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

Procedure & checklist

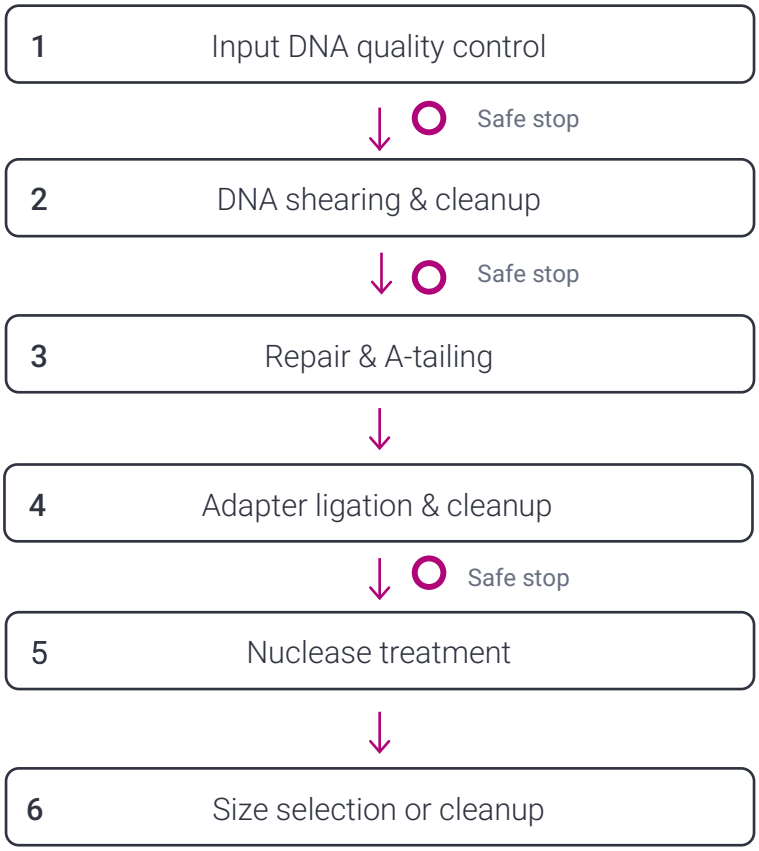
Before you begin

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 systems.

Overview			
Samples per SMRTbell prep kit 3.0	1–24		
Workflow time	4.5 hours for up to 8 samples; 6 hours for 24 samples Time difference is from DNA shearing, which is done in sets of 8 samples. Excludes measuring DNA size on Femto Pulse system.		
DNA input			
Quantity	300 ng–5 µg per library		
	Human, plant, and animal	**Microbes**	**Metagenomes**
DNA size distribution (Femto Pulse system)	50% ≥ 30 kb & 90% ≥ 10 kb	90% ≥ 7 kb	90% ≥ 7 kb
DNA shearing (Megaruptor 3 system)	Speed 31	Speed 40	Speed 40
Target fragment lengths	15–18 kb	7–12 kb	7–12 kb
Size selection required	AMPure® PB beads	none	none

PN 102-166-600 APR2022

Workflow



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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	
Qubit fluorometer	ThermoFisher Scientific Q33238
Qubit 1X dsDNA HS assay kit	ThermoFisher Scientific Q33230
DNA shearing	
Megaruptor 3 system	Diagenode B06010003
Megaruptor 3 shearing kit	Diagenode E07010003
SMRTbell® library preparation	
SMRTbell® prep kit 3.0	PacBio 102-182-700
SMRTbell® barcoded adapter plate 3.0 (optional; for barcoding)	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	
AMPure® PB bead size selection kit	PacBio 102-182-500

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General best practices

DNA Input

For human, animal, and plant genomes, 90% or more of the DNA should be ≥ 10 kb, and 50% or more ≥ 30 kb, as measured on the Femto Pulse system. That corresponds to a genome quality number (GQN) of 9.0 or higher with 10 kb cutoff and 5.0 or higher with 30 kb cutoff.

Size requirements are less stringent for microbial and metagenomic samples. The DNA should be at least as large as the recommended insert lengths of 7–12 kb. Any degradation should be due to shearing from the extraction process (e.g., bead beating) and not from poor sample handling or storage, or biochemical processes.

