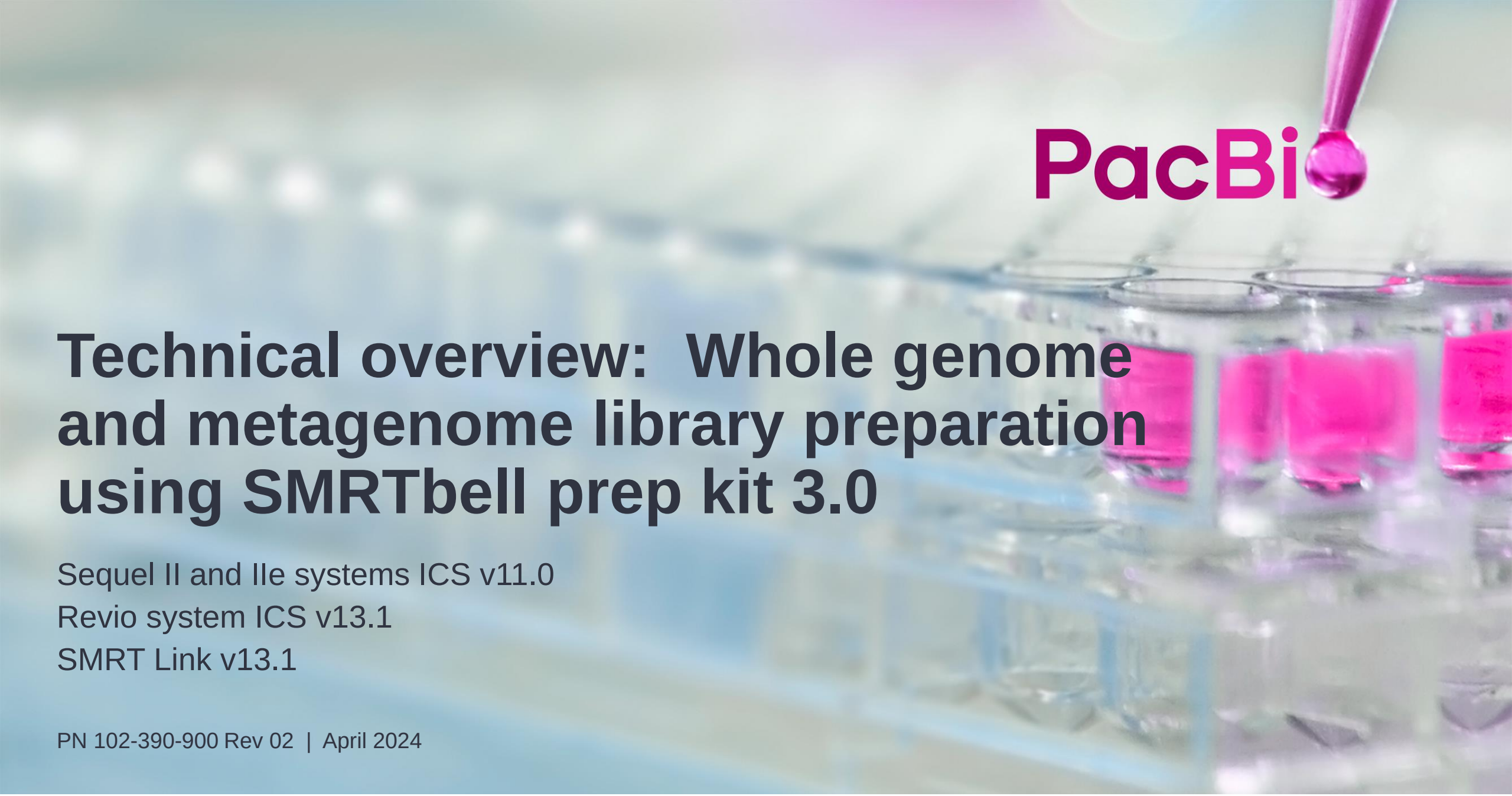


Technical overview: Whole genome and metagenome library preparation using SMRTbell prep kit 3.0

Sequel II and IIe systems ICS v11.0
Revio system ICS v13.1
SMRT Link v13.1

PN 102-390-900 Rev 02 | April 2024

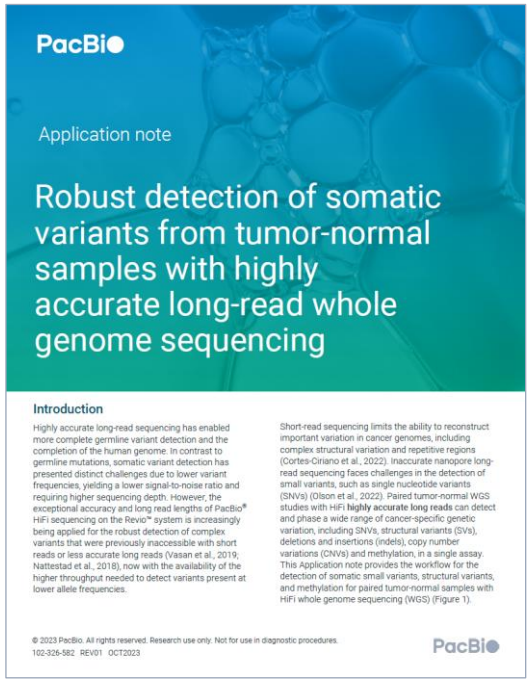
Whole genome and metagenome library preparation using SMRTbell prep kit 3.0

Technical overview

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 – Genomic DNA isolation & QC recommendations for PacBio WGS sample preparation

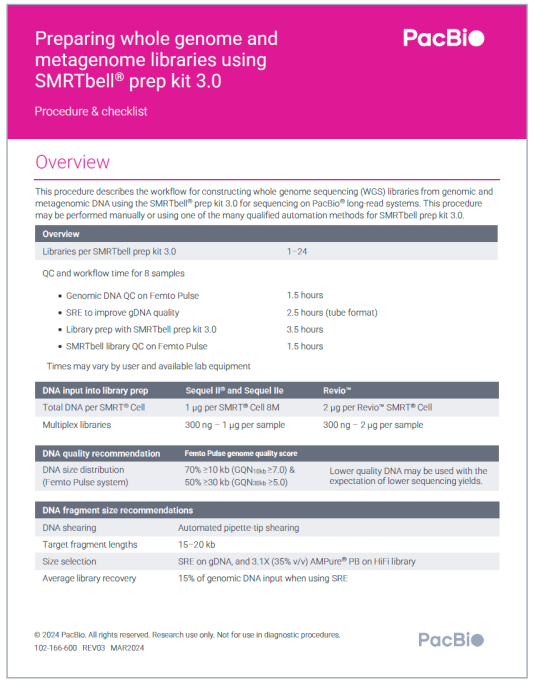
Whole genome and metagenome library preparation using SMRTbell prep kit 3.0: How to get started

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



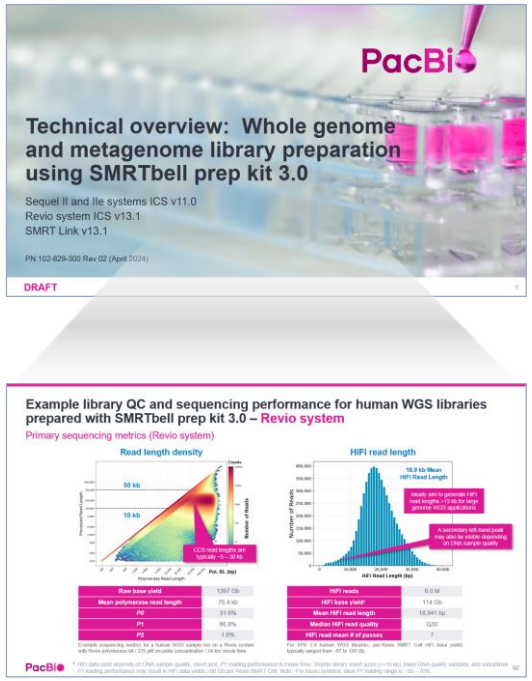
Application notes and Application briefs

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



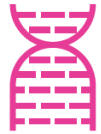
Procedure & Checklist – Preparing whole genome and metagenome libraires using SMRTbell prep kit 3.0 (102-166-600)

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
≥1 µg input gDNA per SMRT Cell 8M
≥2 µg input gDNA per Revio SMRT Cell 8M
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 & Revio systems)

Perform ABC* and sequence WGS libraries on PacBio long-read systems

Data analysis
(SMRT Link or third-party tools)

Genome assembly
Variant detection

* 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
• SRE to improve gDNA quality	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	

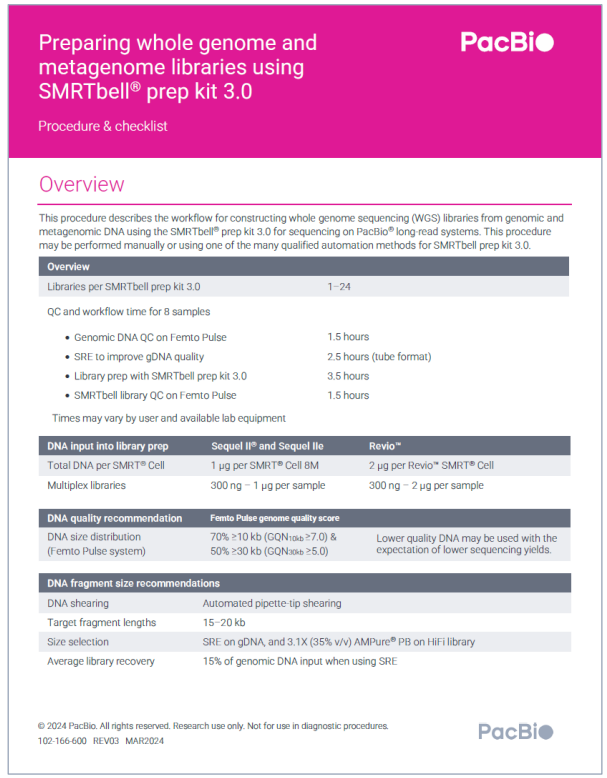
DNA input into library prep	Sequel II® and Sequel IIe	Revio™
Total DNA per SMRT® Cell	1 µg per SMRT® Cell 8M	2 µg per Revio™ SMRT® Cell
Multiplex libraries	300 ng – 1 µg per sample	300 ng – 2 µg per sample

DNA quality recommendation	Femto Pulse genome quality score	
DNA size distribution (Femto Pulse system)	70% ≥10 kb (GQN_{10kb} ≥7.0) & 50% ≥30 kb (GQN_{30kb} ≥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
Target fragment lengths	15–20 kb
Size selection	SRE 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]


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

Procedure & checklist

PacBio

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
• SRE to improve gDNA quality	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	

DNA input into library prep	Sequel II® and Sequel IIe	Revio™
Total DNA per SMRT® Cell	1 µg per SMRT® Cell 8M	2 µg per Revio™ SMRT® Cell
Multiplex libraries	300 ng – 1 µg per sample	300 ng – 2 µg per sample

DNA quality recommendation	Femto Pulse genome quality score	
DNA size distribution (Femto Pulse system)	70% ≥10 kb (GQN_{10kb} ≥7.0) & 50% ≥30 kb (GQN_{30kb} ≥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
Target fragment lengths	15–20 kb
Size selection	SRE 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 REV03 MAR2024

PacBio

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).

PacBio

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

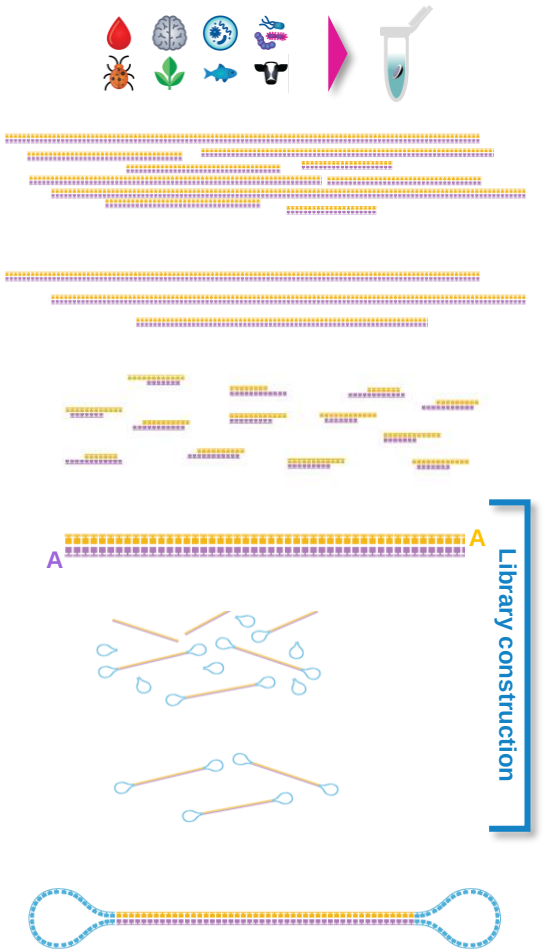
[2] To enable sample multiplexing, SMRTbell adapter index plate 96A (102-009-200) (or adapter index plate 96B/C/D) must be purchased separately from SMRTbell prep kit 3.0 bundle (102-182-700).

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

Optional: Short read eliminator (SRE) kit (102-208-300 / 24 RXN)

Hamilton Microlab Prep
(Pipette-based DNA shearing)[1]

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

Animal/plant/human WGS:[2]
3.1X (35%) AMPure PB SS (>4 kb)

Microbial/metagenomic WGS:
3.1X (35%) AMPure PB SS (>4 kb) or
1X SMRTbell bead cleanup

1 Alternatively, can shear DNA using a Megaruptor system (Diagenode) if a 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[1]
- Kit also includes SMRTbell cleanup beads and low TE buffer
- Barcoded adapters and size-selection reagents are sold separately
- Supports 24 SMRTbell libraries per kit
- Compatible with the Revio system and Sequel II and 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)

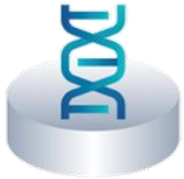
#	Component	Part number	Qty	Color	Volume
1	SMRTbell cleanup beads	102-158-300	1	clear	10 mL

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

Other recommended kits & consumables for DNA sample extraction, DNA size selection, sample multiplexing and SMRT 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)

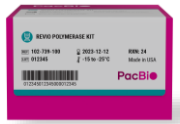
- For HMW DNA extraction from cultured cells, cultured bacteria, whole blood, tissues, insects & plant nuclei[1]

Final library size selection

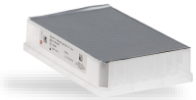
AMPure PB bead size selection kit (102-182-500)

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

SMRT sequencing – Revio



Revio polymerase kit (102-817-600)



Revio sequencing plate (4-rxn: 102-587-400)

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

- Revio polymerase kit supports **24** Revio SMRT Cells for SPK 3.0 WGS and metagenome samples

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

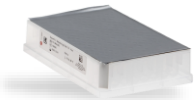
Sample multiplexing

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

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

SMRT sequencing – Sequel II/IIe

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)

- Sequel II binding kit supports **24** Sequel II SMRT Cells for SPK 3.0 WGS and metagenome samples

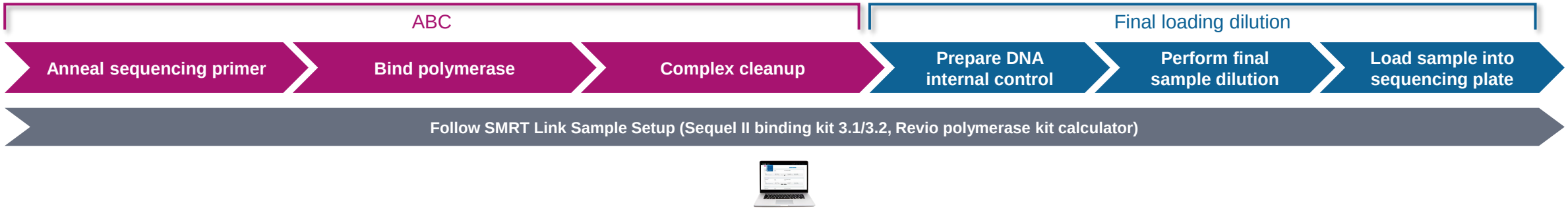
[1] Nanobind CBB kit (102-301-900) may also be used for HMW DNA extraction from cultured mammalian cells, cultured bacteria and whole blood.

Sequencing preparation workflow overview for SPK 3.0 WGS libraries

For SMRTbell prep kit 3.0 WGS libraries bound with Revio polymerase kit or 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	**Revio polymerase kit** (102-817-600) *or* **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

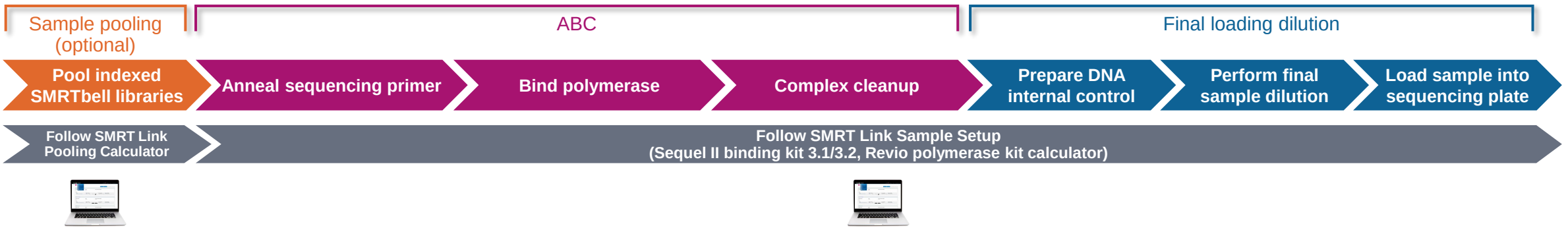


Sequencing preparation workflow overview for SPK 3.0 WGS libraries (cont.)

For SMRTbell prep kit 3.0 WGS libraries bound with Revio polymerase kit or 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 Revio polymerase kit or 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	**Revio polymerase kit** (102-817-600) *or* **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

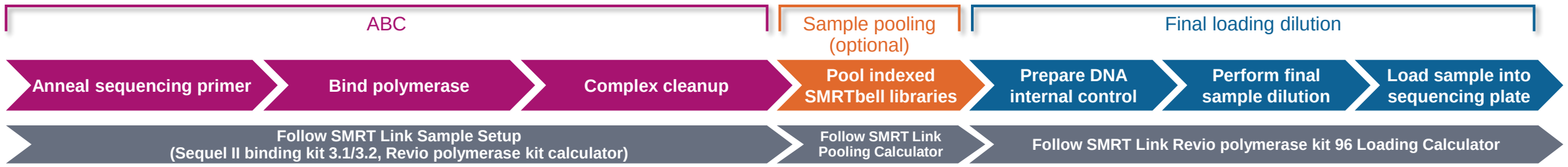


Sequencing preparation workflow overview for SPK 3.0 WGS libraries (cont.)

For SMRTbell prep kit 3.0 WGS libraries bound with Revio polymerase kit or 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 Revio polymerase kit

Library prep kit	Polymerase kit[1]	Sample setup workflow & procedural reference	
SMRTbell prep kit 3.0 102-182-700	**Revio polymerase kit** (102-817-600)	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)

WGS library preparation workflow details

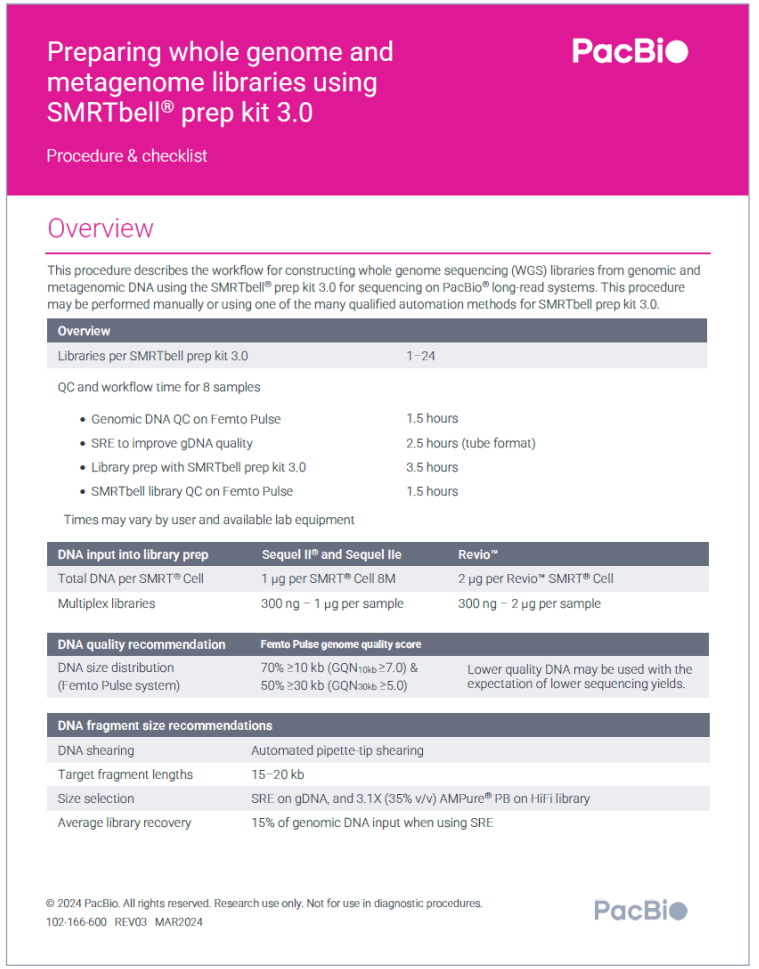
Procedure & checklist – Preparing whole genome libraries using SMRTbell prep kit 3.0 (102-166-600)

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 optional 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]

Note: To prepare SMRTbell prep kit 3.0 samples for sequencing on PacBio long-read systems, follow ABC workflow instructions provided in **SMRT Link Sample Setup** calculator tool



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
• SRE to improve gDNA quality	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	

DNA input into library prep	Sequel II® and Sequel IIe	Revio™
Total DNA per SMRT® Cell	1 µg per SMRT® Cell 8M	2 µg per Revio™ SMRT® Cell
Multiplex libraries	300 ng – 1 µg per sample	300 ng – 2 µg per sample

DNA quality recommendation	Femto Pulse genome quality score	
DNA size distribution (Femto Pulse system)	70% ≥10 kb (GQN_{10kb} ≥7.0) & 50% ≥30 kb (GQN_{30kb} ≥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
Target fragment lengths	15–20 kb
Size selection	SRE 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 REV03 MAR2024

PacBio

PacBio Documentation (102-166-600)

[1] If performing SRE manually, please refer to ***Procedure & checklist – Removing short DNA fragments with the Short Read Eliminator (SRE) kit*** (102-982-300) for additional details. 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.

