Preparing multiplexed amplicon libraries using SMRTbell® prep kit 3.0

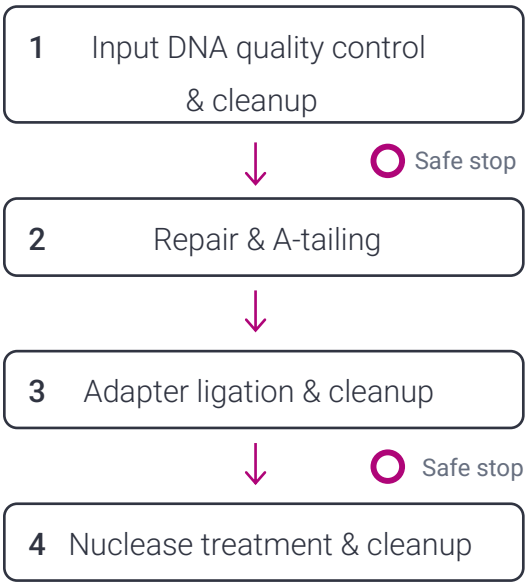
Procedure & checklist

This procedure describes the workflow for constructing amplicon libraries using the SMRTbell® prep kit 3.0 for sequencing on PacBio Sequel ® II and IIe systems. Amplicons may be barcoded during PCR, or during library preparation with SMRTbell barcoded adapters.

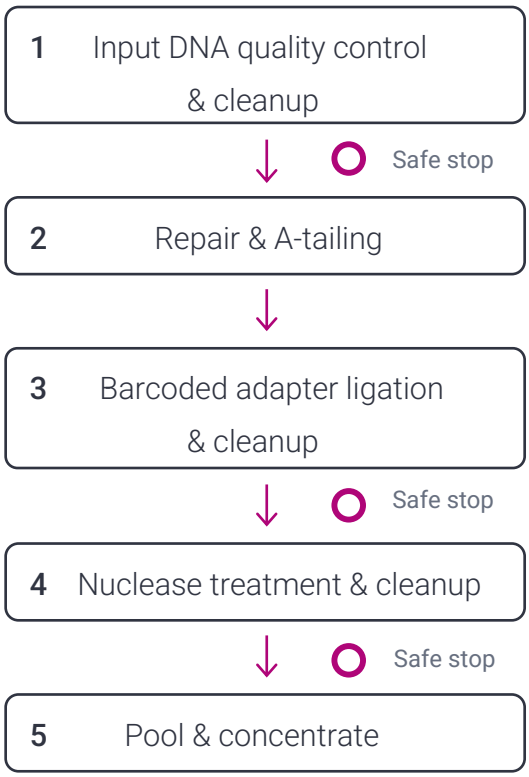
Overview		
	PCR barcoded samples	**Adapter barcoding**
Samples per kit	1 – 24	1 – 24
Workflow time	3.5 hours	4 hours
Size	250 – 25,000 bp	250 – 25,000 bp
DNA input per SMRT Cell 8M	150 – 1000 ng per pool	150 – 1000 ng per sample

Workflow

Primer-barcoded samples



Adapter-barcoded samples



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Required materials and equipment

DNA sizing (one or more of the following)	
1% agarose gel, an electrophoresis unit, and imager	Any Major Lab Supplier (MLS)
2100 Bioanalyzer	Agilent technologies G2939BA
4200 TapeStation	Agilent technologies G2991BA
5300 or 5400 Fragment analyzer	Agilent technologies M5311AA or M5312AA
FEMTO Pulse system	Agilent Technologies M5330AA
DNA quantitation	
Qubit fluorometer	Thermo Fisher Scientific Q33238
Qubit 1X dsDNA HS assay kit	Thermo Fisher Scientific Q33230
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 MLS
Nuclease-free water, molecular biology grade	Any MLS
8-channel Pipettes, P20 and P200	Any MLS
0.2 mL 8-tube strips	USA Scientific TempAssure 1402-4708
Single-channel Pipettes (P10, P20, P100, P200, and P1000)	Any MLS
Microcentrifuge	Any MLS
Magnetic separation rack compatible with 0.2 mL 8-tube strips	Any MLS
Thermocycler compatible with 0.2 mL 8-tube strips	Any MLS
1.5 mL DNA LoBind® Tubes	Eppendorf 022431021

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General best practices

DNA input amount

The total amount of DNA input required for constructing the SMRTbell library is dependent on the mean size of the amplicons, or DNA fragment (e.g., plasmid), being sequenced.

PCR-barcoded sample input

If samples were barcoded during PCR, then the samples can be pooled prior to library prep and **the total DNA input amount will equal the amount of the multiplexed pool**. The per sample input will equal the total DNA input divided by the number of multiplexed samples.

Use no less than 150 ng of total input per SMRT Cell 8M to ensure sufficient library yields for optimal SMRT cell loading when working with targets less than a mean size of 5 kb. Larger amplicons will require more input material to achieve desired molarity for SMRT cell loading. Please refer to the table for the recommended minimum total input amounts per SMRT Cell 8M that will be used for the library.

