

# Preparing multiplexed amplicon libraries using SMRTbell<sup>®</sup> prep kit 3.0

## Procedure & checklist

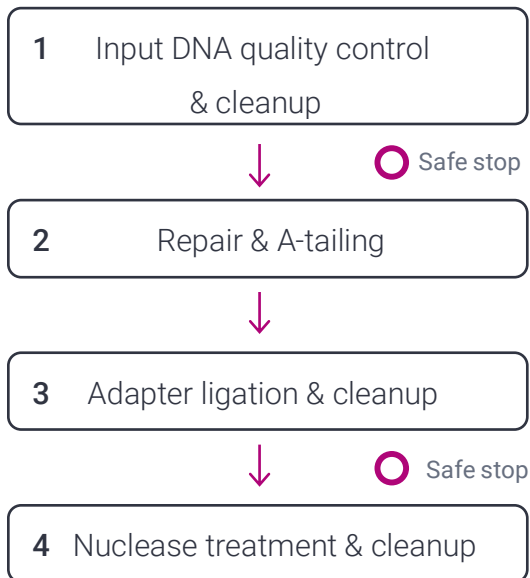
This procedure describes the workflow for constructing amplicon libraries using the SMRTbell prep kit 3.0 for sequencing on PacBio<sup>®</sup> Revio<sup>™</sup>, Sequel<sup>®</sup> II, and IIe systems. Amplicons may be indexed during PCR, or during library preparation with SMRTbell indexed adapters.

Overview		
	PCR-indexed samples	Adapter-indexed samples
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 Revio SMRT <sup>®</sup> Cell	300–2000 ng per pool	300–2000 ng per sample
DNA input per Sequel II SMRT Cell 8M	150–1000 ng per pool	150–1000 ng per sample

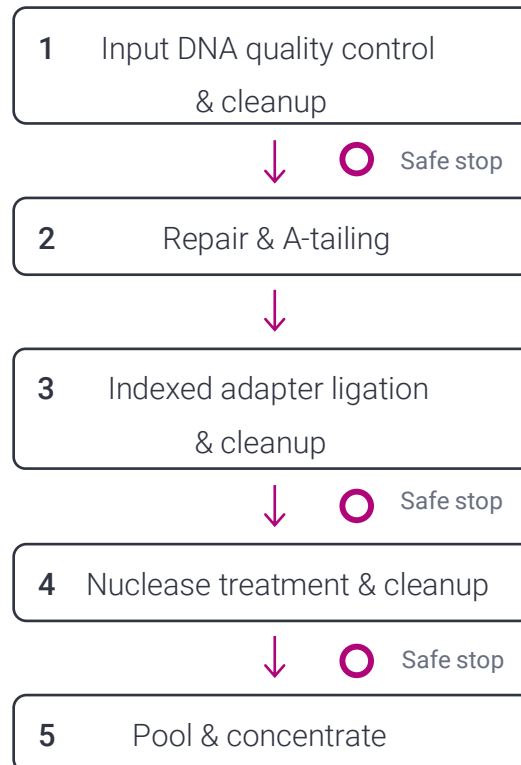
# Workflow

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## Primer-indexed samples



## Adapter-indexed samples



## 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 adapter index plate 96A (optional; for indexing)	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

# 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-indexed sample input

If samples were indexed 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 300 ng of total input per Revio SMRT Cell and no less than 150 ng of total input per Sequel II SMRT Cell 8M to ensure sufficient library yields for optimal SMRT cell loading. Larger amplicons will require more input material to achieve desired molarity for SMRT Cell loading. Please refer to the tables below for the recommended minimum total input amounts per SMRT Cell that will be used for the library. 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.