[3] Size selection is not required for microbial WGS and metagenomic shotgun libraries where retention of shorter fragments is desired.

SPK 3.0 workflow overview for WGS & metagenomic sequencing applications

SPK 3.0 library prep workflow can optionally be automated to enable higher sample throughput

Key workflow steps	Automation	Time[1]	Protocol documentation or reference	Recommended equipment & consumables
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
1 Short Read Eliminator on gDNA		2.5 hrs		 Optional: Short read eliminator (SRE) kit (102-208-300 / 24 RXN)
Safe stop				
2 Pipette DNA shearing		20 min	**SPK 3.0 protocol reference**	 Hamilton Microlab Prep (Pipette-based DNA shearing) *or* Diagenode Megaruptor 3 (Hydropore-based DNA shearing)[2]
Safe stop			 ***Procedure & checklist – Preparing whole genome libraries using SMRTbell prep kit 3.0*** (102-166-600)	
3 Post-shearing cleanup		25 min		Diagenode Megaruptor 3 (Pipette-based DNA shearing)[2]
4 DNA repair & A-tailing		1 hr	**Supported automation systems**  Thermo Fisher KingFisher Apex  Hamilton Microlab NIMBUS Presto  Hamilton Microlab Prep  Hamilton NGS Star	 SMRTbell prep kit 3.0 (102-182-700 / 24 RXN)
5 Adapter ligation & cleanup		1 hr		
6 Nuclease treatment		20 min		 **Animal/plant/human WGS:**[3] 3.1X (35%) AMPure PB SS (>4 kb)
Safe stop				
7 Final library cleanup or size selection		25 min		 **Microbial/metagenomic WGS:** 3.1X (35%) AMPure PB SS (>4 kb); or 1X SMRTbell bead cleanup

Library construction

[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 system (Diagenode) if a Microlab Prep system is unavailable; [3] Can optionally perform gel cassette-based size selection to enrich for SMRTbell library inserts >10 kb.

SPK 3.0 workflow overview for WGS & metagenomic sequencing applications

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

Key workflow steps	Automation	Time[1]
DNA sample extraction		2 – 2.5 hrs
DNA sample QC		2 hrs
1 Short Read Eliminator on gDNA		2.5 hrs
Safe stop		
2 Pipette DNA shearing		20 min
Safe stop		
3 Post-shearing cleanup		25 min
4 DNA repair & A-tailing		1 hr
5 Adapter ligation & cleanup		1 hr
6 Nuclease treatment		20 min
Safe stop		
7 Final library cleanup or size selection		25 min

Library construction

DAY 1

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 – 22 kb library insert size	NGS STAR STARlet STAR V Microlab Prep			DreamPrep Compact
WGS library prep				
SMRTbell prep kit 3.0	NGS STAR	Miro Canvas	SciClone NGSx	DreamPrep Compact
Sequencing preparation (ABC)[1]				
Sequel II binding kit 3.2	NGS STAR			DreamPrep Compact
Revio polymerase kit	NGS STAR			

[1] ABC = Anneal sequencing primer, bind polymerase, clean up polymerase-bound complexes)

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

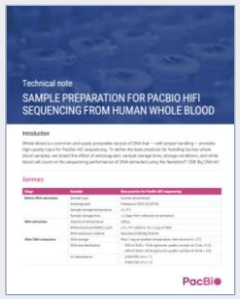
DNA sample extraction

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, 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
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
Saliva	DNA Genotek Oragene OG 500 collection tubes (PN OG-500) • Recommended for collection of saliva samples

[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

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

DNA input amount

DNA Input into SPK 3.0 library prep	Sequel II and IIe systems	Revio system
Total DNA per SMRT Cell	1 μg per SMRT Cell 8M	2 μg per Revio SMRT Cell
Multiplex libraries[1]	300 ng – 1 μg per sample	300 ng – 2 μg per sample

- Recommended DNA input amount for WGS samples will vary by starting genomic DNA quality

DNA quality	90% >10 kb	80% >10 kb	70% >10 kb
gDNA input into SRE size selection step	2 – 3 μg	3 – 4 μg	4 – 5 μg

- **Note 1:** Maximum input gDNA mass tolerated by pipette DNA shearing method is 3 μg
  → Perform parallel shearing reactions if using >3 μg input gDNA
- **Note 2:** Maximum input sheared DNA mass tolerated by library enzymatic reactions in this SPK 3.0 protocol is 5 μg per reaction
  → Perform parallel library prep reactions if using >5 μg input sheared DNA

[1] Minimum input DNA amount per WGS sample required for SPK 3.0 library construction is 300 ng. 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.

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 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 – 25% (includes SRE, shearing, and SMRTbell library preparation)
- Starting with 2 µg of input gDNA (going into SRE size selection step) will, on average, provide enough library to load **1 Revio SMRT Cell**

Mean library insert size	Library mass needed to load one Revio SMRT Cell at 250 pM OPLC
15,000 bp	243 ng
18,000 bp	292 ng
21,000 bp	341 ng

Note: It is recommended to use at least 2 µg of input gDNA even if only using one Revio SMRT Cell the following reasons:

- Ensure adequate SRE recovery
- Final mean library size is not known prior beginning protocol.
- Available library for re-sequencing or topping off coverage if necessary

- If targeting larger insert sizes or working with lower quality DNA, start with at least 3 µg of input gDNA (going into SRE size selection step) to ensure adequate library for optimal SMRT Cell loading

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

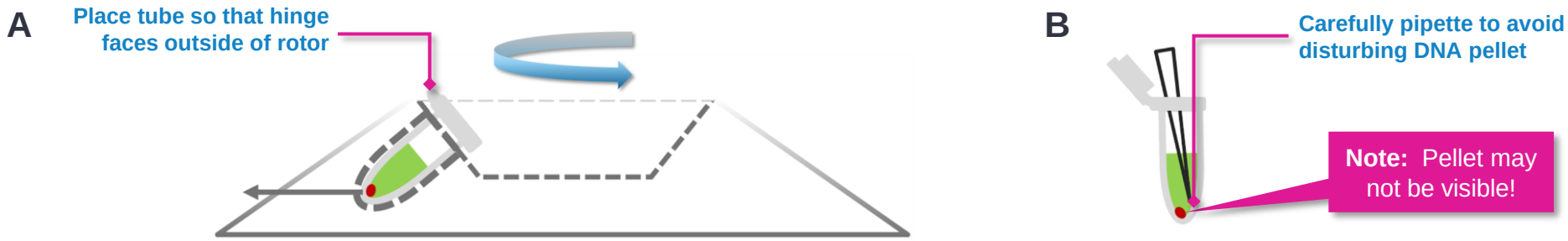
Input genomic DNA size selection using Short read eliminator (SRE) kit (102-208-300)

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

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

DNA shearing

- This protocol recommends shearing genomic DNA to a size between 15–20 kb
- We recommend performing pipette-based DNA shearing 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) may alternatively be used[1]

- 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 if the majority of the starting gDNA is <15 kb
    - If skipping SRE size selection, a lower DNA input amount (less than 2 μg) may be used in this SPK 3.0 library prep procedure[1]
  - → 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 <13 kb, then skip the DNA shearing step in this procedure

PacBio

[1] See Appendix A1 in ***Procedure & checklist – Preparing whole genome and metagenome sequencing libraries using SMRTbell prep kit 3.0*** for Megaruptor 3 system shearing recommendations.

[2] Please note that a 15 to 30% recovery is expected for this SPK 3.0 library prep procedure. Make sure enough input gDNA is used to meet optimal loading amounts on Sequel II or Revio systems.

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

Reagent handling

Thaw these reagents at room temperature	Keep these temperature-sensitive reagents on ice		Bring these reagents to room temperature 30 minutes prior to use
❑ Repair buffer		❑ End repair mix	❑ AMPure PB beads
❑ Nuclease buffer		❑ DNA repair mix	❑ Elution buffer
❑ SMRTbell adapter		❑ Ligation mix	❑ dsDNA quantification reagents
❑ Elution buffer		❑ Ligation enhancer	
		❑ Nuclease mix	
		❑ SMRTbell adapter	

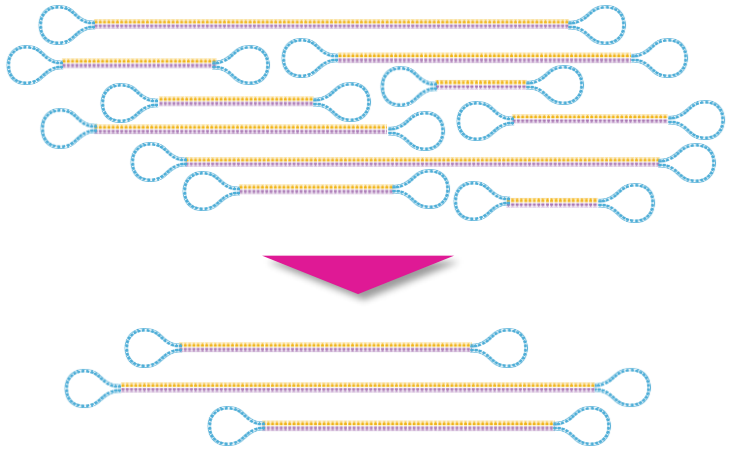
- Room temperature is defined as any temperature in the range of 18 – 25°C for this protocol
- Once thawed, reaction buffers and adapter index plate may be stored on a cold block, at 4°C, or on-ice prior to making master mix or placing on the liquid handler work deck
- Briefly spin down all reagent tubes 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

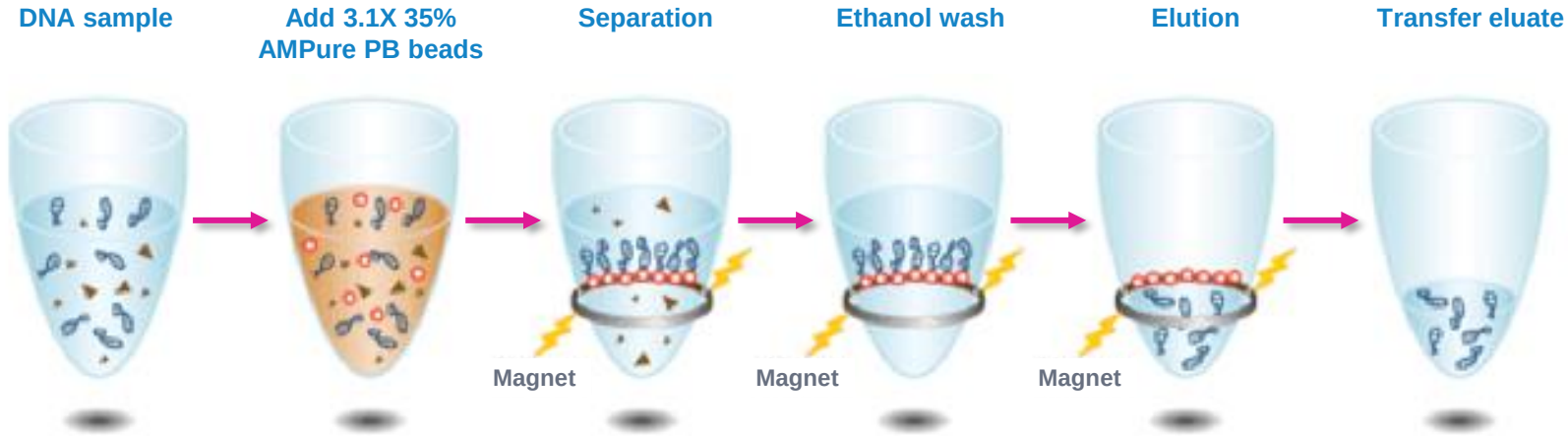
General best practices recommendations for preparing WGS and metagenome libraries using SMRTbell prep kit 3.0 (cont.)

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 remove short DNA fragments (<5 kb) 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

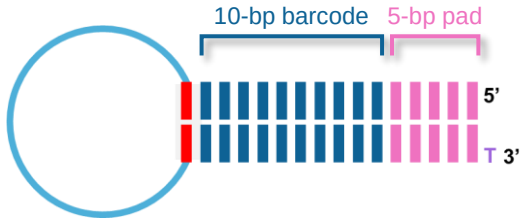
[1]To remove DNA fragments >5 kb using alternative gel-based size selection methods, please see ***Technical Note: Alternative size selection methods for SMRTbell prep kit 3.0*** (102-326-503). Note that use of these tools requires higher per-SMRT Cell DNA input amounts (≥1.5 µg/SMRT Cell 8M or ≥3 µg/Revio SMRT Cell).

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) contains 96 barcoded adapters to support multiplexed SMRTbell library construction for up to 96 samples using SPK 3.0
  - Can be used for microbial assembly and any other WGS or amplicon sequencing application that employs barcoded overhang adapters
  - Kit quantities support a single use of each of the 96 barcoded adapters in the plate for SMRTbell library preparations
- SMRT Link comes pre-installed with the following barcode set FASTA file containing SMRTbell adapter index plate 96A/B/C/D barcode sequences[1]:
  - ❑ `SMRTbell adapters indexes` (for Sequel II/IIe and Revio system run designs)



SMRTbell adapter index 96 structure

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

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

Plate Layout (Excel) [Link]
Barcode Sequences (FASTA) [Link]
Product insert – SMRTbell adapter index plate 96A [Link]

[1] We recommend aiming for a library insert size >15 kb (ideally 15 – 20 kb) to achieve optimum HiFi data yields on the Revio system. **Note:** HiFi read lengths, reads/data per SMRT Cell and other sequencing performance results can vary depending on DNA sample quality, insert size, *P1* loading performance & movie time. Shorter library insert sizes (<15 kb), lower DNA quality samples, and suboptimal *P1* loading performance may result in HiFi data yields <90 Gb per Revio SMRT Cell.

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]
- 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)
- Optionally use the SMRT Link (v13.1+) Sample Setup Pooling Calculator tool to help determine appropriate volumes to use for multiplexing SPK 3.0 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] We recommend aiming for a library insert size >15 kb (ideally 15 – 20 kb) to achieve optimum HiFi data yields on the Revio system. **Note:** HiFi read lengths, reads/data per SMRT Cell and other sequencing performance results can vary depending on DNA sample quality, insert size, *P1* loading performance & movie time. Shorter library insert sizes (<15 kb), lower DNA quality samples, and suboptimal *P1* loading performance may result in HiFi data yields <90 Gb per Revio SMRT Cell.

[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.

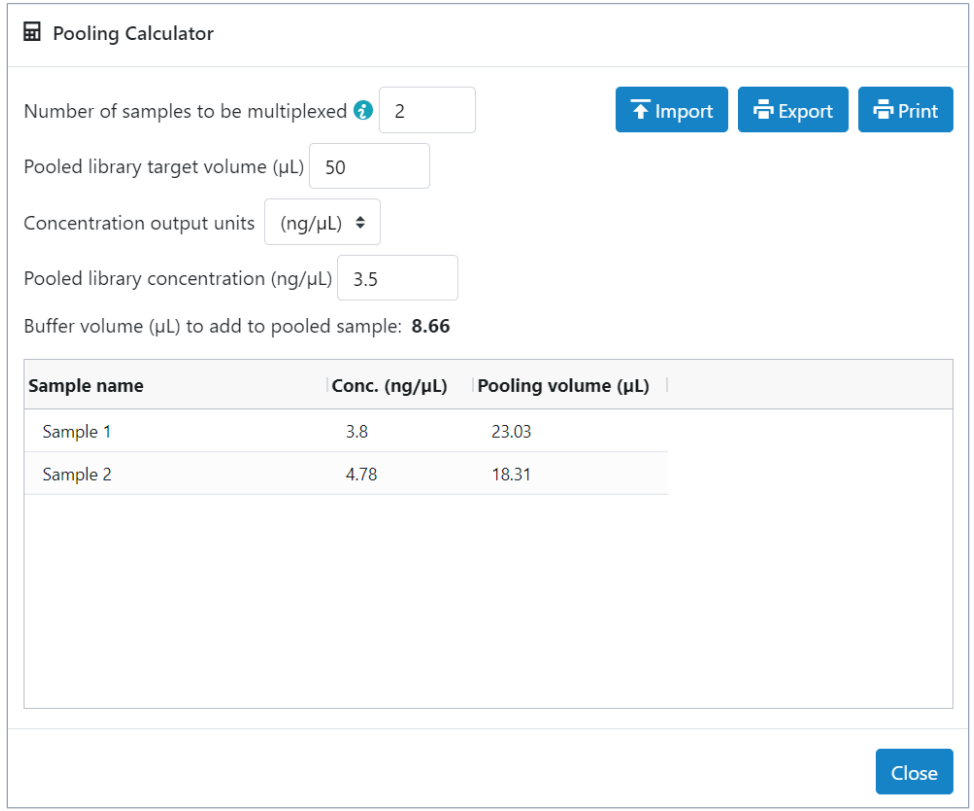
[3] If the difference in average length of your final SMRTbell libraries is >25%, it may be necessary to pool in equal molar amounts to balance the number of reads per library. Equal-mass pooling of barcoded WGS samples can be performed if all samples have similar fragment length profiles and similar 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 (v13.1+) 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:
  - Only pool samples with similar genome sizes to ensure balanced coverage
  - Ensure that samples to be pooled have a similar mean insert size and similar insert length size distribution[1]
  - Aim to pool samples in an equal molar concentration for best balanced coverage
- 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)



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.

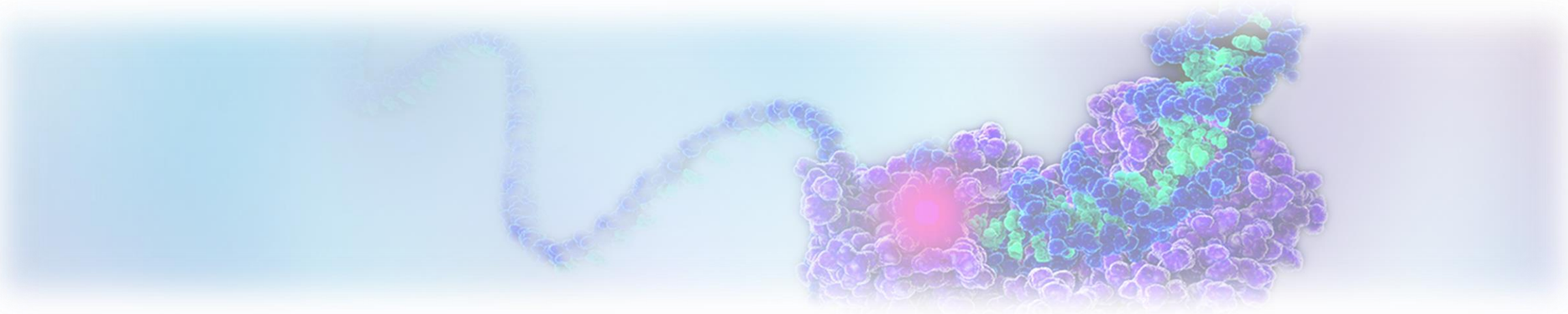
[1] We recommend aiming for a library insert size >15 kb (ideally 15 – 20 kb) to achieve optimum HiFi data yields on the Revio system. **Note:** HiFi read lengths, reads/data per SMRT Cell and other sequencing performance results can vary depending on DNA sample quality, insert size, *P1* loading performance & movie time. Shorter library insert sizes (<15 kb), lower DNA quality samples, and suboptimal *P1* loading performance may result in HiFi data yields <90 Gb per Revio SMRT Cell.

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

- **Note:** ***Procedure & checklist – Preparing whole genome libraries using SMRTbell prep kit 3.0*** (102-166-600) does not include instructions for the primer annealing, polymerase binding, and complex cleanup (ABC) sample setup steps
  - → Follow **SMRT Link Sample Setup Sequel II binding kit 3.1/3.2, Revio polymerase kit** calculator tool to perform ABC and final loading dilution procedure for SPK 3.0 libraries
- 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 library is stable at 4°C for 1 month
  - Frozen polymerase-bound library is stable for at least 6 months
- Please note that the stored polymerase-bound library needs to be protected from light while stored.



[1] Polymerase-bound storage recommendations described here apply to SMRTbell libraries bound to either Revio polymerase or Sequel II polymerase 3.2.

