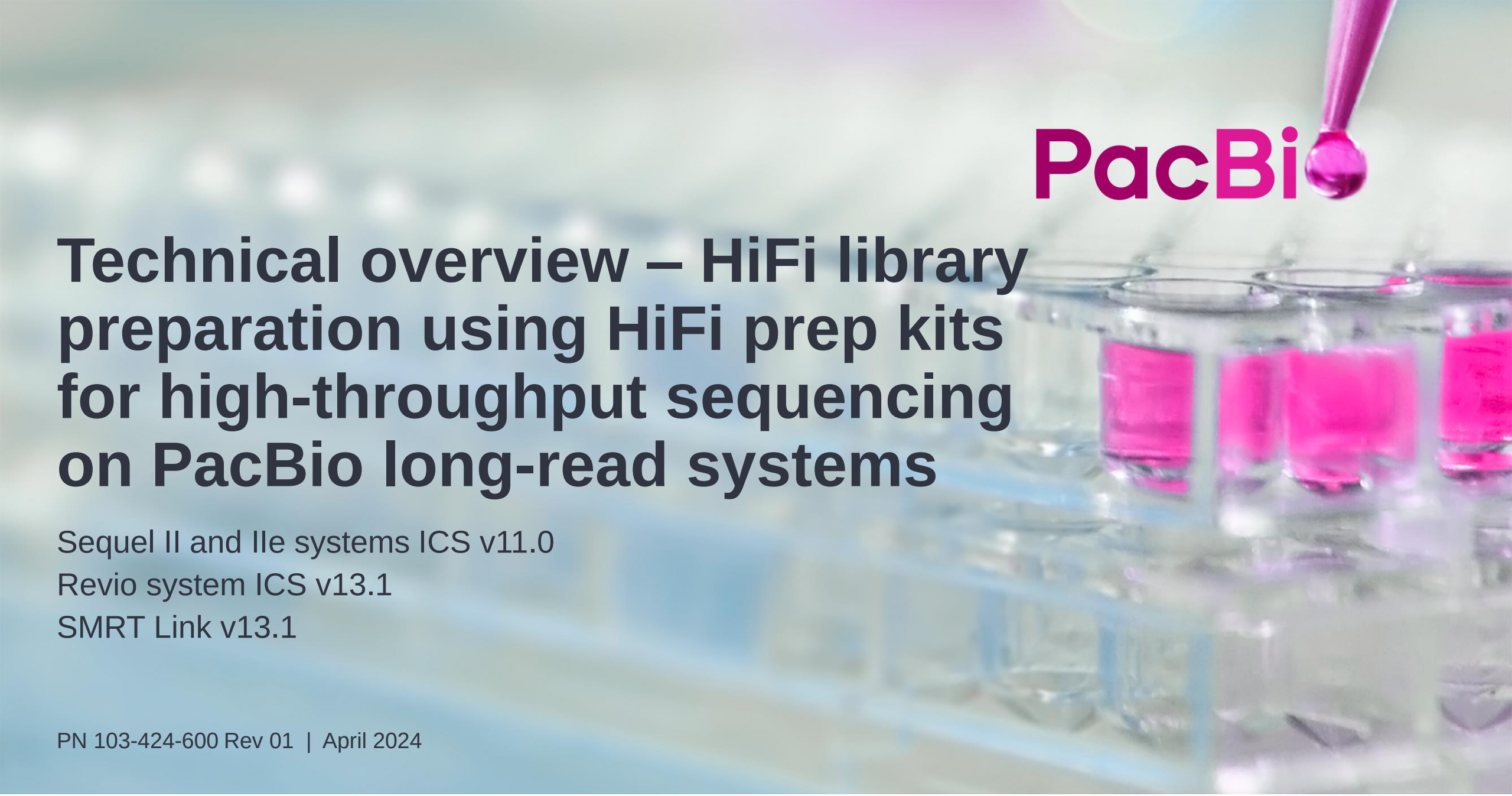


Technical overview – HiFi library preparation using HiFi prep kits for high-throughput sequencing on PacBio long-read systems

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

PN 103-424-600 Rev 01 | April 2024

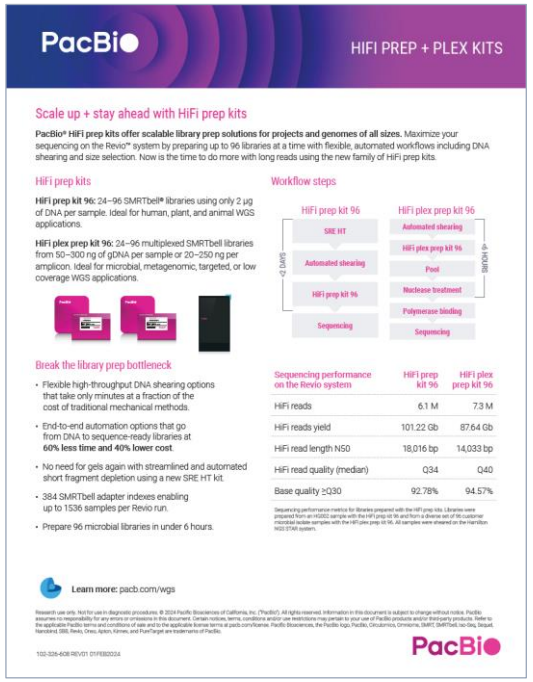
HiFi library preparation using HiFi prep kits for high-throughput sequencing on PacBio long-read systems

Technical Overview

1. HiFi library prep products and workflow options for WGS applications
2. HiFi prep kit 96 workflow overview for WGS applications
3. HiFi prep kit 96 example sequencing performance data
4. HiFi plex prep kit 96 workflow overview for WGS and amplicon sequencing applications
5. HiFi plex prep kit 96 example sequencing performance data
6. Technical documentation & applications support resources

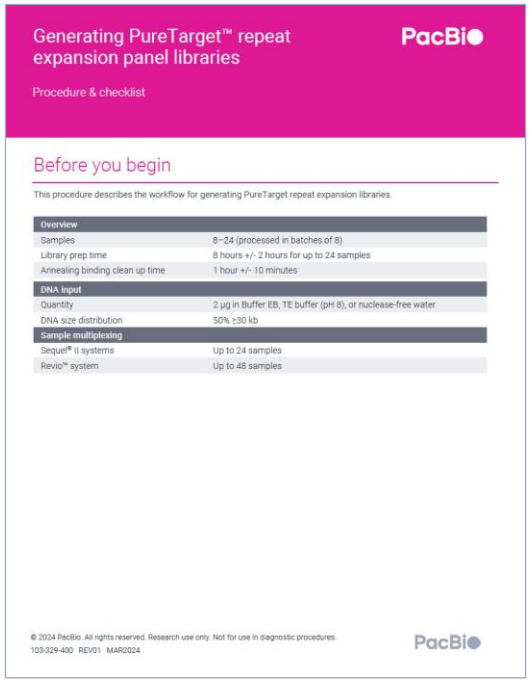
HiFi library preparation using HiFi prep kits for high-throughput sequencing on PacBio long-read systems: Getting started

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



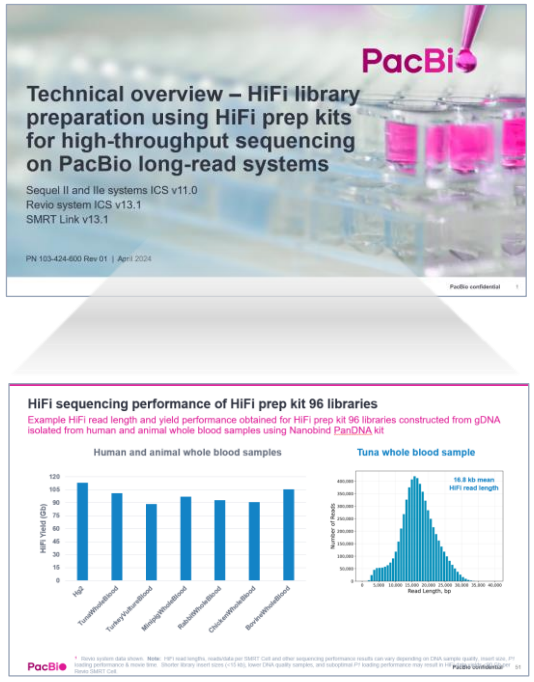
Brochure – HiFi prep kits (102-326-608)

Summary overview of application-specific DNA sample extraction, library preparation and/or data analysis workflow recommendations.



Procedure & checklist – Preparing whole genome libraries using the HiFi prep kit 96 (103-420-700)

Technical documentation containing application-specific library preparation protocol details.



Technical Overview – Technical overview – HiFi library preparation using HiFi prep kits for high-throughput sequencing on PacBio long-read systems (103-424-600)

Technical overview presentations describe workflow details for constructing PacBio SMRTbell libraries for specific applications. Example sequencing performance data for a given application are also summarized.

gDNA sample extraction & QC
(Nanobind kit)

2-3 µg DNA input per sample
Genome quality number (10 kb) ≥7.0
Nanobind PanDNA kit or Nanobind HT CBB kit

HiFi library preparation
(HiFi prep kit 96 / HiFi plex prep kit 96)

SRE size section on input gDNA
Pipette-tip DNA shearing
15 – 20 kb target library insert size for WGS

SMRT sequencing
(Sequel II/IIe & Revio systems)

Perform ABC* and sequence HiFi prep kit libraries on PacBio long-read systems

Data analysis
(SMRT Link)

Use SMRT Link analysis applications to perform *de novo* genome assembly and variant calling

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* ABC = Anneal primer / Bind polymerase / Clean up bound complex

HiFi library prep products and workflow options for WGS applications

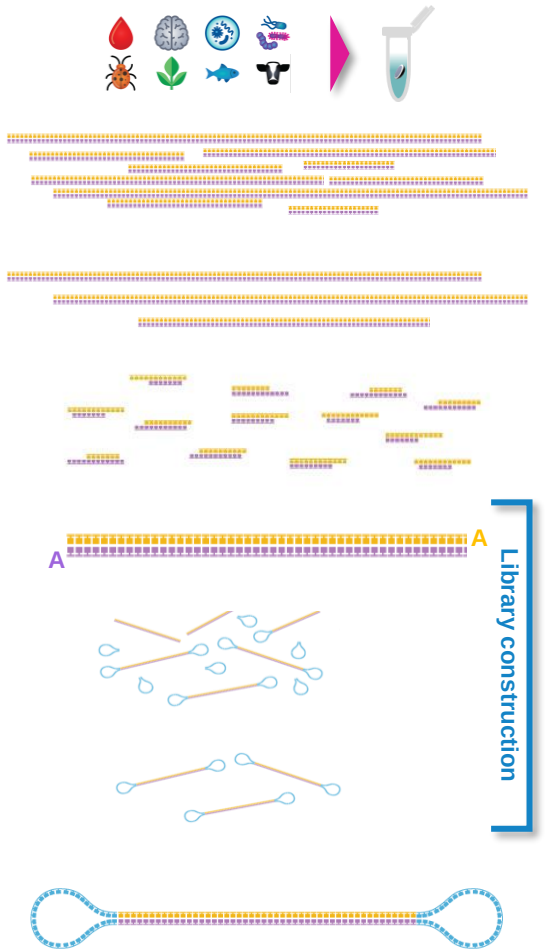
HiFi library prep workflow recommendations for low-throughput whole genome sequencing applications

≤24 samples per month

Low-throughput HiFi WGS workflow

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

Library construction



Protocol documentation or reference

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

Refer to third-party user guide documenation

Procedure & checklist – Removing short DNA fragments with the Short Read Eliminator (SRE) kit (102-982-300) **[For manual workflows]**

Guide and overview – Short Read Eliminator (SRE), DNA shearing, and cleanup for the Hamilton Microlab Prep system (103-424-100)

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 shearing)

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

Animal/plant/human WGS:[1]
3.1X (35%) AMPure PB SS (>4 kb)

Microbial/metagenomic WGS:
1X SMRTbell bead cleanup

[1] Can optionally perform gel cassette-based size selection to enrich for SMRTbell library inserts >10 kb.

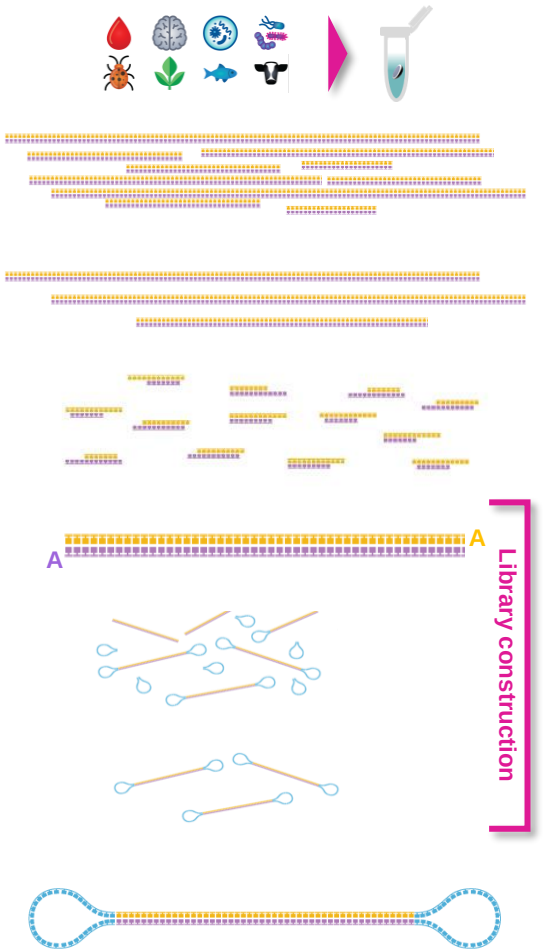
HiFi library prep workflow recommendations for high-throughput whole genome sequencing applications

>24 samples per month

High-throughput HiFi 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 HT kits (103-028-100)[1]

Refer to third-party user guide documenation

HiFi prep kit 96 protocol references for manual & automated workflows

Procedure & checklist – Preparing whole genome libraries using the HiFi prep kit 96 (103-420-700)

Guide & overview – Automated HiFi prep 96 and HiFi annealing, binding, and cleanup for the Hamilton NGS Microlab STAR system (103-425-700)

HiFi plex prep kit 96 protocol references for manual & automated workflows

Procedure & checklist – Preparing multiplexed whole genome and amplicon libraries using the HiFi plex prep kit 96 (103-418-800)

Guide & overview – Automated HiFi plex prep 96 for the Hamilton NGS Microlab STAR system (103-425-800)

Recommended equipment & consumables

Nanobind HT CBB kit /
Nanobind HT 1 mL whole blood kit

Microplate reader + Quant-iT dsDNA HS assay;
Femto Pulse system

SRE HT kit (103-124-500 / 96 RXN) using Hamilton automation system

Pipette-based shearing using Hamilton automation system

HiFi prep kit 96 (103-381-200 / 96 RXN); ***or***

Revio HiFi prep kit 96 (103-382-200 / 96 RXN) [Includes AMPure PB + Revio polymerase kit 96]; ***or***

HiFi plex prep kit 96 (103-381-300 / 96 RXN) + SMRTbell adapter index plate 96A/B/C/D

Animal/plant/human WGS:

3.1X (35%) AMPure PB SS (>4 kb)

Microbial/metagenomic WGS:

1X SMRTbell bead cleanup

[1] Refer to PacBio Documentation website for specific Nanobind HT Procedures & checklists.

Overview of HiFi prep kit product bundles and components

PacBio HiFi prep kits offer scalable library prep solutions for projects and genomes of all sizes

Product	Components	Supported workflow steps	Description
SRE HT kit [96 rxn] 103-124-500 	• Buffer SRE • Buffer LTE	❑ Genomic DNA size selection	• 96 short read eliminator (SRE) reactions for performing rapid high-pass size selection of genomic DNA samples to remove DNA fragments <10 kb (with progressive deletion of fragments <25 kb)
HiFi prep kit 96 bundle [96 rxn] 103-381-200 	• HiFi prep kit 96 • SMRTbell cleanup beads-85mL • SMRTbell adapter index plate 96A • Elution buffer (50 mL) • Buffer LTE HT (50 mL)	❑ Pipette-based DNA shearing ❑ SMRTbell library construction	• 24–96 SMRTbell libraries using only 2 µg of gDNA per sample • Ideal for human, plant, and animal WGS applications
Revio HiFi prep kit 96 bundle [96 rxn] 103-382-200 	• HiFi prep kit 96 • SMRTbell cleanup beads-85mL • SMRTbell adapter index plate 96A • Elution buffer (50 mL) • SRE HT kit (96 RXN) • AMPure PB (5 mL) • Revio polymerase kit 96[1]	❑ gDNA size selection (SRE) ❑ Pipette-based DNA shearing ❑ SMRTbell library construction ❑ SMRTbell library size selection (AMPure PB) ❑ Sequencing preparation (ABC – Anneal sequencing primer / Bind polymerase / Complex cleanup)	• 24–96 SMRTbell libraries using only 2 µg of gDNA per sample • Includes Revio polymerase kit 96 for performing sequencing primer annealing, polymerase binding and complex cleanup reactions to prepare samples for sequencing on a Revio system • Ideal for human, plant, and animal WGS applications
HiFi plex prep kit 96 bundle [96 rxn] 103-381-300 	• HiFi plex prep kit 96 • SMRTbell cleanup beads-52mL • Buffer LTE HT (50 mL) • Elution buffer (50 mL)	❑ Pipette-based DNA shearing ❑ Multiplexed SMRTbell library construction	• 24–96 multiplexed SMRTbell libraries from 50–300 ng of gDNA per sample or 20–250 ng per amplicon sample • Ideal for microbial, metagenomic, targeted, or low coverage WGS (2-3X coverage per sample) applications
SMRTbell adapter index plate 96A/B/C/D [96 rxn]  102-009-200 (A) / 102-547-800 (B) / 102-547-900 (C) / 102-548-000 (D)	• SMRTbell adapter index plate 96A/B/C/D	❑ Indexed adapter ligation (during multiplexed SMRTbell library construction)	• SMRTbell adapter index plate contains 96 indexed adapters in plate format (one sample per indexed adapter) • Plate 96A: bc2001–bc2096 / Plate 96B: bc2097–bc2192 / Plate 96C: bc2193–bc2288 / Plate 96D: bc2289–bc2384

[1] Note: **Revio polymerase kit 96** can only be purchased as part of the Revio HiFi prep kit 96 bundle (103-382-200) and **cannot** be purchased separately. In addition, Revio polymerase kit 96 only includes the Standard sequencing primer and does not include the Kinnex sequencing primer.

Overview of HiFi prep kit bundle supported workflow steps

Revio HiFi prep kit 96 bundle supports library preparation and sequencing preparation (ABC) workflows

Product	Supported library preparation workflow steps
HiFi prep kit 96 bundle 103-381-200 	DNA sample extraction → DNA sample QC → DNA size selection → DNA shearing & cleanup → DNA repair & A-tailing → Adapter ligation → Nuclease treatment → Library size selection → ABC Not supported by kit bundle / Supported by kit bundle / Not supported by kit bundle
Revio HiFi prep kit 96 bundle 103-382-200 	DNA sample extraction → DNA sample QC → DNA size selection → DNA shearing & cleanup → DNA repair & A-tailing → Adapter ligation → Nuclease treatment → Library size selection → ABC Not supported by kit bundle / Supported by kit bundle
HiFi plex prep kit 96 bundle 103-381-300  **SMRTbell adapter index plate 96A/B/C/D**  102-009-200 (A) / 102-547-800 (B) / 102-547-900 (C) / 102-548-000 (D)	DNA sample extraction → DNA sample QC → DNA size selection → DNA shearing & cleanup → DNA repair & A-tailing → Adapter index ligation → Pooling & cleanup → Nuclease Tx & cleanup → ABC Not supported by kit bundle / Supported by kit bundle + SMRTbell adapter index plate / Not supported

HiFi prep kit 96 bundle offers flexibility to use alternative reagents for size selection and ABC steps

Revio HiFi prep kit 96 bundle supports full end-to-end library prep + sequencing prep (ABC) workflow

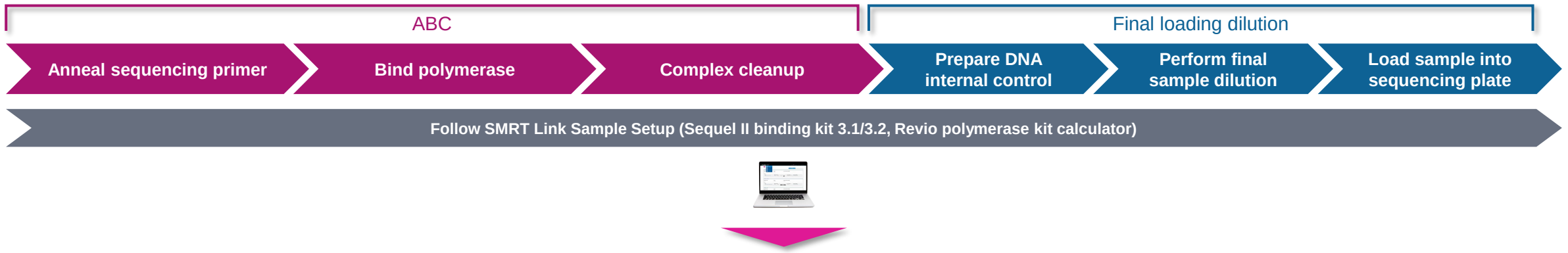
HiFi plex prep kit 96 bundle + SMRTbell adapter index plate supports multiplexed library prep workflow

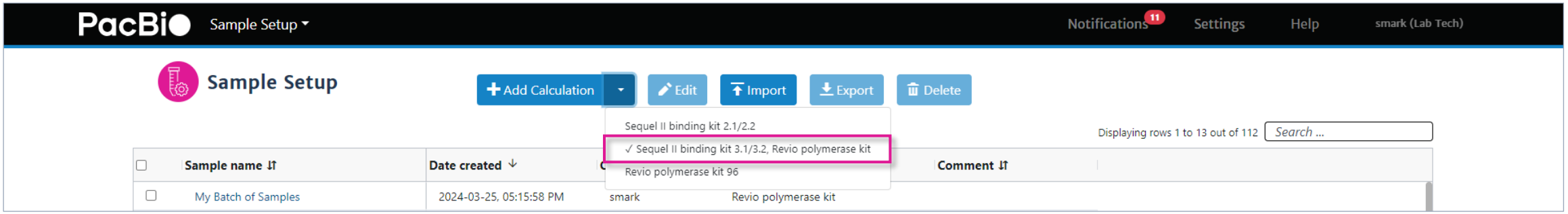
PacBio

Sequencing preparation workflow overview for HiFi prep kit libraries

For HiFi prep kit 96 WGS libraries bound with Revio polymerase kit or Sequel II binding kit 3.2, follow SMRT Link Sample Setup calculator instructions for ABC and final loading dilution procedure

HiFi prep kit bundle	Polymerase kit[1]	Sample setup workflow & procedural reference	
HiFi prep kit 96 bundle 103-381-200	**Revio polymerase kit** (102-817-600) *or* **Sequel II binding kit 3.2** (102-333-300)	Anneal sequencing primer, bind polymerase, complex cleanup (ABC)	❑ Follow SMRT Link v13.1+ Sample Setup
		Final loading dilution procedure	❑ Follow SMRT Link v13.1+ Sample Setup



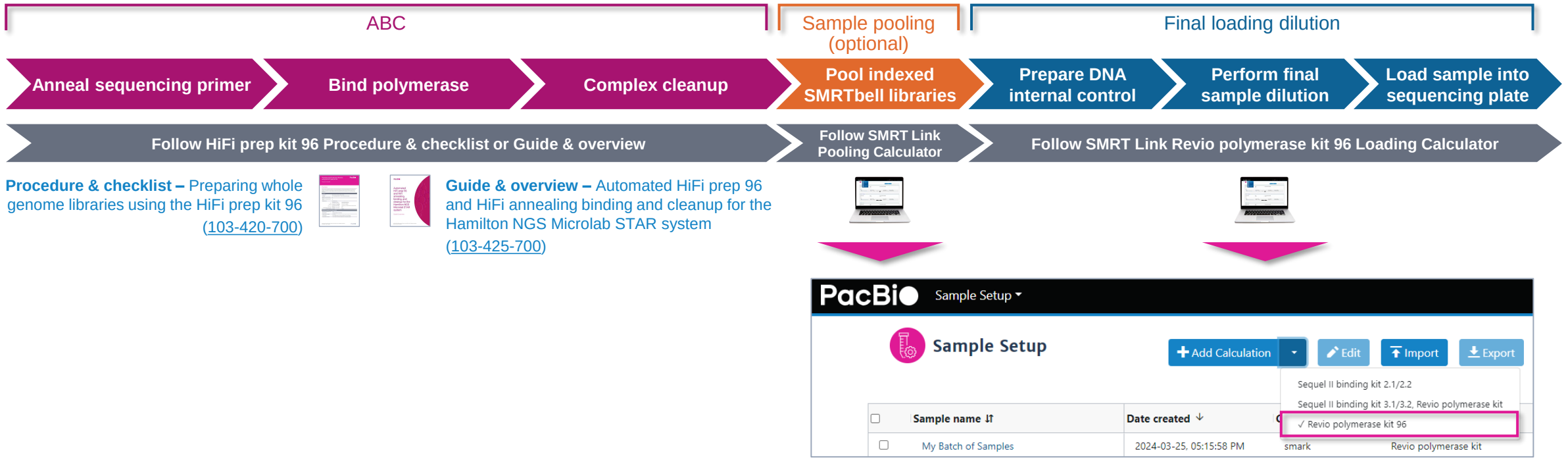


[1] HiFi prep kit 96 bundle (103-381-200) does not include any polymerase kit component; Revio polymerase kit (102-817-600) or Sequel II binding kit 3.2 (102-333-300) must be purchased separately.