Start with a total mass **≥1 µg of DNA per SMRT® Cell 8M** to ensure there is sufficient library to load at concentrations that maximize sequencing yield. This protocol accepts as little as 300 ng of DNA, but the final amount of SMRTbell library may be too little to load at optimal concentrations, resulting in lower sequencing yields.

Use **≥300 ng** of DNA input per sample, with a total mass ≥1 µg across all samples when multiplexing.

Increase DNA input amounts to **≥1.5 µg per SMRT Cell 8M** when using a gel-cassette size selection option. See *Technical Note - Alternative size selection methods for SMRTbell prep kit 3.0* for procedure details.

Reagent and sample handling

Room temperature is defined as any temperature in the range of **18-23°C** for this protocol.

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

Mix reagent buffers and SMRTbell adapter with a brief vortex prior to use. Enzyme mixes do not require vortexing.

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

Bring SMRTbell cleanup beads and Qubit 1X dsDNA HS reagents to room temperature for 30-60 minutes prior to use.

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

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.

Multiplexing

Barcode with SMRTbell barcoded adapter plate 3.0. Quick spin the plate to collect liquid at bottom of the well prior to use.

Shear samples to similar fragment length profiles. This will enable equal mass pooling.

To multiplex, pool an **equal mass** of each final SMRTbell library together. If the molarity of the pool is less than 2 nM, then follow with a concentration step using a 1X (v/v) amount of SMRTbell cleanup beads. Use the conversion calculator in SMRT® Link Sample Setup to determine molarity if necessary.

Thermocycler programs

Program thermocycler(s) prior to beginning the protocol for the first time.

Repair and A-tailing, adapter ligation, and nuclease treatment thermocycler steps can be combined into a single program and paused in between prep treatments if preferred.

Set the lid temperature to 75°C for all programs. If the lid temperature is not programmable, it is acceptable to leave at 95–105°C.

1. Repair and A-tailing program

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

2. Adapter ligation program

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

3. Nuclease treatment program

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

Workflow steps

1. Input DNA quality control

This protocol requires high-quality, high molecular weight (HMW) DNA. Before you begin, evaluate the quantity and size distribution of input DNA to determine whether it is suitable for the protocol.

✔	Step	Instructions
	1.1	Bring the Qubit 1X dsDNA HS working solution and standards to **room temperature**.
	1.2	Pulse vortex or pipette mix each sample to homogenize the DNA in solution.
	1.3	Quick spin each sample to collect liquid.
	1.4	Take a **1 µL** aliquot from each sample and dilute with **9 µL** of **elution buffer or water.**
	1.5	Measure DNA concentration with a Qubit fluorometer using the **1X dsDNA HS kit.**
	1.6	Dilute each aliquot to **250 pg / µL** in Femto Pulse dilution buffer based on the Qubit reading.
	1.7	Measure DNA size distribution with a Femto Pulse system using the **gDNA 165kb analysis kit.**
	1.8	Proceed to the next step of the protocol if sample quality is acceptable.
		SAFE STOPPING POINT - Store at 4°C

2. DNA shearing and cleanup

This protocol utilizes the Megaruptor 3 system for shearing. See *Technical Note - Covaris g-TUBE DNA shearing for SMRTbell prep kit 3.0* for an alternative shearing method that requires only a standard microcentrifuge.

Shear DNA to an appropriate fragment length to optimize HiFi sequencing yield and read accuracy. 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.

Microbial and metagenomics samples may forgo shearing if the DNA is already in the specified fragment length range (7 kb –12 kb). In such cases, proceed to the "cleanup with 1X SMRTbell cleanup beads" to get the appropriate input amount in the correct volume and buffer.