SMRTbell prep kit 3.0 library prep inputs & expected step yields

Final HiFi library yield is typically sufficient to load ≥1 Revio SMRT Cell

		Cleanup	DNA QC	Step input	Step output / Yield (%)
	DNA sample extraction	N/A	N/A	200 μL human or animal whole blood / 1 mL human whole blood / 1x10^6 cells cultured mammalian cells	Nanobind HT CBB kit : 3 – 15 μg HMW DNA Nanobind HT 1 mL blood kit 3 – 70 μg HMW DNA
	DNA sample QC	N/A	Qubit dsDNA HS assay Femto Pulse system	Nanobind HT-extracted HMW DNA	DNA quantification QC → Aim for ≥3 μg HMW DNA DNA sizing QC → Ideally GQN(10 kb) ≥7.0
SRE	**1** Short Read Eliminator on gDNA	N/A	Qubit dsDNA HS assay	40-100 ng/μL DNA in a total volume of 50 μL Input DNA mass depends on expected recovery	Expect ~75% step recovery or 75% total recovery
	Safe stop				
Shear	**2** Pipette DNA shearing	N/A	Qubit dsDNA HS assay (optional) Femto Pulse system	≤10 ng/μL DNA in 300 μL (3 μg total input DNA mass)	≤300 μL sheared DNA Target DNA shear size is ~15-20 kb
	Safe stop				
Library construction	**3** Post-shearing cleanup	1X SMRTbell cleanup beads	Qubit dsDNA HS assay Femto Pulse system	≤300 μL sheared DNA	Expect up to 80% step recovery (60% total recovery)
Library construction	**4** DNA repair & A-tailing	N/A	N/A	46 μL sheared DNA	60 μL repaired & A-tailed DNA
Library construction	**5** Adapter ligation & cleanup	1X SMRTbell cleanup beads	N/A	60 μL post-repaired & A-tailed DNA	Expect ~80% step recovery or 48% total recovery
Library construction	**6** Nuclease treatment	N/A	N/A	40 μL of post-ligation cleanup sample	Expect ~40% step recovery or 19% total recovery
	Safe stop				
Library construction	**7** Final library cleanup or size selection	3.1X (35%) AMPure PB	Qubit dsDNA HS assay Femto Pulse system	15 μL of nuclease-treated sample	Expect 80% step recovery or 15% total recovery (can range from ~10% - 25%)

PacBio

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

↓

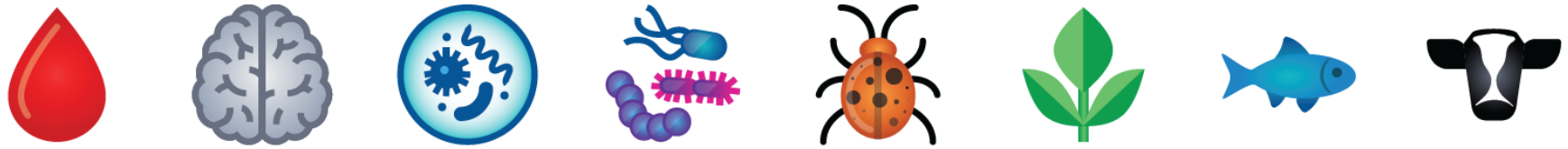
5 Adapter ligation & cleanup

↓

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

Nanobind PanDNA kit
(103-260-000)

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]

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).

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 96 (from Revio polymerase kit 96 PN 103-253-600) or Loading Buffer (from Revio polymerase kit PN 102-817-600) 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.

[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

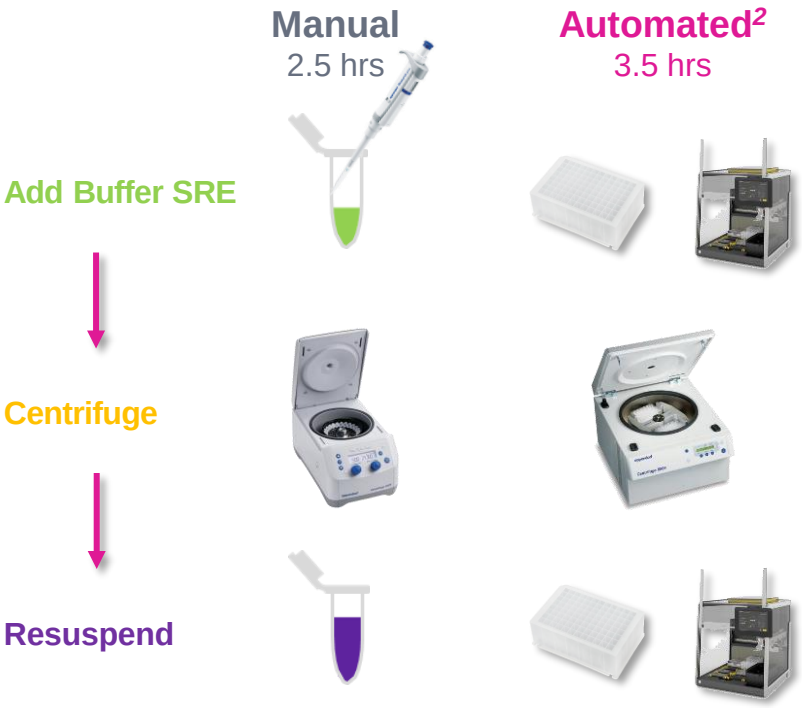
1. Short Read Eliminator

✓	Step	Instructions
	1.1	Bring the DNA samples to a concentration between 40 to 100 ng/µL in a total volume of 50 µL using Buffer LTE in a 1.5 ml Lobind tube or hard-shell plate. **The DNA input requirement for shearing is <3 µg so gDNA input into SRE will depend on expected recovery.**
	1.2	Add 50 µL of **Buffer SRE** to each sample. 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.
	1.5	Centrifuge a tube at 10,000 rcf for 30 minutes Centrifuge a plate at >2250 rcf (max 3220 rcf) for 1 hour • If using a centrifuge with temperature control (i.e., cooling function), turn this function off by specifying a target temperature set point higher than ambient room temperature (e.g., 29°C or 30°C).
	1.6	Carefully remove supernatant without disturbing the pellet. • Leaving up to 10 µL is acceptable to be sure the pellet is not disturbed.
	1.7	Add 300 µL of Buffer LTE to the tube and incubate at room temperature for 10 minutes.
	1.8	After incubation, pipette-mix 20 times and vortex/shake the tube/sealed plate for 15s to ensure that the DNA is properly re-suspended and mixed.
	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

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.

- DNA samples should be between **40 to 100 ng/µL** in a total volume of 50 µL using Buffer LTE
- DNA input requirement into shearing is <3 µg so **gDNA input into SRE will depend on expected recovery**

SRE workflow for SPK 3.0 protocol[1]



[1] If performing SRE step manually, refer to ***Procedure & checklist – Removing short DNA fragments with the Short Read Eliminator (SRE) kit*** (102-982-300) for details on required consumables.

[2] 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.

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 ≤10 ng/µL, if necessary (e.g., if more than 3 µg of gDNA was recovered from SRE). Use Buffer LTE to dilute samples. Bring all samples up to 300 µL 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.

Parameter	Setting
DNA concentration	≤10 ng/µL
Volume of Buffer LTE	300 µL
Number of mixes	300 cycles
Pipette mixing speed	500 µL/sec
Liquid following	83% volume
Pipette tip	300 µL CO-RE II tips (filtered, black, non-sterile)

	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 final SMRTbell library size distribution with a Femto Pulse system to ensure efficient shearing.
	2.5	Proceed to the 1X SMRTbell cleanup bead procedure to concentration samples for library preparation.

- For automated pipette-based shearing using Hamilton systems, DNA samples should be at **<10 ng/ µL** in a total volume of **300 µL** in a 0.8 mL, 96 DeepWell plate (use Buffer LTE to dilute samples)

Hamilton NGS STAR/STARlet/STAR V

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 30 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.

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 = 300 µL • Megaruptor 3 shearing = 100–130 µL
	3.2	Pipette-mix the beads until evenly distributed.
	3.3	Quick-spin the tube strip in a microcentrifuge to collect liquid.
	3.4	Leave 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 pipette off 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

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 75°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	Leave at **room temperature** for **10 minutes** to allow DNA to bind beads.

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

- **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 75°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

7. 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 mix.**
	6.3	Quick-spin **the Nuclease mix** in a microcentrifuge to collect liquid.
	6.4	Add **10 µL** of **Nuclease 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 75°C if programmable.

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

- 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 75°C if programmable

[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

8. 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.
	...	...
	7.14	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a new tube.
	7.15	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. Calculate the total mass. The final overall recovery should be 10–25% as measured from gDNA input to completed SMRTbell library (includes SRE, shearing, and library prep). DNA concentration must be less than 60 ng/µl to proceed to 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.
	7.16	Proceed to SMRT Link Sample Setup for preparing samples for sequencing. Alternatively, libraries can be stored at 4°C if sequencing within 1 month. Store long-term at -20°C. SMRTbell libraries are expected to degrade over time at the same rate as any appropriate buffered pure DNA sample. Minimize freeze/thaw cycles and do not expose to direct sunlight or UV radiation.

PROTOCOL COMPLETE

Note: If performing gel-based size selection to remove fragments >5 kb (or skipping AMPure PB bead size selection altogether), skip AMPure PB bead size selection (Step 8) and perform cleanup using 1X SMRTbell cleanup beads instead[1]

- 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)

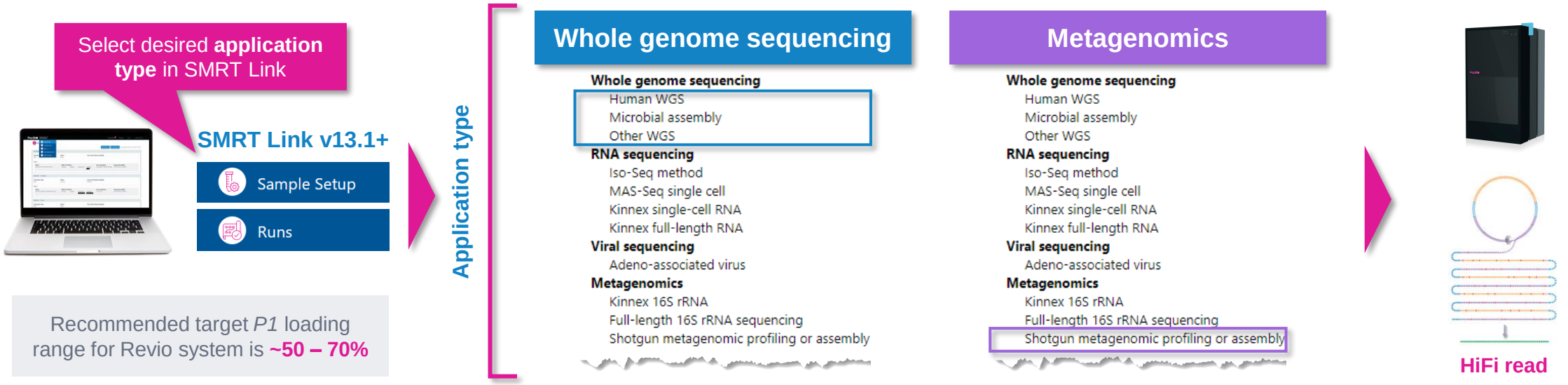
- Perform **DNA concentration QC** on final purified SPK 3.0 SMRTbell library using a Qubit dsDNA HS assay
  - Final overall recovery should be **10 – 25%** as measured from gDNA input to completed SMRTbell library (includes SRE, shearing, and library prep)
- **Note:** Final SPK 3.0 SMRTbell library concentration must be **<60 ng/ µL** to proceed with annealing, binding & cleanup (ABC)
  - → Using a concentration above 60 ng/µL will result in lower *P1* loading during sequencing
- Perform **DNA sizing QC** on final purified SPK 3.0 SMRTbell library using a Femto Pulse system

Note: To prepare SPK 3.0 WGS samples for sequencing, follow annealing, binding & cleanup (ABC) workflow instructions provided in **SMRT Link Sample Setup** calculator tool

[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



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

SMRT Link module	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			
	Concentration on plate	200 – 300 pM			
Runs → 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, then need to specify **Include Base Kinetics = YES**.

SMRT Link Sample Setup procedure for SPK 3.0 libraries – Revio system

Example sample information entry for human WGS library

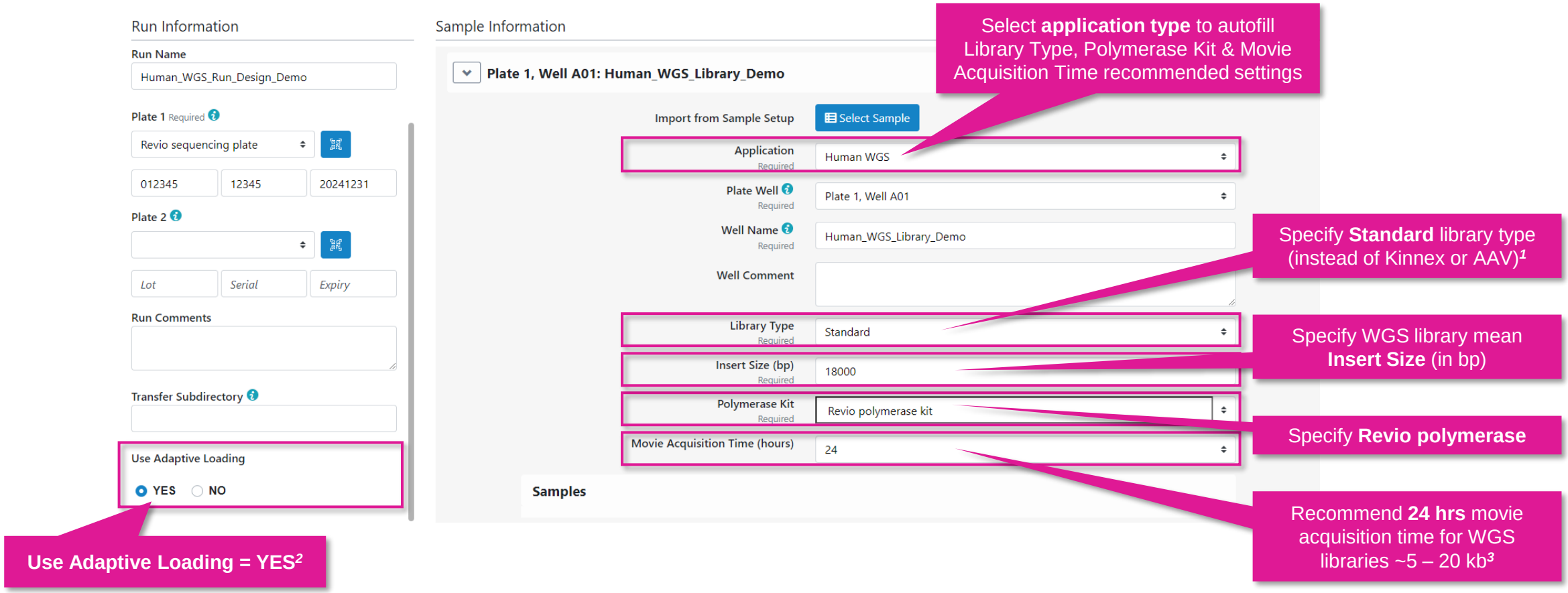
	Sample group
	Copy · Remove · Lock · Download CSV
Name	Human_WGS_library_demo
Application	Human WGS
Library type	Standard
Polymerase / Binding kit	Revio polymerase kit
Number of samples	1 samples
SMRT Cells per sample	1 cells
Available volume per sample	15 uL
Insert size	18000 bp
Sample concentration	40 ng/uL
Cleanup anticipated yield	75 %
Concentration on plate	225 pM Recommended: 200-300 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 **Revio polymerase kit**
- 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 **200 – 300 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 – Revio system

Example run information and sample information entry for human WGS library



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

2 **Note:** In SMRT Link v13.1+, Use Adaptive Loading field is a run-level setting and the default value for new Revio run designs is YES.

3 For WGS libraries ~20 – 25 kb (or larger), can specify to use 30 hrs movie acquisition time.

SMRT Link Run Design procedure for SPK 3.0 libraries – Revio system (cont.)

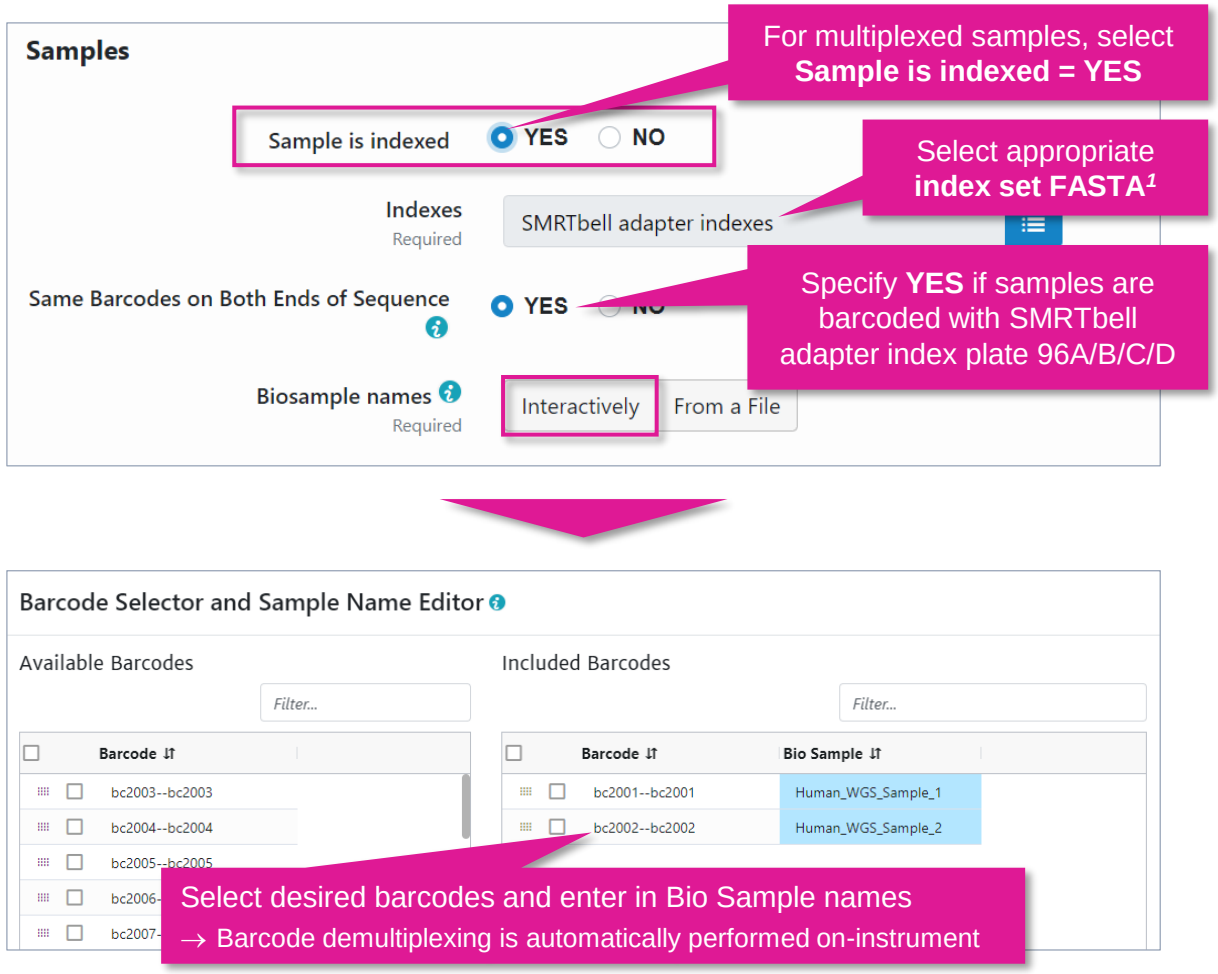
Sample indexing (barcoding) information

SMRT Link Run Design setup procedure for non-multiplexed samples

For non-multiplexed samples:

→ Specify Sample is indexed = NO and directly enter a Bio Sample Name in the adjacent field below

SMRT Link Run Design setup procedure for multiplexed samples



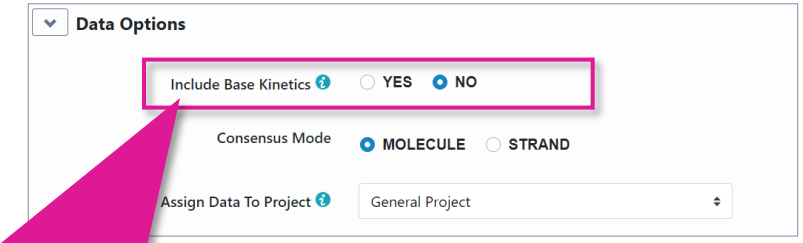
[1] Note: For Revio system, default index (barcode) set in SMRT Link run design is SMRTbell adapter indexes, which can be used to demultiplex SPK 3.0 whole genome or metagenome library samples symmetrically barcoded with SMRTbell indexed adapter plate 96A/B/C/D. To demultiplex amplicon library samples (symmetrically or asymmetrically) barcoded using indexed PCR primers, specify the appropriate index set FASTA.

SMRT Link Run Design procedure for SPK 3.0 libraries – Revio system (cont.)

