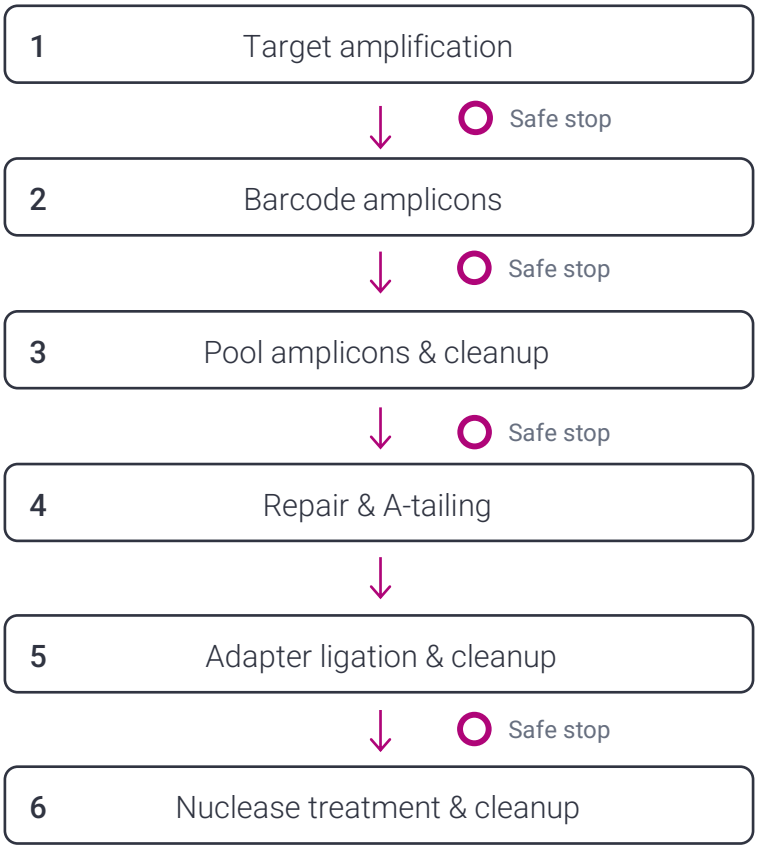
Preparing multiplexed amplicon libraries using PacBio barcoded M13 primers and SMRTbell® prep kit 3.0

Procedure & checklist

This procedure describes the workflow for barcoding amplicons with the barcoded M13 primer plate and constructing SMRTbell libraries using the SMRTbell prep kit 3.0 for sequencing on the Sequel® II and IIe systems. The barcoded M13 primer plate contains 384, 16 bp dual indices.

Overview	
Samples	384
Pooled amplicon input	300–1000 ng
M13 tailed forward primer	/5AmMC6/**GTAAAACGACGGCCAGT**$(N)_n$
M13 tailed reverse primer	/5AmMC6/**CAGGAAACAGCTATGAC**$(N)_n$

Workflow



PN 101-921-300 REV03 MAR2023

Required materials and equipment

PCR		
Forward M13-tailed target-specific primer with 5' block. M13 sequence shown. Target-specific sequence represented by N (any base) n times.	/5AmMC6/GTAAAACGACGGCCAGT$(N)_n$	Any Oligo Synthesis Company (standard desalting)
Reverse M13-tailed target-specific primer with 5' block. M13 sequence shown. Target-specific sequence represented by N (any base) n times.	/5AmMC6/CAGGAAACAGCTATGAC$(N)_n$	Any Oligo Synthesis Company (standard desalting)
Barcoded M13 primer plate 384 well	PacBio® 102-135-500	
KAPA HiFi HotStart ReadyMix PCR Kit	Roche KK2600, KK2601, or KK2602	
96- or 384-block PCR thermocycler with compatible PCR strip tubes, plates, and sealers.	Any Major Lab Supplier (MLS)	
Microcentrifuge tubes for master mixes	Any MLS	
Nuclease-free, water	Any MLS	
SMRTbell library preparation		
SMRTbell prep kit 3.0	PacBio 102-182-700	
200 proof ethanol, molecular biology or ACS grade	Any MLS	
8- or 12-multichannel pipettes (P10, P20, and P200)	Any MLS	
Single channel pipettes (P2, P10, P20, P200, and P1000)	Any MLS	
0.2 mL 8-tube strips	USA Scientific TempAssure 1402-4708	
SMRTbell bead cleanup steps (one or more of the following)		
HDPE 8 place Magnetic Separation Rack for 0.2 mL PCR Tubes (for low throughput)	V&P Scientific Inc. VP772F4-1	
96-well magnetic separator (for high throughput)	Any MLS	
DNA quantification		
Qubit™ Fluorometer	ThermoFisher Scientific Q33238	
Qubit 1x dsDNA HS Assay Kit	ThermoFisher Scientific Q33230	
DNA sizing (one or more of the following)		
1% agarose gel, an electrophoresis unit, and imager	Any MLS	
2100 Bioanalyzer	Agilent technologies G2939BA	
4200 TapeStation	Agilent technologies G2991BA	
5300 or 5400 Fragment Analyzer	Agilent technologies M5311AA or M5312AA	
FEMTO Pulse	Agilent technologies M5330AA	

PN 101-921-300 REV03 MAR2023

General best practices

PCR

Add a 5' block (**5AmMC6**\) and M13 sequence to all first-round, target-specific primers. The 5' block prevents unbarcoded amplicons from ligating to the SMRTbell adapters during library prep.

