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
• Short read eliminator (size-selection)	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 users and available lab equipment

gDNA input mass into library prep	Sequel II® and Sequel IIe	Revio® (non-SPRQ™ chemistry/Vega)	Revio (SPRQ™ chemistry)
Total DNA per SMRT® Cell**	1 µg	2 µg	500 ng

**If multiplexing, the total mass must be equivalent to the numbers indicated above. If using SRE, 500 ng per sample must be used. If bypassing SRE, no less than 20 ng should be used for an individual sample going into library preparation.

DNA quality recommendation	Femto Pulse genome quality number (GQN)	
DNA size distribution (Femto Pulse system)	70% ≥10 kb ($GQN_{10\ kb}$ ≥7.0) & 50% ≥30 kb ($GQN_{30\ kb}$ ≥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 (preferred DNA shearing method)
Target fragment lengths	15–20 kb
Size selection	Short read eliminator 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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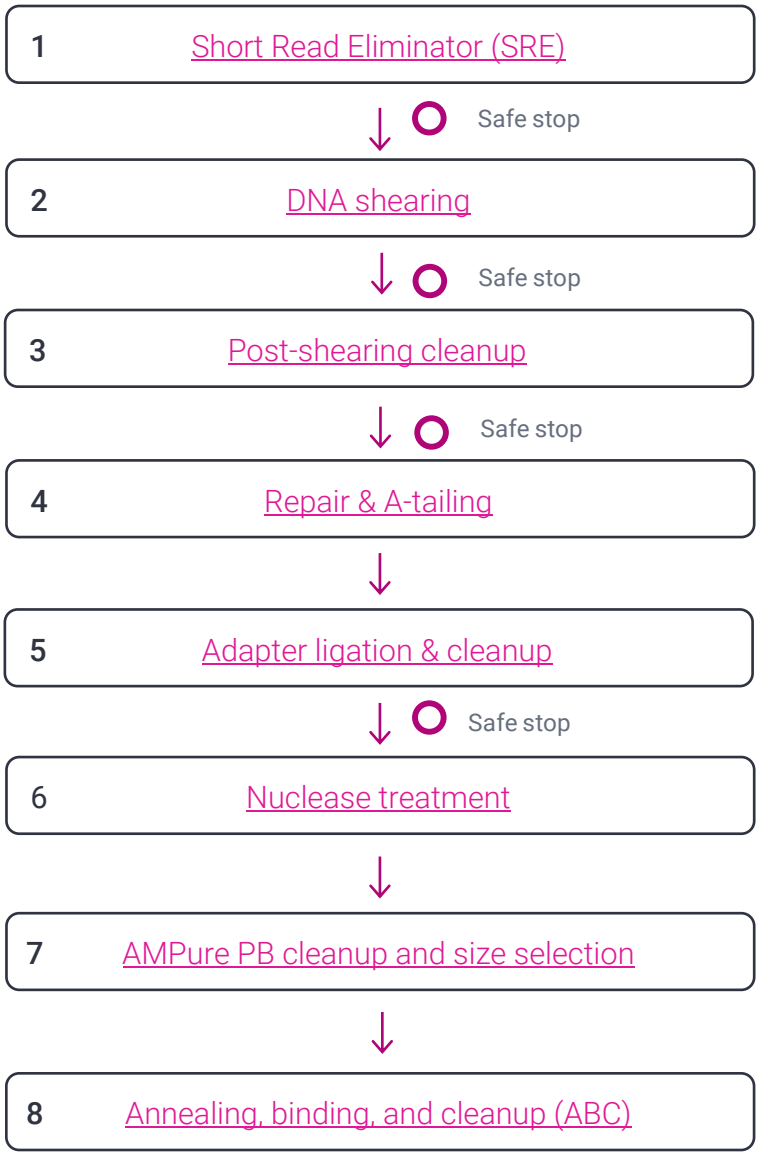
Workflow overview



Required materials and equipment

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	
SRE Kit	PacBio® 102-208-300
Buffer LTE HT (if pipette shearing)	PacBio® 103-306-100
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
AMPure® PB bead size selection kit	PacBio® 102-182-500
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
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

Before you begin

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

Genomic DNA (gDNA) QC and input mass recommendations

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

gDNA quality QC

The Agilent Femto Pulse system is 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. If the GQN at 10 kb is less than 7.0, higher gDNA inputs may be required.
- If the majority of DNA is less than 10 kb, Short read eliminator is not recommended.
- Shearing may be bypassed if the sample is already in the appropriate size-range.