Run options, Data options & Analysis options

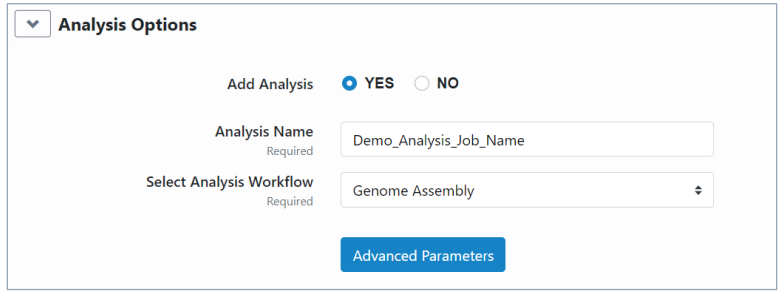
Run options

Data options



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

Analysis options



Run options fields

- **Library Concentration**
  - → Enter in sample on-plate loading concentration (OPLC)
  - → Recommended OPLC range for WGS samples is 200 – 300 pM

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

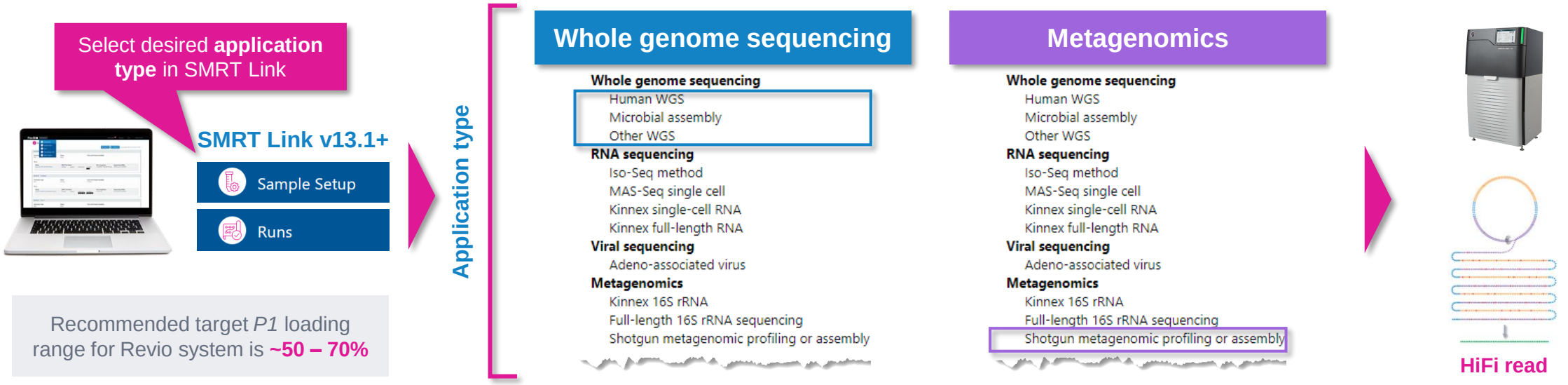
- **Include Base Kinetics**
  - → Default = **NO** for **Human WGS; Other WGS; and Shotgun metagenomic profiling or assembly**
  - → Default = **YES** for **Microbial Assembly**
- **Consensus mode**
  - → Default = **MOLECULE**
- **Assign Data to Project**
  - → Default = **General Project**; select a different project folder if desired

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

[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.

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**.

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

Example sample information entry for human WGS library

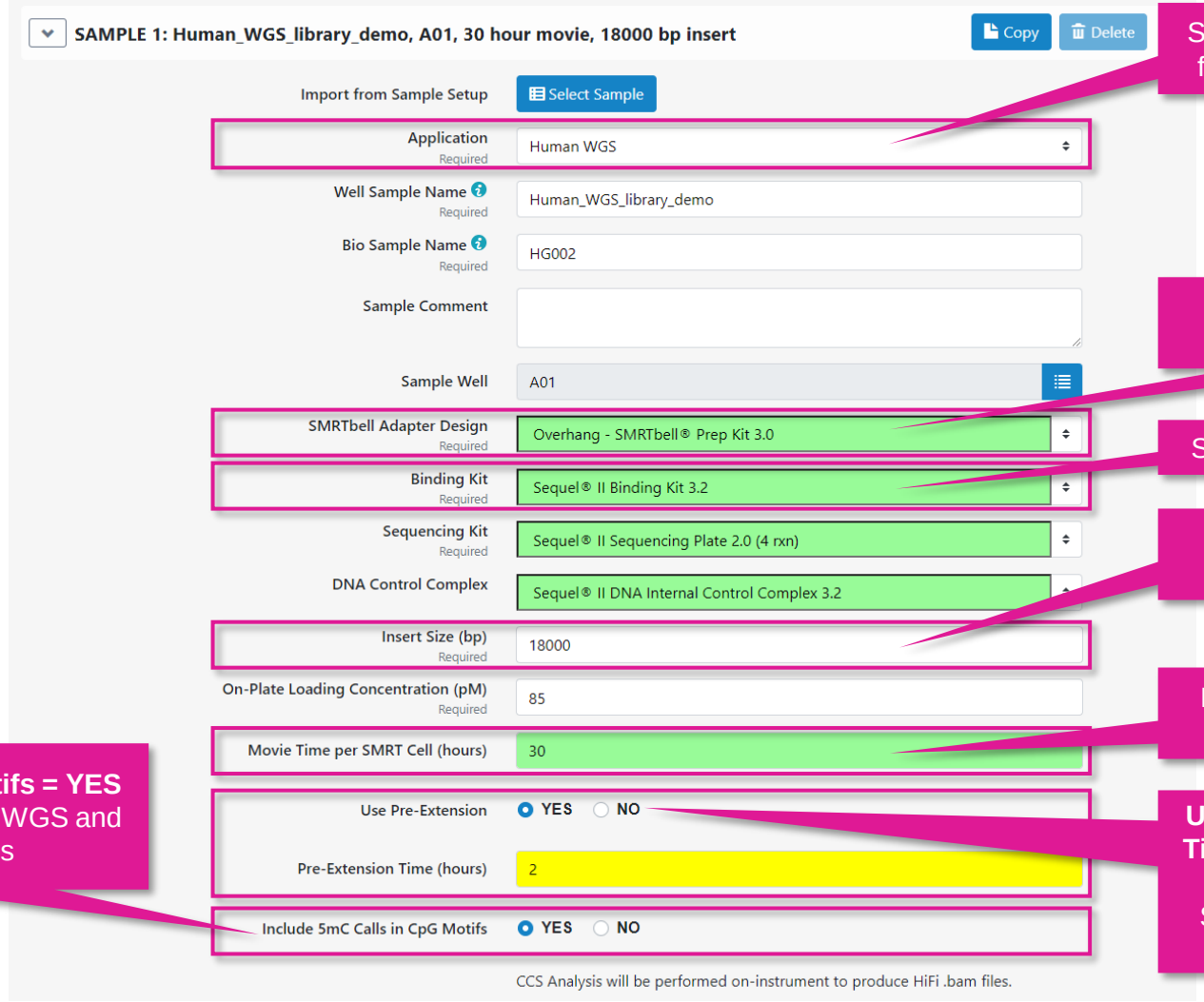
	Sample group
	Copy / Remove / Lock / Download CSV
Name	My Batch of Samples
Application	Human WGS
Library type	Standard
Polymerase / Binding kit	Sequel® II Binding Kit 3.2
Number of samples	1 samples
SMRT Cells per sample	1 cells
Available volume per sample	15 uL
Insert size	18000 bp
Sample concentration	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

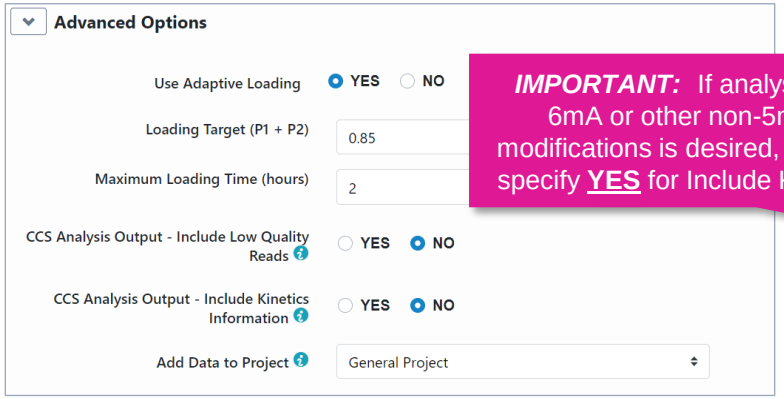
1 **SMRTbell Adapter Design** field determines which adapter finding algorithm is used during post-primary analysis.

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

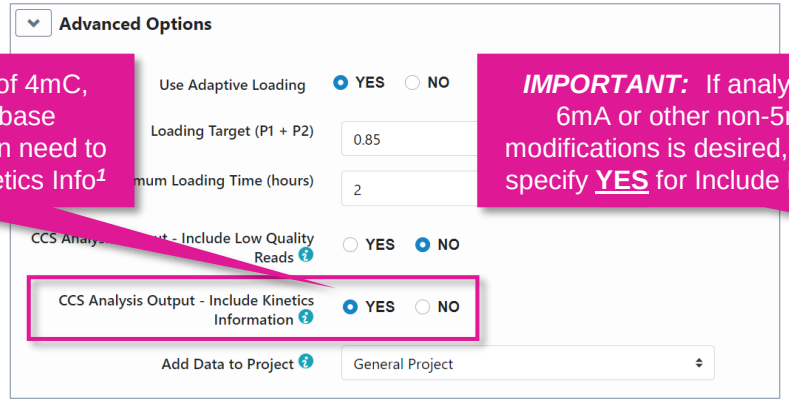
Advanced options

Human WGS or Other WGS



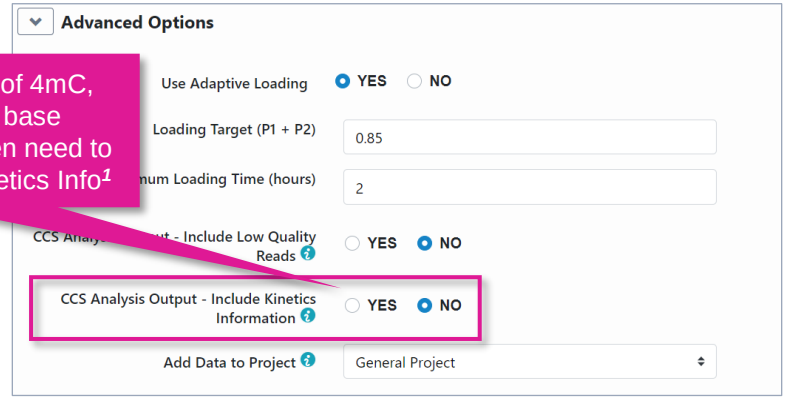
IMPORTANT: If analysis of 4mC, 6mA or other non-5mC base modifications is desired, then need to specify **YES** for Include Kinetics Info[1]

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

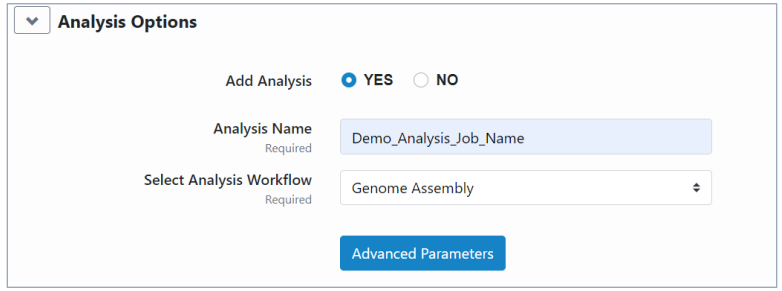
- **CCS Analysis Output – Include Low Quality Reads**
  - → Default = **NO**
- **CCS Analysis Output – Include Kinetics Information**
  - → Default = **NO** if **Human WGS; Other WGS;** or **Shotgun metagenomic profiling or assembly** is selected for application type
  - → Default = **YES** if **WGS – 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

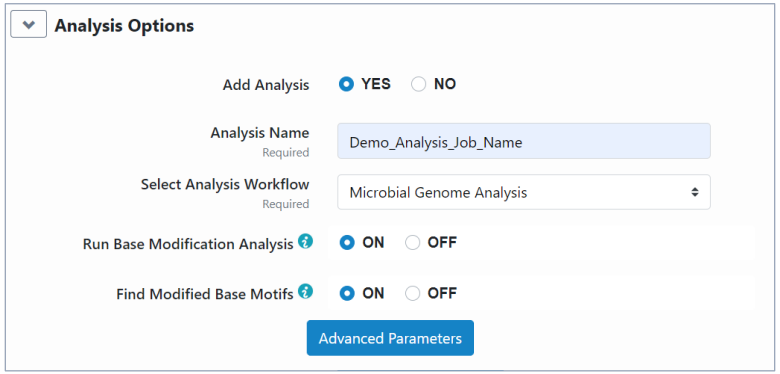
Human WGS or Other WGS



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

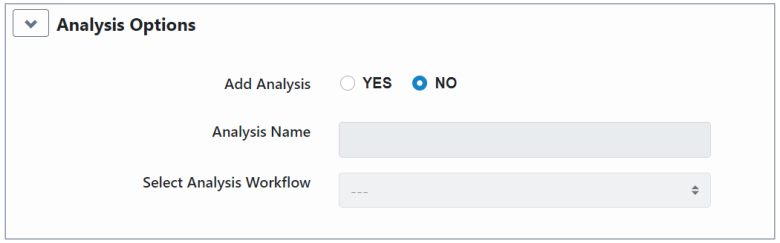
Microbial assembly



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]

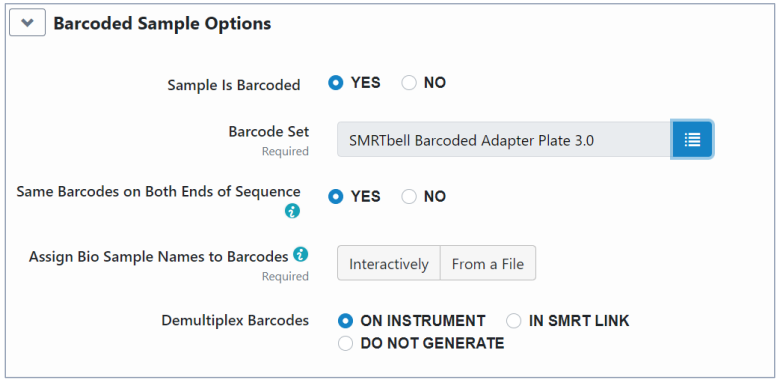
[1] Portik D.M. et al. (2022) Evaluation of taxonomic profiling methods for long-read shotgun metagenomic sequencing datasets. BMC Bioinformatics. 23:541. https://doi.org/10.1186/s12859-022-05103-0

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

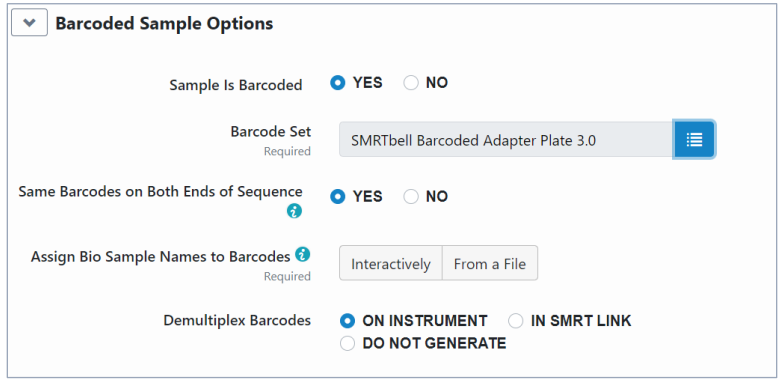
Barcoded sample options

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)

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

HiFi WGS data analysis recommendations for large genomes

Using HiFi reads for *de novo* assembly analysis of large 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 [= 3 SMRT Cells 8M or 1 Revio SMRT Cell]

- Use SMRT Link Genome Assembly analysis application (powered by IPA) or other third-party software for *de novo* assembly analysis using HiFi reads:[1]
  - Hifiasm
  - HiCanu

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 large genomes (cont.)

Using HiFi reads for variant detection analysis of large 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 [= 1 SMRT Cell 8M or <1 Revio SMRT Cell]

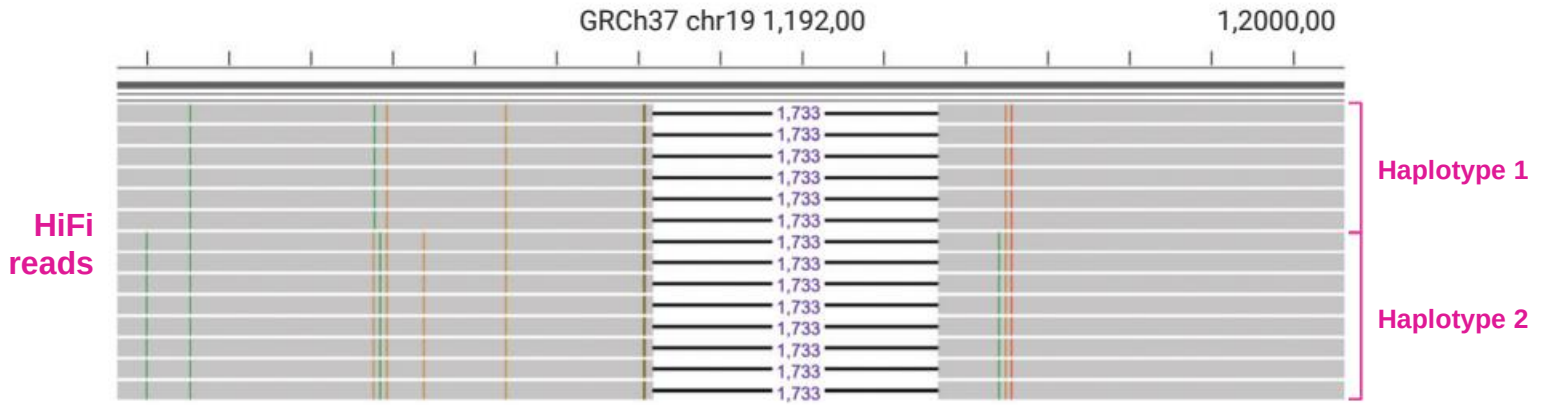
- For detection of all variant classes, we recommend **30-fold HiFi read coverage per sample**

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

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

  Recommended minimum target HiFi base yield = 3 Gb x 30 = 90 Gb [= 3 SMRT Cells 8M or 1 Revio SMRT Cell]

- Use SMRT Link Variant Calling analysis application (powered by Google DeepVariant & PacBio pbsv) for detection of small variants (SNVs, InDels) and structural variants (SVs)[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 small genomes – Microbial multiplexing applications

Using HiFi reads for *de novo* assembly and base modification detection analysis of microbial genomes

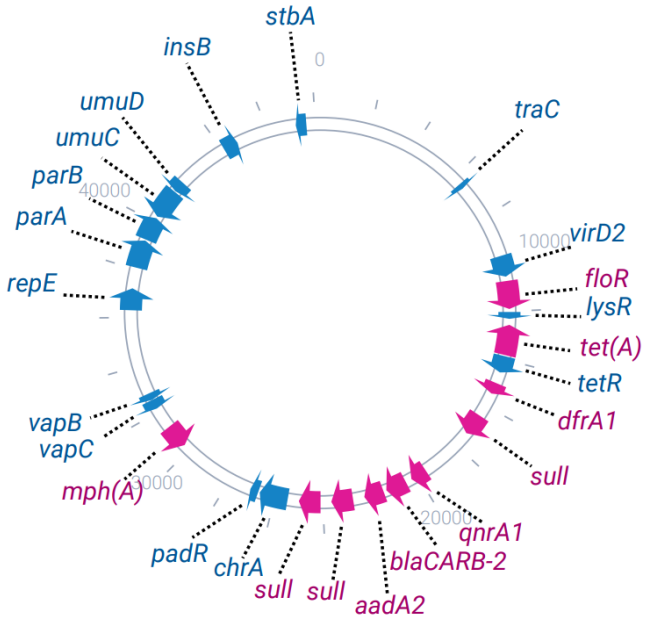
- **15-fold HiFi read coverage per microbe** is recommended for most microbial WGS *de novo* assembly projects

  → $Target\ HiFi\ Base\ Yield = [Microbe\ Genome\ Size\ (Mb)]x\ [Target\ HiFi\ Coverage\ per\ Microbe]$

  E.g., for *de novo* assembly analysis of a 5 Mb microbial genome:

  Recommended minimum target HiFi base yield = 5 Mb x 15 = 75 Mb

- Microbial multiplexing guidance
  - **Sequel II/IIe systems:** Multiplex up to 96 microbial libraries per SMRT Cell 8M (up to a total sum of genome sizes = 375 Mb)
  - **Revio system:** Multiplex up to 384 microbial libraries per Revio SMRT Cell (up to a total sum of genome sizes = 1.2 Gb)[1]
- Use SMRT Link Microbial Genome analysis application for *de novo* assembly and base modification detection analysis using HiFi reads:[2]
  - Easy to use (no requirement for laborious parameter input/optimization)
  - Enables fast and efficient microbial assembly results using HiFi reads (typical time to result is ~20-60 minutes[2] for analysis of a 96-plex microbial data set)
  - Outputs complete, high-quality microbial genome assemblies (including chromosomes and plasmids)



HiFi sequencing of *E. coli* found on retail meats allowed scientists at National Antimicrobial Resistance Monitoring System (NARMS) to fully characterize plasmid-mediated quinolone resistance (PMQR) genes.

