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

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, up to and including 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	
Average time with automation	6 hours (Manual workflow times prior to pooling will vary by user and sample volume)	
	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).

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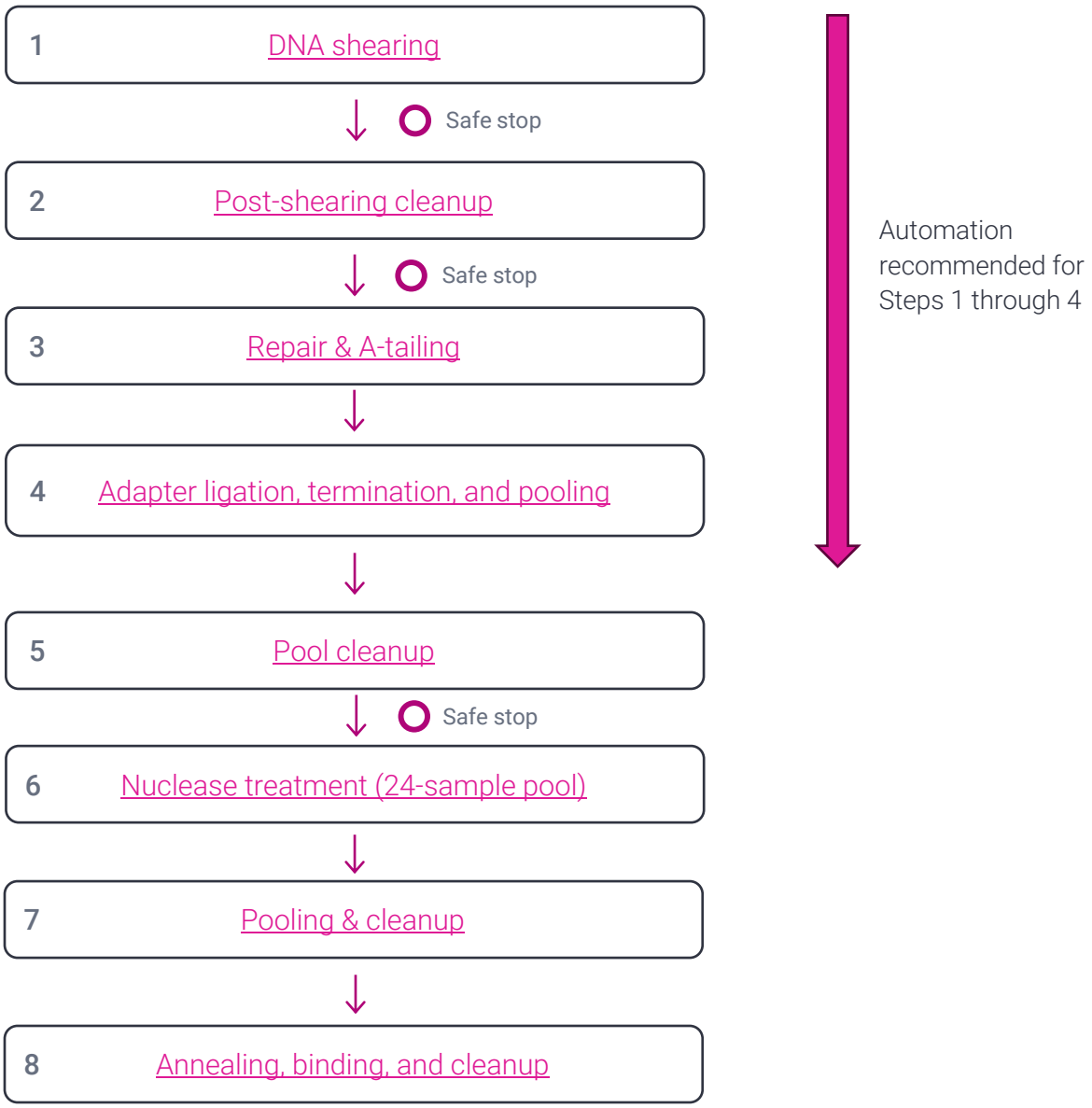
Workflow



