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 (manual time will vary by user and sample volume)	
	Hamilton NGS STAR MOA	**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)

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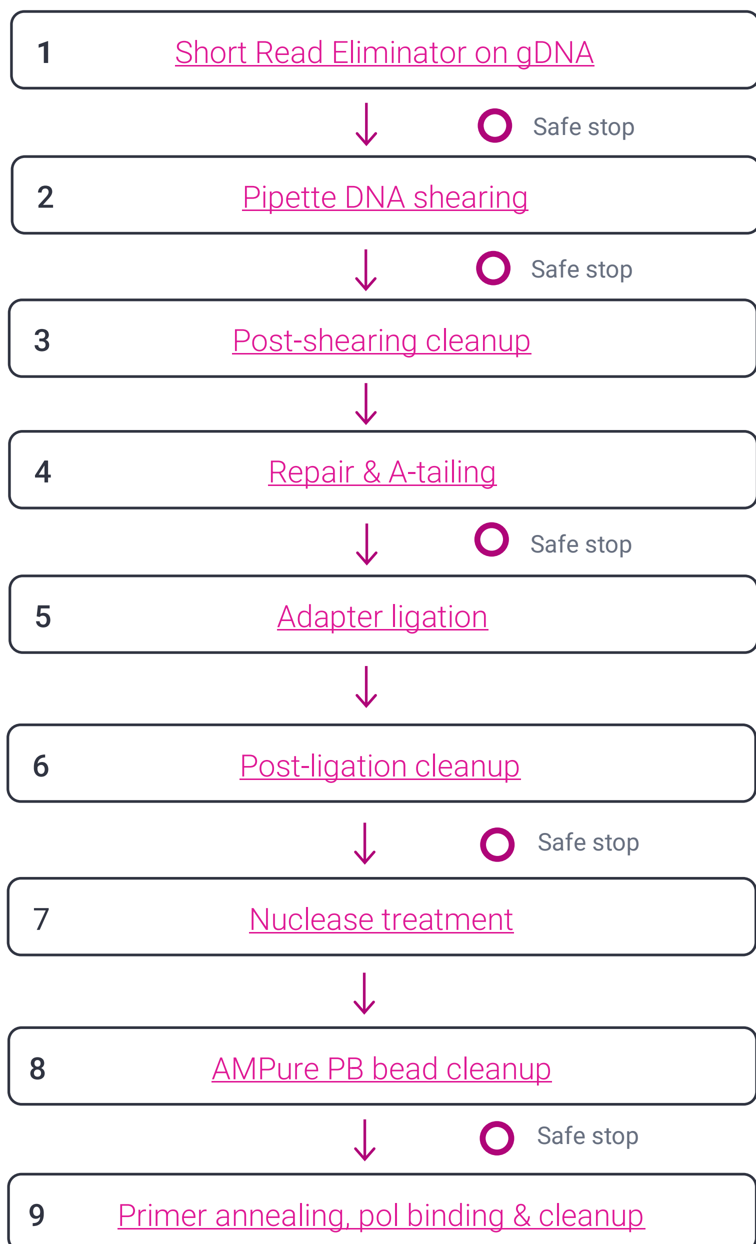
Workflow overview



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Required materials and equipment

DNA sizing	
Femto Pulse system	Agilent Technologies, Inc. M5330AA
Femto Pulse gDNA 165kb analysis kit	Agilent Technologies, Inc. FP-1002-0275
DNA quantitation (one or more of the following may be used)	
Qubit 4 Fluorometer	ThermoFisher Scientific Q33238
Qubit Flex Fluorometer (cannot be used for quantification of polymerase bound library)	ThermoFisher Scientific Q33327
Varioskan LUX multimode microplate reader	ThermoFisher Scientific VL0L00D0
Quant-iT 1X dsDNA HS assay kit (for Varioskan)	ThermoFisher Scientific Q33232
Qubit 1X dsDNA HS assay kit	ThermoFisher Scientific Q33230
DNA shearing (one of the following)	
Hamilton Microlab Prep	PacBio, 103-283-600
Hamilton assay ready workstation	Contact Hamilton
300 µL CO-RE II Tips (Filtered, Conductive)	Hamilton, 235903
HiFi library preparation for Revio	
Revio HiFi prep kit 96, includes: • SRE HT • HiFi prep kit 96 • SMRTbell® cleanup beads 85 mL • SMRTbell® adapter index plate 96A • AMPure® PB • Elution buffer • Revio polymerase kit 96	PacBio® 103-382-200
200 Proof ethanol, molecular biology or ACS grade	Any major lab supplier (MLS)
Nuclease-free water, molecular biology grade	Any MLS
Magnetic bead rack for PCR tubes or plates	Any MLS
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