Sequencing preparation workflow overview for HiFi prep kit libraries (cont.)

For HiFi prep kit 96 WGS libraries bound with Revio polymerase kit 96[1], follow SMRT Link Revio polymerase kit 96 Loading Calculator instructions for final loading dilution procedure

HiFi prep kit bundle	Polymerase kit[1]	Sample setup workflow & procedural reference	
Revio HiFi prep kit 96 bundle 103-382-200	**Revio polymerase kit 96** (103-253-600)	Anneal sequencing primer, bind polymerase, complex cleanup (ABC)	❑ Follow Procedure & checklist or Guide & overview
		Sample pooling (optional)	❑ Follow SMRT Link v13.1+ Pooling Calculator
		Final loading dilution procedure	❑ Follow SMRT Link v13.1+ Loading Calculator

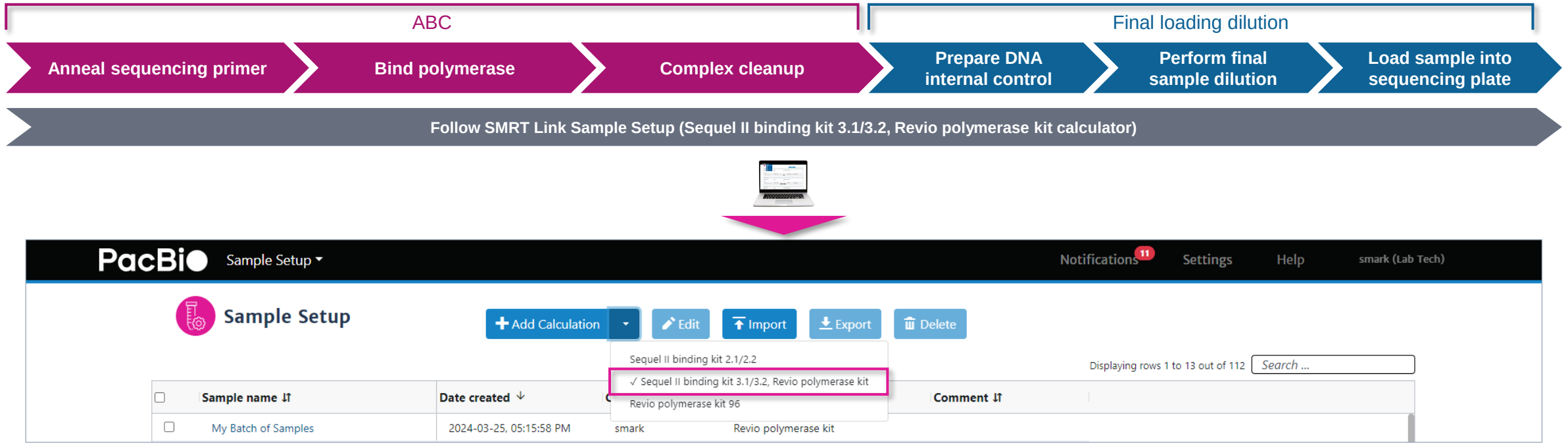


[1] **Revio polymerase kit 96** (103-253-600) is **included** in the Revio HiFi prep kit 96 bundle (103-382-200) and **cannot** be purchased separately.

Sequencing preparation workflow overview for HiFi plex prep kit libraries

For HiFi plex prep kit 96 WGS or amplicon libraries bound with Revio polymerase kit or Sequel II binding kit 3.1/3.2, follow SMRT Link Sample Setup calculator instructions for ABC and final loading dilution procedure

HiFi prep kit bundle	Polymerase kit[2]	Sample setup workflow & procedural reference	
HiFi plex prep kit 96 bundle[1] 103-381-300	**Revio polymerase kit** (102-817-600) *or* **Sequel II binding kit 3.1** (102-333-400) *or* **Sequel II binding kit 3.2** (102-333-300)	Anneal sequencing primer, bind polymerase, complex cleanup (ABC)	❑ Follow SMRT Link v13.1+ Sample Setup
		Final loading dilution procedure	❑ Follow SMRT Link v13.1+ Sample Setup



[1] **Note:** For multiplexed WGS or amplicon libraries prepared using HiFi plex prep kit 96 bundle, samples will typically be pooled prior to proceeding to ABC in SMRT Link Sample Setup.

[1] **Note:** HiFi plex prep kit 96 bundle (103-381-300) does not include any polymerase kit component; Revio polymerase kit or Sequel II binding kit must be purchased separately.

HiFi prep kit 96 bundle (103-381-200) configuration details

Contains HiFi library preparation reagents for processing up to 96 samples

Product	Components		Description
HiFi prep kit 96 bundle (103-381-200)		**HiFi prep kit 96** (103-122-600)	• HiFi library preparation reagents for 96 samples. SMRTbell cleanup beads and low TE buffer included. **Note:** Size-selection reagents are sold separately.[1]
		**SMRTbell cleanup beads-85mL** (103-294-600)	• Paramagnetic beads supplied at 85 mL to selectively bind DNA fragments. The beads are used for: 1) cleanup – removing excess primers, nucleotides, salts, and enzymes; and 2) buffer exchanges.
		**SMRTbell adapter index plate 96A** (102-009-200)	• SMRTbell adapter index plate 96A contains 96 indexed adapters in plate format (one sample per indexed adapter). It includes indexes bc2001–bc2096.
		**Elution buffer (50 mL)** (101-633-500)	• Elution buffer supplied at 50 mL is used with the SMRTbell library template preparation and barcoding associated kits for workflows requiring additional elution buffer and AMPure PB beads cleanup steps.
		**Buffer LTE HT (50 mL)** (103-306-100)	• Low TE buffer supplied at 50 mL to support 96 reactions. This buffer consists of 10 mM Tris-HCl (pH 9.0) and 0.1 mM EDTA.

Supported workflow steps

HiFi prep kit 96 bundle
(103-381-200)

[1] For high-throughput animal/plant/human WGS applications, we recommend performing size selection on input genomic DNA using SRE HT kit (103-124-500) to remove DNA fragments <10 kb and on HiFi SMRTbell libraries using AMPure PB beads (100-265-900) to remove residual library insert fragments <5 kb.

[2] HiFi prep kit 96 bundle includes Buffer LTE for diluting samples for DNA shearing step. For high-throughput animal/plant/human WGS applications, we recommend performing DNA shearing using a pipette-based shearing method with a third-party liquid handling system.

Revio HiFi prep kit 96 bundle (103-382-200) configuration details

Contains HiFi library preparation reagents for processing up to 96 samples and includes Revio polymerase kit

Product	Components	Description
Revio HiFi prep kit 96 bundle (103-382-200)	 **HiFi prep kit 96** (103-122-600)	• HiFi library preparation reagents for 96 samples. SMRTbell cleanup beads and low TE buffer included.
	 **SMRTbell cleanup beads-85mL** (103-294-600)	• Paramagnetic beads supplied at 85 mL to selectively bind DNA fragments. The beads are used for: 1) cleanup – removing excess primers, nucleotides, salts, and enzymes; and 2) buffer exchanges.
	 **SMRTbell adapter index plate 96A** (102-009-200)	• SMRTbell adapter index plate 96A contains 96 indexed adapters in plate format (one sample per indexed adapter). It includes indexes bc2001–bc2096.
	 **Elution buffer (50 mL)** (101-633-500)	• Elution buffer supplied at 50 mL is used with the SMRTbell library template preparation and barcoding associated kits for workflows requiring additional elution buffer and AMPure PB beads cleanup steps.
	 **SRE HT kit** (103-124-500)	• 96 short read eliminator (SRE) reactions for performing rapid high-pass size selection[1] of genomic DNA samples to remove DNA fragments <10 kb (with progressive deletion of fragments <25 kb).
	 **AMPure PB (5 mL)** (100-265-900)	• AMPure PB contains specially formulated paramagnetic beads for the SMRT sequencing workflow.[1]
	 **Revio polymerase kit 96** (103-253-600)	• Reagents for binding sequencing polymerase to SMRTbell library. Includes 96 reactions, each supporting one Revio SMRT Cell.

Supported workflow steps

DNA sample extraction → DNA sample QC → DNA size selection[1] → DNA shearing & cleanup[2] → DNA repair & A-tailing → Adapter ligation → Nuclease treatment → Library size selection[1] → ABC

Not supported by kit bundle

Supported by kit bundle

Revio HiFi prep kit 96 bundle
(103-382-200)

[1] For high-throughput animal/plant/human WGS applications, we recommend performing size selection on input genomic DNA using SRE HT kit (103-124-500) to remove DNA fragments <10 kb and on HiFi SMRTbell libraries using AMPure PB beads (100-265-900) to remove residual library insert fragments <3 kb.

[2] Revio HiFi prep kit 96 bundle includes Buffer LTE for diluting samples for DNA shearing step. For high-throughput animal/plant/human WGS applications, we recommend performing DNA shearing using a pipette-based shearing method with a third-party liquid handling system.

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HiFi plex prep kit 96 bundle (103-381-300) configuration details

Contains HiFi library preparation reagents for multiplexing 96 samples

Product	Components		Description
HiFi plex prep kit 96 bundle (103-381-300)		**HiFi plex prep kit 96** (103-122-800)	• HiFi library preparation reagents for multiplexing 96 samples. SMRTbell® cleanup beads are included. **Note:** SMRTbell adapter indexes and SMRTbell library size-selection reagents are sold separately.
		**SMRTbell cleanup beads-52mL** (103-294-500)	• Paramagnetic beads supplied at 52 mL to selectively bind DNA fragments. The beads are used for: 1) cleanup – removing excess primers, nucleotides, salts, and enzymes; and 2) buffer exchanges.
		**Elution buffer (50 mL)** (101-633-500)	• Elution buffer supplied at 50 mL is used with the SMRTbell library template preparation and barcoding associated kits for workflows requiring additional elution buffer and AMPure PB beads cleanup steps.
		**Buffer LTE HT (50 mL)** 103-306-100	• Low TE buffer supplied at 50 mL to support 96 reactions. This buffer consists of 10 mM Tris-HCl (pH 9.0) and 0.1 mM EDTA.

Supported workflow steps

Note: SMRTbell adapter index plate must be purchased separately

Choose one or more of the following products to perform sample indexing:

- SMRTbell adapter index plate 96A (102-009-200) – Contains indexes bc2001–bc2096
- SMRTbell adapter index plate 96B (102-547-800) – Contains indexes bc2097–bc2192
- SMRTbell adapter index plate 96C (102-547-900) – Contains indexes bc2193–bc2288
- SMRTbell adapter index plate 96D (102-548-000) – Contains indexes bc2289–bc2384

SMRTbell adapter index plate

+

HiFi plex prep kit 96 bundle (103-381-300)

[1] HiFi plex prep kit 96 bundle includes Buffer LTE for diluting samples for DNA shearing step. For high-throughput microbial and shotgun metagenomic WGS applications, we recommend performing DNA shearing using either a pipette-based shearing method (with a third-party liquid handling system) or a plate-based shearing method (e.g., MP Biomedicals FastPrep-96 or SPEX SamplePrep 1600 MiniG).

HiFi prep kit 96 workflow overview for WGS applications

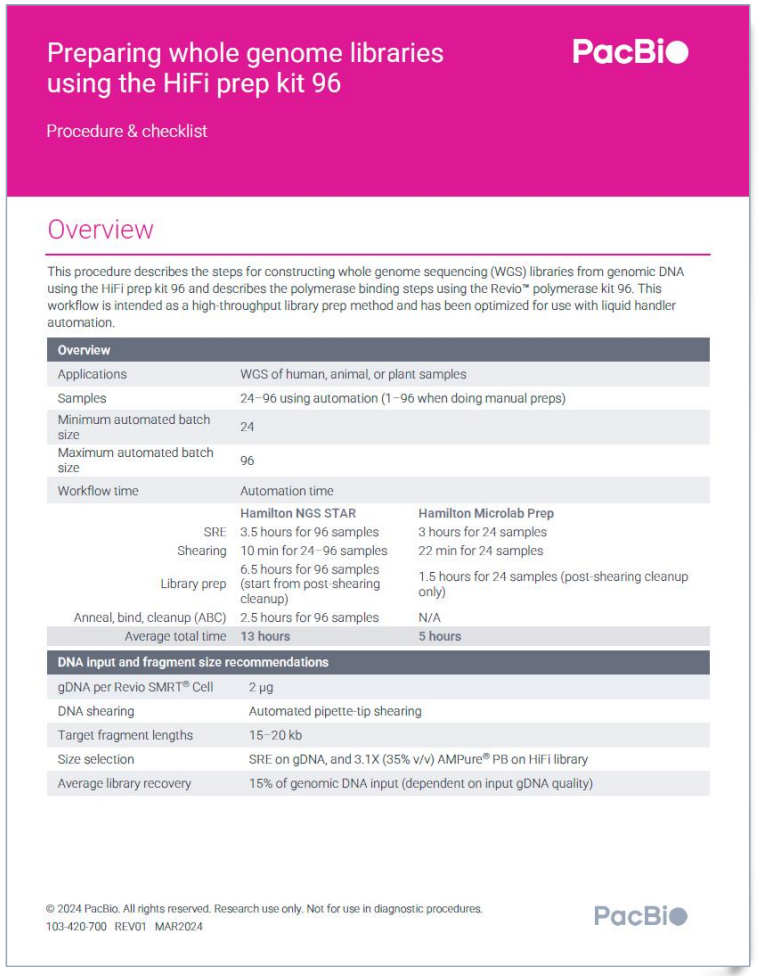
Procedure & checklist – Preparing whole genome libraries using the HiFi prep kit 96 (103-420-700)

Procedure & checklist 103-420-700 describes the steps for constructing whole genome sequencing (WGS) libraries from genomic DNA using HiFi prep kit 96 and describes the polymerase binding steps using Revio polymerase kit 96. This workflow is intended as a high-throughput library prep method and has been optimized for use with liquid handler automation[1].

Overview	
Applications	WGS of human, animal, or plant samples
Samples	24–96 using automation (1–96 when doing manual preps)
Minimum automated batch size	24
Maximum automated batch size	96

DNA input and fragment size recommendations	
gDNA per Revio SMRT® Cell	2 µg
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 (dependent on input gDNA quality)

Note: Procedure & checklist 103-420-700 includes instructions for HiFi prep kit 96 **SMRTbell library construction workflow *and* sequencing preparation (ABC[2]) workflow using Revio polymerase kit 96[2]**



Preparing whole genome libraries using the HiFi prep kit 96

PacBio

Procedure & checklist

Overview

This procedure describes the steps for constructing whole genome sequencing (WGS) libraries from genomic DNA using the HiFi prep kit 96 and describes the polymerase binding steps using the Revio™ polymerase kit 96. This workflow is intended as a high-throughput library prep method and has been optimized for use with liquid handler automation.

Overview		
Applications	WGS of human, animal, or plant samples	
Samples	24–96 using automation (1–96 when doing manual preps)	
Minimum automated batch size	24	
Maximum automated batch size	96	
Workflow time	Automation time	
	Hamilton NGS STAR	Hamilton Microlab Prep
SRE	3.5 hours for 96 samples	3 hours for 24 samples
Shearing	10 min for 24–96 samples	22 min for 24 samples
Library prep	6.5 hours for 96 samples (start from post-shearing cleanup)	1.5 hours for 24 samples (post-shearing cleanup only)
Anneal, bind, cleanup (ABC)	2.5 hours for 96 samples	N/A
Average total time	13 hours	5 hours

DNA input and fragment size recommendations	
gDNA per Revio SMRT® Cell	2 µg
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 (dependent on input gDNA quality)

103-420-700 REV01 MAR2024

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[1] If using the HiFi prep kit 96 or Revio HiFi prep kit 96 in an automated high-throughput library prep workflow with a Hamilton liquid handling system, also refer to ***Guide & overview – Automated HiFi prep 96 and HiFi annealing, binding, and cleanup for the Hamilton NGS Microlab STAR system*** (103-425-700) for specific details surrounding automation equipment setup recommendations.

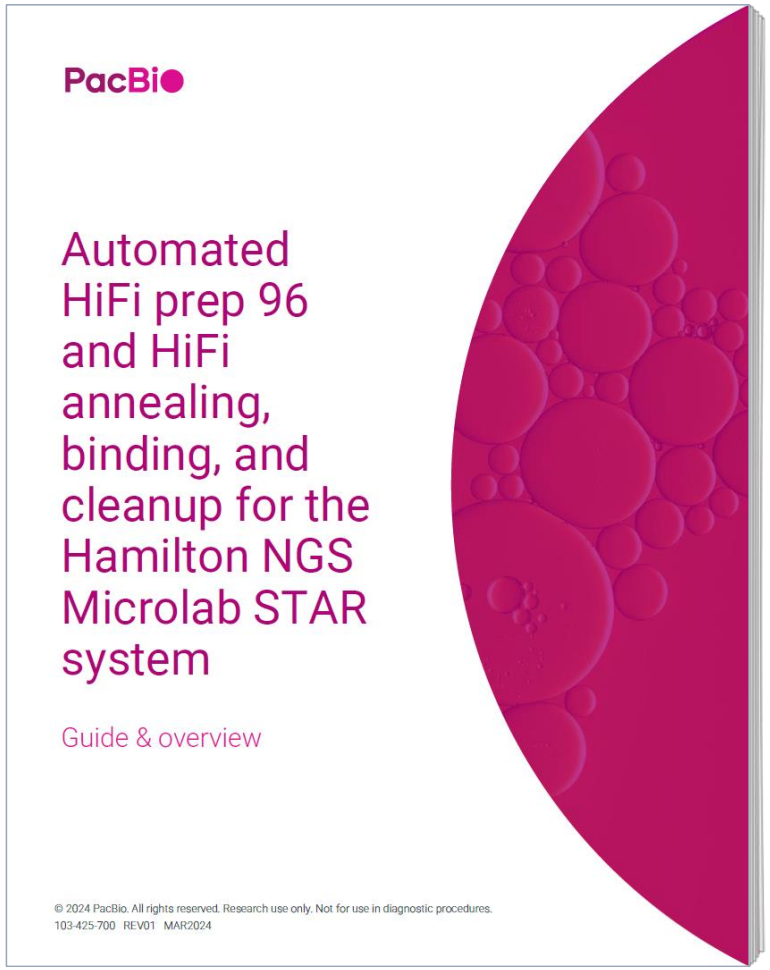
[2] **If using Revio polymerase kit 96 (103-253-600) for polymerase binding:** For primer annealing, polymerase binding & complex cleanup (ABC) steps, follow sample setup instructions for HiFi prep kit 96 libraries in ***Procedure & checklist – Preparing whole genome libraries using the HiFi prep kit 96*** (103-420-700). To perform the final dilution step after completing ABC with Revio polymerase kit 96, follow the instructions provided in the SMRT Link Loading Calculator to prepare HiFi prep kit 96 samples for sequencing. **Note:** If using **Revio polymerase kit (102-817-600)** or **Sequel II binding kit 3.2 (102-333-300)** for polymerase binding, then follow sample setup ABC and final loading dilution instructions provided in SMRT Link Sample Setup Calculator for Sequel II binding kit 3.1/3.2, Revio polymerase kit.

Guide & overview – Automated HiFi prep 96 and HiFi annealing, binding, and cleanup for the Hamilton NGS Microlab STAR system (103-425-700)[1]

Guide & overview 103-425-700 describes the automated workflow for constructing whole genome sequencing (WGS) libraries from genomic DNA using the Revio HiFi prep kit 96 and the Revio polymerase kit 96. The SRE HT kit, HiFi Prep 96 kit and the Revio Polymerase 96 kit are designed for a minimum of 24 and maximum of 96 samples per automated run.

