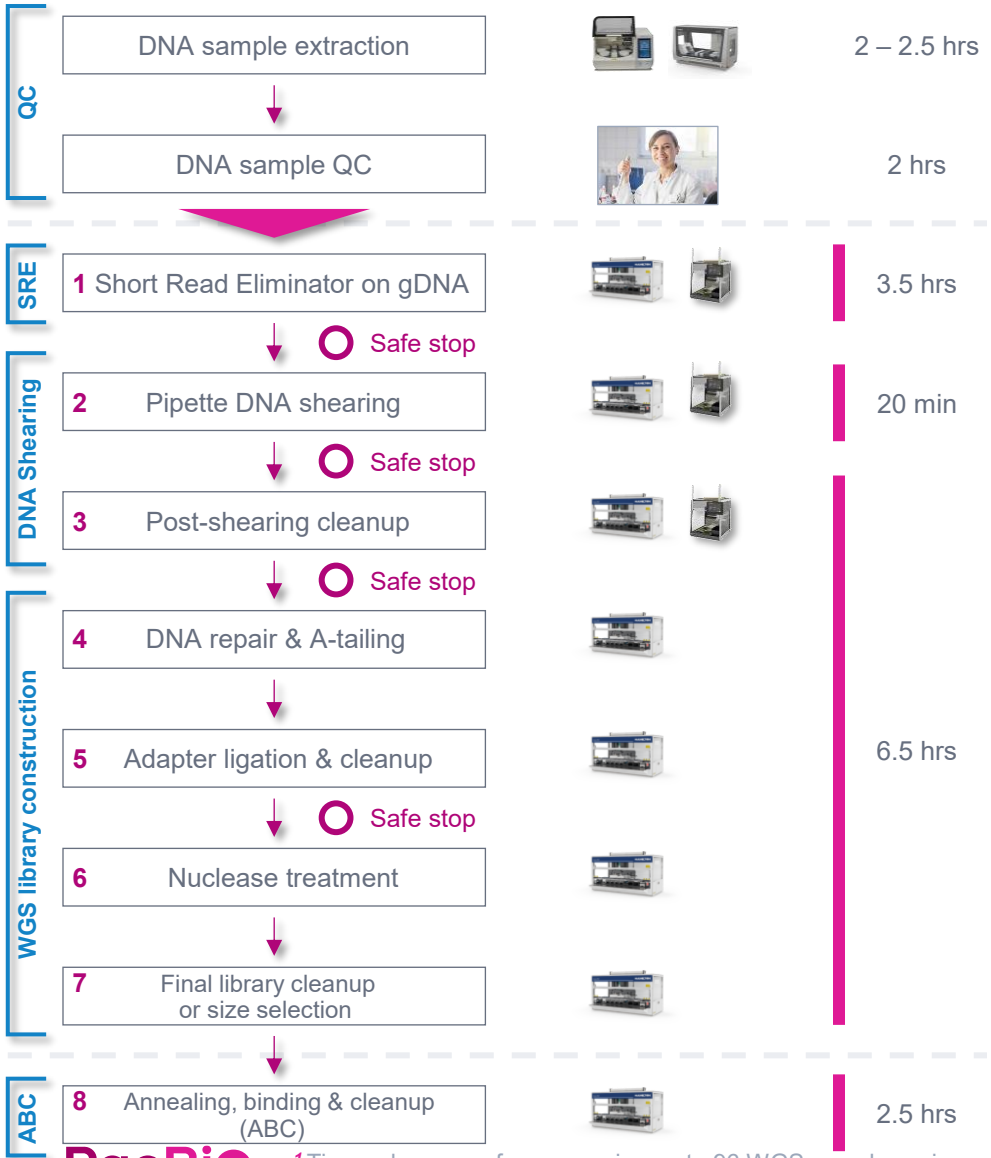
SPK 3.0 library preparation workflow[1] can optionally be automated to enable higher sample throughput

QC	DNA sample extraction		2 – 2.5 hrs	 ***Guide & overview – Nanobind PanDNA kit*** (103-394-800)	 Nanobind PanDNA kit (103-260-000 / 24 RXN)
	DNA sample QC		2 hrs	 Refer to third-party user guide documenation	 Qubit 4 fluorometer system Femto Pulse system
SRE	1 Short Read Eliminator on gDNA		2.5 hrs		 Short read eliminator (SRE) kit (102-208-300 / 24 RXN)
	Safe stop				
DNA Shearing	2 Pipette DNA shearing		20 min	**SPK 3.0 WGS protocol reference**	 Hamilton Microlab Prep (Pipette-based DNA shearing) *or*
	Safe stop			 ***Procedure & checklist – Preparing whole genome libraries using SMRTbell prep kit 3.0*** (102-166-600)	 Diagenode Megaruptor 3 (Hydropore-based DNA shearing)[2] *or*
	3 Post-shearing cleanup		25 min		
	Safe stop				 g-TUBE (Centrifugation-based DNA shearing)[2]
WGS library construction	4 DNA repair & A-tailing		45 min DAY 1	**Example supported automation systems**  Thermo Fisher KingFisher Apex  Hamilton Microlab NIMBUS Presto  Hamilton Microlab Prep  Hamilton NGS STAR MOA	
	5 Adapter ligation & cleanup		45 min		 SMRTbell prep kit 3.0 (102-182-700 / 24 RXN)
	Safe stop				
	6 Nuclease treatment		20 min		 **WGS library insert size >10 kb:**[3] 3.1X (35%) AMPure PB size selection
	7 Final library cleanup or size selection		25 min		 **WGS library insert size <10 kb:** 1X SMRTbell bead cleanup
ABC	8 Annealing, binding & cleanup (ABC)		1 hr DAY 2		 Revio SPRQ polymerase kit *or* Vega polymerase kit

[1] Times shown are for processing up to 8 WGS samples using a manual SPK 3.0 workflow; [2] Alternatively, can shear DNA using a Megaruptor 3 system (Diagenode) or g-TUBEs (Covaris) if a Microlab Prep system is unavailable; [3] Can optionally perform gel cassette-based size selection to enrich for SMRTbell library inserts >10 kb.

Key workflow steps for SPK 3.0 WGS & metagenomic sequencing applications

PacBio qualified automation protocols for WGS library construction and long-read sequencing preparation



Available automation methods for SPK 3.0 WGS workflows

Automation system	Hamilton	Integra	Revvity	Tecan
SRE size selection				
Input gDNA size selection	Microlab Prep			
DNA shearing				
HiFi sequencing using 15 – 20 kb library insert size	NGS STAR MOA STARlet STAR V Microlab Prep			DreamPrep NGS & DreamPrep NGS Compact
WGS library prep				
SMRTbell prep kit 3.0	NGS STAR MOA	Miro Canvas	SciClone NGSx	DreamPrep Compact
HiFi prep kit 96	NGS STAR MOA			
HiFi plex prep kit 96	NGS STAR MOA			
Sequencing preparation (ABC)[1]				
Sequel II binding kit 3.2	NGS STAR MOA			DreamPrep Compact
Revio polymerase kit	NGS STAR MOA			DreamPrep Compact
Revio polymerase kit 96	NGS STAR MOA			

[1] Times shown are for processing up to 96 WGS samples using a Hamilton NGS STAR MOA system – see ***Guide & overview – Automated HiFi prep 96 and HiFi ABC for the Hamilton NGS STAR MOA system*** (103-425-700) for more details.

General best practices recommendations for preparing WGS and metagenome libraries using SMRTbell prep kit 3.0

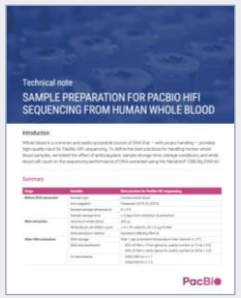
DNA sample extraction

Genomic DNA extraction for WGS applications

PacBio Nanobind DNA extractions kits are recommended to ensure sufficient mass and quality of high-molecular weight (HMW) DNA for use in HiFi WGS library preparation protocols using SPK 3.0

Nanobind PanDNA kit[1] (103-260-000; 24 rxn)	Nanobind HT CBB kit[2] (102-762-700; 96 rxn)
• For HMW DNA extraction from cells, blood, human saliva, bacteria, tissues, insects, and plant nuclei • Expected HMW DNA yield: 3–26 µg	• For high-throughput HMW DNA extraction from up to 200 µL human/mammalian blood, non-mammalian animal blood, cultured cells, and bacteria • Expected HMW DNA yield: 3–15 µg for blood and cultured mammalian cells and 2–10 µg for bacteria

Technical note: Preparing DNA for PacBio HiFi sequencing – Extraction and quality control (102-193-651)



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

Genomic DNA extraction for metagenomic sequencing

Note: The products below have not been tested or validated by PacBio but are listed here as examples of third-party kits used by other PacBio customers for isolating genomic DNA for PacBio metagenomic sequencing applications

Sample type	Third-party product or kit[3]
Fecal and soil	QIAGEN DNeasy PowerSoil Pro (PN 47014)
	QIAGEN PowerFecal Pro (PN 51804)
	QIAGEN DNeasy PowerClean Pro Cleanup Kit (PN 12997-50) • If needed, can be used after extracting DNA with PowerSoil or PowerFecal kits to further improve sequencing performance

[3] Metagenomic DNA can be extracted using commercially available kits from suppliers such as Zymo Research and Qiagen. For metagenome sequencing libraries from sample types that may contain inhibitors or contaminants, it is recommended to further clean up the extracted DNA with a column-based cleanup such as the Dneasy PowerClean Pro Cleanup Kit (cat no. 12997-50).

[1] See ***Guide & overview – Nanobind PanDNA kit*** (103-394-800) for more details.
[2] See ***Guide & overview – Nanobind HT kits*** (103-028-100) for more details.

General best practices recommendations for preparing WGS and metagenome libraries using SMRTbell prep kit 3.0 (cont.)

DNA sizing QC

- Agilent Femto Pulse system[1] is highly recommended for the accurate sizing of genomic DNA samples
  - Femto Pulse system enables simple, rapid sizing QC of genomic DNA and SMRTbell libraries, and conserves sample by using femtogram ranges of input DNA
    - Resolves fragments 1,300 bp to 165 kb using gDNA 165 kb Analysis kit (can resolve 100 – 6,000 bp using Ultra Sensitivity NGS kit)
    - Requires <1 ng of sample DNA
    - Can analyze up to 12 samples in <1.5 hrs
    - Outputs quality metrics such as Genomic Quality Number (GQN)[2] to quickly score integrity of HMW gDNA
- Alternative DNA sizing tools may be used if a Femto Pulse system is unavailable
  - However, caution should be used when interpreting results from other tools that employ constant-field electrophoresis technology
    - These technologies tend to inflate the true size of the gDNA (or library) and should only be used for qualitative assessment of whether an experiment was successful (e.g., intact library) rather than for accurate measurement of fragment size distributions

Femto Pulse system (Agilent Technologies)

DNA quantification QC

- For DNA quantification QC, we recommend using a quantification assay specific for double-stranded DNA (dsDNA) such as the Qubit 1X dsDNA high sensitivity assay kit[3] (Thermo Fisher Scientific)
  - **Note:** We do not recommend quantification with UV-Vis Spectrophotometers (e.g. NanoDrop) that measure all nucleic acids in a sample. For example, measuring all nucleic acid will inflate the true concentration of gDNA in samples.

Qubit 4 fluorometer (Thermo Fisher Scientific)

[1] See ***Product Note – HiFi WGS sequencing with the Agilent Femto Pulse system*** (102-326-561) for more details.
[2] See ***Application Note – Quality Metrics for Nucleic Acids with the Agilent Fragment Analyzer and Femto Pulse Systems*** (Agilent 5994-0521EN)
[3] Alternatively, for high-throughput applications DNA quantification QC may be performed with a microplate reader using the Quant-iT 1X dsDNA high sensitivity assay kit (Thermo Fisher Scientific).

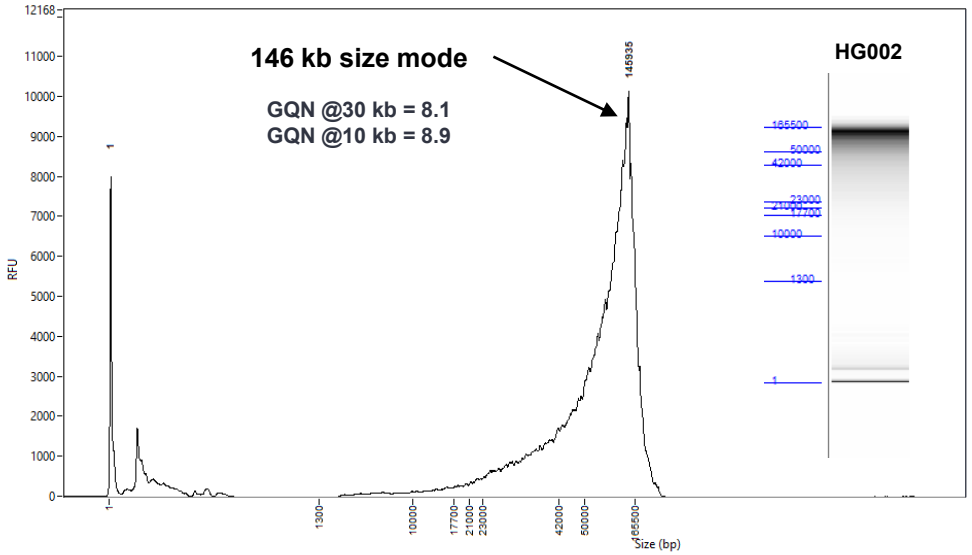
General best practices recommendations for preparing WGS and metagenome libraries using SMRTbell prep kit 3.0 (cont.)

Recommended genomic DNA input amount and quality

DNA input quality

- 70% or more of the DNA should be ≥10 kb for this SPK 3.0 library prep protocol
  - → This corresponds to a genome quality number (GQN) of 7.0 or higher at 10 kb

DNA quality	Femto Pulse genome quality score	Notes
DNA size distribution (Femto pulse system)	70% ≥10 kb (GQN10kb ≥7.0) & 50% ≥30 kb (GQN30kb ≥5.0)	• Lower quality DNA may be used with the expectation of lower sequencing data yields



Any degradation present should be due to shearing from extraction process and **not** from poor sample handling/storage or biochemical processes

Example DNA sizing QC analysis of a high-quality HG002 human genomic DNA sample using a Femto Pulse system with Genomic DNA 165 kb kit.

[1] **Important:** The HiFi yield and HiFi mean read length of a sequencing run are directly proportional to the quality of the genomic DNA input and the fragment lengths generated after shearing. To maximize yield and genome coverage per SMRT Cell, start with high quality gDNA containing minimal DNA below 10 kb, and with >50% mass over 30 kb. High quality gDNA will typically have a higher percent library recovery and HiFi sequencing yield.

General best practices recommendations for preparing WGS and metagenome libraries using SMRTbell prep kit 3.0 (cont.)

Recommended genomic DNA input amount and quality

DNA input amount

DNA Input into SPK 3.0 library prep	Sequel II and IIe systems	Revio non-SPRQ / Vega chemistry	Revio SPRQ chemistry
Total DNA per SMRT Cell[1]	1 µg per SMRT Cell	2 µg per SMRT Cell	500 ng per SMRT Cell

[1] **Note:** If multiplexing, the total mass of input gDNA summed across all samples must be equivalent to the numbers indicated above. **If using SRE, ≥500 ng per sample must be used. If bypassing SRE, no less than 50 ng of input gDNA should be used for an individual sample to go into (13-20 kb) SMRTbell library preparation using SMRTbell prep kit 3.0.**. Also see ***Procedure & checklist – Preparing multiplexed whole genome and amplicon libraries using the HiFi plex prep kit 96*** (103-418-800) for additional details on multiplexing recommendations for WGS applications when using HiFi plex prep kit 96.

Low mass vs high mass DNA input amounts

- Using the Revio system with SPRQ chemistry reduces the gDNA mass required for library preparation
  - Low mass option enables SRE and DNA shearing using 0.5–1.25 µg of gDNA[2]
- It is recommended to stay consistent for both SRE and shearing steps (i.e., use Low ***or*** High mass settings for ***both*** SRE and shearing)
- Note that High mass and Low mass samples cannot be included in a single SRE/shearing automation run
- If targeting larger insert sizes or working with lower quality DNA, start with higher input gDNA amounts (going into SRE size selection step) to ensure adequate library for optimal SMRT Cell loading

gDNA quality	Low mass (compatible with Revio SPRQ)[3]	High mass	Expected SRE recovery (dependent on DNA quality)
70% >10 kb (recommended)	0.5 – 1.25 µg	2 – 4 µg	60 – 95%
<70% >10 kb	1 – 1.75 µg	4 – 5 µg	40 – 60%
<10 kb (no SRE)	0.25 – 1 µg	1 – 3 µg	Not recommended

[2] **Note:** If gDNA mass available is between 1.25 µg and 2 µg, use the "High mass" workflow; however, note that if sequencing on Vega system or Revio system without SPRQ, there may not be enough final library for loading at an optimal on-plate loading concentration for a single SMRT Cell.

[3] If 0.5–1.25 µg gDNA is available and Revio SPRQ chemistry is being used, the Low mass workflow will provide enough library for loading 1 Revio SMRT Cell (+SPRQ); however, High mass parameters can also be used for Revio SPRQ chemistry if excess library is desired. For Revio non-SPRQ chemistry and Vega, 2 µg gDNA input is recommended for loading 1 SMRT Cell.

General best practices recommendations for preparing WGS and metagenome libraries using SMRTbell prep kit 3.0 (cont.)

Recommended genomic DNA input amount and quality

Expected SPK 3.0 WGS library construction yield

- Overall SMRTbell library construction yield is dependent on input gDNA quality and size
  - The recovery from input gDNA to completed SMRTbell library typically ranges between 10 – 32% (includes SRE, shearing, SMRTbell library preparation and ABC)

Protocol step	DNA or SMRTbell step recovery	DNA or SMRTbell overall recovery	Expected size (Femto Pulse)
Starting input	100%	100%	GQN 10kb > 7.0
Post-SRE[1]	65 – 95%	65 – 95%	GQN 10 kb > 9.3
Post-shear SMRTbell bead cleanup	80 – 95%	52 – 90%	15 – 20 kb
Post-ligation SMRTbell bead cleanup	80 – 95%	42 – 86%	15 – 20 kb
Post-nuclease (pre-cleanup)	40 – 50%	17 – 43%	15 – 20 kb
Post-3.1x AMPure PB bead cleanup	75 – 80%	13 – 34%	15 – 20 kb
Post-ABC cleanup	75 – 95%	10 – 32%	15 – 20 kb

[1] Post-SRE recovery will vary with the quality of the DNA input. Higher quality DNA will yield more recovery post-SRE.

General best practices recommendations for preparing WGS and metagenome libraries using SMRTbell prep kit 3.0 (cont.)

Recommended genomic DNA input amount and quality

Minimum polymerase-bound library mass needed to load a SMRT Cell

- Starting with **2 µg** of high-quality input gDNA (going into SRE size selection step) will, on average, provide enough library to load **1 Revio SMRT Cell without SPRQ chemistry** or **1 Vega SMRT Cell**
- Starting with **500 ng** of high-quality input gDNA (going into SRE size selection step) will, on average, provide enough library to load **1 Revio SMRT Cell with SPRQ chemistry**

Mean library insert size	Polymerase-bound library mass needed to load one SMRT Cell (250 pM on-plate loading concentration, OPLC)	
	Revio SPRQ chemistry	Revio non-SPRQ chemistry / Vega chemistry
10,000 bp	41 ng	163 ng
15,000 bp	61 ng	244 ng
18,000 bp	73 ng	293 ng
21,000 bp	85 ng	341 ng

General best practices recommendations for preparing WGS and metagenome libraries using SMRTbell prep kit 3.0 (cont.)

Input genomic DNA size selection using Short read eliminator (SRE) kit (102-208-300)

SRE considerations for use

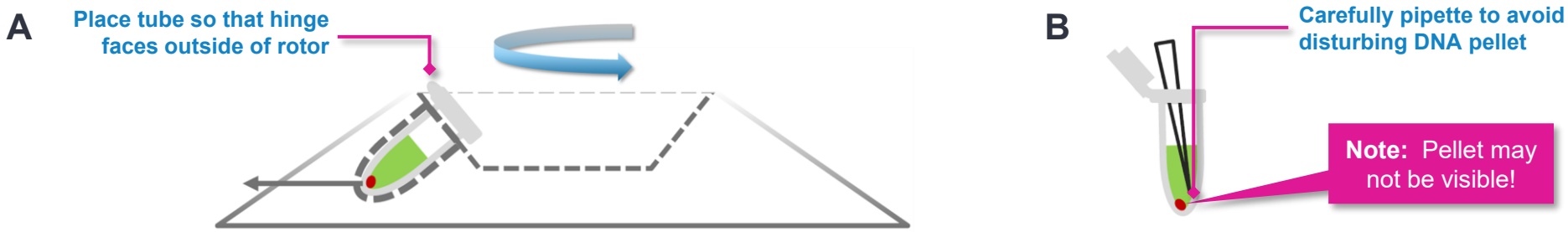
- **SRE kit (102-208-300)** is recommended for rapid size selection of (unsheared) high-molecular weight genomic DNA samples
- Uses a centrifugation procedure similar to standard ethanol precipitation techniques
- SRE kit can significantly enhance mean HiFi read lengths by progressively depleting short DNA fragments
  - SRE will progressively deplete fragments up to 25 kb from gDNA samples → **SRE should not be performed on gDNA samples that are <10 kb**
- When choosing between Low mass and High mass workflows, it is recommended to stay consistent for both SRE and shearing steps (i.e., use Low or High mass settings for **both** SRE ***and*** shearing).

IMPORTANT: Use SRE on only **genomic DNA**

- Attempting to use SRE on sheared DNA or HiFi libraries (post-library construction) will result in poor recoveries

SRE DNA size selection manual pipetting procedure

- Load tube into centrifuge[1] with hinge of tube facing toward the outside of rotor
  - This will help to avoid disturbing the pellet if it cannot be seen
- After centrifugation, the DNA pellet will have formed on the bottom side of the microcentrifuge tube under the hinge region
- Carefully pipette on opposite side towards the thumb lip of tube to avoid disturbing the pellet



[1] Centrifuge at 10,000 x *g* at room temperature (RT) if using tubes. Centrifuge at 2,250 x *g* at room temperature (RT) if using a microplate. Turn off any built-in temp. control function by specifying a target temp. set point higher than ambient RT.

General best practices recommendations for preparing WGS and metagenome libraries using SMRTbell prep kit 3.0 (cont.)

DNA shearing

- ***Important:*** A mean fragment size between 15 to 20 kb with a narrow distribution (typically ~10 – 35 kb) is recommended for this protocol → **If gDNA is within these ranges or lower, the DNA shearing step can be bypassed**
- We recommend performing pipette-based DNA shearing[1] using a Hamilton Microlab Prep liquid handling instrument
  - Refer to ***Technical note – High throughput DNA shearing using Hamilton Microlab Prep*** (102-326-606) or ***Guide and overview – Short Read Eliminator (SRE), DNA shearing, and cleanup for the Hamilton Microlab Prep system*** (103-424-100) for details about third-party consumables requirements
- If a Microlab Prep liquid handling instrument is unavailable for performing pipette-based DNA shearing, then a Megaruptor 3 system (Diagenode) or g-TUBEs (Covaris) may alternatively be used[2]
- When choosing between Low mass and High mass workflows, it is recommended to stay consistent for both SRE and shearing steps (i.e., use Low or High mass settings for **both** SRE ***and*** shearing).

Microlab Prep system (Hamilton)

Metagenomic samples

- Metagenomic samples often have degraded gDNA where the majority of fragments are already <15 kb in length to start
  - → Do not perform SRE size selection on metagenomic samples
  - → The final library insert size for metagenomic samples may be <15 kb
  - → If DNA sizing QC indicates that the average fragment size of the starting gDNA is <18 kb, then skip the DNA shearing step in this procedure
- Because SRE will be skipped, a lower DNA input mass may be used for metagenomic samples. Note that ~14 to 34% recovery (from gDNA to bound SMRTbell) is expected. Ensure enough DNA is used to meet optimal loading concentrations on PacBio long-read systems.

[1] **Note 1:** Maximum input gDNA mass supported by pipette DNA shearing method is 3 μg for High mass workflow and 1 μg for Low mass workflow → Perform parallel shearing reactions if using more than the maximum input gDNA amount. **Note 2:** Maximum input sheared DNA mass tolerated by library enzymatic reactions in this SPK 3.0 WGS protocol is 5 μg per reaction → Perform parallel library prep reactions if using >5 μg of input sheared DNA.

[2] See Appendix A1 in ***Procedure & checklist – Preparing whole genome and metagenome sequencing libraries using SMRTbell prep kit 3.0*** (102-166-600) for Megaruptor 3 system and g-TUBE shearing recommendations.

General best practices recommendations for preparing WGS and metagenome libraries using SMRTbell prep kit 3.0 (cont.)

Reagent handling[1]

PacBio kit or consumable	Thaw these reagents at room temperature	Keep these temperature-sensitive reagents on ice	Bring these reagents to room temperature 30 minutes prior to use
SMRTbell prep kit 3.0 (102-182-700) 	❑ Repair buffer	 ❑ End repair mix	❑ Elution buffer
	❑ Nuclease buffer	 ❑ DNA repair mix	
	❑ SMRTbell adapter	 ❑ Ligation mix	
	❑ Elution buffer	 ❑ Ligation enhancer	
		 ❑ Nuclease mix	
		 ❑ SMRTbell adapter	
AMPure PB bead size selection kit (102-182-500) 	❑ Elution buffer	❑ N/A	❑ Elution buffer
			❑ AMPure PB beads

[1] SRE buffer and Buffer LTE are stored at room temperature.

General best practices recommendations for preparing WGS and metagenome libraries using SMRTbell prep kit 3.0 (cont.)

Reagent handling[1] (cont.)

PacBio reagent handling notes

- Room temperature is defined as any temperature in the range of 18 – 25°C for this protocol
- SRE buffer and Buffer LTE are stored at room temperature
- Once thawed, reaction buffers & adapter index plate may be stored on cold block, at 4°C, or on-ice prior to making master mix or placing on liquid handler work deck
- Mix reagent buffers with a brief vortex prior to use (do not vortex enzymes)
- Briefly spin down all reagent tubes in a microcentrifuge to collect all liquid at the bottom (if using a SMRTbell adapter index plate, briefly vortex and then spin down in a centrifuge with a plate adapter to collect all liquid at the bottom of the wells)
- Shake/vortex SMRTbell cleanup beads and AMPure PB beads immediately before use (failure to do this will result in low recovery)
- Pipette-mix (e.g., up and down 10 times) all bead binding and elution steps until beads are distributed evenly in solution
- It is recommended to pipette-mix all SMRTbell prep reactions by pipetting up and down 10 times
- Samples can be stored at 4°C at all safe stopping points listed in the protocol

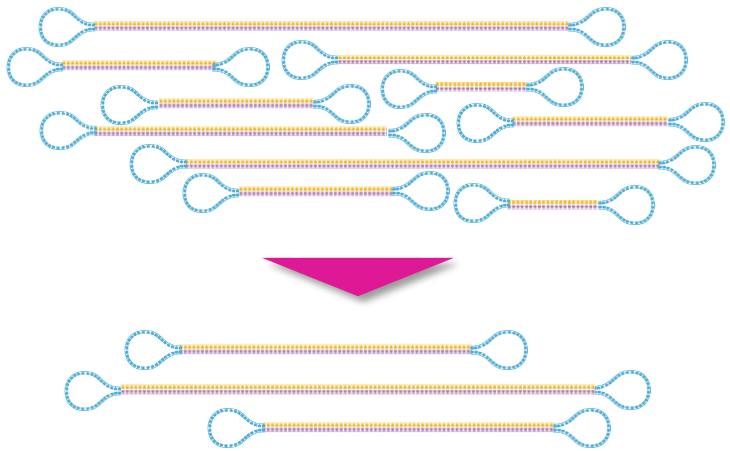
[1] Refer to *Procedure & checklist* protocol for detailed instructions for handling all required library preparation reagents.

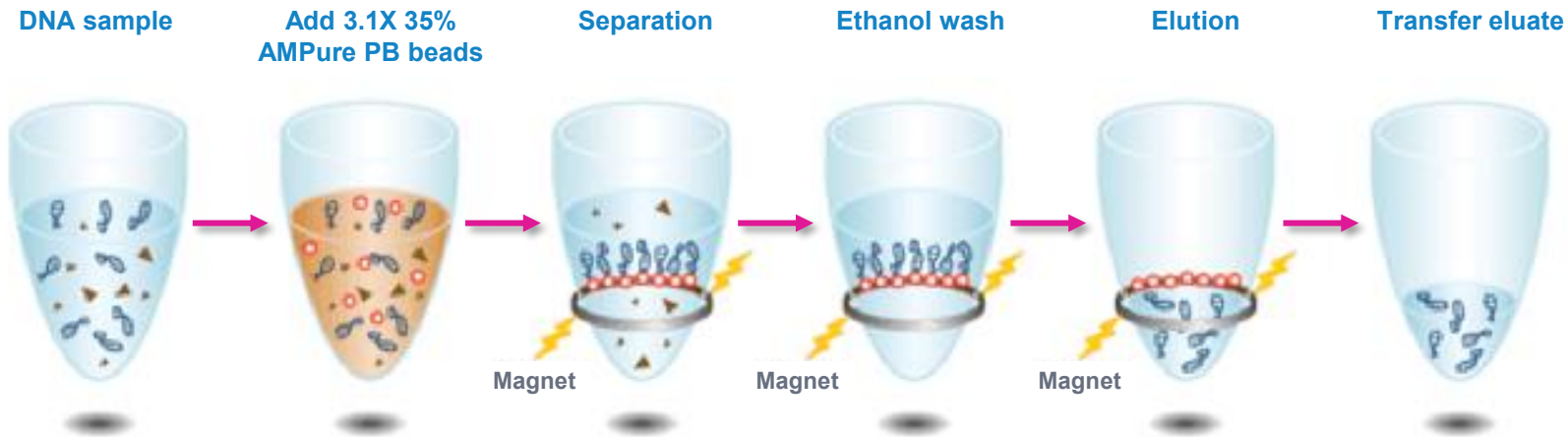
General best practices recommendations for preparing WGS and metagenome libraries using SMRTbell prep kit 3.0 (cont.)

Final library size selection using AMPure PB bead size selection kit (102-182-500)

- AMPure PB beads are used as the default size selection method to progressively deplete short DNA fragments <5 kb[1] from final SPK 3.0 WGS libraries and enrich for long fragments

AMPure PB bead size selection kit
(102-182-500)





AMPure PB bead size selection procedure

1. Prepare a **35% dilution (v/v)** of the AMPure PB bead stock in Elution Buffer (EB)
   - 35% AMPure PB beads solution can be stored at 4°C for 30 days.
2. Add 3.1X of room-temperature 35% AMPure PB beads to each sample and incubate for 20 min at RT
3. Place samples on magnetic rack; wash samples with 80% ethanol 2X; then elute samples in EB for 5 min at RT

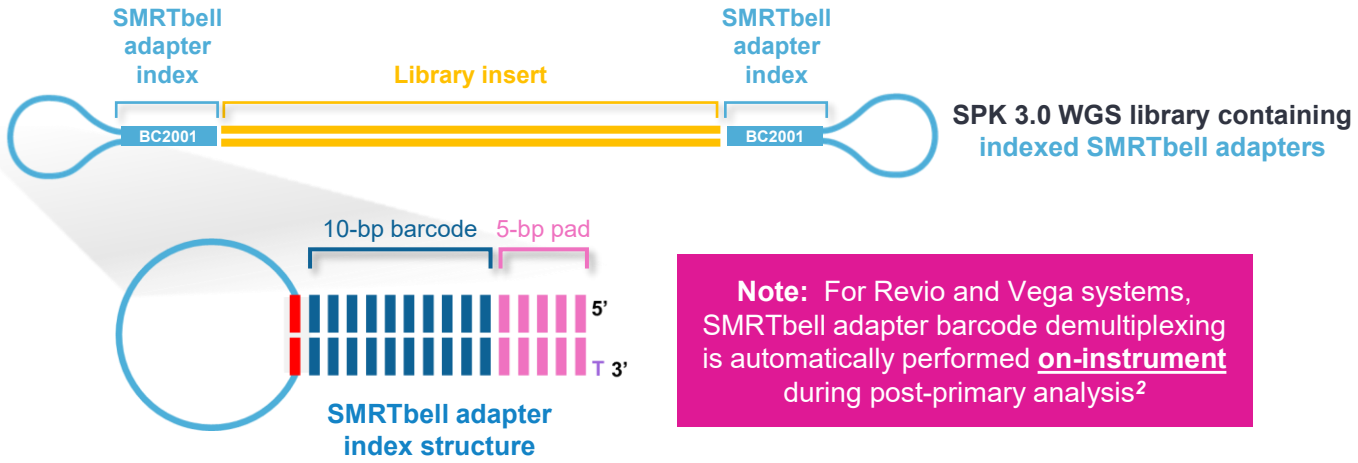
[1] To remove unwanted DNA fragments larger than 5 kb using alternative gel-based size selection methods, please see ***Technical note – Gel cassette size selection methods for WGS HiFi libraries*** (102-326-503). Note that use of these tools requires higher per-SMRT Cell input gDNA amounts (≥1.5 µg/SMRT Cell 8M or ≥3 µg/Revio SMRT Cell without SPRQ chemistry or ≥750 ng/Revio SMRT Cell with SPRQ chemistry).

General best practices recommendations for preparing WGS and metagenome libraries using SMRTbell prep kit 3.0 (cont.)

Multiplexing samples

SMRTbell adapter index plate 96A/B/C/D for construction of indexed SPK 3.0 WGS libraries

- If multiplexing samples, SPK 3.0 libraries must include a SMRTbell adapter index ('barcode')
- SMRTbell adapter index plate 96A (102-009-200)[1] can be used for any WGS or amplicon sequencing application that employs barcoded overhang adapters



Note: For Revio and Vega systems, SMRTbell adapter barcode demultiplexing is automatically performed **on-instrument** during post-primary analysis[2]

- SMRT Link comes preloaded with the following barcode set FASTA file containing SMRTbell adapter index plate 96A/B/C/D barcode sequences:
  - ❑ `SMRTbell adapters indexes` (for Sequel IIe, Revio & Vega system run designs)

	1	2	3	4	5	6	7	8	9	10	11	12
A	BC 2001	BC 2009	BC 2017	BC 2025	BC 2033	BC 2041	BC 2049	BC 2057	BC 2065	BC 2073	BC 2081	BC 2089
B	BC 2002	BC 2010	BC 2018	BC 2026	BC 2034	BC 2042	BC 2050	BC 2058	BC 2066	BC 2074	BC 2082	BC 2090
C	BC 2003	BC 2011	BC 2019	BC 2027	BC 2035	BC 2043	BC 2051	BC 2059	BC 2067	BC 2075	BC 2083	BC 2091
D	BC 2004	BC 2012	BC 2020	BC 2028	BC 2036	BC 2044	BC 2052	BC 2060	BC 2068	BC 2076	BC 2084	BC 2092
E	BC 2005	BC 2013	BC 2021	BC 2029	BC 2037	BC 2045	BC 2053	BC 2061	BC 2069	BC 2077	BC 2085	BC 2093
F	BC 2006	BC 2014	BC 2022	BC 2030	BC 2038	BC 2046	BC 2054	BC 2062	BC 2070	BC 2078	BC 2086	BC 2094
G	BC 2007	BC 2015	BC 2023	BC 2031	BC 2039	BC 2047	BC 2055	BC 2063	BC 2071	BC 2079	BC 2087	BC 2095
H	BC 2008	BC 2016	BC 2024	BC 2032	BC 2040	BC 2048	BC 2056	BC 2064	BC 2072	BC 2080	BC 2088	BC 2096

SMRTbell adapter index plate 96A (102-009-200) contains 96 barcoded adapters to support multiplexed SMRTbell library construction for up to 96 samples using SPK 3.0

SMRTbell adapter index sequences (FASTA) [Link]

Product insert – SMRTbell adapter index plate 96A (contains plate map [Link]

[1] Kit quantities support a single use of each of the 96 barcoded adapters in the plate for SMRTbell library preparations.

[2] **Note:** To enable automatic SMRTbell adapter barcode demultiplexing on-instrument, specify '**Sample is indexed = YES**' in the sequencing run design for Revio and Vega systems.

General best practices recommendations for preparing WGS and metagenome libraries using SMRTbell prep kit 3.0 (cont.)

Multiplexing samples (cont.)

Standard multiplexing recommendations for WGS libraries

- Prior to pooling HiFi libraries together please consider the following guidelines:
  - Ensure that samples to be pooled have a similar mean insert size and similar insert length size distribution[1]
  - Pool samples with similar genome sizes to ensure balanced coverage[2]
  - Pool samples in an equal molar concentration for best balanced coverage[3]
- For low-multiplexing applications, it is recommended to pool HiFi libraries post-ABC (i.e., ***after*** performing primer annealing, polymerase binding and complex cleanup) for the following reasons:
  - Prevent an inhibitor in one sample from affecting the polymerase binding of all samples in a pool
  - Ability to quickly pool different libraries together on additional runs to "top off" coverage (any un-pooled complexed library is available for future sequencing runs without having to re-do ABC)
- For high-multiplexing applications (e.g., ≥24-plex) using HiFi plex prep kit 96 (or other high-throughput kits), adapter-indexed HiFi libraries will typically be pooled ***prior*** to ABC step
- Optionally use the SMRT Link **Sample Setup Pooling Calculator tool** to help determine appropriate volumes to use for multiplexing WGS libraries

DNA shearing

- Shear all samples to similar fragment length profiles[1] and verify using a Femto Pulse system

↓

SMRTbell library construction

- Prepare libraries with SMRTbell prep kit 3.0 and SMRTbell adapter index plate 96A/B/C/D

↓

Sample pooling

- Pool samples with similar genome sizes together
- Pool an equal molar concentration of each SMRTbell library into the pool

[1] For WGS applications, we recommend aiming for a library insert size >15 kb (ideally 15 – 20 kb) to achieve optimum HiFi data yields on PacBio long-read systems. **Note:** HiFi read lengths, reads/data per SMRT Cell and other sequencing performance results can vary depending on DNA sample quality, insert size, sample loading performance & movie time. Shorter library insert sizes (<15 kb), lower DNA quality samples, and suboptimal sample loading performance may result in reduced HiFi data yields.

[2] If fragment length profiles are similar but the estimated genome sizes of the samples are different, then one may consider pooling the libraries with mass proportional to genome size. For example, if pooling a 2 Gb genome sample with a 1 Gb genome sample, add twice the mass of library for the 2 Gb genome sample relative to the 1 Gb genome sample.

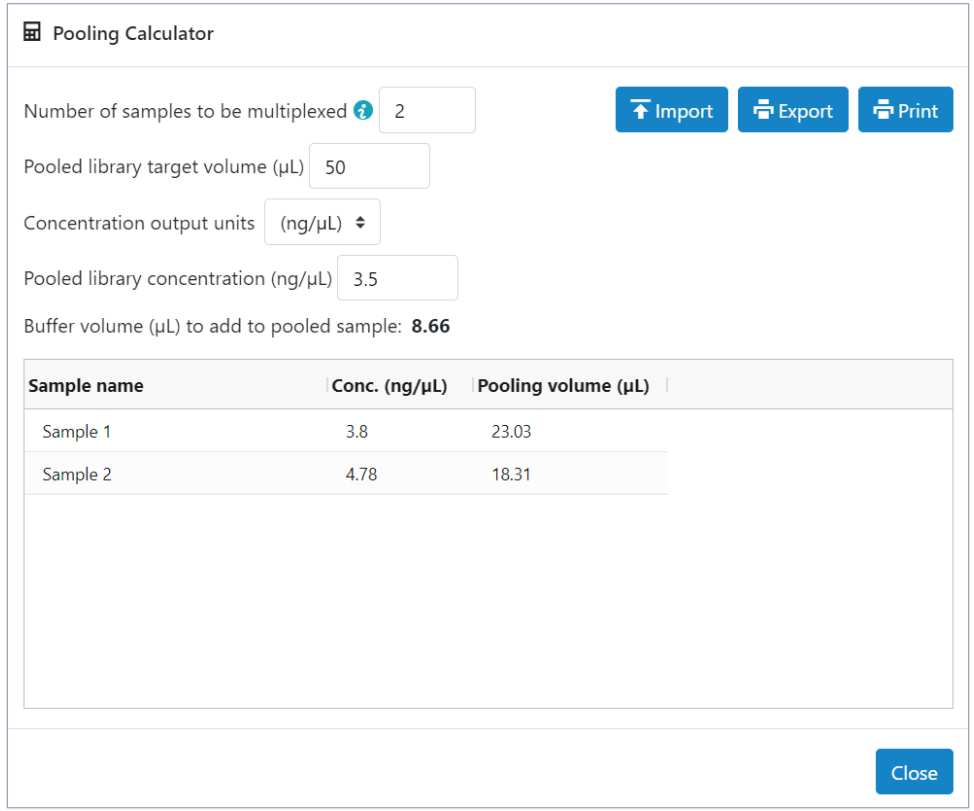
[3] If the difference in average length of your final SMRTbell libraries is >25%, it may be necessary to pool in equal molar amounts to balance the number of reads per library. Equal-mass pooling of barcoded WGS samples can be performed if all samples have similar fragment length profiles and similar estimated genome sizes.

General best practices recommendations for preparing WGS and metagenome libraries using SMRTbell prep kit 3.0 (cont.)

Multiplexing samples (cont.)

SMRT Link Sample Setup Pooling Calculator

- Optionally use the SMRT Link **Sample Setup Pooling Calculator tool** to help determine appropriate volumes to use for multiplexing SPK 3.0 libraries
- Prior to pooling HiFi libraries together please consider the following guidelines:
  - Ensure that samples to be pooled have a similar mean insert size and similar insert length size distribution[1]
  - Pool samples with similar genome sizes to ensure balanced coverage
  - Pool samples in an equal molar concentration for best balanced coverage



SMRT Link Sample Setup Pooling Calculator tool can be used to calculate the required volumes of library samples needed for pooling when performing multiplexed sequencing on a single SMRT Cell.

[1] For WGS applications, we recommend aiming for a library insert size >15 kb (ideally 15 – 20 kb) to achieve optimum HiFi data yields on PacBio long-read systems. **Note:** HiFi read lengths, reads/data per SMRT Cell and other sequencing performance results can vary depending on DNA sample quality, insert size, sample loading performance & movie time. Shorter library insert sizes (<15 kb), lower DNA quality samples, and suboptimal sample loading performance may result in reduced HiFi data yields.

General best practices recommendations for preparing WGS and metagenome libraries using SMRTbell prep kit 3.0 (cont.)