Mean size	Minimum pooled amount per SMRT Cell 8M*
< 5 kb	150 ng
5 – 7 kb	200 ng
> 7 kb	300 ng

*Lower total input amounts are possible but may result in lower sequencing yield if there is not enough SMRTbell library available for optimal SMRT cell loading.

Adapter-barcoded sample input

Use a minimum of 150 ng of DNA input per sample for samples with mean size less than 5 kb, 200 ng between 5 and 7 kb, and 300 ng when greater than 7 kb. This is to ensure sufficient recovery of each sample at the end of library prep for equal mass or equal molar pooling. Using lower per-sample amounts, though possible, may result in low library yields and lead to uneven pooling and sequence coverage. For applications that require lower input amounts, consider using barcoded primers so samples can be pooled prior to library prep.

Mean size	Minimum per sample amount per SMRT Cell 8M
< 5 kb	150 ng
5 – 7kb	200 ng
> 7 kb	300 ng

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Barcoding and multiplexing

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

Pool amplicons of similar size for optimal sequence yields across all samples.

Pool amplicons ≥ 3 kb separately from amplicons ≤ 3 kb for optimal sequencing yields across all samples.

When amplicons are similar in size, pool an equal mass for each sample. Some experiments may require equal molar pooling if the mean size differs between samples and similar coverage levels are required.

Sample quality

Using gel-extracted amplicon products may results in lower sequencing performance due to the damage inherently caused by intercalating dyes such as ethidium bromide and exposure to UV radiation. Sequencing amplicons stained with SYBR dyes from ThermoFisher Scientific is untested, and therefore cannot be recommended. If working with a gel-extracted product that has been stained with a dye, it is recommended to bring it through additional rounds of amplification to remove damage and/or dyes prior to library prep and sequencing.

Reagent 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 & 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.

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 & A-tailing

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

2. Adapter ligation

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

3. Nuclease treatment

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

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Procedure and checklist

1. Input DNA quality control & cleanup

Prior to library preparation, evaluate the quantity and size distribution of input DNA to determine whether it is suitable for the protocol.

✔	Step	Instructions
		DNA QC
	1.1	Measure DNA concentration of each sample with a Qubit fluorometer using the **1X dsDNA HS kit** following manufacturer's instructions.
	1.2	**Recommended**: measure the DNA size distribution with the appropriate sizing technology following the manufacturer's instructions. • Amplicons ≤ 10 kb: Agilent 2100 Bioanalyzer, TapeStation, or Fragment Analyzer. • Amplicons ≥ 10 kb: Agilent FEMTO Pulse system.
	1.3	Proceed to the next step if sample concentration and quality is acceptable.
	1.4	Add the appropriate mass of each sample to a 0.2 mL PCR strip tube. If volume exceeds 100 µL, then use a 1.5 mL DNA LoBind tube instead.
		Cleanup with 1.3X SMRTbell cleanup beads
	1.5	Add **1.3X** volume per volume **(v/v)** of resuspended, room-temperature SMRTbell cleanup beads to each tube.
	1.6	Pipette mix the beads until evenly distributed.
	1.7	Quick spin the tube strip in a microcentrifuge to collect liquid.
	1.8	Leave at **room temperature** for **10 minutes** to allow DNA to bind beads.
	1.9	Place tube strip in a magnetic separation rack until beads separate fully from the solution.
	1.10	Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant.
	1.11	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.
	1.12	Repeat the previous step.
	1.13	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.
	1.14	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.
	1.15	Quick spin the tube strip in a microcentrifuge to collect liquid.
	1.16	Leave at **room temperature** for **5 minutes** to elute DNA.
	1.17	Place tube strip in a magnetic separation rack until beads separate fully from the solution.
	1.18	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a **new 0.2 mL PCR tube strip**. Discard old tube strip with beads.
		SAFE STOPPING POINT – Store at 4°C

2. Repair & A-tailing

✔	Step	Instructions
	2.1	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 (2.2 to 2.4).

Reaction mix 1 (RM1)

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

	2.2	Pipette mix RM1.
	2.3	Quick spin RM1 in a microcentrifuge to collect liquid.
	2.4	Add 14 µL of the RM1 to each sample. Total reaction volume should be 60 µL.
	2.5	Pipette mix each sample.
	2.6	Quick spin the strip tube in a microcentrifuge to collect liquid.
	2.7	Run the repair & A-tailing thermocycler program.
	2.8	Proceed to the next step of the protocol.