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Thermocycler	Any MLS
1.5 mL DNA LoBind tubes	Eppendorf 022431021
0.2 mL 8-tube strips	USA Scientific TempAssure 1402-4708
Plate Centrifuge with 2,250 x *g* force capability (if using plate format)	Any MLS
MicroAmp Clear Adhesive Film (if using plate format)	ThermoFisher Scientific 00146104
ALPS 50 V-Manual Heat Sealer (if using plate format)	Thermo Scientific, AB-1443A
Easy Pierce Heat Sealing Foil (if using plate format)	Thermo Scientifc, AB-0757

Sequel® II/e customers, or those not using Short Read Eliminator or the Revio polymerase kit 96, should order HiFi prep kit 96, which will include:

- HiFi prep kit 96 (103-122-600)
- SMRTbell cleanup beads 85 mL (103-294-600)
- SMRTbell adapter index plate 96A (102-009-200)
- Elution buffer (101-633-500)
- Buffer LTE HT (103-633-500)

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Before you begin

Automation

The HiFi prep kit 96 and workflow were designed for NGS liquid handling automation. As a result, this protocol is intended to describe the SRE, shearing, library prep enzymatic reactions, and bead cleanups to guide automation method development, or in certain instances manual preparation. Because of differences between automation instruments, modifications not described herein may be needed to adapt the protocol to your specific instrumentation. Please visit the WGS page or contact your local support team for a list of instruments with a PacBio qualified method.

This protocol was developed using the Hamilton NGS STAR MOA system.

Genomic DNA (gDNA) QC and input amount recommendations

PacBio Nanobind® DNA extractions 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 this protocol. This corresponds to a genome quality number (GQN) of 7.0 or higher at 10 kb.

Important:

Because HiFi reads are single molecules of DNA, the total base yield and 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. To maximize yield and genome coverage per SMRT Cell, start with high quality gDNA containing little to no DNA below 10 kb, and with >50% mass over 30 kb. In general, the better the quality of gDNA going into the protocol, the higher the percent recovery and HiFi sequencing yield.

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

gDNA input amount

It is highly recommended to use a quantification assay specific for double stranded DNA (dsDNA) such as the Qubit 1X dsDNA high sensitivity assay kit. Alternatively, when a high number of samples will be prepared, we recommend using the Quant-iT 1X dsDNA high sensitivity assay kit with the Varioskan LUX multimode microplate reader. Please follow manufacturer’s instructions for the assay being used.

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.

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Table 1. Recommended DNA input amounts by starting gDNA quality

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

The overall recovery is dependent on gDNA quality and size. **The recovery from gDNA to completed SMRTbell library ranges between 10–25% (includes SRE, shearing, and SMRTbell library preparation).**

Starting with 2 µg of genomic DNA will typically provide enough library to load 1 Revio SMRT Cell (Table 2).

Important: The maximum mass tolerated by shearing and library enzymatic reactions is 3 µg.

Table 2. Polymerase-bound library mass necessary for loading on a Revio SMRT Cell.

Mean insert size	Library at 250 pM
15,000 bp	243 ng
18,000 bp	292 ng
21,000 bp	341 ng

If targeting higher insert sizes or working with lower quality DNA (Table 1), start with at least 3 µg of gDNA to ensure adequate library for optimal SMRT Cell loading.

HiFi prep kit 96 workflow stepwise expected recoveries

Table 3. Expected stepwise recoveries of DNA and SMRTbell library from the HiFi prep kit 96 protocol. Post-SRE recovery will vary with the quality of the DNA input. The better the quality of DNA, the higer the recovery post-SRE.