Sequencing preparation (ABC) and polymerase-bound library storage

- ***Procedure & checklist – Preparing whole genome libraries using SMRTbell prep kit 3.0*** (102-166-600) includes instructions for the primer annealing, polymerase binding & complex cleanup (ABC) sample setup steps for Revio and Vega systems
  - → For sequencing SPK 3.0 WGS libraries on the Revio system with SPRQ chemistry or the Vega system: Follow sample setup instructions stated in the protocol to perform ABC and final loading dilution procedure – Do not use SMRT Link Sample Setup software
- Sequencing polymerase is stable once bound to the SPK 3.0 library and can be stored at 4°C or frozen at -20°C.
- Stored polymerase-bound library shows equivalent loading to freshly prepared bound libraries up to the recommendations listed below.

  Recommended polymerase-bound storage:[1]
  - Polymerase-bound libraries can be stored at 4°C for up to 1 month
  - Polymerase-bound libraries can be stored at -20°C for at least 6 months
  - Polymerase-bound libraries can withstand up to 4 freeze-thaw cycles
- Stored polymerase-bound libraries should be protected from light since Loading buffer is light-sensitive

[1] Polymerase-bound storage recommendations described here apply to SMRTbell libraries bound to either Sequel II polymerases, Vega polymerase, Revio polymerase or Revio SPRQ polymerase.

DNA sample extraction

Nanobind PanDNA kit enables high-quality HMW DNA extraction from cells, blood, bacteria, tissues, insects, and plant nuclei[1]

DNA sample extraction

↓

DNA sample QC

1 Short Read Eliminator on gDNA

↓ Safe stop

2 Pipette DNA shearing

↓ Safe stop

3 Post-shearing cleanup

↓

4 DNA repair & A-tailing

↓

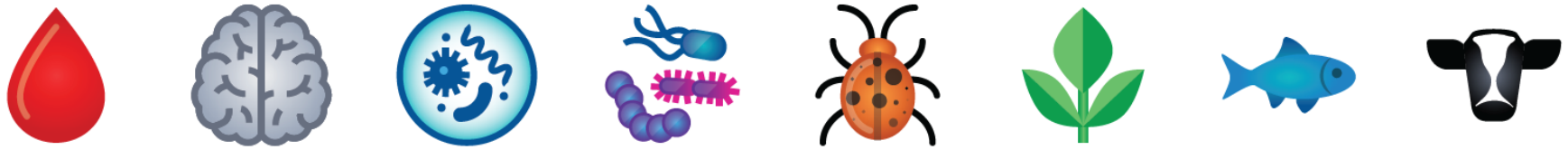
5 Adapter ligation & cleanup

↓

6 Nuclease treatment

↓ Safe stop

7 Final library cleanup or size selection



Easy-to-use solutions built on Nanobind technology

PacBio Nanobind PanDNA kit consolidates the capabilities of our existing sample-specific Nanobind kit product offerings into a single solution for DNA extraction.[1,2]

- Cultured mammalian cells
- Human whole blood
- Animal blood (mammalian & non-mammalian)
- Cultured bacteria
- Animal tissues
- Plant nuclei
- Insects
- RBC lysed human whole blood
- Human saliva

Using the Nanobind PanDNA kit on a diverse set of plant and animal samples demonstrates extraction yields between ~3–26 µg and mode fragment sizes >100 kb for the majority of samples (and >65 kb for insect samples)[3]

Nanobind PanDNA kit
(103-260-000)

Supports 24 reactions per kit and includes:

- Nanobind PanDNA kit RT (103-260-300)
- Nanobind PanDNA kit 4C (103-260-400)

1 See ***Technical overview – HMW DNA sample preparation for PacBio long-read sequencing using Nanobind PanDNA and SRE kits*** (103-401-100).
2 **Note:** Fungal, lichen, algae and microalgae sample types are unsupported with the Nanobind PanDNA kit.
3 See ***Brochure – Nanobind PanDNA kit*** (102-326-604).

PacBio

DNA sample QC

Perform DNA QC using a Qubit dsDNA HS assay and a Femto Pulse system

DNA sample extraction

↓

DNA sample QC

1 Short Read Eliminator on gDNA

↓ Safe stop

2 Pipette DNA shearing

↓ Safe stop

3 Post-shearing cleanup

↓

4 DNA repair & A-tailing

↓

5 Adapter ligation & cleanup

↓

6 Nuclease treatment

↓ Safe stop

7 Final library cleanup or size selection

DNA quantification QC

Qubit 4 fluorometer (Thermo Fisher Scientific)

Qubit fluorometer in conjunction with Qubit 1X dsDNA high-sensitivity assay (Thermo Fisher Scientific) enables rapid, specific and accurate determination of nucleic acid concentrations in a single sample[1,2]

- Assay is highly selective for dsDNA over ssDNA, RNA, protein, and free nucleotides. Contaminants, such as salts, solvents, or detergents are well-tolerated.
- Depending on sample volume, assay kit is designed to be accurate for initial DNA sample concentrations of 5 pg/µL to 120 ng/µL, providing a detection range of 0.1−120 ng.

[1] Alternatively, for high-throughput applications DNA quantification QC may be performed with a microplate reader using the Quant-iT dsDNA high sensitivity assay kit. **Note:** Do not use a Qubit Flex fluorometer when performing DNA quantification QC on polymerase-bound SMRTbell library in Loading buffer since concentration readings will not be accurate.

DNA sizing QC

Femto Pulse system (Agilent Technologies)

Femto Pulse system enables simple, rapid sizing QC of genomic DNA and SMRTbell libraries, and conserves sample by using femtogram ranges of input DNA

- Use the Femto Pulse gDNA 165 kb analysis kit (FP-1002-0275)
- Dilute samples to 250 pg/uL
- 70% or more of the DNA should be ≥10 kb for this protocol. This corresponds to a genome quality number (GQN)[3] of 7.0 or higher at 10 kb

If a Femto Pulse system is unavailable, can consider using alternative DNA sizing QC systems

- Caution should be used when interpreting results from other tools that employ constant-field electrophoresis technology[4]

[4] These technologies tend to inflate the true size of the gDNA (or library) and should only be used for qualitative assessment of whether an experiment was successful (e.g., construction of intact SMRTbell library) rather than for accurate measurement of fragment size distributions

[2] **Note:** We do not recommend quantification with UV-Vis spectrophotometers (e.g., NanoDrop) that measure all nucleic acids in a sample. For example, measuring all nucleic acid will inflate the true concentration of gDNA in samples

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

Short Read Eliminator on gDNA

Perform size selection on input genomic DNA using SRE kit to remove <10 kb fragments

DNA sample extraction

↓

DNA sample QC

1 Short Read Eliminator on gDNA

↓ Safe stop

2 Pipette DNA shearing

↓ Safe stop

3 Post-shearing cleanup

↓

4 DNA repair & A-tailing

↓

5 Adapter ligation & cleanup

↓

6 Nuclease treatment

↓ Safe stop

7 Final library cleanup or size selection

1. Short Read Eliminator

✓	Step	Instructions
	1.1	Dilute gDNA ($GQN_{10\,kb}$ >7.0) to the appropriate concentration in Buffer LTE according to the table below.

	Low mass	High mass
Sample volume	25 µL	50 µL
DNA concentration	20–50 ng/µL	40–80 ng/µL
Recommended max gDNA mass	1.25 µg	4 µg
Elution volume (Buffer LTE)	200 µL	300 µL
Shearing mass limit	≤1 µg	≤3 µg

Note: If working with low quality gDNA with a $GQN_{10\,kb}$ < 7.0, input mass and concentration can be increased if the expected recovery (40 – 60%, Table 1) matches the pipette-tip shearing mass limit for each respective workflow. For example, 2 µg of gDNA can be used with the Low mass workflow if expected recovery is 40% (800 ng).

1.2 Add **Buffer SRE** to each sample.

	Low mass	High mass
Buffer SRE volume	25 µL	50 µL

If working in a plate format, heat seal with foil. Vortex/shake to mix for 5 seconds at max speed.

1.3 Incubate the sample for 1 hour at 50°C in a heat block or thermal cycler. After incubation, if using a plate format, ensure that it is compatible with a 300 µL elution. If not, **transfer to the appropriate deep well plate after incubation and seal with an adhesive seal.**

1.4 Load plate or tube (with the hinge facing toward the outside of the rotor) into the centrifuge.

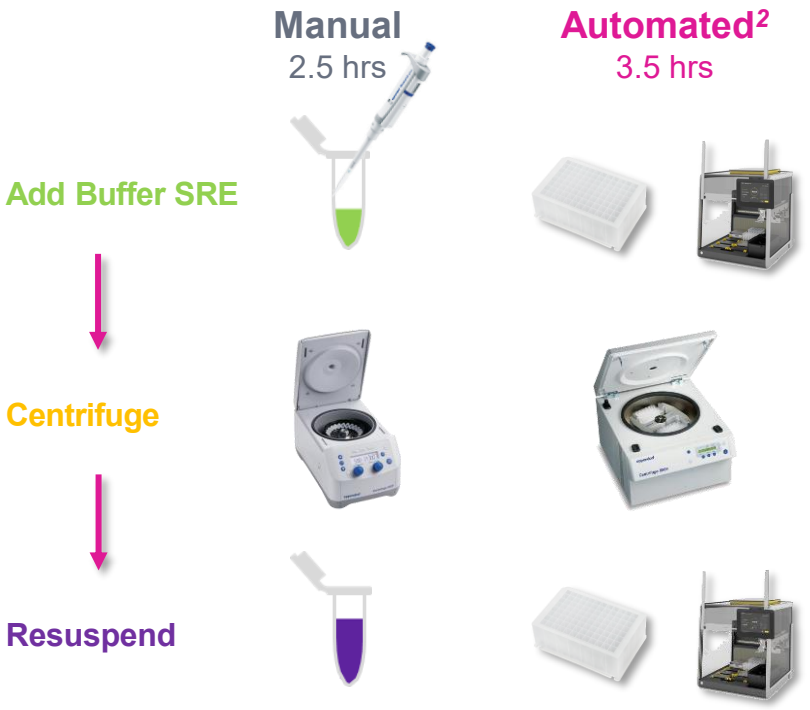
1.9 Quantify the resuspension to measure DNA recovery. If the recovery is lower than 50% repeat pipette-mixing 20 times and vortex/shake. If the recovery is greater than 50%, proceed to next step (DNA shearing).

1.10 Proceed to automated DNA shearing. It is recommended to proceed to DNA shearing within 2 weeks of performing SRE.

SAFE STOPPING POINT - Store at 4°C

- Dilute input gDNA to appropriate concentration in Buffer LTE according to either Low mass or High mass workflow conditions shown in Table.[1]
  - DNA input mass & concentration can be increased if expected recovery yield does not exceed pipette-tip shearing mass limit for each respective workflow.

SRE workflow for SPK 3.0 protocol



[1] ***IMPORTANT!*** Use SRE on only genomic DNA. Attempting to use SRE on HiFi libraries (post-SMRTbell library construction) will result in poor recoveries and potential loss of the entire library.

[2] High mass and Low mass samples cannot be included in the same SRE automation run. Refer to ***Guide & overview – Short Read Eliminator SRE DNA shearing and cleanup for the Hamilton Microlab Prep system*** (103-424-100) for details on required consumables if using automation equipment to perform SRE.

Pipette DNA shearing

Perform automated DNA shearing for WGS samples using Hamilton automation[1]

DNA sample extraction

↓

DNA sample QC

1 Short Read Eliminator on gDNA

↓ Safe stop

2 Pipette DNA shearing

↓ Safe stop

3 Post-shearing cleanup

↓

4 DNA repair & A-tailing

↓

5 Adapter ligation & cleanup

↓

6 Nuclease treatment

↓ Safe stop

7 Final library cleanup or size selection

2. Pipette DNA shearing

✔	Step	Instructions
	2.1	Adjust DNA concentration to **≤5 ng/µL in 200 µL or ≤10 ng/µL in 300 µL**, if necessary (e.g., if more than 1 µg or 3 µg of gDNA was recovered from SRE). Use the Low TE buffer provided with the kit (or buffer LTE from the SRE kit) to dilute samples in a 0.8 mL, 96 DeepWell plate (Thermo Fisher Scientific AB0859).
	2.2	Parameters for shearing on the Microlab Prep, or Hamilton assay-ready workstations are listed below. These parameters should already be part of the installed method on the instrument.
	2.3	Place the plate on the appropriate work deck position and start the shearing procedure.
	2.4	Optional: measure DNA concentration with a Qubit fluorometer using the 1x dsDNA HS kit once the shearing procedure is complete. **Recommended**: Further dilute each aliquot to **250 pg/µL** with the Femto Pulse dilution buffer. Measure the DNA with a Femto Pulse system to ensure efficient shearing.
	2.5	Proceed to the next step of the protocol.

Parameter	Low mass	High mass
DNA concentration	**<5 ng/µL**	**<10 ng/µL**
Volume of Buffer LTE	**200 µL**	**300 µL**
Number of mixes	300 cycles	300 cycles
Pipette mixing speed	**400 µL/sec**	**500 µL/sec**
Liquid following	83% volume	83% volume
Pipette tip	300 µL CO-RE II tips (filtered, black, non-sterile)	300 µL CO-RE II tips (filtered, black, non-sterile)

- Adjust DNA concentration to <5 ng/µL in 200 µL (Low mass workflow) or <10 ng/µL in 300 µL (High mass workflow) using Buffer LTE[2]

Hamilton NGS STAR MOA

Hamilton Microlab Prep

Pipette-based DNA shearing can be completed within ~10 min for up to 96 samples using a Hamilton NGS STAR workstation or within ~20 min for up to 24 samples using a Hamilton Microlab Prep system.

IMPORTANT!

- A mean fragment size between 15 to 20 kb is recommended for this protocol
- In addition, the distribution of fragment sizes should be narrow and generally between ~10 to 35 kb
  - Fragments that are too short produce less yield per read, and fragments that are too long may result in lower read accuracy and are less likely to produce HiFi reads
- Deviating from the concentration and automation settings specified for this SPK 3.0 workflow is not recommended and will result in under-sheared DNA

[1] This section describes the procedure for DNA shearing with the Hamilton Microlab Prep or Hamilton assay ready workstations (NGS STAR MOA, STARlet, and STAR V). It may be possible to shear DNA using other NGS liquid handler systems – Please contact PacBio Technical Support for updated information on all qualified DNA shearing methods.

[2] High mass and Low mass samples cannot be included in the same DNA shearing automation run.

Post-shearing cleanup

Perform post-shearing cleanup using 1X SMRTbell cleanup beads

- DNA sample extraction
- DNA sample QC
- 1 Short Read Eliminator on gDNA
- Safe stop
- 2 Pipette DNA shearing
- Safe stop
- **3 Post-shearing cleanup**
- 4 DNA repair & A-tailing
- 5 Adapter ligation & cleanup
- 6 Nuclease treatment
- Safe stop
- 7 Final library cleanup or size selection

3. Post-shearing cleanup

✔	Step	Instructions post-shear cleanup
	3.1	Add **1.0X** v/v (volume over volume) of resuspended, room-temperature SMRTbell cleanup beads to each tube of sheared DNA. • Automated pipette shearing = 200–300 µL • Megaruptor 3 shearing = 100–130 µL • Megaruptor 3 shearing = 65 µL, skip post-shearing cleanup and proceed to section 4.
	3.2	Pipette-mix the beads until evenly distributed.
	3.3	Quick-spin the tube strip in a microcentrifuge to collect liquid.
	3.4	Incubate at **room temperature** for **10 minutes** to allow DNA to bind beads.
	3.5	Place the tube strip in a magnetic separation rack until beads separate fully from the solution.
	3.6	Slowly remove the cleared supernatant without disturbing the beads. Discard the supernatant.
	3.10	Remove the tube strip from the magnetic rack. **Immediately** add **47 µL** of **low TE buffer** to each tube and resuspend the beads by pipetting 10 times or until evenly distributed.
	3.11	Quick-spin the tube strip in a microcentrifuge to collect liquid.
	3.12	Leave at **room temperature** for **5 minutes** to elute DNA.
	3.13	Place the tube strip in a magnetic separation rack until beads separate fully from the solution.
	3.14	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a **new tube strip**. Discard old tube strip with beads.
	3.15	Recommended: Evaluate sample quality (concentration and size distribution). • Take a **1 µL** aliquot from each tube and dilute with **9 µL** of **elution buffer or water**. • Measure DNA concentration with a Qubit fluorometer using the 1x dsDNA HS kit. • Dilute each aliquot to **250 pg/µL** in Femto Pulse dilution buffer. • Measure DNA size distribution with a Femto Pulse system.
	3.16	Proceed to the next step of the protocol if sample quality is acceptable.
		SAFE STOPPING POINT - Store at 4°C

- ***IMPORTANT!*** Allow SMRTbell cleanup beads to come up to room temperature by bringing them out of 4°C storage at least 30 min prior to use

Note: For **automated** workflows using a liquid handling system to perform steps 1 – 7, the post-shear cleanup step (step 3) should be performed on the **same day** as the library construction steps (steps 4 – 7) to ensure that there is a sufficient volume of SMRTbell cleanup beads to complete the entire library prep workflow[1]

[1] For automated library prep workflows using SPK 3.0 and the Hamilton NGS Microlab Star liquid handling system, the post-shear cleanup step should be performed on the **same day** as the library construction steps (Repair & A-tailing, Adapter ligation, etc.) to ensure that there is a sufficient volume of SMRTbell cleanup beads to complete the entire library prep workflow.

DNA repair & A-tailing

Repair sites of DNA damage and prepare sheared DNA for ligation to SMRTbell adapter

DNA sample extraction

↓

DNA sample QC

1 Short Read Eliminator on gDNA

↓ Safe stop

2 Pipette DNA shearing

↓ Safe stop

3 Post-shearing cleanup

↓

4 DNA repair & A-tailing

↓

5 Adapter ligation & cleanup

↓

6 Nuclease treatment

↓ Safe stop

7 Final library cleanup or size selection

4. DNA repair & A-tailing

✔	Step	Instructions for DNA damage and end repair

4.1 Add the following components in the order and volume listed below to a new microcentrifuge tube. Adjust component volumes for the number of libraries being prepared, plus 15% overage. For individual preps, add components directly to the sample from the previous step at the specified volumes and skip steps (4.2 to 4.4).

Repair mix

Tube	Component	Volume		
		Per library	4 libraries	8 libraries
Purple	Repair buffer	8 µL	36.8 µL	73.6 µL
Blue	End repair mix	4 µL	18.4 µL	36.8 µL
Green	DNA repair mix	2 µL	9.2 µL	18.4 µL
	Total volume	**14 µL**	**64.4 µL**	**128.8 µL**

4.2 Pipette-mix the **Repair mix**.

4.3 Quick-spin the **Repair mix** in a microcentrifuge to collect liquid.

4.4 Add **14 µL** of the **Repair mix** to each sample. Total reaction volume should be **60 µL**.

4.5 Pipette-mix each sample.

4.6 Quick-spin the tube strip in a microcentrifuge to collect liquid.

4.7 Run the Repair and A-tailing thermocycler program. Set the lid temperature to ≥75°C if programmable.

Step	Time	Temperature
1	30 min	37°C
2	5 min	65°C
3	Hold	4°C

4.8 Proceed to the next step of the protocol.

- Prepare a reaction master mix by adding the required components in the order and volume listed to a new microcentrifuge tube[1]
- Adjust component volumes for the number of samples being prepared (e.g., 4, 8, 16 or 24[1]), plus 15% overage

- Run **Repair and A-tailing** thermal cycler program
- Set the lid temperature to ≥75°C if programmable

[1] SMRTbell prep kit 3.0 contains a sufficient volume of reagents to support 24 library prep reactions.

Adapter ligation & cleanup

Ligate SMRTbell adapter to the ends of each DNA fragment

SMRTbell adapter index plate 96A (102-009-200) contains indexes bc2001–bc2096

- DNA sample extraction
- DNA sample QC
- **1** Short Read Eliminator on gDNA
- Safe stop
- **2** Pipette DNA shearing
- Safe stop
- **3** Post-shearing cleanup
- **4** DNA repair & A-tailing
- **5** Adapter ligation & cleanup
- **6** Nuclease treatment
- Safe stop
- **7** Final library cleanup or size selection

5. Adapter ligation & cleanup

✔	Step	Instructions for SMRTbell adapter ligation and reaction cleanup
	5.1	(Optional) If using an adapter index: add **4 µL** of barcoded adapters from the **SMRTbell adapter index plate 96A** to each respective sample from the previous step and exclude the SMRTbell adapter from the ligation mix. Skip this step if not using an adapter index.
	5.2	Add the following components in the order and volume listed below to a new microcentrifuge tube. Adjust component volumes for the number of libraries being prepared, plus 10% overage. For individual preps, add components directly to each sample from the previous step in the order and volume listed below, then skip steps (5.3 to 5.5).

Ligation mix

Tube	Component	Volume		
		Per library	4 libraries	8 libraries
Orange	SMRTbell adapter*	4 µL	17.6 µL	35.2 µL
Yellow	Ligation mix	30 µL	132 µL	264 µL
Red	Ligation enhancer	1 µL	4.4 µL	8.8 µL
	Total volume	**35 µL**	**154 µL**	**308 µL**

5.8 Run the Adapter ligation thermocycler program. Set the lid temperature to ≥30°C if programmable.

Step	Time	Temperature
1	30 min	20°C
2	Hold	4°C

5.9	Add **95 µL** of resuspended, room-temperature SMRTbell cleanup beads to each sample.
5.10	Pipette-mix the beads until evenly distributed.
5.11	Quick-spin the tube strip in a microcentrifuge to collect all liquid from the sides of the tubes.
5.12	Incubate at **room temperature** for **10 minutes** to allow DNA to bind beads.

- **Optional if using an adapter index:** Add 4 µL of indexed adapter (from SMRTbell adapter index plate 96A/B/C/D) to each sample from the previous step.
- Skip this step if not using an adapter index

- Prepare a reaction master mix by adding the required components in the order and volume listed to a new microcentrifuge tube[1]
- Adjust component volumes for the number of samples being prepared (e.g., 4, 8, 16 or 24[1]), plus 10% overage

- Run **Adapter ligation** thermal cycler program
- Set the lid temperature to ≥30°C if programmable

- Perform **1X SMRTbell bead cleanup**
- ***IMPORTANT!*** Allow SMRTbell cleanup beads to come up to room temperature by bringing them out of 4°C storage at least 30 min prior to use

[1] SMRTbell prep kit 3.0 contains a sufficient volume of reagents to support 24 library prep reactions.

Nuclease treatment

Remove unligated DNA fragments and leftover SMRTbell adapters from the sample

DNA sample extraction

↓

DNA sample QC

1 Short Read Eliminator on gDNA

↓ ○ Safe stop

2 Pipette DNA shearing

↓ ○ Safe stop

3 Post-shearing cleanup

↓

4 DNA repair & A-tailing

↓

5 Adapter ligation & cleanup

↓

6 Nuclease treatment

↓ ○ Safe stop

7 Final library cleanup or size selection

6. Nuclease treatment

✔	Step	Instructions for nuclease treatment
	6.1	Add the following components in the order and volume listed below to a new microcentrifuge tube. Adjust component volumes for the number of libraries being prepared, plus 10% overage. For individual preps, add components directly to each sample from the previous step in the order and volume listed below, then skip steps (6.2 to 6.4).

Nuclease mix

✔ Tube	Component	Volume		
		Per library	4 libraries	8 libraries
Light purple	Nuclease buffer	5 µL	22 µL	44 µL
Light green	Nuclease mix	5 µL	22 µL	44 µL
	Total volume	**10 µL**	**44 µL**	**88 µL**

6.2	Pipette-mix **Nuclease master mix.**
6.3	Quick-spin the **Nuclease master mix** in a microcentrifuge to collect liquid.
6.4	Add **10 µL** of **Nuclease master mix** to each sample. Total volume should equal **50 µL**.
6.5	Pipette-mix each sample.
6.6	Quick-spin the tube strip in a microcentrifuge to collect liquid.
6.7	Run the Nuclease treatment thermocycler program. Set the lid temperature to ≥47°C if programmable.

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

	Proceed to the next step of the protocol.
6.8	**Note:** It is necessary to remove the nucleases using either AMPure PB size selection or SMRTbell cleanup beads prior to safely storing the library or libraries.

- Prepare a reaction master mix by adding the required components in the order and volume listed to a new microcentrifuge tube[1]
- Adjust component volumes for the number of samples being prepared (e.g., 4, 8, 16 or 24[1]), plus 10% overage

- Run **Nuclease treatment** thermal cycler program
- Set the lid temperature to ≥47°C if programmable

- **Note:** It is necessary to remove the nucleases using either **AMPure PB size selection *or* SMRTbell cleanup beads** prior to safely storing the library or libraries.

[1] SMRTbell prep kit 3.0 contains a sufficient volume of reagents to support 24 library prep reactions.

Diluted AMPure PB cleanup and size selection

AMPure PB bead size cleanup and selection step will clean the library and deplete DNA fragments <5 kb

DNA sample extraction

↓

DNA sample QC

1 Short Read Eliminator on gDNA

↓ Safe stop

2 Pipette DNA shearing

↓ Safe stop

3 Post-shearing cleanup

↓

4 DNA repair & A-tailing

↓

5 Adapter ligation & cleanup

↓

6 Nuclease treatment

↓ Safe stop

7 Final library cleanup or size selection

7. Diluted AMPure PB cleanup and size selection

✔	Step	Instructions for AMPure PB bead size selection
	7.1	Make a 35% v/v dilution of AMPure PB beads by adding 1.75 mL of resuspended AMPure PB beads to 3.25 mL of elution buffer. The 35% dilution can be stored at 4°C for 30 days. **Note:** The AMPure PB dilution may be scaled as appropriate for smaller/larger scale projects.
	7.2	Add 3.1X v/v (155 µL) of resuspended, room-temperature 35% AMPure PB beads to each sample from the previous step.
	7.3	Pipette-mix the beads until evenly distributed.
	7.4	Leave at **room temperature** for **20 minutes** to allow DNA to bind beads.
	7.5	Place sample on an appropriate magnet and allow beads separate fully from the solution.
	7.6	Slowly pipette off the cleared supernatant without disturbing the beads.
	7.7	Slowly dispense **200 µL**, or enough to cover the beads, of **freshly prepared 80% ethanol** into each sample. After **30 seconds**, pipette off the 80% ethanol and discard.
	7.8	Repeat the previous step.
	7.15	Take a **1 µL** aliquot from each tube and dilute with **9 µL** of **elution buffer or water**. Measure the DNA concentration with a Qubit fluorometer using the 1x dsDNA HS kit. Calculate the total mass. The final overall recovery should be 10–30% as measured from gDNA input mass to completed SMRTbell library (includes SRE, shearing, library prep, and ABC). Recommended: Further dilute each aliquot to **250 pg/µL** with Femto Pulse dilution buffer. Measure final SMRTbell library size distribution with a Femto Pulse system.
		DNA concentration must be less than 60 ng/µL to proceed to ABC. Failure to dilute DNA below 60 ng/µL will result in low P1 loading.
	7.16	Proceed to Section 8 to prepare library for sequencing with Revio +SPRQ or Vega Or Proceed to SMRT Link Sample Setup for preparing samples for Revio non-SPRQ chemistry or Sequel II/e.
		SAFE STOPPING POINT - Store at 4°C for up to 1 month or -20°C for at least 6 months.

- If performing gel-based size selection, skip diluted AMPure PB bead size selection and perform cleanup using 1X SMRTbell cleanup beads instead[1]
- If the DNA library insert size is <10 kb, skip diluted AMPure PB bead size selection and perform cleanup using 1X SMRTbell cleanup beads instead

- Prepare a **35% (v/v) dilution of AMPure PB beads** using elution buffer
  - 35% AMPure PB solution can be stored at **4°C for 30 days**
- **Note:** The AMPure PB dilution procedure may be scaled as appropriate for smaller-/larger-scale projects (each sample requires 155 µL of 35% AMPure PB beads)

- Perform **DNA concentration QC** on final purified SPK 3.0 SMRTbell library using a Qubit dsDNA HS assay

- **Note:** Final SPK 3.0 SMRTbell library concentration must be **<60 ng/ µL** to proceed with annealing, binding & cleanup (ABC)
  - → Using a concentration above 60 ng/µL will result in lower sample loading during sequencing

To prepare SPK 3.0 WGS samples for sequencing using Revio SPRQ or Vega chemistry, follow annealing, binding & cleanup (ABC) workflow instructions in Step 8 in this Procedure & checklist

[1] See Appendix 2 in ***Procedure & checklist – Preparing whole genome libraries using SMRTbell prep kit 3.0*** (102-166-600) for 1X SMRTbell bead cleanup instructions if performing gel-based size selection.

WGS library sequencing preparation workflow details

Sample Setup & Run Design recommendations for SPK 3.0 whole genome sequencing and metagenome libraries – Revio system

Sample setup

Procedure & checklist

Run design

SMRT Link

Recommended target *P1* loading range for Revio system is **~50 – 70%**

Application type

Whole genome sequencing

- **Whole genome sequencing**
  - Human WGS
  - Microbial assembly
  - Other WGS
- **RNA sequencing**
  - Iso-Seq method
  - MAS-Seq single cell
  - Kinnex single-cell RNA
  - Kinnex full-length RNA
- **Viral sequencing**
  - Adeno-associated virus
- **Metagenomics**
  - Kinnex 16S rRNA
  - Full-length 16S rRNA sequencing
  - Shotgun metagenomic profiling or assembly

Metagenomics

- **Whole genome sequencing**
  - Human WGS
  - Microbial assembly
  - Other WGS
- **RNA sequencing**
  - Iso-Seq method
  - MAS-Seq single cell
  - Kinnex single-cell RNA
  - Kinnex full-length RNA
- **Viral sequencing**
  - Adeno-associated virus
- **Metagenomics**
  - Kinnex 16S rRNA
  - Full-length 16S rRNA sequencing
  - Shotgun metagenomic profiling or assembly

HiFi read

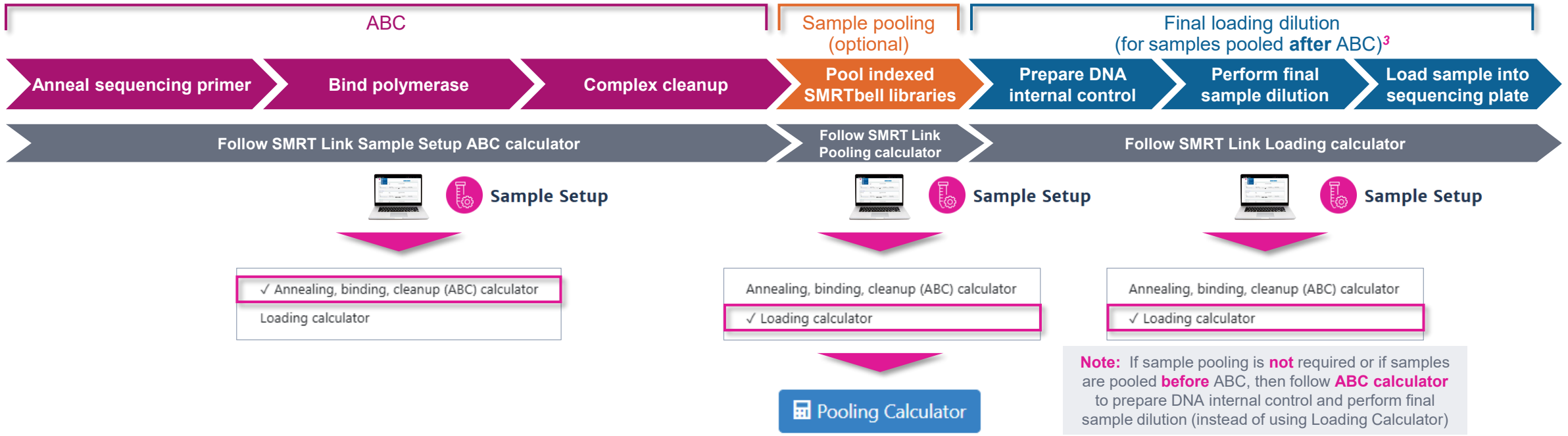
Workflow	Key setup parameters	Revio system recommended settings			
		Human WGS	Microbial assembly	Other WGS	Shotgun metagenomics
Sample setup	Library type	Standard			
	Primer	Standard sequencing primer			
	Polymerase / Binding kit	Revio polymerase kit / Revio SPRQ polymerase kit			
	Concentration on plate	200 – 300 pM			
Run design	Library type	Standard			
	Movie acquisition time	24 hrs (~5 – 20 kb) / 30 hrs (~20 – 25 kb)			
	Use adaptive loading	YES			
	Data options[1]	Include base kinetics = NO Consensus Mode = MOLECULE	Include base kinetics = YES[1] Consensus Mode = MOLECULE	Include base kinetics = NO Consensus Mode = MOLECULE	Include base kinetics = NO Consensus Mode = MOLECULE

[1] **IMPORTANT:** If analysis of 4mC, 6mA or other non-5mC base modifications is desired for **microbial assembly samples**, then need to specify **Include Base Kinetics = YES**.

Sample setup workflow overview for Revio (non-SPRQ) polymerase libraries

For binding libraries with Revio (non-SPRQ) polymerase kit, follow SMRT Link Sample Setup ABC calculator instructions for annealing/binding/complex cleanup steps

Library type[1]	Polymerase kit	Sample setup workflow & procedural reference	
SMRTbell prep kit 3.0 **HiFi plex prep kit 96**	**Revio (non-SPRQ) polymerase kit** (102-817-600)	Anneal sequencing primer, bind polymerase, complex cleanup (ABC)	❑ Follow SMRT Link v13.3+ Sample Setup ABC calculator
		Sample pooling[2] (optional)	❑ Follow SMRT Link v13.3+ Sample Setup Pooling calculator
		Final loading dilution procedure	❑ Follow SMRT Link v13.3+ Sample Setup Loading calculator (if pooling samples **after** ABC)[3]



[1] For binding **HiFi prep kit 96 WGS libraries** with Revio polymerase kit 96, follow HiFi prep kit 96 WGS library prep Procedure & checklist (103-420-700) for sample setup instructions.

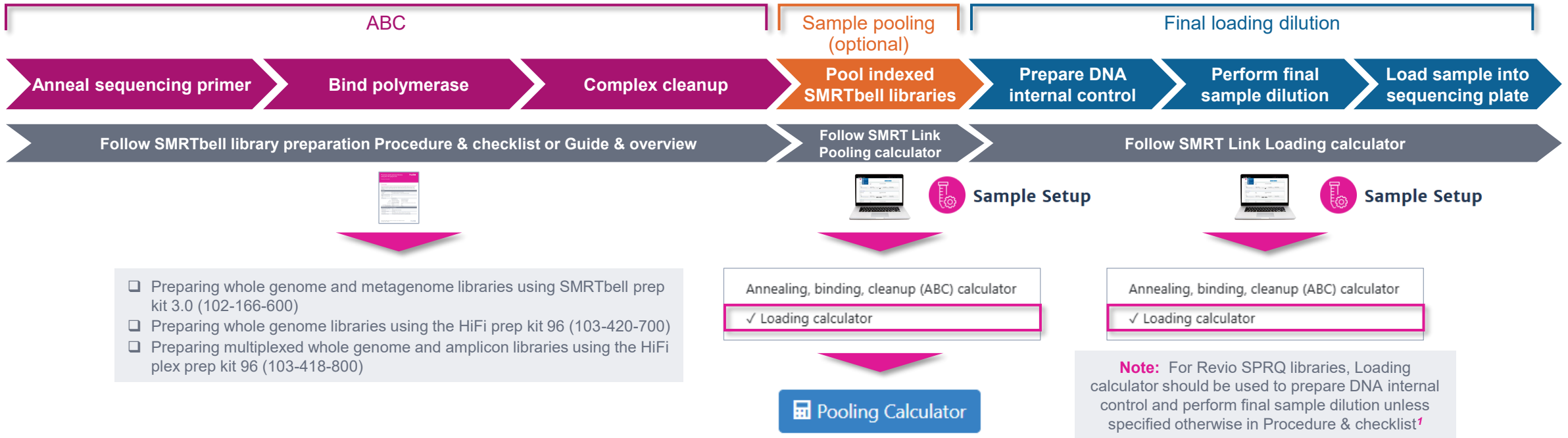
[2] For low-multiplexing applications, it is generally recommended to pool adapter-indexed HiFi libraries post-ABC to prevent any potential inhibitor in one sample from affecting the polymerase binding of all samples in a pool. **Note:** For high-multiplexing applications (e.g., ≥24-plex) using HiFi plex prep kit 96 (or other high-throughput kits), adapter-indexed HiFi libraries will typically be pooled prior to ABC step.

[3] If sample pooling is **not** required or if samples are pooled **before** ABC, then follow **ABC calculator** to prepare DNA internal control and perform final sample dilution (instead of using Loading Calculator).

Sample setup workflow overview for Revio SPRQ polymerase libraries

For binding libraries with Revio SPRQ polymerase kit / Revio SPRQ polymerase kit 96, follow library prep Procedure & checklist instructions for annealing/binding/complex cleanup steps

Library type[1]	Polymerase kit	Sample setup workflow & procedural reference	
SMRTbell prep kit 3.0 **HiFi prep kit 96** **HiFi plex prep kit 96**	**Revio SPRQ polymerase kit** (103-520-100) *or* **Revio SPRQ polymerase kit 96** (for HPK 96 libraries)	Anneal sequencing primer, bind polymerase, complex cleanup (ABC)	❑ Follow library prep Procedure & checklist
		Sample pooling[2] (optional)	❑ Follow SMRT Link v13.3+ Sample Setup Pooling calculator
		Final loading dilution procedure	❑ Follow SMRT Link v13.3+ Sample Setup Loading calculator



[1] If preparing **HiFi prep kit 96 or HiFi plex prep kit libraries using automation**, refer to the appropriate automation Guide & overview documentation for sample setup instructions.

[2] For low-multiplexing applications, it is generally recommended to pool adapter-indexed HiFi libraries post-ABC to prevent any potential inhibitor in one sample from affecting the polymerase binding of all samples in a pool. **Note:** For high-multiplexing applications (e.g., ≥24-plex) using HiFi plex prep kit 96 (or other high-throughput kits), adapter-indexed HiFi libraries will typically be pooled prior to ABC step.

Sample Setup & Run Design recommendations for SPK 3.0 whole genome sequencing and metagenome libraries – Vega system

Sample setup

Procedure & checklist

Run design

SMRT Link

Recommended target loading level range for Vega system is **~50 – 75%**

Application type

Whole genome sequencing

- **Whole genome sequencing**
  - Human WGS
  - Microbial assembly
  - Other WGS
- **RNA sequencing**
  - Iso-Seq method
  - MAS-Seq single cell
  - Kinnex single-cell RNA
  - Kinnex full-length RNA
- **Viral sequencing**
  - Adeno-associated virus
- **Metagenomics**
  - Kinnex 16S rRNA
  - Full-length 16S rRNA sequencing
  - Shotgun metagenomic profiling or assembly

Metagenomics

- **Whole genome sequencing**
  - Human WGS
  - Microbial assembly
  - Other WGS
- **RNA sequencing**
  - Iso-Seq method
  - MAS-Seq single cell
  - Kinnex single-cell RNA
  - Kinnex full-length RNA
- **Viral sequencing**
  - Adeno-associated virus
- **Metagenomics**
  - Kinnex 16S rRNA
  - Full-length 16S rRNA sequencing
  - Shotgun metagenomic profiling or assembly

HiFi read

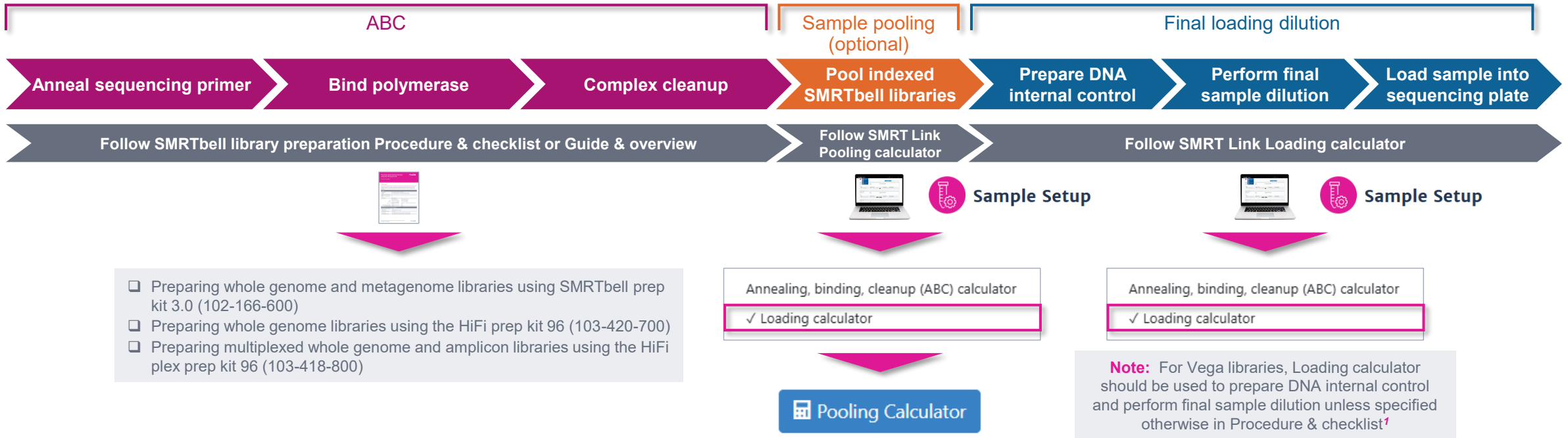
Workflow	Key setup parameters	Vega system recommended settings			
		Human WGS	Microbial assembly	Other WGS	Shotgun metagenomics
Sample setup	Library type	Standard			
	Primer	Standard sequencing primer			
	Polymerase / Binding kit	Vega polymerase kit			
	Concentration on plate	200 – 300 pM			
Run design	Library type	Standard			
	Movie acquisition time	24 hrs (~5 – 20 kb)			
	Use adaptive loading	N/A			
	Data options[1]	Include base kinetics = NO Consensus Mode = MOLECULE	Include base kinetics = YES[1] Consensus Mode = MOLECULE	Include base kinetics = NO Consensus Mode = MOLECULE	Include base kinetics = NO Consensus Mode = MOLECULE

[1] **IMPORTANT:** If analysis of 4mC, 6mA or other non-5mC base modifications is desired for **microbial assembly samples**, then need to specify **Include Base Kinetics = YES**.

Sample setup workflow overview for Vega polymerase libraries

For binding libraries with Vega polymerase kit, follow library prep Procedure & checklist instructions for annealing/binding/complex cleanup steps

Library type[1]	Polymerase kit	Sample setup workflow & procedural reference	
SMRTbell prep kit 3.0 **HiFi prep kit 96** **HiFi plex prep kit 96**	**Vega polymerase kit** (103-520-100)	Anneal sequencing primer, bind polymerase, complex cleanup (ABC)	❑ Follow library prep Procedure & checklist
		Sample pooling[2] (optional)	❑ Follow SMRT Link v13.3+ Sample Setup Pooling calculator
		Final loading dilution procedure	❑ Follow SMRT Link v13.3+ Sample Setup Loading calculator



[1] If preparing **HiFi prep kit 96 or HiFi plex prep kit libraries using automation**, refer to the appropriate automation Guide & overview documentation for sample setup instructions.

