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

Procedure & checklist

Overview

This procedure describes the workflow for constructing whole genome sequencing (WGS) libraries from genomic and metagenomic DNA using the SMRTbell® prep kit 3.0 for sequencing on PacBio® long-read systems. This procedure may be performed manually or using one of the many qualified automation methods for SMRTbell prep kit 3.0.

Overview	
Libraries per SMRTbell prep kit 3.0	1–24

QC and workflow time for 8 samples

• Genomic DNA QC on Femto Pulse	1.5 hours
• SRE to improve gDNA quality	2.5 hours (tube format)
• Library prep with SMRTbell prep kit 3.0	3.5 hours
• SMRTbell library QC on Femto Pulse	1.5 hours

Times may vary by user and available lab equipment

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

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

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

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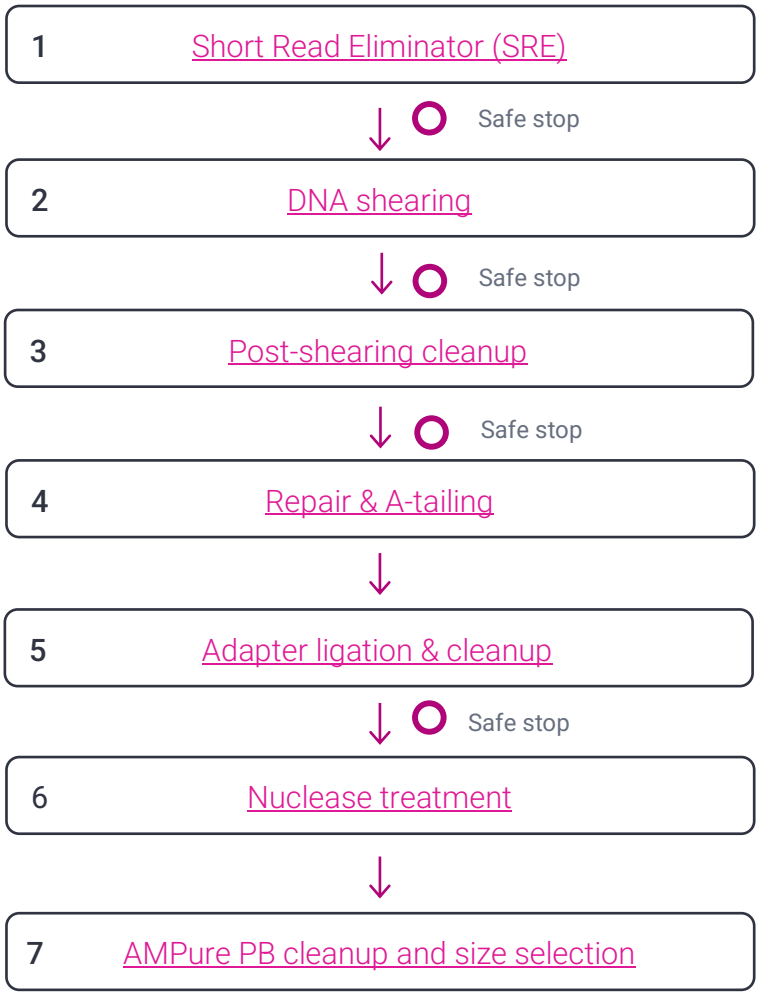
Workflow overview



Required materials and equipment

PCR tube strips are recommended for the enzymatic and bead cleanup steps, but Eppendorf Lo-bind tubes or 0.2 mL 96-well PCR plates are also acceptable. No difference in performance is expected across PCR tube strips, Lo-bind tubes, or plates.