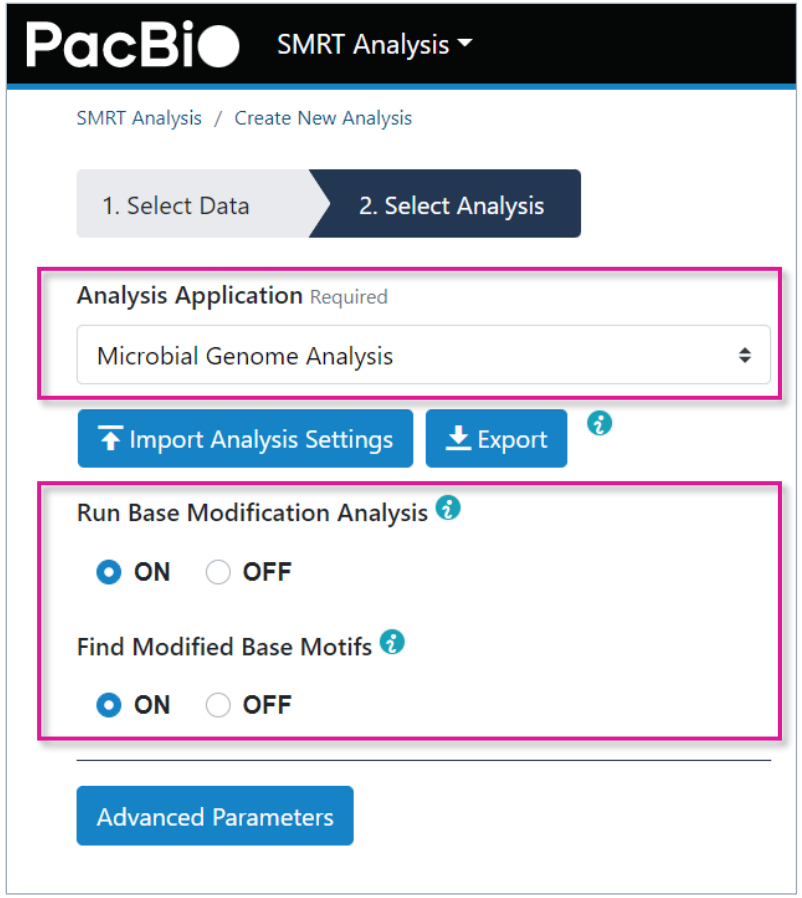
[1] Can use the HiFi plex prep kit 96 (103-381-300) in conjunction with SMRTbell adapter index plate 96A/B/C/D to multiplex up to 384 samples, or up to 375 Mb or 1.2 Gb of total genome per SMRT Cell 8M or Revio SMRT Cell, respectively. See ***Application brief – Microbial whole genome sequencing – Best practices*** (102-193-601).

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

[3] Min. compute requirements: Head Node - Cores: 32, RAM: 64 GB, 1 TB local tmp, 256 GB local db_datadir; Compute Nodes – Cores 64, RAM: 4GB per core, 1 TB local tmp, 256 GB local db_datadir.

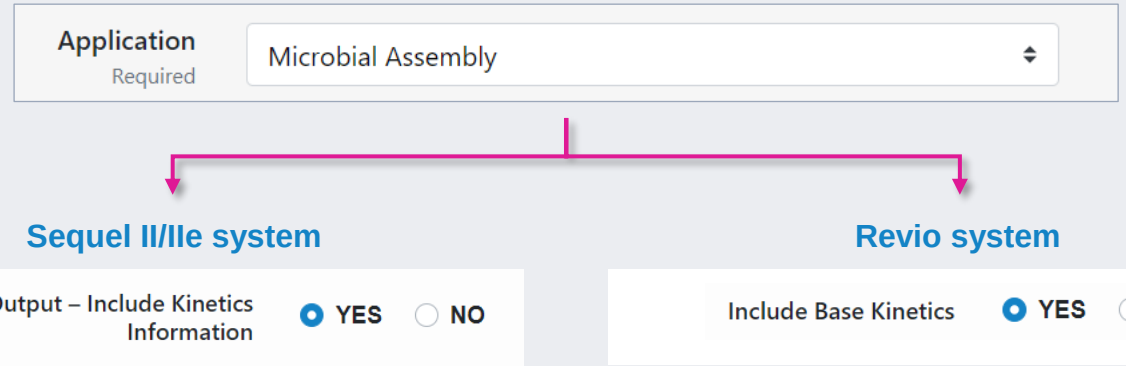
HiFi WGS data analysis recommendations for small genomes – Microbial multiplexing applications (cont.)

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



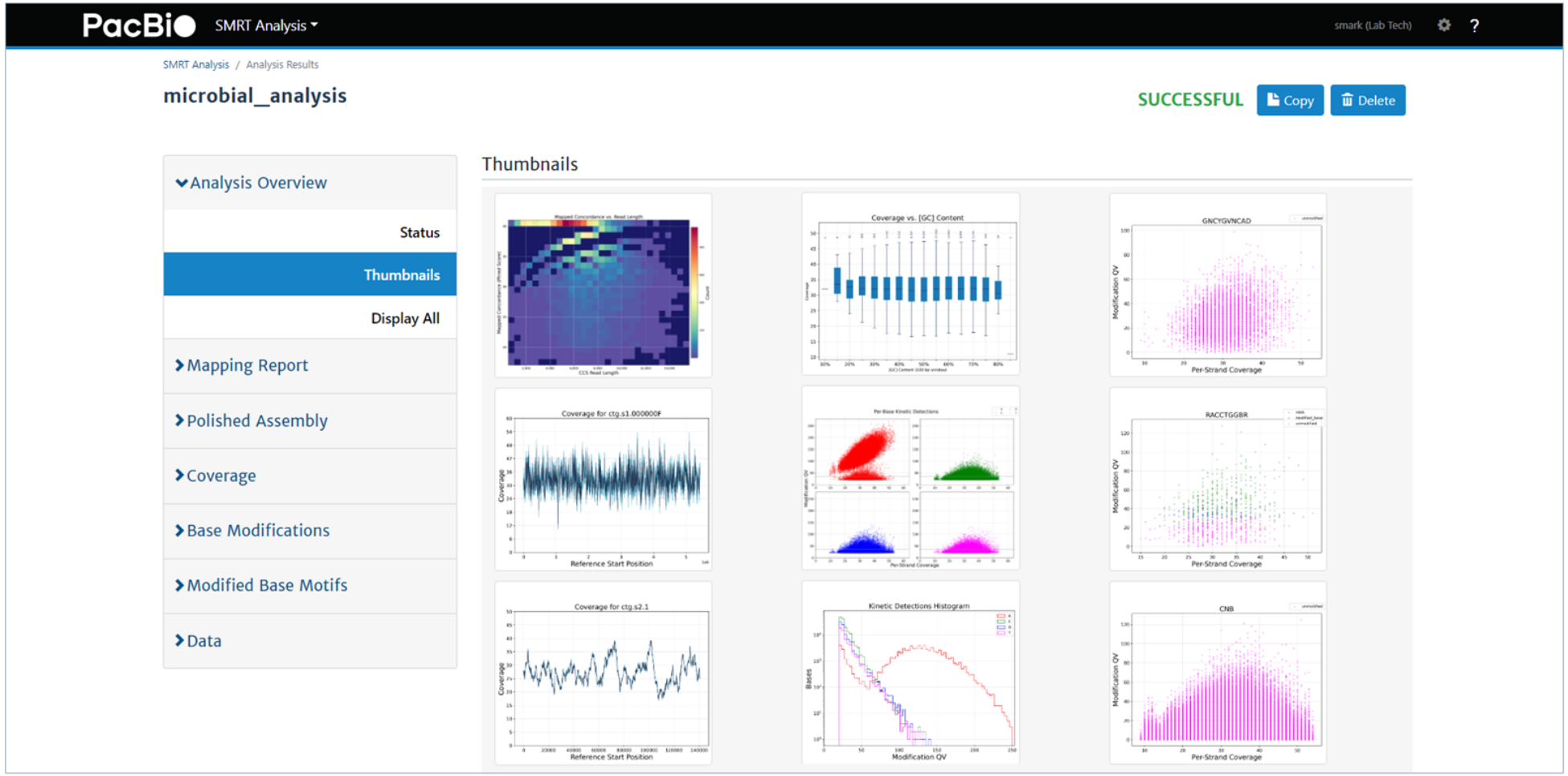
- Generate *de novo* assemblies of small prokaryotic genomes between 1.9-10 Mb and companion plasmids between 2 – 220 kb, and identify methylated bases and associated nucleotide motifs
- Optionally include identification of 6mA & 4mC modified bases and associated DNA sequence motifs.
  - Unlike 5mC calling,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

SMRT Link run design worksheet



HiFi WGS data analysis recommendations for small genomes – Microbial multiplexing applications (cont.)

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

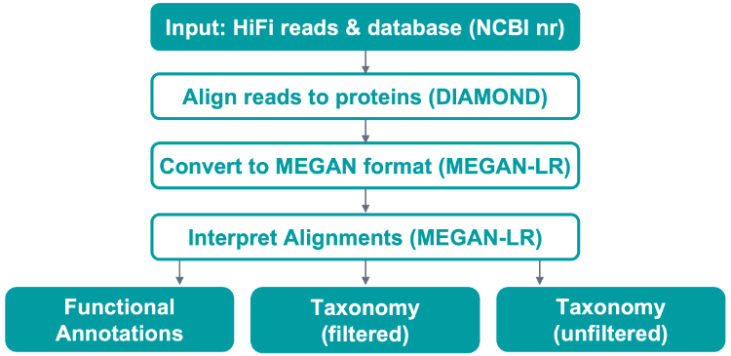


HiFi WGS data analysis recommendations for small genomes – Shotgun metagenomics applications

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

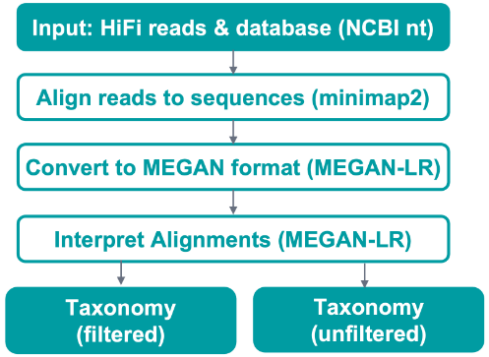
- Use SMRT Link to output HiFi data in standard file formats (BAM and FASTA/Q) for seamless integration with downstream analysis tools
- Can use PacBio metagenomics tools available on GitHub for taxonomic classification and functional gene profiling using HiFi reads[1]

Taxonomic-Profiling-Diamond-Megan



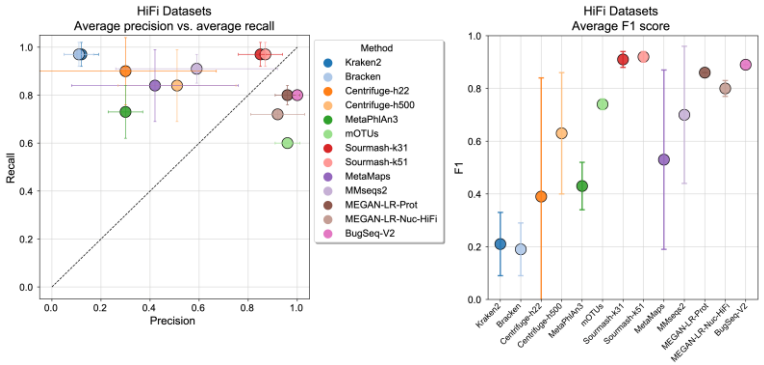
- Perform translation alignment of HiFi reads to a protein database using DIAMOND and summarize with MEGAN-LR, for the purpose of taxonomic and functional profiling.
- Provides access to NCBI and GTDB taxonomic annotations

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.

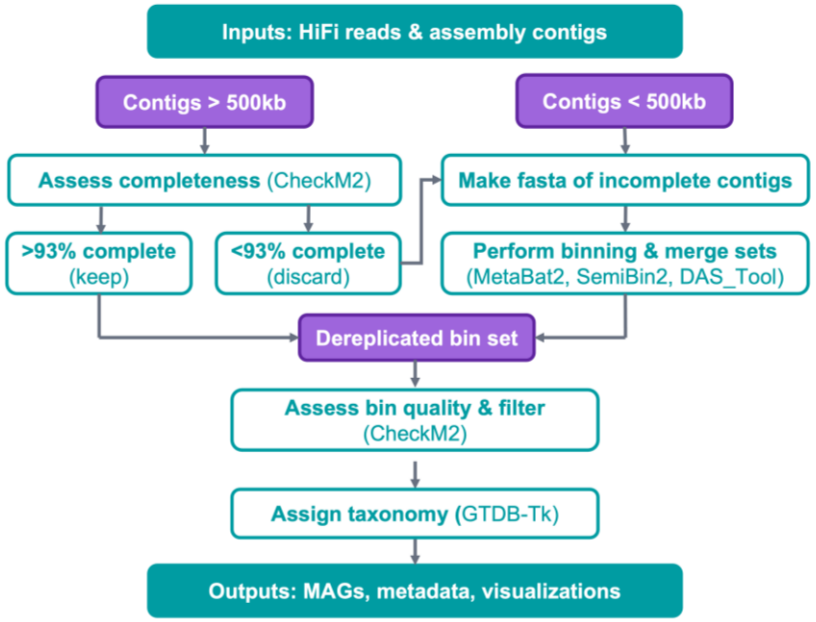
[1] Refer to https://github.com/PacificBiosciences/pb-metagenomics-tools for details about specific PB-metagenomics-tools pipelines.

HiFi WGS data analysis recommendations for small genomes – 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 HiCanu) and evaluate & extract metagenome-assembled genomes using PacBio HiFi-MAG-Pipeline tool available on GitHub (see Portik *et al*.[1])

HiFi-MAG-Pipeline



- 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 96 communities per Revio SMRT Cell or up to 48 communities per Sequel II/II system SMRT Cell 8M
- **Shotgun metagenomic assembly applications:**
  - Multiplex up to 12 communities per Revio SMRT Cell or up to 4 communities per Sequel II/IIe system SMRT Cell 8M

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 SMRT Cell 8M, 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)..

[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]	2.4 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] Typically, ≥99.5% of HiFi reads have recoverable barcodes (for 7 – 10 kb library insert size).

- Average HiFi read length for metagenomics samples is typically ~8 – 9 kb when shearing genomic DNA to ~7 – 10 kb[2] and following our recommended SMRTbell library preparation procedure[3] 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

[2] **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 <13 kb.

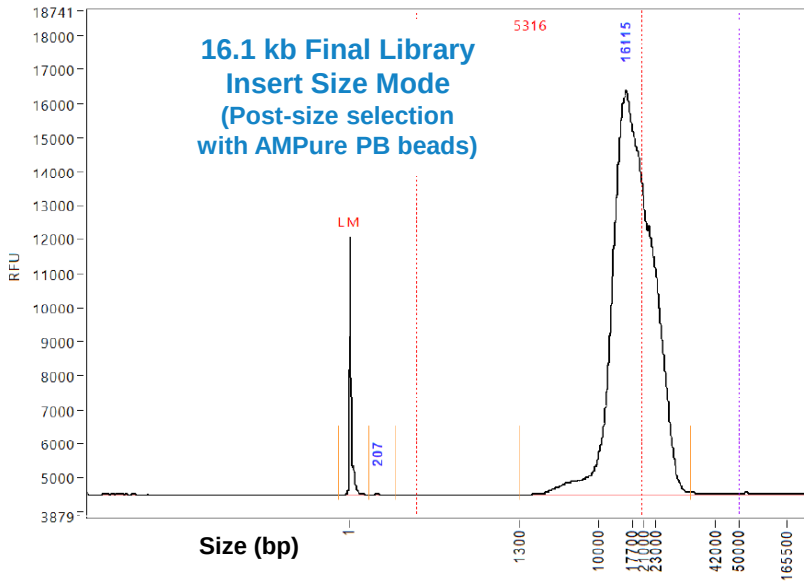
[3] Refer to ***Procedure & checklist – Preparing whole genome and metagenome sequencing libraries using SMRTbell prep kit 3.0*** (102-166-600)

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 shearing	3000 ng
Post-shearing recovery (%)[1]	2400 ng (80%)
Final yield of AMPure PB bead size-selected library (%)[2]	750 ng (25%)

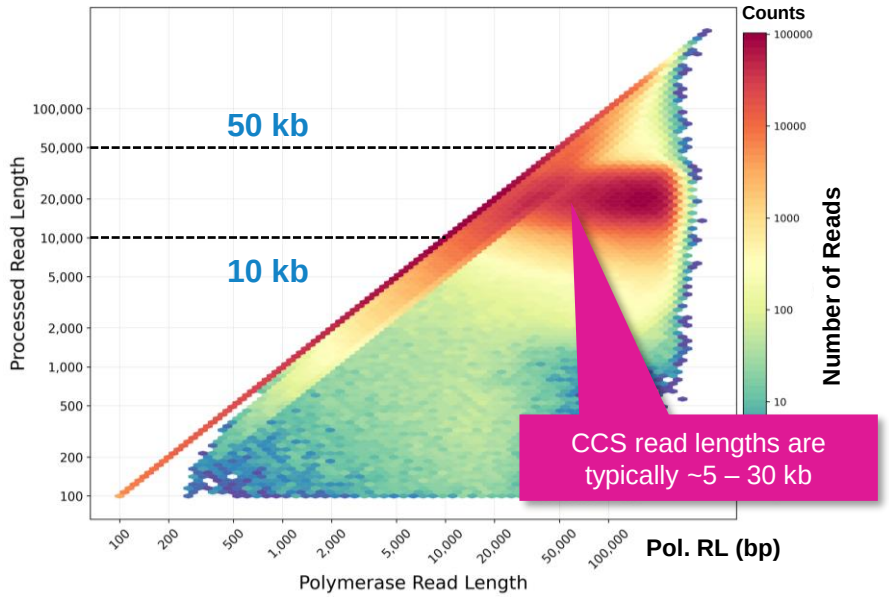
[1] Post-shearing cleanup recoveries typically ranged from ~70% to 80% when using a Hamilton automation system or Megaruptor 3 system to shear input human gDNA samples.

[2] Final post-size selected library yields typically ranged from ~10% to ~25% for human gDNA samples.

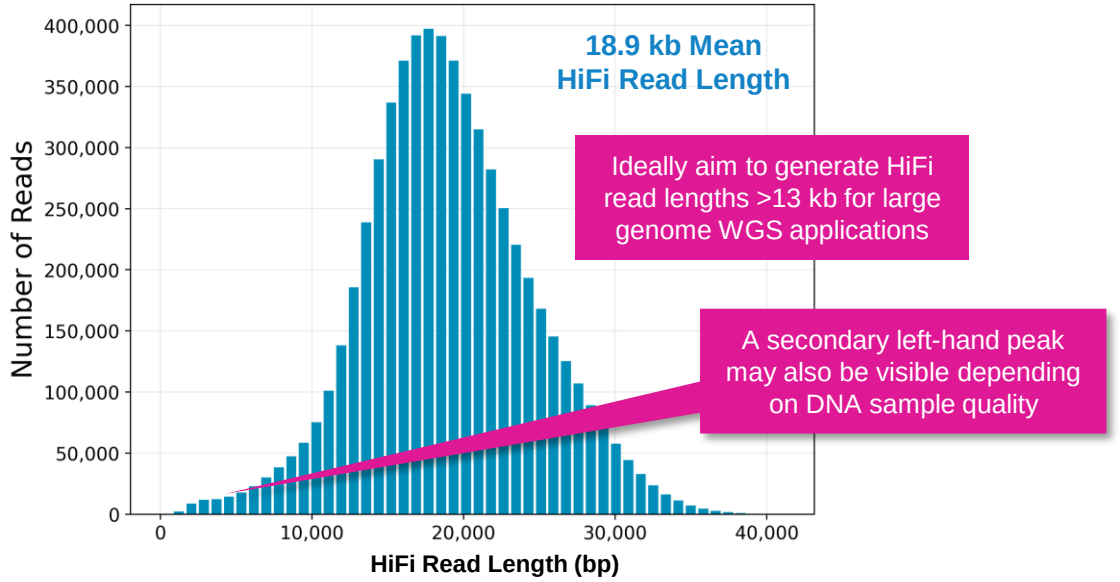
Example sequencing performance for human WGS libraries prepared with SMRTbell prep kit 3.0 – Revio system

Primary sequencing metrics[1] (Revio system)

Read length density



HiFi read length



Raw base yield	1267 Gb
Mean polymerase read length	75.4 kb
P0	31.6%
P1	66.8%
P2	1.6%

Example sequencing metrics for a human WGS sample run on a Revio system with Revio polymerase kit / 225 pM on-plate concentration / 24-hrs movie time.

HiFi reads	6.0 M
HiFi base yield[1]	114 Gb
Mean HiFi read length	18,941 bp
Median HiFi read quality	Q30
HiFi read mean # of passes	7

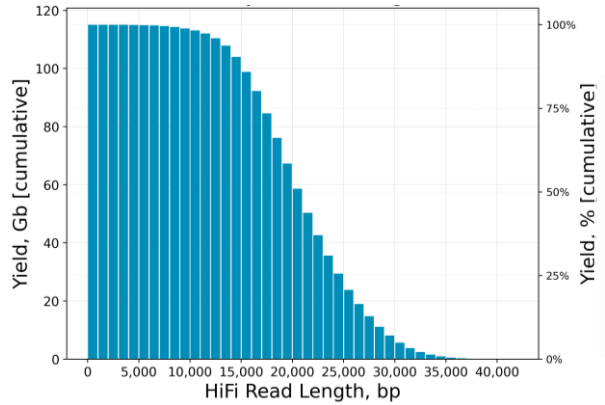
For SPK 3.0 human WGS libraries, per-Revio SMRT Cell HiFi base yields typically ranged from ~87 to 120 Gb.