[2] For low-multiplexing applications, it is generally recommended to pool adapter-indexed HiFi libraries post-ABC to prevent any potential inhibitor in one sample from affecting the polymerase binding of all samples in a pool. **Note:** For high-multiplexing applications (e.g., ≥24-plex) using HiFi plex prep kit 96 (or other high-throughput kits), adapter-indexed HiFi libraries will typically be pooled prior to ABC step.

General best practices recommendations for preparing SPK 3.0 WGS libraries for sequencing on Revio and Vega systems

Polymerase kit thawing procedure[1]

Revio polymerase kit / Revio SPRQ polymerase kit / Vega polymerase kit

Revio SPRQ polymerase kit
(103-520-100)

Vega polymerase kit
(103-517-600)

Thaw these reagents at room temperature		Keep these reagents on a cold block or on ice		Bring these reagents to room temperature 30 minutes prior to use	
	Annealing buffer		Sequencing polymerase		Loading buffer
	Standard sequencing primer		Sequencing control		SMRTbell cleanup beads
	Polymerase buffer				
	Loading buffer				
	Dilution buffer				

- Once thawed, reaction buffers and sequencing primer may be stored on a cold block, at 4°C, or on-ice prior to making master mix or placing on the liquid handler work deck
- Loading buffer should be left at room-temperature
- **Note:** Loading buffer is light sensitive and should be protected from light when not in use

[1] Refer to *Procedure & checklist* protocol for detailed instructions for handling all required sequencing preparation reagents.

PacBio

WGS data analysis recommendations for *de novo* assembly, variant detection and shotgun metagenomics applications

HiFi WGS data analysis recommendations for *de novo* assembly

Using HiFi reads for *de novo* assembly analysis of genomes

- **15-fold HiFi read coverage per haplotype** is recommended for most human/plant/animal WGS *de novo* assembly projects

  → $Target\ HiFi\ Base\ Yield = [Haploid\ Genome\ Size\ (Gb)]\ x\ [Ploidy\ Level]\ x\ [Target\ HiFi\ Coverage\ per\ Haplotype]$

  E.g., for *de novo* assembly analysis of a 3 Gb diploid genome:

  Recommended minimum target HiFi base yield = 3 Gb x 2 x 15 = 90 Gb

- Can use third-party software (e.g., Hifiasm) for *de novo* assembly analysis using HiFi reads[1]

Megabase-sized contigs assembled into a nearly complete human *chromosome one*

[1] Contact PacBio Technical Support (support@pacb.com) or your local Field Applications Bioinformatics Support Scientist for additional information about data analysis recommendations.

HiFi WGS data analysis recommendations for variant detection

Using HiFi reads for variant detection analysis of genomes

- For detection of structural variants, we recommend **10-fold HiFi read coverage per sample**

  → $Target\ HiFi\ Base\ Yield = [Sample\ Haploid\ Genome\ Size\ (Gb)]\ x\ [Target\ Coverage\ per\ Sample]$

  E.g., For structural variant detection analysis of a human genome (3 Gb):

  Recommended minimum target HiFi base yield = 3 Gb x 10 = 30 Gb

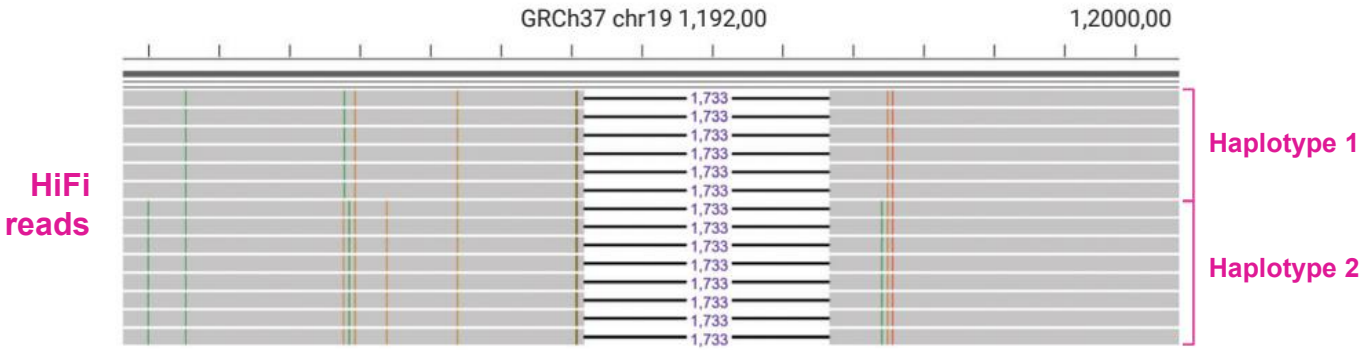
- For detection of all variant classes, we recommend **20-fold HiFi read coverage per sample**

  → $Target\ HiFi\ Base\ Yield = [Sample\ Haploid\ Genome\ Size\ (Gb)]\ x\ [Target\ Coverage\ per\ Sample]$

  E.g., For detection of all variant classes in a human genome (3 Gb):

  Recommended minimum target HiFi base yield = 3 Gb x 20 = 60 Gb

- Recommend using sawfish GitHub tool (available through command line interface) for structural variant calling applications
- Also compatible with SMRT Link Variant Calling analysis application (powered by Google DeepVariant & PacBio pbsv) for detection of small variants (SNVs, InDels)[1]



HiFi reads phased into haplotypes identified a homozygous insertion event and several single nucleotide variants

[1] Contact PacBio Technical Support (support@pacb.com) or your local Field Applications Bioinformatics Support Scientist for additional information about data analysis recommendations.

HiFi WGS data analysis recommendations for human genomics applications

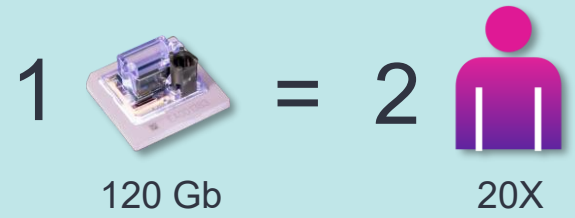
Revio SPRQ chemistry enables 2 human genomes to be sequenced to 20X coverage per Revio SMRT Cell[1]

20× Human WGS

High accuracy SNV, SV calling performance at lower cost per genome; higher throughput + 5mC

- Population genomics (saliva samples)
- Disease cohort studies
- Screening, polygenic risk scores
- Complex disease profiling
- Clinical research

With Revio system SPRQ chemistry



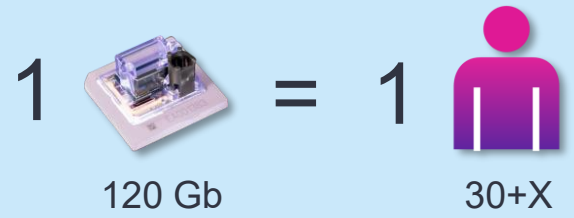
1,250 SMRT Cells/yr → 2,500 samples/yr[2]

30+× Human WGS

Improved power to detect and discover novel variants, now with lower input DNA requirements

- Rare disease studies / NICU (blood samples)
- Reference-grade *de novo* assembled genomes
- *De novo* mutation detection
- Population reference genomes
- Genome benchmarking
- Tumor sequencing

With Revio system SPRQ chemistry



1,250 SMRT Cells/yr → 1,250 samples/yr[2]

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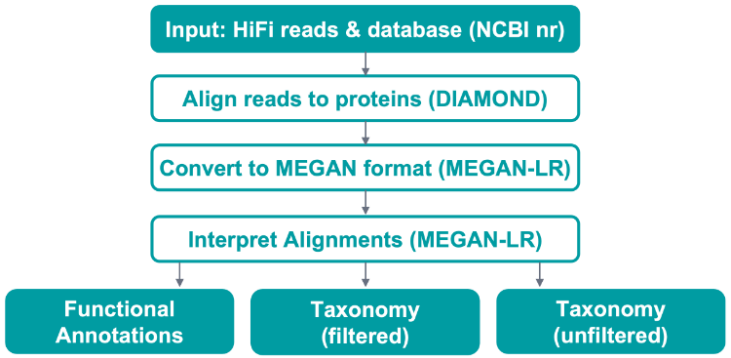
1 Coverage may vary based on sample quality, library quality, and fragment lengths.
2 Annual throughput is estimated and based on 1,250 Revio SMRT Cells for 24 hr runs.

HiFi WGS data analysis recommendations for shotgun metagenomics applications

HiFi reads are compatible with third-party metagenomics data analysis tools for taxonomic & functional profiling

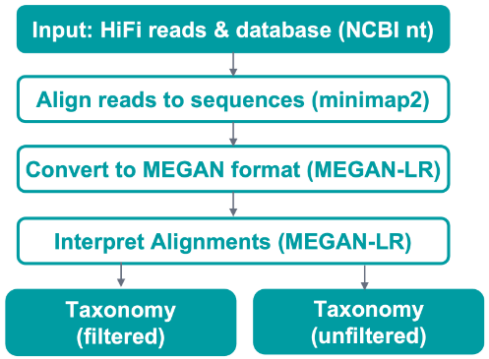
- Use SMRT Link to output HiFi data in standard file formats (BAM and FASTA/Q) for seamless integration with downstream analysis tools
- Can use PacBio metagenomics tools available on GitHub for taxonomic classification and functional gene profiling using HiFi reads[1]

Taxonomic-Profiling-Diamond-Megan



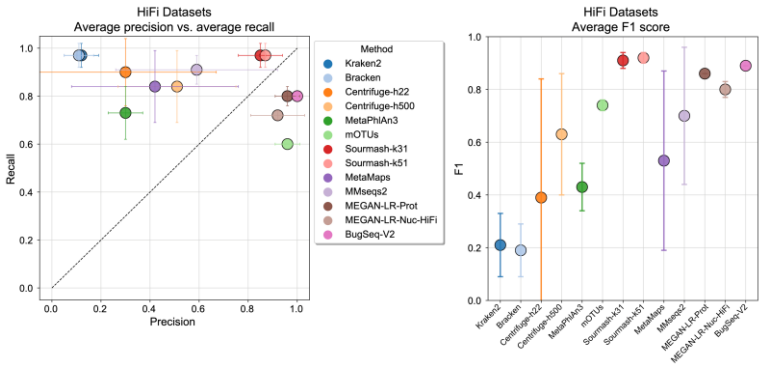
- Perform translation alignment of HiFi reads to a protein database using DIAMOND and summarize with MEGAN-LR, for the purpose of taxonomic and functional profiling.
- Provides access to NCBI and GTDB taxonomic annotations

Taxonomic-Profiling-Minimap-Megan



- Align HiFi reads to a nucleotide database using minimap2 and summarize with MEGAN-LR, for the purpose of taxonomic profiling
- Provides access to NCBI and GTDB taxonomic annotations

Taxonomic-Profiling-Sourmash



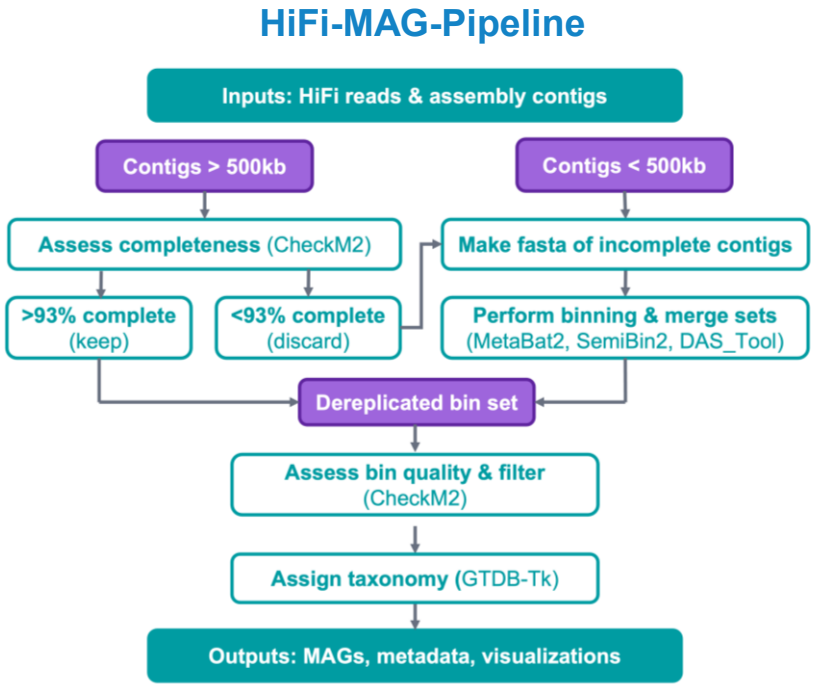
- obtain taxonomic profiles using `sourmash gather --> taxonomy` approach.
- Provides access to NCBI and GTDB taxonomic annotations, or you can build your own database.

[1] Refer to https://github.com/PacificBiosciences/pb-metagenomics-tools for details about specific PB-metagenomics-tools pipelines.

HiFi WGS data analysis recommendations for shotgun metagenomics applications (cont.)

Use HiFi-MAG-Pipeline to obtain high-quality metagenome-assembled genomes (MAGs)

- Can perform metagenomic shotgun assembly directly with HiFi reads using third-party tools (e.g., hifiasm-meta, metaFlye or metaDBG) and evaluate & extract metagenome-assembled genomes using PacBio HiFi-MAG-Pipeline tool available on GitHub (see Portik *et al.*[1])



- Streamlined HiFi-MAG-Pipeline workflow includes a custom "completeness-aware" strategy to identify and protect long & complete contigs
- Binning is performed with MetaBAT2 and SemiBin2; bin merging occurs with DAS_Tool, QC with CheckM2; and taxonomic assignments with GTDB-Tk
- Outputs include high-quality MAG sequences, summary figures, and associated metadata

- Contact PacBio Technical Support (support@pacb.com) or your local Field Applications Bioinformatics Support Scientist for additional information about data analysis recommendations

[1] Portik D.M. et al. (2022) Evaluation of taxonomic profiling methods for long-read shotgun metagenomic sequencing datasets. BMC Bioinformatics. 23:541. https://doi.org/10.1186/s12859-022-05103-0

How many shotgun metagenomic WGS samples can be multiplexed on a single SMRT Cell?

The overall goals of your project will determine the needed coverage depth

General guidance for multiplexing shotgun metagenomic samples[1]

- **Shotgun metagenomic profiling applications:**
  - Multiplex up to 128 samples per Revio SMRT Cell (+SPRQ chemistry), up to 64 samples per Vega SMRT Cell or up to 48 samples per Sequel II SMRT Cell
- **Shotgun metagenomic assembly applications:**
  - Multiplex up to 16 samples per Revio SMRT Cell (+SPRQ chemistry), up 8 samples per Vega SMRT Cell or up to 4 samples per Sequel II SMRT Cell

Project-specific guidance for multiplexing shotgun metagenomic samples

Question 1: ***What is the estimated abundance of the rarest species you want to observe?***

Example: “I want to see species present at 1% abundance."

→ With 1 Sequel II SMRT Cell, you can expect ~24,000 HiFi (≥Q20) reads from a 1% abundant species with an ‘average’ genome size

→ With 1 Revio SMRT Cell, you can expect ~72,000 HiFi (≥Q20) reads from a 1% abundant species with an ‘average’ genome size

Question 2: ***What is your goal?***

In order to achieve…	…You need
Species detection	~100 HiFi reads
Comprehensive gene profiling / discovery[1]	5-Fold coverage; ~3,000 HiFi reads
Complete genome assembly[1]	20-Fold coverage; ~12,000 HiFi reads

[1] See ***Application brief – Metagenomic sequencing with HiFi reads – Best practices*** (102-193-684) for the most up-to-date guidance on multiplexing options and library preparation recommendations.

[2] # Reads Needed = Coverage x 5 Mb Genome / 8.5 kb Median HiFi Read Length. **Note:** Metagenomic samples often have degraded gDNA where the majority of fragments are already <15 kb in length to start. As a result, the final library insert size for metagenomic samples may be <15 kb – and consequently, the mean HiFi read length may be <15 kb.

How many shotgun metagenomic WGS samples can be multiplexed on a single SMRT Cell? (cont.)

Example calculation of estimated coverage levels achievable for rare species at different multiplex levels

	1 Sample / SMRT Cell 8M	1 Sample / Revio SMRT Cell	2 Samples / SMRT Cell 8M	2 Samples / Revio SMRT Cell	3 Samples / SMRT Cell 8M	3 Samples / Revio SMRT Cell
Assignable HiFi (≥Q20) reads per SMRT Cell[1,2]	2.4 M	7.2 M	2.4 M	7.2 M	2.4 M	7.2 M
HiFi reads per sample	2.4 M	7.2 M	1.2 M	3.6 M	800,000	2.4 M
1% of Reads	24,000 → **assembly**	72,000 → **assembly**	12,000 → **assembly**	36,000 → **assembly**	8,000 → **profiling**	24,000 → **assembly**
0.2% of Reads	4,800 → **profiling**	14,000 → **assembly**	2,400 → **detection**	7,200 → **profiling**	1,600 → **detection**	4,800 → **profiling**

[1] Assignable number of HiFi reads per SMRT Cell can vary depending on input genomic DNA sample quality. Typically, ≥99.5% of HiFi reads have recoverable barcodes (for 7 – 10 kb library insert size).
[2] Example assignable number of HiFi reads per Revio SMRT Cell shown in table corresponds to expected performance for a ~8 – 9 kb library insert sequenced using Revio SPRQ chemistry.

- Average HiFi read length for metagenomics samples is typically ~8 – 9 kb or longer (depending on input gDNA quality)[3] when following our recommended SMRTbell library preparation procedure[4] with samples meeting the minimum DNA input quality requirements
- Choose your multiplex level depending on how many reads per rarest-OTU of interest you require for your metagenomic analysis plan

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[3] **Note:** For metagenomic DNA samples, DNA shearing step may be skipped if DNA sizing QC analysis of input gDNA shows that the starting average fragment size is <18 kb.
[4] Refer to ***Procedure & checklist – Preparing whole genome and metagenome sequencing libraries using SMRTbell prep kit 3.0*** (102-166-600) and ***Application brief – Metagenomic sequencing with HiFi reads – Best practices*** (102-193-684) for the most up-to-date guidance on multiplexing options and library preparation recommendations for metagenomic DNA samples.

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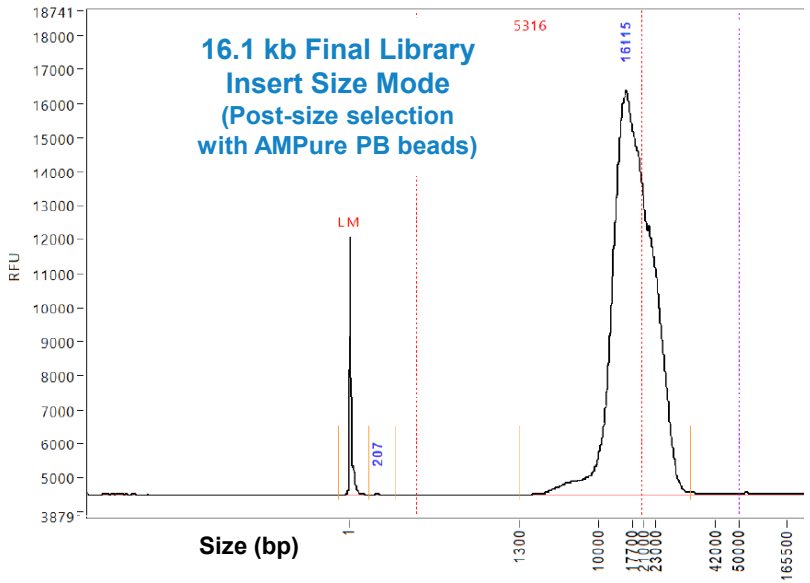
WGS library example sequencing performance data

Example library QC results for human WGS libraries prepared with SMRTbell prep kit 3.0

SMRTbell library DNA sizing and library construction yield QC metrics

Femto Pulse DNA sizing QC electropherogram



Ideally aim for **15 – 20 kb target library size** mode for HiFi whole genome sequencing applications

Input gDNA for SRE	500 ng	2000 ng
Post-SRE recovery (%)[1]	400 ng (80%)	1,500 ng (75%)
Post-shearing recovery (%)[2]	300 ng (60%)	1,120 ng (56%)
Post-AMPure PB bead size-selection (%)[3]	115 ng (23%)	400 ng (20%)
Post-ABC cleanup (%)[4]	95 ng (19%)	300 ng (15%)

1 Post-SRE gDNA size selection yields typically ranged from ~65% to ~95% for human gDNA samples.
2 Post-shearing cleanup recoveries typically ranged from ~70% to 80% when using a Hamilton automation system or Megaruptor 3 system or g-TUBE device to shear human gDNA samples.
3 Post-AMPure PB bead size selected library yields typically ranged from ~13% to ~34% for human gDNA samples.
4 Post-size selected library yields typically ranged from ~10% to ~32% for human gDNA samples.

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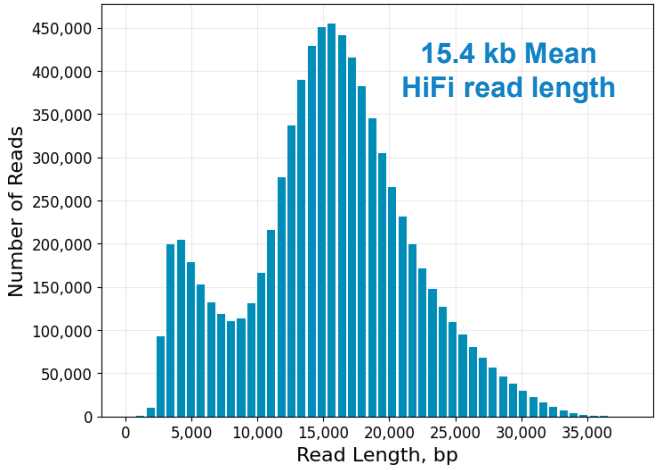
Revio SPRQ whole genome sequencing performance

Human WGS

Example human WGS variant & methylation calling performance data for HG002 using 500 ng of input DNA

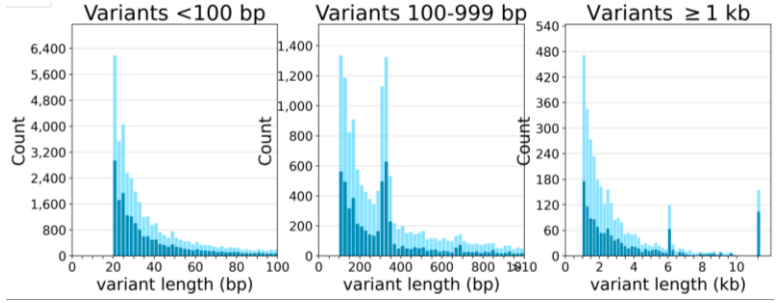
Sequencing metrics

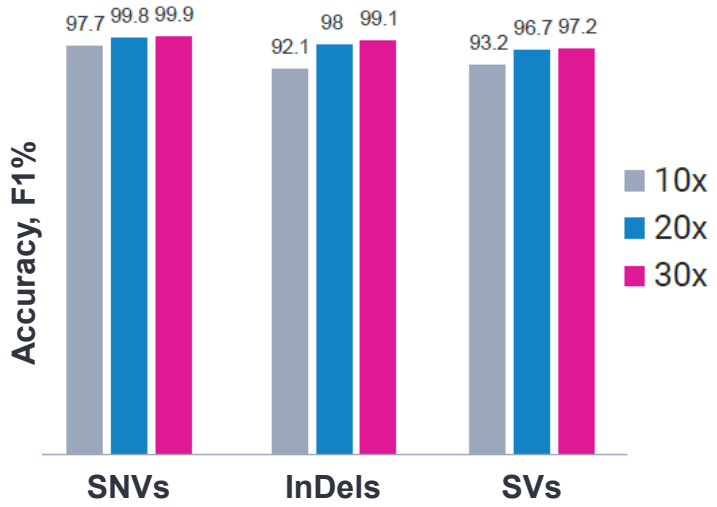


HiFi Reads	8.4 M
HiFi Base Yield	119.7 Gb
Mean HiFi Read Length	15.4 kb
Median HiFi Read Quality	Q33
HiFi Read Mean # of Passes	10

For human HG002 WGS libraries run with Revio SPRQ chemistry, per-SMRT Cell HiFi read counts were typically >7 Million depending on final library insert size and *P1* loading[1]

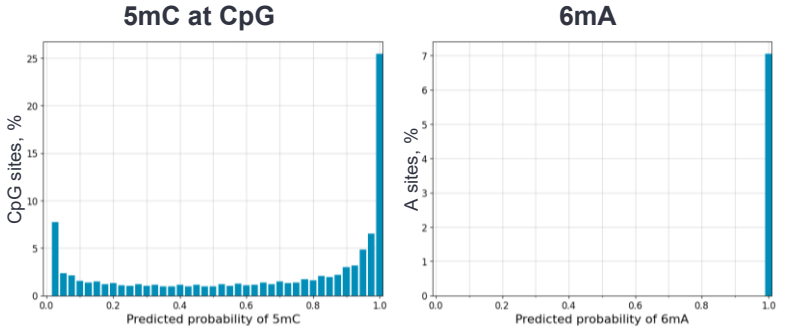
Variant calling performance[2]



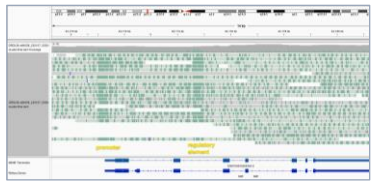


Methylation calling performance

Modificati...	Motif	Scored sites	Modified sites (Pr > 0.5)
5mC	CpG	97.5%	62.3%
6mA	A	7.0%	7.0%



	5mC (CpG)
Sensitivity	93%
Specificity	93%



5mC data show a strongly bimodal profile indicating increased confidence of true positive and true negative 5mC calls

Revio on-instrument calling has high accuracy for 6mA, equivalent to Fibertools for Fiber-seq assays.[3]

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1 Example sequencing metrics shown for human HG002 sample that was loaded at 300 pM OPLC and achieved *P1* = 69% using 500 ng of input gDNA.
2 F1% accuracy score data shown are for a single Revio SMRT Cell for HG002/GM24385 sequenced with Revio SPRQ chemistry.
3 Revio on-instrument 6mA caller has > 90% sensitivity for 6mA in Fiber-seq and < 5 false positive calls per 1,000 A bases in reads.

Technical documentation & applications support resources

Technical resources for WGS library preparation, sequencing & data analysis

DNA sample extraction literature & other resources

- Application note – High-molecular-weight DNA extraction from human blood and saliva using Nanobind kits for HiFi long-read sequencing (102-326-656)
- Nanobind HMW DNA extraction Procedures & checklists [Link]
- Nanobind kit Guides & overviews [Link]
- Overview – Nanobind CBB HMW DNA extraction protocols (103-515-700)
- Overview – Nanobind HT HMW DNA extraction robotic procedures (103-032-000)
- Overview – Nanobind PanDNA HMW DNA extraction protocols (103-510-000)
- Technical note – Preparing DNA for PacBio HiFi sequencing – Extraction and quality control (102-193-651)
- Technical note – Sample preparation for PacBio HiFi sequencing from human whole blood (102-326-500)
- Technical overview – Automated high-throughput HMW DNA extraction for PacBio long-read sequencing using Nanobind HT kits (103-401-700)
- Technical overview – HMW DNA sample preparation for PacBio long-read sequencing using Nanobind PanDNA and SRE kits (103-401-100)

DNA shearing literature & other resources

- Guide & overview – Short Read Eliminator (SRE), DNA shearing, and cleanup for the Hamilton Microlab Prep system (103-424-100)
- Technical note – High-throughput DNA shearing for HiFi whole genome sequencing from whole blood samples [MP Biomedicals FastPrep-96] (102-326-579)
- Technical note – High-throughput DNA shearing for long-read microbial WGS [SPEX SamplePrep 1600 MiniG] (102-326-575)
- Technical note – High throughput DNA shearing using Hamilton Microlab Prep (102-326-606)

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

SMRTbell library preparation literature & other resources

- Application brief – Comprehensive human genomic variant detection with HiFi long-read sequencing (102-326-626)
- Application brief – Metagenomic sequencing with HiFi reads (102-193-684)
- Application brief – Microbial genomics at scale with PacBio HiFi sequencing (102-326-620)
- Application brief – Microbial whole genome sequencing (102-193-601)
- Application brief – Taxonomic and functional profiling with HiFi metagenomics (102-326-574)
- Brochure – Human genomics (102-326-617)
- Brochure – Metagenomics solutions guide (102-326-512)
- Overview – HiFi application options (101-851-300)
- Procedure & checklist – Preparing whole genome and metagenome sequencing libraries using SMRTbell prep kit 3.0 (102-166-600)
- Technical note – Gel cassette size selection methods for WGS HiFi libraries (102-326-503)
- Technical overview – Whole genome and metagenome library preparation using SMRTbell prep kit 3.0 (102-390-900)

Third-party automation instrumentation literature for SMRTbell prep kit 3.0 applications[1]

- Application note – Automation of Long-Read Sequencing Library Preparation with PacBio SMRTbell prep kit 3.0 on Hamilton NGS STAR MOA (Hamilton AN-2305-05)
- Technical note – Automated extraction of High Molecular Weight (HMW) DNA with PacBio Nanobind technology on the Hamilton NIMBUS Presto Assay Ready Workstation (Hamilton AN-2205-05)
- Technical note – Automated Isolation of High Molecular Weight (HMW) DNA from Human Blood Samples with PacBio Nanobind Technology on the Hamilton NIMBUS Presto – Next Level Preparation of Extracts for Long-Read Sequencing (Hamilton AN-2212-03)
- Technical note – DreamPrep NGS and DreamPrep NGS Compact: automated DNA shearing (Tecan 403009)
- Technical note – DreamPrep NGS Compact and PacBio SMRTbell prep kit 3.0 (Tecan 402700)

[1] For third-party automation instrumentation literature references for HiFi prep kit 96 applications, see ***Technical overview – HiFi library preparation using HiFi prep kits for high-throughput sequencing on PacBio long-read systems*** (103-424-600).

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

Publications

- Healey, A.D. et al. (2024) The complex polyploid genome architecture of sugarcane. Nature volume 628, pages 804–810 doi: https://doi.org/10.1038/s41586-024-07231-4
- LeMaster, C. et al. (2024) Mapping structural variants to rare disease genes using long-read whole genome sequencing and trait-relevant polygenic scores. MedRxiv preprint. doi: https://doi.org/10.1101/2024.03.15.24304216
- Mahmoud, M. et al. (2024) Utility of long-read sequencing for All of Us. Nature Communications. 15, Article number:837 doi: https://doi.org/10.1038/s41467-024-44804-3
- Harvey, W.T. et al. (2023) Whole-genome long-read sequencing downsampling and its effect on variant calling precision and recall. Genome Research 33:2029-2040. doi: 10.1101/gr.278070.123
- Cheung, W.A. et al. (2023) Direct haplotype-resolved 5-base HiFi sequencing for genome-wide profiling of hypermethylation outliers in a rare disease cohort. MedRxiv preprint. doi: https://doi.org/10.1101/2022.09.12.22279739
- Nurk S. et al. (2022) The complete sequence of a human genome. Science. 376:44-53. doi: 10.1126/science.abj6987
- Noyes, D.N. et al. (2022) Familial long-read sequencing increases yield of de novo mutations. American journal of human genetics. 109:631-646. doi: https://doi.org/10.1016/j.ajhg.2022.02.014

Webinars

- PacBio Webinar (2024) – Disentangling the complexities of soil communities with highly accurate long-read metagenome assembly [Link]
- PacBio Webinar (2024) – Comprehensive variant detection in pediatric leukemia research with accurate long-read whole genome sequencing [Link]
- PacBio Webinar (2024) – Bioinformatics resources to analyze PacBio HiFi human genomes [Link]
- PacBio Webinar (2023) – Metagenome assembly and characterization of a pooled human fecal reference [Link]
- PacBio Webinar (2022) – Genome and epigenome measured in a single sequencing run [Link]

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

Example PacBio WGS data sets

Whole genome sequencing	Dataset	Data type	PacBio system
Variant detection, assembly, epigenetics	GIAB – HG002	HiFi long read	Vega system
Whole genome sequencing	Various plant & animals – California flannelbush, widow rockfish, and sea otter	HiFi long read	Vega system
Variant detection, assembly, epigenetics	Homo sapiens – GIAB trio HG002-4	HiFi long read	Revio system – SPRQ chemistry
Tumor/normal	Homo sapiens – GIAB HG008 pancreatic	HiFi long read	Revio system – SPRQ chemistry & Vega system
Tumor/normal	COLO829 melanoma	HiFi long read	Revio system – SPRQ chemistry
Fiber-Seq chromatin assay	Homo sapiens – HG002	HiFi long read	Revio system – SPRQ chemistry
Variant detection, assembly, epigenetics	Homo sapiens — GIAB trio HG002-4	HiFi long read	Revio system
Tumor/normal	COLO829 melanoma	HiFi long read	Revio system
Tumor/normal	HCC1395	HiFi long read	Revio system
Whole genome sequencing	Various plant & animals – mouse, ladybug, oak, mistletoe, and maize	HiFi long read	Revio system
Assembly (ultra-low DNA input)	Phlebotomus papatasi, Homo sapiens, Drosophila melanogaster	HiFi long read	Sequel II system
Assembly	Food safety & infectious microbes – 96 plex	HiFi long read	Sequel IIe system

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

Example PacBio microbiome and metagenomics data sets

Metagenomic profiling & assembly	Dataset	Data type	PacBio system
Metagenomic profiling and assembly	ZymoBIOMICS Fecal Reference with TruMatrix Technology (human)	HiFi long read	Revio system
Metagenomic profiling and assembly	ZymoBIOMICS Fecal Reference with TruMatrix Technology (human)	HiFi long read	Sequel IIe system
Metagenomic profiling and assembly	20 strain mock microbial community – ATCC MSA-1003 – shotgun	HiFi long read	Sequel II system
Metagenomic profiling and assembly	Human gut microbiome pooled standards	HiFi long read	Sequel IIe system

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

Data analysis resources

- Application brief – Comprehensive human genomic variant detection with HiFi long-read sequencing (102-326-626)
- Application brief – Taxonomic and functional profiling with HiFi metagenomics (102-326-574)
- Application note – Consolidated analysis tools with the PacBio WGS Variant Pipeline (102-326-588)
- Application brief – HiFi sequencing enables greater accuracy of somatic variant calling (102-326-598)
- Application note – Robust detection of somatic variants from tumor-normal samples with highly accurate long-read whole genome sequencing (102-326-582)
- SMRT Link Cloud v25.3 user guide (103-720-300)
- SMRT Link v25.3 user guide (103-720-100)
- SMRT Link web services API use cases (103-653-100)
- SMRT Tools reference guide (103-653-200)

Revio system v13.3 key specifications

Revio system v13.3 data throughput

Library	Run time[1]	Q30+ bases	HiFi yield per SMRT Cell[2]	Methylation
0.5–5 kb	12 hours	95%	6 – 8 Million reads	5mC at CpG sites and 6mA for native DNA
5–10 kb	24 hours	90%	35 – 70 Gb	
10–15 kb			70 – 100 Gb	
15–20 kb			100 – 120 Gb	
20–25 kb	30 hours	85%	100 – 120 Gb	

1 Run time refers to the data collection step, which determines the time between processing SMRT Cells.

2 HiFi yield is dependent on library quality and sequencing preparation procedures. Specified yield is based on high-quality samples prepared following best practices.

Revio system v13.3 key applications and sample throughput

Library	Sample	Expected coverage[3]	Samples per Revio SMRT Cell	Samples per year[4]
0.5–5 kb	Amplicon	50×	>1,000	>2.5M
5–10 kb	Microbial genome	50×	384	480,000
15–20 kb	PureTarget repeat expansion panel	200×	48	60,000
15–20 kb	Human genome	20×	2	2,500
15–20 kb	Human methylation profiling	5×	8	10,000
15–20 kb	Transcriptome with Kinnex full-length RNA kit	10M reads	6	7,500

3 Expected coverages are estimates.

4 Annual throughput is estimated and based on 2,500 Revio SMRT Cells for 12 hour runs; 1,250 Revio SMRT Cells for 24 hr runs; and 1,050 for 30 hour runs.

Revio system ICS v13.3

See ***Revio system specification sheet*** (102-326-552) for latest Revio system performance specifications.

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Vega system v1.1 key specifications

Vega system v1.1 data throughput

Library	Run time[1]	Q30+ bases	HiFi yield per SMRT Cell[2]	Methylation
0.5–5 kb	12 hours	95%	6 – 8 Million reads	5mC at CpG sites and 6mA for native DNA
5–10 kb	24 hours	90%	25 – 50 Gb	
10–15 kb			50 – 60 Gb	
15–20 kb			50 – 60 Gb	

1 Run time refers to the data collection step, which determines the time between processing SMRT Cells.

2 HiFi yield is dependent on library quality and sequencing preparation procedures. Specified yield is based on high-quality samples prepared following best practices.

Vega system ICS v1.0

Vega system v1.1 key applications and sample throughput

Library	Sample	Expected coverage[3]	Samples per Vega SMRT Cell	Samples per year[4]
0.5–5 kb	Amplicon	50×	>1,000	200,000
5–10 kb	Microbial genome	30×	384	75,000
5 -10 kb	PureTarget repeat expansion panel	200×	48	9,600
15–20 kb	Human genome	20×	1	200
15–20 kb	Human Methylation profiling	5×	4	800
15–20 kb	Transcriptome with Kinnex full-length RNA kit	10 million reads	3	600

3 Expected coverages are estimates.

4 Annual throughput is estimated and based on 200 Vega SMRT Cells.

See ***Vega system specification sheet*** (102-326-625) for the latest Vega system performance specifications.

Vega system v1.1 vs. Revio system v13.3 sample throughput comparison

See ***What can you do with one SMRT Cell*** (102-326-578)

Vega system

Revio system with SPRQ chemistry

Application	Samples per run		
	1 SMRT Cell	1 SMRT Cell	4 SMRT Cells
Whole genome sequencing			
Human genome (20× coverage)	1	2	8
Human methylation profiling (5× coverage)	4	8	32
De novo assembly (1 Gb genome)	2	4	16
Microbial de novo assembly (2 Gb total sum of genomes)	384	384	1,536
Targeted panels			
Amplicon sequencing	>1,000	>1,000	>1,000
Target enrichment			
20 Mb panel	12	16	64
2 Mb panel	72	96	384
100 kb panel	288	384	1,536
PureTarget repeat expansions panel	48	48	192
RNA sequencing			
Kinnex single-cell RNA sequencing	1 (3,000 – 6,000 cells)	1 (6,000 – 10,000 cells)	4 (6,000 – 10,000 cells)
Kinnex full-length RNA sequencing			
5M reads	6	12	48
10M reads	3	6	24
Microbial			
Shotgun metagenomic profiling	64 communities	128 communities	512 communities
Shotgun metagenomic assembly	8 communities	16 communities	64 communities
Kinnex 16S rRNA	1,024 communities	1,536 communities	6,144 communities

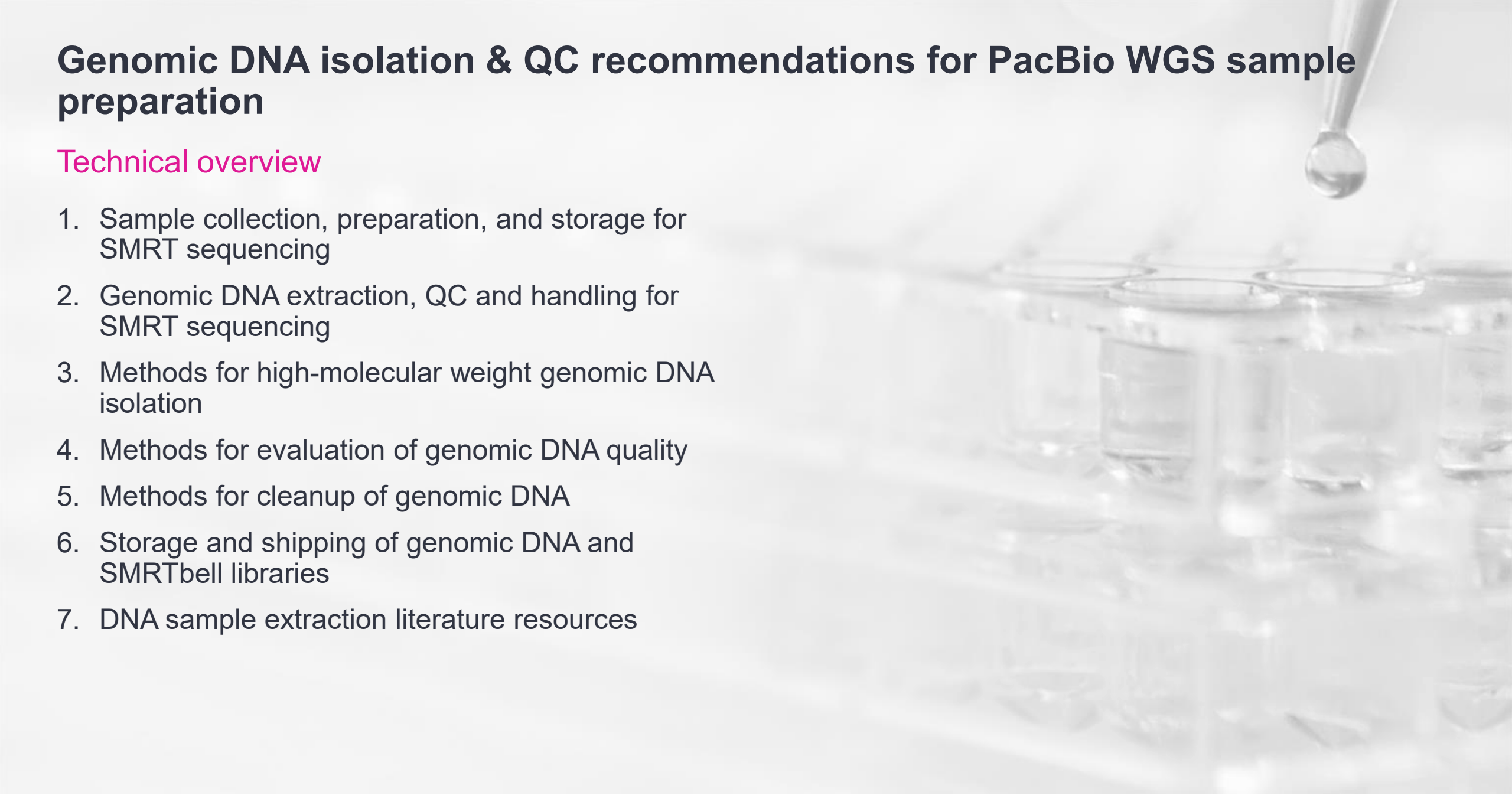
All sample throughputs are estimates for either the Vega system with 1 SMRT Cell or the Revio system using SPRQ chemistry with both 1 or 4 SMRT Cells. Coverage may vary based on sample quality, library quality, and fragment lengths. Currently available SMRTbell® adapter index plates 96A-96D contain a total of 384 SMRTbell barcoded adapters. Microbial de novo assembly assumes microbes with 2 Gb of total genome size at 30x per sample. Single-cell transcriptomics assumes ≥80 million reads per library on the Revio system and ~50-60 million reads per library on the Vega system. Full-length RNA sequencing assumes a total of 60M reads for Revio SPRQ and 30M reads for Vega, regardless of plexity. Amplicon sequencing assumes a 12-hour movie time for 1–5 kb, 24-hour movie time for 5+ kb, and >50× per sample. Target enrichment assumes >50× per sample

APPENDIX 1: Genomic DNA isolation & QC recommendations for PacBio WGS sample preparation

Genomic DNA isolation & QC recommendations for PacBio WGS sample preparation

Technical overview

1. Sample collection, preparation, and storage for SMRT sequencing
2. Genomic DNA extraction, QC and handling for SMRT sequencing
3. Methods for high-molecular weight genomic DNA isolation
4. Methods for evaluation of genomic DNA quality
5. Methods for cleanup of genomic DNA
6. Storage and shipping of genomic DNA and SMRTbell libraries
7. DNA sample extraction literature resources



Sample collection, preparation, and storage for SMRT sequencing

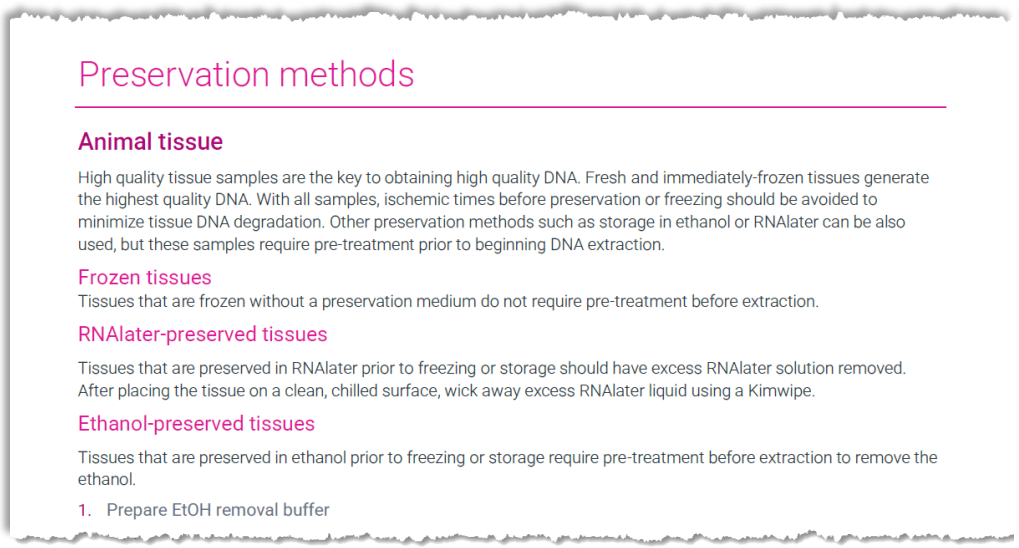
Sample collection, preparation, and storage for SMRT sequencing whole genome sequencing projects

To obtain the highest quality genomic DNA, it is important to start with sample types compatible with high molecular weight (HMW) DNA extraction methods

Nanobind Guides & overviews

Nanobind kit Guides & overviews contain information on HMW DNA isolation kit specifications, general tips, tissue preservation recommendations, extraction and sequencing performance, and troubleshooting tips

- **Guide & overview – Nanobind CBB kit** (102-572-200)
- **Guide & overview – Nanobind PanDNA kit** (103-394-800)



Preservation methods

Animal tissue

High quality tissue samples are the key to obtaining high quality DNA. Fresh and immediately-frozen tissues generate the highest quality DNA. With all samples, ischemic times before preservation or freezing should be avoided to minimize tissue DNA degradation. Other preservation methods such as storage in ethanol or RNAlater can be also used, but these samples require pre-treatment prior to beginning DNA extraction.

