

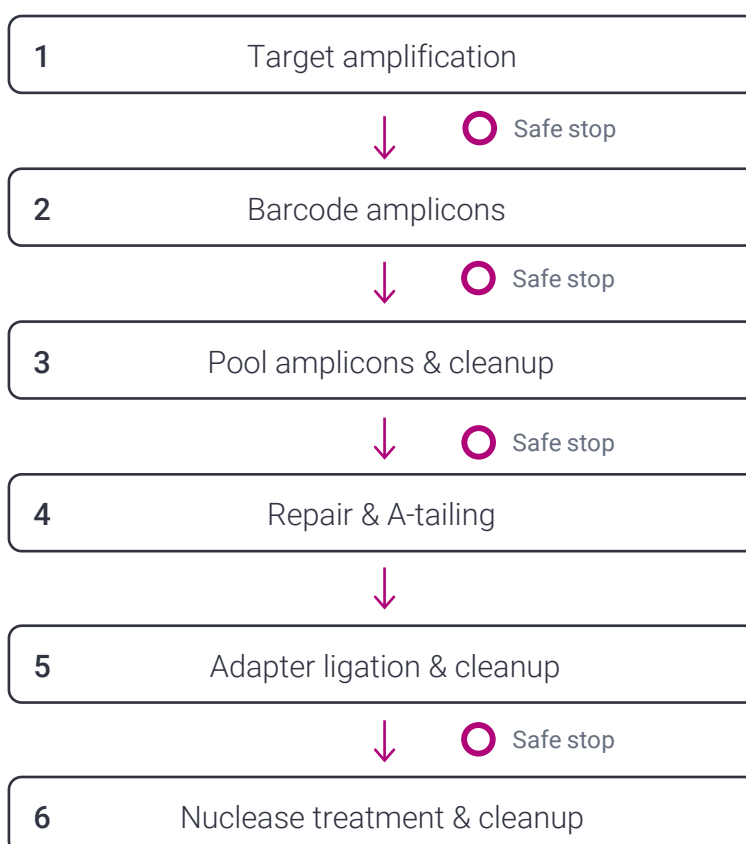
Preparing multiplexed amplicon libraries using PacBio[®] barcoded M13 primers and SMRTbell[®] prep kit

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 PacBio 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



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	
Revio SPRQ™ polymerase kit or Vega™ polymerase kit or Sequel® II binding kit 3.2*	PacBio® 103-520-100 PacBio® 103-517-600 PacBio® 102-333-300	
* Procedure for Sequel II binding kit 3.2 can be found in SMRT® Link Sample Setup.		
General lab supplies and equipment		
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	
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	
DNA quantification		
Qubit™ Fluorometer	ThermoFisher Scientific Q33238	
Qubit 1x dsDNA HS Assay Kit	ThermoFisher Scientific Q33230	
Recommended DNA sizing		
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	

General best practices

PCR

Add a 5' block (\b5AmMC6\b) 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, MgCl₂ concentration, and GC-rich targets to optimize PCR.

Keep all KAPA HiFi HotStart reagents and reactions on ice until PCR, as the high proofreading activity of the enzyme will rapidly degrade primers at room temperature.

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 mass 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.
- Do not vortex enzymes.
- Bring SMRTbell cleanup beads to room temperature. Always vortex immediately prior to use.
- Bring Qubit reagents to room temperature prior to use.
- Thaw frozen reagents at room temperature. Place on ice after thawing.
- Keep master mixes involving temperature-sensitive reagents on ice until use.
- Quick-spin all reagents in a microcentrifuge to collect liquid at the bottom prior to use.
- Samples can be stored at the specified temperature at the safe stopping points listed in the protocol

SMRTbell prep kit 3.0

Kit stored at -20°C.

Component	Tube color
Repair buffer	Purple
End repair mix	Blue

DNA repair mix	Green
Ligation mix	Yellow
Ligation enhancer	Red
Nuclease mix	Light green
Nuclease buffer	Light purple

Polymerase kit

Note: Bring the Loading buffer to room temperature prior to use. The Loading buffer is light sensitive and should be protected from light when not in use.