Important:

The HiFi yield and HiFi mean read length of a sequencing run are 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 minimal DNA below 10 kb, and with >50% mass over 30 kb. High quality gDNA will typically have a higher percent library recovery and HiFi sequencing yield.

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

gDNA input

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 the manufacturer's instructions for the assays being used. We *do not* recommend quantification with UV-Vis Spectrophotometers (e.g. NanoDrop).

Low mass vs High mass

Using the Revio system with SPRQ chemistry reduces the gDNA mass required for library preparation by reducing the SMRT Cell loading volume. **While the "High mass" option is still available, there is now a "Low mass" option with**

updated parameters for SRE and DNA shearing when using 0.5–1.25 µg of gDNA. Low mass SRE has been optimized to provide comparable recoveries to High mass SRE with the same size selection performance. Low mass shearing has been optimized to maintain the size distribution between 15–20 kb. It is recommended to stay consistent for both SRE and shearing steps (i.e. **use Low or High mass settings for *both* SRE and shearing**).

Note that High mass and Low mass samples cannot be included in a single SRE/shearing automation run. **If 0.5–1.25 µg gDNA is available and Revio SPRQ chemistry is being used, the Low mass workflow will provide enough library for a Revio SMRT Cell (+SPRQ)**; however, High mass parameters can also be used for Revio SPRQ chemistry if excess library is desired. **For Revio non-SPRQ chemistry and Vega, 2 µg gDNA input is still recommended for loading of 1 SMRT Cell.**

Table 1. Recommended DNA input mass by starting gDNA quality

gDNA quality	Low mass (compatible with Revio SPRQ)	High mass	Expected SRE recovery (dependent on DNA quality)
70% >10 kb (recommended)	0.5–1.25 µg	2–4 µg	60–95%
<70% >10kb	1–1.75 µg	4–5 µg	40–60%
<10kb (no SRE)	0.25–1 µg	1–3 µg	Not recommended

Starting with 500 ng and 2 µg of high quality genomic DNA will typically provide enough library to load at least 1 Revio SMRT Cell (+SPRQ) and 1 Revio/Vega SMRT Cell (non-SPRQ), respectively (Table 2, Table 3).

If gDNA mass available is between 1.25 µg and 2 µg, use the "High mass" workflow; however, note that if sequencing on Vega or Revio without SPRQ, there may not be enough final library for loading at an optimal on-plate loading concentration for a single SMRT Cell.

Table 2. Polymerase-bound library mass necessary for loading on a Revio SMRT Cell at 250 pM

Mean insert size	Revio +SPRQ (250 pM)	Revio/Vega (non-SPRQ) (250 pM)
10,000 bp	41 ng	163 ng
15,000 bp	61 ng	244 ng
18,000 bp	73 ng	293 ng
21,000 bp	85 ng	341 ng

SMRTbell prep kit 3.0 protocol stepwise recoveries

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, SMRTbell library preparation, and ABC, Table 3).

Table 3. Expected stepwise recoveries of DNA and SMRTbell library from the SMRTbell prep kit 3.0 protocol. Post-SRE recovery will vary with the quality of the DNA input. Higher quality DNA will yield more recovery post-SRE.

Protocol Step	DNA or SMRTbell step recovery	DNA or SMRTbell overall recovery	Expected size (Femto Pulse)
Starting Input	100%	100%	$GQN_{10\ kb}$ >7.0
Post-SRE	65–95%	65–95%	$GQN^{10\ kb}$ >9.3
Post-shear SMRTbell bead cleanup	80–95%*	52–90%	15–20 kb
Post-ligation SMRTbell bead cleanup	80–95%	42–86%	
Post-nuclease (pre-cleanup)	40–50%	17–43%	
Post-3.1x AMPure PB bead cleanup	75–80%	13–34%	
Post-ABC cleanup	75–95%	**10–32%**	

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

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 <18 kb.

Because SRE will be skipped, a lower DNA input mass may be used. Note that 14 to 34% recovery (from gDNA to bound SMRTbell) is expected. Ensure enough DNA is used to meet optimal loading concentrations on Revio, Vega, or Sequel II/e 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 consider the following guidelines:

- 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 cleanup beads and AMPure PB beads

Bring SMRTbell cleanup beads and AMPure PB beads to room temperature prior to use.

Vortex *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.

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

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

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. See recommendations above.

1. Short read eliminator

Short read elminator (SRE) will progressively deplete fragments up to 25 kb from genomic DNA samples. **SRE should not be done on gDNA samples that are <10 kb.**

Important: Use SRE on genomic DNA only. Attempting to use SRE on sheared DNA or HiFi libraries will result in poor recoveries.