DNA sizing	
Femto Pulse system	Agilent Technologies, Inc. M5330AA
Femto Pulse gDNA 165kb analysis kit	Agilent Technologies, Inc. FP-1002-0275
DNA 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
SMRTbell® library preparation	
SMRTbell® prep kit 3.0, includes: • SMRTbell® prep kit 3.0 • SMRTbell® cleanup beads • Low TE buffer	PacBio® 102-182-700
SMRTbell® adapter index plate 96A (optional)	PacBio® 102-009-200
200 Proof ethanol, molecular biology or ACS grade	Any major lab supplier (MLS)
Nuclease-free water, molecular biology grade	Any MLS
8-channel pipettes	Any MLS
0.2 mL 8-tube strips	USA Scientific TempAssure 1402-4708
Microcentrifuge	Any MLS
Magnetic separation rack compatible with 0.2 mL 8-tube strips	V&P Scientific VP 772F4-1
Thermocycler compatible with 0.2 mL 8-tube strips	Any MLS
1.5 mL DNA LoBind tubes	Eppendorf 022431021
Size selection	
SRE	PacBio® 102-208-300
AMPure® PB bead size selection kit	PacBio® 102-182-500

Before you begin

The following are general best practices for whole genome sequencing (WGS) using the Sequel® II/IIe or Revio systems. Please read carefully prior to beginning library prep.

Genomic DNA (gDNA) QC and input amount recommendations

PacBio Nanobind® DNA extractions kits are recommended to ensure sufficient amounts 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 at minimum. 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. 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.

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 at least 1 Revio SMRT Cell (Table 2).

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 (> 20 kb) or working with lower quality DNA (Table 1), start with at least 3 µg of gDNA to ensure enough library is available for optimal Revio SMRT Cell loading at 200 to 300 pM.

Metagenomic samples

If preparing metagenomic samples for shotgun sequencing, the following exceptions to the protocol apply.

- Do not perform SRE
- Average insert sizes may be lower than 15 kb.
- Skip shearing if sample QC shows that the average fragment size is < 13 kb.

Because SRE will be skipped, lower DNA input (less than 2 µg) may be used. Please note that a 15 to 30% recovery is expected. Make sure enough DNA is used to meet optimal loading amounts on Sequel II or Revio systems.

Multiplexing

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 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 of >15 kb.
- Only pool samples with similar genome sizes to ensure balanced coverage.
- 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 and sample handling

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

SRE

SRE buffer and buffer LTE are stored at room temperature.

SMRTbell prep kit 3.0

Thaw the repair buffer, nuclease buffer, SMRTbell adapter and elution buffer at room temperature.

Mix reagent buffers with a brief vortex prior to use. Do not vortex enzymes.

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

Keep all temperature-sensitive reagents on ice.

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

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

It is recommended to pipette-mix all SMRTbell prep reactions by pipetting up and down 10 times.

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

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

Prior to beginning, it is recommended to evaluate the quantity and size distribution of input DNA to determine whether it is suitable for this protocol. Please follow manufacturer instructions as appropriate for the QC instruments being used. See recommendations above.

1. Short read eliminator

Short read elminator (SRE) will progressively deplete fragments up to 25 kb from genomic DNA samples. This means that depletion decreases as the fragment size approaches 25kb. 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 only genomic DNA. Attempting to use SRE on sheared DNA or HiFi libraries (post-library construction) will result in poor recoveries.

If automating this step, please refer to the Microlab Prep Guide & overview for details on consumables.

If performing SRE manually, please see the SRE protocol for additional details.

This step is optional. If not performing SRE, then please proceed to the DNA shearing step.