Follow the manufacturer's instructions and any necessary adjustments to annealing temperature, $MgCl_2$ concentration, and GC-rich targets to optimize PCR.

Keep all KAPA HiFi HotStart reagents and reactions on ice until PCR; the high proofreading activity of the enzyme will rapidly degrade primers at room temperature. This is generally true for all high-fidelity polymerases.

Use high-quality DNA and work in a PCR-clean environment to avoid contamination.

Use non-template control (NTC) to check for contamination.

Optimize PCR parameters to enable equal volume pooling and prevent off-target amplification and primer-dimers. Off-target products and high levels of primer dimers may reduce sequencing yields and performance.

Use the fewest number of PCR cycles required for obtaining adequate yields (ng).

Avoid using gel-extraction and intercalating dyes such as ethidium bromide on the 2nd round (barcoded) amplicons because this causes DNA damage which will impact sequencing yields.

Amplicon input into library prep

Use a total pooled amplicon amount of **300–1000 ng** to ensure optimal loading and sequencing yields. Larger amplicons require higher input amounts relative to smaller amplicons to achieve the required molarity for SMRT® cell loading.

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 a 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

PN 101-921-300 REV03 MAR2023

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

Pool amplicons ≤3 kb separately from those >3 kb in length for optimal loading and sequencing performance.

Normalizing DNA input into PCR and optimizing PCR will improve sequence coverage balance across samples when pooling amplicons in an equal volume fashion.

Pooling amplicons of different sizes will increase sequence coverage variability because of differences in molarity between those samples. Differences in the number of molecules in a sample will translate to the differences in the number of molecules loaded and sequenced on the SMRT Cell 8M.

Thermocycler programs

PCR programs

1. Target amplification using the KAPA HiFi HotStart Ready Mix program

   Parameters may be adjusted as needed to optimize PCR.

Step	Time	Temperature
1	3 min	95°C
2	20 sec	98°C
3	15 sec	Tm of target-specific primers
4	15–60 sec/kb	72°C
5	Repeat steps 2 to 4	-
6	20 sec	98°C
7	15 sec	65°C
8	15–60 sec/kb	72°C
9	Repeat steps 6 to 8 for 20 cycles	-
10	5 min	72°C
11	Hold	4°C

2. Barcoded M13 primer program

   Parameters may be adjusted as needed to optimize PCR.

Step	Time	Temperature
1	3 min	95°C
2	20 sec	98°C
3	15 sec	60°C
4	15–60 sec/kb	72°C
5	Repeat steps 2 to 4	-
6	20 sec	98°C
7	15 sec	65°C
8	15–60 sec/kb	72°C
9	Repeat steps 6 to 8 (20 cycles total)	-
10	5 min	72°C
11	Hold	4°C

SMRTbell prep kit 3.0 programs

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 SMRTbell prep kit programs. If the lid temperature is not programmable, it is acceptable to leave at 95–105°C.

1. Repair & 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

Procedure and checklist

1. Target amplification with M13-tailed primers

✔	Step	Instructions

1.1 Determine the volume of DNA that will used and make a PCR master mix by adding the following components to a microcentrifuge tube (1.5 or 2.0 mL) in the order listed below. Volume of nuclease-free water may be adjusted to account for higher volumes of DNA input. Higher than 10% overage may be necessary for automation or high number of samples to account for the volume loss during pipetting.

PCR master mix		
Component	**1X volume**	**Volume for N samples**
Nuclease-free water	6.50 – (DNA) µL	(6.50 - DNA µL) x N x 1.1
3 µM M13 tailed target-specific forward primer	2.50 µL	2.50 µL x N x 1.1
3 µM M13 tailed target-specific reverse primer	2.50 µL	2.50 µL x N x 1.1
2X KAPA HiFi HotStart ReadyMix	12.5 µL	12.5 µL x N x 1.1
Total volume	**Up to 24.0 µL**	**Up to 26.4 µL x N**

1.2 Pipette-mix and quick-spin in microcentrifuge to collect liquid.

1.3 **Optional:** distribute the PCR master mix volume evenly across a 0.2 mL PCR strip tube. This will enable using a multichannel pipet to add the master mix to the 96- or 384-well PCR plate.