Kit stored at -20°C.

Component	Tube color
Annealing buffer	Light blue
Standard sequencing primer	Light green
Polymerase buffer	Yellow
Loading buffer	Green
Dilution buffer	Blue
Sequencing polymerase	Purple
Sequencing control	Red

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	T _m 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 incubate 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	<p>Determine the volume of DNA that will be 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.</p> <table border="1"> <thead> <tr> <th colspan="3">PCR master mix</th> </tr> <tr> <th>Component</th> <th>1X volume</th> <th>Volume for N samples</th> </tr> </thead> <tbody> <tr> <td>Nuclease-free water</td> <td>6.50 – (DNA) μL</td> <td>(6.50 - DNA μL) x N x 1.1</td> </tr> <tr> <td>3 μM M13 tailed target-specific forward primer</td> <td>2.50 μL</td> <td>2.50 μL x N x 1.1</td> </tr> <tr> <td>3 μM M13 tailed target-specific reverse primer</td> <td>2.50 μL</td> <td>2.50 μL x N x 1.1</td> </tr> <tr> <td>2X KAPA HiFi HotStart ReadyMix</td> <td>12.5 μL</td> <td>12.5 μL x N x 1.1</td> </tr> <tr> <td>Total volume</td> <td>Up to 24.0 μL</td> <td>Up to 26.4 μL x N</td> </tr> </tbody> </table>	PCR master mix			Component	1X volume	Volume for N samples	Nuclease-free water	6.50 – (DNA) μ L	(6.50 - DNA μ L) x N x 1.1	3 μ M M13 tailed target-specific forward primer	2.50 μ L	2.50 μ L x N x 1.1	3 μ M M13 tailed target-specific reverse primer	2.50 μ L	2.50 μ L x N x 1.1	2X KAPA HiFi HotStart ReadyMix	12.5 μ L	12.5 μ L x N x 1.1	Total volume	Up to 24.0 μL	Up to 26.4 μL x N
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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	<p>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.</p> <table border="1"> <thead> <tr> <th>Component</th> <th>Volume</th> </tr> </thead> <tbody> <tr> <td>PCR master mix</td> <td>17.5–24.0 μL</td> </tr> <tr> <td>DNA sample (>1 ng/μL)</td> <td>1.00–6.50 μL</td> </tr> <tr> <td>Total volume</td> <td>25.0 μL</td> </tr> </tbody> </table>	Component	Volume	PCR master mix	17.5–24.0 μ L	DNA sample (>1 ng/ μ L)	1.00–6.50 μ L	Total volume	25.0 μL													
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PCR master mix	17.5–24.0 μ L																					
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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															
		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.															
2.1		<table border="1"> <thead> <tr> <th colspan="3">Barcoding PCR master mix</th> </tr> <tr> <th>Component</th> <th>1X volume</th> <th>Volume for N samples</th> </tr> </thead> <tbody> <tr> <td>Nuclease-free water</td> <td>9.00 μL</td> <td>9.00 μL x N x 1.1</td> </tr> <tr> <td>2X KAPA HiFi HotStart ReadyMix</td> <td>12.5 μL</td> <td>12.5 μL x N x 1.1</td> </tr> <tr> <td>Total volume</td> <td>21.5 μL</td> <td>23.7 μL x N</td> </tr> </tbody> </table>	Barcoding PCR master mix			Component	1X volume	Volume for N samples	Nuclease-free water	9.00 μ L	9.00 μ L x N x 1.1	2X KAPA HiFi HotStart ReadyMix	12.5 μ L	12.5 μ L x N x 1.1	Total volume	21.5 μL	23.7 μL x N
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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.															
		Combine the components listed below into a new PCR well or tube. Total volume should equal to 25 μL .															
2.4		<table border="1"> <thead> <tr> <th>Component</th> <th>Volume</th> </tr> </thead> <tbody> <tr> <td>Barcoding PCR master mix</td> <td>21.5 μL</td> </tr> <tr> <td>Barcoded M13 primer pair</td> <td>2.5 μL</td> </tr> <tr> <td>M13-tailed amplicon (previous PCR)</td> <td>1.0 μL</td> </tr> <tr> <td>Total volume</td> <td>25.0 μL</td> </tr> </tbody> </table>	Component	Volume	Barcoding PCR master mix	21.5 μ L	Barcoded M13 primer pair	2.5 μ L	M13-tailed amplicon (previous PCR)	1.0 μ L	Total volume	25.0 μL					
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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.															