✔	Step	Instructions
	1.1	Bring the DNA samples to a concentration between 40 to 100 ng/µL in a total volume of 50 µL using Buffer LTE in a 1.5 ml Lobind tube or hard-shell plate. **The DNA input requirement for shearing is <3 µg so gDNA input into SRE will depend on expected recovery.**
	1.2	Add 50 µL of **Buffer SRE** to each sample. If working in a plate format, heat seal with foil. Vortex/shake to mix for 5 seconds at max speed.
	1.3	Incubate the sample for 1 hour at 50°C in a heat block or thermal cycler. After incubation, if using a plate format, ensure that it is compatible with a 300 µL elution. If not, **transfer to the appropriate deep well plate after incubation and seal with an adhesive seal.**
	1.4	Load plate or tube (with the hinge facing toward the outside of the rotor) into the centrifuge.
	1.5	Centrifuge a tube at 10,000 rcf for 30 minutes Centrifuge a plate at >2250 rcf (max 3220 rcf) for 1 hour • If using a centrifuge with temperature control (i.e., cooling function), turn this function off by specifying a target temperature set point higher than ambient room temperature (e.g., 29°C or 30°C).
	1.6	Carefully remove supernatant without disturbing the pellet. • Leaving up to 10 µL is acceptable to be sure the pellet is not disturbed.
	1.7	Add 300 µL of Buffer LTE to the tube and incubate at room temperature for 10 minutes.
	1.8	After incubation, pipette-mix 20 times and vortex/shake the tube/sealed plate for 15s to ensure that the DNA is properly re-suspended and mixed.
	1.9	Quantify the resuspension to measure DNA recovery. If the recovery is lower than 50% repeat pipette-mixing 20 times and vortex/shake. If the recovery is greater than 50%, proceed to next step (DNA shearing).

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

SAFE STOPPING POINT - Store at 4°C

2. DNA shearing for WGS using automation

Please see Appendix for instructions on shearing with the Megaruptor 3 system.

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.

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, STARlet, and STAR V systems

✔	Step	Instructions
	2.1	Adjust DNA concentration to ≤10 ng/µL, if necessary (e.g., if more than 3 µg of gDNA was recovered from SRE). Use Buffer LTE to dilute samples. Bring all samples up to 300 µL in a 0.8 mL, 96 DeepWell plate (Thermo Fisher Scientific AB0859).
	2.2	Parameters for shearing on the Microlab Prep, or Hamilton assay-ready workstations are listed below. These parameters should already be part of the installed method on the instrument.

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

	2.3	Place the plate on the appropriate work deck position and start the shearing procedure.
	2.4	Optional: measure DNA concentration with a Qubit fluorometer using the 1x dsDNA HS kit once the shearing procedure is complete. **Recommended**: Further dilute each aliquot to **250 pg/µL** with the Femto Pulse dilution buffer. Measure the final SMRTbell library size distribution with a Femto Pulse system to ensure efficient shearing.

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

3. Post-shearing cleanup

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

✔	Step	Instructions post-shear cleanup
	3.1	Add **1.0X** v/v (volume over volume) of resuspended, room-temperature SMRTbell cleanup beads to each tube of sheared DNA. • Automated pipette shearing = 300 µL • Megaruptor 3 shearing = 100–130 µL
	3.2	Pipette-mix the beads until evenly distributed.
	3.3	Quick-spin the tube strip in a microcentrifuge to collect liquid.
	3.4	Leave at **room temperature** for **10 minutes** to allow DNA to bind beads.
	3.5	Place the tube strip in a magnetic separation rack until beads separate fully from the solution.
	3.6	Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant.
	3.7	Slowly dispense **200 µL**, or enough to cover the beads, of **freshly prepared 80% ethanol** into each tube. After **30 seconds**, pipette off the 80% ethanol and discard.
	3.8	Repeat the previous step.
	3.9	Remove residual 80% ethanol: • Remove the tube strip from the magnetic separation rack. • Quick-spin the tube strip in a microcentrifuge. • Place the tube strip back in a magnetic separation rack until beads separate fully from the solution. • Pipette off residual 80% ethanol and discard.
	3.10	Remove the tube strip from the magnetic rack. **Immediately** add **47 µL** of **low TE buffer** to each tube and resuspend the beads by pipetting 10 times or until evenly distributed.
	3.11	Quick-spin the tube strip in a microcentrifuge to collect liquid.
	3.12	Leave at **room temperature** for **5 minutes** to elute DNA.
	3.13	Place the tube strip in a magnetic separation rack until beads separate fully from the solution.
	3.14	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a **new tube strip**. Discard old tube strip with beads.