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

If not performing SRE, proceed to the DNA shearing step.

✔	Step	Instructions
	1.1	Dilute gDNA ($GQN_{10\,kb}$ >7.0) to the appropriate concentration in Buffer LTE according to the table below. **Note:** If working with low quality gDNA with a $GQN_{10\,kb}$ < 7.0, input mass and concentration can be increased if the expected recovery (40 – 60%, Table 1) matches the pipette-tip shearing mass limit for each respective workflow. For example, 2 µg of gDNA can be used with the Low mass workflow if expected recovery is 40% (800 ng).
	1.2	Add **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.

	Low mass	High mass
Sample volume	25 µL	50 µL
DNA concentration	20–50 ng/µL	40–80 ng/µL
Recommended max gDNA mass	1.25 µg	4 µg
Elution volume (Buffer LTE)	200 µL	300 µL
Shearing mass limit	≤1 µg	≤3 µg

	Low mass	High mass
Buffer SRE volume	25 µL	50 µL

1.5	**Tube**: centrifuge at 10,000 rcf for 30 minutes. **Plate**: centrifuge at >2250 rcf (max 3220 rcf) for 1 hour. **Important:** 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 5 µL and 10 µL supernatant with the low and High mass SRE workflow, respectively, is acceptable to ensure the pellet is not aspirated.
1.7	If pipette shearing, add Buffer LTE to the tube and incubate at room temperature for 10 minutes. If alternative shearing options will be used, elute in the appropriate volume for that method. See appendix for recommended elutions volumes if using Megaruptor 3 DNA shearing
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

Shearing option	Low mass	High mass
Pipette shearing	200 µL	300 µL

2. DNA shearing for WGS using automation

Please see Appendix for instructions on shearing with the Megaruptor 3 system (A1) or Covaris g–TUBE (A4) .

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 with a narrow distribution (typically ~10 – 35 kb) is recommended for this protocol. **If gDNA is within these ranges or lower, the DNA shearing step can be bypassed**. Deviating from the concentration and automation settings is not recommended and will result in undersheared DNA.

These shearing parameters are specific for the Hamilton Microlab Prep, or assay ready workstations (Hamilton NGS STAR, STARlet, and STAR V systems)

<table>
<tr><th>✔</th><th>Step</th><th>Instructions</th></tr>
<tr><td></td><td>2.1</td><td>Adjust DNA concentration to ≤5 ng/µL in 200 µL or ≤10 ng/µL in 300 µL, if necessary (e.g., if more than 1 µg or 3 µg of gDNA was recovered from SRE). Use the Low TE buffer provided with the kit (or buffer LTE from the SRE kit) to dilute samples in a 0.8 mL, 96 DeepWell plate (Thermo Fisher Scientific AB0859).</td></tr>
<tr><td></td><td>2.2</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>Low mass</th><th>High mass</th></tr>
<tr><td>DNA concentration</td><td><5 ng/µL</td><td><10 ng/µL</td></tr>
<tr><td>Volume of Buffer LTE</td><td>200 µL</td><td>300 µL</td></tr>
<tr><td>Number of mixes</td><td>300 cycles</td><td>300 cycles</td></tr>
<tr><td>Pipette mixing speed</td><td>400 µL/sec</td><td>500 µL/sec</td></tr>
<tr><td>Liquid following</td><td>83% volume</td><td>83% volume</td></tr>
<tr><td>Pipette tip</td><td>300 µL CO-RE II tips (filtered, black, non-sterile)</td><td>300 µL CO-RE II tips (filtered, black, non-sterile)</td></tr>
</table></td></tr>
<tr><td></td><td>2.3</td><td>Place the plate on the appropriate work deck position and start the shearing procedure.</td></tr>
<tr><td></td><td>2.4</td><td>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 DNA with a Femto Pulse system to ensure efficient shearing.</td></tr>
<tr><td></td><td>2.5</td><td>Proceed to the next step of the protocol.</td></tr>
</table>

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 = 200–300 µL • Megaruptor 3 shearing = 100–130 µL • Megaruptor 3 shearing = 65 µL, skip post-shearing cleanup and proceed to section 4.
	3.2	Pipette-mix the beads until evenly distributed.
	3.3	Quick-spin the tube strip in a microcentrifuge to collect liquid.
	3.4	Incubate 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 remove 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**, remove 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. • Remove 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	Incubate 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 remove the cleared eluate without disturbing the beads. Transfer eluate 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.