Mean size	Minimum pooled amount per Revio SMRT Cell
<3 kb	300 ng
3–12 kb	500 ng
>12 kb	750 ng

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

## Adapter-indexed sample input

Use no less than 300 ng of DNA input per sample per Revio SMRT Cell and no less than 150 ng of total DNA input per sample per Sequel II SMRT Cell 8M to ensure sufficient library yields for optimal SMRT Cell loading. Larger amplicons will require more input material to achieve desired molarity for SMRT Cell loading. Please refer to the tables below for the recommended minimum total input amounts per SMRT Cell that will be used for the library. 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 indexed primers so samples can be pooled prior to library prep.

Mean size	Minimum per sample amount per Revio SMRT Cell
<3 kb	300 ng
3–12 kb	500 ng
>12 kb	750 ng

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

## Indexing and multiplexing

Use the SMRTbell adapter index plates to index 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  $\geq 3$  kb separately from amplicons  $\leq 3$  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 result 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

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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

# 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
<b>DNA QC</b>		
	1.1	Measure DNA concentration of each sample with a Qubit fluorometer using the <b>1X dsDNA HS kit</b> following manufacturer's instructions.
	1.2	<p><b>Recommended:</b> measure the DNA size distribution with the appropriate sizing technology following the manufacturer's instructions.</p> <ul style="list-style-type: none"> <li>• Amplicons <math>\leq 10</math> kb: Agilent 2100 Bioanalyzer, TapeStation, or Fragment Analyzer.</li> <li>• Amplicons <math>\geq 10</math> kb: Agilent FEMTO Pulse system.</li> </ul>
	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 $\mu$ L, then use a 1.5 mL DNA LoBind tube instead.
<b>Cleanup with 1.3X SMRTbell cleanup beads</b>		
	1.5	Add <b>1.3X</b> volume per volume ( <b>v/v</b> ) 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 <b>room temperature</b> for <b>10 minutes</b> 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 <b>200 <math>\mu</math>L</b> , or enough to cover the beads, of <b>freshly prepared 80% ethanol</b> into each tube. After <b>30 seconds</b> , pipette off the 80% ethanol and discard.
	1.12	Repeat the previous step.
	1.13	<p>Remove residual 80% ethanol:</p> <ul style="list-style-type: none"> <li>• Remove the tube strip from the magnetic separation rack.</li> <li>• Quick-spin the tube strip in a microcentrifuge.</li> <li>• Place the tube strip back in a magnetic separation rack until beads separate fully from the solution.</li> <li>• Pipette off residual 80% ethanol and discard.</li> </ul>
	1.14	Remove the tube strip from the magnetic rack. <b>Immediately</b> add <b>47 <math>\mu</math>L</b> of <b>low TE buffer</b> 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 <b>room temperature</b> for <b>5 minutes</b> to elute DNA.
	1.17	Place the 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 <b>new 0.2 mL PCR tube strip</b> . Discard the old tube strip with beads.
<b>SAFE STOPPING POINT – Store at 4°C</b>		



## 2. Repair & A-tailing

✓	Step	Instructions																								
		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 (Steps 2.2 to 2.4).																								
2.1		<table border="1"> <thead> <tr> <th colspan="4">Reaction mix 1 (RM1)</th> </tr> <tr> <th>✓</th> <th>Tube</th> <th>Component</th> <th>Volume per sample</th> </tr> </thead> <tbody> <tr> <td></td> <td>Purple</td> <td>Repair buffer</td> <td>8 <math>\mu</math>L</td> </tr> <tr> <td></td> <td>Blue</td> <td>End repair mix</td> <td>4 <math>\mu</math>L</td> </tr> <tr> <td></td> <td>Green</td> <td>DNA repair mix</td> <td>2 <math>\mu</math>L</td> </tr> <tr> <td colspan="3"><b>Total volume</b></td> <td><b>14 <math>\mu</math>L</b></td> </tr> </tbody> </table>	Reaction mix 1 (RM1)				✓	Tube	Component	Volume per sample		Purple	Repair buffer	8 $\mu$ L		Blue	End repair mix	4 $\mu$ L		Green	DNA repair mix	2 $\mu$ L	<b>Total volume</b>			<b>14 <math>\mu</math>L</b>
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2.2		Pipette-mix <b>RM1</b> .																								
2.3		Quick-spin <b>RM1</b> in a microcentrifuge to collect liquid.																								
2.4		Add <b>14 <math>\mu</math>L</b> of the <b>RM1</b> to each sample. The total reaction volume should be <b>60 <math>\mu</math>L</b> .																								
2.5		Pipette-mix each sample.																								
2.6		Quick-spin the strip tube in a microcentrifuge to collect liquid.																								
2.7		Run the <a href="#">repair &amp; A-tailing thermocycler program</a> .																								
2.8		Proceed to the next step of the protocol.																								