SAFE STOPPING POINT – Store at 4°C

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 μ L. <ul style="list-style-type: none"> • 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		Pipette mix until evenly distributed.
3.4		Quick-spin the tube in a microcentrifuge to collect liquid.
3.5		Incubate 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 remove 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 , remove the 80% ethanol and discard.
3.9		Repeat the previous step.
3.10		Remove residual 80% ethanol: <ul style="list-style-type: none"> • 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. • Remove 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 pipette-mix until evenly distributed.
3.12		Quick-spin the tube in a microcentrifuge to collect liquid.
3.13		Incubate 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 aspirate the cleared eluate without disturbing the beads. Transfer eluate 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

SAFE STOPPING POINT – Store at 4 °C

4. Repair & a-tailing

✓	Step	Instructions																								
		Add the following components to the sample in the order and volume listed below.																								
		<table border="1"> <thead> <tr> <th>✓</th> <th>Tube</th> <th>Component</th> <th>Volume per sample</th> </tr> </thead> <tbody> <tr> <td></td> <td>Previous</td> <td>Contents from previous step</td> <td>46 μL</td> </tr> <tr> <td>4.1</td> <td>Purple</td> <td>Repair buffer</td> <td>8 μL</td> </tr> <tr> <td></td> <td>Blue</td> <td>End repair mix</td> <td>4 μL</td> </tr> <tr> <td></td> <td>Green</td> <td>DNA repair mix</td> <td>2 μL</td> </tr> <tr> <td colspan="3">Total volume</td> <td>60 μL</td> </tr> </tbody> </table>	✓	Tube	Component	Volume per sample		Previous	Contents from previous step	46 μ L	4.1	Purple	Repair buffer	8 μ L		Blue	End repair mix	4 μ L		Green	DNA repair mix	2 μ L	Total volume			60 μ L
✓	Tube	Component	Volume per sample																							
	Previous	Contents from previous step	46 μ L																							
4.1	Purple	Repair buffer	8 μ L																							
	Blue	End repair mix	4 μ L																							
	Green	DNA repair mix	2 μ L																							
Total volume			60 μ L																							
	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																										
		Add the following components to the sample in the order and volume listed below.																								
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✓	Tube	Component	Volume per sample																							
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5.1	Orange	SMRTbell adapter	4 μ L																							
	Yellow	Ligation mix	30 μ L																							
	Red	Ligation enhancer	1 μ L																							
Total volume			95 μ L																							
	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	Incubate 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 remove 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**, remove 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.
 - Remove 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 pipette-mix until evenly distributed.
- 5.15 Quick-spin the tube strip in a microcentrifuge.
- 5.16 Incubate 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 aspirate the cleared eluate without disturbing the beads. Transfer the eluate 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																	
Add the following components to the sample in the order and volume listed below.																	
6.1	✓	<table border="1"> <thead> <tr> <th>Tube</th> <th>Component</th> <th>Volume per sample</th> </tr> </thead> <tbody> <tr> <td>Previous</td> <td>Contents from previous step</td> <td>40 µL</td> </tr> <tr> <td>Light purple</td> <td>Nuclease buffer</td> <td>5 µL</td> </tr> <tr> <td>Light green</td> <td>Nuclease mix</td> <td>5 µL</td> </tr> <tr> <td colspan="2" style="text-align: center;">Total volume</td> <td>50 µL</td> </tr> </tbody> </table>	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
	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 Incubate 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 remove 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**, remove the 80% ethanol and discard.
- 6.12 Repeat the previous step.
- 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.
- 6.13
- 6.14 Remove the tube strip from the magnetic rack. **Immediately** add **25 μL** of **Elution buffer** to each tube and pipette-mix until evenly distributed.
- 6.15 Quick-spin the tube strip in a microcentrifuge.
- 6.16 Incubate 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 aspirate the cleared eluate without disturbing the beads. Transfer the eluate 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.
- If necessary, dilute 25 μL of SMRTbell library to the concentrations indicated below.** Failure to normalize libraries to the appropriate concentration prior to ABC may result in low sequencing yield.
- | SMRTbell library size | Concentration (ng/ μL) |
|-----------------------|------------------------------------|
| >10 kb | <60 ng/ μL |
| 3–10 kb | <20 ng/ μL |
| <3 kb | <10 ng/ μL |
| <1.5 kb | < 2 ng/ μL |
- 6.20
- 6.21 Proceed to Section 7 to prepare library for sequencing with Revio SPRQ/SPRQ-Nx or Vega
Or
Proceed to SMRT® Link Sample Setup for preparing library for sequencing on Sequel II/e.