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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 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 master 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 master mix**.
4.3	Quick-spin the **Repair master mix** in a microcentrifuge to collect liquid.
4.4	Add **14 µL** of the **Repair master 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 master mix**.

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

5.5	**Indexed samples:** add **31 µL** of the **Ligation master mix** to each sample from the previous step. **Non-indexed samples:** add **35 µL** of the **Ligation master 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 ≥30°C if programmable.

Step	Time	Temperature
1	30 min	20°C
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	Incubate 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 remove 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**, remove 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. • Remove 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	Incubate 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.
5.22	Slowly aspirate the cleared eluate without disturbing the beads. Transfer eluate 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 master mix.**
6.3	Quick-spin the **Nuclease master mix** in a microcentrifuge to collect liquid.
6.4	Add **10 µL** of **Nuclease master 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 ≥47°C if programmable.

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

6.8	Proceed to the next step of the protocol. **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 removes contaminants and progressively depletes 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 the DNA library is <10 kb, proceed with a 1x SMRTbell bead cleanup.**

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	Incubate 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 remove 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**, remove 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. • Remove residual 80% ethanol and discard.
	7.10	Remove samples from the magnet and **immediately** add **26 µL** of **elution buffer** to each sample.
	7.11	Pipette-mix the beads until evenly distributed.
	7.12	Incubate at **room temperature** for **5 minutes** to elute DNA off of the beads.
	7.13	Place samples on the magnet and allow the beads to separate fully from the solution.
	7.14	Slowly remove the cleared eluate without disturbing the beads. Transfer eluate to a new tube.

Take a **1 µL** aliquot from each tube and dilute with **9 µL** of **elution buffer or water**. Measure the DNA concentration with a Qubit fluorometer using the 1x dsDNA HS kit. Calculate the total mass. The final overall recovery should be 10–30% as measured from gDNA input mass to completed SMRTbell library (includes SRE, shearing, library prep, and ABC).

7.15 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.

DNA concentration must be less than 60 ng/µL to proceed to ABC. Failure to dilute DNA below 60 ng/µL will result in low P1 loading.

Proceed to Section 8 to prepare library for sequencing with Revio +SPRQ or Vega

Or

7.16 Proceed to SMRT Link Sample Setup for preparing samples for Revio non-SPRQ chemistry or Sequel II/e.

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 SMRTbell library (25 µL) for sequencing on Revio +SPRQ or Vega. If samples are pooled prior to ABC or a custom volume is required, see Appendix section A3. **The Polymerase kit used will depend on which sequencer or chemistry is being used (see below).**

System	Kit	PN
Revio	Revio SPRQ polymerase kit	103-520-100
Vega	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 1X SMRTbell cleanup beads

8.13 Add **100 µL** of resuspended, room-temperature SMRTbell cleanup beads to each 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 1**5 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 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.

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. Optimal conditions may vary by sample.

✔	Step	Instructions DNA shearing and cleanup
		DNA shearing
	A1.1	Dilute DNA to the appropriate concentration in **low TE buffer**. Low mass: Volume 65 µL; Target concentration range 4–15 ng/µL High mass: Volume 100–130 µL; Target concentration range 10–39 ng/µL
	A1.2	Shear DNA on the Megaruptor 3 system. Recommended settings are below. Human, plant, or animal: Shear speed 31; Target insert length 15–18 kb Microbe: Shear speed 40; Target insert length 7–10 kb
	A1.3	Transfer sheared DNA into a tube strip for a 1x SMRTbell cleanup bead step. If shearing volume is 65 µl, proceed directly to Repair and A-tailing. Typical volume loss during shearing is between 5–10 µL.

A2. 1X SMRTbell cleanup 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	Incubate 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 remove 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**, remove 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. • Remove 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	Incubate 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 remove the cleared eluate without disturbing the beads. Transfer eluate 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.

A3. Annealing, binding, and cleanup (ABC) for custom volumes

This step is for preparing libraries for sequencing on PacBio HiFi sequencers. Libraries or pools of libraries must be at a concentration of <60 ng/µL. 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. Use the calculations below to determine reagent volumes based on input sample volume:

	SMRTbell library	Annealing buffer	Standard sequencing primer	Polymerase dilution
Volume (µL)	x	x/2	x/2	x*2
Example	100	50	50	200

See Section 8 Annealing, binding, and cleanup (ABC) for full protocol.