Required materials and equipment

DNA QC sizing	
Femto Pulse system	Agilent Technologies, Inc. M5330AA
Femto Pulse gDNA 165kb analysis kit	Agilent Technologies, Inc. FP-1002-0275
DNA QC quantitation	
Qubit fluorometer	ThermoFisher Scientific Q33238
Qubit 1X dsDNA HS assay kit	ThermoFisher Scientific Q33230
DNA shearing	
Hamilton Microlab Prep	PacBio® 103-283-600
Hamilton assay ready workstation	Contact Hamilton
300 µL CO-RE II Tips (Filtered, Conductive)	Hamilton, 235903
Library preparation	
HiFi plex prep kit 96, includes: • HiFi plex prep kit 96 • SMRTbell cleanup beads—52 mL • Buffer LTE HT • Elution buffer	PacBio® 103-381-300
SMRTbell® adapter index plate 96 (A, B, C, or D)	Plate A – PacBio® 102-009-200 Plate B – PacBio® 102-547-800 Plate C – PacBio® 102-547-900 Plate D – PacBio® 102-548-000
Revio® SPRQ™ polymerase kit or Vega™ polymerase kit or Revio® polymerase kit* or Sequel® II binding kit 3.2*	PacBio® 103-520-100 PacBio® 103-517-600 PacBio® 102-817-600 PacBio® 102-333-300

* Procedure for Revio polymerase kit (non-SPRQ) and Sequel II binding kit 3.2 can be found in SMRT® Link Sample Setup.

Other supplies	
200 Proof ethanol, molecular biology or ACS grade	Any major lab supplier (MLS)
Nuclease-free water, molecular biology grade	Any MLS
Thermocycler	Any MLS
0.2 mL 8-tube strips	USA Scientific TempAssure 1402-4708

Magnetic bead rack for PCR tubes or plates	Any MLS
2 mL DNA LoBind tubes	Eppendorf 022431048
Hard-shell 96-Well PCR Plates, low profile, thin wall, skirted	Bio-Rad HSP9601
Abgene 96 Well 0.8mL Polypropylene Deepwell Plate	ThermoFisher Scientific, AB0859
Magnetic bead rack	ThermoFisher Scientific 12321D

Before you begin

Automation

The HiFi plex prep kit 96 and its 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. Because of differences between automation instruments, modifications not described herein may be needed to be adapted to the protocol relevant to your specific instrumentation. Please visit the PacBio WGS page or contact your local support team for a list of instruments with qualified PacBio methods.

This protocol was developed using the Hamilton NGS STAR MOA system. Please refer to the Guide & overview for HiFi plex prep kit 96 for step-by-step instructions for running this protocol on the NGS STAR MOA system.

The HiFi plex prep kit 96 contains enough fill volume to support up to 4 x 24 sample automated runs. If setting up fewer than 24 samples, there may not be enough volume to support 96 samples due to automation dead volume requirements.

Genomic DNA (gDNA) QC and input amount recommendations

PacBio Nanobind® DNA extraction kits are recommended to ensure sufficient mass and quality of high molecular weight DNA for this protocol.

gDNA quality QC

The Agilent Femto Pulse system is highly recommended for the accurate sizing of gDNA. Please see the PacBio Technical note for more details.

Recommended guidelines for evaluating gDNA quality for this protocol:

- Use the Femto Pulse gDNA 165 kb analysis kit (Agilent FP-1002-0275)
- Dilute samples to 250 pg/µL
- 70% or more of the DNA should be 10 kb for best results. This corresponds to a genome quality number (GQN) of 7.0 or higher at 10 kb.
- Shearing may be bypassed if the sample is in the appropriate size-range.

Important:

The HiFi yield and HiFi mean read length of a sequencing run is directly proportional to the quality of the genomic DNA input and the fragment lengths generated after shearing. In general, the better the quality of gDNA going into the protocol, the higher the HiFi sequencing yield.

Please see the Revio spec sheet for more information on yield expectations by insert size.

gDNA and amplicon input amount

It is highly recommended to use a quantification assay specific for double stranded DNA (dsDNA) such as the Qubit dsDNA high sensitivity assays. Please follow manufacturer instructions for the assay being used.

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

Remove all RNA from genomic DNA samples prior to beginning. Residual RNA can inhibit sequencing polymerase binding and can therefore lead to low loading.

The following input masses are recommended for library preparation:

gDNA	Amplicons
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.
- At least 24 samples need to be used when using lower input amounts (e.g., 50 ng).
- Samples must be pooled after ligation.

HiFi plex prep kit 96 stepwise recoveries

Expected DNA and SMRTbell library recovery after each protocol step. The overall yield applies to both an individual sample and to the cumulative mass of all samples being prepared.