3.15	Recommended: Evaluate sample quality (concentration and size distribution). • Take a **1 µL** aliquot from each tube and dilute with **9 µL** of **elution buffer or water**. • Measure DNA concentration with a Qubit fluorometer using the 1x dsDNA HS kit. • Dilute each aliquot to **250 pg/µL** in Femto Pulse dilution buffer. • Measure DNA size distribution with a Femto Pulse system.
3.16	Proceed to the next step of the protocol if sample quality is acceptable.

SAFE STOPPING POINT - Store at 4°C

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 DNA damage and end repair

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

Repair mix

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

4.2	Pipette-mix the **Repair mix**.
4.3	Quick-spin the **Repair mix** in a microcentrifuge to collect liquid.
4.4	Add **14 µL** of the **Repair mix** to each sample**.** Total reaction volume should be **60 µL**.
4.5	Pipette-mix each sample.
4.6	Quick-spin the tube strip in a microcentrifuge to collect liquid.

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

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

4.8 Proceed to the next step of the protocol.

5. Adapter ligation and cleanup

This step ligates the SMRTbell adapter to the ends of each DNA fragment. Please ensure SMRTbell cleanup beads have been brought up to room temperature before proceeding to the cleanup steps.

✔	Step	Instructions for SMRTbell adapter ligation and reaction cleanup

SMRTbell adapter ligation

5.1 (Optional) If using an adapter index: add **4 µL** of barcoded adapters from the **SMRTbell adapter index plate 96A** to each respective sample from the previous step and exclude the SMRTbell adapter from the ligation mix.

Skip this step if not using an adapter index.

5.2 Add the following components in the order and volume listed below to a new microcentrifuge tube. Adjust component volumes for the number of libraries being prepared, plus 10% overage. For individual preps, add components directly to each sample from the previous step in the order and volume listed below, then skip steps (5.3 to 5.5).

Ligation mix

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

*Exclude the SMRTbell adapter if using the SMRTbell adapter index plate 96A

5.3 Pipette-mix the **Ligation mix**.

5.4 Quick-spin the **Ligation mix** in a microcentrifuge to collect liquid.

✔	Step	Instructions for SMRTbell adapter ligation and reaction cleanup
	5.5	**Indexed samples:** add **31 µL** of the **Ligation mix** to each sample from the previous step. **Non-indexed samples:** add **35 µL** of the **Ligation mix** containing the SMRTbell adapter to each sample from the previous step. The total volume per sample should be **95 µL.**
	5.6	Pipette-mix each sample.
	5.7	Quick-spin the tube strip in a microcentrifuge to collect liquid.
	5.8	Run the **adapter ligation** thermocycler program. Set the lid temperature to 75°C if programmable. Step 1: 30 min, 20°C Step 2: Hold, 4°C
		Cleanup with 1X SMRTbell cleanup beads
	5.9	Add **95 µL** of resuspended, room-temperature SMRTbell cleanup beads to each sample.
	5.10	Pipette-mix the beads until evenly distributed.
	5.11	Quick-spin the tube strip in a microcentrifuge to collect all liquid from the sides of the tubes.
	5.12	Leave at **room temperature** for **10 minutes** to allow DNA to bind beads.
	5.13	Place the tube strip in a magnetic separation rack until beads separate fully from the solution.
	5.14	Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant.
	5.15	Slowly dispense **200 µL**, or enough to cover the beads, of **freshly prepared 80% ethanol** into each tube. After **30 seconds**, pipette off the 80% ethanol and discard.
	5.16	Repeat the previous step.
	5.17	Remove residual 80% ethanol: • Remove the tube strip from the magnetic separation rack. • Quick-spin the tube strip in a microcentrifuge. • Place the tube strip back in a magnetic separation rack until beads separate fully from the solution. • Pipette off residual 80% ethanol and discard.
	5.18	Remove the tube strip from the magnetic rack. **Immediately** add **40 µL** of **elution buffer** to each tube and resuspend the beads.
	5.19	Quick-spin the tube strip in a microcentrifuge.
	5.20	Leave at **room temperature** for **5 minutes** to elute DNA.
	5.21	Place the tube strip in a magnetic separation rack until beads separate fully from the solution.