[1] HiFi data yield depends on DNA sample quality, insert size, *P1* loading performance & movie time. Shorter library insert sizes (<<15 kb), lower DNA quality samples, and suboptimal *P1* loading performance may result in HiFi data yields <90 Gb per Revio SMRT Cell. Note: For Revio systems, ideal *P1* loading range is ~50 – 70%.

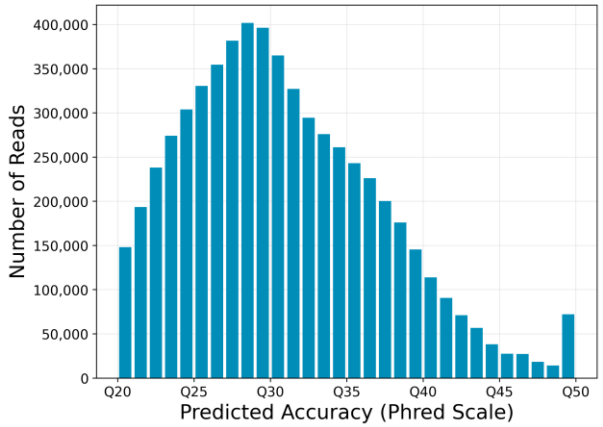
Example sequencing performance for human WGS libraries prepared with SMRTbell prep kit 3.0 – Revio system (cont.)

Primary sequencing metrics (Revio system)

Yield by HiFi Read Length



Read Quality Distribution



HiFi Read Length Summary

Read Length (bp)	Reads	Reads (%)	Yield (bp)	Yield (%)
≥ 0	6,064,580	100	114,873,966,093	100
≥ 5,000	6,014,067	99	114,702,702,517	100
≥ 10,000	5,790,033	95	112,893,558,634	98
≥ 15,000	4,696,928	77	98,540,172,331	86
≥ 20,000	2,404,855	40	58,380,785,446	51
≥ 25,000	843,391	14	23,712,877,910	21
≥ 30,000	175,604	3	5,632,859,224	5
≥ 35,000	13,685	0	499,258,512	0
≥ 40,000	346	0	14,820,110	0

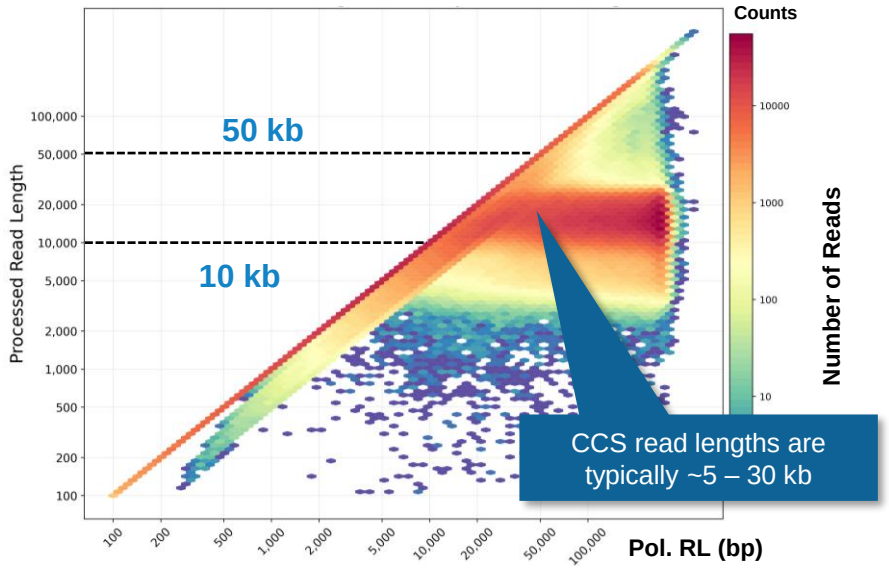
HiFi Read Quality Summary

Read Quality (Phred)	Reads	Reads (%)	Yield (bp)	Yield (%)
≥ Q20	6,064,580	100	114,873,966,093	100
≥ Q30	3,044,988	50	51,987,813,962	45
≥ Q40	533,339	9	6,723,697,325	6
≥ Q50	60,814	1	459,612,867	0

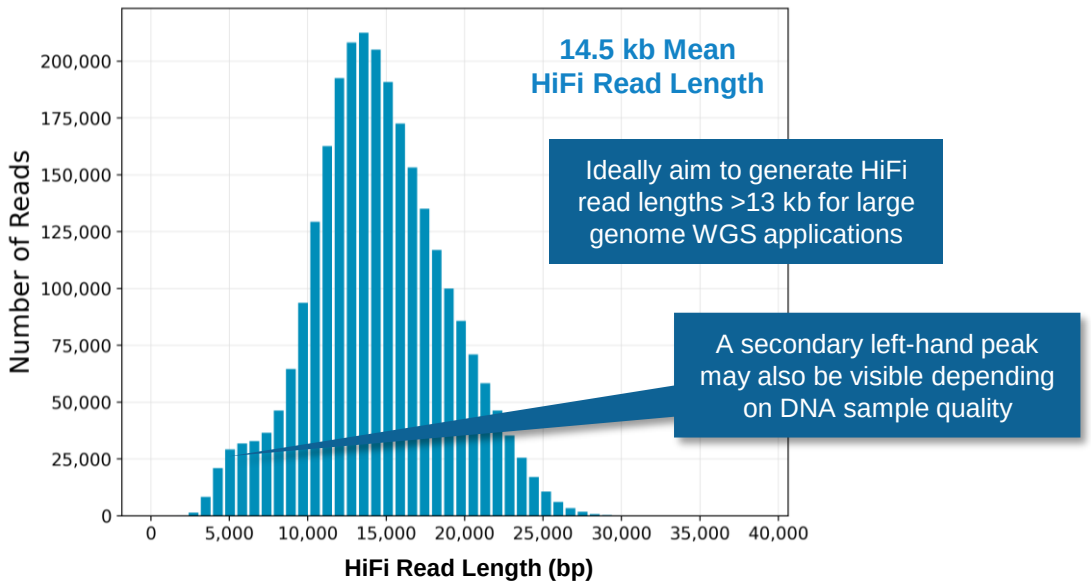
Example sequencing performance for human WGS libraries prepared with SMRTbell prep kit 3.0 – Sequel IIe system

Primary sequencing metrics[1] (Sequel IIe system)

Read length density



HiFi read length



Raw base yield	617.6 Gb
Mean polymerase read length	102.8 kb
P0	23.4%
P1	75.0%
P2	1.6%

Example sequencing metrics for a human WGS sample run on a Sequel IIe system with Binding Kit 3.2 (Polymerase 2.2) / 85 pM on-plate concentration / 30-hrs movie time / 2-hrs pre-extension time / Adaptive loading target = 0.85

HiFi reads	2.7 M
HiFi base yield[1]	39.2 Gb
Mean HiFi read length	14,490 bp
Median HiFi read quality	Q34
HiFi read mean # of passes	12

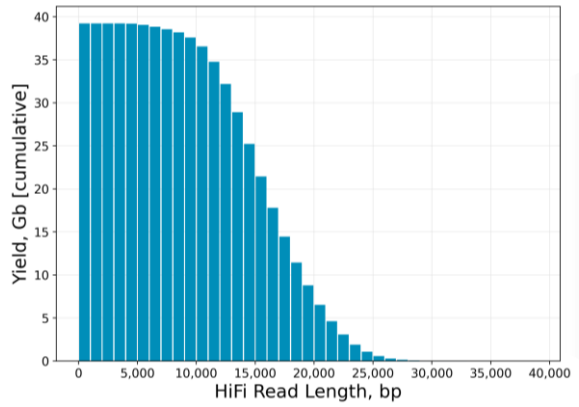
For SPK 3.0 human WGS libraries, per-SMRT Cell 8M HiFi base yields typically ranged from ~28 to 39 Gb.

[1] HiFi data yield depends on DNA sample quality, insert size, *P1* loading performance & movie time. Shorter library insert sizes (<<15 kb), lower DNA quality samples, and suboptimal *P1* loading performance may result in HiFi data yields <30 Gb per SMRT Cell 8M. Note: For Sequel II/IIe systems, ideal *P1* loading range is ~50 – 80%.

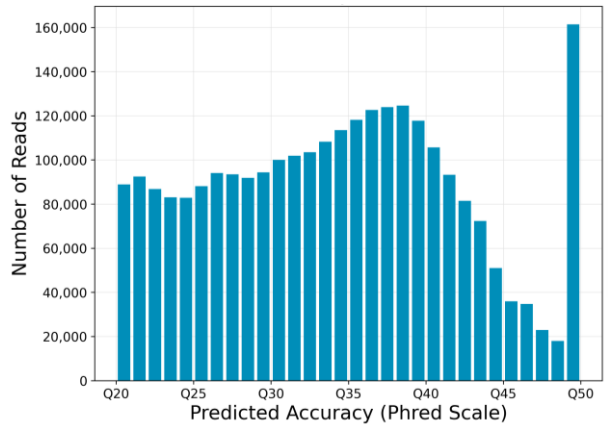
Example sequencing performance for human WGS libraries prepared with SMRTbell prep kit 3.0 – Sequel IIe system (cont.)

Primary sequencing metrics (Sequel IIe system)

Yield by HiFi Read Length



Read Quality Distribution



HiFi Read Length Summary

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

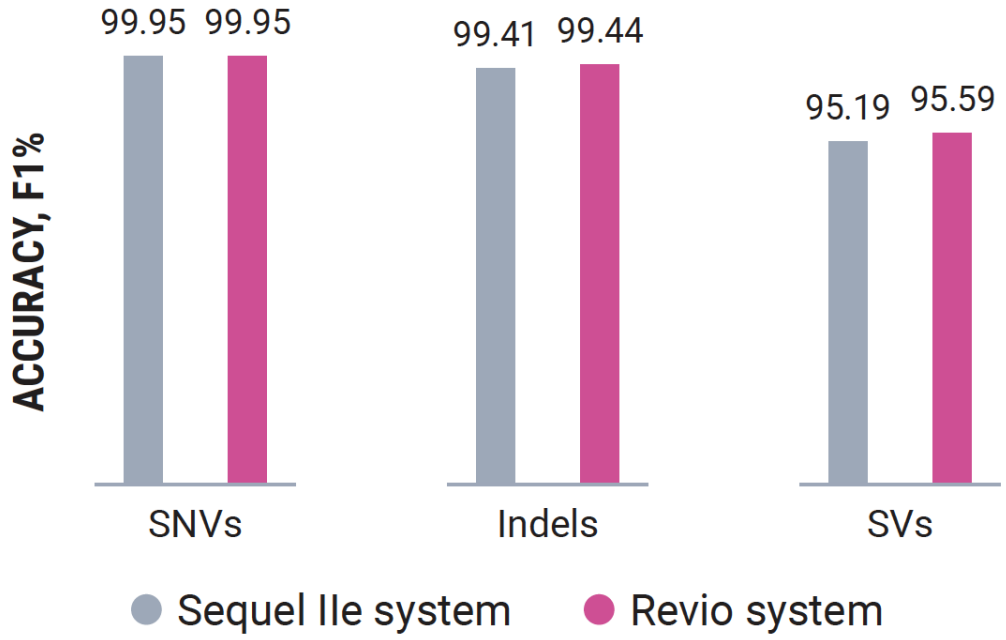
HiFi Read Quality Summary

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

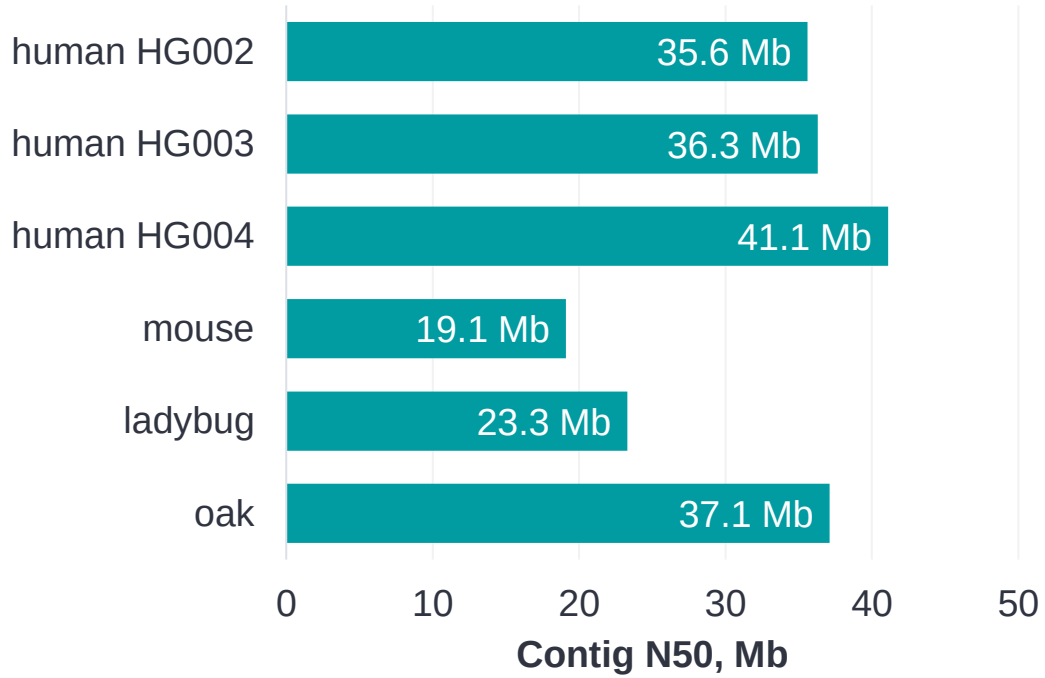
Example SPK 3.0 WGS application performance

Sequel IIe & Revio systems demonstrate exceptional human variant detection and genome assembly application performance

Revio system **matches *precisionFDA*-winning variant calling performance** of Sequel IIe systems



Revio system has **excellent genome assembly** performance



Example SPK 3.0 WGS application performance (cont.)

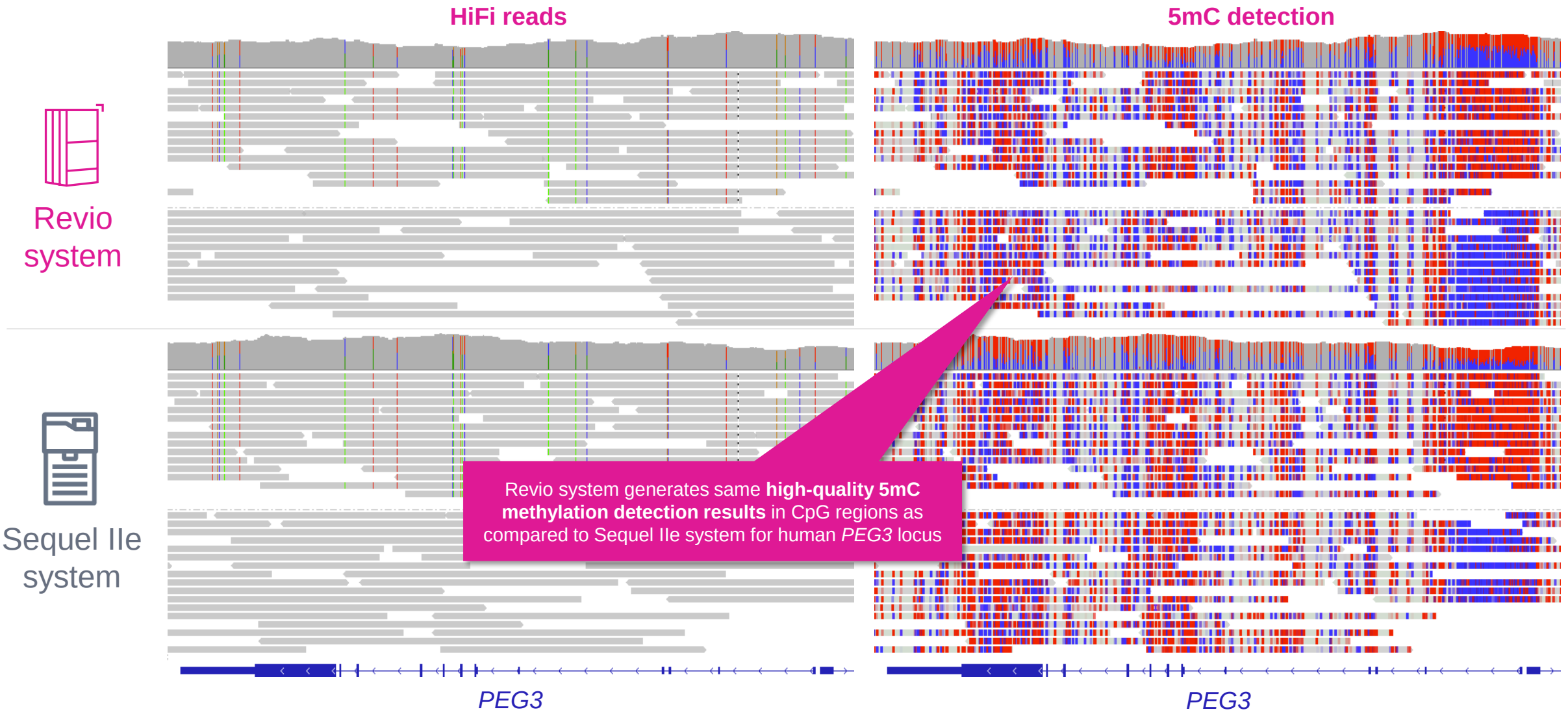
Example high-quality plant and animal genome assemblies produced with the Revio system in collaboration with the Darwin Tree of Life project at the Sanger Institute

Revio SMRT Cell	HiFi yield	>Q30 bases	HiFi yield	Average HiFi insert length	Assembly size (hap1 / hap2)	Contig N50 (hap1 / hap2)
ladybug	91.2 Gb	91%	20.8 Gb	11.6 kb	550 / 525 Mb	23.9 / 15.5 Mb
+						
mouse			70.4 Gb	16.4 kb	2,880 Mb	22.0 Mb
oak	92.6 Gb	92%	41.0 Gb	14.3 kb	825 / 797 Mb	33.9 / 33.7 Mb
+						
mistletoe			51.6 Gb	17.3 kb	n.a.	n.a.

Two samples were pooled and sequenced on a single Revio SMRT Cell

Example SPK 3.0 methylation detection performance

Example 5mC methylation detection results for a human HG002 sample



HG002 at maternally imprinted *PEG3* locus

https://downloads.pacbcloud.com/public/revio/2022Q4/HG002-rep3/analysis/HG002.m84005_220827_014912_s1.GRCh38.bam

https://downloads.pacbcloud.com/public/dataset/HG002-CpG-methylation-202202/HG002.GRCh38.haplotagged.bam

PacBio

Example SPK 3.0 microbial WGS application performance (96-plex) – Sequel II system

Experiment design & multiplexed SMRTbell library preparation workflow

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

Experiment design

- 24 different microbes; each ligated independently to 4 different SMRTbell adapter indexes for 96-plex
- Selected microbes relevant to food safety and human health represent a range of genome sizes, GC content, and plasmid composition
- Total sum of genome sizes = 375 Mb

SMRTbell library construction

- 1 µg of input gDNA per microbe for Megaruptor 3 shearing
- For this data set, target shear size = 7 kb – 10 kb
- 500 ng of sheared DNA per sample for library prep
- Symmetrically barcoded samples using SMRTbell adapter index plate 96A (102-009-200)
- No size-selection performed on final libraries

Microbial genome assembly complexity[1]

Class I – Have few repeats except for the rDNA operon sized 5 to 7 kb

Class II – Class II genomes have many repeats, such as insertion sequence elements, but none greater than 7 kb.

Class III – Contain large, often phage-related, repeats >7 kb.