Protocol step	DNA or SMRTbell recovery
Starting Input	100%
Post-shear SMRTbell bead cleanup	80–95%
Post ligation termination pooling (40/60 µL pooled)	67%
Post-ligation SMRTbell bead cleanup	80–95%*
Post-nuclease (pre-cleanup)	25–35%
Post-nuclease SMRTbell bead cleanup recovery	80–95%
Post-ABC SMRTbell bead cleanup recovery	80–95%
Overall Recovery	**7–19%**

*This can vary based on extraction methods. As low as 60% step recovery has been observed.

Multiplexing

The procedure requires one of four available SMRTbell adapter index plates:

- SMRTbell adapter index plate 96A
- SMRTbell adapter index plate 96B
- SMRTbell adapter index plate 96C
- SMRTbell adapter index plate 96D

To balance the number of reads per sample, please 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 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 SMRT Cell. A total of 1,536 (4 cells x 384 samples) can be sequenced on a single Revio™ run.

DNA shearing

This protocol recommends shearing gDNA using automated liquid handler systems to a size between 13–20 kb.

For more details, please refer to the Microlab Prep Guide & overview for details on consumables.

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 below <15 kb, we recommend the following equipment:

SPEX SamplePrep *1600 MiniG* homogenizer:

- Speed = 1500 RPM
- Time = 3 minutes
- DNA input = 300 ng – 3µg
- Volume = Up to 300 µL

For more details, please see the PacBio Technical note describing experimental conditions for shearing using the 1600 MiniG.

FastPrep96 (contact MP Bio for latest protocol on shearing for microbial samples). PacBio recommendations below. Conditions may need to be adjusted based on DNA input and volume used.

- Speed = 1800 RPM
- Time = 60 seconds
- DNA input = 300 ng
- Volume = 50 µL

Reagent and sample handling

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

SMRTbell cleanup beads

Bring SMRTbell cleanup beads to room temperature for at least 1.5 hours prior to use. Alternatively, beads can be left out overnight if being used the next morning.

Vortex or resuspend *immediately* before any addition to sample. Failure to do this will result in low recovery.

Pipette-mix all bead binding and elution steps until beads are distributed evenly in solution.

HiFi plex prep kit 96

Thaw the repair buffer M96, nuclease buffer M96, index plate, and stop solution M96 at room temperature. Once thawed, place on ice.

Quick-spin all reagents in a microcentrifuge to collect liquid at bottom prior to use.

Keep all temperature-sensitive reagents on ice.

Temperature-sensitive reagents		
Step used	**Tube color**	**Reagent**
Repair and A-tailing	Blue	End repair mix M96
	Green	DNA repair mix M96
Adapter ligation	Yellow	Ligation mix M96
	Red	Ligation enhancer M96
Nuclease treatment	Light green	Nuclease mix M96

Bring 1X dsDNA HS reagents to room temperature for 30 minutes prior to use.

Samples can be stored at 4°C at all safe stopping points listed in the protocol.

Anneal, bind, and cleanup

Thaw the following reagents at room temperature:

Component	Tube color
Annealing buffer	Light blue
Standard sequencing primer	Light green
Polymerase buffer	Yellow
Loading buffer	Green
Dilution buffer	Blue

Once thawed, place reaction buffers and sequencing primer on-ice prior to making master mix. The Loading buffer should be left at room-temperature.

Note: The Loading buffer is light sensitive and should be protected from light when not in use.

Keep the following reagents on a cold block or ice:

- Sequencing polymerase
- Sequencing control

Bring the following reagents up to room temperature 30 minutes prior to use:

- Loading buffer
- SMRTbell cleanup beads

Safety precautions

Refer to the Safety Data Sheet (SDS) for information on reagent hazards and protocols for safe handling, use, storage, and disposal.

Procedure and checklist

1. Automated DNA shearing for WGS using Hamilton automation

If starting with amplicon DNA, skip this step and proceed to the post-shear cleanup step (if cleanup is required) or the Repair and A-tailing step.

This protocol utilizes the Hamilton Microlab Prep or Hamilton NGS STAR MOA system for pipette shearing. For more details, please refer to the Microlab Prep Guide & overview for details on consumables.

Microbial and metagenomics samples may forgo shearing if the DNA is in the fragment length range of 7–18 kb. In such cases, proceed to the 1X SMRTbell cleanup bead step to get the appropriate input amount in the correct volume and buffer.

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.