A4. DNA shearing with Covaris g-TUBE

DNA shearing	PN
g-TUBE	Covaris 520079

This step describes the procedure for shearing DNA using Covaris g-TUBE. **Optimal conditions will vary by sample**.

<table>
<tr><th>✔</th><th>Step</th><th>Instructions DNA shearing and cleanup</th></tr>
<tr><td></td><td>A4.1</td><td>Dilute DNA to the appropriate concentration in low TE buffer or Buffer LTE.
<table>
<tr><th>gDNA mass</th><th>Volume</th><th>Target concentration range</th></tr>
<tr><td>500 ng – 1000 ng</td><td>150 µL</td><td>3.3 ng/µL - 6.7 ng/µL</td></tr>
<tr><td>>1000 – 2000 ng</td><td>150 µL</td><td>>6.7 ng/µL – 13.3 ng/µL</td></tr>
</table></td></tr>
<tr><td></td><td>A4.2</td><td>Centrifuge according to the below conditions for 5 minutes per pass.
<table>
<tr><th>gDNA mass</th><th>Number of passes</th><th>Centrifugation speed</th><th>Target insert length</th></tr>
<tr><td>500 ng – 1000 ng</td><td>2</td><td>1000 xg (rcf)</td><td rowspan="2">15–18 kb</td></tr>
<tr><td>>1000 – 2000 ng</td><td>2</td><td>1500 xg (rcf)</td></tr>
</table></td></tr>
<tr><td></td><td>A4.3</td><td>Transfer sheared DNA into a tube strip for a 1x (150 µL) SMRTbell bead cleanup step. Final elution volume is 47 µL.</td></tr>
</table>

Troubleshooting FAQs

1. **The "Low mass" workflow specifies an input mass of 0.5 – 1.25 µg and the "High mass" workflow specifies an input mass of 2 – 4 µg gDNA. What should I use if I have 1.25 – 2 µg of gDNA?**
   - If you have 1.25 – 2 µg of gDNA input mass for SRE, you can use the "High mass" workflow. This corresponds to a concentration range of 25 ng/µL – 40 ng/µL in a 50 µL volume. Please note that if sequencing on Vega or Revio (non-SPRQ), you may not have enough final library for loading at an optimal on-plate loading concentration for a single SMRT Cell.

2. **How should gDNA be quanted to ensure that the concentration is accurate?**
   - Use a quantification method specific to dsDNA, such as Qubit. High molecular weight (HMW) DNA will be in-homogenous and will therefore give inaccurate results depending on where the sample was pulled from in the tube. Vigorously vortexing gDNA prior to quantifyng will improve quant accuracy. The vortexing will not adversely affect the DNA.

3. **What are possible error modes if low SRE recovery is observed?**
   - If Buffer SRE is not appropriately mixed with the sample prior to incubation and centrifugation, recovery will be low. The DNA will remain in the supernatant and not efficiently pellet. It is critical that the Buffer SRE and the sample are well mixed by vortexing. In some cases, pipette mixing prior to vortexing improves performance.
   - Try to mix your final eluted sample again. If the final pellet is not properly resuspended, your quant reading will be low. Try pipette mixing and vortexing followed by a re-quant.
   - You may have aspirated the DNA pellet during supernatant removal. If you still have the supernatant, you can re-vortex/mix, incubate at 55°C for 1 hour, and re-centrifuge to re-pellet the sample and follow the procedure to remove the supernatant and re-suspend the pellet.
   - Was the sample immediately retrieved after centrifugation? With time, the pellet may detach or diffuse and will therefore be aspirated during supernatant removal.

4. **What happens if the library is not diluted to the recommended concentration going into ABC?**
   - The primer and polymerase concentration remains constant, regardless of sample input concentration. The recommended concentrations keep the SMRTbell libraries below a certain molarity so that there is an appropriate ratio of primer and polymerase to library. If a concentration above the recommended range is used, there will be a higher proportion of library without a primer/polymerase, which will result in a lower P1 value.

5. **What should I do if I made a mistake during ABC and my whole library was prepped?**
   - Run a 1x SMRTbell bead cleanup (50 µL SMRTbell cleanup beads in 50 µL of bound library, for example) according to standard procedure (with EtOH) and elute in 25 µL of EB. Repeat ABC according to standard procedure.

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
Updated with stepwise recovery table	04	April 2024
Updated for SPRQ chemistry and the Vega system	05	December 2024
Corrected error in target DNA concentration range	06	January 2025
Added ABC reagents to the Reagent handling section, clarified polymerase kit part numbers	07	June 2025

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