✔	Step	Instructions
		DNA shearing
	2.1	Bring DNA up to a final volume of **100 µL–130 µL** with **low TE buffer**. Target a concentration of **30 ng/µL** (range: **3 ng/µL–39 ng/µL**).
	2.2	Shear DNA on the Megaruptor 3 system. Genome / Shear speed / Insert length Human, plant, or animal / **31** / 15 kb–18 kb Microbe / **40** / 7 kb–10 kb
	2.3	Recover sheared DNA into a tube strip. Typical volume loss is 5 µL–10 µL.
		Cleanup with 1X SMRTbell cleanup beads
	2.4	Add **1.0X** v/v (volume over volume) of resuspended, room-temperature SMRTbell cleanup beads to each tube of sheared DNA.
	2.5	Pipette mix the beads until evenly distributed.
	2.6	Quick spin the tube strip in a microcentrifuge to collect liquid.
	2.7	Leave at **room temperature** for **10 minutes** to allow DNA to bind beads.
	2.8	Place tube strip in a magnetic separation rack until beads separate fully from the solution.
	2.9	Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant.
	2.10	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.
	2.11	Repeat the previous step.
	2.12	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.
	2.13	Remove 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.
	2.14	Quick spin the tube strip in a microcentrifuge to collect liquid.

2.15	Leave at **room temperature** for **5 minutes** to elute DNA.
2.16	Place tube strip in a magnetic separation rack until beads separate fully from the solution.
2.17	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a **new tube strip**. Discard old tube strip with beads.
2.18	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.
2.19	Proceed to the next step of the protocol if sample quality is acceptable.

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3. Repair and A-tailing

✔	Step	Instructions
	3.1	Add the following components in the order and volume listed below to a new microcentrifuge tube. Adjust component volumes for the number of samples being prepared, plus 10% overage. For individual preps, add components directly to the sample from the previous step at the specified volumes and skip RM1 steps (3.2 to 3.4).

Reaction Mix 1 (RM1)

✔	Tube	Component	Volume
	Purple	Repair buffer	8 µL
	Blue	End repair mix	4 µL
	Green	DNA repair mix	2 µL
		Total volume	**14 µL**

	3.2	Pipette mix **RM1**.
	3.3	Quick spin **RM1** in a microcentrifuge to collect liquid.
	3.4	Add **14 µL** of the **RM1** to each sample**.** Total reaction volume should be **60 µL**.
	3.5	Pipette mix each sample.
	3.6	Quick spin the strip tube in a microcentrifuge to collect liquid.
	3.7	Run the **repair and A-tailing** thermocycler program.
	3.8	Proceed to the next step of the protocol.

4. Adapter ligation and cleanup

✔	Step	Instructions
		Adapter ligation
	4.1	Add **4 µL** of SMRTbell adapter (non-barcoded) or SMRTbell barcoded adapter 3.0 to each sample from the previous step.
	4.2	Add the following components in the order and volume listed below to a new microcentrifuge tube. Adjust component volumes for the number of samples 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 RM2 steps (4.3 to 4.5).
	4.3	Pipette mix **RM2**.
	4.4	Quick spin **RM2** in a microcentrifuge to collect liquid.
	4.5	Add **31 µL** of **RM2** to each sample from previous step. Total volume should be **95 µL.**
	4.6	Pipette mix each sample.
	4.7	Quick spin the strip tube in a microcentrifuge to collect liquid.
	4.8	Run the **adapter ligation** thermocycler program.
		Cleanup with 1X SMRTbell cleanup beads
	4.9	Add **95 µL** of resuspended, room-temperature SMRTbell cleanup beads to each sample.
	4.10	Pipette mix the beads until evenly distributed.
	4.11	Quick spin the tube strip in a microcentrifuge to collect all liquid from the sides of the tubes.
	4.12	Leave at **room temperature** for **10 minutes** to allow DNA to bind beads.
	4.13	Place tube strip in a magnetic separation rack until beads separate fully from the solution.
	4.14	Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant.
	4.15	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.
	4.16	Repeat the previous step.
	4.17	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.
	4.18	Remove tube strip from the magnetic rack. **Immediately** add **40 µL** of **elution buffer** to each tube and resuspend the beads.
	4.19	Quick spin the tube strip in a microcentrifuge.
	4.20	Leave at **room temperature** for **5 minutes** to elute DNA.
	4.21	Place tube strip in a magnetic separation rack until beads separate fully from the solution.
	4.22	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a **new tube strip**. Discard old tube strip with beads.
	4.23	Proceed to the next step of the protocol.