Overview	
Applications	WGS of human, animal, or plant samples
Samples	24–96 using automation
Minimum automated batch size	24
Maximum automated batch size	96
Workflow time	Automation time
SRE	3.5 hours for 96 samples
Shearing	10 min for 24–96 samples
Library prep	6.5 hours for 96 samples (start from post-shearing cleanup)
Anneal, bind, cleanup (ABC)	2.5 hours for 96 samples
Average total time	13 hours

Note: Guide & overview 103-425-700 includes instructions for **automated** HiFi prep kit 96 **SMRTbell library construction workflow *and* sequencing preparation (ABC[2]) workflow using Revio polymerase kit 96[2]**



[1] If using the HiFi prep kit 96 or Revio HiFi prep kit 96 in a manual high-throughput library prep workflow, refer to ***Procedure & checklist – Preparing whole genome libraries using the HiFi prep kit 96*** (103-420-700).

[2] **If using Revio polymerase 96 kit (103-253-600) for polymerase binding:** For primer annealing, polymerase binding & complex cleanup (ABC) steps, follow sample setup instructions for HiFi prep kit 96 libraries in ***Guide & overview – Automated HiFi prep 96 and HiFi annealing, binding, and cleanup for the Hamilton NGS Microlab STAR system*** (103-425-700). To perform the final dilution step after completing ABC with Revio polymerase kit 96, follow the instructions provided in the SMRT Link Loading Calculator to prepare HiFi prep kit 96 samples for sequencing. **Note:** If using **Revio polymerase kit (102-817-600)** or **Sequel II binding kit 3.2 (102-333-300)** for polymerase binding, then follow sample setup ABC and final loading dilution instructions provided in SMRT Link Sample Setup Calculator for Sequel II binding kit 3.1/3.2, Revio polymerase kit.

HiFi prep kit 96 workflow overview for human/animal/plant WGS applications

Automation of HiFi library prep workflow enables high-throughput processing of 24 to 96 samples in 2 days

Key workflow steps	Automation	Time	Protocol documentation or reference	Recommended equipment & consumables
DNA sample extraction		2 – 2.5 hrs	 ***Guide & overview – Nanobind HT kits*** (103-028-100) **[UPDATED]**	 Nanobind HT CBB kit / Nanobind HT 1 mL whole blood kit
DNA sample QC		2 hrs	 Refer to third-party user guide documenation	 DNA concentration QC: • Varioskan LUX microplate reader DNA sizing QC: • Femto Pulse system
1 Short Read Eliminator on gDNA		DAY 1 3.5 hrs	**Protocol for manual & automated workflows**	 SRE HT kit (103-124-500) with Hamilton Microlab prep or NGS Star
Safe stop				
2 Pipette DNA shearing		10 min	 ***Procedure & checklist – Preparing whole genome libraries using the HiFi prep kit 96*** (103-420-700)	 Pipette-based shearing with Hamilton Microlab Prep or NGS STAR
Safe stop				
Library construction: **3** Post-shearing cleanup[1] / **4** Repair & A-tailing		DAY 1 1.5/0.5 hrs	**Guide & overview for automated workflows**	
Safe stop				
Library construction: **5** Adapter ligation / **6** Post-ligation cleanup		1.0/1.5 hrs	 ***Guide & overview – Automated HiFi prep 96 and HiFi annealing, binding, and cleanup for the Hamilton NGS Microlab STAR system*** (103-425-700)	
Safe stop				
Library construction: **7** Nuclease treatment		DAY 2 0.5 hrs	**Supported automation systems**  Thermo Fisher KingFisher Apex  Hamilton Microlab NIMBUS Presto  Hamilton Microlab Prep  Hamilton NGS Star	 Revio HiFi prep kit 96 (103-382-200) [includes AMPure PB + Revio polymerase kit 96] with Hamilton NGS Star
8 AMPure PB bead cleanup		1.5 hrs		 3.1X (35%) AMPure PB cleanup & size selection (>4 kb) with Hamilton NGS Star
Safe stop				
9 Annealing, binding & cleanup		2.5 hrs		 Revio polyerase kit 96 with Hamilton NGS Star

[1] For automated library prep workflows using the HiFi prep kit 96 and Hamilton NGS Microlab Star liquid handling system, the post-shear cleanup step should be performed on the **same day (Day 2)** 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.

General best practices recommendations for preparing WGS libraries using HiFi prep kit 96

Automation

- The HiFi prep kit 96 and workflow was designed for use with NGS liquid handling automation sys
- As a result, this protocol (***Preparing whole genome libraries using the HiFi prep kit 96*** (103-4 is intended to describe the SRE, shearing, library prep enzymatic reactions, and bead cleanups t automation method development, or in certain instances manual preparation
- **Note:** This protocol was developed using the Hamilton NGS STAR MOA 96 system
  - Refer to ***Guide & overview – Automated HiFi prep 96 and HiFi annealing, binding, and cleanup for the Hamilton NGS Microlab STAR system*** (103-425-700)
- Because of differences between automation instruments, modifications not described herein may be needed to adapt the protocol to user-specific instrumentation
  - Please visit www.pacb.com or contact your local support team for a list of instruments with a PacBio qualified method

Hamilton NGS Star workstation

Genomic DNA sample extraction

- PacBio Nanobind DNA extractions kits are recommended to ensure sufficient mass and quality of high-molecular weight (HMW) DNA for this protocol

Nanobind HT CBB kit (102-762-700; 96 rxn)	Nanobind HT 1 mL blood kit (102-762-800; 96 rxn)	Nanobind PanDNA kit (103-260-000; 24 rxn)
• For high-throughput HMW DNA extraction from up to 200 µL human/mammalian blood, non-mammalian animal blood1, cultured cells, and bacteria • Expected HMW DNA yield: 3–15 µg for blood and cultured mammalian cells and 2–10 µg for bacteria	• For high-throughput HMW DNA extraction from 1 mL human blood • Expected HMW DNA yield: 3–70 µg	• For HMW DNA extraction from cells, blood, bacteria, tissues, insects, and plant nuclei • Expected HMW DNA yield: 3–26 µg

PacBio Nanobind kits

General best practices recommendations for preparing WGS libraries using HiFi prep kit 96

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 high-throughput DNA quantification QC workflows, we recommend using the Quant-iT 1X dsDNA high sensitivity assay kit[3] (Thermo Fisher Scientific) with the Varioskan LUX multimode microplate reader (Thermo Fisher Scientific) (or similar instrument)
  - Varioskan LUX multimode microplate reader enables rapid, specific and accurate determination of nucleic acid concentrations in a wide range
  - Quant-iT assay is well-adapted to high-throughput use (typically in 96-well or 384-well plates) and is highly selective for dsDNA over RNA (in the 0.2–100 ng range, the fluorescence signal is linear with the amount of DNA)
  - **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

Varioskan LUX multimode microplate reader (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 lower-throughput applications DNA quantification QC may be performed without a microplate reader using the **Qubit 1X dsDNA high sensitivity assay kit** with a **Qubit fluorometer**.
Note: Do not use a Qubit Flex fluorometer when performing DNA quantification QC on polymerase-bound SMRTbell library in Loading Buffer 96 since concentration readings will not be accurate.

General best practices recommendations for preparing WGS libraries using HiFi prep kit 96

Recommended genomic DNA input amount and quality

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

- Recommended DNA input amounts will vary by starting gDNA 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: The maximum input gDNA mass tolerated by shearing and library enzymatic reactions in this HiFi prep kit 96 protocol is 3 μg per reaction

→ Perform parallel library prep reactions if using >3 μg input gDNA

- 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%

- 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
20,000 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 libraries using HiFi prep kit 96

Reagent handling

- Room temperature is defined as any temperature in the range of 18 – 25°C for this protocol

HiFi prep kit 96

Thaw these reagents at room temperature	Keep these temperature-sensitive reagents on ice		Bring these reagents to room temperature 30 minutes prior to use	Bring these reagents to room temperature 1.5 hours prior to use
❑ Repair buffer 96		❑ End repair mix 96	❑ AMPure PB beads	❑ SMRTbell cleanup beads-85 mL[1]
❑ Nuclease buffer 96		❑ DNA repair mix 96	❑ Elution buffer	
❑ SMRTbell adapter index plate		❑ Ligation mix 96	❑ dsDNA quantification reagents	
		❑ Ligation enhancer 96		
		❑ Nuclease mix 96		

[1] Alternatively, can bring SMRTbell cleanup beads-85 mL to room temperature the night before if starting protocol in the morning.

- 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
- Briefly vortex then spin down SMRTbell adapter index plate 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 libraries using HiFi prep kit 96

Reagent handling (cont.)

Revio polymerase kit 96

Thaw these reagents at room temperature		Keep these reagents on a cold block or on ice		Bring these reagents to room temperature 30 minutes prior to use		Bring these reagents to room temperature 1.5 hours prior to use
	Annealing buffer 96		Sequencing polymerase 96		Loading buffer 96	SMRTbell cleanup beads-85 mL[1]
	Standard sequencing primer 96		Sequencing control 96			
	Polymerase buffer 96					
	Loading buffer 96					
	Dilution buffer 96					

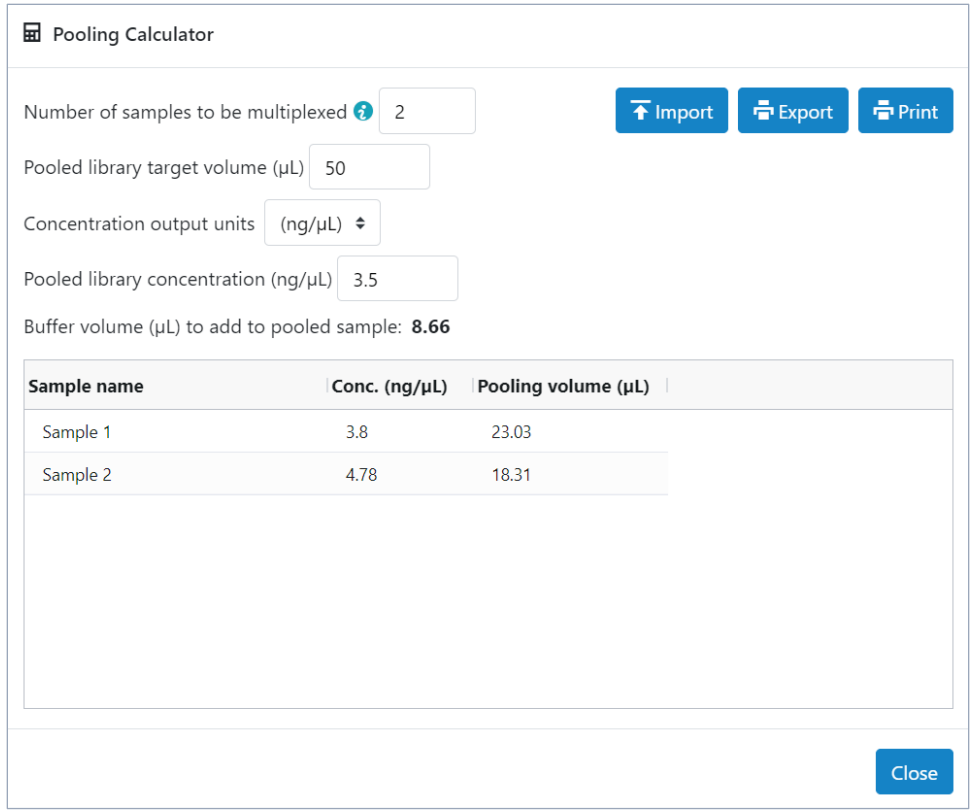
[1] Alternatively, can bring SMRTbell cleanup beads-85 mL to room temperature the night before if starting protocol in the morning.

- Once thawed, reaction buffers and sequencing primer may be stored on a cold block, at 4°C, or on-ice prior to making master mix or placing on the liquid handler work deck
- Loading buffer 96 should be left at room-temperature
- **Note:** Loading buffer 96 is light sensitive and should be protected from light when not in use

General best practices recommendations for preparing WGS libraries using HiFi prep kit 96

Multiplexing samples

- All libraries constructed for each DNA sample using this protocol will include a SMRTbell adapter index ('barcode')
- Use the SMRT Link (v13.1+) Sample Setup Pooling Calculator tool to help determine appropriate volumes to use for multiplexing libraries prepared with HiFi prep kit 96 and bound with Revio polymerase kit 96
- 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 HiFi prep kit library samples needed for pooling when performing multiplexed sequencing on a single SMRT Cell.

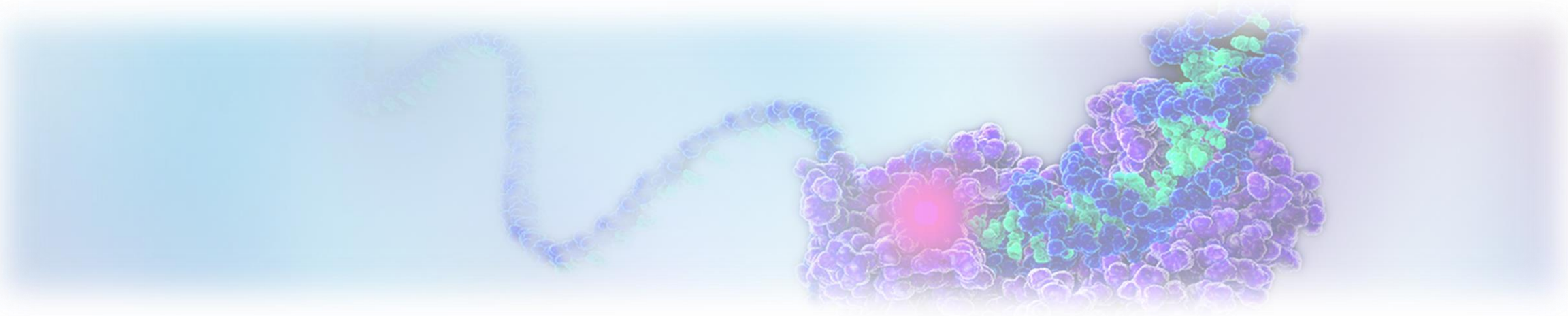
[1] We recommend aiming for a library insert size >15 kb (ideally 15 – 20 kb) to achieve optimum HiFi data yields on the Revio system. **Note:** HiFi read lengths, reads/data per SMRT Cell and other sequencing performance results can vary depending on DNA sample quality, insert size, *P1* loading performance & 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 libraries using HiFi prep kit 96

Sequencing preparation (ABC) and polymerase-bound library storage

- **Note:** *Procedure & checklist – Preparing whole genome libraries using HiFi prep kit 96* (103-420-700) brings the final SMRTbell library through the primer annealing, polymerase binding, and complex cleanup (ABC) sample setup steps
  - → Do not need to use SMRT Link Sample Setup to perform ABC calculations for HiFi prep kit 96 libraries.
  - → Follow SMRT Link Revio polymerase kit 96 Loading Calculator tool to perform final loading procedure for HiFi prep kit 96 libraries
- Sequencing polymerase is stable once bound to the HiFi prep kit 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
- Stored polymerase-bound library needs to be protected from light while stored.



[1] Polymerase-bound storage recommendations described here also apply to SMRTbell libraries bound with either Revio polymerase kit or Sequel II binding kit polymerase 3.1/3.2.

HiFi prep kit 96 library prep inputs & expected step yields

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

		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	Quant-iT/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	N/A	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 3 Post-shearing cleanup	1X SMRTbell cleanup beads	Post-shearing QC with Quant-iT/Qubit (optional) & Femto Pulse	≤10 ng/µL DNA in 300 µL (3 µg total input DNA mass) into Step 2	Expect ~80% step recovery or 60% total recovery; target DNA shear size is ~15-20 kb
	Safe stop				
Library construction	4 DNA repair & A-tailing	N/A	N/A	49 µL sheared DNA	60 µL repaired & A-tailed DNA
Library construction	5 Adapter ligation 6 Post-ligation cleanup	1X SMRTbell cleanup beads	N/A	60 µL post-repaired & A-tailed DNA into Step 5	Expect ~80% step recovery or 48% total recovery
	Safe stop				
Library construction	7 Nuclease treatment	N/A	N/A	40 µL of post-ligation cleanup sample	Expect ~40% step recovery or 19% total recovery
Library construction	8 AMPure PB bead cleanup	3.1X (35%) AMPure PB	Quant-iT/Qubit dsDNA HS assay Femto Pulse system (optional)	50 µL of nuclease-treated sample	Expect 80% step recovery or 15% total recovery (can range from ~10% - 25%)
	Safe stop				
ABC	9 Annealing, binding & cleanup	1X SMRTbell cleanup beads	Quant-iT/Qubit dsDNA assay[1]	DNA concentration must be less than 60 ng/µl to proceed with ABC	Expect ~70% step recovery relative to starting library input mass for ABC

[1] **Note:** After performing ABC step, do not use a Qubit Flex fluorometer when performing DNA quantification QC on polymerase-bound SMRTbell library in Loading Buffer 96 since concentration readings will not be accurate.

DNA sample extraction

Perform automated high-throughput HMW DNA extraction using Nanobind HT kits[1]

DNA sample extraction

↓

DNA sample QC

1 Short Read Eliminator on gDNA

↓ Safe stop

2 Pipette DNA shearing

3 Post-shearing cleanup

↓ Safe stop

4 DNA repair & A-tailing

↓

5 Adapter ligation

6 Post-ligation cleanup

↓ Safe stop

7 Nuclease treatment

↓

8 AMPure PB bead cleanup

↓ Safe stop

9 Annealing, binding & cleanup

ThermoFisher SCIENTIFIC

KingFisher Apex

HAMILTON

Microlab NIMBUS Presto

Automated solutions built on Nanobind technology

Nanobind HT kits use magnetic disk processing to automate lysis, binding, washing and elution steps and are compatible with instruments from Hamilton and Thermo Fisher

- Hamilton NIMBUS Presto is a walkaway solution with automated plate filling
- Thermo Fisher KingFisher instruments are semi-automated with manual plate filling and limited user interaction during the run

Available Nanobind HT kits

Nanobind HT CBB kit (102-762-700; 96 rxn)

- For up to 200 µL human/mammalian blood, non-mammalian animal blood[2], cultured cells, and bacteria
- Expected HMW DNA yield: 3–15 µg for blood and cultured mammalian cells and 2–10 µg for bacteria

Nanobind HT 1 mL blood kit (102-762-800; 96 rxn)

- For 1 mL human blood
- Expected HMW DNA yield: 3–70 µg

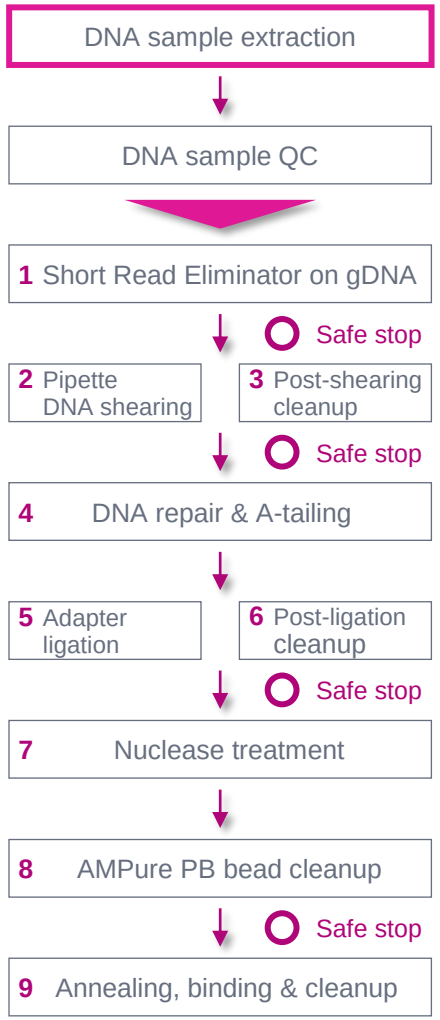
PacBio

1 See ***Technical overview — Automated high-throughput HMW DNA extraction for PacBio long-read sequencing using Nanobind HT kits*** (103-401-700).

2 Note: For animals with nucleated red blood cells, only 2.5 – 20 µL of blood is needed for HMW DNA extraction using Nanobind HT CBB kit.

DNA sample extraction (cont.)

Available Nanobind HT procedures



Automation system	Procedure & checklist	Sample type
KingFisher Apex	Extracting HMW DNA using the Nanobind HT CBB kit for mammalian cultured cells on KingFisher Apex system [102-996-100]	Mammalian cells
	Extracting HMW DNA using the Nanobind HT CBB kit for 200 µL human whole blood on KingFisher Apex system [102-995-700]	Human whole blood
	Extracting HMW DNA using the Nanobind HT CBB kit for bacteria on the KingFisher Apex system [103-377-600]	Cultured bacteria
	Extracting HMW DNA using the Nanobind HT CBB kit for non-human mammalian blood (NHMB) on the KingFisher Apex system [103-397-300]	Non-human mammalian blood
	Extracting HMW DNA using the Nanobind HT CBB kit for nucleated red blood cells (nRBCs) on the KingFisher Apex system [103-377-800]	Non-mammalian blood (nucleated RBCs)
	Extracting HMW DNA using Nanobind HT 1 mL blood kit for human whole blood on KingFisher Apex system [102-995-300]	Human whole blood
Hamilton NIMBUS Presto	Extracting HMW DNA using the Nanobind HT CBB kit for mammalian cultured cells on Hamilton NIMBUS Presto system [102-996-400]	Mammalian cells
	Extracting HMW DNA using the Nanobind HT CBB kit for 200 µL human whole blood on Hamilton NIMBUS Presto system [102-996-000]	Human whole blood
	Extracting HMW DNA using the Nanobind HT CBB kit for bacteria on Hamilton NIMBUS Presto system [103-397-400]	Cultured bacteria
	Extracting HMW DNA using the Nanobind HT CBB kit for non-human mammalian blood (NHMB) on the Hamilton NIMBUS Presto system [103-377-700]	Non-human mammalian blood
	Extracting HMW DNA using the Nanobind HT CBB kit for nucleated red blood cells (nRBCs) on the Hamilton NIMBUS Presto system [103-397-500]	Non-mammalian blood (nucleated RBCs)
	Extracting HMW DNA using the Nanobind HT 1 mL blood kit for human whole blood on Hamilton NIMBUS Presto system [102-995-600]	Human whole blood

[1] See PacBio's Documentation website for the most up-to-date list of Nanobind HT HMW DNA sample preparation protocols.

