Procedure & Checklist - Preparing HiFi SMRTbell® Libraries from Ultra-Low DNA Input

This procedure describes preparing HiFi SMRTbell libraries from 5 ng of input genomic DNA (gDNA) for sequencing on the Sequel® II/IIe System. Genomic DNA is sheared to approximately 10 kb using a g-TUBE or a Megaruptor system, amplified by PCR, constructed to a SMRTbell library and size-selected using the BluePippin system. This workflow enables de novo assembly of insect genomes of up to 500 Mb (>500 Mb genome size is not supported) and human variant detection from as low as 5 ng gDNA.

Required gDNA Input Amount	Required Quality of Input gDNA	gDNA Shearing Method	Target Sheared Fragment Size Distribution Mode	Amplification Target Size Distribution Mode	Total Mass of Pooled PCR Product Required for Library Construction	Required SMRTbell Library Input for BluePippin Size-Selection
5-20 ng	Majority of gDNA >20 kb	Megaruptor or g-TUBE	10 kb sheared DNA is optimal	8-10 kb	≥500 ng	≥400ng

Table1: Library construction requirements for ultra-low DNA input samples.

PacBio recommends using high molecular weight gDNA with majority >20 kb for library construction. A DNA sample containing significant amounts of <10 kb fragments will result in preferential amplification of short fragments thereby resulting in short reads that are not ideal for de novo assembly and resequencing for variant detection. Therefore, it is critical to understand the quality of your gDNA sample prior to shearing.

The Femto Pulse system is highly recommended because it requires significantly lower amounts of DNA (200 - 500 picograms) than other systems. Complementary to Femto Pulse, the Qubit High Sensitivity (HS) is highly recommended for measuring DNA concentration.

For constructing a SMRTbell library, a minimum 500 ng of amplified sample (PCR reaction 1 + PCR reaction 2) is required to generate sufficient SMRTbell library for 1 Sequel II SMRT® Cell 8M. To generate 2 Sequel SMRT Cells 8M, we recommend starting with approximately 800 ng of pooled amplified sample for library construction.

If DNA availability is not a constraint (e.g. >150 ng DNA), we recommend using the low DNA input procedure (found here) for up to 1Gb genome size. For large and complex genomes that require multiple SMRT Cells and where DNA can be extracted in abundant quantities from a single individual sample, we recommend constructing a HiFi library using the standard workflow found here.

Required Materials

Item	Vendor	Part Number
DNA QC (one of the following)		
Femto Pulse®	Agilent	M5330AA
Fragment Analyzer®	Agilent	M5311AA
DNA Quantitation		
Qubit™ Fluorometer	ThermoFisher Scientific	Q33226
Qubit™ 1X dsDNA HS Assay Kit	ThermoFisher Scientific	Q33230
DNA Shearing		
Megaruptor	Diagenode	B06010001
Long Hydropores	Diagenode	E07010002
Hydrotubes	Diagenode	C30010018
g-TUBE	Covaris	520104
SMRTbell Library Preparation		
SMRTbell® Express Template Prep Kit 2.0	PacBio	100-938-900
SMRTbell® gDNA Sample Amplification Kit	PacBio	101-980-000
Eppendorf MiniSpin Plus or other equivalent benchtop centrifuge model	Eppendorf	22620100
HDPE 8 place Magnetic Separation Rack for 0.2 ml PCR Tubes	V&P Scientific Inc.	VP772F4-1
0.2 mL PCR 8-strip tubes	USA Scientific	1402-4708
Wide Orifice Tips (Tips LTS W-O 200 UL Fltr RT-L200WFLR)	Rainin	17014294
ProNex® Beads	Promega	NG2002 – 125 mL
Elution Buffer	PacBio	101-633-500
100% Ethanol, Molecular Biology Grade	Any MLS	
Thermal Cycler that is 100 µL and 8-tube strip compatible	Any MLS	
Size-Selection		
BluePippin Size-Selection System	Sage Science	BLU0001
BluePippin with dye free, 0.75% Agarose Cassettes and S1 Marker	Sage Science	BLF7510

Table 2: List of Required Materials and Equipment for constructing HiFi Libraries from as low as 5 ng gDNA.