Frozen tissues

Tissues that are frozen without a preservation medium do not require pre-treatment before extraction.

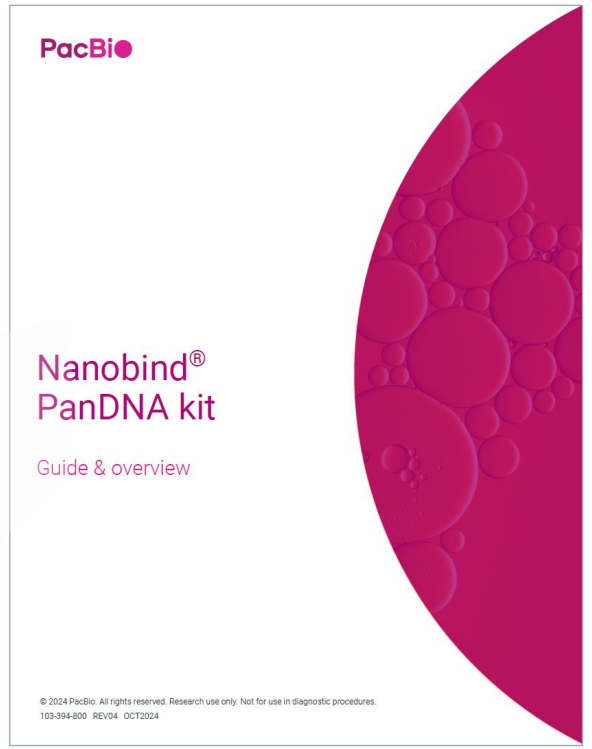
RNAlater-preserved tissues

Tissues that are preserved in RNAlater prior to freezing or storage should have excess RNAlater solution removed. After placing the tissue on a clean, chilled surface, wick away excess RNAlater liquid using a Kimwipe.

Ethanol-preserved tissues

Tissues that are preserved in ethanol prior to freezing or storage require pre-treatment before extraction to remove the ethanol.

1. Prepare EtOH removal buffer



Visit PacBio's Documentation website to find the latest resources for using Nanobind kits for HMW DNA extraction and recommended tissue preservation methods.

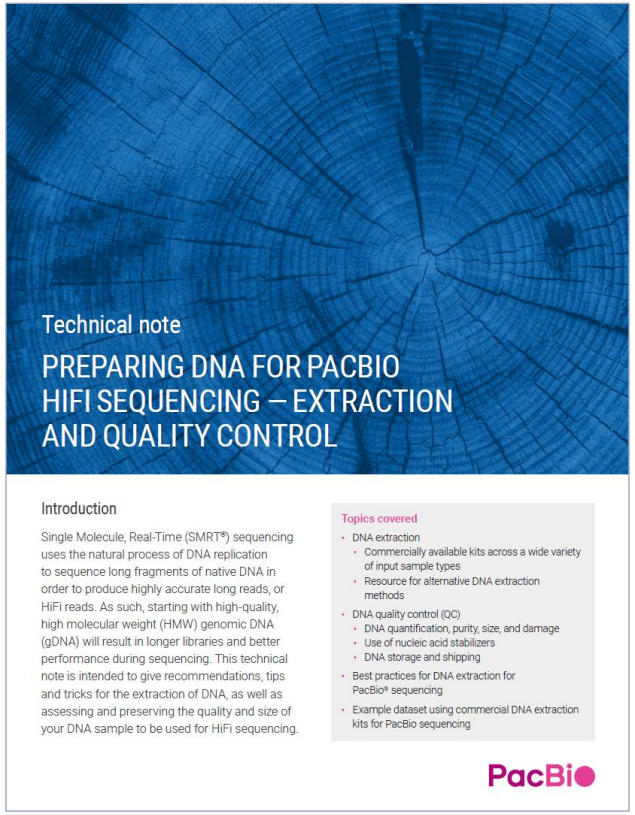
Genomic DNA extraction, QC and handling for SMRT sequencing

DNA extraction, QC and handling for SMRT sequencing whole-genome sequencing projects

Starting with high-quality, high molecular weight (HMW) genomic DNA (gDNA) will result in longer libraries and better performance during sequencing

PacBio Technical note: DNA prep (102-193-651)

- Provides recommendations, tips and tricks for the extraction of genomic DNA, as well as assessing and preserving the quality and size of your DNA sample to be used for PacBio HiFi sequencing for *de novo* assembly and variant detection
- Topics covered include:
  - DNA extraction
    - Commercially available kits across a wide variety of input sample types
    - Resources for alternative DNA extraction methods
  - DNA quality control (QC)
    - DNA quantification, purity, size, and damage
    - Use of nucleic acid stabilizers
    - DNA storage and shipping
  - General best practices for DNA extraction for PacBio sequencing



PacBio Technical note: Preparing DNA for PacBio HiFi sequencing – Extraction and quality control (102-193-651)

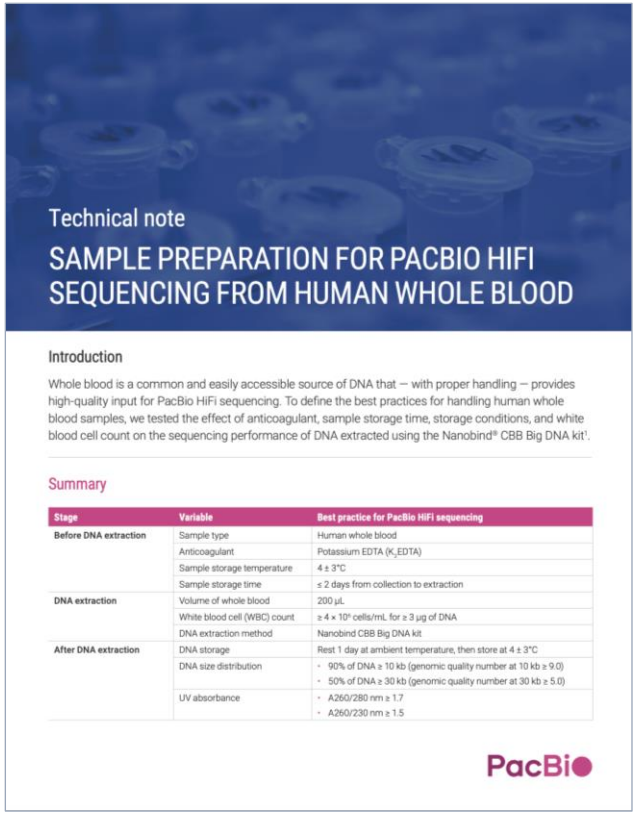
DNA extraction, QC and handling for SMRT sequencing whole-genome sequencing projects (cont.)

Whole blood is a common and easily accessible source of DNA that – with proper handling – provides high-quality input for PacBio HiFi sequencing

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

- To define the best practices for handling human whole blood samples, we tested the effect of anticoagulant, sample storage time, storage conditions, and white blood cell count on the sequencing performance of DNA extracted using Nanobind kits.

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



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

DNA extraction, QC and handling for SMRT sequencing whole-genome sequencing projects (cont.)

Refer to PacBio's extensive Nanobind literature to learn how to extract the highest-quality high-molecular weight (HMW) DNA using Nanobind DNA extraction kits

Nanobind kit Guide & overviews

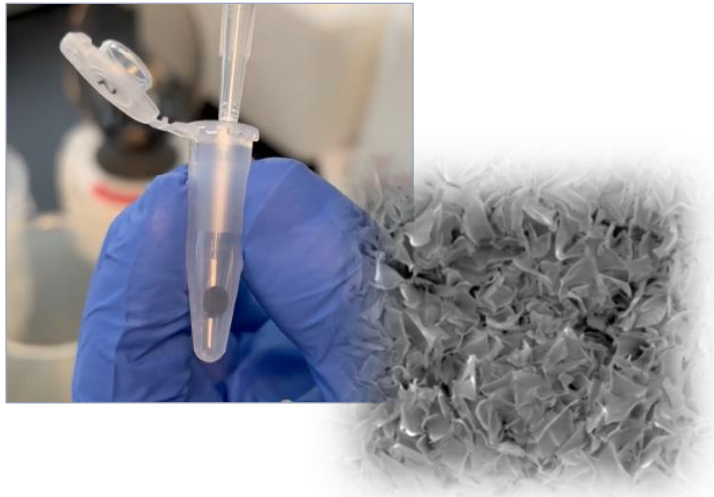
- Nanobind kit Guide & overviews contain information on HMW DNA isolation kit specifications, general tips, tissue preservation recommendations, extraction & sequencing performance, and troubleshooting tips.

Nanobind Procedure & checklists

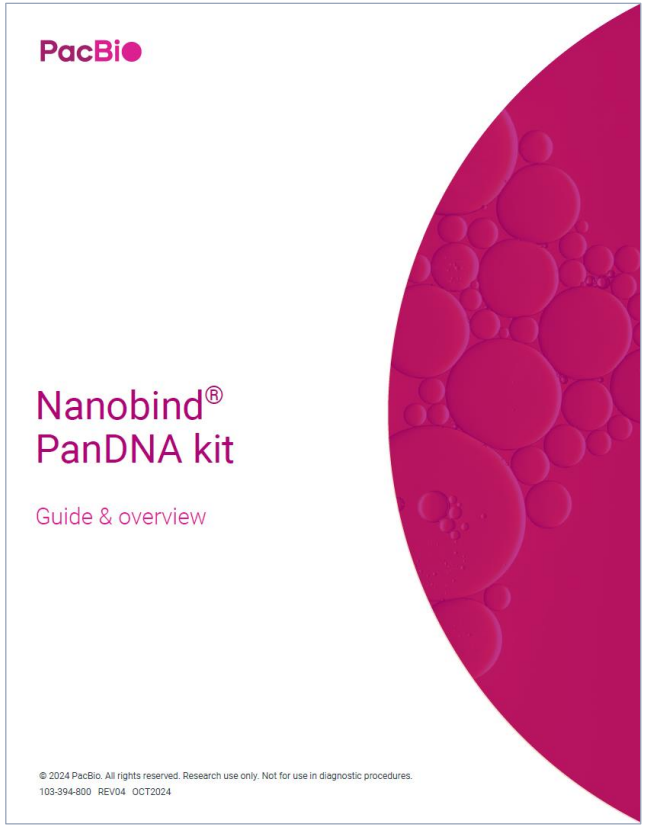
- Nanobind protocols are always our most up-to-date versions and should take precedence over the Guides & overviews.

Nanobind protocol Overviews

- Overview – Nanobind CBB HMW DNA extraction protocols (103-515-700)
- Overview – Nanobind HT HMW DNA extraction robotic procedures (103-032-000)
- Overview – Nanobind PanDNA HMW DNA extraction protocols (103-510-000)



SEM image of Nanobind's silica surface structure.



Visit the PacBio's Documentation website to find the latest resources for using Nanobind kits for HMW DNA extraction.

General recommendations for isolating high-molecular weight (HMW) genomic DNA

Before gDNA extraction:

- Use fresh or flash-frozen tissue
- Store flash-frozen tissue at -80°C and avoid freeze-thaw cycles
- Do not store blood samples longer than 2 days at 4–8°C before DNA extraction
- Microbial gDNA Isolation:
  - ❑ Avoid culture incubation in complex or rich media
  - ❑ Harvesting from several replicate cultures rather than a single, high-density culture is preferred
  - ❑ Extraction of small culture volumes is preferred over large volumes to avoid accumulating high concentrations of potentially inhibiting secondary components

During gDNA extraction:

- Mechanically disrupt tissues using TissueRuptor (QIAGEN), Dounce homogenizer or liquid nitrogen grinding
- Inactivate nucleases and DNA binding proteins with a protease, such as proteinase K
- Remove all RNA with RNase A
- Avoid oxidative agents such as phenol and/or chloroform if possible to minimize DNA damage
- Resuspend, or elute, DNA in a low salt buffer, such as 10 mM Tris-HCl pH 8.0 – 9.0 + 0.1 mM EDTA

General recommendations for isolating high-molecular weight (HMW) genomic DNA (cont.)

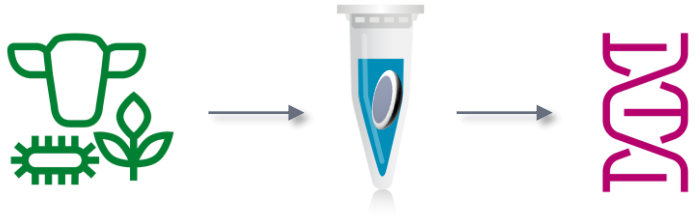
After gDNA extraction:

- Check DNA concentration using both NanoDrop and Qubit systems for concordance
- High-quality, pure DNA typically shows a A260/280 ratio ≥1.8 and A260/230 ratio ≥2.0
- To help resuspend HWM DNA, pipette mix 1–10 times with a standard P200 pipette tip. Allow DNA to rest overnight at 25°C.
- Inactivate DNase as recommended by the vendor kit
  - → Avoid heat inactivation when possible since overheating samples can introduce DNA damage.
- DNA storage conditions: 4°C (short-term); -20°C / -80°C (long-term)
- Ideally proceed to SMRTbell library preparation with freshly isolated DNA whenever possible

Example protocols and kit solutions for high-molecular weight genomic DNA isolation

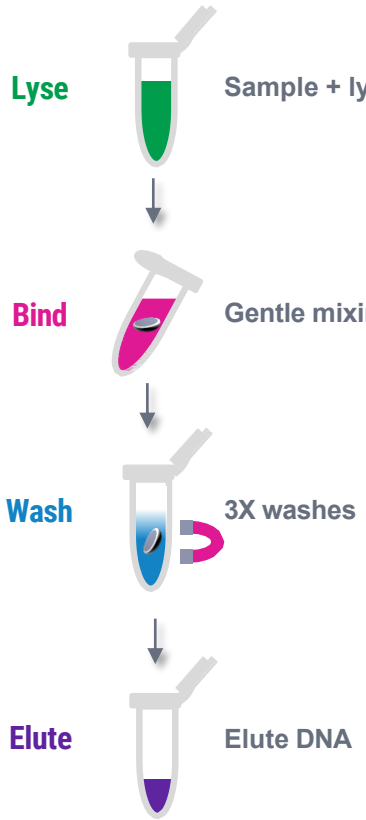
PacBio Nanobind products for HMW genomic DNA extraction

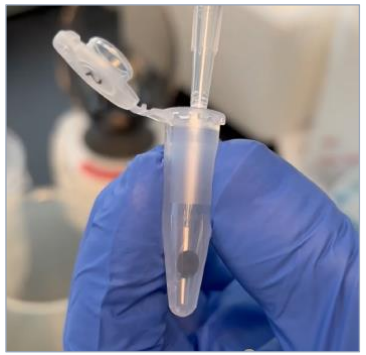
Nanobind kits can be used to extract high-quality, high-molecular weight DNA suitable for HiFi sequencing



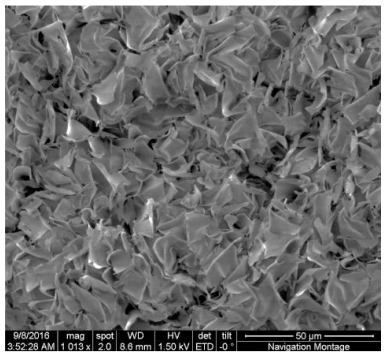
- Nanobind is a novel magnetic disk covered with a micro- and nanostructured silica
- Nanobind disks bind and release DNA without fragmentation to yield DNA up to megabase pairs in length

Rapid magnetic purification process is automatable

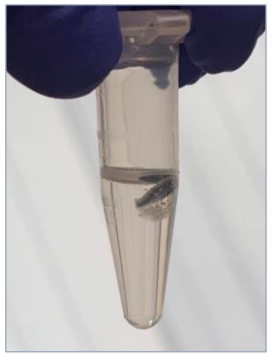




A single Nanobind disk can be processed in a 1.5 mL tube.



SEM image of Nanobind's silica surface structure.



Extracted HMW DNA bound to a Nanobind disk

For manual workflows

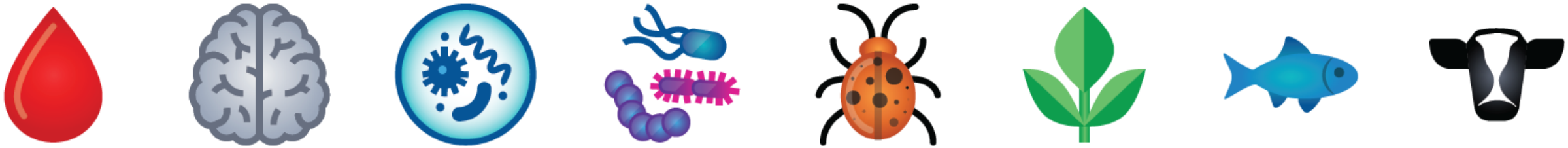
Nanobind PanDNA kit 103-260-000 (24 RXN)	Nanobind CBB kit 102-301-900 (24 RXN)
• HMW DNA extraction from cultured cells, blood, cultured bacteria, tissues, human saliva, insects, and plant nuclei	• HMW DNA extraction from cultured cells, blood, human saliva and cultured bacterial

For high-throughput automated workflows

Nanobind HT CBB kit 102-762-700 (96 RXN)	Nanobind HT 1 mL whole blood kit 102-762-800 (96 RXN)
• High-throughput HMW DNA extraction from human/animal blood, mammalian cells, and cultured bacteria	• High-throughput HMW DNA extraction from 1 mL of whole blood

Nanobind PanDNA kit provides an all-in-one DNA extraction kit that can be used with a wide range of sample types for PacBio HiFi sequencing

Nanobind PanDNA kit enables high-quality HMW DNA extraction from cells, blood, saliva, bacteria, tissues, insects, and plant nuclei



Easy-to-use solutions built on Nanobind technology

The all-new PacBio Nanobind PanDNA kit consolidates the capabilities of our existing sample-specific Nanobind kit product offerings into a single solution for DNA extraction.[1,2]

- Cultured mammalian cells
- Human whole blood
- Animal blood (mammalian & non-mammalian)
- Cultured bacteria
- Animal tissues
- Plant nuclei
- Insects
- RBC lysed human whole blood
- Human saliva

Nanobind PanDNA kit (103-260-000)

Supports 24 reactions per kit and includes:

- Nanobind PanDNA kit RT (103-260-300)
- Nanobind PanDNA kit 4C (103-260-400)

Using the Nanobind PanDNA kit on a diverse set of plant and animal samples demonstrates extraction yields between ~3–26 µg and mode fragment sizes >100 kb for the majority of samples (and >65 kb for insect samples)[3]

[1] Nanobind CBB kit (102-301-900) is also available for HMW DNA extraction from cultured mammalian cells, blood, and bacterial samples.
[2] **Note:** Fungal, lichen, algae and microalgae sample types are unsupported with the Nanobind PanDNA kit.
[3] See ***Brochure – Nanobind PanDNA kit*** (102-326-604).

Available Nanobind PanDNA HMW DNA extraction protocols (cont.)

Select the appropriate Nanobind Procedure & checklist to use based on sample type and starting material[1]

Sample type	Starting material	Sample input	Workflow time	Procedure & checklist
Blood	Human whole blood	200 µL	~1 hr	*Extracting HMW DNA from human whole blood using Nanobind kits* (102-573-500)[2]
	Nucleated red blood cells (nRBCs)	2.5 – 20 µL	~1 hr	*Extracting HMW DNA from nucleated red blood cells using Nanobind kits* (102-574-000)
	Human whole blood with RBC lysis	400 µL	<1.5 hrs	*Extracting HMW DNA from human whole blood with RBC lysis using Nanobind kits* (103-377-500)
Saliva	Human saliva collected in DNA Genotek™ Oragene™ devices	500 µL	~2 hrs	*Extracting HMW DNA from saliva using Nanobind kits* (103-544-000)
Animal tissue	Diverse tissue types	2 – 100 mg	~2.5 hrs	*Extracting HMW DNA from animal tissue* (102-574-600)[3]
Insect tissue	Insect whole body or segment	>20 mg	~2.5 hrs	*Extracting HMW DNA extraction from insects using the Nanobind PanDNA kit* (102-377-400)
Plant tissue	Isolated plant nuclei	0.25 – 5 g	~1.5 hrs[4]	*Extracting HMW DNA from plant nuclei using Nanobind kits* (103-378-200)
Mammalian cultured cells	Suspension cell culture	1 x 10^6 – 5 x 10^6 diploid human cells	~1 hr	*Extracting HMW DNA from cultured suspension cells using Nanobind kits* (103-394-500)
	Adherent cell culture	1 x 10^6 – 5 x 10^6 diploid human cells	~1 hr	*Extracting HMW DNA from cultured adherent cells using Nanobind kits* (102-573-600)
Cultured bacteria	Gram-negative bacteria	5 x 10^8 – 5 x10^9 bacterial cells	~1 hr	*Extracting HMW DNA from Gram-negative bacteria using Nanobind kits* (102-573-800)
	Gram-positive bacteria	5 x 10^8 – 5 x10^9 bacterial cells	~1 hr	*Extracting HMW DNA from Gram-positive bacteria using Nanobind kits* (102-573-900)

[3] ***Procedure & checklist – Extracting HMW DNA from animal tissue*** (102-574-600) describes the extraction of HMW DNA from animal tissues using a TissueRuptor tool for tissue disruption. If a TissueRuptor tool is unavailable, then ***Procedure & checklist – Extracting HMW DNA from standard Dounce homogenizer tissue using Nanobind kits*** (102-573-700) may alternatively be used.

[1] For a complete list of supported Nanobind HMW DNA extraction procedures, refer to the PacBio Documentation website.
[2] Note that this manual Nanobind procedure (102-573-500) does not support processing of other types of (non-human) mammalian blood samples that are non-nucleated.
[4] Upstream plant nuclei prep isolation procedure (e.g., 102-574-900 or 102-574-800) typically take ~3 hours to complete.

Nanobind PanDNA HMW DNA extraction workflow overview

Key DNA extraction workflow processing steps for standard Nanobind PanDNA procedures

Cells, blood and bacteria

Add lysis buffer

Step 1 Lyse — Incubate

Add Nanobind + isopropanol

Step 2 Bind — Gentle mixing

Add wash buffers (CW1, CW2, CW2)

Step 3 Wash — Wash 3X

Discard supernatant and add Buffer LTE

Step 4 Elute — HMW DNA

Insect samples

Step 1 Disrupt insect tissue — Pellet pestle

Homogenize sample in cold buffer (CT)

Step 2 Lyse and pellet debris — Incubate

Add Nanobind + isopropanol

Step 3 Bind — Gentle mixing

Add wash buffers (CW1, CW2, CW2)

Step 4 Wash — Wash 4X

Discard supernatant and add Buffer LTE

Step 5 Elute — HMW DNA

RBC lysis samples

Step 1 Lyse red blood cells — RBC lysis buffer

Pellet WBC and discard supernatant containing lysed RBC

Step 2 Lyse white blood cells — Incubate

Add Nanobind + isopropanol

Step 3 Bind — Gentle mixing

Add wash buffers (CW1, CW2, CW2)

Step 4 Wash — Wash 3X

Discard supernatant and add Buffer LTE

Step 5 Elute — HMW DNA

Human saliva

Step 1 Pre-extraction QC —  Qubit assay

Determine if DNA content is sufficient to proceed with extraction

Step 2 Lyse — Incubate

Add Nanobind + isopropanol

Step 3 Bind — Gentle mixing

Add wash buffers (CW1, CW2, CW2)

Step 4 Wash — Wash 3X

Discard supernatant and add Buffer LTE

Step 5 Elute — HMW DNA

Nanobind PanDNA HMW DNA extraction workflow overview

Key DNA extraction workflow processing steps for standard Nanobind PanDNA procedures

Animal tissue samples

Step 1
Disrupt animal tissue

Tissue homogenization with TissueRuptor

Step 2
Lyse and pellet debris

Incubate

Add Nanobind + isopropanol

Step 3
Bind

Gentle mixing

Add wash buffers (CW1, CW2, CW2)

Step 4
Wash

Wash 4X

Discard supernatant and add Buffer LTE

Step 5
Elute

HMW DNA

Plant tissue samples

Plant nuclei isolation

Step 1
Disrupt plant tissue

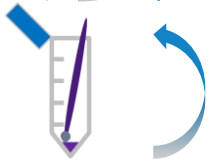
Tissue homogenization with TissueRuptor

Step 2
Lyse and filter

Lyse ground plant tissue

Centrifuge to pellet nuclei

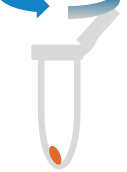
Step 3
Wash



Resuspend nuclei pellet and wash 3X

Centrifuge to pellet nuclei

Step 4
Pellet nuclei



Proceed to DNA extraction

Nanobind DNA extraction

Step 1
Lyse

Step 2
Transfer supernatant

Add Nanobind + isopropanol

Step 3
Bind

Gentle mixing in cap

Add wash buffer (PW1)

Step 4
Wash

Wash 2X

Discard supernatant and add Buffer LTE

Step 5
Elute

HMW DNA

PacBio

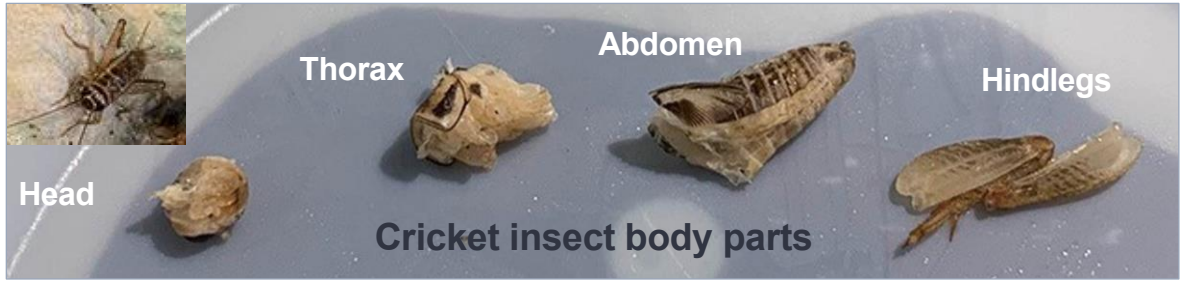
Nanobind HMW DNA extraction procedure for insect samples

Procedure & checklist – Extracting HMW DNA from insects using the Nanobind PanDNA kit (102-377-400)

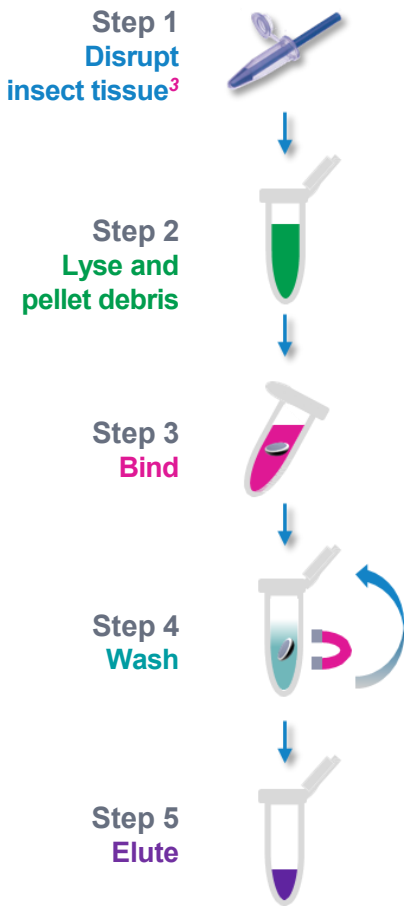
Procedure & checklist 102-377-400 describes the extraction of HMW DNA from insects and other arthropods[1] using the **Nanobind PanDNA kit** for PacBio HiFi sequencing workflows.

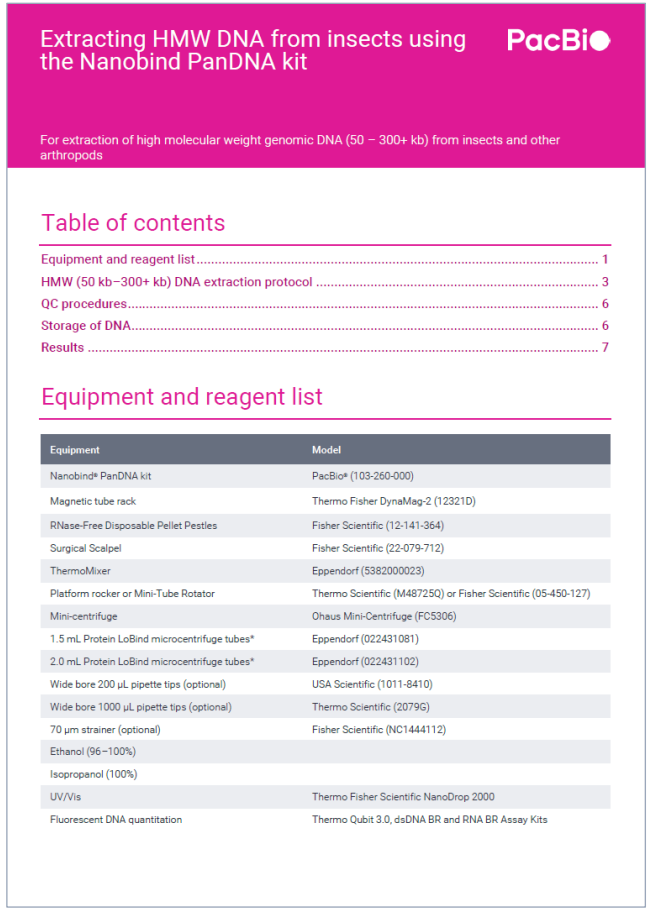
Sample input requirements

- Use a sample input amount (~20 – 100 mg[2]) that yields 3–30 µg of DNA (optimal input mass will vary by tissue type and by insect species)
  - Using too high of an input amount will overload lysis chemistries and negatively impact DNA yield & quality
- For large insects: Ideally use thorax body part → Remove any wings or legs
- Use pupa(e) or larva(e) stage insects → Have less chitin and typically less pigment than adults
- Use fresh or flash frozen insects → Avoid storage in ethanol for insects



If the insect is big enough to dissect into body parts, we first recommend using the thorax and then the head (but be aware that eye pigments may interfere with DNA binding during processing). Use abdomen only if there is no other option available since this body part has a higher concentration of gut microbes compared to the thorax or head. See ***Technical note – Insect DNA extraction*** (102-326-612).




Extracting HMW DNA from insects using the Nanobind PanDNA kit

PacBio

For extraction of high molecular weight genomic DNA (50 – 300+ kb) from insects and other arthropods

Table of contents

Equipment and reagent list

Equipment	Model
Nanobind® PanDNA kit	PacBio® (103-260-000)
Magnetic tube rack	Thermo Fisher DynaMag-2 (12321D)
RNase-Free Disposable Pellet Pestles	Fisher Scientific (12-141-364)
Surgical Scalpel	Fisher Scientific (22-079-712)
ThermoMixer	Eppendorf (5382000023)
Platform rocker or Mini-Tube Rotator	Thermo Scientific (M48725Q) or Fisher Scientific (05-450-127)
Mini-centrifuge	Ohaus Mini-Centrifuge (FC5306)
1.5 mL Protein LoBind microcentrifuge tubes*	Eppendorf (022431081)
2.0 mL Protein LoBind microcentrifuge tubes*	Eppendorf (022431102)
Wide bore 200 µL pipette tips (optional)	USA Scientific (1011-8410)
Wide bore 1000 µL pipette tips (optional)	Thermo Scientific (2079G)
70 µm strainer (optional)	Fisher Scientific (NC1444112)
Ethanol (96–100%)	
Isopropanol (100%)	
UV/Vis	Thermo Fisher Scientific NanoDrop 2000
Fluorescent DNA quantitation	Thermo Qubit 3.0, dsDNA BR and RNA BR Assay Kits

PacBio Documentation (102-377-400)

[1] This procedure is also suitable for DNA extraction from other types of arthropod species such as crustaceans (e.g., shrimps, crabs, lobsters, etc.)

[2] Note that the exoskeleton (largely composed of chitin, wax, and protein) may contribute significantly to sample mass but does not contain any DNA.

[3] Use a pellet pestle (do not use TissueRuptor or Dounce homogenizer) to break open exoskeleton so that inner tissue (containing DNA) will be exposed to lysis and digestion reagents.

PacBio

Nanobind HMW DNA extraction procedure for RBC lysis samples

Procedure & checklist – Extracting HMW DNA from human whole blood with RBC lysis using Nanobind kits (103-377-500)

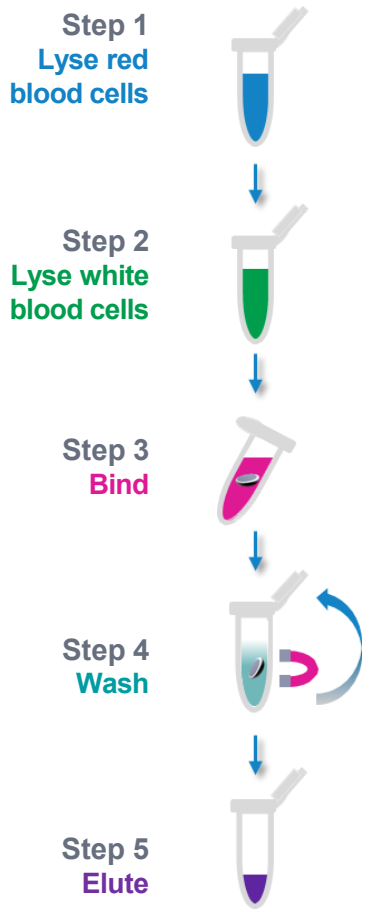
Procedure & checklist 103-377-500 describes the extraction of HMW DNA from RBC lysed human whole blood using the **Nanobind PanDNA kit** for PacBio HiFi sequencing workflows.

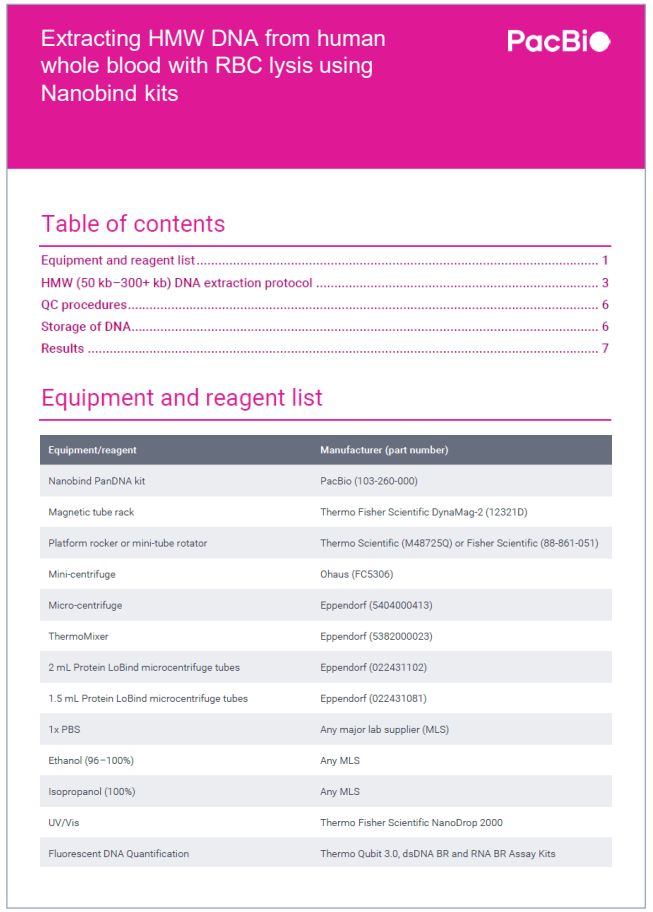
Sample input requirements

- Sample input volume: 400 µL of human whole blood → Typical DNA yield is ~3–25 µg based on donor WBC concentration
- Stored blood should be frozen as quickly as possible after being drawn
  - Storage at 4°C should be limited to 2 days or fewer to prevent sample degradation
  - Blood samples should be aliquoted to avoid repeated freeze-thaws
- No systematic difference has been observed in DNA QC or sequencing results between fresh and frozen blood samples
- For frozen blood samples, we recommend thawing at 37°C for 15 minutes
- K2 EDTA is the recommended anticoagulant[2]

Red blood cell lysis step

- Mammalian red blood cells (RBCs) typically do not contain nuclei and thus cannot be used for DNA extraction
- In RBC lysis method, RBCs are first lysed and removed from the blood sample and then DNA is extracted from the white blood cells (WBCs)
- DNA extracted using RBC lysis method allowing for extraction from higher volumes and amounts of blood without having to use large quantities of DNA extraction reagents





Extracting HMW DNA from human whole blood with RBC lysis using Nanobind kits

PacBio

Table of contents

Equipment and reagent list

Equipment/reagent	Manufacturer (part number)
Nanobind PanDNA kit	PacBio (103-260-000)
Magnetic tube rack	Thermo Fisher Scientific DynaMag-2 (12321D)
Platform rocker or mini-tube rotator	Thermo Scientific (M48725Q) or Fisher Scientific (88-861-051)
Mini-centrifuge	Ohaus (FC5306)
Micro-centrifuge	Eppendorf (5404000413)
ThermoMixer	Eppendorf (5382000023)
2 mL Protein LoBind microcentrifuge tubes	Eppendorf (022431102)
1.5 mL Protein LoBind microcentrifuge tubes	Eppendorf (022431081)
1x PBS	Any major lab supplier (MLS)
Ethanol (96–100%)	Any MLS
Isopropanol (100%)	Any MLS
UV/Vis	Thermo Fisher Scientific NanoDrop 2000
Fluorescent DNA Quantification	Thermo Qubit 3.0, dsDNA BR and RNA BR Assay Kits

PacBio Documentation (103-377-500)

[1] Samples stored in sodium heparin (NaHep) and citrate (NaCit) also performed well in limited testing.