### 3. Adapter ligation & cleanup

✓	Step	Instructions																				
<b>Adapter ligation</b>																						
3.1		Add <b>4 µL</b> of SMRTbell adapter (primer-indexed amplicon pools) or indexed SMRTbell adapter (non-indexed amplicons) to each sample tube from the 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 <b>RM2</b> steps (steps 3.3 to 3.5).																				
		<table border="1"> <thead> <tr> <th colspan="4">Reaction mix 2 (RM2)</th> </tr> <tr> <th>✓</th> <th>Tube</th> <th>Component</th> <th>Volume per sample</th> </tr> </thead> <tbody> <tr> <td></td> <td>Yellow</td> <td>Ligation mix</td> <td>30 µL</td> </tr> <tr> <td></td> <td>Red</td> <td>Ligation enhancer</td> <td>1 µL</td> </tr> <tr> <td colspan="3"><b>Total volume</b></td> <td><b>31 µL</b></td> </tr> </tbody> </table>	Reaction mix 2 (RM2)				✓	Tube	Component	Volume per sample		Yellow	Ligation mix	30 µL		Red	Ligation enhancer	1 µL	<b>Total volume</b>			<b>31 µL</b>
Reaction mix 2 (RM2)																						
✓	Tube	Component	Volume per sample																			
	Yellow	Ligation mix	30 µL																			
	Red	Ligation enhancer	1 µL																			
<b>Total volume</b>			<b>31 µL</b>																			
3.3		Pipette-mix <b>RM2</b> .																				
3.4		Quick-spin <b>RM2</b> in a microcentrifuge to collect liquid.																				
3.5		Add <b>31 µL</b> of <b>RM2</b> to each sample from previous step. The total volume should be <b>95 µL</b> .																				
3.6		Pipette-mix each sample.																				
3.7		Quick-spin the strip tube in a microcentrifuge to collect liquid.																				
3.8		Run the <a href="#">adapter ligation thermocycler program</a> .																				
<b>Cleanup with 1.3X SMRTbell cleanup beads</b>																						
3.9		Add <b>124 µL</b> 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 <b>room temperature</b> for <b>10 minutes</b> to allow DNA to bind beads.																				
3.13		Place the 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 <b>200 µL</b> , or enough to cover the beads, of <b>freshly prepared 80% ethanol</b> into each tube. After <b>30 seconds</b> , pipette off the 80% ethanol and discard.																				
3.16		Repeat the previous step.																				