Protocol Step	DNA or SMRTbell yield
Starting Input	100%
Post-SRE	65-95%
Post-shear SMRTbell bead cleanup	80-95%
Post-ligation SMRTbell bead cleanup	80-95%
Post-nuclease (pre-cleanup)	40-50%
Post-3.1x AMPure PB bead cleanup	70-80%
Overall Recovery	**12-34%**

Multiplexing

All libraries constructed using this protocol will include a SMRTbell adapter index. Starting with SMRT Link v13.1, there will be a pooling calculator in Sample Setup to help determine the appropriate volumes to use for multiplexing libraries.

Prior to pooling HiFi libraries together please consider the following guidelines:

- Each Revio SMRT Cell is expected to yield ≥ 90 Gb of HiFi data, on average, when using a mean insert size >15 kb.
- Only pool samples with similar genome sizes to ensure balanced coverage.

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- Ensure that the samples to be pooled have a similar mean insert size and insert length size distribution.
- Pool samples in an equal molar concentration for best balanced coverage.

It is recommended to pool HiFi libraries post-ABC (annealing, binding, cleanup) for the following reasons:

- Ability to use only the amount of polymerase-bound library needed for that sequencing run and thus preserving un-pooled library for future sequencing runs.
- Ability to quickly pool different libraries together on additional runs to “top off” coverage.
- Prevent an inhibitor in one sample from affecting the polymerase binding of all samples in a pool.

Reagent handling

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

SRE HT kit

Buffer SRE and Buffer LTE are room temperature reagents.

HiFi prep kit 96

Thaw the Repair buffer 96, Nuclease buffer 96, and adapter index plate at room temperature. 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.

Keep the following temperature-sensitive reagents on ice.

Temperature-sensitive reagents HiFi prep kit 96		
Step used	**Tube**	**Reagent**
Repair and A-tailing	Blue	End repair 96
	Green	DNA repair 96
Adapter ligation	Yellow	Ligation mix 96
	Red	Ligation enhancer 96
Nuclease treatment	Light green	Nuclease mix 96

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

- AMPure PB beads
- Elution buffer
- dsDNA quantification reagents

Bring the following reagents to room temperature 1.5 hours prior to use (or the night before if starting protocol in the morning):

- SMRTbell cleanup beads 85 mL

Shake/vortex SMRTbell cleanup beads and AMPure PB beads immediately before use.

Briefly spin down all reagent tubes in a microcentrifuge to collect all liquid at the bottom.

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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.

Anneal, bind, and cleanup using the Revio polymerase kit 96

Thaw the following reagents at room temperature:

Component	Tube color
Annealing buffer 96	Light blue
Standard sequencing primer 96	Light green
Polymerase buffer 96	Yellow
Loading buffer 96	Green
Dilution buffer 96	Blue

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. The loading buffer 96 should be left at room-temperature.

Please note that the Loading buffer 96 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 96
- Sequencing control 96

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

- Loading buffer 96

Bring the following reagents up to room temperature 1.5 hours prior to use (or the night before if starting protocol in the morning):

- SMRTbell cleanup beads 85 mL

Polymerase-bound library stability

This protocol brings the entire library through the anneal, bind, and cleanup (ABC) steps. The sequencing polymerase is stable once bound to the HiFi library and can be stored at **4°C for 1 month** or at **-20°C for at least 6 months**. Stored polymerase-bound library shows equivalent loading to freshly prepared bound libraries up to the recommendations listed below.

Please note that the stored polymerase-bound library needs to be protected from light while stored.

Safety precautions

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

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Procedure and checklist

1. Short Read Eliminator

The Short read eliminator (SRE) will progressively deplete fragments up to 25 kb from genomic DNA samples. This means that depletion decreases as the fragment size approaches 25 kb. The depletion occurs in an unbiased manner and results in improved sample quality for HiFi sequencing with most fragments under 10 kb removed.

Important: Use SRE on genomic DNA only. Attempting to use SRE on HiFi libraries (following library construction) will result in poor recoveries and potential loss of the entire library.

Please note that this protocol may differ from the manual protocol for using the 24 rxn SRE kit (102-208-300).

Please refer to the NGS STAR MOA Guide & overview for the HiFi prep kit 96 or the Microlab Prep Guide & overview for details on consumables for automation.

Estimated automated time for this step (plate format): 3.5 hours. Estimated manual time for this step (tube format): 2.5 hours

✔	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).

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

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2. Automated DNA shearing for WGS using Hamilton robots

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 check with your local PacBio support team for updated information on all qualified DNA shearing methods. Estimated time for the shearing step is 10 minutes for 96 samples on the NGS STAR MOA system, or 22 minutes for 24 samples on the Microlab Prep.