Nanobind HMW DNA extraction procedure for saliva samples

Procedure & checklist – Extracting HMW DNA from saliva using Nanobind kits (103-544-000)

Procedure & checklist 103-544-000 describes the extraction of HMW DNA (50 – 300+ kb) from saliva collected with a Genotek Oragene DNA saliva collection device (e.g., OG-500 or OG-600) using Nanobind kits [Nanobind CBB kit (102-301-900) or Nanobind PanDNA kit (103-260-000)]

Saliva sample input requirements

- Use 500 µL of input saliva collected and stabilized in a Oragene device (DNA Genotek)[1]
  - Saliva collected in Oragene devices is stable at RT for up to 5 years[2]
- Perform pre-extraction QC using Qubit BR assay to verify saliva sample contains >2 µg of DNA in 500 µL for efficient extraction using Nanobind kits[3]
- DNA yield for saliva samples extracted using Nanobind kits can vary from ~1 to ~45 µg (per 500 µL) depending on sample properties

Collect saliva sample using Oragene device following DNA Genotek instructions

Use 500 µL input saliva stabilized in Oragene device buffer

Verify input DNA >2 µg in 500 µL saliva sample using Qubit BR assay

Perform saliva HMW DNA extraction using Nanobind PanDNA kit or Nanobind CBB kit

Step 1
Saliva collection
Pre-extraction QC

Step 2
Lyse white blood cells

Step 3
Bind

Step 4
Wash

Step 5
Elute

Extracting HMW DNA from saliva collected in DNA Genotek™ Oragene™ devices using Nanobind® kits

PacBio

Procedure & checklist

This protocol describes the extraction of HMW DNA (50–300+ kb) from saliva collected with a DNA Genotek Oragene•DNA saliva collection device using Nanobind kits. Applicable collection devices are the Oragene•Dx (OGD-500, OGD-510, OGD-600, OGD-610), Oragene•DISCOVER (OGR-500, OGR-600) and Oragene•DNA (OG-500, OG-510, OG-600, OG-610). This protocol is recommended for PacBio® HiFi sequencing and requires the Nanobind CBB kit (102-301-900) or the Nanobind PanDNA kit (103-260-000).

Required material and equipment

Equipment	Model
Nanobind® CBB kit or Nanobind® PanDNA kit	PacBio® (102-301-900 or 103-260-000)
Magnetic tube rack	Thermo Fisher DynaMag-2 (12321D)
ThermoMixer	Eppendorf (5382000023)
Platform rocker or Mini-Tube Rotator	Thermo Scientific (M48725Q) or Fisher Scientific (05-450-127)
Mini-centrifuge	Ohaus Mini-Centrifuge (FC5306)
1.5 mL Protein LoBind microcentrifuge tubes*	Eppendorf (022431081)
Ethanol (96–100%)	Any major lab supplier (MLS)
Isopropanol (100%)	Any MLS
UV/Vis	Thermo Fisher Scientific NanoDrop 2000
Fluorescent DNA quantitation	Thermo Qubit 3.0, dsDNA BR and RNA BR Assay Kits
Air or water incubator capable of maintaining 50°C	Any MLS

Before you begin

For all protocols

Eppendorf Protein LoBind tubes (Eppendorf #022431081) are highly recommended for all extractions to reduce protein contamination from tube carryover. Protein LoBind tubes are more effective in reducing carryover contamination than DNA LoBind tubes or other tubes and will result in improved UV purity.

Prior to starting

The PanDNA kit contains 3 wash buffers (CW1, CW2 and, PW1) to extract various sample types. The CBB kit only contains 2 wash buffers (CW1 and CW2). Buffers CW1, CW2, and PW1 are supplied as concentrates. CW1 and

103-544-000 REV01 OCT2024

PacBio Documentation (103-544-000)

1 Suitable collection devices are the Oragene•Dx (OGD-500, OGD-510, OGD-600, OGD-610), Oragene•DISCOVER (OGR-500, OGR-600) and Oragene•DNA (OG-500, OG-510, OG-600, OG-610).
2 DNA GenoTek white paper – *Long-term stability of DNA from saliva samples stored in the Oragene® self-collection kit* (PD-WP-005)
3 If pre-extraction DNA concentration measurement is <4 ng/µL (<2 µg in 500 µL), Nanobind extraction recovery may be low and we recommend recollecting the saliva sample for extraction.

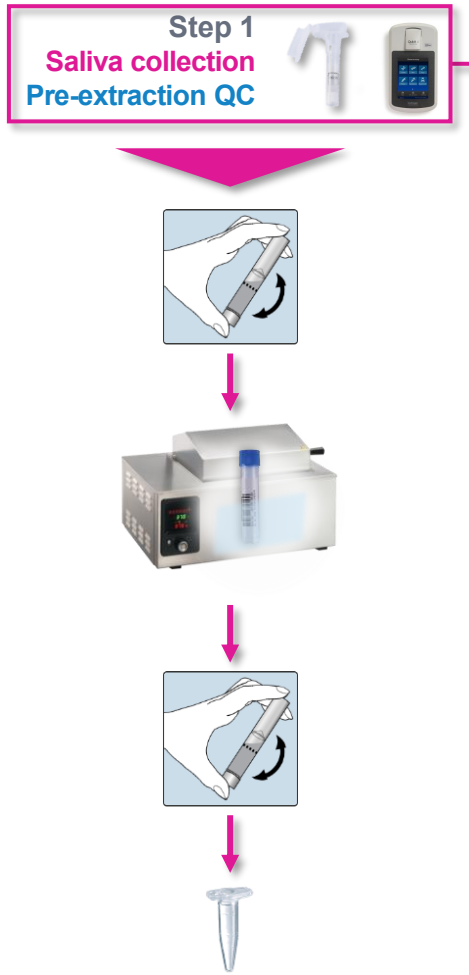
Nanobind HMW DNA extraction procedure for saliva samples (cont.)

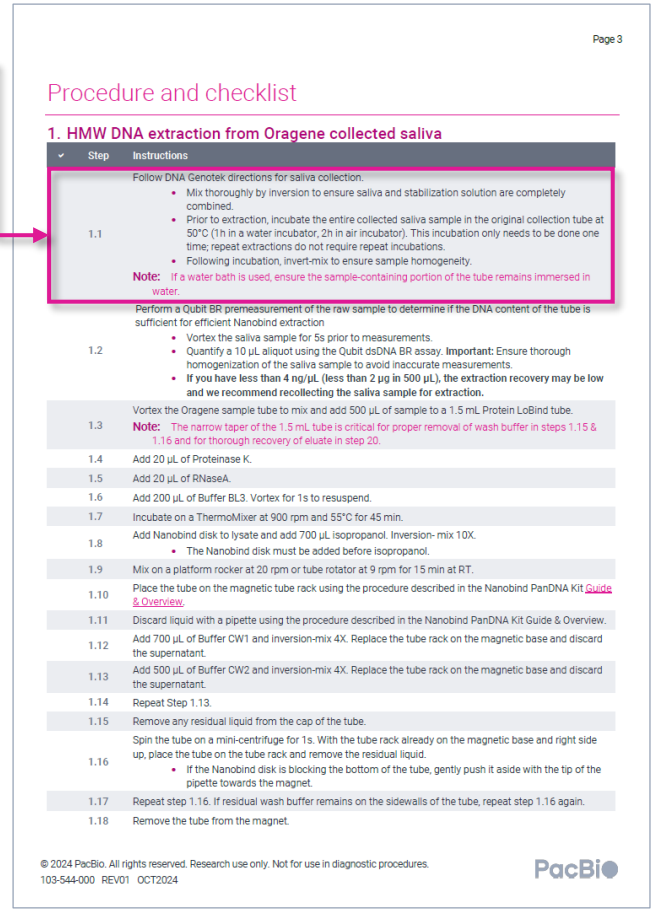
Procedure & checklist – Extracting HMW DNA from saliva using Nanobind kits (103-544-000)

Procedure & checklist describes the extraction of HMW DNA (50 – 300+ kb) from saliva collected with a Genotek OrageneDNA saliva collector (e.g., OG-500 or OG-600) using Nanobind kits.

Saliva sample collection procedure

- Follow DNA Genotek directions for saliva collection using Oragene kits:
  - ❑ During sample collection, inversion mix thoroughly to ensure saliva and storage solution are completely combined
  - ❑ Prior to beginning extraction for **first time**, incubate Oragene collection tube at 50°C for 1 hr in water incubator or 2 hrs in air incubator
  - ❑ This incubation only needs to be done one time; repeat extractions do not require repeat incubations.
  - ❑ **Note:** If a water bath is used, ensure the sample-containing portion of the tube remains immersed in water.
  - ❑ Following incubation, invert-mix to ensure sample homogeneity.
- Transfer 500 µL of saliva solution to new 1.5 mL Protein LoBind tube[1]





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Procedure and checklist

1. HMW DNA extraction from Oragene collected saliva

Step	Instructions
1.1	Follow DNA Genotek directions for saliva collection. • Mix thoroughly by inversion to ensure saliva and stabilization solution are completely combined. • Prior to extraction, incubate the entire collected saliva sample in the original collection tube at 50°C (1h in a water incubator, 2h in air incubator). This incubation only needs to be done one time; repeat extractions do not require repeat incubations. • Following incubation, invert-mix to ensure sample homogeneity. **Note:** If a water bath is used, ensure the sample-containing portion of the tube remains immersed in water.
1.2	Perform a Qubit BR premeasurement of the raw sample to determine if the DNA content of the tube is sufficient for efficient Nanobind extraction • Vortex the saliva sample for 5s prior to measurements. • Quantify a 10 µL aliquot using the Qubit dsDNA BR assay. **Important:** Ensure thorough homogenization of the saliva sample to avoid inaccurate measurements. • **If you have less than 4 ng/µL (less than 2 µg in 500 µL), the extraction recovery may be low and we recommend recollecting the saliva sample for extraction.**
1.3	Vortex the Oragene sample tube to mix and add 500 µL of sample to a 1.5 mL Protein LoBind tube. **Note:** The narrow taper of the 1.5 mL tube is critical for proper removal of wash buffer in steps 1.15 & 1.16 and for thorough recovery of eluate in step 20.
1.4	Add 20 µL of Proteinase K.
1.5	Add 20 µL of RNaseA.
1.6	Add 200 µL of Buffer BL3. Vortex for 1s to resuspend.
1.7	Incubate on a ThermoMixer at 900 rpm and 55°C for 45 min.
1.8	Add Nanobind disk to lysate and add 700 µL isopropanol. Inversion- mix 10X. • The Nanobind disk must be added before isopropanol.
1.9	Mix on a platform rocker at 20 rpm or tube rotator at 9 rpm for 15 min at RT.
1.10	Place the tube on the magnetic tube rack using the procedure described in the Nanobind PanDNA Kit Guide & Overview.
1.11	Discard liquid with a pipette using the procedure described in the Nanobind PanDNA Kit Guide & Overview.
1.12	Add 700 µL of Buffer CW1 and inversion-mix 4X. Replace the tube rack on the magnetic base and discard the supernatant.
1.13	Add 500 µL of Buffer CW2 and inversion-mix 4X. Replace the tube rack on the magnetic base and discard the supernatant.
1.14	Repeat Step 1.13.
1.15	Remove any residual liquid from the cap of the tube.
1.16	Spin the tube on a mini-centrifuge for 1s. With the tube rack already on the magnetic base and right side up, place the tube on the tube rack and remove the residual liquid. • If the Nanobind disk is blocking the bottom of the tube, gently push it aside with the tip of the pipette towards the magnet.
1.17	Repeat step 1.16. If residual wash buffer remains on the sidewalls of the tube, repeat step 1.16 again.
1.18	Remove the tube from the magnet.

PacBio Documentation (103-544-000)

[1] Eppendorf Protein LoBind tubes (Eppendorf #022431081) are highly recommended for all extractions to reduce protein contamination from tube carryover. Protein LoBind tubes are more effective in reducing carryover contamination than DNA LoBind tubes or other tubes and will result in improved UV purity.

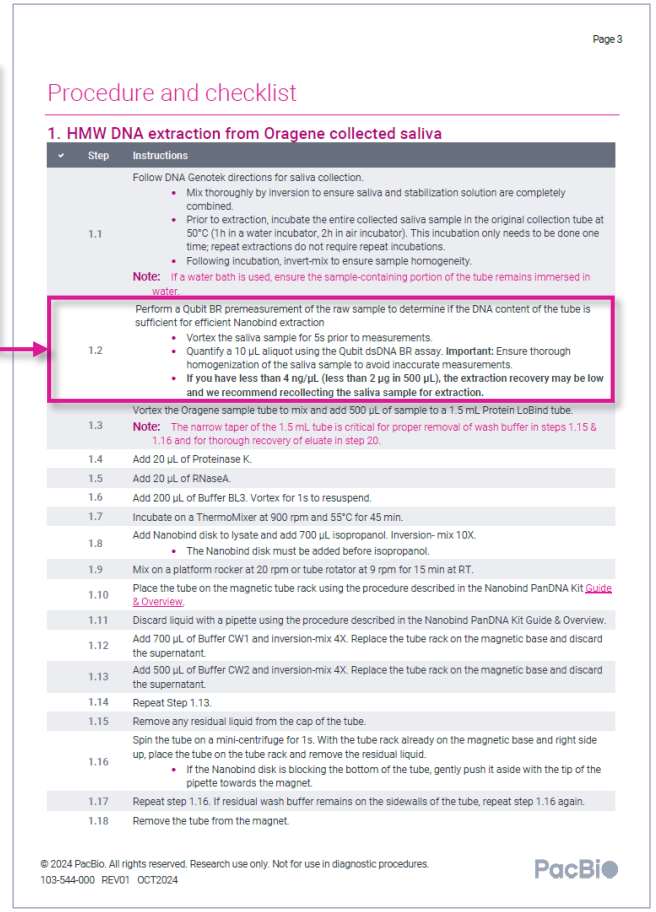
Nanobind HMW DNA extraction procedure for saliva samples (cont.)

Procedure & checklist – Extracting HMW DNA from saliva using Nanobind kits (103-544-000)

Procedure & checklist describes the extraction of HMW DNA (50 – 300+ kb) from saliva collected with a Genotek OrageneDNA saliva collector (e.g., OG-500 or OG-600) using Nanobind kits.

Saliva sample pre-extraction DNA QC procedure

- Perform a Qubit broad range (BR) dsDNA assay premeasurement of the raw sample to determine if DNA content in the tube is sufficient for efficient Nanobind extraction:
  - ❑ Vortex the saliva sample for 5 sec prior to measurements.
  - ❑ Quantify a 10 µL aliquot using the Qubit dsDNA BR assay.
    - **Important:** Ensure thorough homogenization of the saliva sample to avoid inaccurate measurements
  - ❑ If pre-extraction DNA concentration measurement is <4 ng/µL (<2 µg in 500 µL), Nanobind extraction recovery may be low and we recommend recollecting the saliva sample for extraction[1]



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Procedure and checklist

1. HMW DNA extraction from Oragene collected saliva

Step	Instructions
1.1	Follow DNA Genotek directions for saliva collection. • Mix thoroughly by inversion to ensure saliva and stabilization solution are completely combined. • Prior to extraction, incubate the entire collected saliva sample in the original collection tube at 50°C (1h in a water incubator, 2h in air incubator). This incubation only needs to be done one time; repeat extractions do not require repeat incubations. • Following incubation, invert-mix to ensure sample homogeneity. **Note:** If a water bath is used, ensure the sample-containing portion of the tube remains immersed in water.
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1.3	Vortex the Oragene sample tube to mix and add 500 µL of sample to a 1.5 mL Protein LoBind tube. **Note:** The narrow taper of the 1.5 mL tube is critical for proper removal of wash buffer in steps 1.15 & 1.16 and for thorough recovery of eluate in step 20.
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1.8	Add Nanobind disk to lysate and add 700 µL isopropanol. Inversion- mix 10X. • The Nanobind disk must be added before isopropanol.
1.9	Mix on a platform rocker at 20 rpm or tube rotator at 9 rpm for 15 min at RT.
1.10	Place the tube on the magnetic tube rack using the procedure described in the Nanobind PanDNA Kit Guide & Overview.
1.11	Discard liquid with a pipette using the procedure described in the Nanobind PanDNA Kit Guide & Overview.
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1.14	Repeat Step 1.13.
1.15	Remove any residual liquid from the cap of the tube.
1.16	Spin the tube on a mini-centrifuge for 1s. With the tube rack already on the magnetic base and right side up, place the tube on the tube rack and remove the residual liquid. • If the Nanobind disk is blocking the bottom of the tube, gently push it aside with the tip of the pipette towards the magnet.
1.17	Repeat step 1.16. If residual wash buffer remains on the sidewalls of the tube, repeat step 1.16 again.
1.18	Remove the tube from the magnet.

PacBio Documentation (103-544-000)

[1] If pre-extraction DNA concentration measurement is <4 ng/µL (<2 µg in 500 µL) and re-collection of saliva samples is not possible, contact PacBio Technical Support to inquire about possible alternative extraction methods.

Methods for evaluation of genomic DNA quality

Methods for evaluation of DNA quality

Use appropriate tools for evaluation of DNA quality to generate optimal HiFi sequencing data quality

DNA sizing QC

Example: Use a Femto Pulse system[1] or other DNA sizing tool to evaluate input genomic DNA quality and final SMRTbell DNA library size

- ☑ **High-quality, high-molecular weight DNA** → Longer read lengths / higher data yields
- ☒ **Low-quality, degraded/damaged DNA** → Shorter read lengths / lower data yields / lower library synthesis yields

DNA purity QC

Example: Use a NanoDrop instrument or other spectrophotometer device to determine DNA purity

- ☑ **High-quality, pure DNA** → Longer read lengths / higher data yields
- ☒ **Low-quality, contaminated DNA** → Shorter read lengths / lower data yields / lower library synthesis yields

DNA quantification QC

Use a Qubit fluorometric assay for accurate dsDNA quantitation

- ☑ **Accurate dsDNA quantitation** → Optimal library construction yields / higher data yields
- ☒ **Inaccurate dsDNA quantitation** → Lower library construction yields / lower data yields

PacBio

1 Femto Pulse system remains the best practice for whole genome sequencing (WGS) library QC – however, users may consider lower-cost alternatives for sizing, depending on application-specific needs.

Methods for evaluation of DNA quality (cont.)

Use an appropriate DNA sizing tool to perform QC analysis of input genomic DNA samples & final libraries

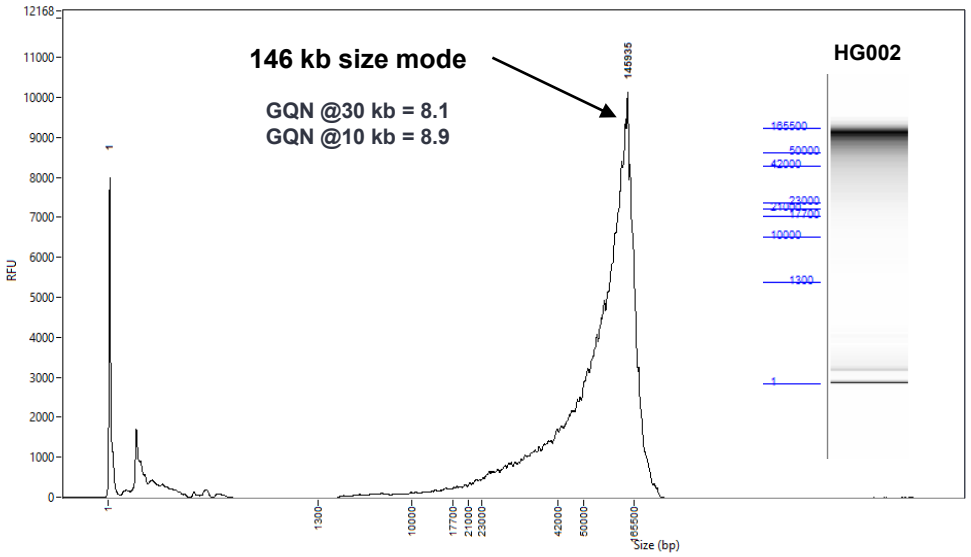
DNA sizing QC

Application	Input DNA fragment size	Requirement[1]	Notes
Human/animal/plant/other WGS **Microbial WGS** **Shotgun metagenomic profiling** **Shotgun metagenomic assembly**	>30 kb	≥50%	• Femto Pulse GQN at 30 kb ≥5.0
	>10 kb	≥70%	• Femto Pulse GQN at 10 kb ≥7.0

[1] Lower quality DNA may be used with the expectation of lower HiFi sequencing data yields.

DNA purity QC

DNA quantification QC



Example DNA sizing QC analysis of a high-quality HG002 human genomic DNA sample using a Femto Pulse system with Genomic DNA 165 kb kit.

Any degradation present should be due to shearing from extraction process and **not** from poor sample handling/storage or biochemical processes[2]

Femto Pulse system

- Resolves up to ~165 kb
- Requires <1 ng of sample
- <1.5-hr analysis time

[2] Use a wide-bore pipette when preparing sample dilutions for DNA sizing QC analysis of high-molecular weight gDNA using a **Femto Pulse system**.

Methods for evaluation of DNA quality (cont.)

Use a NanoDrop instrument or other spectrophotometer device to determine DNA purity QC[1]

DNA sizing QC

Absorbance ratio metric[1]	Recommended range	Potential causes for low absorbance ratios
A260/A280	~1.8 – 2.0	Protein / phenol / other contaminants that absorb strongly at or near 280 nm
A260/A230	≥2.0	Protein / carbohydrate (often a problem with plants) / residual phenol from nucleic acid extraction / residual guanidine (often used in column-based kits) / glycogen

[1] Ideally perform triplicate NanoDrop UV/VIS measurements from top, middle, and bottom of tube to determine purity of HMW gDNA samples

DNA purity QC

- High UV absorbance values are *not* always a guarantee of optimal sequencing performance
  - Not all inhibitors absorb at 230, 260, and 280 nm
- Conversely, low UV absorbance values are *not* always a guarantee that non-optimal sequencing performance will be obtained
  - gDNA samples with A260/280 ≥1.7 and A260/230 ratios ≥1.5 can still generate excellent HiFi sequencing performance[2]

DNA quantification QC

If A260/280 and A260/230 readings are out of recommended ranges, perform one or more rounds of purification using **AMPure PB beads** or **SMRTbell cleanup beads** followed by re-assessment of quantity and purity of input DNA sample.[3]

[2] *Technical Note: Sample preparation for PacBio HiFi sequencing from human whole blood* (102-326-500)
[3] *Technical Note: Preparing DNA for PacBio HiFi sequencing – Extraction and quality control* (102-193-651)

Methods for evaluation of DNA quality (cont.)

Use a Qubit fluorometric assay for accurate dsDNA quantitation QC

DNA sizing QC

DNA purity QC

DNA quantification QC

gDNA input mass into library prep	Sequel IIe system	Vega system	Revio system with SPRQ chemistry
Total DNA per SMRT Cell	≥1,000 ng per SMRT Cell 8M	≥2,000 ng per Vega SMRT Cell	≥500 ng per Revio SMRT Cell
Multiplexed library[1]	150 ng – 1 μg per sample	300 ng – 2 μg per sample	300 ng – 2 μg per sample

[1] If multiplexing, total **combined** mass of multiplexed (barcoded and pooled) samples should be ≥ minimum input DNA amount required per SMRT Cell for a non-multiplexed (single-sample) library. **Note:** If using short read eliminator (SRE) to clean up input genomic DNA, 500 ng per sample must be used. If bypassing SRE, no less than 20 ng of input gDNA should be used for an individual sample going into library preparation.

- Use Qubit dsDNA high sensitivity (HS) assay kit [Thermo Fisher Scientific] for routine DNA quantitation during SMRTbell library construction and final QC[2]
  - If measured NanoDrop value is significantly **different** (>50%) from Qubit value, try performing a bead-based purification step (using AMPure PB beads or SMRTbell cleanup beads)
- Can use a Qubit RNA BR assay kit to measure levels of any RNA contamination[3]
  - Samples should be **free of RNA** before beginning library prep
  - If RNA is detected, treat genomic DNA sample with RNase A according to manufacturer's instructions (e.g., 37°C for 15 min[4]), followed by 1X SMRTbell cleanup beads (or AMPure PB beads) before proceeding

[1] Alternatively, to remove RNA from samples, can add 1 μL of NEB Monarch RNase A into the DNA repair & A-tailing reaction tube during SMRTbell library construction.

Note: Starting with DNA input amounts lower than the recommended minimum may produce **insufficient** amounts of SMRTbell library to load at concentrations that optimize HiFi sequencing data yield

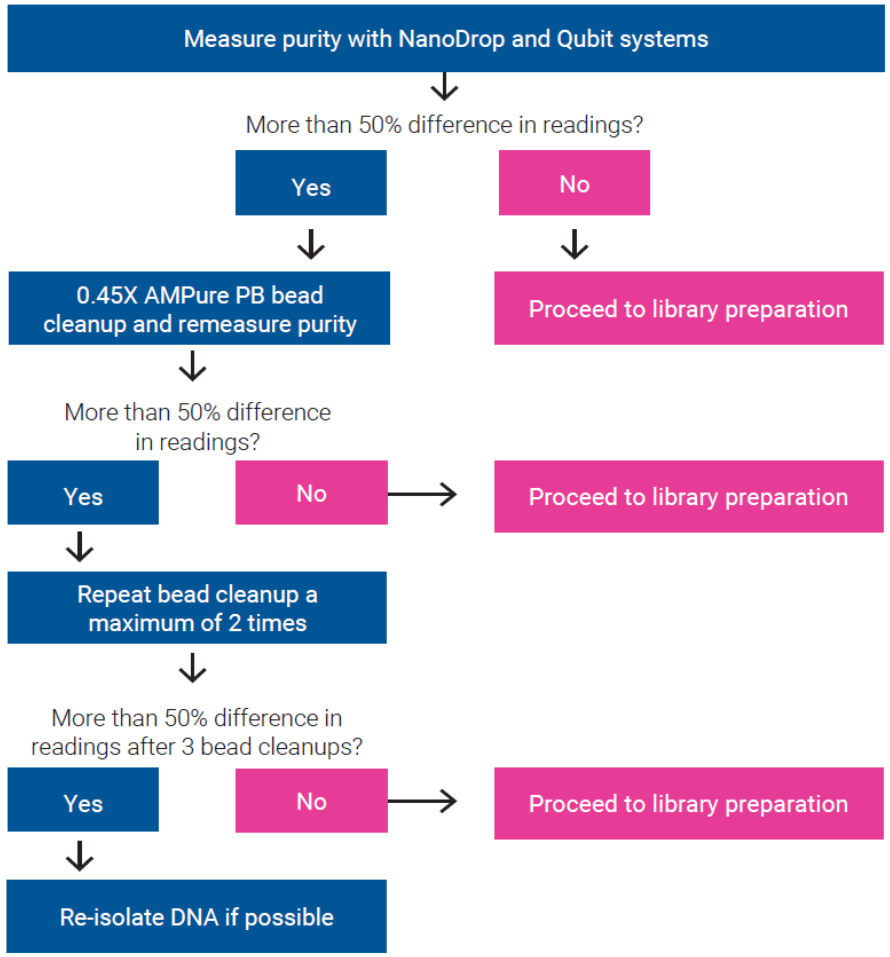
[2] Alternatively, use a Qubit dsDNA broad range (BR) assay kit for DNA concentration QC of HMW genomic DNA if Qubit HS assay kit does not provide reproducible results.
[3] Contaminant RNA can negatively impact library construction yields (by competing with DNA substrates during enzymatic steps) and reduce sequencing data yields (by inhibiting annealing of sequencing primers to SMRTbell templates and/or competing with templates for binding to polymerase).

Methods for cleanup of genomic DNA

General recommendations for cleanup of genomic DNA

PacBio SMRTbell cleanup beads or AMPure PB beads can be used for general cleanup of gDNA to remove contaminants[1]

- A quick and very effective check for sample purity is to compare concentration readings between NanoDrop Spectrophotometer and Qubit Fluorometer: High-quality DNA should show relative agreement in concentration measurements.
- If you observe a large difference in concentration readings between the NanoDrop and Qubit systems (e.g., a difference of ≥50%), check for RNA contamination using the Qubit RNA broad range assay.
- If there is no RNA contamination, then we recommend performing at least one to three rounds of bead purification until concentrations are <50% different.
- If agreement does not improve after three rounds of purification, try using either a commercial DNA cleanup kit, isopropanol precipitation, or a new DNA extraction method to obtain a cleaner DNA sample.
- If there is RNA in the sample, then treat with RNase A followed by a round of bead purification.



Recommended cleanup process for isolated gDNA using 0.45X AMPure PB beads.[2] (1X AMPure PB beads or SMRTbell cleanup beads may also be used.)

[1] SMRTbell cleanup beads or AMPure PB beads may also be used for concentrating DNA samples prior to starting SMRTbell library prep – refer to specific ***Procedure & checklist*** documentation for recommended volumetric ratio of beads to use for cleanup and/or concentration of DNA samples (e.g., for DNA fragments >10 kb, use 1X bead ratio; for DNA fragments <10 kb, use 1.3X bead ratio)

[2] See ***Technical Note: Preparing DNA for PacBio HiFi sequencing – Extraction and quality control*** (102-193-651).

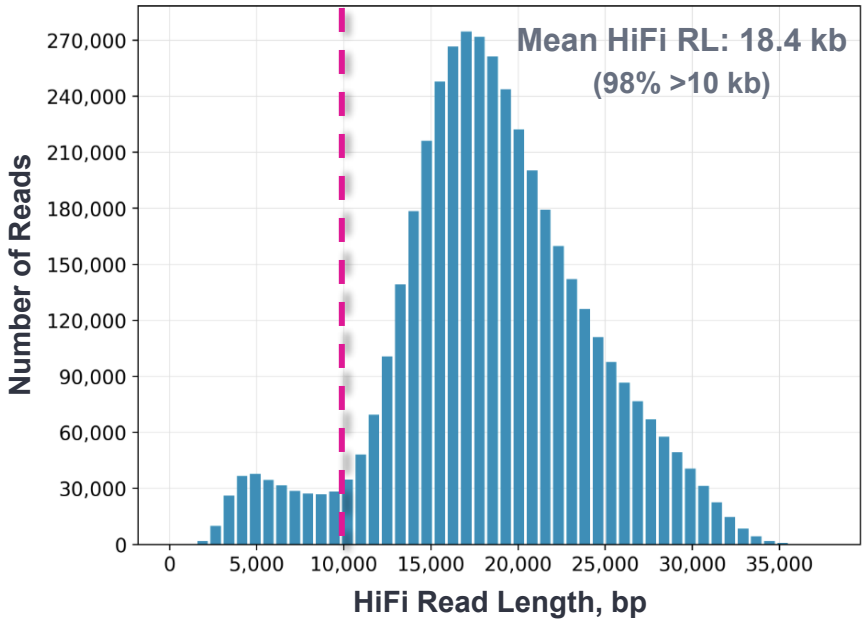
Short read eliminator (SRE) kits for genomic DNA sample cleanup for PacBio HiFi sequencing

PacBio Short read eliminator kits[1] can be used can be used for rapid high-pass size selection of gDNA samples

- **SRE kit (102-208-300)** can be used for easy and rapid size selection of (unsheared) HMW gDNA samples[2] prior to HiFi library preparation
- Uses a simple centrifugation procedure similar to standard ethanol precipitation techniques

Short read eliminator (SRE) kit (102-208-300) contains reagents for removal of DNA fragments <10 kb using size-selective precipitation.

HiFi read length distribution



Example HiFi read length distribution profile for a HG002 WGS library prepared by performing selection using the SRE kit (102-208-300) on the unsheared starting input HMW DNA.

SRE kit (102-208-300) can significantly enhance mean HiFi read lengths by depleting short DNA fragments <10 kb

PacBio

1 See ***Guide & overview – Nanobind short read eliminator kit family*** (102-582-400) for recommended use cases and input DNA quality & DNA concentration requirements.
2 SRE kit is recommended for HiFi library prep workflows – refer to application-specific ***Procedure & checklist*** protocol documentation for instructions on how to perform SRE size selection.

Storage and shipping of genomic DNA and SMRTbell libraries

Guidelines for storage and shipping of genomic DNA & SMRTbell libraries

Minimize any heat exposure and freeze/thaw cycles during storage and transport of gDNA & SMRTbell libraries

High-molecular weight genomic DNA storage

- Very clean HMW gDNA extracted with Nanobind kits can be stored at 4°C for weeks with no degradation
  - Clean HMW gDNA can also be stored a few days at 25°C
- HMW gDNA can also be frozen at -20/-80°C for extended storage
  - Avoid freeze/thaw cycles

SMRTbell library storage

- Use SMRTbell library immediately for sequencing or store at -20°C
  - Like most DNA, SMRTbell libraries will slowly degrade over time[1]
- Reduce or eliminate freeze/thaw cycles of your SMRTbell library to prevent damage

Shipping

- Heat exposure to DNA should be minimized or eliminated during transport
- Lyophilized DNA may be used as long as heat is not applied during the process
- PacBio generally recommends shipping genomic DNA[2] and SMRTbell libraries in a frozen state on dry ice with overnight shipping priority
  - Place primary sample tube(s) inside a secondary form of containment (e.g., 50 mL conical tube) and surround it with bubble wrap to help ensure that primary sample tube does not become damaged during transport

PacBio

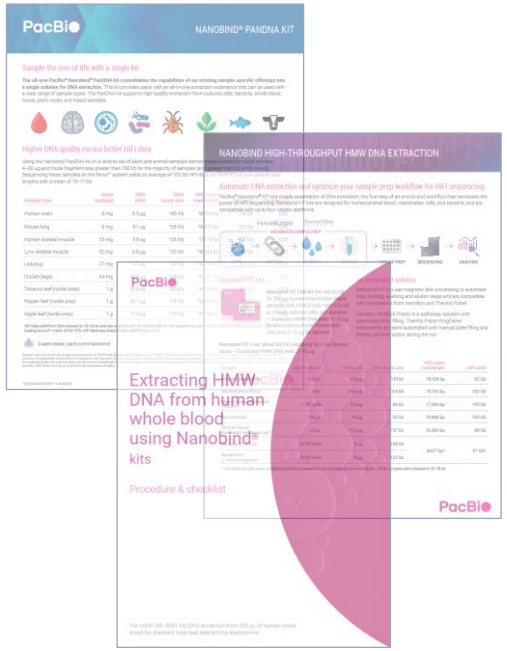
[1] See ***Quick Reference Card – Long-read sequencing workflow product storage recommendations*** (001-534-424).
[2] **Note:** HMW genomic DNA extracted with Nanobind kits can also be shipped in liquid form on wet ice.

DNA sample extraction literature resources

DNA sample extraction documentation & other literature

Brochures

- Brochure – Nanobind high-throughput HMW DNA extraction (102-326-565)
- Brochure – Nanobind PanDNA kit (102-326-604)



Application & Technical notes

- Application note – High-molecular-weight DNA extraction from human blood and saliva using Nanobind kits for HiFi long-read sequencing (102-326-656)
- Technical note – High-throughput DNA extraction (102-326-611)
- Technical note – Insect DNA extraction (102-326-612)
- Technical note – Preparing DNA for PacBio HiFi sequencing – Extraction and quality control (102-193-651)
- Technical note – Sample preparation for PacBio HiFi sequencing from human whole blood (102-326-500)

Nanobind kit protocols and Guides & overviews

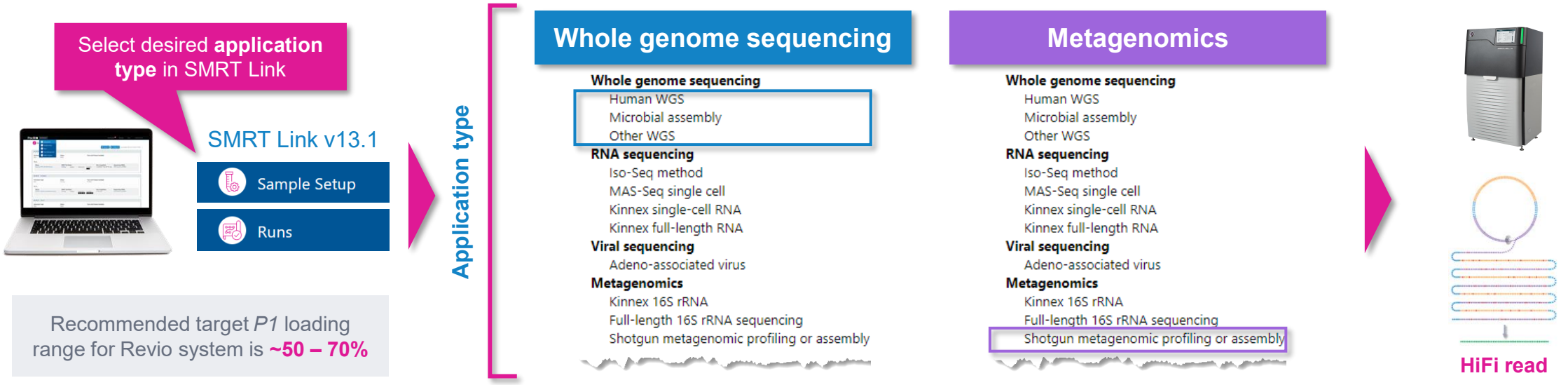
- Guide & overview – Nanobind CBB kit (102-572-200)
- Guide & overview – Nanobind PanDNA kit (103-394-800)
- Nanobind Procedures & checklists – see PacBio Documentation
- Overview – Nanobind CBB HMW DNA extraction protocols (103-515-700)
- Overview – Nanobind HT HMW DNA extraction robotic procedures (103-032-000)
- Overview – Nanobind PanDNA HMW DNA extraction protocols (103-510-000)
- Technical overview – HMW DNA sample preparation for PacBio long-read sequencing using Nanobind PanDNA and SRE kits (103-401-100)

Nanobind high-throughput (HT) automation kit protocols and Guides & overviews

- Guide & overview – Nanobind HT kits (103-028-100)
- Nanobind HT Procedures & checklists – see PacBio Documentation
- Technical overview – Automated high-throughput HMW DNA extraction for PacBio long-read sequencing using Nanobind HT kits (103-401-700)

APPENDIX 2: Sequencing preparation workflow overview for Sequel II/IIe systems

Sample Setup & Run Design recommendations for SPK 3.0 whole genome sequencing and metagenome libraries – Sequel II/IIe system



SMRT Link module	Key setup parameters	Sequel II/IIe system recommended settings			
		Human WGS	Microbial assembly	Other WGS	Shotgun metagenomics
Sample setup	Library type	Standard			
	Primer	Standard sequencing primer			
	Polymerase / Binding kit	Sequel II binding kit 3.2			
	Concentration on plate	50 - 90 pM			
Runs → Run design	SMRTbell adapter design	Overhang – SMRTbell prep kit 3.0			
	Movie time per SMRT Cell	30 hrs	15 hrs (<10 kb) / 30 hrs (≥10 kb)[1]	30 hrs	30 hrs
	Use pre-extension	YES (Pre-extension time = 2hrs)			
	Data options[2]	Use adaptive loading = YES Include Low Quality Reads = NO Include base kinetics = NO	Use adaptive loading = YES Include Low Quality Reads = NO Include base kinetics = YES[2]	Use adaptive loading = YES Include Low Quality Reads = NO Include base kinetics = NO	Use adaptive loading = YES Include Low Quality Reads = NO Include base kinetics = NO

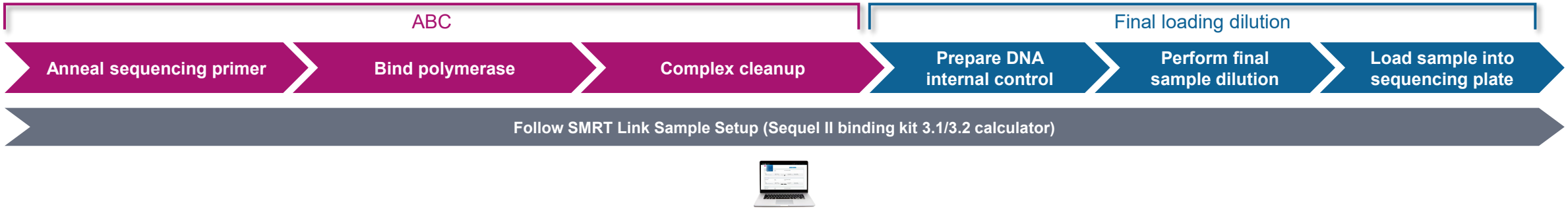
[1] **Note:** For microbial assembly applications, a 15 hrs movie time can be used if library insert size is <10 kb. If library size is ≥10 kb, we recommend using a 30 hrs movie time.
[2] **IMPORTANT:** If analysis of 4mC, 6mA or other non-5mC base modifications is desired, then need to specify **Include Base Kinetics = YES**.

Sample setup workflow overview for Sequel II polymerase libraries

For SMRTbell prep kit 3.0 WGS libraries bound with Sequel II binding kit 3.2, follow sample setup instructions provided in SMRT Link

Sequencing preparation workflow for non-multiplexed samples

Library prep kit	Polymerase kit[1]	Sample setup workflow & procedural reference	
SMRTbell prep kit 3.0 102-182-700	**Sequel II binding kit 3.2** (102-333-300)	1. Anneal sequencing primer, bind polymerase, complex cleanup (ABC)	❑ Follow SMRT Link v13.1 Sample Setup
		2. Final loading dilution procedure	❑ Follow SMRT Link v13.1 Sample Setup

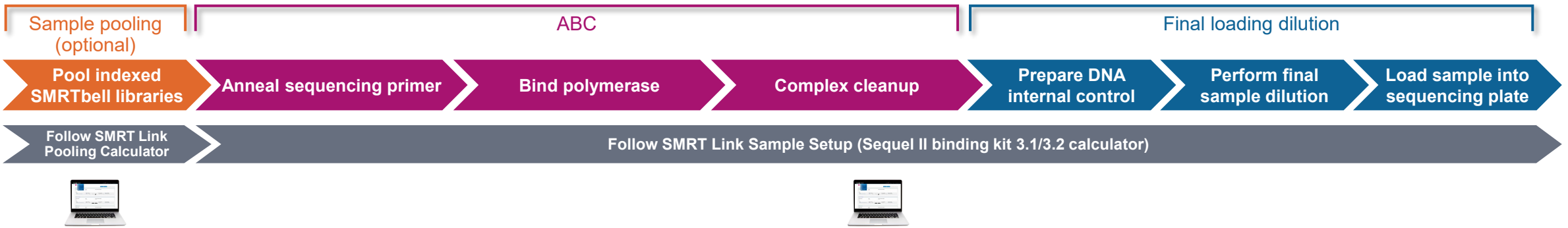


Sample setup workflow overview for Sequel II polymerase libraries (cont.)

For SMRTbell prep kit 3.0 WGS libraries bound with Sequel II binding kit 3.2, follow sample setup instructions provided in SMRT Link

Optional sequencing preparation workflow for multiplexed samples if pooling SPK 3.0 libraries before ABC with Sequel II binding kit 3.2

Library prep kit	Polymerase kit[1]	Sample setup workflow & procedural reference	
SMRTbell prep kit 3.0 102-182-700	**Sequel II binding kit 3.2** (102-333-300)	1. Sample pooling (optional)	❑ Follow SMRT Link v13.1 Pooling Calculator
		2. Anneal sequencing primer, bind polymerase, complex cleanup (ABC)	❑ Follow SMRT Link v13.1 Sample Setup
		3. Final loading dilution procedure	❑ Follow SMRT Link v13.1 Sample Setup

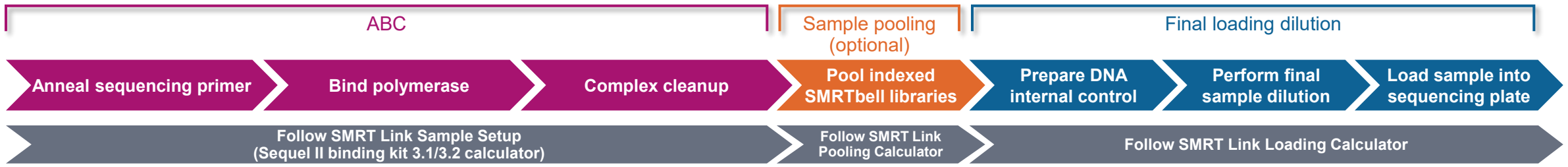


Sample setup workflow overview for Sequel II polymerase libraries (cont.)