DNA sample QC

Perform high-throughput DNA QC using a microplate reader[1] and a Femto Pulse system

DNA sample extraction

↓

DNA sample QC

↓

1 Short Read Eliminator on gDNA

↓ Safe stop

2 Pipette DNA shearing

3 Post-shearing cleanup

↓ Safe stop

4 DNA repair & A-tailing

↓

5 Adapter ligation

6 Post-ligation cleanup

↓ Safe stop

7 Nuclease treatment

↓

8 AMPure PB bead cleanup

↓ Safe stop

9 Annealing, binding & cleanup

DNA quantification QC

Varioskan LUX multimode microplate reader (Thermo Fisher Scientific)

Varioskan LUX multimode microplate reader enables rapid, specific and accurate determination of nucleic acid concentrations in high-throughput workflows[1]

- Perform DNA concentration measurements using Quant-iT dsDNA assay[2] (Thermo Fisher Scientific)
- Quant-iT assay is well-adapted to high-throughput use, typically in 96-well or 384-well plates
- Quant-iT assay is highly selective for dsDNA over RNA, and in the range of 0.2–100 ng, the fluorescence signal is linear with the amount of DNA

[1] Alternatively, for lower-throughput applications DNA quantification QC may be performed without a microplate reader using the Qubit 1X 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] See ***How can low DNA concentrations be reliably measured in microplate format?*** (Thermo Fisher Scientific SNLUXQuant-iT_0617). 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 high-throughput size selection on input genomic DNA using SRE HT kit to remove <10 kb fragments

DNA sample extraction

↓

DNA sample QC

↓

1 Short Read Eliminator on gDNA

↓ Safe stop

2 Pipette DNA shearing

↓

3 Post-shearing cleanup

↓ Safe stop

4 DNA repair & A-tailing

↓

5 Adapter ligation

↓

6 Post-ligation cleanup

↓ Safe stop

7 Nuclease treatment

↓

8 AMPure PB bead cleanup

↓ Safe stop

9 Annealing, binding & cleanup

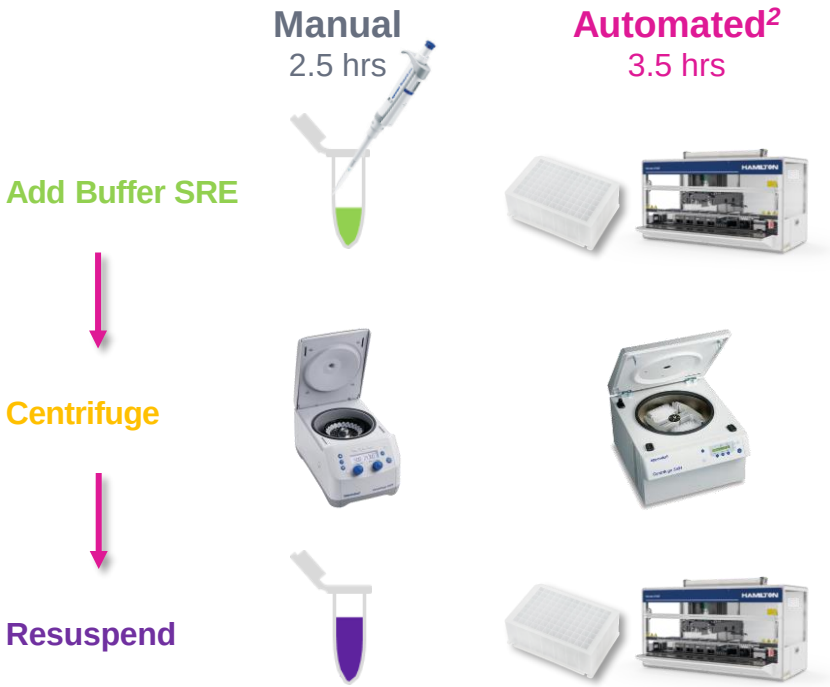
1. Short Read Eliminator

✔	Step	Instructions for SRE on gDNA
	1.1	Bring the DNA samples to a concentration between 40 and 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 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 HiFi prep kit 96 protocol[1]



PacBio

[1] **Note:** SRE size selection procedure described in high-throughput HiFi prep kit 96 protocols (103-420-700 and 103-425-700) uses slightly different starting sample concentration and volume ranges from the low-throughput SRE protocol described in ***Procedure & checklist – Removing short DNA fragments with the Short Read Eliminator (SRE) kit*** (102-982-300).

[2] Please refer to ***Automated HiFi prep 96 and HiFi annealing, binding, and cleanup for the Hamilton NGS Microlab STAR system*** (103-425-700) for details on required consumables.

Pipette DNA shearing

Perform automated high-throughput 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 | **3** Post-shearing cleanup

↓ Safe stop

4 DNA repair & A-tailing

↓

5 Adapter ligation | **6** Post-ligation cleanup

↓ Safe stop

7 Nuclease treatment

↓

8 AMPure PB bead cleanup

↓ Safe stop

9 Annealing, binding & cleanup

2. Pipette DNA shearing

✔	Step	Instructions for automated DNA shearing on Hamilton systems
	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.
	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 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 concentrate samples for library preparation.

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

- **Note:** Post-shear cleanup step should be performed on the **same day** as the library construction steps to ensure there is sufficient volume of SMRTbell cleanup beads to complete the entire library prep workflow

- 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 Hamilton liquid handling instruments.

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 HiFi prep kit 96 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

3 Post-shearing cleanup

↓ Safe stop

4 DNA repair & A-tailing

↓

5 Adapter ligation

6 Post-ligation cleanup

↓ Safe stop

7 Nuclease treatment

↓

8 AMPure PB bead cleanup

↓ Safe stop

9 Annealing, binding & cleanup

3. Post-shearing cleanup

✔	Step	Instructions for SMRTbell cleanup bead step
	3.1	Add 300 µL (1.0X) of resuspended, room-temperature SMRTbell cleanup beads to each sample. **Note:** If using less than 300 µL, add 1.0X (v/v) concentration of SMRTbell cleanup beads.
	3.2	Pipette-mix the sample until the beads are evenly distributed.
	3.3	Leave at **room temperature** for **10 minutes** to allow DNA to bind to the beads.
	3.4	Place samples on an appropriate magnet and allow beads to separate fully from the solution. The solution should be clear and beads pelleted to the magnet.
	3.5	Slowly pipette off the supernatant without disturbing the beads. Discard the supernatant.
	3.6	Slowly dispense **200 µL**, or enough to cover the beads, of **freshly prepared 80% ethanol** to each sample. After **30 seconds**, pipette off the 80% ethanol and discard.
	3.7	Repeat the previous step.
	3.8	Remove residual 80% ethanol: • Remove the samples from the magnet • Quick-spin to collect liquid at the bottom of the tube or well. • Place the tube or plate back in a magnetic separation rack until beads separate fully from the solution. • Pipette off residual 80% ethanol and discard. Alternatively, air dry samples for 1 minute to allow residue ethanol to evaporate. Do not let the bead pellet completely dry out.
	3.9	Remove samples from the magnet and immediately resuspend the beads with **49 µL of elution buffer.**
	3.10	Resuspend by pipetting mixing until beads are evenly distributed in solution.
	3.11	Leave samples at **room temperature** for **5 minutes** to elute DNA off beads.
	3.12	Place samples back on the magnet and allow beads to separate fully from the solution. The solution should be clear and beads pelleted to the magnet before aspirating the supernatant.
	3.13	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a new tube strip or plate. Discard old tube strip or plate with beads.

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 1.5 hrs prior to use
- If performing cleanup in the morning, beads may be left out at room temperature overnight

Note: Post-shear cleanup step should be performed on the **same day** as the library construction steps 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 the HiFi prep kit 96 and Hamilton NGS Microlab Star liquid handling system, the post-shear cleanup step should be performed on the **same day (Day 2)** 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 | 3 Post-shearing cleanup

↓ Safe stop

4 DNA repair & A-tailing

↓

5 Adapter ligation | 6 Post-ligation cleanup

↓ Safe stop

7 Nuclease treatment

↓

8 AMPure PB bead cleanup

↓ Safe stop

9 Annealing, binding & cleanup

4. DNA repair & A-tailing

✔	Step	Instructions for repair and A-tailing step
	4.1	Prepare the appropriate volume of master mix with 15% overage using the per reaction volumes listed below.

Repair mix

Tube	Component	Per rxn vol.
Purple	Repair buffer 96	8 µL
Blue	End repair mix 96	2 µL
Green	DNA repair mix 96	1 µL
	Total volume	11 µL

	4.2	*Slowly* pipette mix the **Repair mix** and quick-spin to collect liquid at the bottom of the tube. If bubbles form during mixing, pulse spin to remove.
	4.3	Add **11 µL** of the **Repair mix** to each sample. Total reaction volume should be **60 µL.**
	4.4	Pipette-mix the reactions and quick-spin to collect liquid at the bottom of the well/tube. If using a plate format, seal with a heated foil seal.
	4.5	Run the Repair and A-tailing thermocycler program. Set lid temperature to 75°C if programmable.

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

	4.6	Proceed directly 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., 24, 48, 72 or 96[1]), plus 15% overage

- Run **Repair and A-tailing** on-deck thermal cycler program
- Set the lid temperature to 75°C

[1] HiFi prep kit 96 contains a sufficient volume of reagents to support 4 automated runs using a minimum batch size of 24 samples per run.

Adapter ligation & cleanup

Ligate SMRTbell adapter to the ends of each DNA fragment

Revio HiFi prep kit 96 includes SMRTbell adapter index plate 96A (102-009-200), which contains indexes bc2001–bc2096

- DNA sample extraction
- DNA sample QC
- **1** Short Read Eliminator on gDNA
- Safe stop
- **2** Pipette DNA shearing
- **3** Post-shearing cleanup
- Safe stop
- **4** DNA repair & A-tailing
- **5** Adapter ligation
- **6** Post-ligation cleanup
- Safe stop
- **7** Nuclease treatment
- **8** AMPure PB bead cleanup
- Safe stop
- **9** Annealing, binding & cleanup

5. Adapter ligation

✔	Step	Instructions for adapter ligation
	5.1	Add **4 µL** of indexed adapter to each sample from the previous step. Any of the adapters from the four SMRTbell adapter index plates can be used (e.g. 96A, 96B, 96C, or 96D).

Component	Per rxn vol.
SMRTbell adapter index plate 96(A, B, C, or D)	4 µL

	Step	Instructions for adapter ligation
	5.2	Prepare the appropriate volume of master mix with 10% overage using the per reaction volumes listed below.

Ligation mix

Tube	Component	Volume
Yellow	Ligation mix 96	20 µL
Red	Ligation enhancer 96	1 µL
	Total volume	**21 µL**

	5.5	Pipette mix each reaction to ensure reagents are thoroughly mixed. Quick spin plate if necessary to collect liquid at the bottom of the well.
	5.6	Run the **Adapter ligation** thermocycler program. Set lid temperature to 75°C if programmable.

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

	5.7	Proceed to post-ligation cleanup step

- Add 4 µL of indexed adapter to each sample from the previous step.
- Any of the adapters from the four SMRTbell adapter index plates can be used (e.g. 96A, 96B, 96C, or 96D)

- 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., 24, 48, 72 or 96[1]), plus 10% overage

- Run **Adapter ligation** on-deck thermal cycler program
- Set the lid temperature to 75°C

6. Post-ligation 1X SMRTbell bead cleanup

✔	Step	Instructions for SMRTbell cleanup bead step
	6.1	Add 85 µL (1.0X) of resuspended, room-temperature SMRTbell cleanup beads to each sample.
	6.2	Pipette mix the sample until the beads are evenly distributed.

- ***IMPORTANT!*** Allow SMRTbell cleanup beads to come up to room temperature by bringing them out of 4°C storage at least 1.5 hours prior to beginning

[1] HiFi prep kit 96 contains a sufficient volume of reagents to support 4 automated runs using a minimum batch size of 24 samples per run.

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 | **3** Post-shearing cleanup

↓ Safe stop

4 DNA repair & A-tailing

↓

5 Adapter ligation | **6** Post-ligation cleanup

↓ Safe stop

7 Nuclease treatment

↓

8 AMPure PB bead cleanup

↓ Safe stop

9 Annealing, binding & cleanup

7. Nuclease treatment

✔	Step	Instructions
	7.1	Prepare the appropriate volume of master mix with 10% overage using the per reaction volumes listed below. **Prepare the master mix immediately before use to ensure optimal activity.**

Nuclease mix

✔	Tube	Component	Volume
	Light purple	Nuclease buffer 96	5 µL
	Light green	Nuclease mix 96	5 µL
		Total volume	**10 µL**

7.2	Pipette mix **Nuclease mix** and quick spin to collect liquid.
7.3	Add **10 µL** of **Nuclease mix** to each sample. Total volume should equal **50 µL**.
7.4	Pipette-mix each sample and quick-spin to collect liquid.
7.5	Run the **Nuclease treatment** thermocycler program. Set lid temperature to 75°C if programmable.

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

7.6	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., 24, 48, 72 or 96[1]), plus 10% overage

- Run **Nuclease treatment** on-deck thermal cycler program
- Set the lid temperature to 75°C

[1] HiFi prep kit 96 contains a sufficient volume of reagents to support 4 automated runs using a minimum batch size of 24 samples per run.

Diluted AMPure PB cleanup and size selection

AMPure PB bead size cleanup and selection step will clean the library and deplete DNA fragments <3 kb

DNA sample extraction

↓

DNA sample QC

1 Short Read Eliminator on gDNA

↓ Safe stop

2 Pipette DNA shearing | 3 Post-shearing cleanup

↓ Safe stop

4 DNA repair & A-tailing

↓

5 Adapter ligation | 6 Post-ligation cleanup

↓ Safe stop

7 Nuclease treatment

↓

8 AMPure PB bead cleanup

↓ Safe stop

9 Annealing, binding & cleanup

8. Diluted AMPure PB cleanup and size selection

✔	Step	Instructions for AMPure PB bead cleanup
	8.1	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.
	8.2	Add 3.1X v/v (155 µL) of resuspended, room-temperature 35% AMPure PB beads to each sample from the previous step.
	8.3	Pipette-mix the beads until evenly distributed.
	8.4	Leave at **room temperature** for **20 minutes** to allow DNA to bind beads.
	8.5	Place sample on an appropriate magnet and allow beads separate fully from the solution.
	8.6	Slowly pipette off the cleared supernatant without disturbing the beads.
	8.7	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.
	8.8	Repeat the previous step.
	8.10	Remove samples from the magnet and **immediately** add **25 µL** of **elution buffer** to each sample.
	8.11	Pipette-mix the beads until evenly distributed.
	8.12	Leave at **room temperature** for **5 minutes** to elute DNA of the beads.
	8.13	Place samples on the magnet and allow the beads separate fully from the solution.
	8.14	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a new tube.
	8.15	**Optional:** 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; however, libraries typically are at <40 ng/µL after the SMRTbell library preparation process. **Optional:** Further dilute each aliquot to **250 pg/µL** with Femto Pulse dilution buffer. Measure final SMRTbell library size distribution with a Femto Pulse system.

SAFE STOPPING POINT - Store at 4°C

- 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)

- **Optional:** Perform **DNA concentration QC** on final purified HiFi prep kit 96 SMRTbell library using a Qubit dsDNA HS assay or Quant-iT ds DNA 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 HiFi prep kit 96 SMRTbell library concentration must be **<60 ng/ µL** to proceed with annealing, binding & cleanup (ABC)[1]
  - → Using a concentration above 60 ng/µL will result in lower *P1* loading during sequencing
- **Optional:** Perform **DNA sizing QC** on final purified HiFi prep kit 96 SMRTbell library using a Femto Pulse system

Varioskan LUX multimode microplate reader (Thermo Fisher Scientific)

Femto Pulse system (Agilent Technologies)

[1] **Note:** HiFi prep kit 96 libraries are typically already at <40 ng/µL after the SMRTbell library preparation process if using ≤3 µg of input gDNA per sample (thus DNA quantification prior to ABC is optional) → No need to normalize all sample concentrations prior to ABC.

Annealing, Binding, and Cleanup (ABC)

Perform primer annealing, polymerase binding and complex cleanup to prepare samples for sequencing

DNA sample extraction

↓

DNA sample QC

↓

1 Short Read Eliminator on gDNA

↓ Safe stop

2 Pipette DNA shearing

3 Post-shearing cleanup

↓ Safe stop

4 DNA repair & A-tailing

↓

5 Adapter ligation

6 Post-ligation cleanup

↓ Safe stop

7 Nuclease treatment

↓

8 AMPure PB bead cleanup

↓ Safe stop

9 Annealing, binding & cleanup

9.1. Anneal sequencing primer

Step	Instructions
9.1	Prepare the appropriate volume of master mix with 10% overage using the per reaction volumes listed below.

Annealing mix

Tube	Component	Volume
Light blue	Annealing buffer 96	12.5 µL
Light green	Standard sequencing primer 96	12.5 µL
	Total volume	**25 µL**

9.2	Pipette-mix the **Annealing mix** and quick spin to collect liquid.
9.3	Add **25 µL** of the **Annealing mix** to each library. Total volume should equal **50 µL.**
9.4	Pipette-mix each sample and quick spin to collect liquid.
9.5	Incubate at room temperature for **15 minutes.**
9.6	During primer incubation, prepare the polymerase dilution (see below) and store on ice.

- For primer annealing reaction, each sample requires **25 µL of Annealing mix** solution
- Scale up the appropriate volume of Annealing mix solution required to process all samples by adding 1 volume of **Standard sequencing primer 96** + 1 volume of **Annealing buffer 96** to a new tube

Annealed SMRTbell library

9.7. Bind polymerase

Step	Instructions
9.7	To prepare the polymerase, add the following components to a new microcentrifuge tube on ice. Adjust component volumes for the number of samples being prepared, plus 10% overage.

Polymerase Dilution

Tube	Component	Volume
Yellow	Polymerase buffer 96	47 µL
Purple	Sequencing polymerase 96	3 µL
	Total volume	**50 µL**

9.8	Pipette mix the **polymerase dilution** and quick-spin to collect liquid.
9.9	Add **50 µL of polymerase dilution** to primer annealed sample. Total volume should equal **100 µL**.
9.10	Pipette-mix each sample and quick-spin to collect liquid.
9.11	Incubate at **room temperature for 15 minutes.**
9.12	Proceed immediately to the next step of the protocol to remove excess polymerase.

- For binding reaction, each annealed sample requires **50 µL of Revio sequencing polymerase 96 working solution**
- Scale up the appropriate volume of Revio polymerase 96 working solution required to process all samples by diluting the **Revio polymerase 96 stock** 16.7-fold in **Polymerase buffer 96**

Polymerase-bound SMRTbell library

PacBio

Annealing, Binding, and Cleanup (ABC) (cont.)

Perform primer annealing, polymerase binding and complex cleanup to prepare samples for sequencing

DNA sample extraction

↓

DNA sample QC

1 Short Read Eliminator on gDNA

↓ Safe stop

2 Pipette DNA shearing | **3** Post-shearing cleanup

↓ Safe stop

4 DNA repair & A-tailing

↓

5 Adapter ligation | **6** Post-ligation cleanup

↓ Safe stop

7 Nuclease treatment

↓

8 AMPure PB bead cleanup

↓ Safe stop

9 Annealing, binding & cleanup

9.13. Clean up polymerase-bound complexes

✔	Step	Instructions
	9.13	Add **100 µL** of resuspended, room-temperature SMRTbell cleanup beads to each sample
	9.14	Pipette-mix the beads until evenly distributed and quick-spin if necessary to collect all liquid from the sides of the tube.
	9.15	Leave at **room temperature** for **10 minutes** to allow DNA to bind beads
	9.16	Place sample on an appropriate magnet and allow beads to separate fully from the solution
	9.17	Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant. **DO NOT USE EtOH.** Proceed immediately to the elution. It is important not to let the beads dry out.
	9.18	Remove sample from the magnet and **immediately** add **50 µL** of **Loading Buffer 96** to each tube and resuspend the beads by pipette mixing.
	9.19	Quick-spin the samples to collect any liquid from the sides of the tube.
	9.20	Leave at **room temperature** for **5 minutes** to elute DNA
	9.21	Place sample on magnet and allow beads to separate fully from the solution.
	9.22	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a **new tube**. Discard the old tube with beads
	9.23	Use **1 µL** of sample to measure DNA concentration with a Qubit fluorometer using the 1x dsDNA HS kit. **Important:** The **Qubit Flex** instrument is not compatible with measuring polymerase-bound library in Loading Buffer 96. Concentration readings will not be accurate.
	9.24	Proceed to the **Loading Calculator** in SMRT Link v13.1 or higher to calculate the final dilution for adding the sample to the Revio sequencing plate.
		PROTOCOL COMPLETE

Storing polymerase-bound HiFi prep kit 96 libraries

- Polymerase-bound libraries can be stored at 4°C for 1 month, or at -20°C for >6 months prior to sequencing.
- Polymerase-bound libraries can withstand >4 freeze-thaw cycles without affecting sequencing performance.

- For complex cleanup reaction, each sample requires **100 µL of SMRTbell cleanup beads**

Purified polymerase-bound library

- Each sample is eluted in **50 µL of Loading Buffer 96**

- Perform **DNA concentration QC** on final purified polymerase-bound HiFi prep kit 96 SMRTbell library using a Qubit dsDNA HS assay or Quant-iT ds DNA HS assay
  - Post-ABC recovery yield (relative to library input amount for primer annealing reaction) should be ~**70%**
- **IMPORTANT!** The Qubit Flex instrument is not compatible with measuring polymerase-bound library in Loading Buffer 96 → Concentration readings will not be accurate.