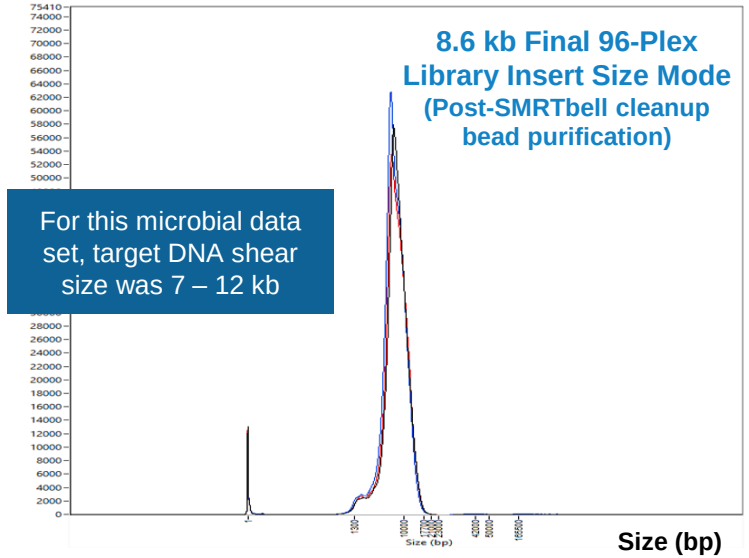
Microbial species	Genome size (bp)	GC content (%)	Microbial genome complexity	Barcode names
Acinetobacter baumannii AYE	3,960,239	39.35	Class 3	bc2001 / bc2025 / bc2049 / bc2073
Bacillus cereus 971	5,430,163	35.29	Class 1	bc2002 / bc2026 / bc2050 / bc2074
Bacillus subtilis W23	4,045,592	43.5	Class 1	bc2003 / bc2027 / bc2051 / bc2075
Burkholderia cepacia UCB 717	8,569,621	66.6	Class 3	bc2004 / bc2028 / bc2052 / bc2076
Burkholderia multivorans 249	7,008,277	66.68	Class 3	bc2005 / bc2029 / bc2053 / bc2077
Enterococcus faecalis OG1RF	2,739,503	37.75	Class 1	bc2006 / bc2030 / bc2054 / bc2078
Escherichia coli H10407	5,393,109	50.71	Class 1	bc2007 / bc2031 / bc2055 / bc2079
Escherichia coli K12 MG1655	4,642,522	50.79	Class 1	bc2008 / bc2032 / bc2056 / bc2080
Helicobacter pylori J99	1,645,141	39.19	Class 1	bc2009 / bc2033 / bc2057 / bc2081
Klebsiella pneumoniae BAA-2146	5,780,684	56.97	Class 2	bc2010 / bc2034 / bc2058 / bc2082
Listeria monocytogenes Li2	2,950,984	37.99	Class 1	bc2011 / bc2035 / bc2059 / bc2083
Listeria monocytogenes Li23	2,979,685	38.19	Class 1	bc2012 / bc2036 / bc2060 / bc2084
Methanocorpusculum labreanum Z	1,804,962	50.5	Class 1	bc2013 / bc2037 / bc2061 / bc2085
Neisseria meningitidis FAM18	2,194,814	51.62	Class 3	bc2014 / bc2038 / bc2062 / bc2086
Neisseria meningitidis Serogroup B	2,304,579	51.44	Class 1	bc2015 / bc2039 / bc2063 / bc2087
Rhodopseudomonas palustris CGA009	5,459,213	64.9	Class 3	bc2016 / bc2040 / bc2064 / bc2088
Salmonella enterica LT2	4,950,860	52.24	Class 1	bc2017 / bc2041 / bc2065 / bc2089
Salmonella enterica Ty2	4,791,947	52.05	Class 1	bc2018 / bc2042 / bc2066 / bc2090
Staphylococcus aureus Seattle 1945	2,806,348	32.86	—	bc2019 / bc2043 / bc2067 / bc2091
Staphylococcus aureus USA300_TCH1516	2,872,915	32.7	Class 1	bc2020 / bc2044 / bc2068 / bc2092
Streptococcus pyogenes Bruno	1,844,942	38.48	—	bc2021 / bc2045 / bc2069 / bc2093
Thermanaerovibrio acidaminovorans DSM6589	1,852,980	63.78	Class 1	bc2022 / bc2046 / bc2070 / bc2094
Treponema denticola A	2,842,721	37.87	—	bc2023 / bc2047 / bc2071 / bc2095
Vibrio parahaemolyticus EB101	5,146,979	45.33	Class 1	bc2024 / bc2048 / bc2072 / bc2096

[1] *Koren, S. et al. (2013) Reducing assembly complexity of microbial genomes with single-molecule sequencing. Genome Biol 14, R101*

Example microbial WGS application performance (96-plex) – Sequel II system

SMRTbell library QC & primary sequencing metrics[1] (Sequel II system)

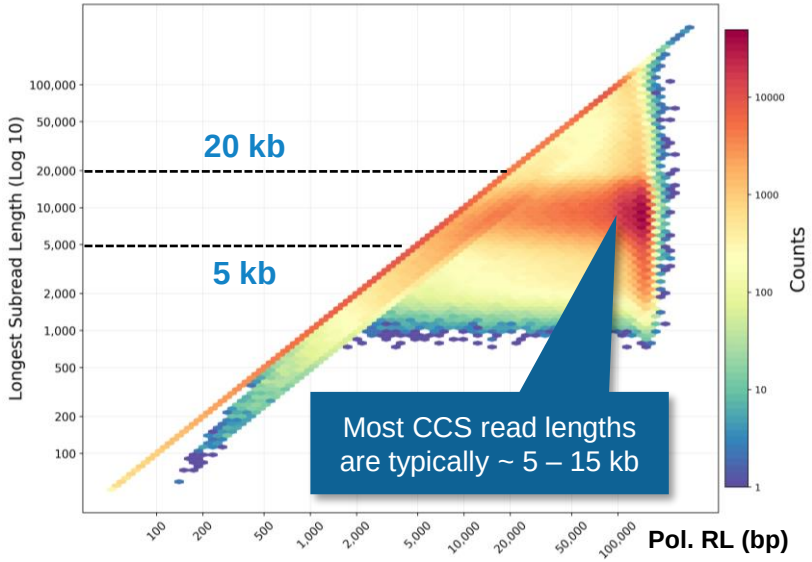
Size-selected Library QC



Input gDNA per microbe for DNA shearing	1 µg
Input sheared DNA per microbe for library construction	500 ng
Mean SMRTbell library construction yield per microbe before pooling (%)*	32%

* LC yields ranged from 17 – 52% across 96 microbes. Microbial libraries were barcoded with SMRTbell adapter index plate 96A, independently purified with SMRTbell cleanup beads after nuclease treatment, and then pooled for sequencing on a single SMRT Cell 8M.

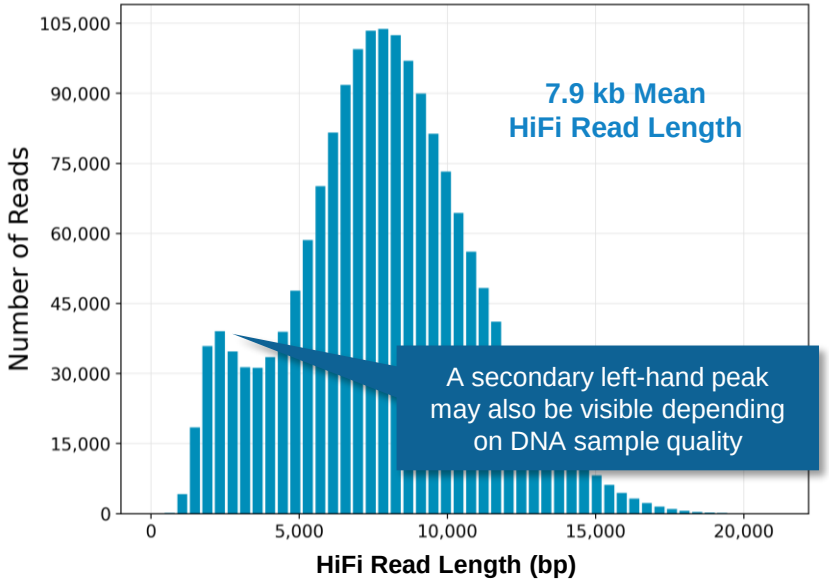
Raw Data Report



Raw Base Yield	224.9 Gb
Mean Polymerase Read Length	79.2 kb
P0	63.3
P1	35.6
P2	1.1

90 pM on-plate concentration / 15-hrs movie time / 2-hrs pre-extension time / Adaptive loading target = 0.85

CCS Analysis Report



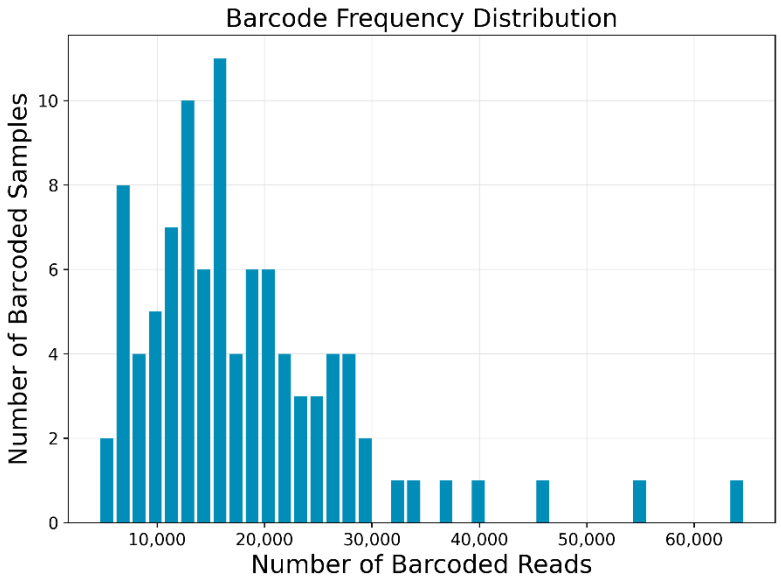
HiFi Reads	1.8 M
HiFi Base Yield	13.8 Gb
Mean HiFi Read Length	7,881 bp
Median HiFi Read Quality	Q38
HiFi Read Mean # of Passes	14

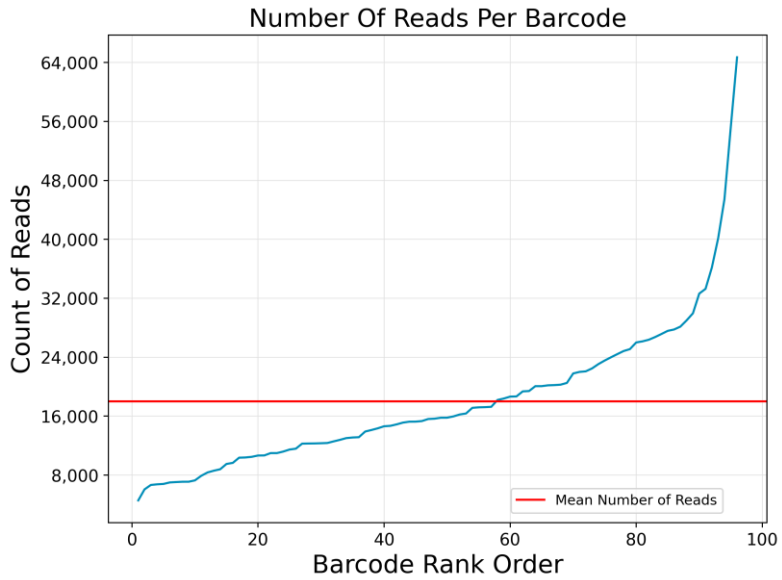
[1] HiFi data yield depends on DNA sample quality, insert size, *P1* loading performance & movie time. Shorter library insert sizes (<<15 kb), lower DNA quality samples, and suboptimal *P1* loading performance may result in HiFi data yields <30 Gb per SMRT Cell 8M. Note: For Sequel II/IIe systems, ideal *P1* loading range is ~50 – 80%.

Example microbial WGS application performance (96-plex) – Sequel II system

Barcode demultiplexing results (Sequel II system)

Value	Analysis Metric
96	Unique Barcodes
1,731,704	Barcoded Reads
18,038	Mean Reads
64,709	Max. Reads
4,565	Min. Reads
7,856	Mean Read Length
24,632	Unbarcoded Reads
98.66%	Percent Bases in Barcoded Reads
98.59%	Percent Barcoded Reads

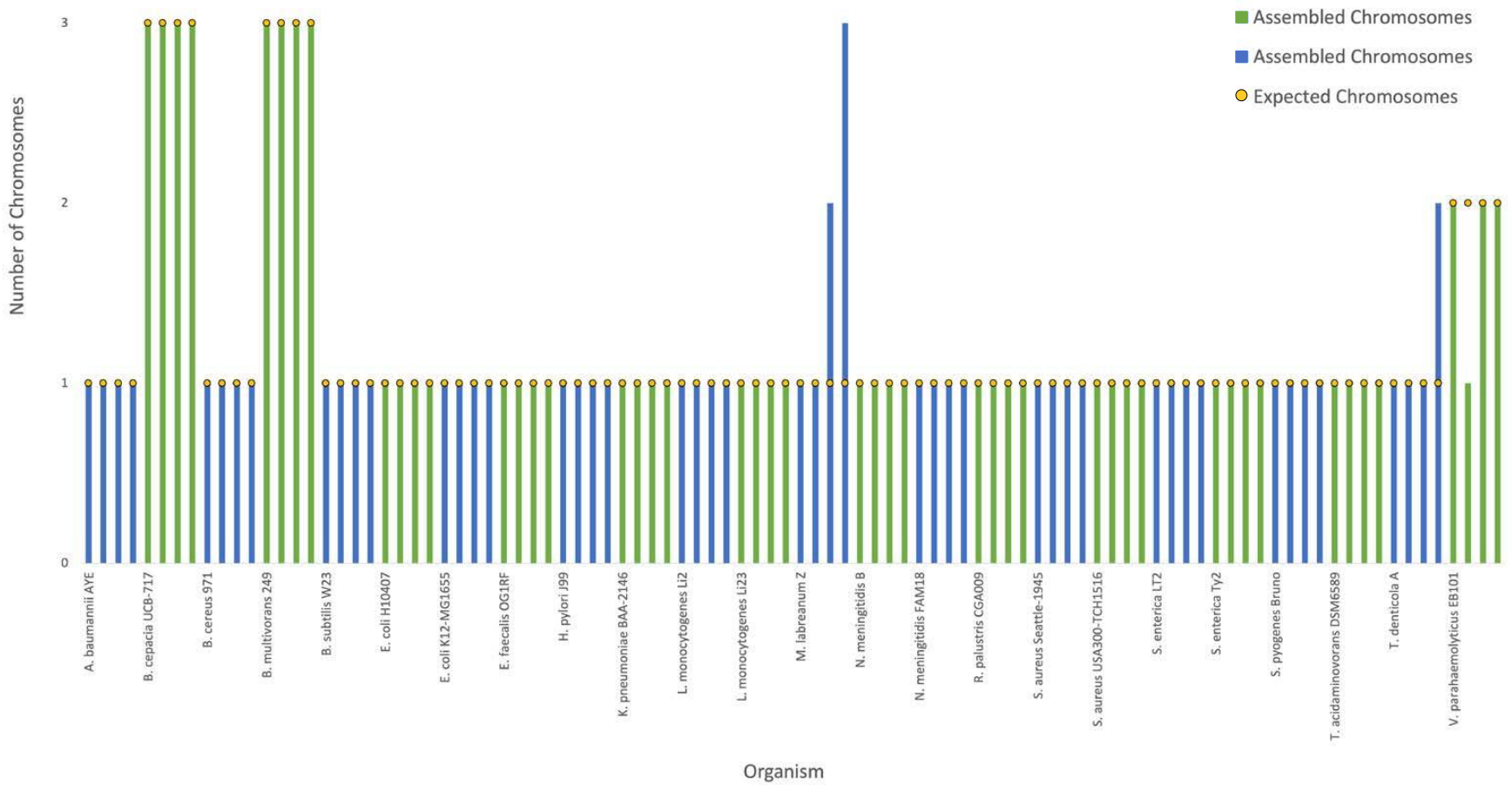




- **All 96 barcodes detected**
- Mean # of barcoded HiFi reads per microbe is ~18,000
- Mean HiFi base coverage per microbe is 36-fold (Range is 19- to 63-fold)

Example microbial WGS application performance (96-plex) – Sequel II system

HiFi de *novo* assembly results – assembled chromosomes (Sequel II system)

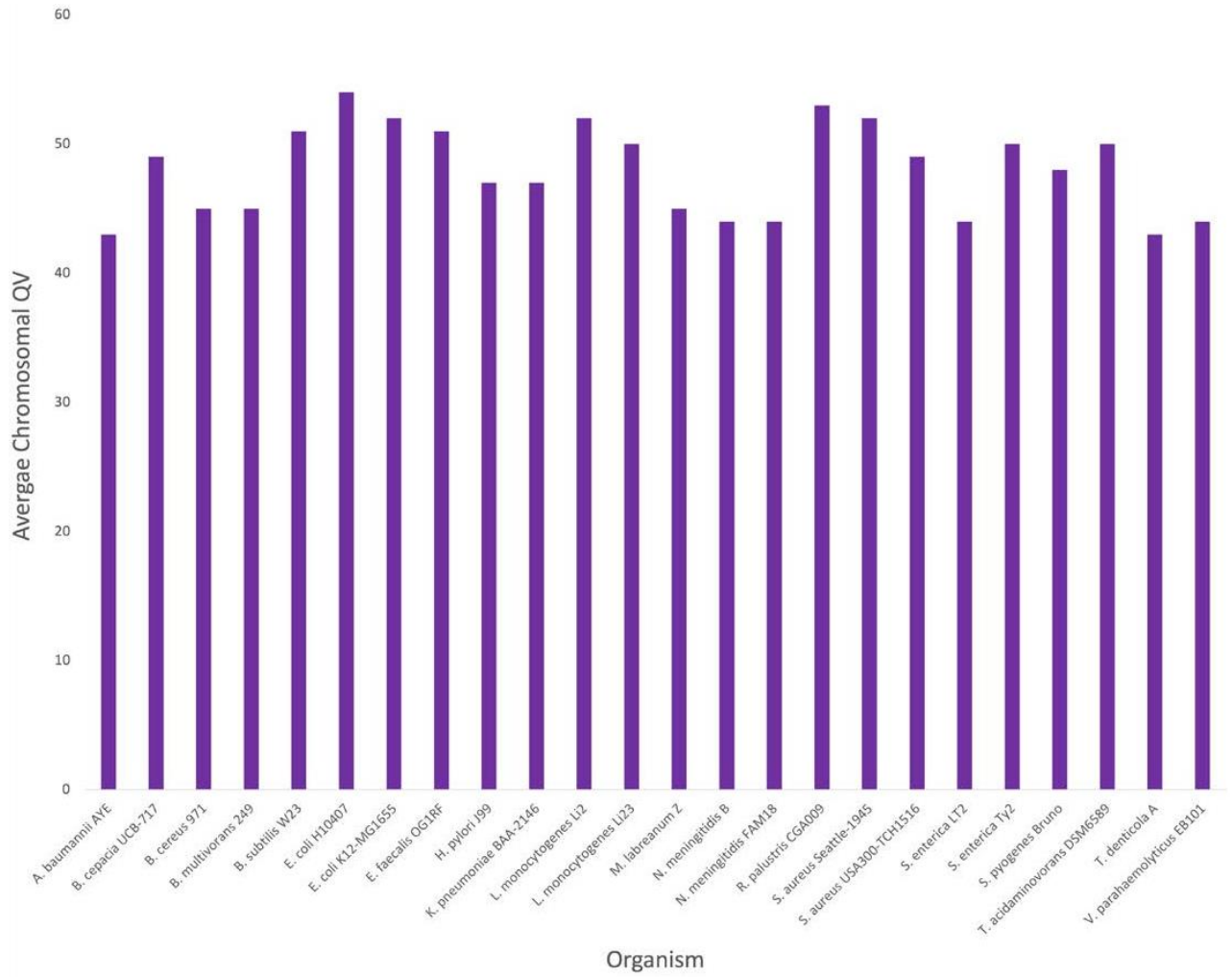


- Achieved **1 Contig / Chromosome** for 92 out of 96 assemblies
- For all 96 microbes, **most or all chromosomal assemblies were complete and of the expected sizes**

Microbial assembly statistics from a 96-plex pool of bacteria relevant to food safety and human health. These data were generated on the Sequel II system and assembled with the fully automated HiFi-based Microbial Assembly application in SMRT Link using the default parameters, without any manual curation. Download and explore the data yourself.

Example microbial WGS application performance (96-plex) – Sequel II system

HiFi *de novo* assembly results – representative assembly accuracies (Sequel II system)



- Accuracy of representative samples from a 96-plex microbial whole genome sequencing run on a Sequel II system
- With HiFi data and the Microbial Assembly application in SMRT Link, **genome assemblies are consistently >99.99% accurate**

Technical documentation & applications support resources

Technical resources for WGS library preparation, sequencing & data analysis

DNA sample extraction literature & other resources

- Nanobind HMW DNA extraction Procedures & checklists [Link]
- Nanobind kit Guides & overviews [Link]
- Procedure & checklist – Removing short DNA fragments with the Short Read Eliminator (SRE) kit (102-982-300)
- 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 – Metagenomic sequencing with HiFi reads – Best practices (102-193-684)
- Application brief – Microbial whole genome sequencing – Best practices (102-193-601)
- Application brief – Whole genome sequencing (WGS) for *de novo* assembly – Best practices (102-193-627)
- Application brief – Variant detection using whole genome sequencing with HiFi reads – Best practices (102-193-604)
- 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 – Alternative size selection methods for SMRTbell prep kit 3.0 (TN103-110921)
- Technical note – Covaris g-TUBE DNA shearing for SMRTbell prep kit 3.0 (102-326-501)
- 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)

[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

- 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
- Bickhart, D.M. et al. (2022) Generating lineage-resolved, complete metagenome-assembled genomes from complex microbial communities. Nature biotechnology. 40:711-719. doi: 10.1038/s41587-021-01130-z

Webinars

- 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]
- PacBio Webinar (2022) – HiFi metagenomics: more samples, more species, more MAGs [Link]
- PacBio Webinar (2022) – Unlocking the genome with long-read sequencing in genetic disease research [Link]

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

Data analysis resources

- 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)
- SMRT Link user guide [Link]
- SMRT Tools reference guide [Link]

Example PacBio data sets

Whole genome sequencing application	Dataset	Data type	PacBio system
Assembly, variant detection and epigenetics	Homo sapiens - GIAB trio HG002-4	HiFi reads	Revio system
Tumor/normal	COLO829 melanoma	HiFi reads	Revio system
Tumor/normal	HCC1395	HiFi reads	Revio system
Whole genome sequencing	Various plant & animals – maize, mouse, and others	HiFi reads	Revio system
Assembly	Food safety and infectious microbes – 96 plex	HiFi reads	Sequel II system

APPENDIX: 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

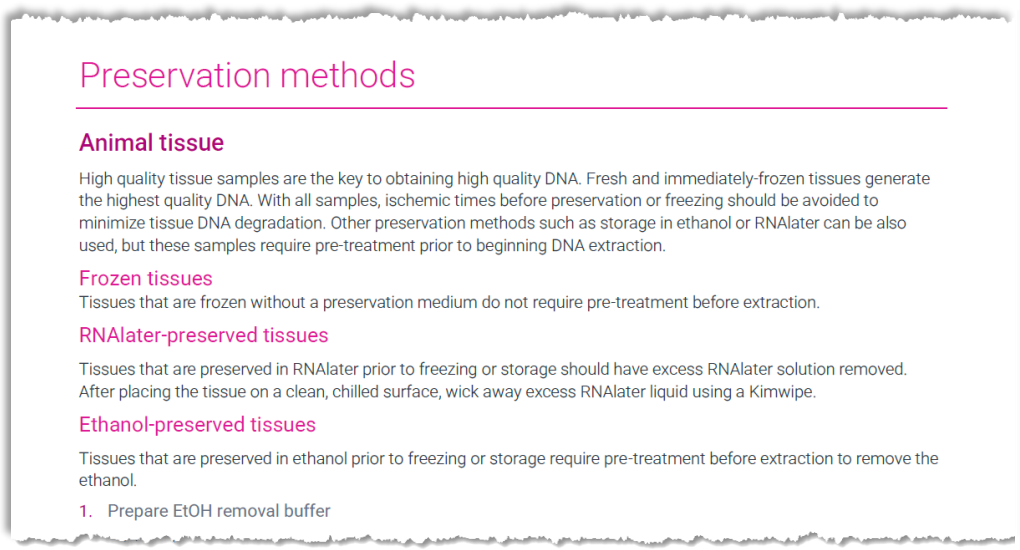
Sample collection, preparation, and storage for SMRT sequencing whole genome sequencing projects

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.