7. Annealing, binding, and cleanup (ABC)

This step is for preparing the SMRTbell library (25 μL) for sequencing on supported platforms.

✓	Step	Instructions
		Prepare the appropriate volume of master mix with 10% overage using the per reaction volumes listed below.
		Annealing mix
7.1	✓ Tube	Component Volume
	Light blue	Annealing buffer 12.5 μL
	Light green	Standard sequencing primer 12.5 μL
		Total volume 25 μL
7.2		Pipette-mix the Annealing mix and quick spin to collect liquid.
7.3		Add 25 μL of the Annealing mix to each library for a total volume of 50 μL .
7.4		Pipette-mix each sample and quick spin to collect liquid.
7.5		Incubate at room temperature for 15 minutes .
7.6		During primer incubation, prepare the polymerase dilution (see below) and store on ice.
		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
7.7	✓ Tube	Component Volume
	Yellow	Polymerase buffer 47 μL
	Purple	Sequencing polymerase 3 μL
		Total volume 50 μL
7.8		Pipette mix the polymerase dilution and quick-spin to collect liquid.
7.9		Add 50 μL of polymerase dilution to primer annealed sample for a total volume of 100 μL .
7.10		Pipette-mix each sample and quick-spin to collect liquid.
7.11		Incubate at room temperature for 15 minutes .
7.12		Proceed immediately to the next step of the protocol to remove excess polymerase.
		1.3X SMRTbell bead cleanup
7.13		Add 130 μL of resuspended, room-temperature SMRTbell cleanup beads to each sample
7.14		Pipette-mix the beads until evenly distributed and quick-spin if necessary to collect all liquid from the sides of the tube.
7.15		Incubate at room temperature for 10 minutes to allow DNA to bind beads
7.16		Place sample on an appropriate magnet and allow beads to separate fully from the solution
7.17		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.

Remove sample from the magnet and **immediately** add **Loading buffer** to each tube and resuspend the beads by pipette mixing.

7.18	Revio SPRQ polymerase kit	Vega polymerase kit
	Loading buffer	23 μ L

7.19 Quick-spin the samples to collect any liquid from the sides of the tube.

7.20 Incubate at **room temperature** for **15 minutes** to elute DNA

7.21 Place sample on magnet and allow beads to separate fully from the solution.

7.22 Slowly transfer the cleared eluate without disturbing the beads to a **new tube protected from light**. Discard the old tube with beads. The sample is now ready for sequencing (see Section 10).

PROTOCOL COMPLETE

Important: Polymerase-bound libraries can be stored at 4°C for up to 1 week, or at -20°C for up to 6 months prior to sequencing. Note that the Loading buffer is light sensitive.

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
Updated for Revio SPRQ-Nx	04	May 2026

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