- Prepare DNA internal control and perform final loading dilution procedure using **SMRT Link Loading Calculator** tool

SMRT Link v13.1+

SMRT Link Loading Calculator & Pooling Calculator for HiFi prep kit 96 libraries

Use **SMRT Link Revio polymerase kit 96 Loading Calculator** to calculate amount of polymerase-bound library to add to Revio sequencing plate and optionally use SMRT Link **Pooling Calculator**[1,2] to calculate sample pooling volumes

Sample setup workflow for preparing Revio HiFi prep kit 96 libraries for sequencing

ABC | Sample pooling (optional) | Final loading dilution

Anneal sequencing primer → **Bind polymerase** → **Complex cleanup** → **Pool indexed SMRTbell libraries** → **Prepare DNA internal control** → **Perform final sample dilution** → **Load sample into sequencing plate**

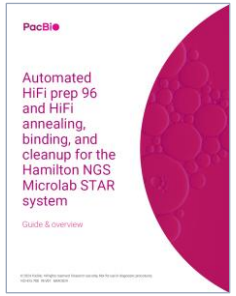
Follow HiFi prep kit 96 Procedure & checklist *or* Guide & overview | **Follow SMRT Link Pooling Calculator** | **Follow SMRT Link Revio polymerase kit 96 Loading Calculator**

Procedure & checklist
Preparing whole genome libraries using the HiFi prep kit 96 (103-420-700)

ABC instructions for manual HiFi prep kit 96 workflows

Or

Guide & overview
Automated HiFi prep 96 and HiFi annealing binding and cleanup for the Hamilton NGS Microlab STAR system (103-425-700)



ABC instructions for automated HiFi prep kit 96 workflows

SMRT Link v13.1+

- Optional: Use **SMRT Link Pooling Calculator** to calculate required sample pooling volumes if performing multiplexed sequencing[1,2]

- Use **SMRT Link Loading Calculator** to calculate final sample dilution when you are starting with Revio polymerase kit 96-bound SMRTbell libraries prepared using liquid handler automation or previously prepared manually

1 If sequencing HiFi plex prep kit 96 SMRTbell libraries, **you do not need to use** this SMRT Link Pooling Calculator tool since samples are typically **already pooled** during SMRTbell library construction.

1 If sequencing HiFi prep kit 96 SMRTbell libraries, this SMRT Link Pooling Calculator tool can optionally be used for pooling samples **either before** performing ABC **or after** performing ABC.

SMRT Link Pooling Calculator & Loading Calculator workflow overview

Pooling & Loading Calculator workflow overview for SMRTbell libraries bound with Revio polymerase kit 96

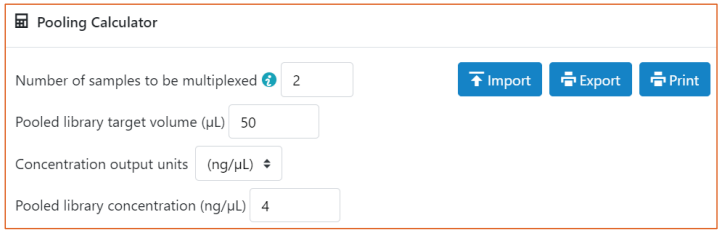
Use SMRT Link Revio polymerase kit 96 Loading Calculator & Pooling Calculator

Specify all required information in SMRT Link calculator tools

SMRT Link Pooling Calculator

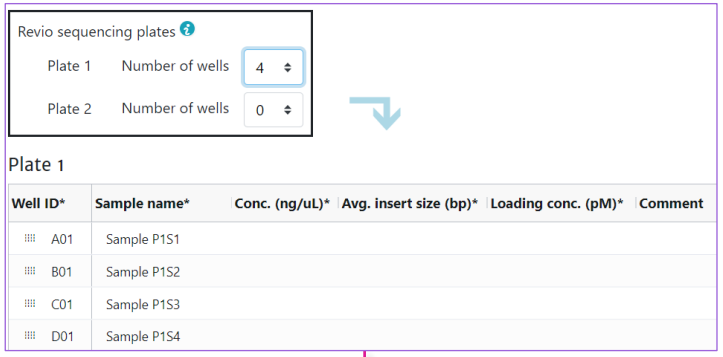
Optional if using the Revio polymerase kit 96 ***and*** pooling multiple biological samples together in one well

Specify # **of samples to be multiplexed**, **pooled library target volume**, **pooled library concentration** and other parameters



SMRT Link Loading Calculator

Specify the **# of sample wells** per Revio sequencing plate, followed by the **concentration**, **average insert size** & **loading concentration** of each sample



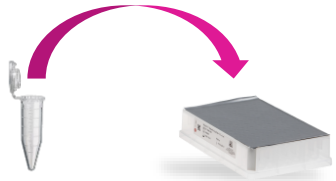
Follow provided instructions to process samples in the lab

Pool samples (OPTIONAL)

Prepare DNA internal control

Perform final sample dilution at target loading concentration

Load sample into Revio sequencing plate[1]



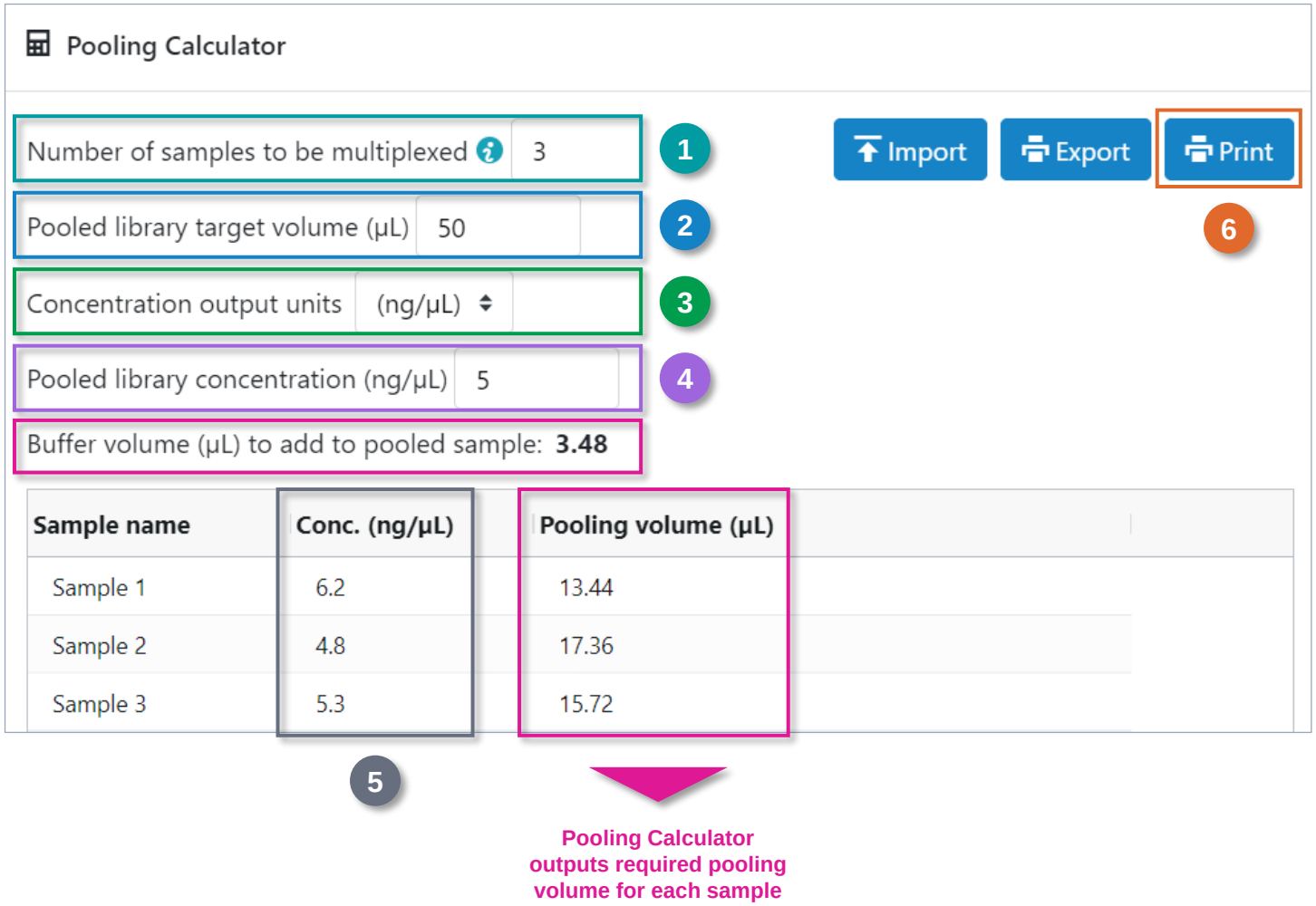
Final diluted sample

[1] If sequencing HiFi prep kit 96 SMRTbell libraries on a Sequel II/IIe system, load final diluted samples into the appropriate wells of a Sequel II system sample plate.

SMRT Link Pooling Calculator procedure for Revio HiFi prep kit 96 libraries

Pooling Calculator procedure for performing equal-mass pooling of HiFi prep kit 96 libraries bound with Revio polymerase kit 96

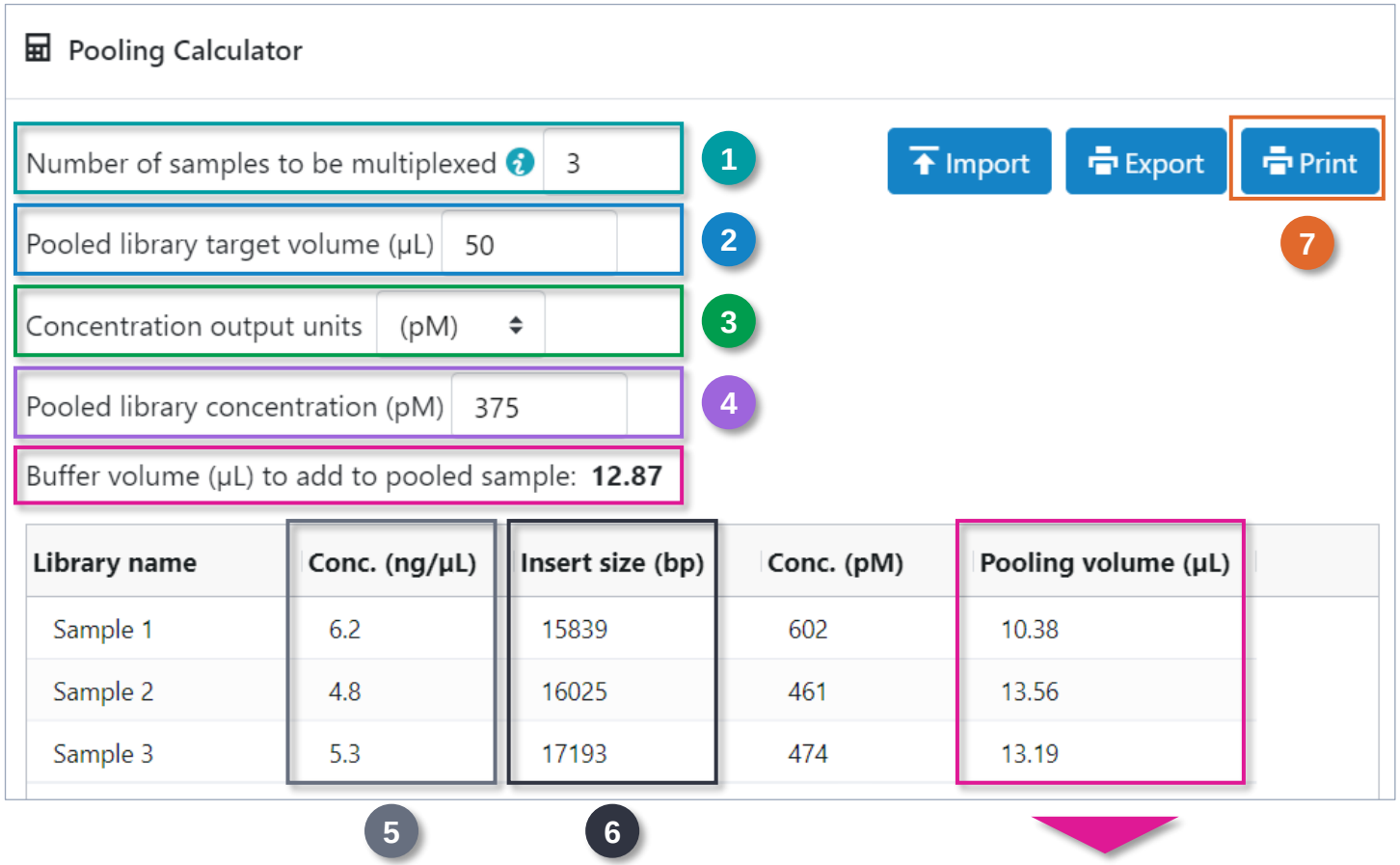
1. **Specify number of samples to be multiplexed**
   - Can specify a value between 2 and 384 samples
2. **Specify pooled library target volume (µL)**
   - For post-ABC library samples, typically specify a pooled library target volume ≥50 µL
   - For pre-ABC library samples, typically specify a pooled library target volume ≥25 µL
3. **Specify concentration output units**
   - Specify ng/µL to perform equal-mass pooling
4. **Specify pooled library concentration**
   - Typically, specify a pooled library concentration value that is lower than the least concentrated sample
5. **Specify sample concentrations**
   - Specify the concentration of each sample in ng/µL
6. **Print or export instructions (optional)**
   - To print the calculation(s) and instructions, click the Print button.



SMRT Link Pooling Calculator procedure for Revio HiFi prep kit 96 libraries (cont.)

Pooling Calculator procedure for performing equal-molar pooling of HiFi prep kit 96 libraries bound with Revio polymerase kit 96

1. **Specify number of samples to be multiplexed**
   - Can specify a value between 2 and 384 samples
2. **Specify pooled library target volume (µL)**
   - For post-ABC library samples, typically specify a pooled library target volume ≥50 µL
   - For pre-ABC library samples, typically specify a pooled library target volume ≥25 µL
3. **Specify concentration output units**
   - Specify nM or pM to perform equal-molar pooling
4. **Specify pooled library concentration**
   - Typically, specify a pooled library concentration value that is lower than the least concentrated sample
5. **Specify sample concentrations**
   - Specify the concentration of each sample in nM or pM
6. **Specify sample insert sizes**
   - Specify the insert size of each sample in bp
7. **Print or export instructions (optional)**
   - To print the calculation(s) and instructions, click the Print button.



SMRT Link Loading Calculator procedure for Revio HiFi prep kit 96 libraries

Entering sample information

1. Specify number of samples to use

- Specify the number (1-4) of sample wells to use per Revio sequencing plate
- **Note:** If you are using only one Revio sequencing plate, specify 0 for Plate 2

2. Enter information for first sample well

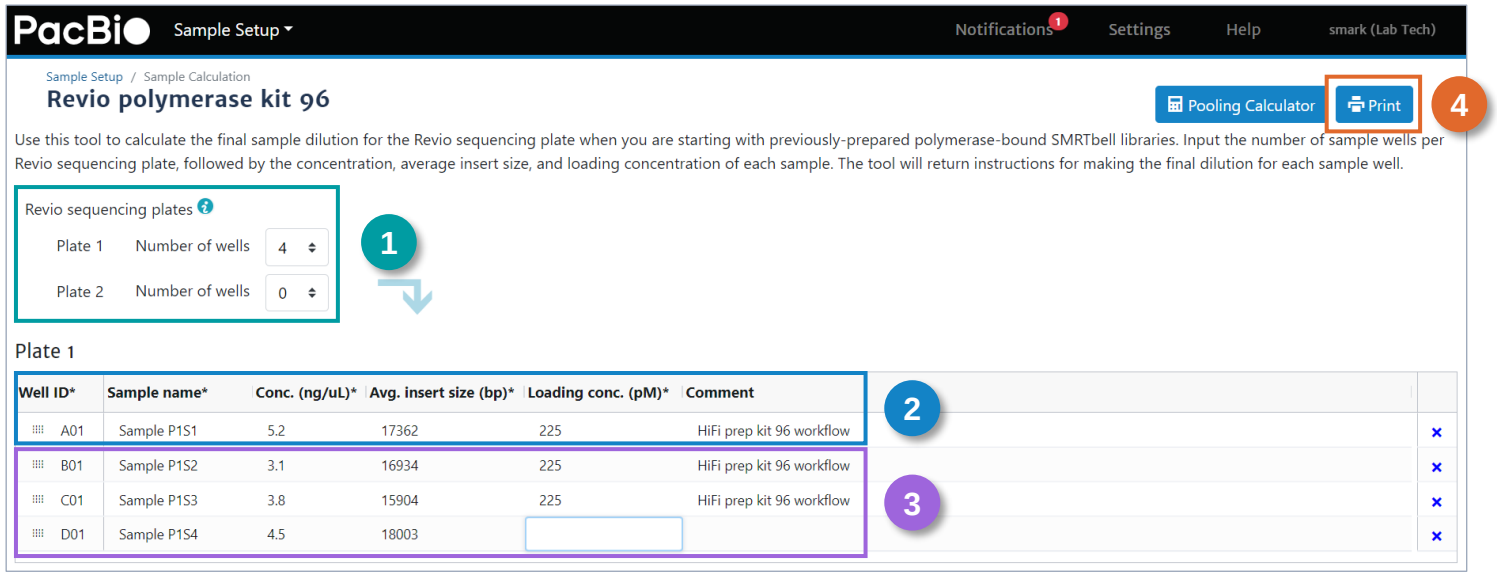
- Sample name
- Concentration (ng/μL)
- Average insert size (in base pairs)
- Loading concentration (in pM)
- Comments (optional)
- **Note:** If using a partially-used Revio sequencing plate, can delete a Well ID by clicking on the '**x**' button at right-hand side of table

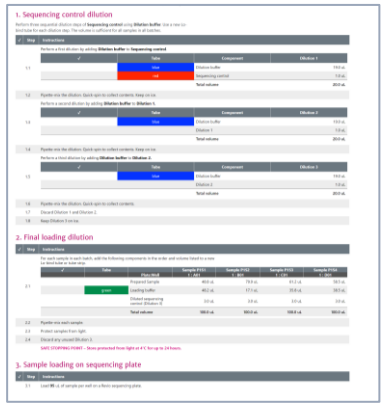
3. Repeat Step 2 for additional sample wells

- **Note:** All sample wells must be filled in for the instructions to display.

4. Print instructions (optional)

- To print the calculation(s) and instructions, click the Print button.





Loading Calculator outputs instructions for final loading dilution procedure

SMRT Link Loading Calculator procedure for Revio HiFi prep kit 96 libraries (cont.)

Calculator outputs and instructions

1. Sequencing control dilution

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

1. Sequencing control dilution

Perform three sequential dilution steps of **Sequencing control** using **Dilution buffer**. Use a new Lo-bind tube for each dilution step. The volume is sufficient for all samples in all batches.

✓	Step	Instructions
	1.1	Perform a first dilution by adding **Dilution buffer** to **Sequencing control**.

✓	Tube	Component	Dilution 1
	blue	Dilution buffer	19.0 uL
	red	Sequencing control	1.0 uL
		Total volume	20.0 uL

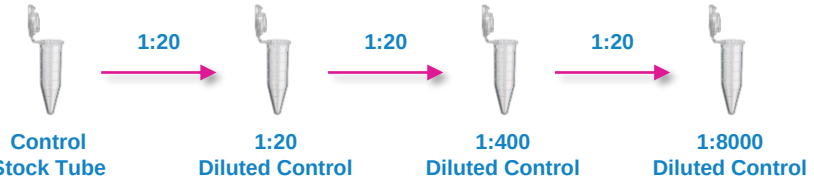
1.2	Pipette-mix the dilution. Quick-spin to collect contents. Keep on ice.	
	Perform a second dilution by adding **Dilution buffer** to **Dilution 1.**	

✓	Tube	Component	Dilution 2
1.3	blue	Dilution buffer	19.0 uL
		Dilution 1	1.0 uL
		Total volume	20.0 uL

1.4	Pipette-mix the dilution. Quick-spin to collect contents. Keep on ice.	
	Perform a third dilution by adding **Dilution buffer** to **Dilution 2.**	

✓	Tube	Component	Dilution 3
1.5	blue	Dilution buffer	19.0 uL
		Dilution 2	1.0 uL
		Total volume	20.0 uL

1.6	Pipette-mix the dilution. Quick-spin to collect contents.
1.7	Discard Dilution 1 and Dilution 2.
1.8	Keep Dilution 3 on ice.



Final diluted samples should be **used within 24 hrs**

2. Final loading dilution

Add **3 µL** (per Revio SMRT Cell) of serially diluted (1:8000) Revio sequencing control to final on-plate sample dilution

2. Final loading dilution

✓	Step	Instructions
	2.1	For each sample in each batch, add the following components in the order and volume listed to a new Lo-bind tube or tube strip.

✓	Tube	Plate:Well	Sample P1S1 1 : A01	Sample P1S2 1 : B01	Sample P1S3 1 : C01	Sample P1S4 1 : D01
		Prepared Sample	48.8 uL	79.9 uL	61.2	58.5 uL
	green	Loading buffer	48.2 uL	17.1 uL	35.	38.5 uL
		Diluted sequencing control (Dilution 3)	3.0 uL	3.0 uL	3.0 uL	3.0 uL
		Total volume	100.0 uL	100.0 uL	100.0 uL	100.0 uL

2.2	Pipette-mix each sample.	
2.3	Protect samples from light.	
2.4	Discard any unused Dilution 3.	

SAFE STOPPING POINT – Store protected from light at 4°C for up to 24 hours.

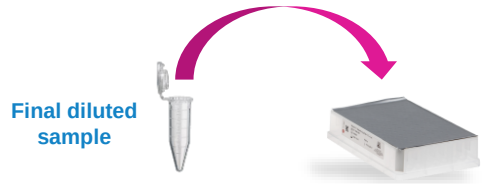
Final loading dilution total volume (per Revio SMRT Cell) is **100 µL**

3. Sample loading on sequencing plate

Load **95 µL** of final diluted sample per well on a Revio sequencing plate.

3. Sample loading on sequencing plate

✓	Step	Instructions
	3.1	Load **95** uL of sample per well on a Revio sequencing plate.