For SMRTbell prep kit 3.0 WGS libraries bound with Sequel II binding kit 3.2, follow sample setup instructions provided in SMRT Link

Optional sequencing preparation workflow for multiplexed samples if pooling SPK 3.0 libraries after ABC with Sequel II binding kit 3.2

Library prep kit	Polymerase kit[1]	Sample setup workflow & procedural reference	
SMRTbell prep kit 3.0 102-182-700	**Sequel II binding kit 3.2** (102-333-300)	1. Anneal sequencing primer, bind polymerase, complex cleanup (ABC)	❑ Follow SMRT Link v13.1 Sample Setup
		2. Sample pooling (optional)	❑ Follow SMRT Link v13.1 Pooling Calculator
		3. Final loading dilution procedure	❑ Follow SMRT Link v13.1 Loading Calculator



- It is recommended to pool HiFi libraries post-ABC (i.e., ***after*** performing primer annealing, polymerase binding and complex cleanup) for the following reasons:
  - Prevent an inhibitor in one sample from affecting the polymerase binding of all samples in a pool
  - Ability to quickly pool different libraries together on additional runs to "top off" coverage (any un-pooled complexed library is available for future sequencing runs without having to re-do ABC)

General best practices recommendations for preparing SPK 3.0 WGS libraries for sequencing on Sequel II and IIe systems

Binding kit thawing procedure

Sequel II binding kit 3.2

Sequel II binding kit 3,2 and cleanup beads
(102-333-300)

Thaw these reagents at room temperature		Keep these reagents on a cold block or on ice		Bring these reagents to room temperature 30 minutes prior to use	
	Sequel II Annealing buffer		Sequel II DNA polymerase 2.2		Sequel II loading buffer
	Sequel II sequencing primer 3.2		Sequel II DNA internal control complex 3.2		SMRTbell cleanup beads
	Sequel II polymerase dilution buffer				
	Sequel II loading buffer				
	Sequel II ABC buffer				

- Once thawed, reaction buffers and sequencing primer may be stored on a cold block, at 4°C, or on-ice prior to making master mix or placing on the liquid handler work deck
- Loading buffer should be left at room-temperature
- **Note:** Loading buffer is light sensitive and should be protected from light when not in use

SMRT Link Sample Setup procedure for SPK 3.0 libraries – Sequel II/IIe system

Example SMRT Link Sample Setup information entry for human WGS library

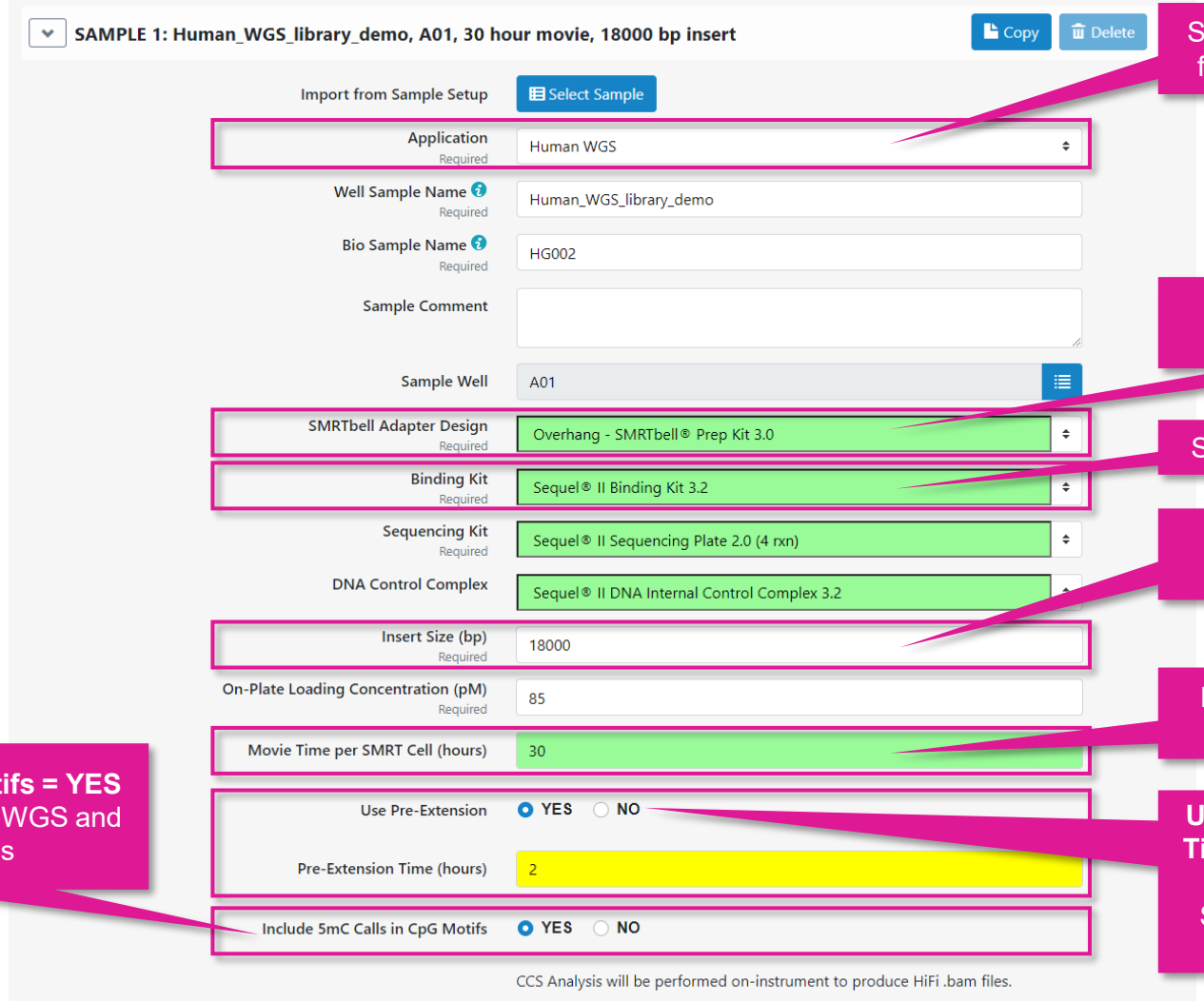
	Sample group
	Copy · Remove · Lock · Download CSV
Name	My Batch of Samples
Application	Human WGS
Library type	Standard
Polymerase / Binding kit	Sequel® II Binding Kit 3.2
Number of samples	1 samples
SMRT Cells per sample	1 cells
Available volume per sample	15 uL
Insert size	18000 bp
Sample concentration	40 ng/uL
Cleanup anticipated yield	75 %
Concentration on plate	85 pM Recommended: 50-90 pM
Minimum pipetting volume	1 uL
Comment	

- Select **application type** to autofill fields in green
- Specify **Library type = Standard**
  - Library type field specifies structure of SMRTbell library and determines sequencing primer type to use for annealing reaction
- Specify **Sequel II binding kit 3.2**
- Recommended WGS sample concentration range:[1]
  - For WGS libraries ≥10 kb: **20 – 60 ng/μL**
  - For WGS libraries 3 – 10 kb: **6 – 20 ng/μL**
- Recommended on-plate loading concentration (OPLC) range for WGS samples is **50 – 90 pM**

[1] ***IMPORTANT:*** Sample concentration for SPK 3.0 whole genome or metagenome libraries must not exceed the stated maximum values (i.e., 60 ng/μL for libraries ≥10 kb and 20 ng/μL for libraries 3 – 10 kb) in order to proceed with annealing, binding & cleanup (ABC) → Using a concentration above the stated maximum values will result in lower *P1* loading during sequencing.

SMRT Link Run Design procedure for SPK 3.0 libraries – Sequel IIe system

Example run information and sample information entry for human WGS library



Select **application type** to autofill fields highlighted in green below

Specify **Overhang – SMRTbell prep kit 3.0**[1]

Specify **Sequel II binding kit 3.2**

Specify WGS library mean **Insert Size** (in bp)

Recommend **30 hrs** movie time for WGS libraries ≥10 kb[2]

Use Pre-Extension = YES and **Pre-Extension Time = 2 hrs** are the default settings for Human WGS, Microbial assembly, Other WGS and Shotgun metagenomics profiling or assembly applications

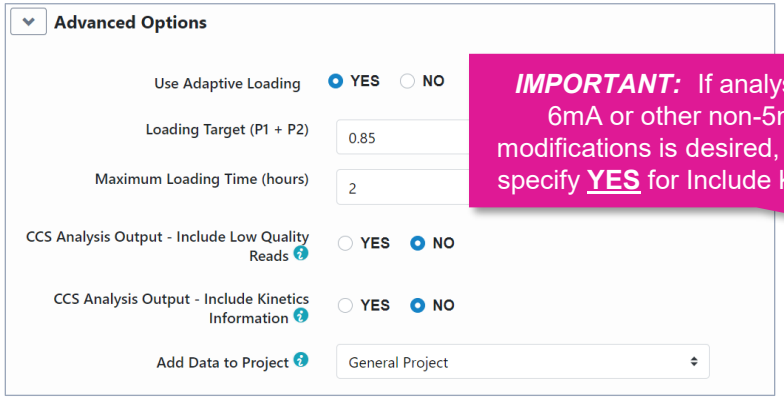
Include 5mC calls in CpG motifs = YES is the default setting for Human WGS and Other WGS applications

1 **SMRTbell Adapter Design** field determines which adapter finding algorithm is used during post-primary analysis.
2 For WGS libraries <10 kb, can specify to use 15 hrs movie time.

SMRT Link Run Design procedure for SPK 3.0 libraries – Sequel IIe system

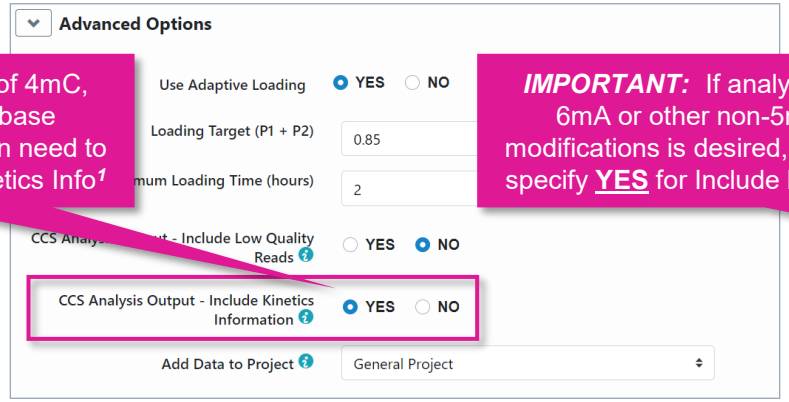
Advanced options

Human WGS or Other WGS



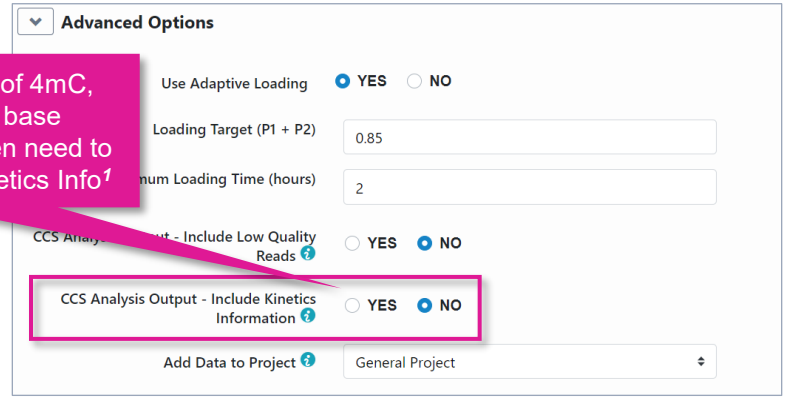
IMPORTANT: If analysis of 4mC, 6mA or other non-5mC base modifications is desired, then need to specify **YES** for Include Kinetics Info[1]

Microbial assembly



IMPORTANT: If analysis of 4mC, 6mA or other non-5mC base modifications is desired, then need to specify **YES** for Include Kinetics Info[1]

Shogun metagenomic profiling or assembly



For all WGS applications, leave Adaptive Loading, Loading Target & Maximum Loading Time fields at their **default** values

If needed, the following fields can be changed from their default values

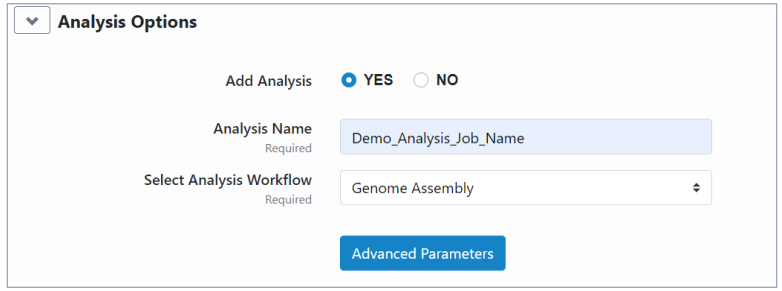
- **CCS Analysis Output – Include Low Quality Reads**
  - → Default = **NO**
- **CCS Analysis Output – Include Kinetics Information**
  - → Default = **NO** if **Human WGS; Other WGS;** or **Shotgun metagenomic profiling or assembly** is selected for application type
  - → Default = **YES** if **WGS – Microbial Assembly** is selected for application type
- **Add Data to Project**
  - → Default project folder = **General Project**; select a different project folder if desired

[1] **Note:** Adding kinetics information can increase the amount of storage used by the output BAM files by up to 5 times, and is not needed for 5mC methylation detection.

SMRT Link Run Design procedure for SPK 3.0 libraries – Sequel IIe system

Analysis options

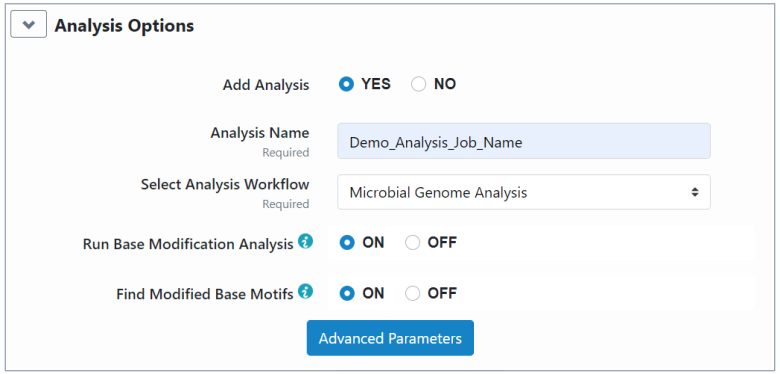
Human WGS or Other WGS



If needed, the following fields can be changed from their default values

- ❑ **Add Analysis**
  - → Default = **NO**; if specifying YES then fill out the fields below
- ❑ **Analysis Name**
  - → Specify an analysis job name
- ❑ **Select Analysis Workflow**
  - → Select desired analysis application, e.g., Genome Assembly for de novo assembly analysis or Variant Calling for variant detection analysis

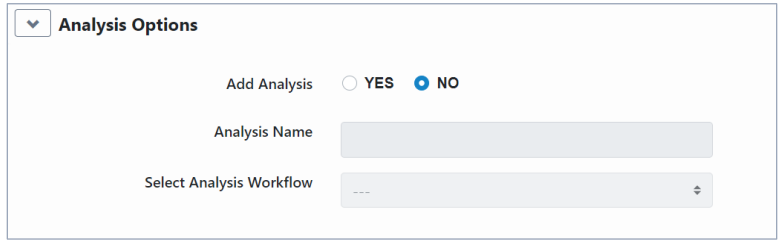
Microbial assembly



If needed, the following fields can be changed from their default values

- ❑ **Add Analysis**
  - → Default = **NO** ; if specifying YES then fill out the fields below
- ❑ **Analysis Name**
  - → Specify an analysis job name
- ❑ **Select Analysis Workflow**
  - → Select desired analysis application, e.g., Microbial Genome Analysis
- ❑ **Run Base Modification Analysis**
  - → Default = **ON**
- ❑ **Find Modified Base Motifs**
  - → Default = **ON**

Shogun metagenomic profiling or assembly



If needed, the following fields can be changed from their default values

- ❑ **Add Analysis**
  - → Default = **NO**
- ❑ **Analysis Name**
  - → Required only if an analysis is added
- ❑ **Select Analysis Workflow**
  - → Required only if an analysis is added

- Use PacBio GitHub metagenomics tools for taxonomic classification & functional gene profiling using HiFi reads
- Perform metagenomic shotgun assembly directly with HiFi reads using Hifiasm and evaluate & extract metagenome-assembled genomes using PacBio HiFi-MAG-Pipeline tool[1]

PacBio

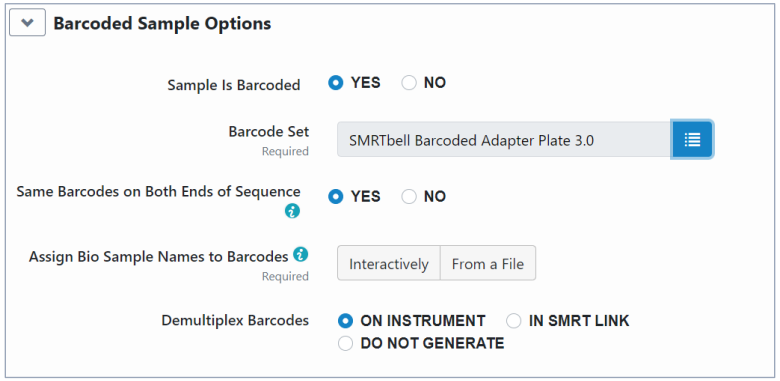
[1] Portik D.M. et al. (2022) Evaluation of taxonomic profiling methods for long-read shotgun metagenomic sequencing datasets. BMC Bioinformatics. 23:541. https://doi.org/10.1186/s12859-022-05103-0

SMRT Link Run Design procedure for SPK 3.0 libraries – Sequel IIe system

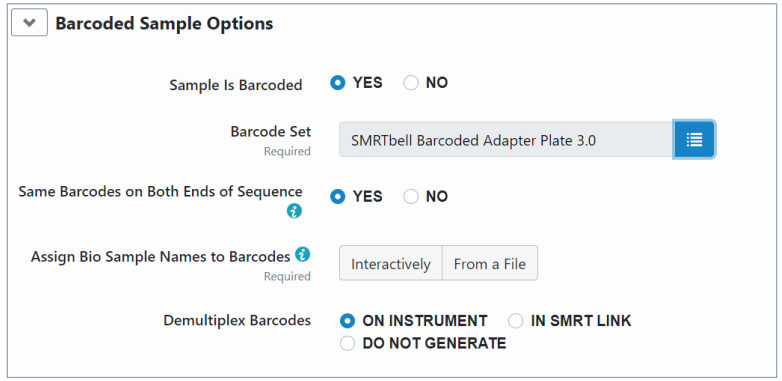
Barcoded sample options

Human WGS or Other WGS

Microbial assembly



Shogun metagenomic profiling or assembly



If needed, the following fields can be changed from their default values

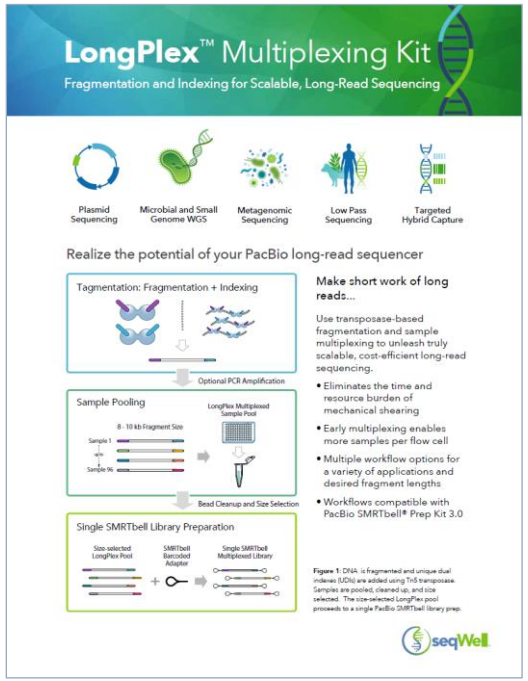
- **Sample is Barcoded**
  - → Default = **NO**; if specifying YES then fill out the fields below
- **Barcode Set**
  - → Specify barcode set FASTA file to use
- **Same Barcodes on Both Ends of Sequence**
  - → Specify YES or No
- **Assign Bio Sample Names to Barcodes**
  - → Use interactive method or import a CSV file
- **Demultiplex Barcodes**
  - → Specify where the demultiplexing analysis is to be performed (on- or off-instrument)

APPENDIX 3: PacBio compatible WGS library preparation workflows

seqWell LongPlex WGS workflow overview

Whole genome and metagenome library preparation using SeqWell LongPlex Multiplexing kit with PacBio SMRTbell prep kit 3.0: Getting started

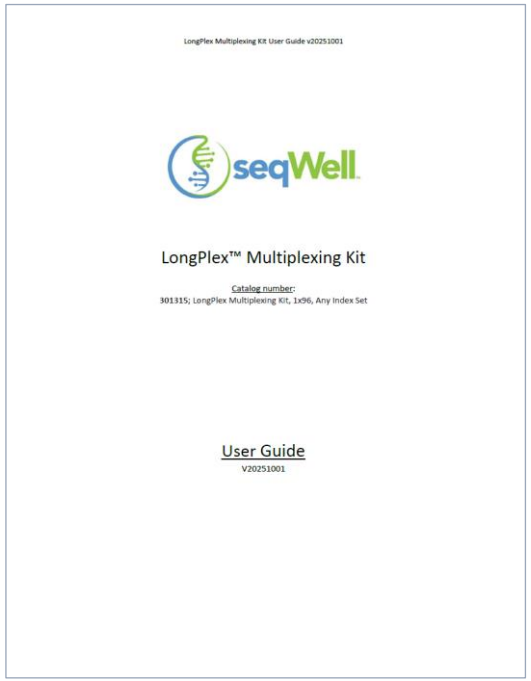
Application-specific educational literature



seqWell / PacBio literature website [Link]

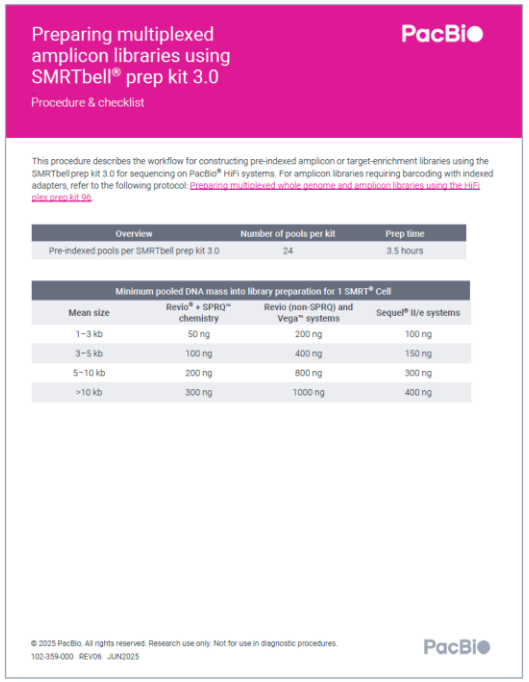
Application-specific brochures, informational guides and other product literature containing best practices recommendations for library preparation and data analysis workflows.

Application-specific protocol documentation



seqWell LongPlex Multiplexing Kit user guide (LP20251001) [seqWell]

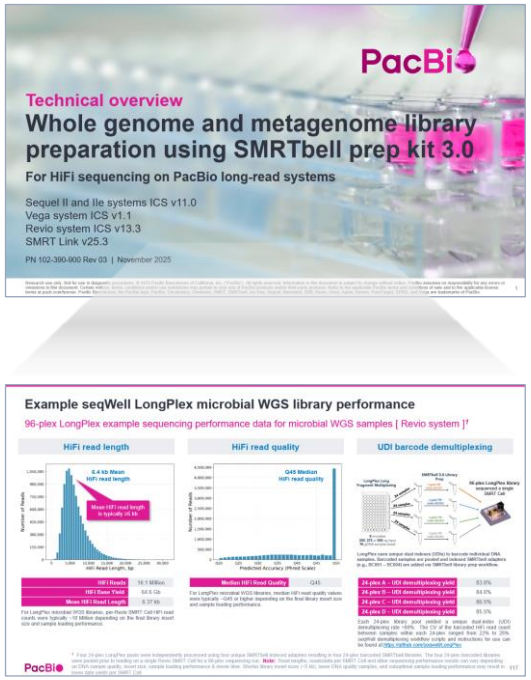
Technical documentation containing seqWell LongPlex Multiplexing kit protocol details.



Procedure & checklist – Preparing multiplexed amplicon libraries using SMRTbell Prep Kit 3.0 (102-359-000) [PacBio]

Technical documentation containing PacBio SMRTbell library construction details.

Application-specific technical overviews



Technical overview: Whole genome and metagenome library preparation using SMRTbell prep kit 3.0 (102-390-900)

Technical overview presentations describe sample preparation details for constructing HiFi libraries for specific applications. Example sequencing performance data for a given application are also summarized.

HiFi sequencing workflow overview for seqWell LongPlex WGS PCR-plus / PCR-free libraries

Follow seqWell LongPlex Multiplexing Kit User Guide (LP20251001) to generate multiplexed, indexed DNA samples suitable for SMRTbell library construction and HiFi sequencing on PacBio long-read systems

Tagmentation

↓

OPTIONAL: PCR amplification

↓

Pooling, cleanup & size selection

↓

SMRTbell library construction with SMRTbell prep kit 3.0

↓

PacBio HiFi long-read sequencing

↓

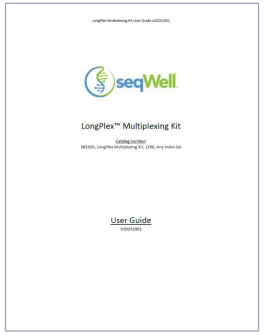
Demultiplex LongPlex HiFi reads

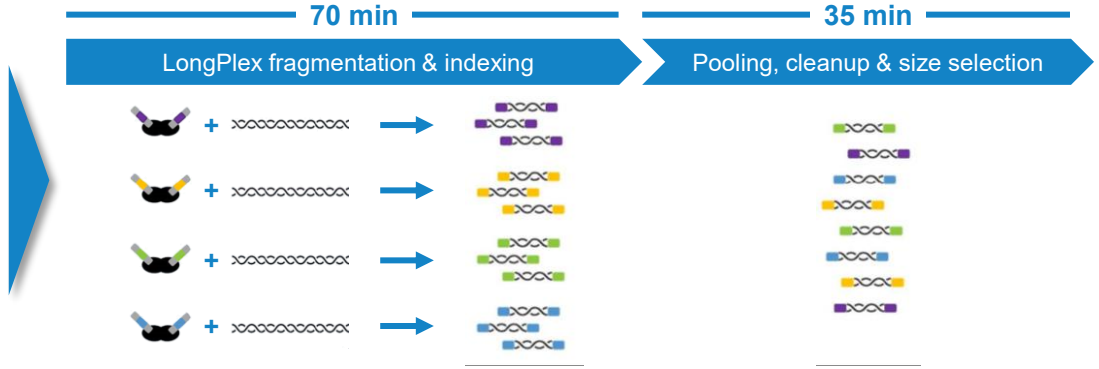
Workflow for sequencing indexed WGS samples generated with seqWell LongPlex Multiplexing Kit.

seqWell reference for LongPlex kit procedure[1]

LongPlex Multiplexing Kit User Guide (LP20251001)

seqWell





LongPlex WGS library	LongPlex WGS PCR-plus	LongPlex WGS PCR-free
Description	PCR-amplified library preparation for microbial, small genome WGS, and large genome low-pass WGS	PCR-free library preparation for microbial, small genome WGS, and large genome low-pass WGS
Library insert size	7 – 10 kb	7 – 10 kb
Input DNA amount	150 – 500 ng	250 – 500 ng
Batch range	8 – 96 samples	8 – 96 samples

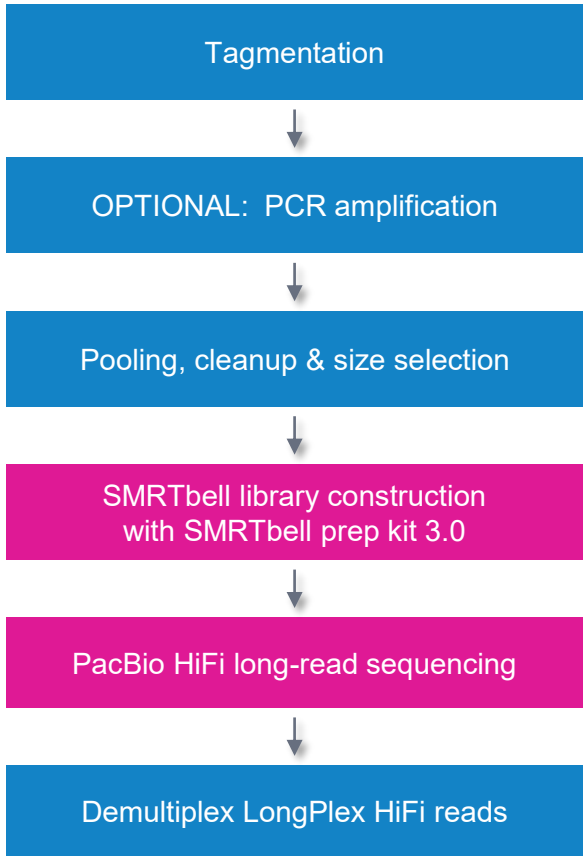
- **LongPlex Multiplexing Kit** fragments HMW genomic DNA to the range of 7-10 kb while simultaneously adding unique dual-indexed barcodes in a rapid transposase-based enzymatic method.
- **WGS PCR-Plus application** is best suited for lower quality or degraded DNA with a DIN ≥6.5, if less than 250 ng is available, if methylation data is not required, or if downstream inhibitors such polysaccharides are likely present
- **WGS PCR-Free application** is best suited for high quality DNA (DIN ≥8), if methylation data is required, and if downstream inhibitors are unlikely to be present.
- After DNA fragmentation and barcoding, samples are pooled together prior to bead-based clean up and size selection. This pool can then be processed in a single SMRTbell library prep for downstream sequencing on PacBio long-read systems.

PacBio

[1] For detailed instructions on preparing LongPlex WGS samples, refer to seqWell **LongPlex Multiplexing Kit User Guide** (LP20251001).

HiFi sequencing workflow overview for seqWell LongPlex WGS PCR-plus / PCR-free libraries (cont.)

Indexed LongPlex DNA samples may be pooled for SMRTbell library construction by following PacBio *Procedure & checklist – Multiplexed amplicon library preparation using SMRTbell Prep Kit 3.0* (102-359-000)

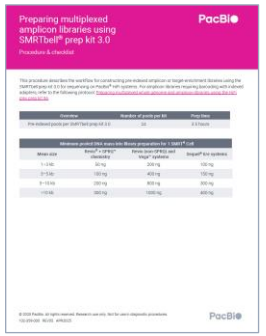


Workflow for sequencing indexed WGS samples generated with seqWell LongPlex Multiplexing Kit.

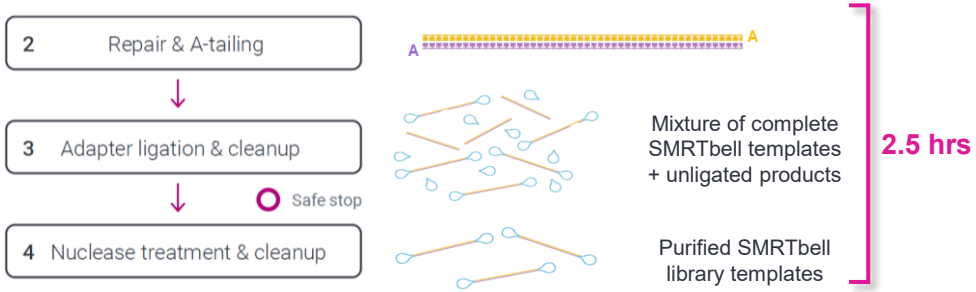
PacBio reference for SMRTbell library construction procedure[1]

Procedure & checklist – Preparing multiplexed amplicon libraries using SMRTbell Prep Kit 3.0 (102-359-000)

PacBio



LongPlex libraries are already fragmented, barcoded, and pooled → thus can be treated as "amplicons" in downstream PacBio library preparation starting at Step 2 (Repair & A-tailing)



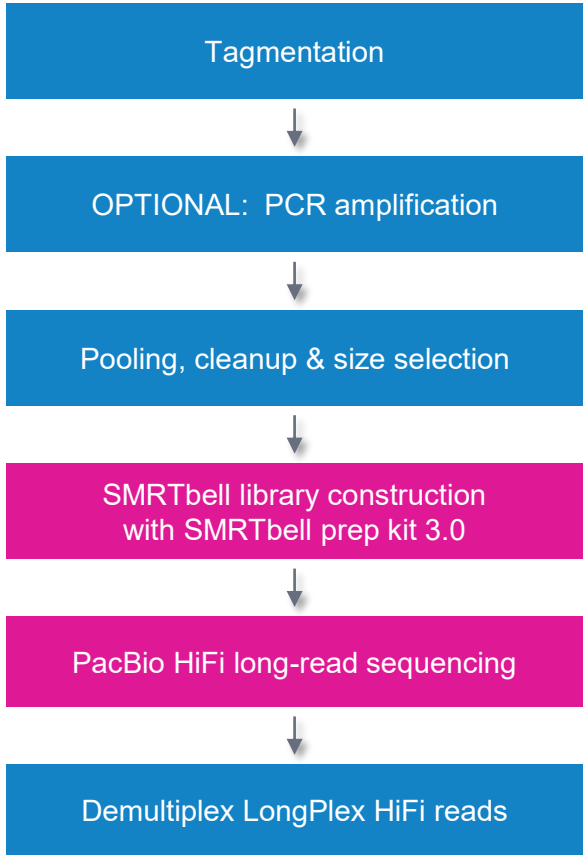
- Indexed LongPlex DNA samples may be pooled for SMRTbell library construction by following PacBio Procedure & checklist – Multiplexed amplicon library preparation using SMRTbell Prep Kit 3.0 (102-359-000)
- Recommended LongPlex input DNA mass for SMRTbell library construction = **1 µg** (up to 2 µg) in 46 µL.
- Multiplexed LongPlex SMRTbell library may be sequenced on a single SMRT Cell using Sequel IIe, Vega or Revio system using 24-hour data acquisition (movie collection) time
- **Note:** Sample demultiplexing[1] should be performed using seqWell demultiplexing workflow:
  - Demultiplexing of PacBio HiFi BAM files obtained by sequencing of LongPlex libraries can be performed with `lima` using specific workflow parameters and barcode manifest files that correspond to transposase-based barcode sequences utilized in the LongPlex kit
  - Demultiplexing workflow scripts and instructions for use can be downloaded and viewed at https://github.com/seqwell/LongPlex

PacBio

[1] For detailed instructions on SMRTbell library preparation, refer to PacBio **Procedure & checklist – Preparing multiplexed amplicon libraries using SMRTbell Prep Kit 3.0** (102-359-000).

HiFi sequencing workflow overview for seqWell LongPlex WGS PCR-plus / PCR-free libraries (cont.)

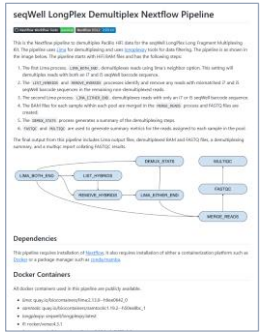
Barcode demultiplexing guidelines for indexed LongPlex HiFi sequencing datasets



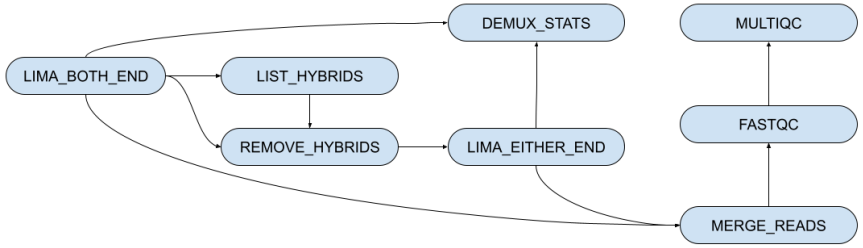
Workflow for sequencing indexed WGS samples generated with seqWell LongPlex Multiplexing Kit.

seqWell reference for LongPlex demultiplexing workflow

seqWell LongPlex Demultiplex Nextflow Pipeline [Link]



Demultiplexing[1] of LongPlex samples containing i5 + i7 unique dual indexes (UDIs) should be performed using **seqWell demultiplexing workflow**



- Demultiplexing of PacBio HiFi BAM files obtained by sequencing of LongPlex libraries can be performed with `lima` using specific workflow parameters and barcode manifest files that correspond to transposase-based barcode sequences utilized in the LongPlex kit
- Demultiplexing workflow scripts and instructions for use can be downloaded and viewed at https://github.com/seqwell/LongPlex
  - **Note:** Standard lima settings look for fragments that have an i7/P7 adapter on one side and an i5/P5 adapter on the other. However, due to the nature of transposase-based fragmentation and tagging with LongPlex workflows, there is some proportion of fragments that will have P5-P5 and P7-P7 adapter instead.
  - While PCR amplification will enrich for P7-P5 ends, there is still a proportion of P5-P5 and P7-P7 fragments that varies based on PCR efficiency, etc. Adjusting the settings in lima to look for fragments from all 3 populations (P7-P5, P5-P5, and P7-P7) will increase the total yield of properly demultiplexed reads
- Final output from this pipeline includes Lima output files, demultiplexed BAM and FASTQ files, a demultiplexing summary, and a multiqc report collating FASTQC results.

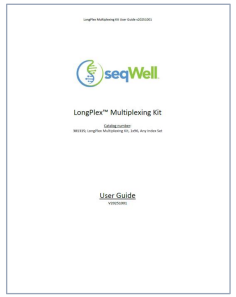
[1] To demultiplex LongPlex samples barcoded with SMRTbell adapter index plate 96A/B/C/D, configure SMRT Link run design to specify **Sample is indexed = YES** to enable on-instrument demultiplexing. PacBio indexed SMRTbell adapters may be used to increase the number of samples to be pooled on a single SMRT Cell. Combining 96 LongPlex UDIs + 96 PacBio barcoded SMRTbell adapters provides a theoretical maximum of 9,216 samples that can be pooled on a single SMRT Cell See **LongPlex Multiplexing Kit User Guide** (LP20251001) and **FAQs** (LP080824).

Example library QC results for seqWell LongPlex microbial WGS libraries prepared with SMRTbell prep kit 3.0

SMRTbell library DNA sizing and library construction yield QC metrics

seqWell LongPlex kit procedure

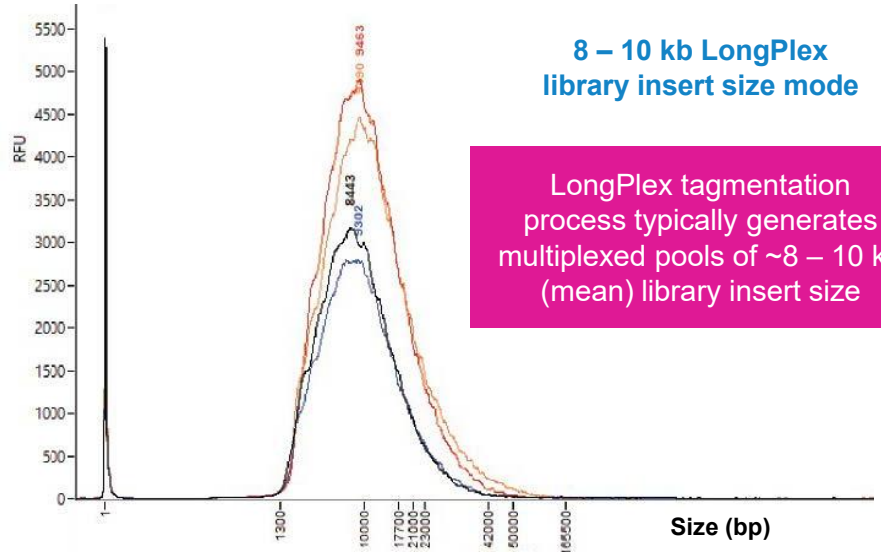
LongPlex Multiplexing Kit User Guide (LP20251001)



PacBio SMRTbell library construction procedure

Procedure & checklist – Preparing multiplexed amplicon libraries using SMRTbell Prep Kit 3.0 (102-359-000)

Femto Pulse DNA sizing QC electropherogram



8 – 10 kb LongPlex library insert size mode

LongPlex tagmentation process typically generates multiplexed pools of ~8 – 10 kb (mean) library insert size

Per-sample input gDNA for LongPlex tagmentation[1]	**250 – 500 ng**
Post-LongPlex tagged DNA pooling & size selection (%) [24-plex][2]	1965 ng (22%)
Total sample input DNA for SPK 3.0 SMRTbell library prep [24-plex][3]	**1965 ng**
Post-SPK 3.0 nuclease treatment and cleanup (%) [24-plex]	467 ng (24%)

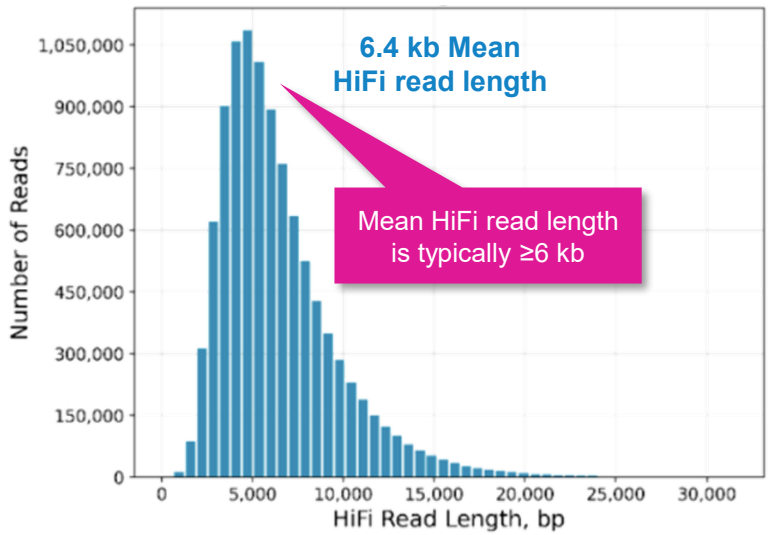
Top: Electropherograms show fragment sizes for four (4) 24-plex pools following LongPlex and SMRTbell library prep processing as assessed using Agilent Femto Pulse gDNA 165 kb Analysis kit. **Bottom:** Example recovery yields for LongPlex tagmentation and SPK 3.0 SMRTbell library prep workflows.