1.4 Combine the following components into each respective PCR well or tube in the order listed below. Total volume should be 25.0 µL per PCR well/tube.

Component	Volume
PCR master mix	17.5–24.0 µL
DNA sample (>1 ng/µL)	1.00–6.50 µL
Total volume	**25.0 µL**

1.5 Seal PCR plate or strip tubes.

1.6 Quick-spin PCR plate or strip tubes in a microcentrifuge to collect liquid at bottom.

1.7 Place PCR plate or strip tubes on a thermocycler and run a program optimized for target(s) amplification. Start with the recommended **1st round PCR conditions using the KAPA HiFi HotStart Ready Mix** thermal program listed at the beginning of the protocol if performing PCR for the first time with the M13-tagged primers. Please refer to the KAPA HotStart ReadyMix technical data sheet for specific guidance on annealing and extension parameters.

1.8 **Optional:** perform a quality control on the amplified samples to verify expected target size was amplified using an agarose gel or DNA sizing technology (e.g., TapeStation).

1.9 Proceed to the next step of the protocol.

SAFE STOPPING POINT – Store at 4°C

2. Barcode amplicons with barcoded M13 primers

✔	Step	Instructions
	2.1	Make a barcoding PCR master mix by adding the following components to a microcentrifuge tube in the order listed below. Please note that higher than 10% overage may be necessary for automation or a high number of samples to account volume loss during pipetting.

Barcoding PCR master mix

Component	1X volume	Volume for N samples
Nuclease-free water	9.00 µL	9.00 µL x N x 1.1
2X KAPA HiFi HotStart ReadyMix	12.5 µL	12.5 µL x N x 1.1
Total volume	**21.5 µL**	**23.7 µL x N**

	2.2	Pipette-mix and quick-spin to collect liquid.
	2.3	**Optional:** distribute the PCR master mix volume evenly across a 0.2 mL PCR strip tube. This will enable using a multichannel pipet to add the master mix to the 96- or 384-well PCR plate.
	2.4	Combine the components listed below into a new PCR well or tube. Total volume should equal to 25 µL.

Component	Volume
Barcoding PCR master mix	21.5 µL
Barcoded M13 primer pair	2.5 µL
M13-tailed amplicon (previous PCR)	1.0 µL
Total volume	**25.0 µL**

	2.5	Seal PCR plate or strip tubes.
	2.6	Quick-spin PCR plate or strip tubes in a microcentrifuge to collect liquid at bottom.
	2.6	Place PCR plate or strip tubes on the thermocycler and run the **barcoded M13 primer program** listed at the beginning of the protocol. Adjust the extension time as appropriate for the amplicon size (15–60 sec/kb). The number of cycles can be increased up to 25 as necessary.
	2.7	Perform a quality control on the amplified samples using an agarose gel or DNA sizing technology. If off-target amplification is observed, PCR parameters may need to be optimized. Primer dimers will be removed using the SMRTbell cleanup beads.
	2.8	Proceed to the next step of the protocol if amplification was successful.

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3. Pool and cleanup

✔	Step	Instructions
	3.1	Pool an **equal volume** of each barcoded sample together in a 2.0 mL DNA LoBind tube. Do not exceed a total volume of 800 µL. • 96 samples use 8 µL per sample (for a total of approximately 800 µL) • 384 samples use 2 µL per sample (for a total of approximately 800 µL)
	3.2	Add **1.3X v/v** of resuspended, room-temperature SMRTbell cleanup beads to pooled sample.
	3.3	Mix beads by pipetting until evenly distributed.
	3.4	Quick-spin the tube in a microcentrifuge to collect liquid.
	3.5	Leave at **room temperature** for **10 minutes** to allow DNA to bind beads.
	3.6	Place the tube in a magnetic separation rack until beads separate fully from the solution.
	3.7	Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant.
	3.8	Slowly dispense **500 µL**, or enough to cover the beads, of **freshly prepared 80% ethanol** into the tube. After **30 seconds**, pipette off the 80% ethanol and discard.
	3.9	Repeat the previous step.
	3.10	Remove residual 80% ethanol: • Remove the tube from the magnetic separation rack. • Quick-spin the tube in a microcentrifuge. • Place the tube back in a magnetic separation rack until beads separate fully from the solution. • Pipette off residual 80% ethanol and discard.
	3.11	Remove the tube from the magnetic rack. **Immediately** add **47 µL** of **low TE buffer** to the tube and resuspend the beads by pipetting 10 times or until evenly distributed.
	3.12	Quick-spin the tube in a microcentrifuge to collect liquid.
	3.13	Leave at **room temperature** for **5 minutes** to elute DNA.
	3.14	Place the tube in a magnetic separation rack until beads separate fully from the solution.
	3.15	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a **0.2 mL strip tube**. Discard the old tube with beads.
	3.16	Measure the concentration of the sample on a Qubit fluorometer using the 1x dsDNA HS Kit to verify there is at least **300–1000 ng** of DNA for SMRTbell library prep.
	3.17	Proceed to the next step of the protocol

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4. Repair & a-tailing

✔	Step	Instructions
	4.1	Add the following components to the sample in the order and volume listed below.