HiFi prep kit 96 example sequencing performance data

HiFi sequencing performance of HiFi prep kit 96 libraries

Example HiFi read length and yield performance obtained for HiFi prep kit 96 libraries constructed from human HG002 genomic DNA samples[1]

Plate A 96 pool

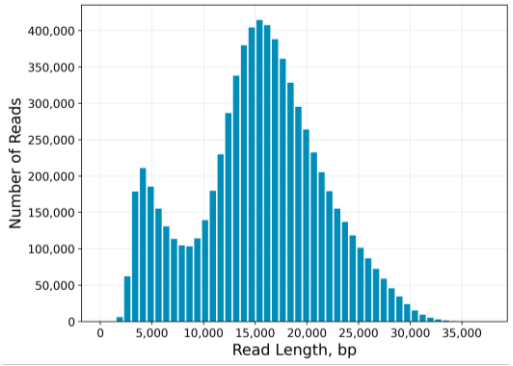


Plate B 96 pool

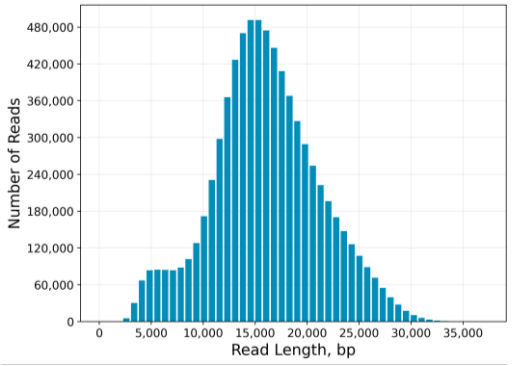


Plate C 96 pool

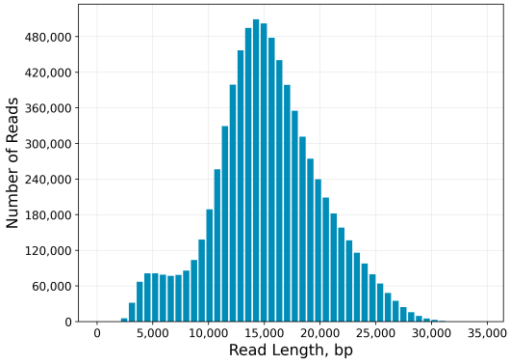


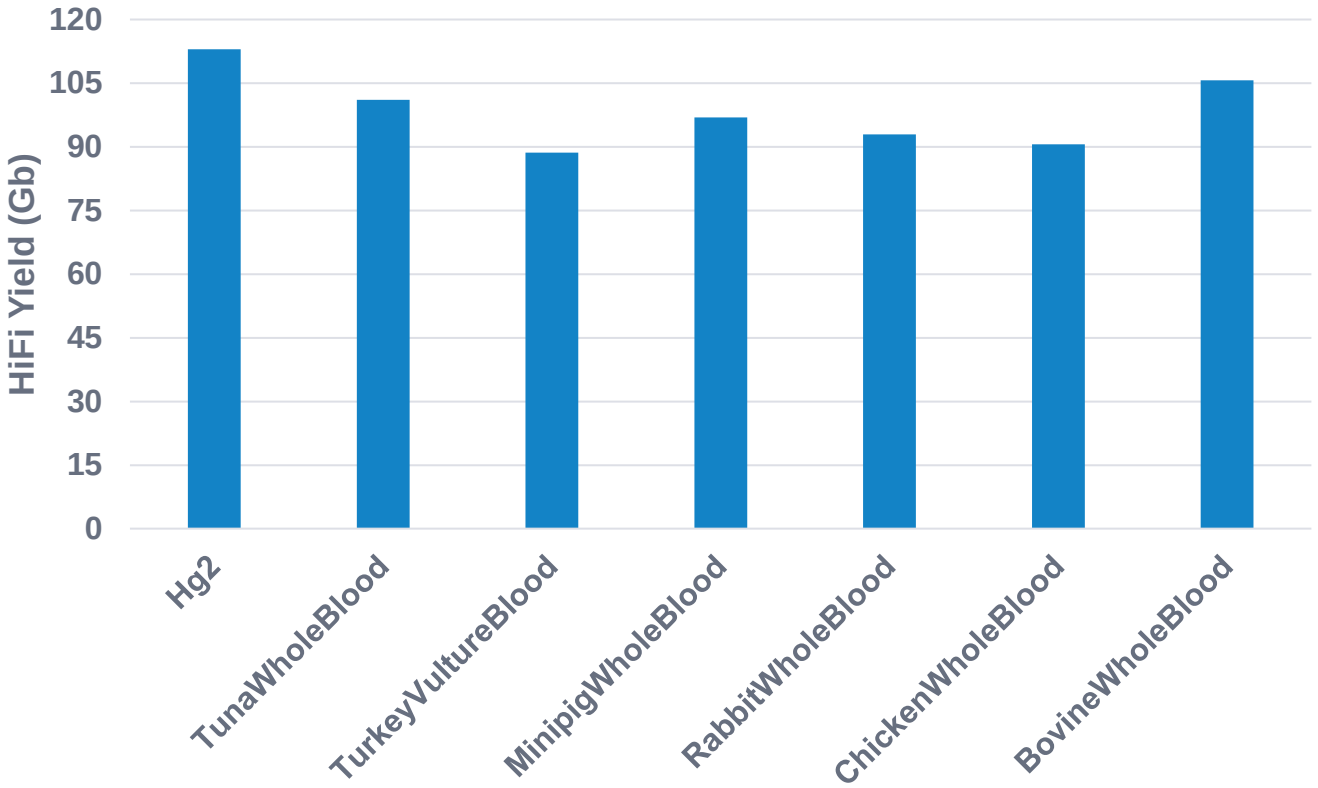
	Plate A	Plate B	Plate C
SMRTbell adapter index plate	96A	96B	96C
HiFi yield	111 Gb	121 Gb	117 Gb
HiFi read length	15 kb	16 kb	15 kb
Average Revio SMRT Cells/2 µg gDNA	2.1 ± 0.4	1.9 ± 0.3	1.8 ± 0.3
Automation workflow time (gDNA → ABC)	12.4 hrs	11.6 hrs	12 hrs

[1] Revio system data shown. **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.

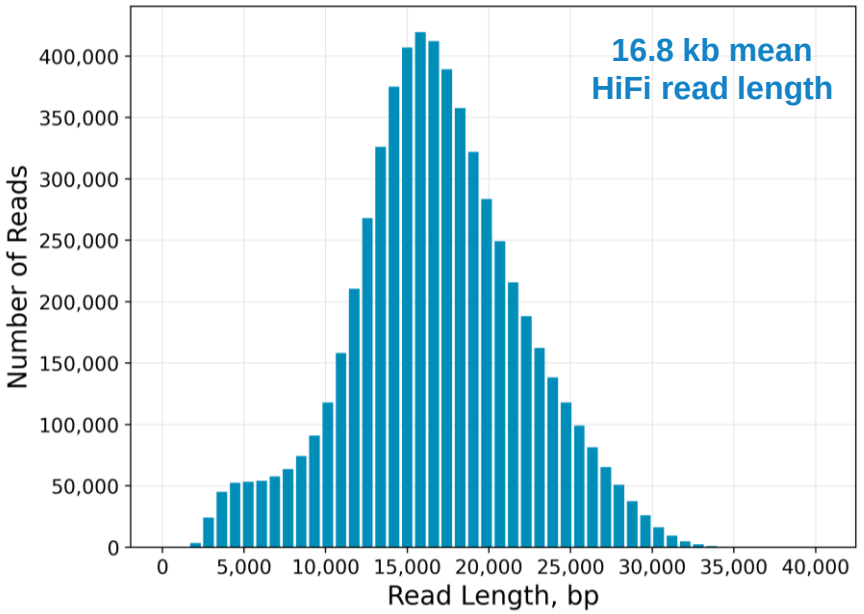
HiFi sequencing performance of HiFi prep kit 96 libraries

Example HiFi read length and yield performance obtained for HiFi prep kit 96 libraries constructed from gDNA isolated from human and animal whole blood samples using Nanobind PanDNA kit[1]

Human and animal whole blood samples



Tuna whole blood sample



[1] Revio system data shown. **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.

HiFi plex prep kit 96 workflow overview for WGS and amplicon sequencing applications

Procedure & checklist – Preparing multiplexed whole genome and amplicon libraries using the HiFi plex prep kit 96 (103-418-800)

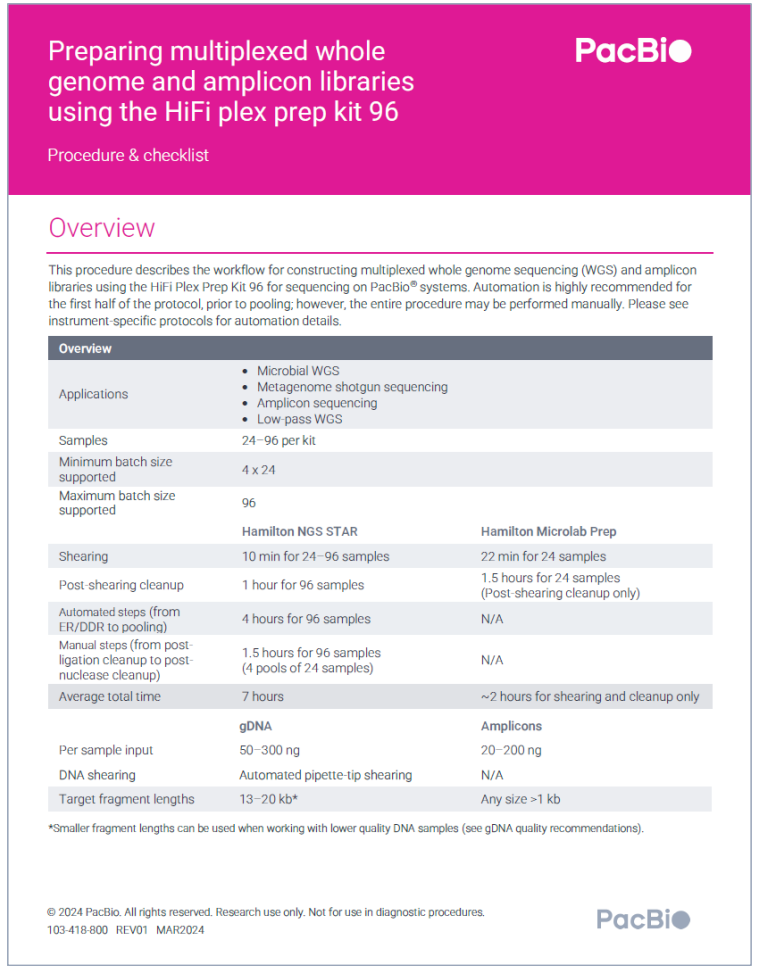
Procedure & checklist 103-418-800 describes the workflow for constructing multiplexed whole genome sequencing (WGS) and amplicon libraries using the HiFi Plex Prep Kit 96 for sequencing on PacBio systems. Automation is highly recommended for the first half of the protocol up to and including pooling; however, the entire procedure may be performed manually. Please see instrument-specific protocols for automation details[1].

Overview	
Applications	• Microbial WGS • Metagenome shotgun sequencing • Amplicon sequencing • Low-pass WGS
Samples	24–96 per kit
Minimum batch size supported	4 x 24
Maximum batch size supported	96

DNA input and fragment size recommendations		
	gDNA	Amplicons
Per sample input	50–300 ng	20–200 ng
DNA shearing	Automated pipette-tip shearing	N/A
Target fragment lengths	13–20 kb*	Any size >1 kb

*Smaller fragment lengths can be used when working with lower quality DNA samples (see gDNA quality recommendations).

Note: To prepare HiFi plex prep kit 96 samples for sequencing on PacBio long-read systems, follow ABC[2] workflow instructions provided in **SMRT Link Sample Setup** calculator tool



Preparing multiplexed whole genome and amplicon libraries using the HiFi plex prep kit 96

PacBio

Procedure & checklist

Overview

This procedure describes the workflow for constructing multiplexed whole genome sequencing (WGS) and amplicon libraries using the HiFi Plex Prep Kit 96 for sequencing on PacBio® systems. Automation is highly recommended for the first half of the protocol, prior to pooling; however, the entire procedure may be performed manually. Please see instrument-specific protocols for automation details.

Overview		
Applications	• Microbial WGS • Metagenome shotgun sequencing • Amplicon sequencing • Low-pass WGS	
Samples	24–96 per kit	
Minimum batch size supported	4 x 24	
Maximum batch size supported	96	
	Hamilton NGS STAR	Hamilton Microlab Prep
Shearing	10 min for 24–96 samples	22 min for 24 samples
Post-shearing cleanup	1 hour for 96 samples	1.5 hours for 24 samples (Post-shearing cleanup only)
Automated steps (from ER/DDR to pooling)	4 hours for 96 samples	N/A
Manual steps (from post-ligation cleanup to post-nuclease cleanup)	1.5 hours for 96 samples (4 pools of 24 samples)	N/A
Average total time	7 hours	~2 hours for shearing and cleanup only
	gDNA	Amplicons
Per sample input	50–300 ng	20–200 ng
DNA shearing	Automated pipette-tip shearing	N/A
Target fragment lengths	13–20 kb*	Any size >1 kb

*Smaller fragment lengths can be used when working with lower quality DNA samples (see gDNA quality recommendations).

103-418-800 REV01 MAR2024

PacBio

[1] If using the HiFi plex prep kit 96 in an automated high-throughput library prep workflow with a Hamilton liquid handling system, refer to ***Guide & overview – Automated HiFi plex prep 96 for the Hamilton NGS Microlab STAR system*** (103-425-800).

[2] If using **Revio polymerase kit (102-817-600)** or **Sequel II binding kit 3.2 (102-333-300)** for polymerase binding, follow sample setup ABC instructions provided in SMRT Link Sample Setup calculator tool for Sequel II binding kit 3.1/3.2, Revio polymerase kit.to perform primer annealing, polymerase binding & complex cleanup (ABC) steps and final loading dilution procedure to prepare HiFi plex prep kit 96 libraries for sequencing (do not use SMRT Link Loading Calculator).

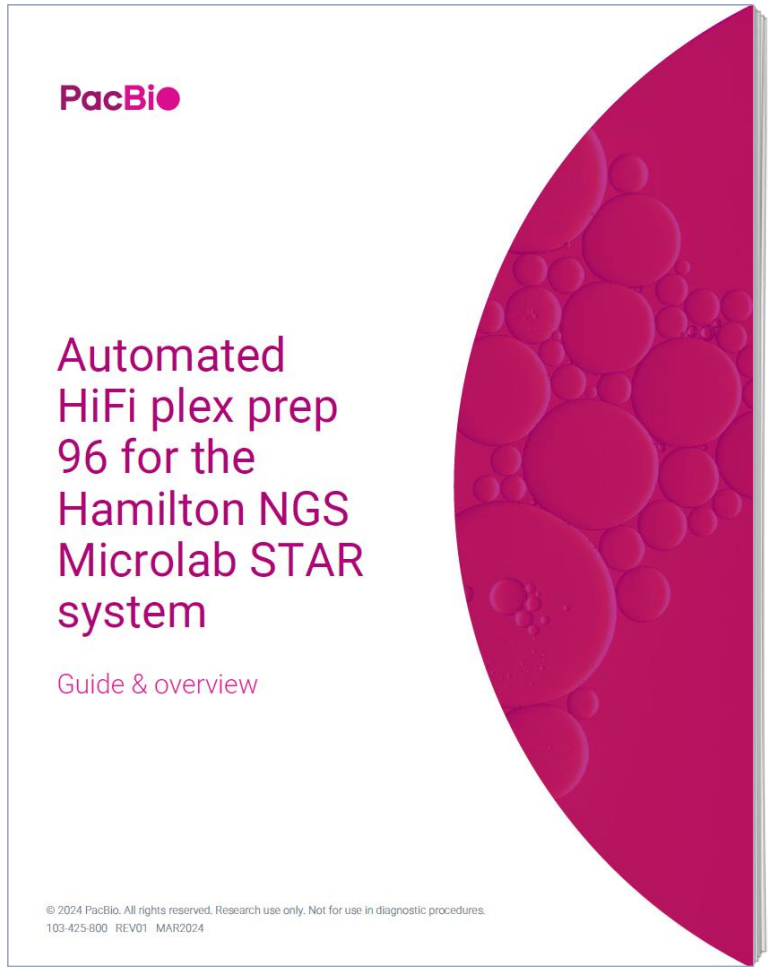
PacBio

Guide & overview – Automated HiFi plex prep 96 for the Hamilton NGS Microlab STAR system (103-425-800)[1]

Guide & overview 103-425-800 describes the workflow for PacBio high-throughput semi-automated long read sample prep utilizing the HiFi Prep Plex 96 kit that is designed for a minimum of 24 and maximum of 96 samples per automated run.

Overview	
Applications	• Microbial WGS • Metagenome shotgun sequencing • Amplicon sequencing • Long read low pass WGS
Samples	24 – 96 per kit
Minimum batch size supported	4 x 24
Maximum batch size supported	96
	Hamilton NGS STAR
Shearing	10 min for 24–96 samples
Post-shearing cleanup	1 hour for 96 samples
Automated steps (ER/DDR to pooling)	4 hours for 96 samples
Manual steps (Post-ligation cleanup to post-nuclease cleanup)	1.5 hours for 96 samples (4 pools of 24 samples)
Average total time	7 hours

Note: To prepare HiFi plex prep kit 96 samples for sequencing on PacBio long-read systems, follow ABC[2] workflow instructions provided in **SMRT Link Sample Setup** calculator tool



[1] If using the HiFi plex prep kit 96 in an automated high-throughput library prep workflow with a liquid handling system, refer to ***Guide & overview – Automated HiFi plex prep 96 for the Hamilton NGS Microlab STAR system*** (103-425-800).

[2] If using **Revio polymerase kit (102-817-600)** or **Sequel II binding kit 3.2 (102-333-300)** for polymerase binding, follow sample setup ABC instructions provided in SMRT Link Sample Setup calculator tool for Sequel II binding kit 3.1/3.2, Revio polymerase kit.to perform primer annealing, polymerase binding & complex cleanup (ABC) steps and final loading dilution procedure to prepare HiFi plex prep kit 96 libraries for sequencing (do not use SMRT Link Loading Calculator).

HiFi plex prep kit 96 workflow overview for multiplexed WGS & amplicon applications

Automation of HiFi library prep workflow enables high-throughput processing of 24 to 96 samples in 2 days

Key workflow steps	Automation	Time	Protocol documentation or reference	Recommended equipment & consumables
DNA sample extraction		2 – 2.5 hrs	 ***Guide & overview – Nanobind HT kits*** (103-028-100) **[UPDATED]**	 Nanobind HT CBB kit / Nanobind HT 1 mL whole blood kit
DNA sample QC		2 hrs	 Refer to third-party user guide documenation	 DNA concentration QC: • Varioskan LUX microplate reader DNA sizing QC: • Femto Pulse system
1 Pipette DNA shearing		10 min	**Protocol for manual & automated workflows**	 Pipette-based shearing with Hamilton Microlab Prep or NGS STAR
Safe stop				
2 Post-shearing cleanup[1]		1 hr	 ***Procedure & checklist – Preparing multiplexed whole genome and amplicon libraries using the HiFi plex prep kit 96*** (103-418-800)	
Safe stop			**Guide & overview for automated workflows**	
3 DNA repair & A-tailing		0.5 hrs	 ***Guide & overview – Automated HiFi plex prep 96 for the Hamilton NGS Microlab STAR system*** (103-425-800)	
4 Adapter ligation, termination & pooling		3.5 hrs	**Supported automation systems**  Thermo Fisher KingFisher Apex  Hamilton Microlab NIMBUS Presto  Hamilton Microlab Prep  Hamilton NGS Star	 HiFi plex prep kit 96 (103-381-300 / 96 RXN) + SMRTbell adapter index plate 96A/B/C/D with Hamilton
5 Pool cleanup		45 min		
6 Nuclease treatment		15 min		
Safe stop				
7 Final cleanup		45 min		 1X SMRTbell bead cleanup

Library construction

DAY 1

[1] For automated library prep workflows using the HiFi plex prep kit 96 and 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.

General best practices recommendations for preparing multiplexed WGS and amplicon libraries using HiFi plex prep kit 96

Automation

- The HiFi plex prep kit 96 and workflow was designed to work with NGS liquid handling automation **up to the sample pooling step**
  - Once samples are pooled, the remaining steps are prepared manually
- **Note:** This protocol was developed using the Hamilton NGS STAR MOA 96 system
  - Refer to ***Guide & overview – Automated HiFi plex prep 96 for the Hamilton NGS Microlab STAR system*** (103-425-800)
- Because of differences between automation instruments, modifications not described herein may be needed to adapt the protocol to your specific instrumentation
  - Please visit www.pacb.com or contact your local support team for a list of instruments with a PacBio qualified method

Hamilton NGS Star workstation

General best practices recommendations for preparing multiplexed WGS and amplicon libraries using HiFi plex prep kit 96 (cont.)

DNA sample extraction

Genomic DNA extraction from cultured bacteria

PacBio Nanobind DNA extractions kits are recommended to ensure sufficient mass and quality of high-molecular weight (HMW) DNA for use in HiFi plex prep kit 96 protocol

Nanobind HT CBB kit (102-762-700; 96 rxn)	Nanobind PanDNA kit (103-260-000; 24 rxn)
• For high-throughput HMW DNA extraction from up to 200 µL human/mammalian blood, non-mammalian animal blood1, cultured cells, and bacteria • Expected HMW DNA yield: 3–15 µg for blood and cultured mammalian cells and 2–10 µg for bacteria	• For HMW DNA extraction from cells, blood, bacteria, tissues, insects, and plant nuclei • Expected HMW DNA yield: 3–26 µg

Genomic DNA extraction from metagenomic samples

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

Amplicon DNA generation

- **Note:** Using gel-extracted amplicon products may results in lower sequencing performance due to the damage inherently caused by intercalating dyes such as ethidium bromide and exposure to UV radiation.
- Sequencing amplicons stained with SYBR dyes from ThermoFisher Scientific is untested, and therefore cannot be recommended.
- If working with a gel-extracted product that has been stained with a dye, it is recommended to bring it through additional rounds of amplification to remove damage and/or dyes prior to library prep and sequencing.

General best practices recommendations for preparing multiplexed WGS and amplicon libraries using HiFi plex prep kit 96 (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 high-throughput DNA quantification QC workflows, we recommend using the Quant-iT 1X dsDNA high sensitivity assay kit[3] (Thermo Fisher Scientific) with the Varioskan LUX multimode microplate reader (Thermo Fisher Scientific)
  - Varioskan LUX multimode microplate reader enables rapid, specific and accurate determination of nucleic acid concentrations in a wide range
  - Quant-iT assay is well-adapted to high-throughput use (typically in 96-well or 384-well plates) and is highly selective for dsDNA over RNA (in the 0.2–100 ng range, the fluorescence signal is linear with the amount of DNA)
  - **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

Varioskan LUX multimode microplate reader (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 lower-throughput applications DNA quantification QC may be performed without a microplate reader using the **Qubit 1X dsDNA high sensitivity assay kit** with a **Qubit fluorometer**.

General best practices recommendations for preparing multiplexed WGS and amplicon libraries using HiFi plex prep kit 96

Recommended DNA input amount and quality

- Recommended DNA input amounts will vary by starting DNA sample type

Genomic DNA	Amplicon DNA
50–300 ng per sample	20–200 ng per sample

IMPORTANT!