[1] In these studies, three DNA input levels were tested (250, 375, and 500 ng) in quadruplicate on 8 microbial species.
[2] Following tagging reaction, 24 samples were pooled and underwent purification and size selection according to seqWell ***LongPlex Multiplexing Kit User Guide***, creating four 24-plex LongPlex long fragment pools labeled with seqWell UDIs.
[3] Each of the four 24-plex LongPlex pools independently underwent SMRTbell library preparation following PacBio ***Procedure & checklist – Preparing multiplexed amplicon libraries using SMRTbell Prep Kit 3.0***.

Example seqWell LongPlex microbial WGS library performance

96-plex LongPlex example sequencing performance data for microbial WGS samples [Revio system][1]

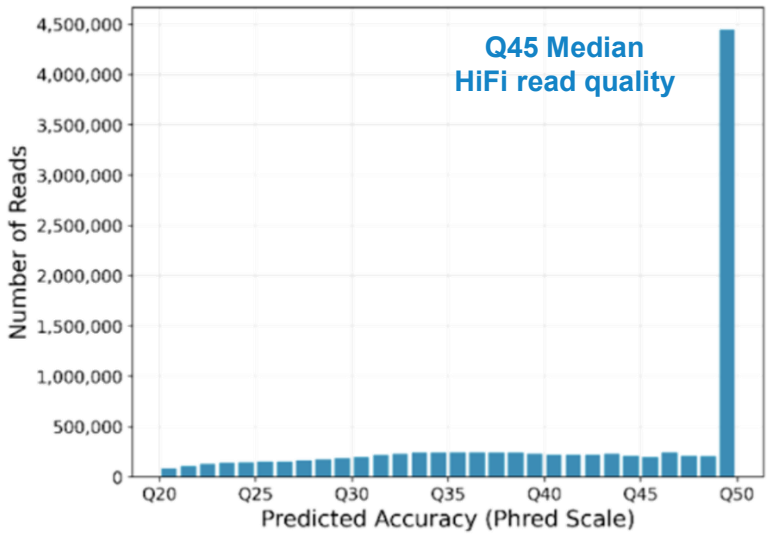
HiFi read length



HiFi Reads	10.1 Million
HiFi Base Yield	64.6 Gb
Mean HiFi Read Length	6.37 kb

For LongPlex microbial WGS libraries, per-Revio SMRT Cell HiFi read counts were typically ~10 Million depending on the final library insert size and sample loading performance.

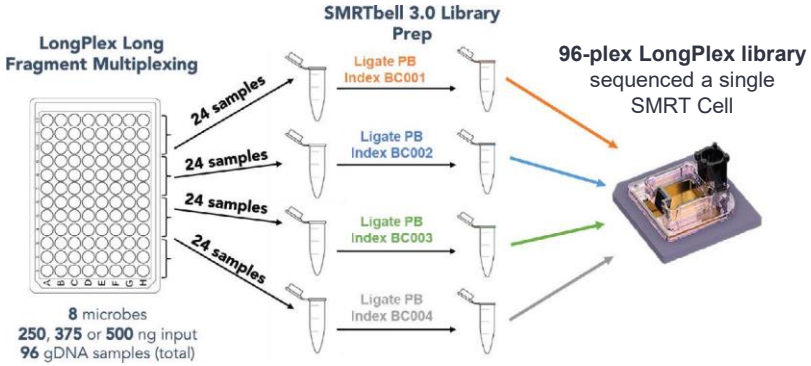
HiFi read quality



Median HiFi Read Quality	Q45

For LongPlex microbial WGS libraries, median HiFi read quality values were typically ~Q45 or higher depending on the final library insert size and sample loading performance.

UDI barcode demultiplexing



LongPlex uses unique dual indexes (UDIs) to barcode individual DNA samples. Barcoded samples are pooled and indexed SMRTbell adapters (e.g., BC001 – BC004) are added *via* SMRTbell library prep workflow.

24-plex A – UDI demultiplexing yield	83.8%
24-plex B – UDI demultiplexing yield	84.6%
24-plex C – UDI demultiplexing yield	86.5%
24-plex D – UDI demultiplexing yield	85.5%

Each 24-plex library pool yielded a unique dual-index (UDI) demultiplexing rate >80%. The CV of the barcoded HiFi read count between samples within each 24-plex ranged from 22% to 28%. seqWell demultiplexing workflow scripts and instructions for use can be found at https://github.com/seqwell/LongPlex.

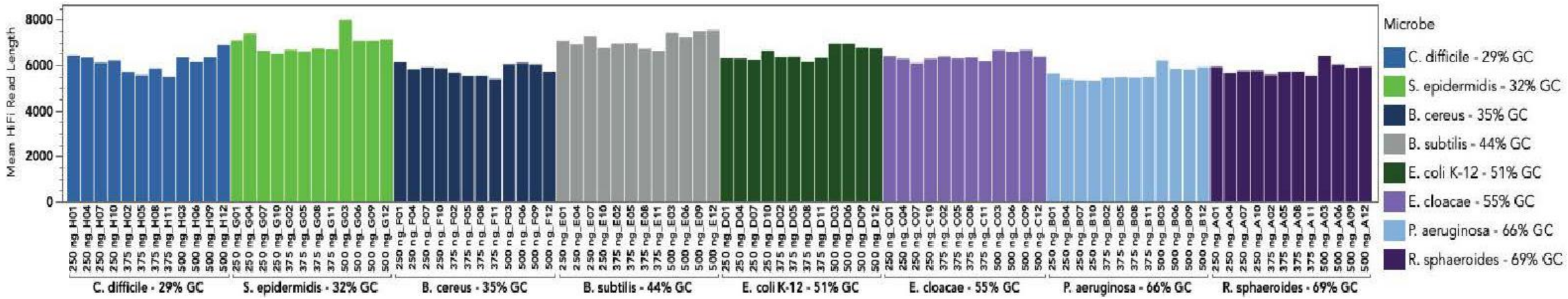
[1] Four 24-plex LongPlex pools were independently processed using four unique SMRTbell indexed adapters resulting in four 24-plex barcoded SMRTbell libraries. The four 24-plex barcoded libraries were pooled prior to loading on a single Revio SMRT Cell for a 96-plex sequencing run. **Note:** Read lengths, reads/data per SMRT Cell and other sequencing performance results can vary depending on DNA sample quality, insert size, sample loading performance & movie time. Shorter library insert sizes (<5 kb), lower DNA quality samples, and suboptimal sample loading performance may result in lower data yields per SMRT Cell.

Example seqWell LongPlex microbial WGS library performance (cont.)

96-plex LongPlex example coverage & assembly performance data for microbial WGS samples [Revio system][1]

Summary of coverage and maximum contig length for 8 different organisms varied in genome size and GC content.

Organism	%GC	Genome size (Mb)	Genome contig count	Assembled contig count	Max contig size (Mb)	Max coverage
Clostridioides difficile	29	4.3	3 (All circularized)	3	4.1	113
Staphylococcus epidermidis	32	2.6	5 (1 circularized)	3	2.6	289
Bacillus cereus	35	5.4	2 (1 circularized)	2	5.4	55
Bacillus subtilis	44	4.2	1 (All circularized)	1	4.2	180
Escherichia coli	51	4.6	2 (All circularized)	2	4.6	108
Enterobacter cloacae	55	5.3	4 (3 circularized)	3	5.3	107
Pseudomonas aeruginosa	66	6.8	1 (All circularized)	1	6.8	73
Rhodobacter sphaeroides	69	4.5	10 (6 circularized)	10	2.4	118



Uniformity of coverage upon sample multiplexing. Uniformity of mean HiFi read length of the four 24-plex across 8 different microbes with GC content ranging from 29 – 69%. A uniform coverage helps produce more accurate and contiguous genome assemblies by providing consistent data across the entire genome.

EpiCypher Fiber-seq assay workflow overview

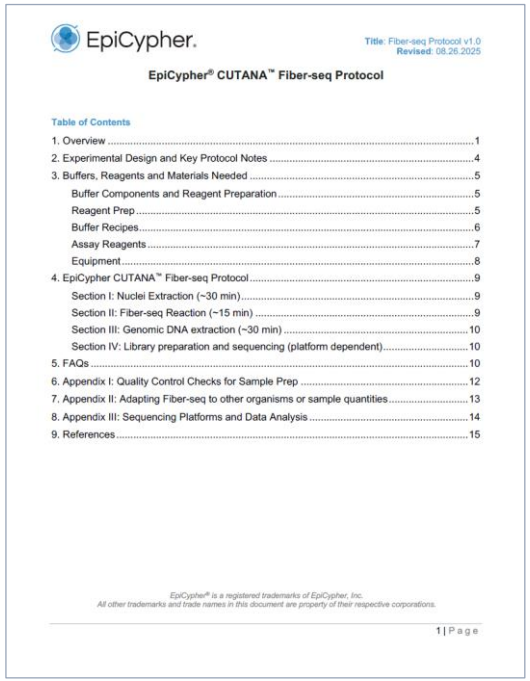
Fiber-seq assay using EpiCypher Fiber-seq assay kit and PacBio SMRTbell prep kit 3.0: Getting started

Application-specific educational literature

EpiCypher / PacBio literature website [Link]

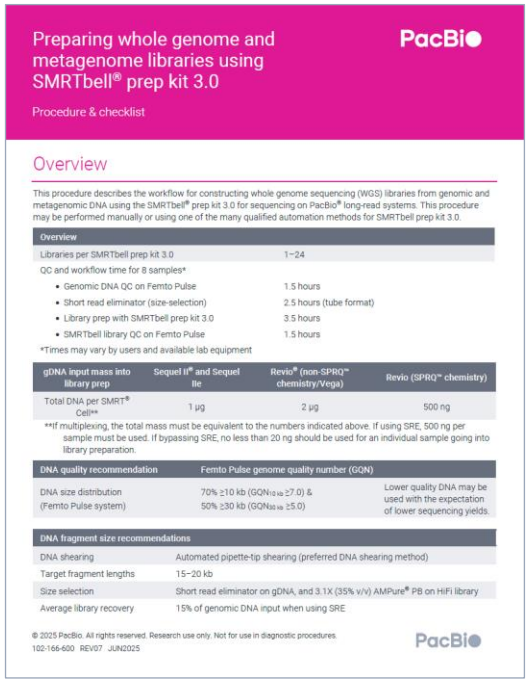
Application-specific brochures, informational guides and other product literature containing best practices recommendations for library preparation and data analysis workflows.

Application-specific protocol documentation



CUTANA™ Fiber-seq Protocol [EpiCypher]

Technical documentation containing EpiCypher CUTANA Fiber-seq kit protocol details.



Procedure & checklist – Preparing whole genome and metagenome libraries using SMRTbell prep kit 3.0 (102-166-600) [PacBio]

Technical documentation containing PacBio SMRTbell library construction details.

Application-specific technical overviews

Technical overview: Whole genome and metagenome library preparation using SMRTbell prep kit 3.0 (102-390-900)

Technical overview presentations describe sample preparation details for constructing HiFi libraries for specific applications. Example sequencing performance data for a given application are also summarized.

HiFi sequencing workflow overview for Fiber-seq libraries prepared with EpiCypher Fiber-seq assay kit and PacBio SMRTbell prep kit 3.0

Follow EpiCypher CUTANA Fiber-seq Protocol [Link] to generate Fiber-seq DNA samples suitable for SMRTbell library construction and HiFi sequencing on PacBio long-read systems

Nuclei extraction

↓

Fiber-seq reaction

↓

Genomic DNA extraction

↓

SMRTbell library construction with SMRTbell prep kit 3.0

↓

PacBio HiFi long-read sequencing

↓

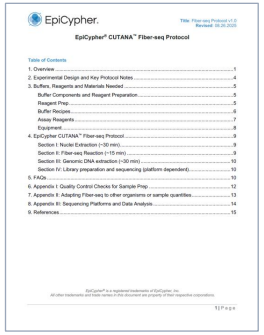
Data analysis using third-party software (e.g., `fibertools`, FIRE)

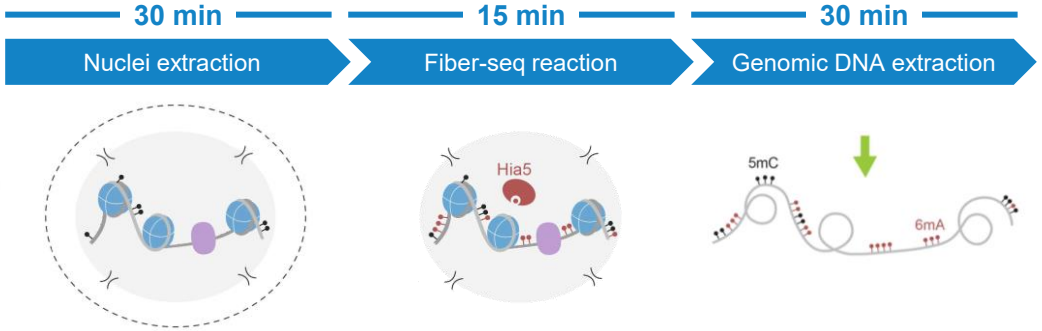
Workflow for sequencing Fiber-seq samples generated with EpiCypher Fiber-seq assay procedure.

EpiCypher Fiber-seq assay procedure[1]

EpiCypher CUTANA Fiber-seq Protocol [Link]

EpiCypher





Fiber-seq assay	Materials and methods
Protocol reference	EpiCypher CUTANA Fiber-seq Protocol [Link]
Reagent kit	EpiCypher CUTANA Fiber-seq Kit (# 14-2001-8RXN or 14-2001-24RXN)
Recommended sample types	Eukaryotic organisms without endogenous adenine methylation
Sample input requirements	Recommended input is 1,000,000 human nuclei per Hia5 reaction → Harvest 2,000,000 cells per reaction (plus 10% excess when possible) to account for sample loss during nuclei prep
DNA extraction method	EpiCypher CUTANA Fiber-seq Kit includes Monarch Spin gDNA Extraction Kit (NEB) for isolating genomic DNA from Fiber-seq-treated nuclei samples[2]

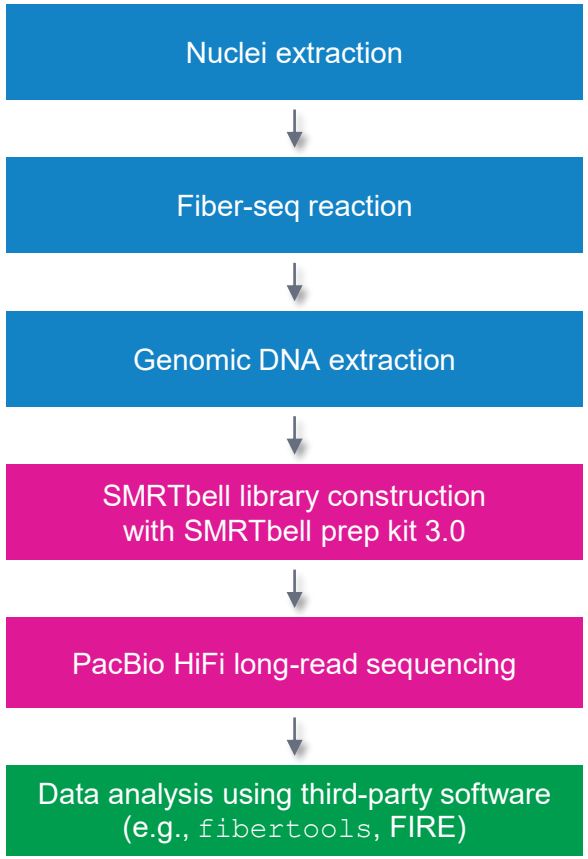
- **EpiCypher CUTANA Fiber-seq Kit** (# 14-2001-8RXN or 14-2001-24RXN) includes all key reagents needed to perform nuclei extraction, Fiber-seq reaction and genomic DNA extraction (**Note:** SAM ((S-adenosylmethionine) reagent is highly sensitive to freeze-thaw cycles and may be inactivated over time. To avoid this concern, make single-use aliquots to avoid freeze/thaws.)
- Genomic DNA extracted from Fiber-seq-treated nuclei can be processed in a single SMRTbell library prep for downstream sequencing on PacBio long-read systems

PacBio

1 For detailed instructions on preparing Fiber-seq samples, refer to **EpiCypher CUTANA Fiber-seq Protocol [Link]**.
2 Fiber-seq genomic DNA samples extracted with either NEB Monarch Spin gDNA kit or PacBio Nanobind kits are compatible with PacBio HiFi sequencing.

HiFi sequencing workflow overview for Fiber-seq libraries prepared with EpiCypher Fiber-seq assay kit and PacBio SMRTbell prep kit 3.0 (cont.)

Fiber-seq DNA samples may be used as input for SMRTbell library construction by following PacBio *Procedure & checklist – Preparing whole genome and metagenome libraries using SMRTbell Prep Kit 3.0* (102-166-600)

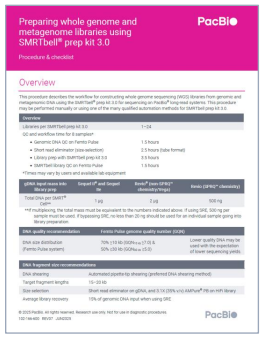


Workflow for sequencing Fiber-seq samples generated with EpiCypher Fiber-seq assay procedure.

PacBio SMRTbell library construction procedure[1]

Procedure & checklist – Preparing whole genome and metagenome libraries using SMRTbell prep kit 3.0 (102-166-600)

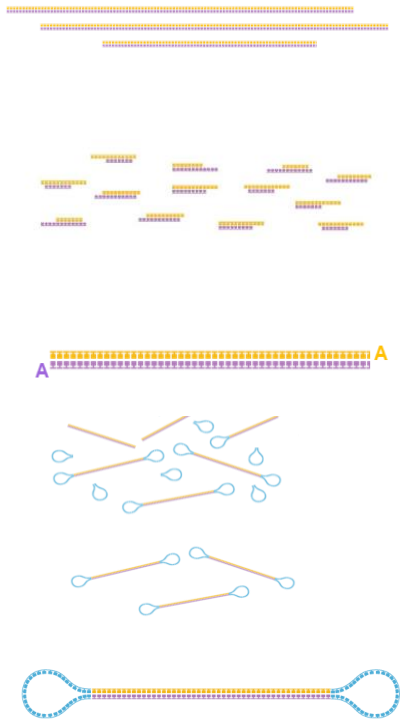
PacBio



- Recommended Fiber-seq DNA sample mass for SMRTbell library construction = **500 ng for Revio system** or **2 µg for Vega system**
- Fiber-seq library may be sequenced on Vega or Revio system using **24-hour** data acquisition (movie collection) time
- For human genome samples, we recommend generating **90 Gb of sequencing data per Fiber-seq sample** to achieve approximately **30X** genome coverage.

SMRTbell library construction procedure [1 day]

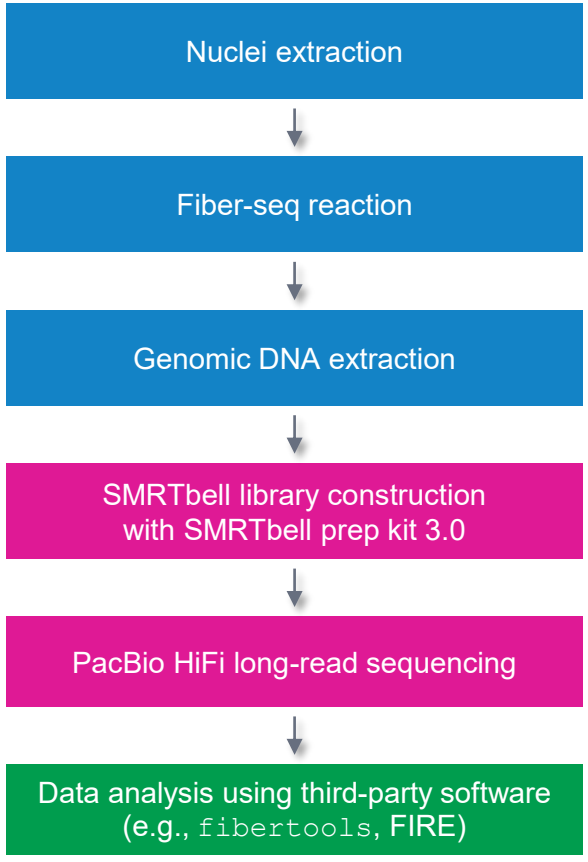
1. Short Read Eliminator (SRE)
   - Safe stop
2. DNA shearing
   - Safe stop
3. Post-shearing cleanup
   - Safe stop
4. Repair & A-tailing
5. Adapter ligation & cleanup
   - Safe stop
6. Nuclease treatment
7. AMPure PB cleanup and size selection



[1] For detailed instructions on SMRTbell library preparation, refer to **Procedure & checklist – Preparing whole genome and metagenome libraries using SMRTbell Prep Kit 3.0** (102-166-600)

HiFi sequencing workflow overview for Fiber-seq libraries prepared with EpiCypher Fiber-seq assay kit and PacBio SMRTbell prep kit 3.0 (cont.)

Analyze Fiber-seq HiFi sequencing datasets using Fiber-seq software tools available on GitHub[1]



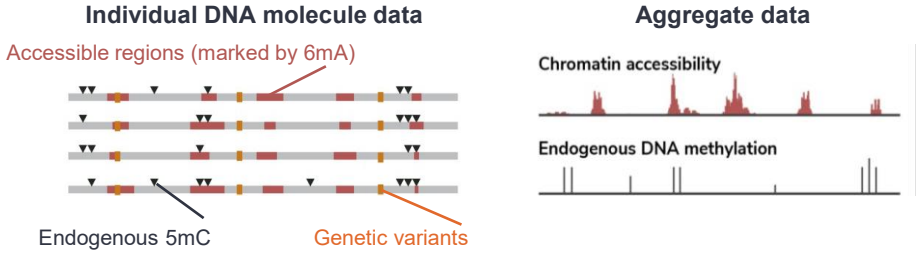
Workflow for sequencing Fiber-seq samples generated with EpiCypher Fiber-seq assay procedure.

GitHub Fiber-seq analysis tools

Fiber-seq computational guide [Link]

GitHub

Use **Fiber-seq tools** to classify methylation-sensitive patches (MSPs) and Fiber-seq Inferred Regulatory Elements (FIREs) from each HiFi read



FIBER TOOLS

- `Fibertools`[2] software tool provides Fiber-seq-specific quality control metrics, detection of accessible DNA regions (methylation-sensitive patches), and nucleosome positioning
  - **Methyltransferase sensitive patch (MSP):** A stretch of a Fiber-seq read that has a high density of m6A sites. Specifically, it is defined as a region that is inferred to not be occluded by a nucleosome.
- **Fiber-seq Inferred Regulatory Elements**[3] software tool is a method for identifying regulatory elements on individual fibers and peak calling
  - **Fiber-seq Inferred Regulatory Element (FIRE):** An MSP that is inferred to be a regulatory element based on features of the MSP including m6A density.

1 For Fiber-seq data analysis workflow recommendations, refer to ***Fiber-seq Computational Guide*** [Link] available on GitHub.
2 Jha, A. et al. (2024) Genome Res. 34:1976-1986 doi:10.1101/gr.279095.124.
3 Vollger, M. et al. (2025) BioRxiv preprint doi: 0.1101/2024.06.14.599122.

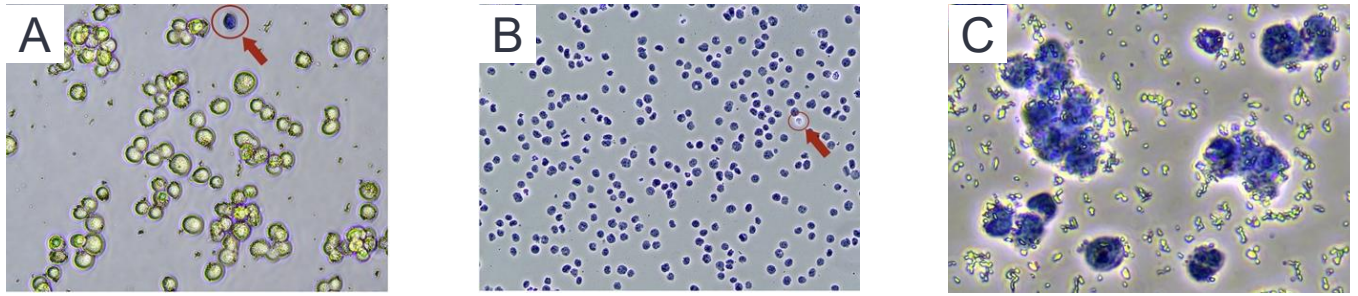
PacBio

Fiber-seq sample input requirements for EpiCypher Fiber-seq assay kit

Recommended sample types, input amount and quality for Fiber-seq experiments

Recommended sample input amount and quality

- Recommended input for Fiber-seq assays is **1,000,000 freshly isolated, native (unfixed) nuclei per Hia-5 reaction**
- Harvest 2,000,000 cells per reaction (plus 10% excess when possible) to account for sample loss during nuclei prep
- To confirm high quality sample prep, perform the Trypan Blue staining QC procedure in Appendix I: Quality Control Checks for Sample Prep
  - Samples are assessed at two points: At initial cell harvest and after nuclei isolation
  - These quality control checks can improve experimental success
- **Note:** EpiCypher Fiber-seq protocol is designed for using fresh, native human suspension cells as input material[1,2]



Representative images of cell and nuclei samples for Fiber-seq. Samples were stained with Trypan Blue and visualized under brightfield microscope. **(A)** Cells before nuclei extraction. A dead cell (blue; Trypan positive) is circled in red, whereas the remaining live cells are bright white and round. **(B)** Nuclei after extraction. An intact cell (Trypan negative) is circled in red, whereas isolated nuclei are Trypan stained. . **(C) Note:** Isolated nuclei can be "sticky" and create small clusters (this is acceptable).

Recommended sample types

- Recommended sample types for the Fiber-seq assay include eukaryotic organisms **without** endogenous adenine methylation
- Fiber-seq is **not** recommended for organisms known to naturally contain high levels of 6mA, such as bacteria
- Unlabelled libraries are generally not necessary as a negative control unless you suspect the presence of endogenous 6mA in your model system
  - However, if background labeling is a concern, we suggest including a Hia5 enzyme-negative control in parallel to assess endogenous 6mA levels and distinguish true accessibility signals from background

Note: Some cell types are more challenging for isolating high-quality nuclei

Easy to isolate cell types are usually:

- Suspension or semi-adherent cell lines
- Cells with fewer cellular interactions and more surface area exposed to the nuclei extraction buffer

Difficult to isolate cell types are usually:

- Adherent cell lines (e.g., fibroblast/epithelial origin)
- Invasive cancer cell lines
- Cells that grow in a 3D structure
- Cell types with many cell-cell interactions

PacBio

[1] **Note:** If starting with whole tissue samples, they must first be broken down into a single-nuclei suspension. Once nuclei are isolated, they can be input into the Fiber-seq protocol workflow.

[2] Flash frozen nuclei and crosslinked nuclei are also compatible with EpiCypher Fiber-seq protocol. Flash frozen nuclei show %6mA levels ~1-2% higher than fresh controls. Crosslinked nuclei typically have 1-3% less %6mA levels compared to native controls (it is recommended to crosslink cells (protocol) first, and then isolate nuclei.)

Fiber-seq sample input requirements for EpiCypher Fiber-seq assay kit (cont.)

Adapting Fiber-seq assay input requirements to support other non-human organisms or sample quantities

- The Fiber-seq protocol can be adjusted to support the following use cases
  - Analysis using sample input amounts other than the standard 1,000,000 human nuclei (or human nuclei equivalents)
  - Analysis of organisms with genome sizes that differ significantly from human (~3.2 Gb)

Scaling Fiber-seq reactions to support different sample input amounts

- The standard Fiber-seq labeling reaction (60 µL) is optimized to achieve ~6% 6mA labeling in 1,000,000 human nuclei
- The Hia5 reaction generally follows Michaelis-Menten kinetics, meaning it scales proportionally with the amount of substrate (i.e., DNA)
- If using more than the equivalent of 1,000,000 human nuclei (e.g., more than ~1,200,000 mouse nuclei) in a Fiber-seq reaction, the reaction volume and reagents should be increased proportionally to the additional nuclei to be used
  - **Example:** For 2,000,000 human nuclei → Double the reaction volume and all reagents used
- **Important:** We **do not** recommend scaling the reaction down below 1,000,000 human nuclei (or equivalent DNA content)
  - Reducing the number of input nuclei used may result in insufficient DNA recovery, making it difficult or impossible to load your sample onto PacBio long-read sequencing systems at a suitable on-plate loading concentration (OPLC)

Adjusting Fiber-seq sample input amounts to support different genome sizes

- For organisms with smaller genomes, higher nuclei input amounts are needed to match the total DNA content of 1,000,000 human nuclei
  - This ensures consistent labeling and yields enough DNA for long-read sequencing
  - **Recommendation:** Keep the reaction volume and enzyme concentration the same but adjust the number of input nuclei to match the total DNA input of 1,000,000 human nuclei.

$$\frac{[\text{Human Genome in } Mb]}{[\text{New Genome in Mb}]} \text{ x 1,000,000 = The number of nuclei required per Hia5 reaction}$$

Organism	Genome size	Genome size relative to human	Recommended Nuclei per 60 µL Reaction
Human (Hs)	3,200 Mb	100%	1,000,000
Mouse (Mm)	2,700 Mb	84%	1,185,000
Drosophila (Dm)	143.7 Mb	4.5%	22,270,000
Yeast (Sc)	12.07 Mb	0.38%	265,120,000

Fiber-seq assay optimization

Recommended conditions for optimal Fiber-seq assay performance

- For optimal performance, we recommend using Fiber-seq reaction conditions that will result in labeling **~5-7%** of the genome with 6mA
  - This is validated to effectively resolve nucleosome position and protein footprinting
  - Outside this range can still be useful (**Note:** Less than 5% will give poor resolution and greater than 7% will artificially decrease footprint sizes)
- EpiCypher CUTANA Fiber-seq protocol (Link) is optimized to achieve ~6% 6mA labeling, which supports confident detection of accessible chromatin and high-resolution protein footprinting
  - → Significant deviation from this target may indicate a need for further protocol optimization before proceeding to deeper sequencing
- If needed, a 3-point sample input titration may be performed as described below to determine the conditions for optimal 6mA labeling

Fiber-seq reaction optimization procedure [optional]

Step 1: Perform a 3-point Fiber-seq sample input titration

i. 500,000 input nuclei (= 0.5-fold more than the standard input)
ii. 1,000,000 input nuclei (= Standard input)
iii. 2,000,000 input nuclei (= 2-fold more than the standard input)

Step 2: Perform a shallow sequencing run (~1x coverage)

Use the 6mA labeling results to determine how many nuclei are needed for the final reaction to label ~5-7% of the genome with 6mA

Notes

- Run the final Fiber-seq assay experiment using reaction conditions determined by your sample input titration results
- For precious samples, a similar cell type can be used in the titration reactions
- A titration experiment can also be performed with 0.5-fold, standard, and 2-fold concentrations of Hia5 enzyme

Fiber-seq sample preparation QC workflow recommendations

For every Fiber-seq experiment, always check the quality of starting input cell samples and isolated nuclei

Cell harvesting and QC

- Spin down input cell sample and resuspend cells in 1X PBS
- Stain cells with 0.4% Trypan Blue viability indicator stain
- Evaluate viable cell count & sample integrity using Hemacytometer & microscope tools

Nuclei extraction and QC

- Spin down required number of input cells and resuspend in 1X PBS
- Spin down cells and resuspend in Nuclei Extraction Buffer (10 min on ice)
- Spin down extracted nuclei and resuspend in 1X Reaction Buffer
- Evaluate nuclei count & sample integrity using Hemacytometer & microscope tools

Protocol: Harvesting cells and nuclei for Fiber-seq with built-in quality control checks[1]

We recommend performing these quality control checks for <u>every</u> Fiber-seq experiment

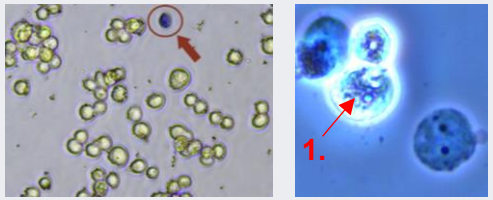
→ This is a **key step** in the Fiber-seq protocol, and if nuclei are of poor quality, DNA yields may be reduced and Fiber-seq data quality may be affected

Sample	Figure reference	Success metric (Trypan Blue staining)	Troubleshooting tips
Input cells	Figure A	Cells should be bright white (Trypan Blue excluded), round, unclumped, and ideally show >80% viability. Excess dead cells may reduce DNA yields and impact Fiber-seq data quality.	Optimize cell culture conditions; use fresh media, evaluate potential contamination issues.
Extracted nuclei	Figure B	Nuclei should be >95% Trypan Blue positive and unclumped.	Monitor cells during nuclei extraction by Trypan Blue staining to optimize incubation time. Increase spin time if you are losing nuclei during spins (keep at 600 x g).

Good quality samples are critical to Fiber-seq success

To obtain good quality nuclei, you need good quality cells as input → ≥80% viable at harvest is ideal

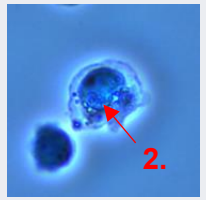
Before nuclei extraction



Healthy cells do not stain with Trypan Blue. Red arrow indicates a dead cell.

1. Alive, healthy cell with non-permeable membrane (ideally ≥80% viable cells)

Failed nuclei extraction



2. Permeable cell (dead, dying) with membrane remnants still attached

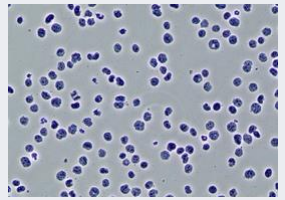
Questions about isolating nuclei or sample quality? Contact EpiCypher at techsupport@epicypher.com

Include the following info:

- Input cell type
- Extraction protocol used
- Images of the sample

Successful nuclei extraction

3. Swollen nuclei[2]
4. Completely isolated nuclei



Isolated nuclei should be >95% Trypan Blue positive and mostly unclumped.

PacBio

[1] The Fiber-seq assay protocol starts with the Nuclei Isolation Protocol step (~30-minute protocol using CUTANA™ Nuclei Extraction Buffer is recommended). For detailed instructions on cell harvesting and nuclei isolation procedure, to **EpiCypher CUTANA Fiber-seq Protocol [Link]**.

[2] Isolated nuclei can swell in solution leading to the appearance of different-sized nuclei. Swollen nuclei are acceptable when present in a minority of the total population of isolated nuclei.

Fiber-seq assay QC workflow recommendations

While there is currently no supported method to directly verify successful 6mA labeling prior to sequencing, the actions below can help assess whether your Fiber-seq experiment is on track before committing to a deeper coverage sequencing run

Fiber-seq assay QC workflow

Perform Fiber-seq reaction

Perform Fiber-seq reaction and genomic DNA extraction as described in EpiCypher CUTANA Fiber-seq Protocol [Link]

Evaluate total DNA yield after genomic DNA extraction

Qubit 4 fluorometer (Thermo Fisher Scientific)

Evaluate total DNA yield after genomic DNA extraction using a Qubit assay

- DNA recovery will vary depending on sample type and nuclei quantity. This protocol was optimized using 1,000,000 human K562 nuclei, which typically yield 3–4 µg of DNA when extracted with the New England Biolabs® (NEB®) Monarch® Spin gDNA Extraction Kit (Cat. No. T3010S / T3010L).
- If your yield is significantly lower, it may affect library prep and sequencing success.

Evaluate genomic DNA quality

Femto Pulse system (Agilent Technologies)

Assess genomic DNA quality using a Femto Pulse system or other appropriate DNA sizing tool

- High molecular weight DNA should show a dominant band or peak >60 kb, minimal smearing, and a high Genomic Quality Number (GQN) or DNA Integrity Number (DIN) or other equivalent metric.
- Lower quality DNA may be used with the expectation of lower HiFi sequencing data yields and lower Fiber-seq data quality

Perform a shallow sequencing run to estimate 6mA labeling efficiency

Revio or Vega system (PacBio)

Performing a shallow sequencing run (e.g., 5–10× genome coverage) to estimate 6mA labeling efficiency

- Shallow sequencing run results can indicate if further optimization is needed before committing to deeper sequencing.
- This Fiber-seq protocol is optimized to achieve ~6% 6mA labeling, which supports confident detection of accessible chromatin and high-resolution protein footprinting.
- Significant deviation from this target may indicate a need for further protocol optimization before proceeding to deeper sequencing.

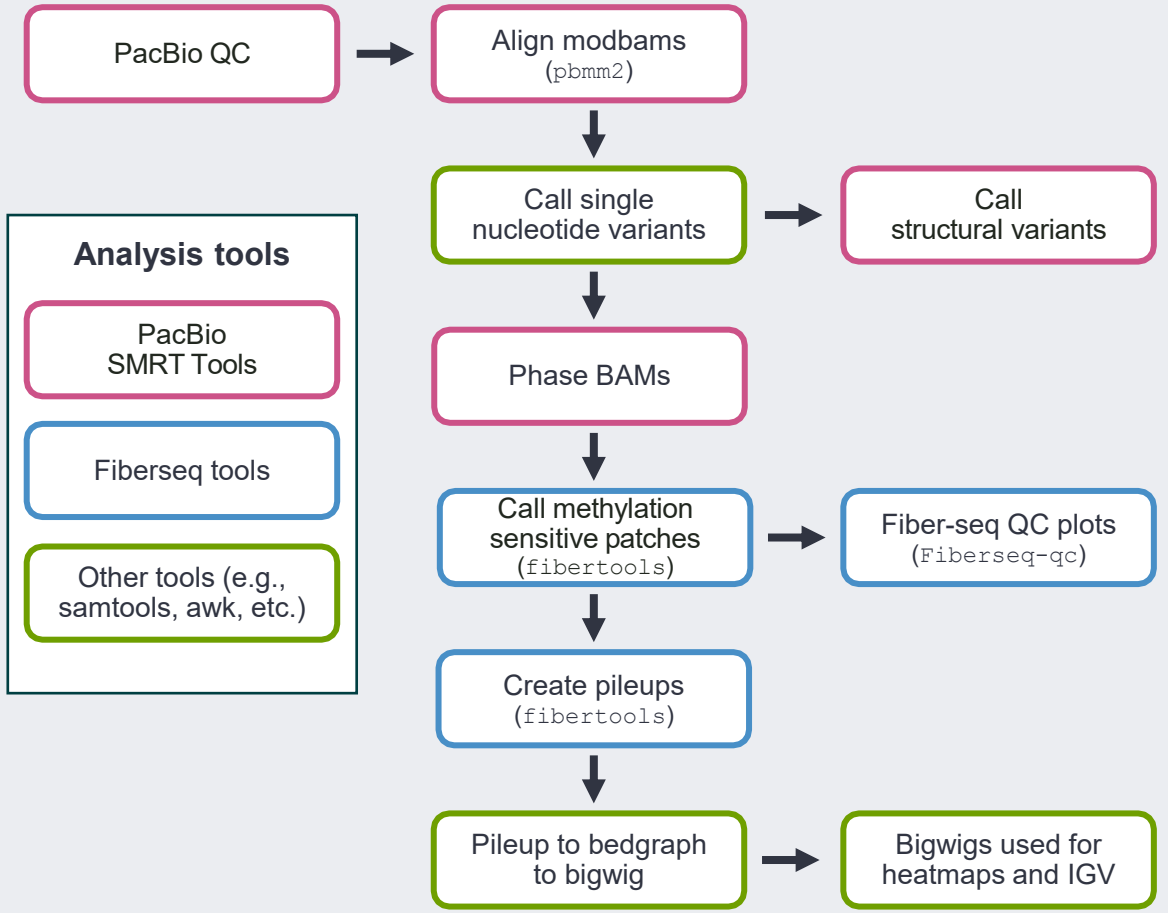
Fiber-seq data analysis QC workflow recommendations

Data analysis QC can be performed using PacBio SMRT Link and other third-party tools available through GitHub

- Key metrics to evaluate in SMRT Link run details QC reports (per SMRT Cell)
  - HiFi reads: ~10M
  - HiFi yield: 90-100Gb
  - Read Length (mean): 15-20Kb
  - P1 Productivity: >60%
  - Read Quality: >Q30
- **Note:** SMRT Link run details QC report displays %6mA metrics on a per-SMRT Cell basis (not by sample)
- Use PacBio SMRT Tools for read alignment
- Use GitHub Fiberseq tools to analyze (demultiplexed) Fiber-seq BAM data files
  - Use `fiberseq-qc` to generate key Fiber-seq data QC metrics
    - 6mA per read
    - Methyltransferase sensitive patch (MSP) length
    - Autocorrelation plot
    - Nucleosome Size
  - Use `fibertools` to analyze Fiber-seq data to generate 6mA labeling stats and pileups, and to also infer protein footprints

Refer to Fiberseq GitHub page for guidance on how to use Fiberseq tools for fiber-seq dataset QC evaluation[1]

Generalized pipeline for processing Fiber-seq HiFi sequencing datasets



[1] For detailed instructions on processing Fiber-seq data and performing secondary analysis to identify regulatory elements on individual fibers and peak calling using the FIRE pipeline, refer to the Fiber-seq Computation Guide on GitHub (see Jha, A. et al. (2024) Genome Res. 34:1976-1986 doi:10.1101/gr.279095.124 and Vollger, M. et al. (2025) BioRxiv preprint doi: 0.1101/2024.06.14.599122).

Fiber-seq data analysis QC workflow recommendations (cont.)