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 is not recommended and will result in undersheared DNA.

These shearing parameters are not universal and are specific for only the Hamilton Microlab Prep, or assay ready workstations like the NGS STAR MOA, STARlet, and STAR V systems.

✔	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.

Step 2.2 parameters:

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)

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2.5	Proceed to the 1X SMRTbell cleanup bead procedure to concentrate samples for library preparation.

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3. Post-shearing cleanup

Estimated time for this step is 1.5 hours when processing 96 samples using the NGS STAR MOA system or 24 samples on the Microlab Prep. Times will vary when preparing samples manually. This step concentrates the DNA for library prep.

Important: Please allow the 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. If performing the cleanup in the morning, beads may be left out at room temperature overnight.

✔	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.

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	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.

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4. Repair and A-tailing

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

✔	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.
	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.
	4.6	Proceed directly to the next step of the protocol.

Repair mix (step 4.1)

✔	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

Repair and A-tailing program (step 4.5)

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

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5. Adapter ligation

This step ligates the SMRTbell adapter to the ends of each DNA fragment.

✔	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**

✔	Step	Instructions for adapter ligation
	5.3	Pipette mix the **ligation mix** and quick spin to collect liquid.
	5.4	Add **21 µL** of **ligation mix** to each sample. Total reaction volume should be **85 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

✔	Step	Instructions for adapter ligation
	5.7	Proceed to post-ligation cleanup step

6. Post-ligation cleanup

Please ensure SMRTbell cleanup beads have been brought up to room temperature before proceeding to the cleanup steps.

✔	Step	Instructions for SMRTbell cleanup bead step
	6.1	Add 85 µL (1.0X) of resuspended, room-temperature SMRTbell cleanup beads to each sample.

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6.2	Pipette mix the sample until the beads are evenly distributed.
6.3	Leave at **room temperature** for **10 minutes** to allow DNA to bind to the beads.
6.4	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.
6.5	Slowly pipette off the supernatant without disturbing the beads. Discard the supernatant.
6.6	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.
6.7	Repeat the previous step.
6.8	Remove residual 80% ethanol: • Remove the sample from the magnet. • Quick-spin to collect liquid at the bottom. • Place sample back on the magnet and allow beads separate fully from the solution. • Pipette off residual 80% ethanol and discard.
6.9	Remove samples from the magnet and immediately resuspend the beads with **40 µL of elution buffer.**
6.10	Resuspend by pipetting mixing until beads are evenly distributed in solution.
6.11	Leave samples **at room temperature** for **5 minutes** to elute DNA off beads.
6.12	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.
6.13	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.

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7. Nuclease treatment

This step removes unligated DNA fragments and leftover SMRTbell adapter from the sample.

✔	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.**
	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.
	7.6	Proceed to the next step of the protocol.

Nuclease mix

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

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

8. Diluted AMPure PB cleanup and size selection

The AMPure PB bead cleanup removes potential contaminants and depletes DNA fragments shorter than 5 kb. Size selection performance is sensitive to bead concentrations; therefore, ensure accurate pipetting volumes when diluting the beads and adding them to the library.

✔	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.

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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.
8.9	Remove residual 80% ethanol: • Remove the sample from the magnet. • Quick-spin to collect liquid at the bottom. • Place sample back on the magnet and allow beads separate fully from the solution. • Pipette off residual 80% ethanol and discard.
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	**Recommended:** 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.

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9. Annealing, binding, and cleanup (ABC)

This step is for preparing the libraries for sequencing on Revio systems. The sequencing polymerase is stable once bound to the HiFi library and can be stored at 4°C for 1 month or at -20°C for at least 6 months.

✔	Step	Instructions
	9.1	Prepare the appropriate volume of master mix with 10% overage using the per reaction volumes listed below.
	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.
	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.
	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.
		Post-binding cleanup with 1X SMRTbell cleanup beads
	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

Annealing mix (step 9.1)

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

Polymerase Dilution (step 9.7)

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

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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.
	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.

PROTOCOL COMPLETE

Revision history (description)	Version	Date
Initial release	01	March 2024
Updated to correct reference to the Hamilton NGS STAR MOA system and include stepwise recovery table	02	Aptril 2024

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