- Do not exceed >300 ng of gDNA per sample going into the Repair and A-tailing steps.
  - → Too much DNA may overwhelm enzymatic reactions and lead to poor library recovery.
- At least 24 samples need to be used when using lower input amounts (e.g., 50 ng).
- Samples must be pooled after ligation

- For genomic DNA samples, 70% or more of the DNA should be ≥10 kb for this protocol
  - This corresponds to a genome quality number (GQN) of 7.0 or higher at 10 kb
- Remove all RNA from genomic DNA samples prior to beginning
  - Residual RNA can inhibit sequencing polymerase binding and can therefore lead to low loading

General best practices recommendations for preparing multiplexed WGS and amplicon libraries using HiFi plex prep kit 96

DNA shearing

- This protocol recommends shearing genomic DNA using automated liquid handler systems to a size between 13–20 kb
  - If shearing DNA samples 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 shearing DNA samples using a Hamilton NGS Star MOA liquid handling workstation, refer to ***Guide & overview – Automated HiFi plex prep 96 for the Hamilton NGS Microlab STAR system*** (103-425-800) for details about third-party consumables requirements
- Microbial and metagenomic samples often have degraded DNA where the majority of fragments are already <15 kb in length
  - → To better balance the number of reads between samples it may be necessary to shear all samples to a mean size of ~10 kb
- For shearing DNA samples to a target fragment size below <15 kb for PacBio HiFi sequencing, we recommend using the following equipment options:

SPEX SamplePrep *1600 MiniG* homogenizer[1]	
Sheared fragment size	7–10 kb
Parameter	**Settings**
Speed	1500 RPM
Time	3 minutes
DNA input	300 ng – 3 µg
Volume	Up to 300 µL

MP Bio *FastPrep 96* homogenizer[2]	
Sheared fragment size	7–10 kb
Parameter	**Settings**
Speed	1800 RPM
Time	60 seconds
DNA input	300 ng – 3 µg
Volume	50 µL

PacBio

1 See ***Technical note – HT plate shearing microbial inserts*** (102-326-575) for description of SamplePrep 1600 MiniG system for PacBio workflows.

2 See ***Technical note – HT DNA shearing for HiFi whole genome sequencing from whole blood samples*** (102-326-579) for description of FastPrep 96 system for PacBio workflows.

General best practices recommendations for preparing multiplexed WGS and amplicon libraries using HiFi plex prep kit 96

Reagent handling

- Room temperature is defined as any temperature in the range of 18 – 25°C for this protocol

HiFi plex prep kit 96

Thaw these reagents at room temperature	Keep these temperature-sensitive reagents on ice	Bring these reagents to room temperature 30 minutes prior to use	Bring these reagents to room temperature 1.5 hours prior to use
❑ Repair buffer M96	❑ End repair mix M96	❑ 1X dsDNA HS assay reagents	❑ SMRTbell cleanup beads-85 mL[1]
❑ Nuclease buffer M96	❑ DNA repair mix M96		
❑ SMRTbell adapter index plate	❑ Ligation mix M96		
❑ Stop solution M96	❑ Ligation enhancer M96		
	❑ Nuclease mix M96		

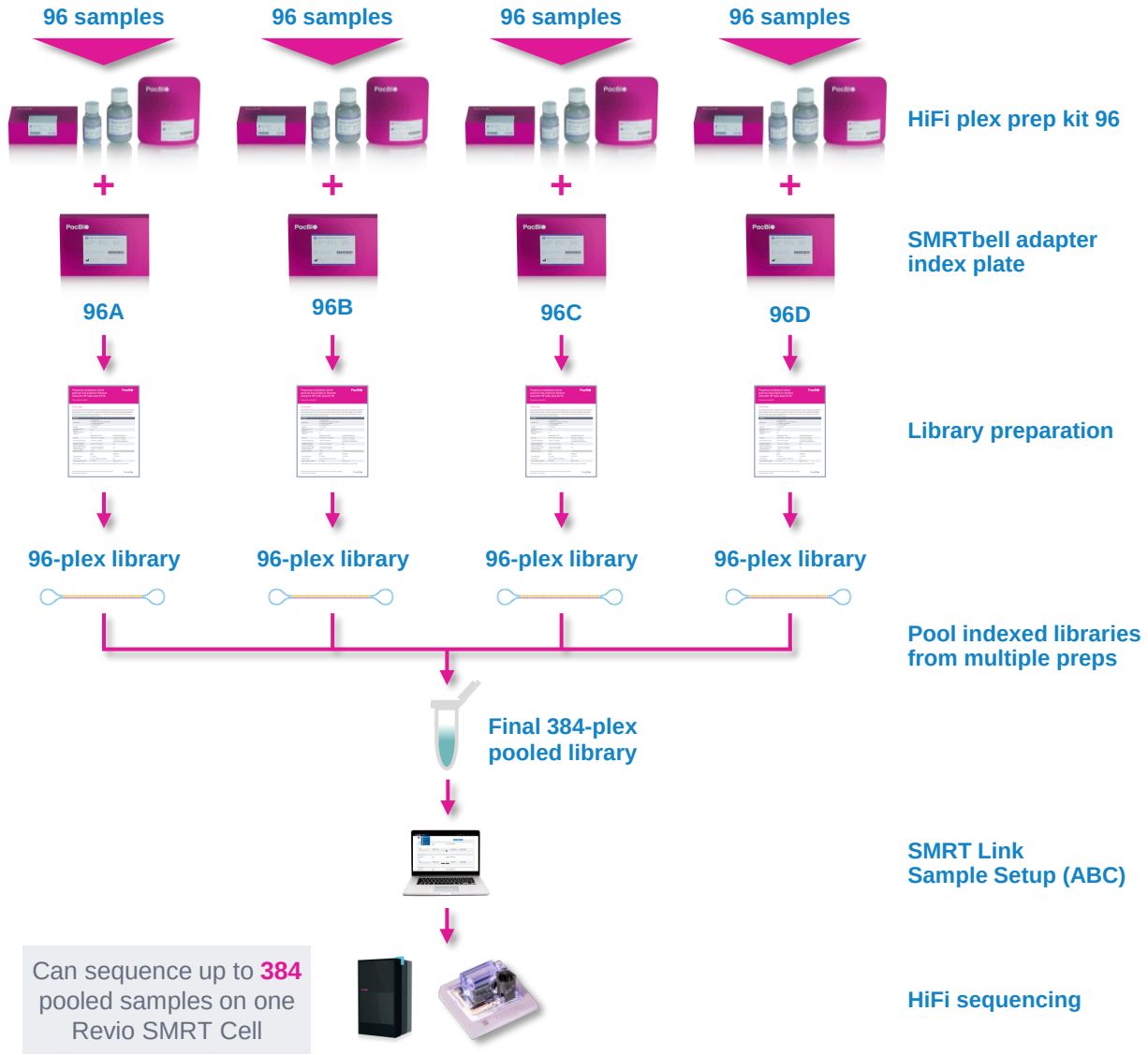
[1] Alternatively, can bring SMRTbell cleanup beads-85 mL to room temperature the night before if starting protocol in the morning.

- Once thawed, reaction buffers and adapter index plate may be stored on ice
- Briefly spin down all reagent tubes to collect all liquid at bottom
- Briefly vortex then spin down SMRTbell adapter index plate in a centrifuge with a plate adapter to collect all liquid at the bottom of the wells
- Shake/vortex SMRTbell cleanup beads immediately before use
- Pipette-mix bead binding and elution steps until beads are distributed evenly
- Pipette-mix all SMRTbell prep reactions by pipetting up and down 10 times.
- Samples can be stored at 4°C at all safe stopping points listed in the protocol.

General best practices recommendations for preparing multiplexed WGS and amplicon libraries using HiFi plex prep kit 96

Multiplexing samples

- **Note:** The procedure requires any of the four available SMRTbell adapter index plates:
  - ❑ SMRTbell adapter index plate 96A (bc2001–bc2096)
  - ❑ SMRTbell adapter index plate 96B (bc2097–bc2192)
  - ❑ SMRTbell adapter index plate 96C (bc2193–bc2288)
  - ❑ SMRTbell adapter index plate 96D (bc2289–bc2384)
- To balance the number of reads per sample, consider the following:
  - Shear all gDNA samples to similar mean fragment sizes and distributions
  - Normalize DNA input across all samples
- To pool more than 96 samples per SMRT Cell, combine indexed libraries constructed using multiple HiFi plex prep kits following the final cleanup
  - → Each prep kit needs to use a different SMRTbell adapter index plate
- A total of 384 samples can be pooled for sequencing on a single Revio SMRT Cell[1]
  - → A total of 1,536 (4 cells x 384 samples) can be sequenced in a single Revio system 4-cell run



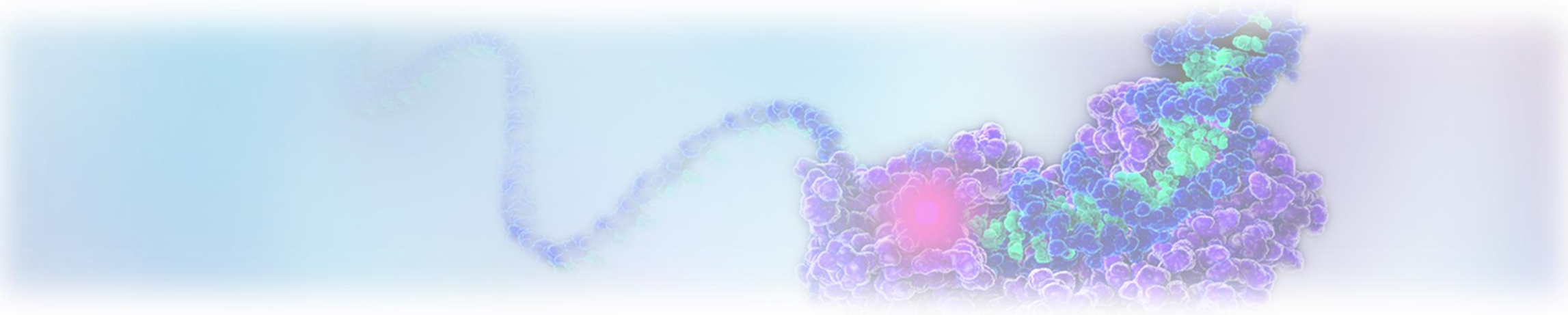
[1] For Sequel II/IIe systems, up to 96 samples can be pooled on a single SMRT Cell 8M.

General best practices recommendations for preparing multiplexed WGS and amplicon libraries using HiFi plex prep kit 96

Sequencing preparation (ABC) and polymerase-bound library storage

- **Note:** ***Procedure & checklist – Preparing multiplexed whole genome and amplicon libraries using the HiFi plex prep kit 96*** *(103-418-800)* 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 HiFi plex prep kit 96 libraries
- Sequencing polymerase is stable once bound to the HiFi prep kit 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.

HiFi plex prep kit 96 library prep inputs & expected step yields

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

		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	Quant-iT/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
DNA shearing	1 Pipette DNA shearing	N/A	Post-shearing QC with Quant-iT/Qubit (optional) & Femto Pulse	≤10 ng/µL DNA in 300 µL (3 µg total input DNA mass) into Step 2	Expect up to 80% step recovery (60% total recovery) Target DNA shear size is ~15-20 kb
	Safe stop				
DNA shearing	2 Post-shearing cleanup	1X SMRTbell cleanup beads	N/A	≤300 µL sheared DNA	4.5 µL purified sheared DNA
	Safe stop				
Library construction	3 DNA repair & A-tailing	N/A	N/A	4.5 µL purified sheared DNA	30 µL repaired & A-tailed DNA
Library construction	4 Adapter ligation & pooling	N/A	N/A	30 µL post-repaired & A-tailed DNA	960 µL for 24-plex of pooled libraries (24 x 40 µL per sample)
Library construction	5 Pool cleanup	1X SMRTbell cleanup beads	N/A	960 µL for 24-plex of pooled libraries	40 µL of purified pooled libraries
Library construction	6 Nuclease treatment	N/A	N/A	40 µL of purified pooled libraries	50 µL of nuclease-treated sample
	Safe stop				
Library construction	7 Final cleanup	1X (for >3 kb) or 1.3X (for <3 kb) SMRTbell cleanup beads	Quant-iT/Qubit dsDNA HS assay Femto Pulse system	50 µL of nuclease-treated sample	Final DNA concentration must be <60 ng/µl for >10 kb libraries, <20 ng/µL for 3-10 kb libraries and <10 ng/µL for <3 kb libraries to proceed with ABC[1]

[1] **Note:** Failure to dilute library to the recommended DNA concentration prior to ABC may result in lower *P1* loading during sequencing.

DNA sample extraction

Perform automated high-throughput HMW DNA extraction using Nanobind HT kits[1]

DNA sample extraction

↓

DNA sample QC

1 Pipette DNA shearing

↓ Safe stop

2 Post-shearing cleanup

↓ Safe stop

3 DNA repair & A-tailing

↓

4 Adapter ligation & pooling

↓

5 Pool cleanup

↓

6 Nuclease treatment

↓ Safe stop

7 Final cleanup

ThermoFisher SCIENTIFIC

KingFisher Duo/Flex/Apex

HAMILTON

Microlab NIMBUS Presto

Automated solutions built on Nanobind technology

Nanobind HT kits use magnetic disk processing to automate lysis, binding, washing and elution steps and are compatible with instruments from Hamilton and Thermo Fisher

- Hamilton NIMBUS Presto is a walkaway solution with automated plate filling
- Thermo Fisher KingFisher instruments are semi-automated with manual plate filling and limited user interaction during the run

Available Nanobind HT kits

Nanobind HT CBB kit (102-762-700; 96 rxn)

- For up to 200 µL human/mammalian blood, non-mammalian animal blood[2], cultured cells, and bacteria
- Expected HMW DNA yield: 3–15 µg for blood and cultured mammalian cells and 2–10 µg for bacteria

Nanobind HT 1 mL blood kit (102-762-800; 96 rxn)

- For 1 mL human blood
- Expected HMW DNA yield: 3–70 µg

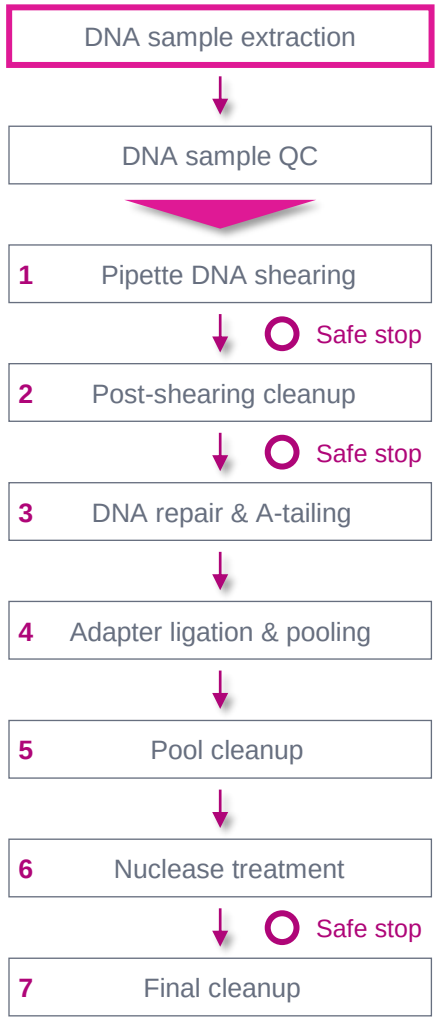
PacBio

[1] See ***Technical overview — Automated high-throughput HMW DNA extraction for PacBio long-read sequencing using Nanobind HT kits*** (103-401-700).

[2] Note: For animals with nucleated red blood cells, only 2.5 – 20 µL of blood is needed for HMW DNA extraction using Nanobind HT CBB kit.

DNA sample extraction (cont.)

Available Nanobind HT procedures for high-throughput HMW DNA extraction from bacteria



Automation system	Procedure & checklist	Sample type
KingFisher Apex	Extracting HMW DNA using the Nanobind HT CBB kit for mammalian cultured cells on KingFisher Apex system [102-996-100]	Mammalian cells
	Extracting HMW DNA using the Nanobind HT CBB kit for 200 μL human whole blood on KingFisher Apex system [102-995-700]	Human whole blood
	Extracting HMW DNA using the Nanobind HT CBB kit for bacteria on the KingFisher Apex system [103-377-600]	Cultured bacteria
	Extracting HMW DNA using the Nanobind HT CBB kit for non-human mammalian blood (NHMB) on the KingFisher Apex system [103-397-300]	Non-human mammalian blood
	Extracting HMW DNA using the Nanobind HT CBB kit for nucleated red blood cells (nRBCs) on the KingFisher Apex system [103-377-800]	Non-mammalian blood (nucleated RBCs)
	Extracting HMW DNA using Nanobind HT 1 mL blood kit for human whole blood on KingFisher Apex system [102-995-300]	Human whole blood
Hamilton NIMBUS Presto	Extracting HMW DNA using the Nanobind HT CBB kit for mammalian cultured cells on Hamilton NIMBUS Presto system [102-996-400]	Mammalian cells
	Extracting HMW DNA using the Nanobind HT CBB kit for 200 μL human whole blood on Hamilton NIMBUS Presto system [102-996-000]	Human whole blood
	Extracting HMW DNA using the Nanobind HT CBB kit for bacteria on Hamilton NIMBUS Presto system [103-397-400]	Cultured bacteria
	Extracting HMW DNA using the Nanobind HT CBB kit for non-human mammalian blood (NHMB) on the Hamilton NIMBUS Presto system [103-377-700]	Non-human mammalian blood
	Extracting HMW DNA using the Nanobind HT CBB kit for nucleated red blood cells (nRBCs) on the Hamilton NIMBUS Presto system [103-397-500]	Non-mammalian blood (nucleated RBCs)
	Extracting HMW DNA using the Nanobind HT 1 mL blood kit for human whole blood on Hamilton NIMBUS Presto system [102-995-600]	Human whole blood

[1] See PacBio's Documentation website for the most up-to-date list of Nanobind HT HMW DNA sample preparation protocols.

DNA sample QC

Perform high-throughput DNA QC using a microplate reader[1] and a Femto Pulse system

DNA sample extraction

↓

DNA sample QC

1 Pipette DNA shearing

↓ Safe stop

2 Post-shearing cleanup

↓ Safe stop

3 DNA repair & A-tailing

↓

4 Adapter ligation & pooling

↓

5 Pool cleanup

↓

6 Nuclease treatment

↓ Safe stop

7 Final cleanup

DNA quantification QC

Varioskan LUX multimode microplate reader (Thermo Fisher Scientific)

Varioskan LUX multimode microplate reader enables rapid, specific and accurate determination of nucleic acid concentrations in high-throughput workflows[1]

- Perform DNA concentration measurements using Quant-iT dsDNA assay[2] (Thermo Fisher Scientific)
- Quant-iT assay is well-adapted to high-throughput use, typically in 96-well or 384-well plates
- Quant-iT assay is highly selective for dsDNA over RNA, and in the range of 0.2–100 ng, the fluorescence signal is linear with the amount of DNA

[1] Alternatively, for lower-throughput applications DNA quantification QC may be performed without a microplate reader using the Qubit 1X dsDNA high sensitivity assay kit.

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 optimal results. This corresponds to a genome quality number (GQN)[3] of 7.0 or higher at 10 kb.

[2] See ***How can low DNA concentrations be reliably measured in microplate format?*** (Thermo Fisher Scientific SNLUXQuant-iT 0617). 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).

Pipette DNA shearing

Perform automated high-throughput shearing for HMW gDNA samples using Hamilton automation[1,2]

DNA sample extraction

↓

DNA sample QC

1 Pipette DNA shearing

↓ Safe stop

2 Post-shearing cleanup

↓ Safe stop

3 DNA repair & A-tailing

↓

4 Adapter ligation & pooling

↓

5 Pool cleanup

↓

6 Nuclease treatment

↓ Safe stop

7 Final cleanup

1. Pipette DNA shearing

✔	Step	Instructions
	1.1	Use Buffer LTE HT to bring all samples up to 300 µL total volume in a 0.8 mL, 96 DeepWell plate (Thermo Fisher Scientific AB0859). **The concentration of each sample must be <10 ng/µL.**
	1.2	Parameters for shearing on the Microlab Prep, or Hamilton assay-ready workstations. 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)

	1.3	Place the plate on the appropriate work deck position and start the shearing procedure.
	1.4	Optional: measure DNA concentration with a Qubit fluorometer using the 1x dsDNA HS kit once shearing procedure is complete. **Recommended:** Further dilute each aliquot to **250 pg/µL** with Femto Pulse dilution buffer. Measure the final SMRTbell library size distribution with a Femto Pulse system to ensure efficient shearing.
	1.5	Proceed to the 1X SMRTbell cleanup bead procedure to concentrate 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 Hamilton liquid handling instruments.

IMPORTANT!

- Please follow the exact shearing conditions outlined below for the automated DNA shearing method using Hamilton automation.
- Deviating from these conditions may result in undersheared or unsheared DNA.

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

[2] **Note:** If using a SPEX SamplePrep 1600 MiniG homogenizer or MP Bio FastPrep 96 homogenizer to shear lower-quality gDNA samples to a target fragment size <15 kb, follow the alternative equipment settings provided in ***Procedure & checklist – Preparing multiplexed whole genome and amplicon libraries using the HiFi plex prep kit 96*** (103-418-800).

Post-shearing cleanup

Perform post-shearing cleanup using 1X SMRTbell cleanup beads

DNA sample extraction

↓

DNA sample QC

1 Pipette DNA shearing

↓ Safe stop

2 Post-shearing cleanup

↓ Safe stop

3 DNA repair & A-tailing

↓

4 Adapter ligation & pooling

↓

5 Pool cleanup

↓

6 Nuclease treatment

↓ Safe stop

7 Final cleanup

2. Post-shearing cleanup

✔	Step	Instructions for SMRTbell cleanup bead step
	2.1	**Sheared genomic DNA**: add 300 µL (1.0X) of resuspended, room-temperature SMRTbell cleanup beads to each sample. **Amplicon DNA**: if clean-up or concentration is required for amplicons, add 1.0X (amplicons >3 kb) or 1.3X (amplicons <3 kb) (v/v) concentration of SMRTbell cleanup beads.
	2.2	Pipette-mix the sample until the beads are evenly distributed. If necessary, quick-spin the samples to collect liquid.
	2.3	Leave at **room temperature** for **10 minutes** to allow DNA to bind beads.
	2.4	Place samples on a magnet and allow beads to separate fully from the solution. The solution should be clear and beads pelleted to the magnet.
	2.5	Slowly pipette off the supernatant without disturbing the beads. Discard the supernatant.
	2.6	Slowly dispense **200 µL**, or enough to cover the beads, of **freshly prepared 80% ethanol** to each sample. After **30 seconds**, pipette off the 80% ethanol and discard.
	2.7	Repeat the previous step.
	2.8	Remove residual 80% ethanol: • Remove the samples from the magnet and quick-spin to collect liquid. • Place samples back on the magnet and wait until beads separate fully from the solution. • Carefully pipette-off the residual 80% ethanol without disturbing the bead pellet and discard.
	2.9	Remove samples from the magnet and immediately add **25.5 µL of elution buffer.** Resuspend by pipette mixing until beads are evenly distributed in the solution. Quick-spin samples if necessary to collect liquid.
	2.10	Leave samples at **room temperature** for **5 minutes** to elute DNA off beads.
	2.11	Place samples back on the magnet and allow beads to separate fully from the solution. The solution should be clear, and beads pelleted to the magnet before proceeding.
	2.12	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a new plate or tube strip. Discard old plate or tube with beads.