✔	Step	Instructions for SMRTbell adapter ligation and reaction cleanup
	5.22	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a **new tube strip**. Discard old tube strip with beads.
	5.23	Proceed to the next step of the protocol.

SAFE STOPPING POINT - Store at 4°C

6. Nuclease treatment

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

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

Nuclease mix

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

	6.2	Pipette-mix **Nuclease mix.**
	6.3	Quick-spin **the Nuclease mix** in a microcentrifuge to collect liquid.
	6.4	Add **10 µL** of **Nuclease mix** to each sample. Total volume should equal **50 µL.**
	6.5	Pipette-mix each sample.
	6.6	Quick-spin the tube strip in a microcentrifuge to collect liquid.
	6.7	Run the **nuclease treatment** thermocycler program. Set the lid temperature to 75°C if programmable.

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

Proceed to the next step of the protocol.

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

7. Diluted AMPure PB bead cleanup and size selection

The AMPure PB bead size selection step will clean the library and deplete DNA fragments shorter than 5 kb. Please see the Technical note – HiFi WGS Performance with AMPure PB bead size selection for more information on performance of this method.

If performing gel-based size selection, skip the AMPure PB cleanup and perform a cleanup using the 1X SMRTbell cleanup beads described in the Appendix.

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 size selection
	7.1	Make a 35% v/v dilution of AMPure PB beads by adding 1.75 mL of resuspended AMPure PB beads to 3.25 mL of elution buffer. The 35% dilution can be stored at 4°C for 30 days. **Note:** The AMPure PB dilution may be scaled as appropriate for smaller/larger scale projects.
	7.2	Add 3.1X v/v (155 µL) of resuspended, room-temperature 35% AMPure PB beads to each sample from the previous step.
	7.3	Pipette-mix the beads until evenly distributed.
	7.4	Leave at **room temperature** for **20 minutes** to allow DNA to bind beads.
	7.5	Place sample on an appropriate magnet and allow beads separate fully from the solution.
	7.6	Slowly pipette off the cleared supernatant without disturbing the beads.
	7.7	Slowly dispense **200 µL**, or enough to cover the beads, of **freshly prepared 80% ethanol** into each sample. After **30 seconds**, pipette off the 80% ethanol and discard.
	7.8	Repeat the previous step.
	7.9	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.
	7.10	Remove samples from the magnet and **immediately** add **15 µL** of **elution buffer** to each sample.
	7.11	Pipette-mix the beads until evenly distributed.
	7.12	Leave at **room temperature** for **5 minutes** to elute DNA of the beads.

7.13	Place samples on the magnet and allow the beads to separate fully from the solution.
7.14	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a new tube.
7.15	Take a **1 µL** aliquot from each tube and dilute with **9 µL** of **elution buffer or water**. Measure DNA concentration with a Qubit fluorometer using the 1x dsDNA HS kit. Calculate the total mass. The final overall recovery should be 10–25% as measured from gDNA input to completed SMRTbell library (includes SRE, shearing, and library prep). DNA concentration must be less than 60 ng/µl to proceed to ABC. Recommended: Further dilute each aliquot to **250 pg/µL** with Femto Pulse dilution buffer. Measure final SMRTbell library size distribution with a Femto Pulse system.
7.16	Proceed to SMRT Link Sample Setup for preparing samples for sequencing. Alternatively, libraries can be stored at 4°C if sequencing within 1 month. Store long-term at -20°C. SMRTbell libraries are expected to degrade over time at the same rate as any appropriate buffered pure DNA sample. Minimize freeze/thaw cycles and do not expose to direct sunlight or UV radiation.