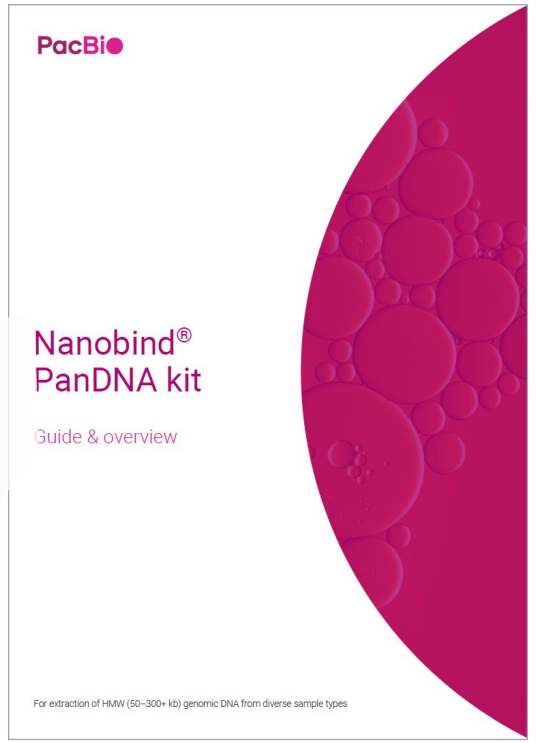
RNAlater-preserved tissues

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.

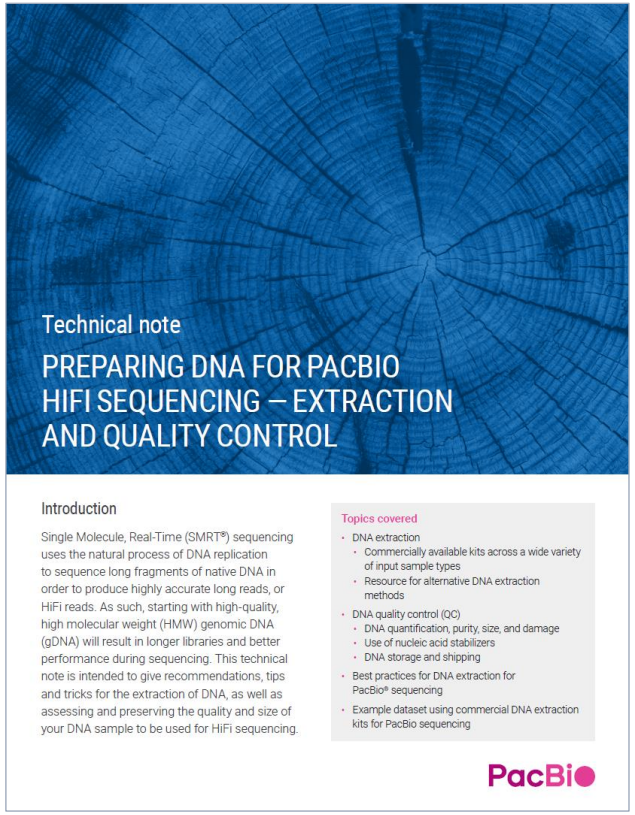
Genomic DNA extraction, QC and handling for SMRT sequencing

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
- Topics covered include:
  - DNA extraction
    - Commercially available kits across a wide variety of input sample types
    - Resource 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
- This technical note also includes an example dataset for a California Redwood tree DNA sample that was isolated using Nanobind kits.



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

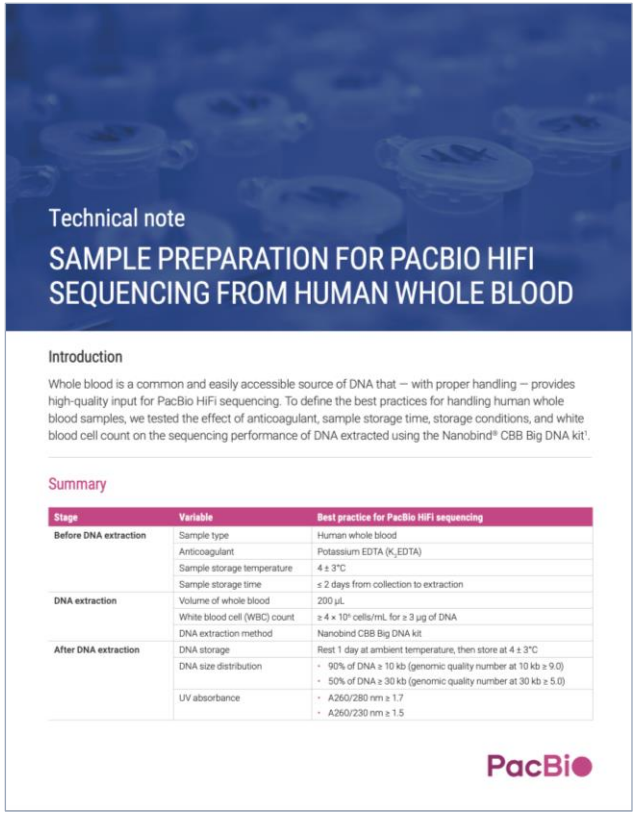
DNA extraction, QC and handling for SMRT sequencing whole-genome sequencing projects (cont.)

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.)

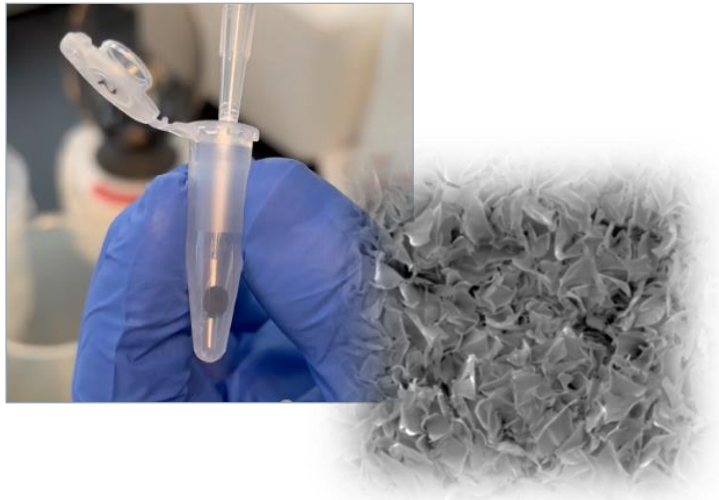
Refer to PacBio's extensive Nanobind literature to learn how to extract the highest-quality high-molecular weight (HMW) DNA using Nanobind DNA extraction kits

Nanobind kit Guide & overviews

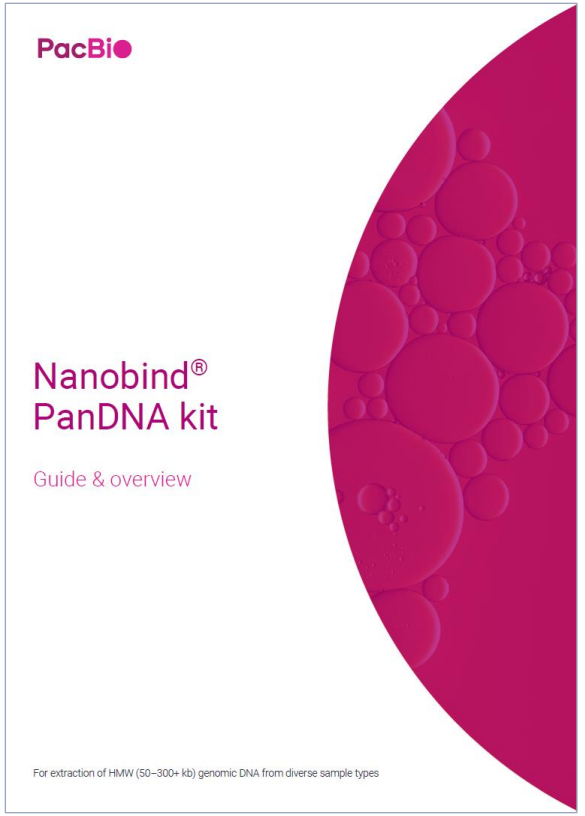
- Nanobind kit Guide & overviews contain information on HMW DNA isolation kit specifications, general tips, tissue preservation recommendations, extraction & sequencing performance, and troubleshooting tips.

Nanobind Procedure & checklists

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



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.)

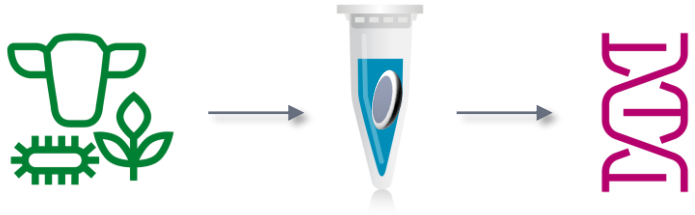
After gDNA extraction:

- 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

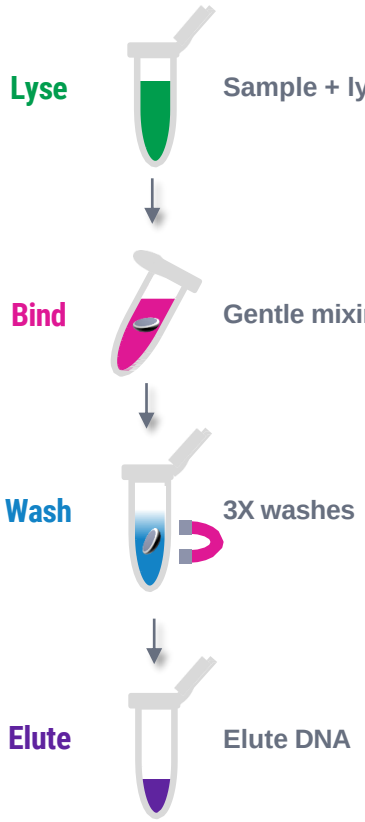
PacBio Nanobind products for HMW genomic DNA extraction

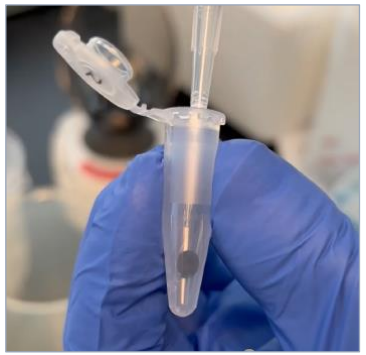
Nanobind kits can be used to extract high-quality, high-molecular weight DNA



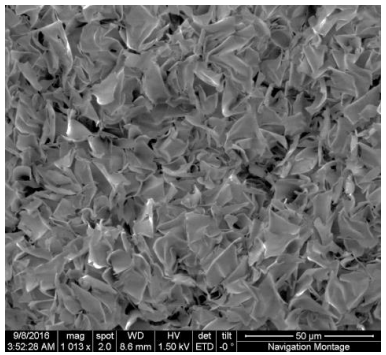
- Nanobind is a novel magnetic disk covered with a micro- and nanostructured silica
- Nanobind disks bind and release DNA without fragmentation to yield DNA up to megabase pairs in length

Rapid magnetic purification process is automatable

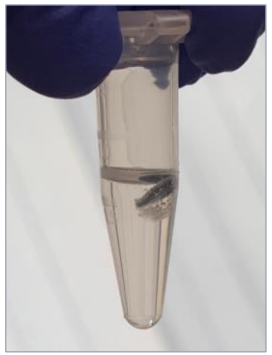




A single Nanobind disk can be processed in a 1.5 mL tube.



SEM image of Nanobind's silica surface structure.



Extracted HMW DNA bound to a Nanobind disk

For manual workflows

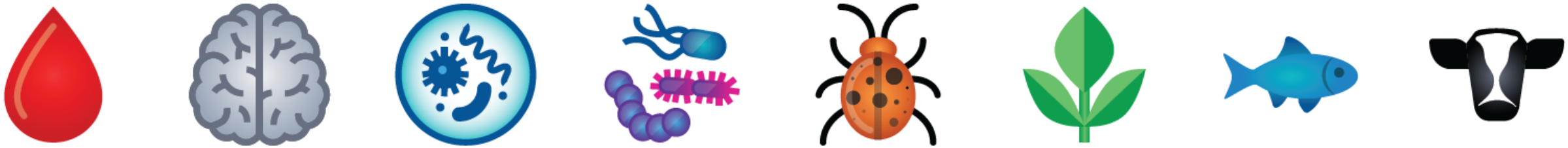
Nanobind PanDNA kit 103-260-000 (24 RXN)	Nanobind CBB kit 102-301-900 (24 RXN)
• HMW DNA extraction from cultured cells, blood, cultured bacteria, tissues, insects, and plant nuclei	• HMW DNA extraction from cultured cells, blood and cultured bacterial

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, bacteria, tissues, insects, and plant nuclei



Easy-to-use solutions built on Nanobind technology

PacBio's Nanobind PanDNA kit consolidates the capabilities of our existing 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

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 cultured 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) for example DNA extraction performance data.

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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)
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.

Methods for evaluation of genomic DNA quality

Methods for evaluation of DNA quality

Use recommended tools for evaluation of DNA quality to generate optimal SMRT sequencing data quality

DNA sizing QC

Use a Femto Pulse system for accurate DNA sizing QC of genomic DNA samples and final SMRTbell libraries

- ☑ **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

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

Methods for evaluation of DNA quality (cont.)

Use a Femto Pulse or PFGE system[1] for accurate DNA sizing QC of input genomic DNA samples & final libraries

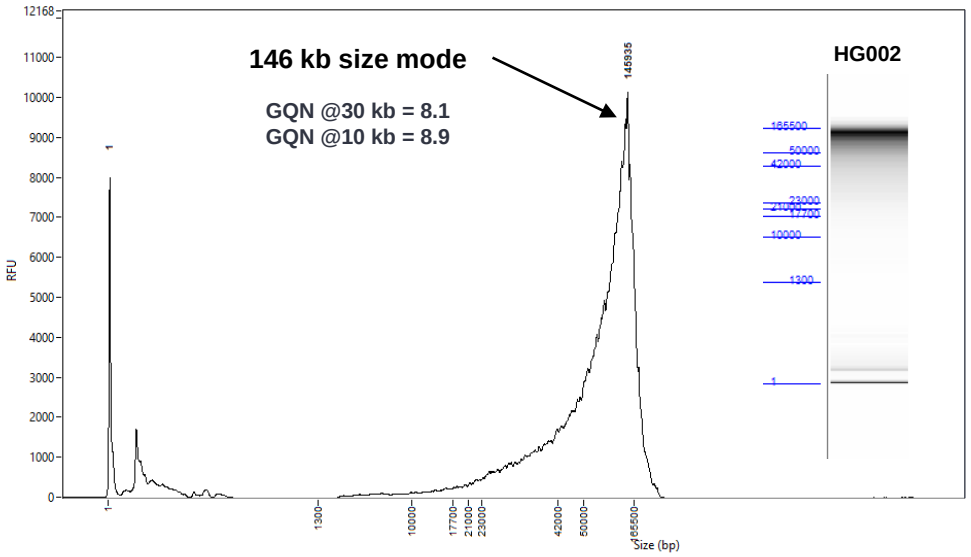
DNA sizing QC

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 ≥9.0

[1] Lower quality DNA may be used with the expectation of lower 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

Minimum input DNA required	Sequel IIe system v11.0	Revio system v13.1
Single library	≥1,000 ng per SMRT Cell 8M	≥2,000 ng per Revio SMRT Cell
Multiplexed library[1]	300 ng – 1 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.

- Use Qubit dsDNA high sensitivity (HS) assay kit [Thermo Fisher Scientific] for routine DNA quantitation during SMRTbell library construction and final QC
- Can 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
  - 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[2]
  - Samples should be **free of RNA** before beginning library prep
  - If RNA is detected, treat with RNase A (37°C for 15 min), followed by 1X SMRTbell cleanup beads (or AMPure PB) before proceeding

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

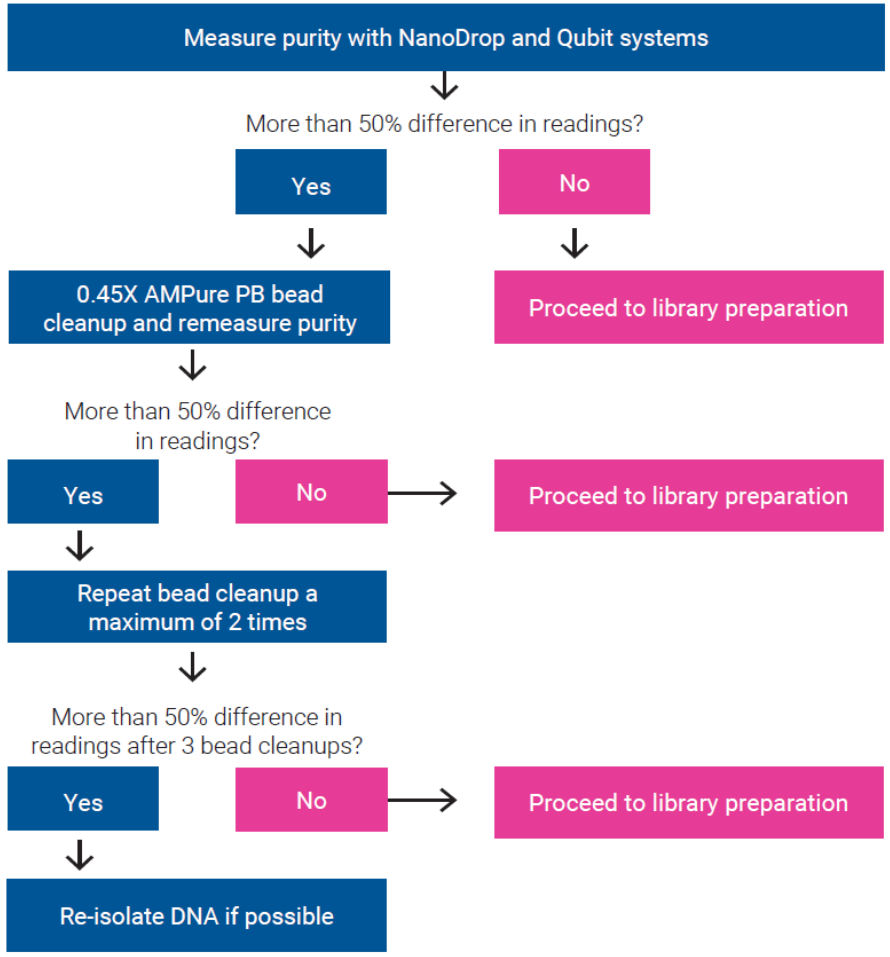
[2] Contaminant RNA can negatively impact library construction yields (by competing with DNA substrates during enzymatic steps) and reduce sequencing data yields (by inhibiting annealing of sequencing primers to SMRTbell templates and/or competing with templates for binding to polymerase).

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

- 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.[1] (1X AMPure PB beads or SMRTbell cleanup beads may also be used.)

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

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

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[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

Technical notes

- 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)

Short Read Eliminator (SRE) kit protocols and Guides & overviews

- Guide & overview – Short Read Eliminator (SRE) XS and XL kits (102-582-400)
- Procedure & checklist – Removing short DNA fragments with the Short Read Eliminator (SRE) kit (102-982-300)

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
- 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[1] Guides & overviews

- Brochure – Nanobind high-throughput HMW DNA extraction (102-326-565)
- Guide & overview – Nanobind HT kits (103-028-100)
- Technical overview – Automated high-throughput HMW DNA extraction for PacBio long-read sequencing using Nanobind HT kits (103-401-700)

[1] Contact PacBio Technical Support for information about the latest available Nanobind HT robotic automation scripts.

www.pacb.com