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 1.5 hrs prior to beginning

Note: Post-shear cleanup step should be performed on the **same day** as the library construction steps 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 the HiFi prep kit 96 and Hamilton NGS Microlab Star liquid handling system, the post-shear cleanup step should be performed on the **same day (Day 2)** 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 Pipette DNA shearing
- Safe stop
- 2 Post-shearing cleanup
- Safe stop
- **3 DNA repair & A-tailing**
- 4 Adapter ligation & pooling
- 5 Pool cleanup
- 6 Nuclease treatment
- Safe stop
- 7 Final cleanup

3. DNA repair & A-tailing

✔	Step	Instructions
	3.1	Prepare the appropriate volume of master mix with 15% overage using the per reaction volumes listed below.

Repair mix

Tube	Component	Volume
Purple	Repair buffer M96	4 µL
Blue	End repair mix M96	1 µL
Green	DNA repair mix M96	0.5 µL
	Total volume	**5.5 µL**

3.2	Slowly pipette-mix the **repair mix** and quick spin to collect liquid at the bottom of the tube. If bubbles form during mixing, pulse-spin to remove bubbles.
3.3	Add **5.5 µL** of the repair mix to each sample. The total reaction volume should be **30 µL**.
3.4	Pipette-mix the reactions and quick-spin to collect liquid at the bottom of the well.
3.5	Run the Repair and A-tailing thermocycler program. Set lid temperature to 75°C if programmable.

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

3.6	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., 24-plex, 48-plex, 72-plex or 96-plex[1]), plus 15% overage

- Run **Repair and A-tailing** on-deck thermal cycler program
- Set the lid temperature to 75°C

PacBio

[1] HiFi plex prep kit 96 contains a sufficient volume of reagents to support 4 automated runs using a minimum batch size of 24 samples per run.

Adapter ligation, termination & pooling

In this step, ligate SMRTbell indexed adapter to the ends of each DNA fragment, stop the ligation reaction and then pool indexed samples together

DNA sample extraction

↓

DNA sample QC

1 Pipette DNA shearing

↓ Safe stop

2 Post-shearing cleanup

↓ Safe stop

3 DNA repair & A-tailing

↓

4 Adapter ligation & pooling

↓

5 Pool cleanup

↓

6 Nuclease treatment

↓ Safe stop

7 Final cleanup

4. Adapter ligation, termination & pooling

✔	Step	Instructions for adapter index ligation
	4.1	Add **4 µL** of indexed adapter to each sample from the previous step.

Component	Volume
SMRTbell adapter index plate 96 (A, B, C, or D)	4 µL

	Step	Instructions for adapter index ligation
	4.2	Prepare the appropriate volume of master mix using the per reaction volumes listed below. Add 10% overage if preparing the reactions manually.

Ligation mix

Tube	Component	Volume
Yellow	Ligation mix M96	10 µL
Red	Ligation enhancer M96	0.5 µL
	Total volume	**10.5 µL**

	4.3	Pipette-mix the ligation mix and quick-spin to collect liquid.
	4.4	Add **10.5 µL** of ligation mix to each sample from previous step. The total volume should be **44.5 µL.**
	4.5	Pipette-mix each sample thoroughly to ensure the mix is evenly distributed.
	4.6	Quick-spin to collect liquid.
	4.7	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

Proceed to the next step to terminate the ligation reaction prior to pooling.

	4.8	Terminate the ligation reaction by adding **15.5 µL** of **Stop Solution M96** to each sample. Pipette mix thoroughly.
	4.9	Pool **40 µL** from up to 24 reactions into a **2 mL LoBind tube**. The total pooled volume for 24 samples should be **960µL**. If processing 96 samples, use four 2 mL LoBind tubes.
	4.10	Proceed to the next step of the protocol.

- Add 4 µL of indexed adapter to each sample from the previous step.
- Any of the adapters from the four SMRTbell adapter index plates can be used (e.g. 96A, 96B, 96C, or 96D)

- 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., 24-plex, 48-plex, 72-plex or 96-plex[1]), plus 10% overage

- Run **Adapter ligation** on-deck thermal cycler program
- Set the lid temperature to 75°C

- **Terminate** ligation reaction by adding 15.5 µL of Stop Solution M96 to each sample and mixing thoroughly

- **Pool** 40 µL from up to 24 reactions into a 2 mL LoBind tube (total pooled volume for 24 samples = 960 µL)
- If processing 96 samples, use four 2 mL LoBind tubes

[1] HiFi plex prep kit 96 contains a sufficient volume of reagents to support 4 automated runs using a minimum batch size of 24 samples per run.

Pool cleanup

Perform 1X SMRTbell cleanup bead purification of pooled libraries

- DNA sample extraction
- DNA sample QC
- 1 Pipette DNA shearing
- Safe stop
- 2 Post-shearing cleanup
- Safe stop
- 3 DNA repair & A-tailing
- 4 Adapter ligation & pooling
- **5 Pool cleanup**
- 6 Nuclease treatment
- Safe stop
- 7 Final cleanup

5. SMRTbell cleanup bead purification of pooled libraries

✔	Step	Instructions
	5.1	Add **960 µL** (1.0X) of resuspended, room-temperature SMRTbell cleanup beads to each pool. If using less than 960 µL, add 1.0X (v/v) concentration of SMRTbell cleanup beads.
	5.2	Pipette-mix or invert the sample until the beads are evenly distributed. Quick-spin the samples to collect liquid.
	5.3	Leave at **room temperature** for **10 minutes** to allow DNA to bind beads.
	5.4	Place samples on a magnet and allow beads to separate fully from the solution. The solution should be clear and beads pelleted to the magnet.
	5.5	Slowly pipette off the supernatant without disturbing the beads. Discard the supernatant.
	5.6	Slowly dispense 200 µL, or enough to cover the beads, of freshly prepared 80% ethanol to each sample. After 30 seconds, pipette off the 80% ethanol and discard.
	5.7	Repeat the previous step.
	5.8	Remove residual 80% ethanol: • Remove the samples from the magnet and quick-spin to collect liquid. • Place samples back on the magnet and wait until beads separate fully from the solution. • Carefully pipette off the residual 80% ethanol without disturbing the bead pellet and discard.
	5.9	Remove samples from the magnet and immediately **add 40 µL of elution buffer.** Resuspend by pipetting mixing until beads are evenly distributed in solution. Quick-spin samples if necessary to collect liquid.
	5.10	Leave samples at **room temperature** for **5 minutes** to elute DNA off beads.
	5.11	Place samples back on the magnet and allow beads to separate fully from the solution. The solution should be clear, and beads pelleted to the magnet before proceeding.
	5.12	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a new tube strip. Discard the old tube with beads.
	5.13	Proceed to the next step (nuclease treatment), or store samples at 4°C.

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 1.5 hours prior to beginning

PacBio

Nuclease treatment

Remove unligated DNA fragments and leftover SMRTbell adapters from the sample

- DNA sample extraction
- DNA sample QC
- 1 Pipette DNA shearing
- Safe stop
- 2 Post-shearing cleanup
- Safe stop
- 3 DNA repair & A-tailing
- 4 Adapter ligation & pooling
- 5 Pool cleanup
- 6 Nuclease treatment
- Safe stop
- 7 Final cleanup

6. Nuclease treatment

✔	Step	Instructions for nuclease treatment
	6.1	Add the following components in the order and volume listed below to each of the pools from the previous step.

Nuclease mix

Tube	Component	Volume
Light purple	Nuclease buffer M96	5 µL
Light green	Nuclease mix M96	5 µL
	Total volume	**10 µL**

6.2	Pipette-mix and spin down to collect liquid. The total volume should equal **50 µL**.
6.3	Run the **Nuclease treatment** thermocycler program. Set lid temperature to 75°C if programmable.

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

- For Nuclease treatment reaction, each sample requires 10 µL of **Nuclease mix** solution
- Prepare the appropriate volume of Nuclease mix solution required to process all samples by adding 1 volume of **Nuclease buffer M96** and 1 volume of **Nuclease mix M96** to a new tube[1]

- Run **Nuclease treatment** thermocycler program
- Set the lid temperature to 75°C (if lid temp. is not programmable, leave at 95–105°C)

[1] HiFi plex prep kit 96 contains a sufficient volume of reagents to support 4 automated runs using a minimum batch size of 24 samples per run.

Final cleanup

Perform SMRTbell cleanup bead purification of final libraries

- DNA sample extraction
- DNA sample QC
- 1 Pipette DNA shearing
- Safe stop
- 2 Post-shearing cleanup
- Safe stop
- 3 DNA repair & A-tailing
- 4 Adapter ligation & pooling
- 5 Pool cleanup
- 6 Nuclease treatment
- Safe stop
- **7 Final cleanup**

7. SMRTbell cleanup bead purification of final libraries

✔	Step	Instructions
	7.1	For **DNA >3 kb, add 50 µL (1.0X)** of resuspended, room-temperature SMRTbell cleanup beads to each sample. **For DNA <3kb, add 65 µL (1.3x)** of resuspended, room-temperature SMRTbell cleanup beads to the sample.
	7.2	Pipette-mix the sample until the beads are evenly distributed. Quick spin the samples to collect liquid.
	7.3	Leave at **room temperature** for **10 minutes** to allow DNA to bind beads.
	7.4	Place samples on a magnet and allow beads to separate fully from the solution. The solution should be clear and beads pelleted to the magnet.
	7.5	Slowly pipette off the supernatant without disturbing the beads. Discard the supernatant.
	7.10	Leave samples at **room temperature** for **5 minutes** to elute DNA off beads.
	7.11	Place samples back on the magnet and allow beads to separate fully from the solution. The solution should be clear, and beads pelleted to the magnet before proceeding.
	7.12	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant t… new tube strip. Discard tube with beads.
	7.13	**Recommended:** quality control checkpoint. Use 1 µL from each pool to measure the concentration and size distribution of the completed library.
	7.14	Please note: elutions from the bead cleanup can be pooled together at this stage prior to proceeding to ABC in SMRT® Link Sample Setup. The input library concentration into ABC must be <60 ng/µL for >10 kb libraries, <20 ng/µL for 3–10 kb libraries, and <10 ng/µL for <3 kb libraries. Failure to dilute library prior to ABC may result in low loading.
		PROTOCOL COMPLETE

Note: To prepare HiFi plex prep kit 96 samples for sequencing on PacBio long-read systems, follow annealing, binding & cleanup (ABC) workflow instructions provided in **SMRT Link Sample Setup** calculator tool

- **For DNA >3 kb**
  - → Add 50 µL (1.0X) of resuspended, room-temperature SMRTbell cleanup beads to sample
- **For DNA <3kb**
  - → Add 65 µL (1.3x) of resuspended, room-temperature SMRTbell cleanup beads to sample

- Perform **DNA concentration QC** on final purified HiFi plex prep kit 96 SMRTbell library using a Qubit dsDNA HS assay or Quant-iT ds DNA HS assay
- Perform **DNA sizing QC** on final purified HiFi plex prep kit 96 SMRTbell library using a Femto Pulse system

Varioskan LUX multimode microplate reader (Thermo Fisher Scientific)

Femto Pulse system (Agilent Technologies)

- **Note:** Final HiFi plex prep kit 96 SMRTbell library concentration must be **<60 ng/µL for >10 kb; <20 ng/µL for 3-10 kb** and **<10 ng/L for <3 kb** to proceed with ABC
  - → Failure to dilute libraries to the recommended concentration range will result in lower *P1* loading during sequencing

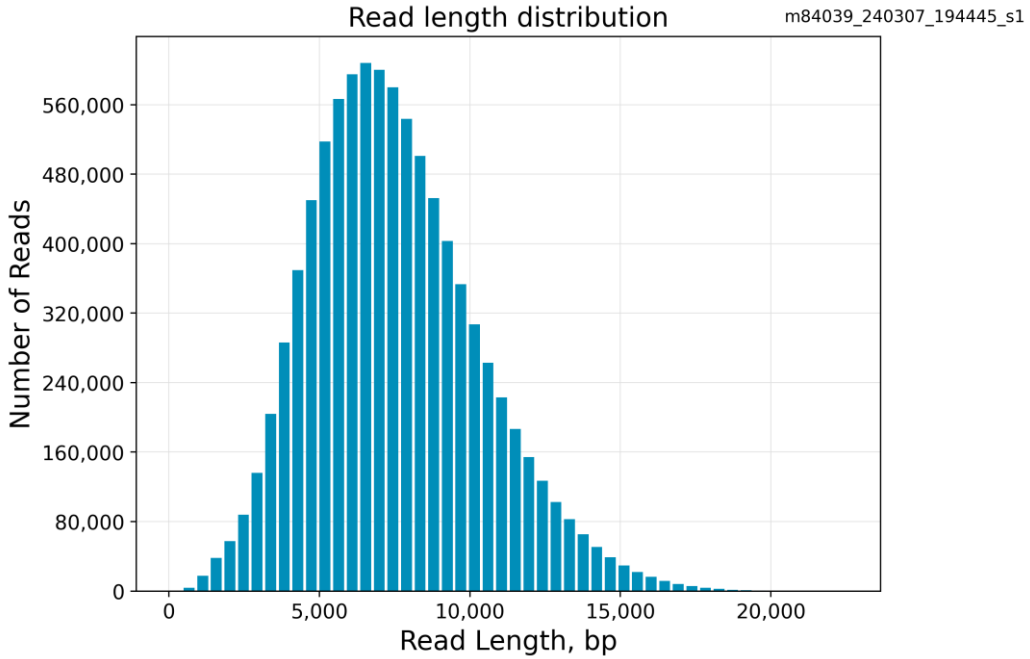
PacBio

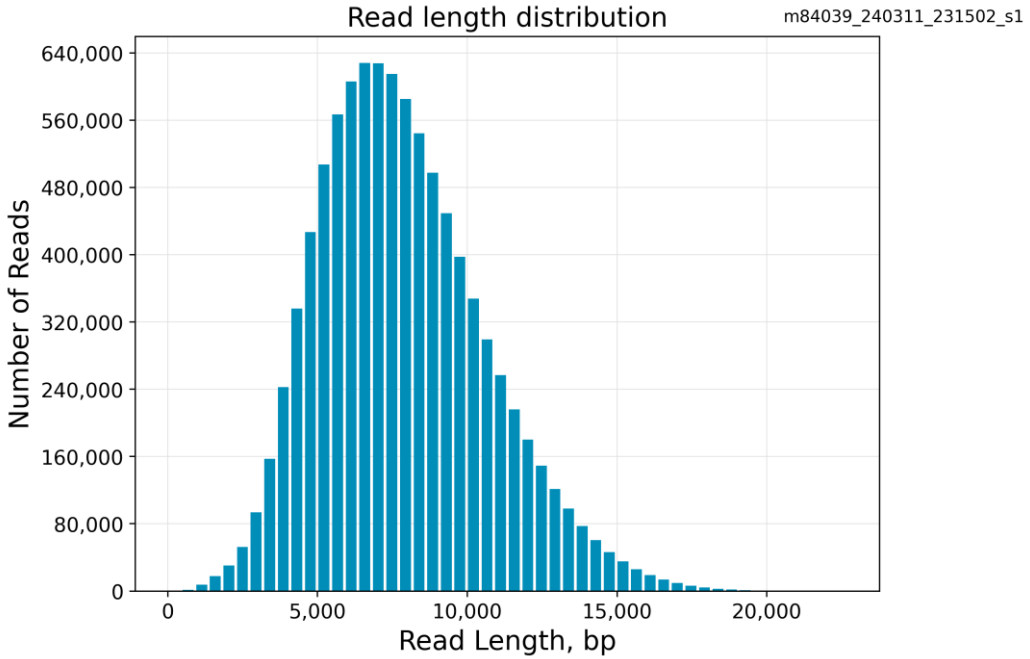
HiFi plex prep kit 96 example sequencing performance data

HiFi sequencing performance of HiFi plex prep kit 96 libraries

Example HiFi sequencing performance obtained for HiFi plex prep kit 96 libraries constructed from *E. coli* gDNA sheared using SPEX SamplePrep *1600 MiniG* homogenizer[1]

Sample[2]	Polymerase RL (bp)	Yield (Gb)	HiFi Mean Length (bp)	Mean QV	*P1* %
96_plex E.coli _Cell 1 (250 pM)	81,529	67.8	7,477	Q45	61
96_plex E.coli_Cell 2 (250 pM)	96,977	73.1	7,810	Q46	59





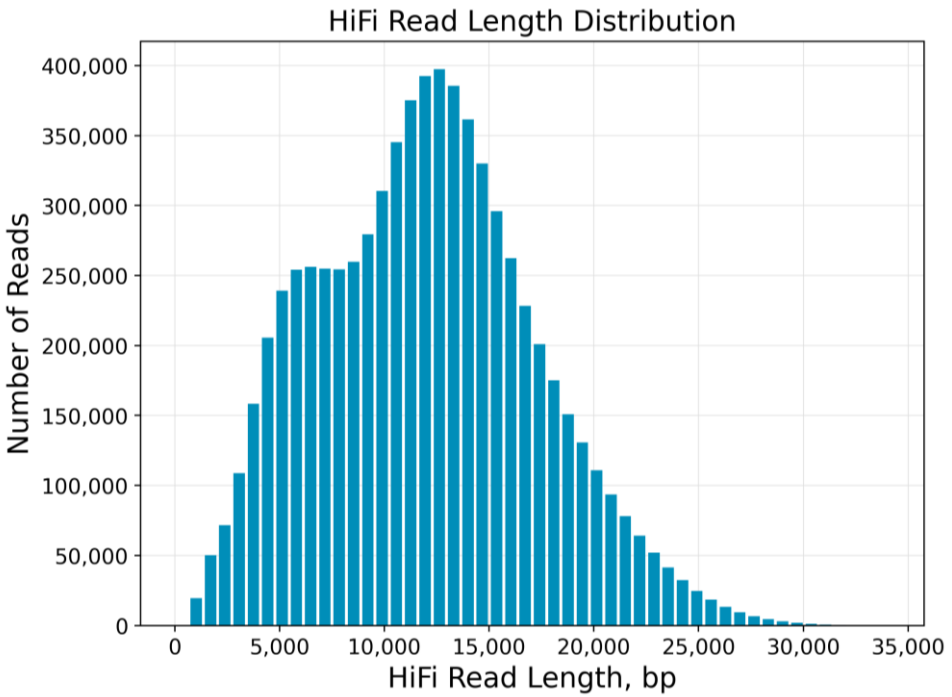
[1] Revio system data shown. **Note:** HiFi read lengths, reads/data per SMRT Cell and other sequencing performance results can vary depending on DNA sample quality, insert size, *P1* loading performance & movie time. Shorter library insert sizes (<15 kb), lower DNA quality samples, and suboptimal *P1* loading performance may result in HiFi data yields <90 Gb per Revio SMRT Cell.

[2] Samples were sequenced on a Revio system using 250 pM OPLC with 24-hours movie time.

HiFi sequencing performance of HiFi plex prep kit 96 libraries (cont.)

Example HiFi sequencing performance obtained for HiFi plex prep kit 96 libraries constructed from microbial gDNA samples sheared using Hamilton NGS STAR workstation[1]

Metric	Result
HiFi reads	7.3 M
HiFi reads yield	**87.64 Gb**
HiFi read length (mean)	11.99 kb
HiFi read length (N50)	**14,033 kb**
Median read quality	Q40
Base quality ≥Q30	**94.57%**
Barcodes detected	**96**
Barcoded HiFi reads %	**99.81%**



- HMW DNA isolated from 96 different microbial isolates and sheared on Hamilton NGS STAR
- All samples prepared with HiFi plex prep kit 96 with no size selection used
- Single 96-plex sample pool was processed through ABC workflow with Revio polymerase kit
- Sequenced on Revio system using 200 pM on-plate loading concentration with 24 hrs movie time

[1] Revio system data shown. **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.

Technical documentation & applications support resources

Technical resources for high-throughput HiFi library preparation, sequencing & data analysis

DNA extraction literature & other resources

- Guide & overview – Nanobind HT kits for automated HMW DNA extraction (103-028-100)
- Guide & overview – Nanobind PanDNA kit (103-394-800)
- Technical note – High-throughput DNA extraction (102-326-611)
- Technical overview – Automated high-throughput HMW DNA extraction for PacBio long-read sequencing using Nanobind HT kits (103-401-700)

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)

HiFi SMRTbell library preparation literature & other resources

- Brochure – HiFi prep kits (102-326-608)
- Guide & overview – Automated HiFi plex prep 96 for the Hamilton NGS Microlab STAR system (103-425-800)
- Guide & overview – Automated HiFi prep 96 and HiFi annealing, binding, and cleanup for the Hamilton NGS Microlab STAR system (103-425-700)
- Procedure & checklist – Preparing multiplexed whole genome and amplicon libraries using the HiFi plex prep kit 96 (103-418-800)
- Procedure & checklist – Preparing whole genome libraries using the HiFi prep kit 96 (103-420-700)
- Procedure & checklist – Preparing whole genome and metagenome sequencing libraries using SMRTbell prep kit 3.0 (102-166-600)
- Technical Overview – Technical overview – HiFi library preparation using HiFi prep kits for high-throughput sequencing on PacBio long-read systems (103-424-600)

Technical resources for high-throughput HiFi library preparation, sequencing & data analysis (cont.)

Third-party automation instrumentation literature

- 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)

HiFi data analysis literature & other resources

- SMRT Link software installation guide [Link]
- SMRT Link user guide [Link]
- SMRT Tools reference guide [Link]

HiFi WGS applications literature & other resources

- Application note – Consolidated analysis tools with the PacBio WGS Variant Pipeline (102-326-588)
- Application note – Robust detection of somatic variants from tumor-normal samples with highly accurate long-read whole genome sequencing (102-326-582)
- Overview – Human genomics: Unlock your next great discovery with HiFi sequencing (102-326-536)

Webinars

- PacBio webinar (2024) – More samples, lower costs, less time: New PacBio HIFI prep kits + microbial WGS and antimicrobial resistance [Link]

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