PROTOCOL COMPLETE

Appendix

A1. DNA shearing with the Megaruptor 3 system

DNA shearing	
Megaruptor 3 system	Diagenode B06010003
Megaruptor 3 shearing kit	Diagenode E07010003

This step describes the procedure for shearing DNA using the Megaruptor 3 system.

✔	Step	Instructions DNA shearing and cleanup
		DNA shearing
	A1.1	Bring DNA up to a final volume of **100–130 µL** with **low TE buffer**. Target a concentration of **30 ng/µL** (range: **3–39 ng/µL**).
	A1.2	Shear DNA on the Megaruptor 3 system. Recommended settings are below. Genome / Shear speed / Target insert length Human, plant, or animal / 31 / 15–18 kb Microbe / 40 / 7–10 kb
	A1.3	Transfer sheared DNA into a tube strip or other appropriate tube for the 1x SMRTbell cleanup bead step. Typical volume loss during shearing is between 5–10 µL.
	A1.4	Proceed to the post-shearing cleanup step (section 3) using 1X SMRTbell cleanup beads.

A2. 1X cleaunup for performing gel-based size selection.

This step describes the cleanup prior to performing gel-based size selection. Please see the Technical Note - Alternative size selection methods for SMRTbell prep kit 3.0 for procedural details.

✔	Step	Instructions for bead binding, washing, and sample elution
	A2.1	Add **50 µL** of SMRTbell cleanup beads to each nuclease treated library.
	A2.2	Pipette-mix the beads until evenly distributed.
	A2.3	Quick-spin the tube strip in a microcentrifuge to collect all liquid.
	A2.4	Leave at **room temperature** for **10 minutes** to allow DNA to bind beads.

A2.5	Place tube strip in a magnetic separation rack until beads separate fully from the solution.
A2.6	Slowly pipette off the cleared supernatant without disturbing the beads. It is recommended to save the supernatant in another tube strip in case of poor DNA recovery.
A2.7	Slowly dispense **200 µL**, or enough to cover the beads, of **freshly prepared 80% ethanol** into each tube. After **30 seconds**, pipette off the 80% ethanol and discard.
A2.8	Repeat the previous step.
A2.9	Remove residual 80% ethanol: • Remove tube strip from the magnetic separation rack. • Quick-spin tube strip in a microcentrifuge. • Place tube strip back in a magnetic separation rack until beads separate fully from the solution. • Pipette off residual 80% ethanol and discard.
A2.10	Remove tube strip from the magnetic rack. **Immediately** add **21 µL** of **elution buffer** to each tube and resuspend the beads by pipetting 10 times or until evenly distributed.
A2.11	Quick-spin the tube strip in a microcentrifuge to collect liquid.
A2.12	Leave at **room temperature** for **5 minutes** to elute DNA.
A2.13	Place tube strip in a magnetic separation rack until beads separate fully from the solution.
A2.14	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a **new tube strip**. Discard old tube strip with beads.
A2.15	Take a **1 µL** aliquot from each tube and dilute with **9 µL** of **elution buffer or water**. Measure DNA concentration with a Qubit fluorometer using the 1x dsDNA HS kit. Calculate the total mass. **Recommended:** Further dilute each aliquot to **250 pg/µL** with Femto Pulse dilution buffer. Measure final SMRTbell library size distribution with a Femto Pulse system.
A2.16	Store libraries at 4°C if sequencing within 1 month. Store long-term at -20°C. SMRTbell libraries are expected to degrade over time at the same rate as any appropriate buffered pure DNA sample. Minimize freeze/thaw cycles and do not expose to direct sunlight or UV radiation.

Revision history (description)	Version	Date
Initial release.	01	April 2022
Updated to include new information for the Revio system	02	February 2023
Updated with new reccomendations for WGS library prep	03	March 2024

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