3. Adapter ligation & cleanup

✔	Step	Instructions
		Adapter ligation
	3.1	Add **4 µL** of SMRTbell adapter (primer-barcoded amplicon pools) or SMRTbell barcoded adapter 3.0 (non-barcoded amplicons) to each sample tube from previous step.
	3.2	Add the following components in the order and volume listed below to a microfuge 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 (3.3 to 3.5).
	3.3	Pipette mix **RM2**.
	3.4	Quick spin **RM2** in a microcentrifuge to collect liquid.
	3.5	Add **31 µL** of **RM2** to each sample from previous step. Total volume should be **95 µL.**
	3.6	Pipette mix each sample.
	3.7	Quick spin the strip tube in a microcentrifuge to collect liquid.
	3.8	Run the **adapter ligation** thermocycler program.
		Cleanup with 1.3X SMRTbell cleanup beads
	3.9	Add **124 µL** of resuspended, room-temperature SMRTbell cleanup beads to each sample.
	3.10	Pipette mix the beads until evenly distributed.
	3.11	Quick spin the tube strip in a microcentrifuge to collect all liquid from the sides of the tubes.
	3.12	Leave at **room temperature** for **10 minutes** to allow DNA to bind beads.
	3.13	Place 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. Discard the supernatant.
	3.15	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.
	3.16	Repeat the previous step.

Reaction mix 2 (RM2)

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

3.17	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.
3.18	Remove tube strip from the magnetic rack. **Immediately** add **40 µL** of e**lution buffer** to each tube and resuspend the beads by pipetting 10 times or until evenly distributed.
3.19	Quick spin the tube strip in a microcentrifuge.
3.20	Leave at **room temperature** for **5 minutes** to elute DNA.
3.21	Place tube strip in a magnetic separation rack until beads separate fully from the solution.
3.22	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a **new tube strip**. Discard old tube strip with beads.
3.23	Proceed to the next step of the protocol.

SAFE STOPPING POINT – Store at 4°C

4. Nuclease treatment & cleanup

✔	Step	Instructions
		Nuclease treatment
	4.1	Add the following components in the order and volume listed below to a microfuge 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 (4.2 to 4.4).

Reaction mix 3 (RM3)

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

4.2	Pipette mix **RM3**.
4.3	Quick spin **RM3** in a microcentrifuge to collect liquid.
4.4	Add **10 µL** of **RM3** to each sample. Total volume should equal **50 µL.**
4.5	Pipette mix each sample.
4.6	Quick spin the strip tube in a microcentrifuge to collect liquid.
4.7	Run the **nuclease treatment** thermocycler program.

	Cleanup with 1.3X SMRTbell cleanup beads
4.8	Add **65 µL** of resuspended, room-temperature SMRTbell cleanup beads to each sample.
4.9	Pipette mix the beads until evenly distributed.
4.10	Quick spin the tube strip in a microcentrifuge to collect all liquid from the sides of the tubes.
4.11	Leave at **room temperature** for **10 minutes** to allow DNA to bind beads.
4.12	Place tube strip in a magnetic separation rack until beads separate fully from the solution.
4.13	Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant.
4.14	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.
4.15	Repeat the previous step.
4.16	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.
4.17	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.
4.18	Quick spin the tube strip in a microcentrifuge.
4.19	Leave at **room temperature** for **5 minutes** to elute DNA.
4.20	Place tube strip in a magnetic separation rack until beads separate fully from the solution.
4.21	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a **new tube strip**. Discard old tube strip with beads.
4.22	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.
4.23	For **primer-barcoded samples** that don't require any additional pooling, proceed to SMRT Link Sample Setup to prepare sample(s) for sequencing. Protocol is complete. 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. For samples **barcoded with SMRTbell barcoded adapters** and require pooling, proceed to the next section for instructions on pooling and concentrating.

5. Pooling & concentrating samples with SMRTbell barcoded adapters

✓	Step	Instructions
		Pooling
	5.1	Combine an **equal mass** of each SMRTbell barcoded adapter sample together into a single pool using a 1.5 mL DNA LoBind Tube. Proceed to the next step to concentrate the pool. Alternatively, samples can be pooled in **equal molar** portions for better sequence coverage balance. Total SMRTbell library mass should be ≥ **100 ng** when amplicon sizes are less than 10 kb, and ≥ **300 ng** when greater or equal to 10 kb.
		Concentrate with 1.3X SMRTbell cleanup beads
	5.2	Add **1.3X v/v** SMRTbell cleanup beads to each pool.
	5.3	Pipette mix the beads until evenly distributed.
	5.4	Quick spin the tube in a microcentrifuge to collect all liquid.
	5.5	Leave at room temperature for **10 minutes** to allow DNA to bind beads.
	5.6	Place tube in a magnetic separation rack until beads separate fully from the solution.
	5.7	Slowly pipette off the cleared supernatant without disturbing the beads.
	5.8	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.
	5.9	Repeat the previous step.
	5.10	Remove residual 80% ethanol: • Remove tube from the magnetic separation rack. • Quick spin tube in a microcentrifuge. • Place tube back in a magnetic separation rack until beads separate fully from the solution. • Pipette off residual 80% ethanol and discard.
	5.11	Remove tube 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.
	5.12	Quick spin the tube in a microcentrifuge to collect liquid.
	5.13	Leave at **room temperature** for **5 minutes** to elute DNA.
	5.14	Place tube in a magnetic separation rack until beads separate fully from the solution.
	5.15	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a **new tube**. Discard old tube with beads.
	5.16	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.
	5.17	Proceed to SMRT Link Sample Setup to prepare sample(s) for sequencing. 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	Apr 2022
Clarify minimum DNA input requirements and best practices.	02	Sep 2022