Estimated time for this step is: 10 minutes for up to 96 samples using the Hamilton NGS STAR MOA system or 22 minutes for 24 samples using the Microlab Prep system.

<table>
<tr><th>✔</th><th>Step</th><th>Instructions</th></tr>
<tr><td></td><td>1.1</td><td>Use Buffer LTE HT to bring samples up to 300 µL (<300 ng gDNA mass, <1 ng/µL) in a 0.8 mL, 96 DeepWell plate (Thermo Fisher Scientific AB0859). A range of 50–300 ng gDNA per sample is recommended for pipette shearing and library preparation.</td></tr>
<tr><td></td><td>1.2</td><td>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.
<table>
<tr><th>Parameter</th><th></th></tr>
<tr><td>DNA concentration</td><td>≤1 ng/µL*</td></tr>
<tr><td>Volume of Buffer LTE</td><td>300 µL</td></tr>
<tr><td>Number of mixes</td><td>300 cycles</td></tr>
<tr><td>Pipette mixing speed</td><td>500 µL/sec</td></tr>
<tr><td>Liquid following</td><td>83% volume</td></tr>
<tr><td>Pipette tip</td><td>300 µL CO-RE II tips (filtered, black, non-sterile)</td></tr>
</table>
*DNA shearing is effective at ≤ 10 ng/µL; however, the maximum recommended input into library prep is 300 ng gDNA.</td></tr>
<tr><td></td><td>1.3</td><td>Place the plate on the appropriate work deck position and start the shearing procedure.</td></tr>
<tr><td></td><td>1.4</td><td>Optional: measure DNA concentration with a Qubit fluorometer using the 1x dsDNA HS kit once shearing procedure is complete.</td></tr>
</table>

	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.

2. Post-shearing cleanup

This step concentrates the sheared gDNA or amplicon samples for the Repair and A-tailing step. Before beginning, ensure the SMRTbell cleanup beads are at room temperature.

✔	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 a bead clean-up or concentration step 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	Incubate 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 remove 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**, remove 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 **24.5 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	Incubate 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 aspirate the cleared eluate without disturbing the beads. Transfer eluate to a new plate or tube strip. Discard old plate or tube with beads.

3. Repair and A-tailing

This step repairs sites of DNA damage and prepares the sheared DNA for ligation to the SMRTbell adapter.

✔	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.

4. Adapter ligation, termination, and pooling

This step ligates the adapter index, stops the ligation reaction, and pools samples together.

✔	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

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 master mix** and quick-spin to collect liquid.
4.4	Add **10.5 µL** of **Ligation master 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 **960 µL**. If processing 96 samples, use four 2 mL LoBind tubes.
4.10	Proceed to the next step of the protocol.

5. SMRTbell cleanup bead purification of pooled libraries

Bring SMRTbell cleanup beads to room temperature prior to the purification step.

✔	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	Incubate 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 remove the supernatant without disturbing the beads. Discard the supernatant.
5.6	Slowly dispense 2000 µL, or enough to cover the beads, of freshly prepared 80% ethanol to each sample. After 30 seconds, remove 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 remove 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	Incubate 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 aspirate the cleared eluate without disturbing the beads. Transfer eluate 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

6. Nuclease treatment

This step removes unligated or damaged DNA from the library to improve sequencing performance.

✔	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

7. Pooling (optional) and SMRTbell cleanup bead purification

Bring the SMRTbell cleanup beads to room temperature prior to the purification step.

Pool the completed nuclease reactions based on the number of SMRTcells to be run. For example, if 96 samples will be sequenced on a single SMRTcell, pool four 24-sample nuclease reactions prior to SMRTbell cleanup.

Nuclease reactions pooled	Total volume	SMRTbell cleanup bead volume for 1.0x	SMRTbell cleanup bead volume for 1.3x	Elution volume
1	50 µL	50 µL	65 µL	25 µL
2	100 µL	100 µL	130 µL	25 µL
3	150 µL	150 µL	195 µL	25 µL
4	200 µL	200 µL	260 µL	25 µL

✔	Step	Instructions
	7.1	For **DNA >3 kb, add 50–200 µL** (**1.0X,** see table above) of resuspended, room-temperature SMRTbell cleanup beads to each sample. For **DNA <3 kb, add 65 µL–260** (**1.3x,** see table above) 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	Incubate 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 remove the supernatant without disturbing the beads. Discard the supernatant.
	7.6	Slowly dispense 200 µL, or enough to cover the beads, of freshly prepared 80% ethanol to each sample. After 30 seconds, remove the 80% ethanol and discard.