Reaction Mix 2 (RM2)

✔	Tube	Component	Volume
	Yellow	Ligation mix	30 µL
	Red	Ligation enhancer	1 µL
		Total volume	**31 µL**

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5. Nuclease treatment

✔	Step	Instructions
	5.1	Add the following components in the order and volume listed below to a new microcentrifuge tube. Adjust component volumes for the number of samples 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 **RM3** steps (5.2 to 5.4).

Reaction Mix 3 (RM3)

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

	5.2	Pipette mix **RM3**.
	5.3	Quick spin **RM3** in a microcentrifuge to collect liquid.
	5.4	Add **10 µL** of **RM3** to each sample. Total volume should equal **50 µL**.
	5.5	Pipette mix each sample.
	5.6	Quick spin the strip tube in a microcentrifuge to collect liquid.
	5.7	Run the **nuclease treatment** thermocycler program.
	5.8	Proceed to the next step of the protocol.

6. AMPure PB beads size selection or cleanup with SMRTbell cleanup beads

AMPure PB beads size selection effectively removes fragments shorter than 5 kb. Size selection is sensitive to bead concentrations; therefore, dilute carefully and retain supernatant in case of poor DNA recovery.

✔	Step	Instructions for AMPure PB bead size selection
	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.
	2	Add 3.1X v/v (155 µL) of resuspended, room-temperature 35% AMPure PB beads to each sample from the previous step.
	3	Proceed to step 6.1 in the table below.

OR

Standard cleanup with 1X v/v SMRTbell cleanup beads. Use when performing an alternative size selection method, or for applications where fragments shorter than 5 kb are desired (*e.g.* microbial or metagenomic samples). See *Technical Note - Alternative size selection methods for SMRTbell prep kit 3.0* for procedural details on gel-cassette based size selection methods.

✔	Step	Instructions for cleanup with 1X (v/v) SMRTbell cleanup beads
	1	Add **50 µL** SMRTbell cleanup beads to each sample from the previous step.
	2	Proceed to step 6.1 in the table below

Bind, wash, and elution steps.

✔	Step	Instructions for bead binding, washing, and sample elution
	6.1	Pipette mix the beads until evenly distributed.
	6.2	Quick spin the tube strip in a microcentrifuge to collect all liquid.
	6.3	Leave at **room temperature** for **20 minutes** for AMPure PB beads and **10 minutes** for SMRTbell cleanup beads to allow DNA to bind beads.
	6.4	Place tube strip in a magnetic separation rack until beads separate fully from the solution.
	6.5	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.
	6.6	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.
	6.7	Repeat the previous step.
	6.8	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.
	6.9	Remove tube strip from the magnetic rack. **Immediately** add **15 µL** of **elution buffer** to each tube and resuspend the beads by pipetting 10 times or until evenly distributed.
	6.10	Quick spin the tube strip in a microcentrifuge to collect liquid.

6.11	Leave at **room temperature** for **5 minutes** to elute DNA.
6.12	Place tube strip in a magnetic separation rack until beads separate fully from the solution.
6.13	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a **new tube strip**. Discard old tube strip with beads.
6.14	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.
	To multiplex libraries follow step 6.15, otherwise skip, and proceed to step 6.16
6.15	Multiplex samples by combining an **equal mass** of each barcoded SMRTbell library in a DNA LoBind microcentrifuge tube. • Total combined mass should be ≥300 ng. • Determine molarity from the concentration and average insert size using the conversion calculator provided in SMRT Link Sample Setup. • If the molarity of the multiplexed pool is **≥2 nM**, then it is safe to proceed to step 6.17. • Otherwise, concentrate the multiplexed pool using another round of 1X (v/v) SMRTbell cleanup bead purification as described above in steps 6.1 through 6.14.
6.16	Proceed to **SMRT Link Sample Setup** to prepare the SMRTbell library for sequencing.
6.17	Store SMRTbell libraries at 4°C if sequencing within the week. Long-term storage should be at -20°C. Minimize freeze-thaw cycles when handling SMRTbell libraries.

PROTOCOL COMPLETE

Revision history (description)	Version	Date
Initial release.	01	April 2022