- 3.17 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.
  - Pipette off residual 80% ethanol and discard.
- 3.18 Remove the tube strip from the magnetic rack. **Immediately** add **40  $\mu\text{L}$**  of **elution 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 the 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																				
<b>Nuclease treatment</b>																					
<p>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 <b>RM3</b> steps (steps 4.2 to 4.4).</p>																					
4.1	<table border="1"> <thead> <tr> <th colspan="4">Reaction mix 3 (RM3)</th> </tr> <tr> <th>✓</th> <th>Tube</th> <th>Component</th> <th>Volume per sample</th> </tr> </thead> <tbody> <tr> <td></td> <td>Light purple</td> <td>Nuclease buffer</td> <td>5 <math>\mu\text{L}</math></td> </tr> <tr> <td></td> <td>Light green</td> <td>Nuclease mix</td> <td>5 <math>\mu\text{L}</math></td> </tr> <tr> <td colspan="3"><b>Total volume</b></td> <td><b>10 <math>\mu\text{L}</math></b></td> </tr> </tbody> </table>	Reaction mix 3 (RM3)				✓	Tube	Component	Volume per sample		Light purple	Nuclease buffer	5 $\mu\text{L}$		Light green	Nuclease mix	5 $\mu\text{L}$	<b>Total volume</b>			<b>10 <math>\mu\text{L}</math></b>
Reaction mix 3 (RM3)																					
✓	Tube	Component	Volume per sample																		
	Light purple	Nuclease buffer	5 $\mu\text{L}$																		
	Light green	Nuclease mix	5 $\mu\text{L}$																		
<b>Total volume</b>			<b>10 <math>\mu\text{L}</math></b>																		
4.2	Pipette-mix <b>RM3</b> .																				
4.3	Quick-spin <b>RM3</b> in a microcentrifuge to collect liquid.																				
4.4	Add <b>10 <math>\mu\text{L}</math></b> of <b>RM3</b> to each sample. Total volume should equal <b>50 <math>\mu\text{L}</math></b> .																				
4.5	Pipette-mix each sample.																				
4.6	Quick-spin the strip tube in a microcentrifuge to collect liquid.																				
4.7	Run the <a href="#">nuclease treatment thermocycler program</a> .																				

### Cleanup with 1.3X SMRTbell cleanup beads

- 4.8 Add **65  $\mu\text{L}$**  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 the 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  $\mu\text{L}$** , 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.
- 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.
- 4.16
- 4.17 Remove the tube strip from the magnetic rack. **Immediately** add **15  $\mu\text{L}$**  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 the 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  $\mu\text{L}$**  aliquot from each tube and dilute with **9  $\mu\text{L}$**  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-indexed samples** that don't require any additional pooling, proceed to the SMRT<sup>®</sup> Link Sample Setup to prepare sample(s) for sequencing. The 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 **indexed with SMRTbell adapters** that require pooling, proceed to the next section for instructions on pooling and concentrating.

## 5. Pooling & concentrating samples with indexed SMRTbell adapters

✓	Step	Instructions
<b>Pooling</b>		
5.1		Combine an <b>equal mass</b> sampled indexed with SMRTbell adapters 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 <b>equal molar</b> portions for better sequence coverage balance.
		The total SMRTbell library mass should be <b>≥100 ng</b> when amplicon sizes are less than 10 kb, and <b>≥300 ng</b> when greater or equal to 10 kb.
<b>Concentrate with 1.3X SMRTbell cleanup beads</b>		
5.2		Add <b>1.3X v/v</b> 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 <b>10 minutes</b> to allow DNA to bind beads.
5.6		Place the 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 <b>200 µL</b> , or enough to cover the beads, of <b>freshly prepared 80% ethanol</b> into each tube. After <b>30 seconds</b> , pipette off the 80% ethanol and discard.
5.9		Repeat the previous step.
5.10		Remove residual 80% ethanol: <ul style="list-style-type: none"> <li>• Remove the tube from the magnetic separation rack.</li> <li>• Quick-spin the tube in a microcentrifuge.</li> <li>• Place the tube back in a magnetic separation rack until beads separate fully from the solution.</li> <li>• Pipette off residual 80% ethanol and discard.</li> </ul>
5.11		Remove the tube from the magnetic rack. <b>Immediately</b> add <b>15 µL</b> of <b>elution buffer</b> 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 <b>room temperature</b> for <b>5 minutes</b> to elute DNA.
5.14		Place the 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 <b>new tube</b> . Discard old tube with beads.
5.16		Take a <b>1 µL</b> aliquot from each tube and dilute with <b>9 µL</b> of <b>elution buffer or water</b> . 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.
<b>PROTOCOL COMPLETE</b>		

Revision history (description)	Version	Date
Initial release	01	Apr 2022
Clarify minimum DNA input requirements and best practices	02	Sep 2022
Updated to include recommendations for the Revio system	03	Dec 2023

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