7.7	Repeat the previous step.
7.8	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 remove the residual 80% ethanol without disturbing the bead pellet and discard.
7.9	Remove samples from the magnet and immediately **add 26 µL of elution buffer.** Resuspend by pipette mixing until beads are evenly distributed in solution. Quick-spin samples if necessary to collect liquid.
7.10	Incubate 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 aspirate the cleared eluate without disturbing the beads. Transfer eluate to a 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	**If necessary, dilute 25 µL of SMRTbell library to the concentrations indicated below**. Failure to normalize libraries to the appropriate concentration prior to ABC may result in low sequencing yield. **Proceed to Section 8 to prepare library for sequencing on the Vega or Revio (+SPRQ chemistry).** **or** **Proceed to SMRT Link Sample Setup for preparing samples for sequencing with Revio (non-SPRQ) or Sequel II/e.**

SMRTbell library size	Concentration (ng/µL)
>10 kb	<60 ng/µL
3–10 kb	<20 ng/µL
<3 kb	<10 ng/µL

SAFE STOPPING POINT - Store at 4°C for up to 1 month or -20°C for at least 6 months.

8. Annealing, binding, and cleanup (ABC)

This step is for preparing the libraries (25 µL) for sequencing on Revio or Vega systems. **The Polymerase kit used will depend on which sequencer or chemistry is being used (see below).**

Kit	PN
Revio SPRQ polymerase kit	103-520-100
Vega polymerase kit	103-517-600

✔	Step	Instructions
	8.1	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	12.5 µL
	Light green	Standard sequencing primer	12.5 µL
		Total volume	**25 µL**

	8.2	Pipette-mix the **Annealing mix** and quick spin to collect liquid.
	8.3	Add **25 µL** of the **Annealing mix** to each library. Total volume should equal **50 µL.**
	8.4	Pipette-mix each sample and quick spin to collect liquid.
	8.5	Incubate at room temperature for **15 minutes.**
	8.6	During primer incubation, prepare the polymerase dilution (see below) and store on ice.
	8.7	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	47 µL
	Purple	Sequencing polymerase	3 µL
		Total volume	**50 µL**

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

Post-binding cleanup with SMRTbell cleanup beads

8.13	For **DNA >3 kb, add 100 µL (1.0X)** of resuspended, room-temperature SMRTbell cleanup beads to each sample. **For DNA <3kb, add 130 µL (1.3x)** of resuspended, room-temperature SMRTbell cleanup beads to the sample.
8.14	Pipette-mix the beads until evenly distributed and quick-spin if necessary to collect all liquid from the sides of the tube.
8.15	Incubate at **room temperature** for **10 minutes** to allow DNA to bind beads
8.16	Place sample on an appropriate magnet and allow beads to separate fully from the solution
8.17	Slowly remove 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.
8.18	Remove sample from the magnet and **immediately** add **Loading Buffer** to each tube and resuspend the beads by pipette mixing.

	Revio SPRQ polymerase kit	Vega polymerase kit
Loading buffer	25 µL	50 µL

8.19	Quick-spin the samples to collect any liquid from the sides of the tube.
8.20	Incubate at **room temperature** for **15 minutes** to elute DNA
8.21	Place sample on magnet and allow beads to separate fully from the solution.
8.22	Slowly remove the cleared eluate without disturbing the beads. Transfer eluate to a **new tube**. Discard the old tube with beads
8.23	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.
8.24	Proceed to the **Loading Calculator** in SMRT Link v13.3 or higher to calculate the final dilution for adding the sample to the sequencing reagent plate. **The recommended loading concentration is 200 – 300 pM.**

PROTOCOL COMPLETE

Important: Polymerase-bound libraries can be stored at 4°C for up to 1 month, or at -20°C for at least 6 months prior to sequencing. Polymerase-bound libraries can withstand up to 4 freeze-thaw cycles. Note that the Loading buffer is light sensitive.

Revision history (description)	Version	Date
Initial release	01	March 2024
Updated to correct reference to the Hamilton NGS STAR MOA system, workflow time in overview table, ethanol volume in section 5 for post-ligation cleanup, and to include stepwise recovery table	02	April 2024
Updated for SPRQ chemistry and the Vega system	03	December 2024
Added ABC reagents to the Reagent handling section, clarified polymerase kit part numbers	04	June 2025

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