Data analysis QC can be performed using PacBio SMRT Link and other third-party tools available through GitHub

Data analysis QC workflow details[1]

Aligning BAMs – Creating a reference
- Use PacBio `pbmm2` to create a custom index
- The .mmi reference output will be used for alignment
- Align BAMS (PacBio includes presets for HiFi data)

Sequencing QC
- First step is to create a PacBio-specific index of BAM file
- After creating the index, you must create a dataset with each individual bam
- This will create individual reports for each barcode, providing per-sample QC

Add- nucleosomes
- Adds MSP/nucleosome calls to aligned BAMs using Fibertools add-nucleosomes

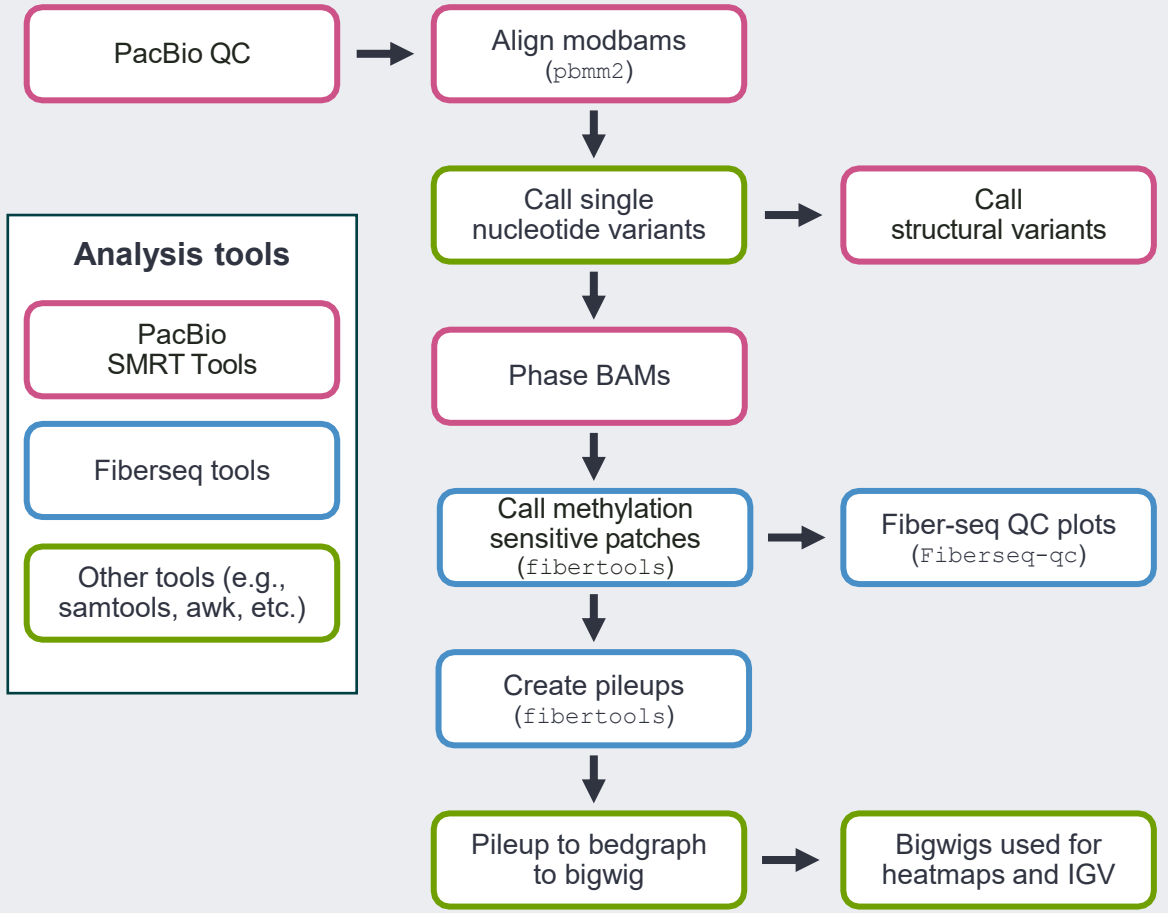
Fiber-seq QC
- Creates a variety of plots focused on assessing the success of the Fiber-seq experiment
- Uses `fiberseq-qc` tool

Create pileups
- Creates a pileup of 6mA, 5mC, and MSPs
- Calculates percentage of coverage of each methylation type
- Can also separate pileup output by haplotype if the input BAM has been phased
- Uses fibertools `ft-pileup` command

Pileups to bedgraph to bigwigs
- First step is to split the pileup output by mark
- The first three columns must always be included, as they mark the genomic coordinates
- The fourth column is then the mark of choice (e.g., 6mA, 5mC, nucleosome calls)

Input bigwigs into IGV or use to create heatmaps

Generalized pipeline for processing Fiber-seq HiFi sequencing datasets

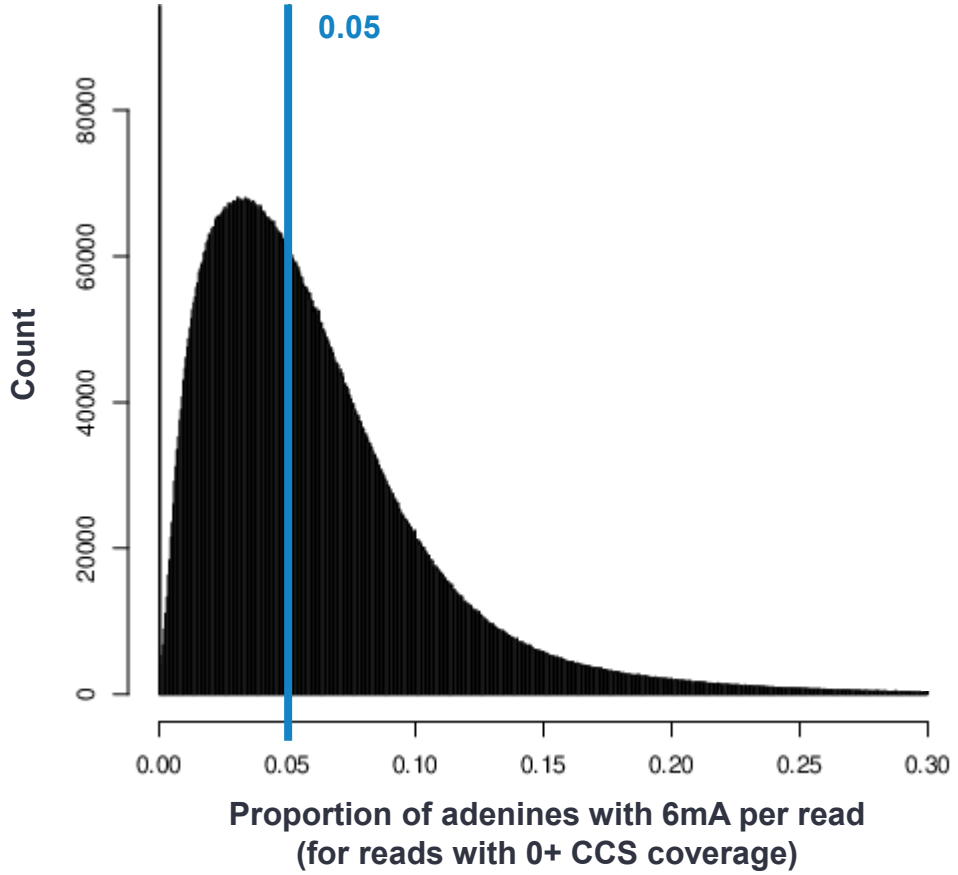


[1] For detailed instructions on processing Fiber-seq data and performing secondary analysis to identify regulatory elements on individual fibers and peak calling using the FIRE pipeline, refer to the Fiber-seq Computation Guide on GitHub (see Jha, A. et al. (2024) Genome Res. 34:1976-1986 doi:10.1101/gr.279095.124 and Vollger, M. et al. (2025) BioRxiv preprint doi: 0.1101/2024.06.14.599122).

Fiber-seq data analysis QC workflow recommendations (cont.)

fiberseq-qc analysis of 6mA labeling efficiency

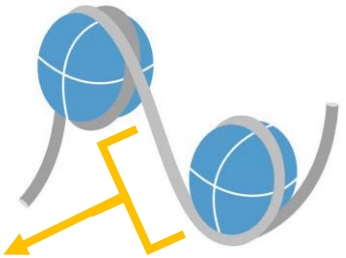
- `fiberseq-qc` calculates the density of %6mA per read
- Median %6mA value should be ~5-7%
- Ideally <1% of reads with no 6mA
- Greater than 1% of reads with no 6mA could be indicative that there was an issue with the 6mA labeling reaction
  - Not enough Hia5 enzyme
  - Too many nuclei
  - Inactive SAM reagent



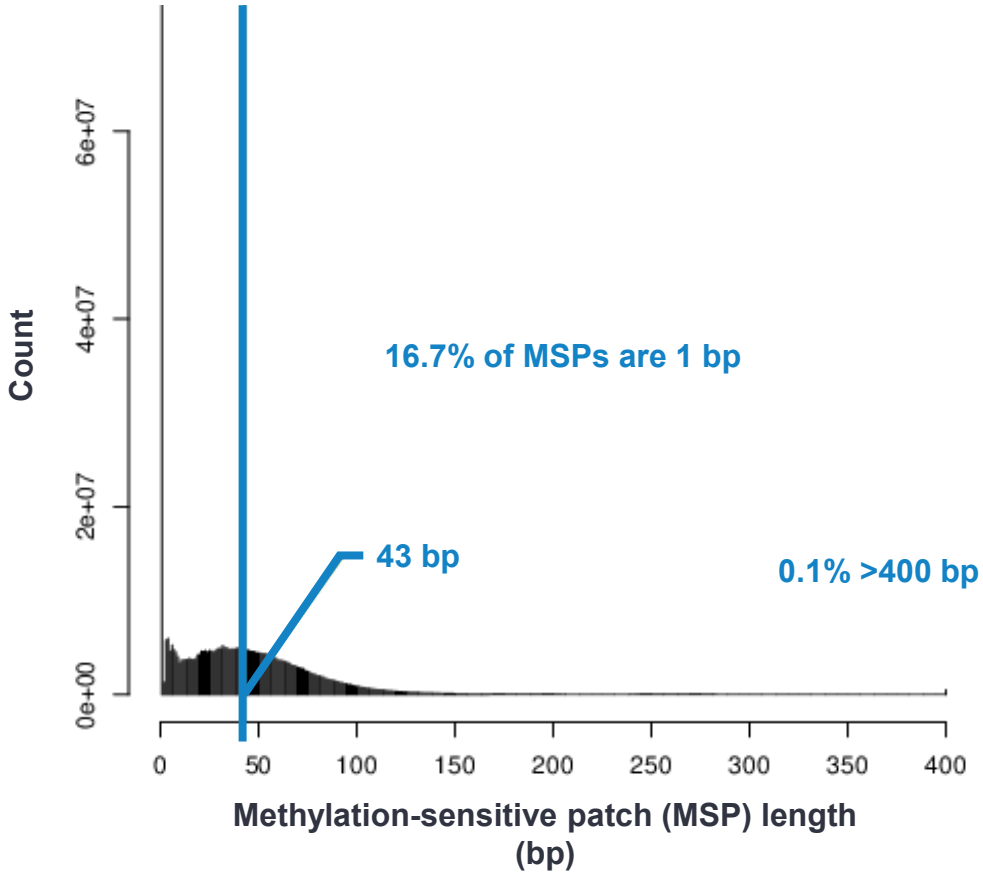
Fiber-seq data analysis QC workflow recommendations (cont.)

fiberseq-qc analysis of methyltransferase-sensitive patch (MSP) length

- `fiberseq-qc` calculates methyltransferase-sensitive patch (MSP) lengths
- A MSP region has high density of 6mA and is inferred to be linker DNA between nucleosomes
- MSP length should correspond to the average length (20-90 bp) of the linker DNA for your species of interest
  - For human samples, expected MSP length is 47 bp
- If median MSP length is outside the expected range, this can be due to poor 6mA labeling
  - Linker DNA length resolution is generally reduced when 6mA labeling efficiency is poor
- There should typically be little to no MSP lengths >400 bp
  - Higher-than-expected MSP lengths suggests a large amount of "naked" or unwrapped DNA molecules



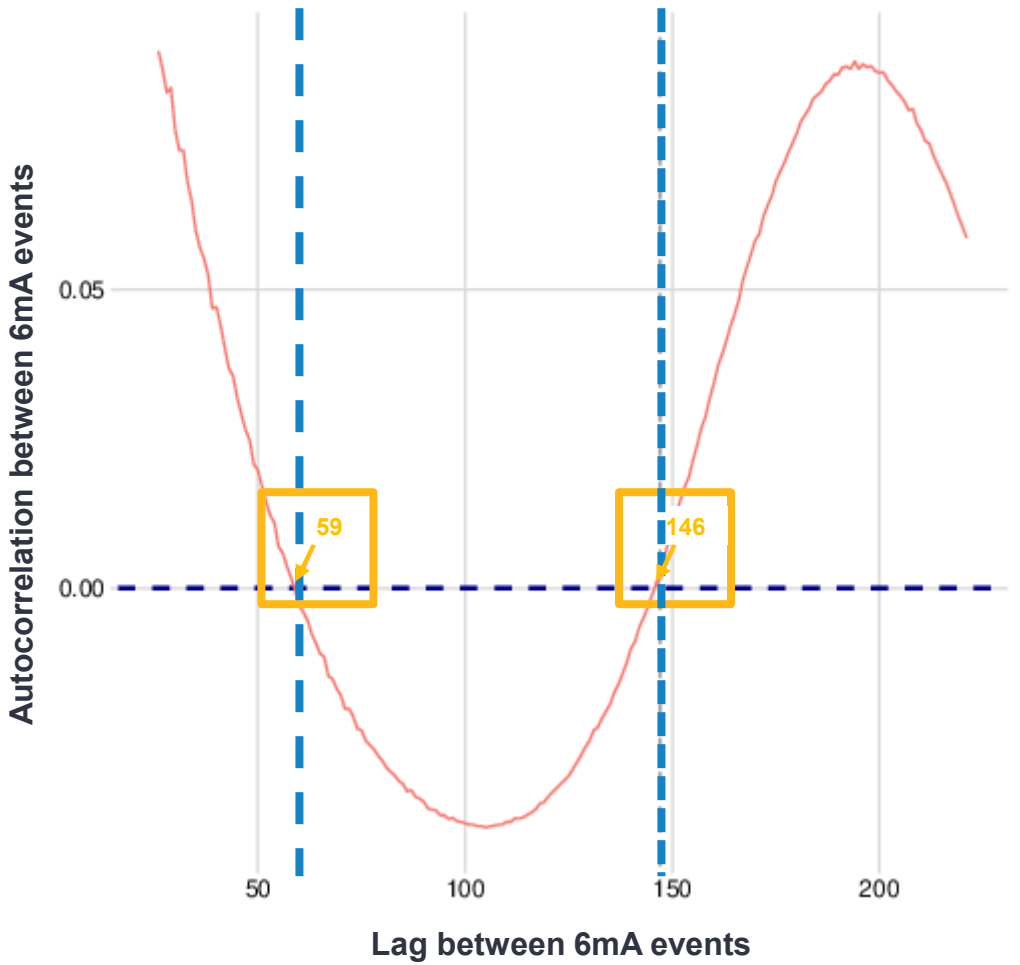
Linker DNA captured in the MSP



Fiber-seq data analysis QC workflow recommendations (cont.)

fiberseq-qc autocorrelation analysis

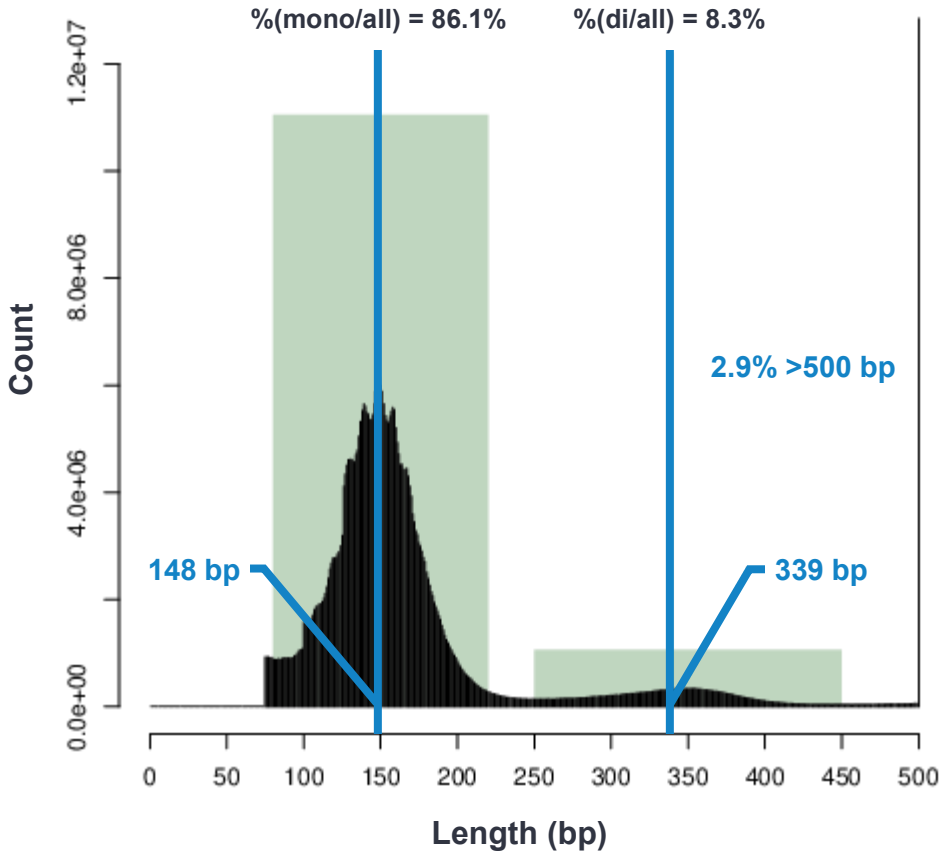
- `fiberseq-qc` autocorrelation analysis plots the lags (distance) between 6mA bases
- Autocorrelation plot has two intercepts
  - First intercept indicates length of the linker DNA (~47 bp)
  - Second intercept indicates length of DNA wrapped around the nucleosome (~147 bp)
- If there is a large variation from expected sizes, then the 6mA labeling reaction likely failed
  - Not enough Hia5 enzyme
  - Too many nuclei
  - Inactive SAM reagent



Fiber-seq data analysis QC workflow recommendations (cont.)

fiberseq-qc analysis of nucleosomal DNA lengths

- `fiberseq-qc` plots the length of DNA wrapped around mononucleosomes and dinucleosomes
- A peak should be observed at the expected sizes for mononucleosomes and dinucleosomes (varies by species)
  - ~147 bp for human mononucleosomes
  - ~340 bp for human dinucleosomes (includes linker DNA length)
- Mononucleosome-sized peak may appear choppy due to nucleosome "breathing"
  - Nucleosome "breathing" refers is the dynamic unwrapping and rewrapping of DNA from the histone core



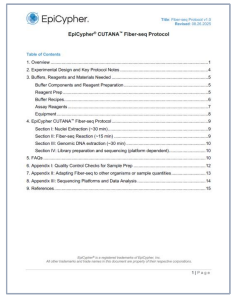
Example library QC results for Fiber-seq libraries prepared with SMRTbell prep kit 3.0

SMRTbell library DNA sizing and library construction yield QC metrics for human Fiber-seq WGS samples

EpiCypher Fiber-seq assay procedure

EpiCypher CUTANA Fiber-seq Protocol [Link]

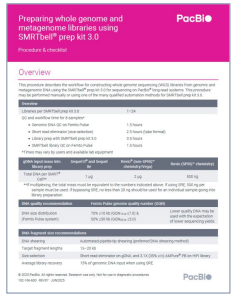
EpiCypher



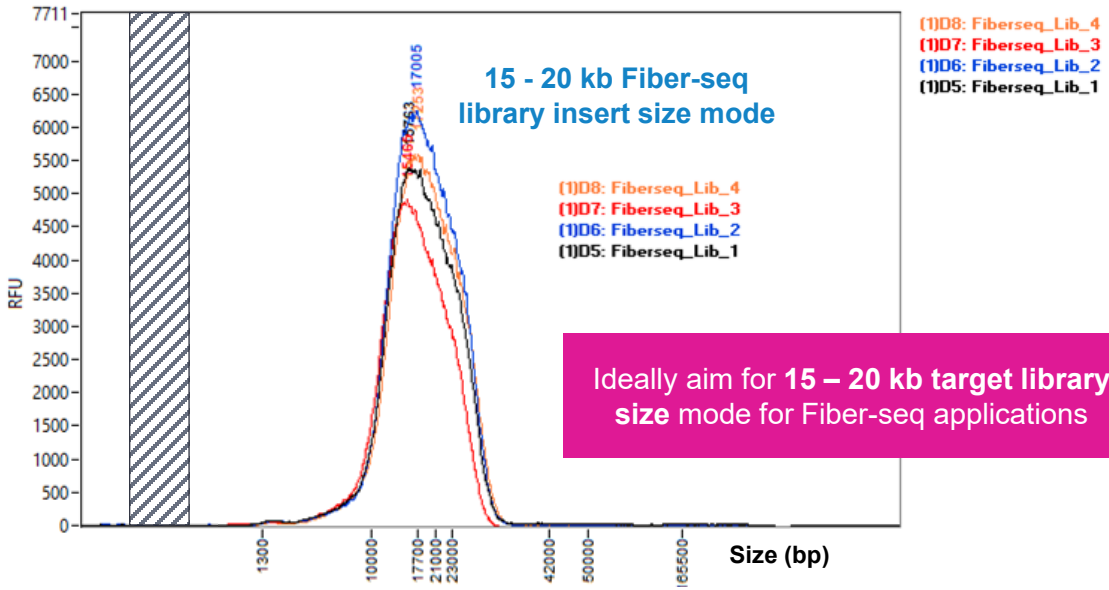
PacBio SMRTbell library construction procedure

Procedure & checklist – Preparing whole genome and metagenome libraries using SMRTbell prep kit 3.0 (102-166-600)

PacBio



Femto Pulse DNA sizing QC electropherogram



Ideally aim for **15 – 20 kb target library size** mode for Fiber-seq applications

Input Fiber-seq sample DNA for SRE	500 ng	2000 ng
Post-SRE recovery (%)[1]	430 (86%)	1820 ng (91%)
Post-shearing recovery (%)[2]	345 (69%)	1440 ng (72%)
Post-AMPure PB bead size-selection (%)[3]	150 (30%)	720 ng (36%)

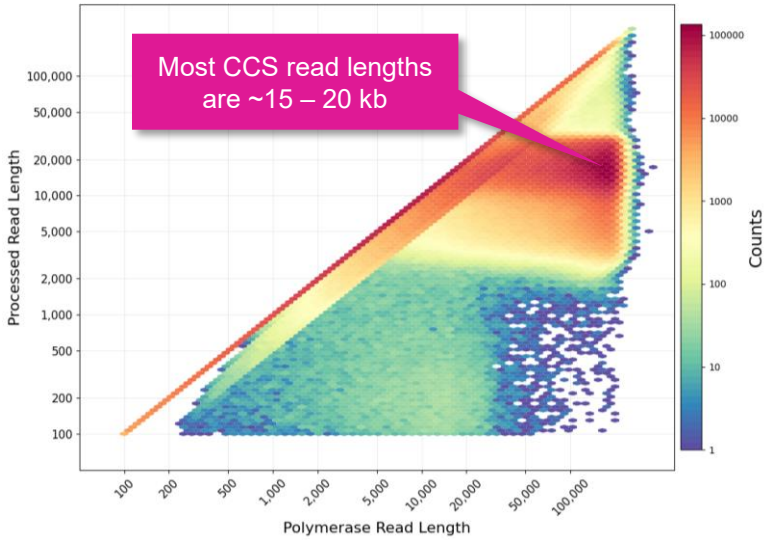
PacBio

[1] Post-SRE gDNA size selection yields typically ranged from ~65% to ~95% for human Fiber-seq DNA samples.
[2] Post-shearing cleanup recoveries typically ranged from ~65% to 80% when using a Hamilton automation system or Megaruptor 3 system or g-TUBE device to shear human Fiber-seq DNA samples.
[3] Post-AMPure PB bead size selected library yields typically ranged from ~20% to ~40% for human Fiber-seq DNA samples.

Example Fiber-seq WGS library performance

Example sequencing performance data for human Fiber-seq WGS samples – Revio system + SPRQ chemistry[1]

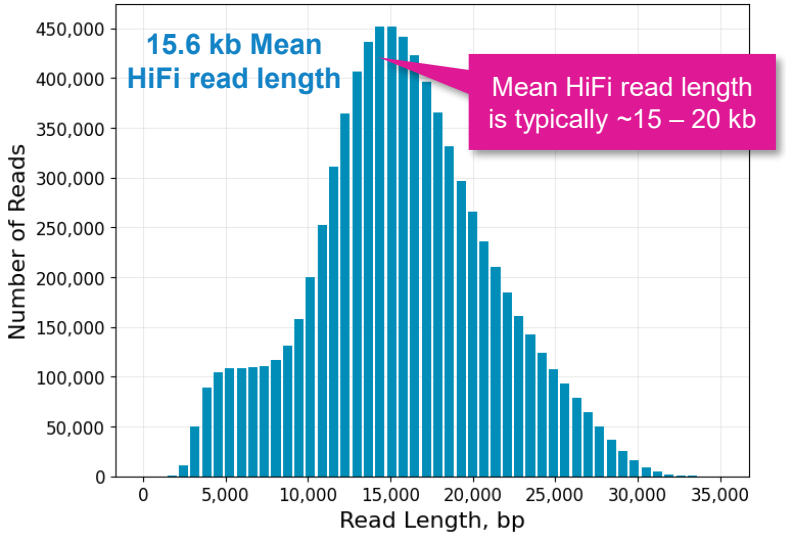
Raw data report



Raw Base Yield	1307 Gb
Mean Polymerase Read Length	83.1 kb
P0	37%
P1	63%
P2	0%

Example metrics for a human Fiber-seq WGS library run on a Revio system with SPRQ chemistry using a 24-hrs movie time. Revio system *P1* range for Fiber-seq libraries was typically ~60%–75%.

HiFi read length

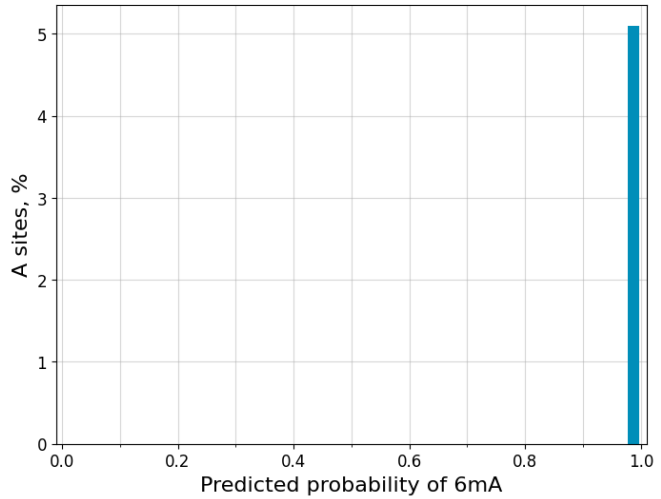


HiFi Reads	8.0 Million
HiFi Base Yield	125.5 Gb
Mean HiFi Read Length	15.6 kb
Median HiFi Read Quality	Q34
HiFi Read Mean # of Passes	9

For human Fiber-seq WGS libraries, per-Revio SMRT Cell HiFi read counts were typically ~8-10 Million depending on the final library insert size and sample loading performance.

Methylation report[2]

Modification	Motif	Scored sites	Modified sites (Pr > 0.5)
6mA	A	5.1%	5.1%



Note: On-instrument %6mA calling results are reported on a **per-SMRT Cell** basis (not by sample) in SMRT Link run details QC reports

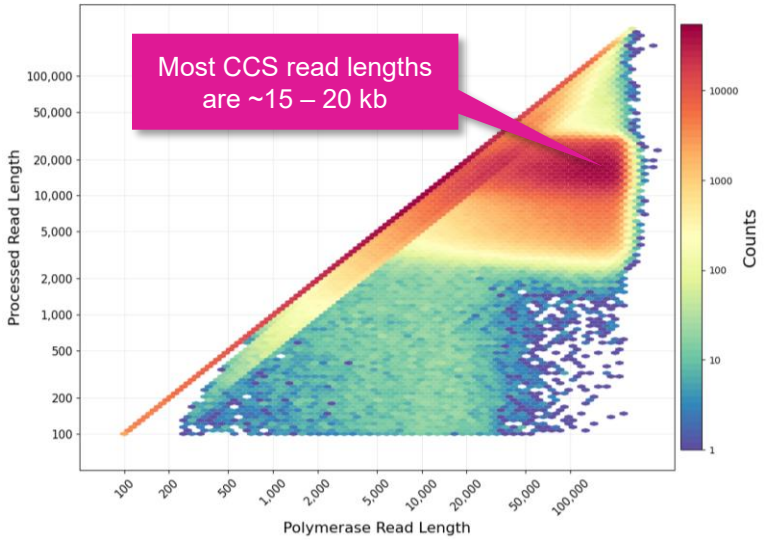
→ If needed, perform barcode demultiplexing prior to performing downstream (per-sample) data analysis using GitHub Fiberseq tools

[1] **Note:** Read lengths, reads/data per SMRT Cell and other sequencing performance results can vary depending on DNA sample quality, insert size, sample loading performance & movie time. Shorter library insert sizes (<5 kb), lower DNA quality samples, and suboptimal sample loading performance may result in lower data yields per SMRT Cell.

Example Fiber-seq WGS library performance

Example sequencing performance data for human Fiber-seq WGS samples – Vega system][1]

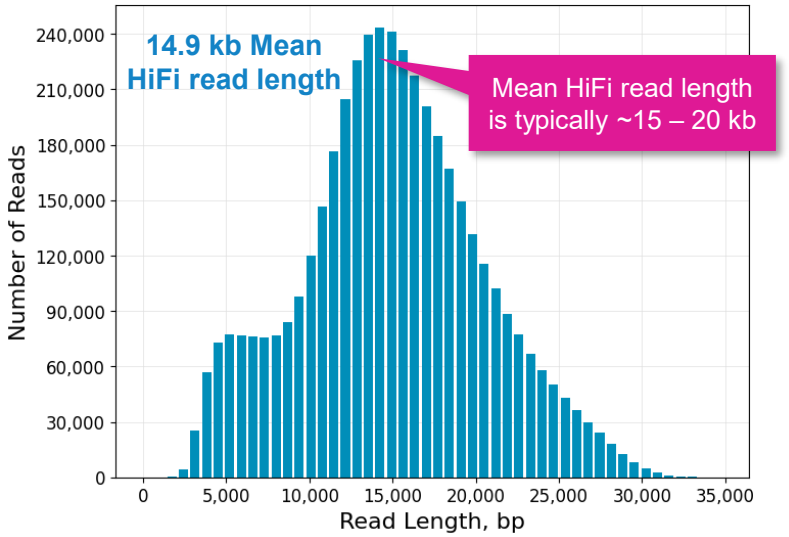
Raw data report



Mean Polymerase Read Length	73.6 kb
Loading level	75%

Example metrics for a human Fiber-seq WGS library run on a Vega system using a 24-hrs movie time. Vega system Loading level range for Fiber-seq libraries was typically ~65%–80%.

HiFi read length

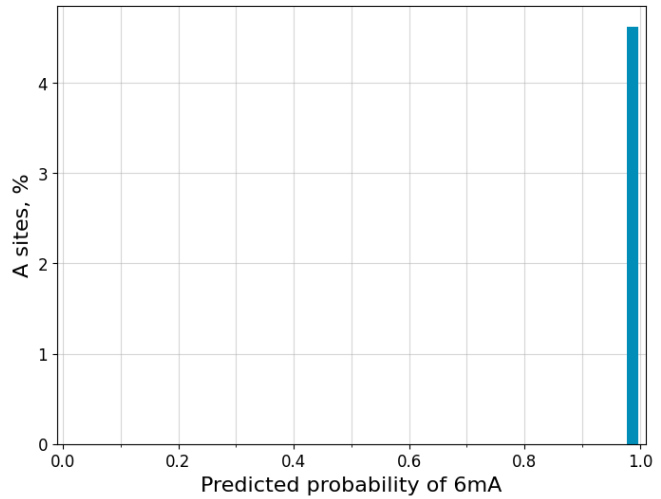


HiFi Reads	4.3 Million
HiFi Base Yield	64.9 Gb
Mean HiFi Read Length	14.9 kb
Median HiFi Read Quality	Q34
HiFi Read Mean # of Passes	9

For human Fiber-seq WGS libraries, per-Vega SMRT Cell HiFi read counts were typically ~4 – 4.5 Million depending on the final library insert size and sample loading performance.

Methylation report[2]

Modification	Motif	Scored sites	Modified sites (Pr > 0.5)
6mA	A	4.6%	4.6%



Note: On-instrument %6mA calling results are reported on a **per-SMRT Cell** basis (not by sample) in SMRT Link run details QC reports

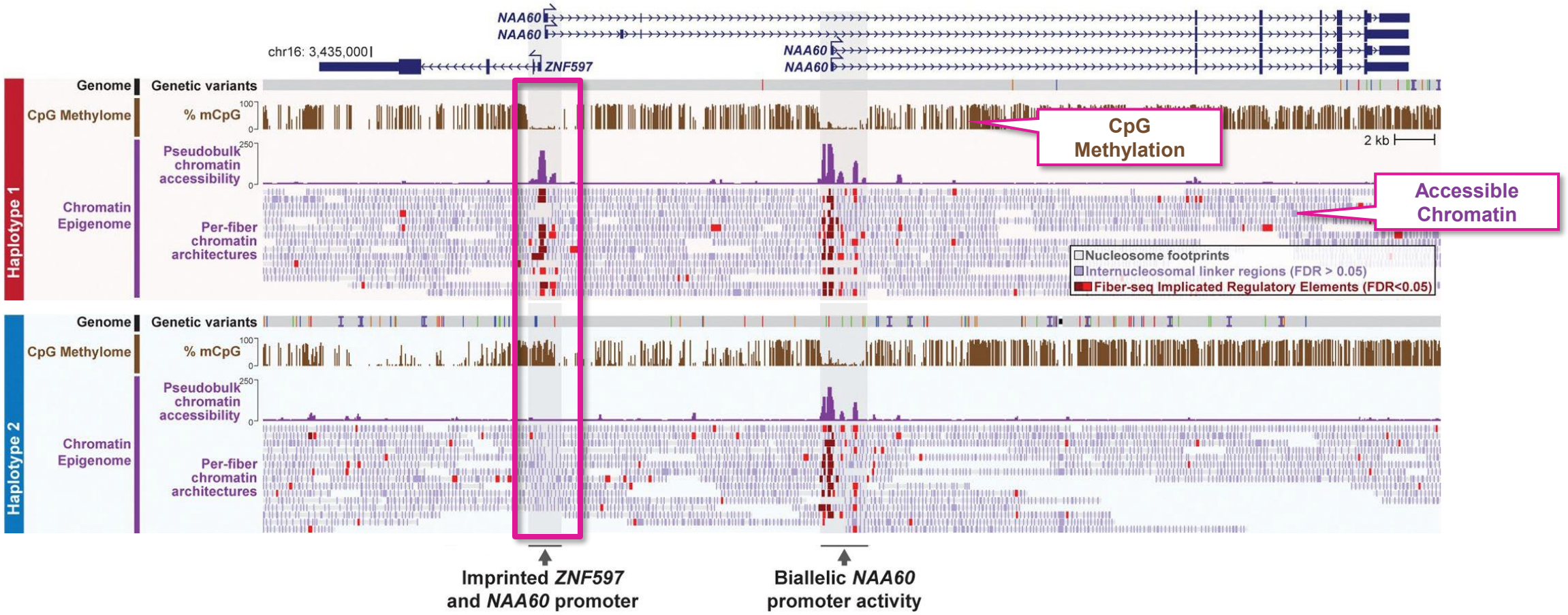
→ If needed, perform barcode demultiplexing prior to performing downstream (per-sample) data analysis using GitHub Fiberseq tools

[1] **Note:** Read lengths, reads/data per SMRT Cell and other sequencing performance results can vary depending on DNA sample quality, insert size, sample loading performance & movie time. Shorter library insert sizes (<5 kb), lower DNA quality samples, and suboptimal sample loading performance may result in lower data yields per SMRT Cell.

Example Fiber-seq WGS library performance (cont.)

Example Fiber-seq assay results for human WGS samples

Fiber-seq assay can resolve haplotypes, with different accessibility[1]



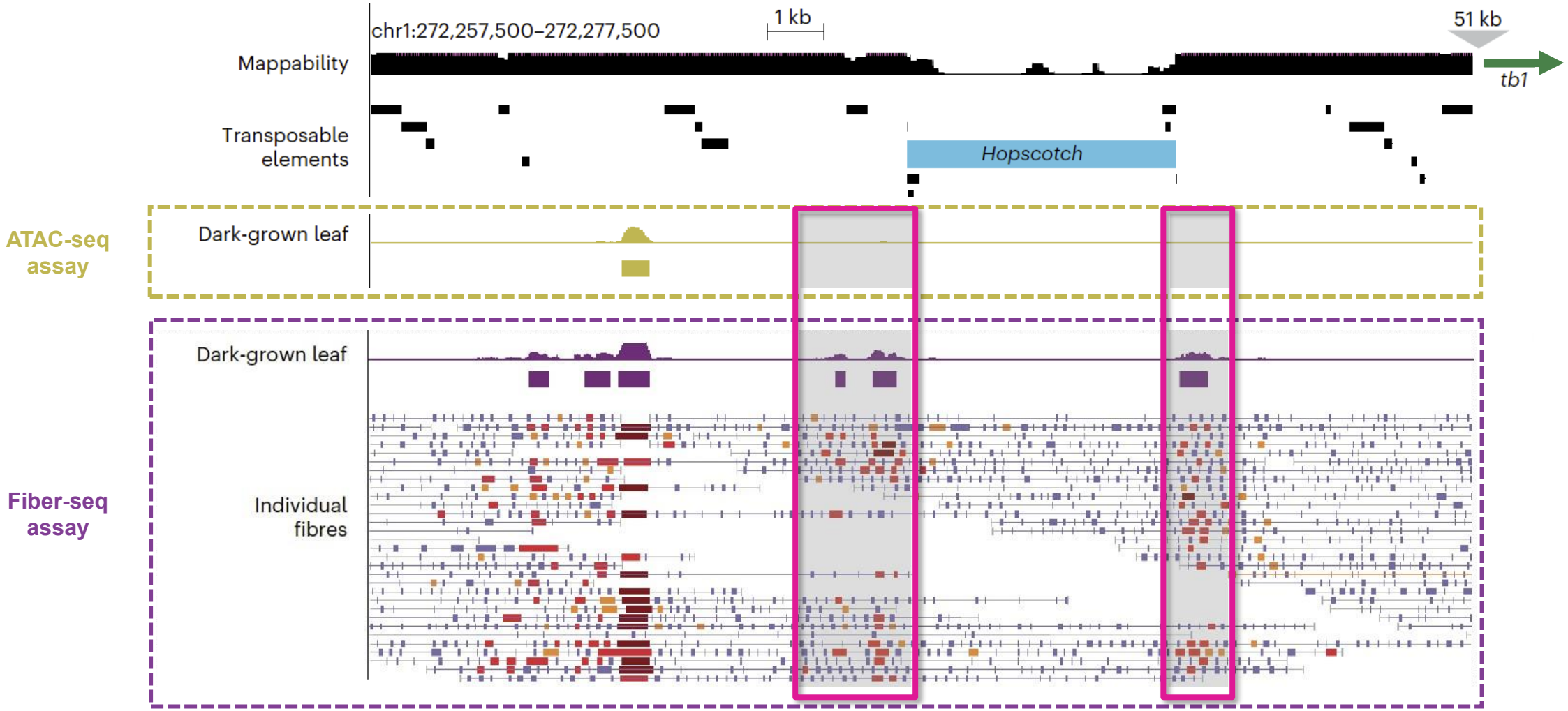
Example genomic region showing the haplotype-resolved genome, CpG methylome, and chromatin epigenome from GM12878 cells at a known imprinted locus.

[1] Figure adapted from Figure 1 Vollger et al. (2025) Synchronized long-read genome, methylome, epigenome and transcriptome profiling resolve a Mendelian condition. Nature Genetics. 57(2):469 [Link].

Example Fiber-seq WGS library performance (cont.)

Example Fiber-seq assay results for maize plant WGS samples

Fiber-seq assay can capture more accessible regions than the ATAC-seq method[1]



[1] Figure adapted from Figure 1 Bubb et al. (2025) The regulatory potential of transposable elements in maize. Nature Plants. 11(6):1181. [Link].

Technical resources for Fiber-seq library preparation, sequencing & data analysis

EpiCypher technical support resources

- Contact **EpiCypher Technical Support** at techsupport@epicypher.com for inquiries about CUTANA Fiber-seq Assay protocol workflow details and for general questions about Fiber-seq data analysis recommendations
  - Please include information about the specific EpiCypher products you are using (e.g., Hia5 enzyme standalone product or the complete Fiber-seq kit) and the protocol/experimental design being followed (e.g., titration experiment)
- Refer to **EpiCypher Technical Support** page for Fiber-seq FAQs and helpful information about protocol optimization & troubleshooting

Fiber-seq data analysis resources

- Transcription factor footprinting
  - FIMO: Find TF motif genomic coordinates [Link]
  - ft footprint: Using motif coordinates, assess accessibility at those locations [Link]
- Variant calling and haplotype phasing
  - Short nucleotide variant calling: DeepVariant – Google variant caller [Link]
  - Structural variant calling: `pbsv` – Part of PacBio SMRT Tools [Link]
  - Haplotype phasing: HiPhase – Maintained by PacBio; requires outputs from at least one of the above variant calling tools [Link]

Technical resources for Fiber-seq library preparation, sequencing & data analysis

Fiber-seq publications

- Stergachis, A.B. et al. (2020) Single-molecule regulatory architectures captured by chromatin fiber sequencing. Science. 368:1449. [Link]
  - Original Fiber-seq method development publication
- Boltengagen, M. et al.(2024) A single fiber view of the nucleosome organization in eukaryotic chromatin. Nucleic Acids Research. 52(1):166 [Link]
  - Nucleosome organization in human
- Jha, A. et al. (2024) DNA-m6A calling and integrated long-read epigenetic and genetic analysis with fibertools. Genome Research. 34:1976. [Link]
  - Description of fibertools data analysis workflow
- Peter, C.J. et al. (2024) Single chromatin fiber profiling and nucleosome position mapping in the human brain. Cell Rep Methods. 4(12). [Link]
  - Mapped nucleosome positions in the human brain to find haplotype-specific chromatin regulatory elements
- Tullius, TW et al. (2024) RNA polymerases reshape chromatin architecture and couple transcription on individual fibers. Molecular Cell. 84(17):3209. [Link]
  - Footprinting of RNA Pol II in Drosophila to find transcription events
- Bubb, K.L. et al. (2025) The regulatory potential of transposable elements in maize. Nature Plants. 11(6):1181. [Link]
  - Mapped repetitive regions in corn.
- Dubocanin, D. et al. (2025) Conservation of dichromatin organization along regional centromeres. Cell Genomics. 5:100819 [Link]
  - Developed/used FiberFold to predict 3D chromatin 3D contact maps.
- Vollger, M.R. et al. (2025) Synchronized long-read genome, methylome, epigenome and transcriptome profiling resolve a Mendelian condition. Nature Genetics. 57(2):469 [Link]
  - Uses human genome, methylome , and epigenome analysis to diagnose a Mendelian gene disorder.

See EpiCypher Product Reference webpage for the latest publications using EpiCypher products

www.pacb.com

Revision history (description)	Version	Date
Added information about PacBio compatible WGS library preparation workflows using seqWell LongPlex multiplexing kit and EpiCypher Fiber-seq assay kit	03	November 2025