✔	Tube	Component	Volume per sample
	Previous	Contents from previous step	46 µL
	Purple	Repair buffer	8 µL
	Blue	End repair mix	4 µL
	Green	DNA repair mix	2 µL
		Total volume	**60 µL**

	4.2	Pipette-mix.
	4.3	Quick-spin the strip tube in a microcentrifuge to collect liquid.
	4.4	Run the **repair & A-tailing** thermocycler program.
	4.5	Proceed to the next step of the protocol.

5. Adapter ligation & cleanup

✔	Step	Instructions
		Adapter ligation
	5.1	Add the following components to the sample in the order and volume listed below.

✔	Tube	Component	Volume per sample
	Previous	Contents from previous step	60 µL
	Orange	SMRTbell adapter	4 µL
	Yellow	Ligation mix	30 µL
	Red	Ligation enhancer	1 µL
		Total volume	**95 µL**

	5.2	Pipette-mix.
	5.3	Quick-spin the strip tube in a microcentrifuge to collect liquid.
	5.4	Run the **adapter ligation** thermocycler program.
		Cleanup with 1.3X SMRTbell cleanup beads
	5.5	Add **124 µL** of resuspended, room temperature SMRTbell cleanup beads to the sample.
	5.6	Pipette-mix the beads until evenly distributed.
	5.7	Quick-spin the tube strip in a microcentrifuge to collect all liquid from the sides of the tubes.
	5.8	Leave at **room temperature** for **10 minutes** to allow DNA to bind beads.

5.9	Place the tube strip in a magnetic separation rack until beads separate fully from the solution.
5.10	Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant.
5.11	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.
5.12	Repeat the previous step.
5.13	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.
5.14	Remove the tube strip from the magnetic rack. **Immediately** add **40 µL** of **elution buffer** to each tube and resuspend the beads by pipetting 10 times or until evenly distributed.
5.15	Quick-spin the tube strip in a microcentrifuge.
5.16	Leave at **room temperature** for **5 minutes** to elute DNA.
5.17	Place the tube strip in a magnetic separation rack until beads separate fully from the solution.
5.18	Slowly pipette-off the cleared supernatant without disturbing the beads. Transfer the supernatant to a **new tube strip**. Discard the old tube strip with beads.
5.19	Proceed to the next step of the protocol.

SAFE STOPPING POINT – Store at 4°C

6. Nuclease treatment & cleanup

✔	Step	Instructions
		Nuclease treatment
	6.1	Add the following components to the sample in the order and volume listed below.

✔	Tube	Component	Volume per sample
	Previous	Contents from previous step	40 µL
	Light purple	Nuclease buffer	5 µL
	Light green	Nuclease mix	5 µL
		Total volume	**50 µL**

6.2	Pipette-mix.
6.3	Quick-spin the strip tube in a microcentrifuge to collect liquid.
6.4	Run the **nuclease treatment** thermocycler program.
	Cleanup with 1.3X SMRTbell cleanup beads
6.5	Add **65 µL** of resuspended, room temperature SMRTbell cleanup beads to the sample.

6.6	Pipette-mix the beads until evenly distributed.
6.7	Quick-spin the tube strip in a microcentrifuge to collect all liquid from the sides of the tubes.
6.8	Leave at **room temperature** for **10 minutes** to allow DNA to bind beads.
6.9	Place the tube strip in a magnetic separation rack until beads separate fully from the solution.
6.10	Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant.
6.11	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.
6.12	Repeat the previous step.
6.13	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.
6.14	Remove the 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.
6.15	Quick-spin the tube strip in a microcentrifuge.
6.16	Leave at **room temperature** for **5 minutes** to elute the DNA.
6.17	Place the tube strip in a magnetic separation rack until beads separate fully from the solution.
6.18	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer the supernatant to a **new tube**. Discard old tube strip with beads.
6.19	Dilute a **1 µL** aliquot with **9 µL** of **elution buffer or water**. Measure DNA concentration with a Qubit fluorometer using the 1x dsDNA HS kit.
6.20	Proceed to SMRT® Link Sample Setup to prepare sample for sequencing, or store SMRTbell library 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	December 2020
Updated for compatibility with SMRTbell preparation kit 3.0	02	April 2022
Updated to clarify that protocol is for Sequel II and IIe systems only	03	March 2023

PN 101-921-300 REV03 MAR2023