Ultra-Low DNA Input Library Construction Workflow

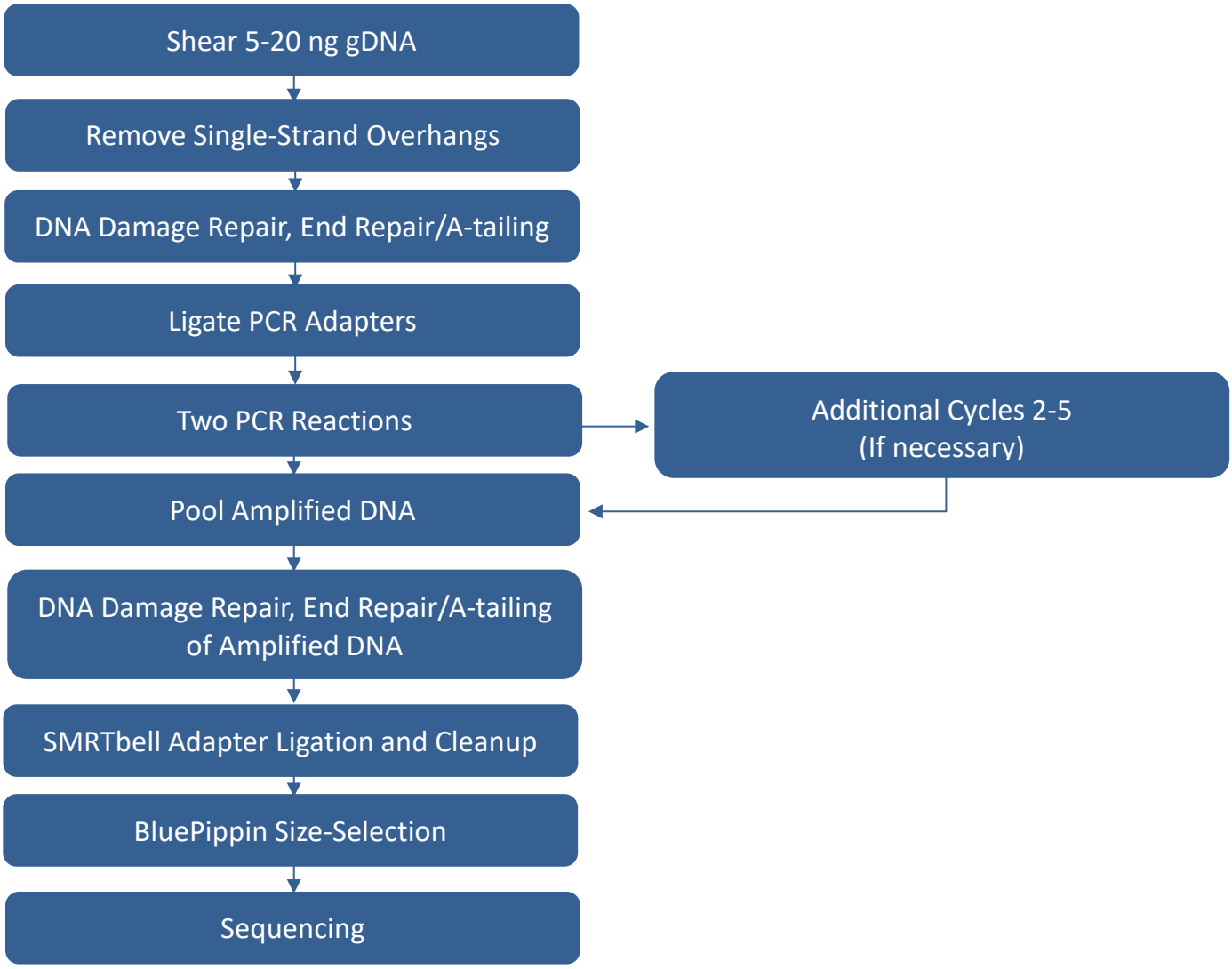

Figure 1: Workflow for preparing Ultra-Low DNA Input using the SMRTbell Express Template Prep Kit 2.0.

Best Practice Recommendations

1. Use wide-bore tips for all pipette mixing steps.
2. Always follow Qubit best practices:
    - Prepare the Qubit HS working solution by diluting the Qubit reagent 1:200 in Qubit HS buffer. Prepare 200 µL of working solution for each standard and sample. Always make new standards for each assay.
    - Set up two 190 µL assay tubes for the standards and one 199 µL assay tube for each sample. Add 10 µL of standard (from kit) and 1 µL of sample to the respective assay tubes. Both the standard and sample DNAs should be at room temperature.
    - Vortex all tubes for 2 seconds.
    - Incubate the tubes for 2 minutes at room temperature prior to measurement.
3. Always set your thermocyclers to the appropriate temperature for incubations before proceeding with the procedure.
4. Always allow ProNex beads to equilibrate to room temperature before use.

Recommended Tools for gDNA Quantification and Qualification

When working with small amounts of input DNA, accurate sizing and quantification is critical for generating sufficient coverage of long reads to produce a high-quality genome assembly.
For quantification, we recommend using the Qubit fluorometer and Qubit HS DNA assay reagents.

To accurately determine the size distribution of your gDNA sample, we highly recommend the use of the Femto Pulse system because of its ability to rapidly evaluate size distributions using only ~200-500 picograms of DNA. Two commercially available systems that may be used to evaluate gDNA size distribution are listed in Table 3 below with links to recommended procedures.

DNA Sizing QC Method	Comments	Procedure
Femto Pulse	Highly recommended (Requires 200-500 picograms)	Agilent Technologies, Inc.
Fragment Analyzer	Requires 2 ng	Agilent Technologies, Inc.

Table 3. gDNA Size Evaluation Methods and Procedures.

Evaluation of gDNA Samples for Ultra Low-Input Library Construction

The size distribution of the starting input gDNA sample is critical to successful library construction and sequencing on the Sequel II Systems. Always evaluate the quality of the gDNA samples before proceeding with library construction.

PacBio recommends starting with high molecular weight gDNA samples where the majority of the fragments are >20 kb. Figure 2 below shows examples of individual insect gDNA samples of varying quality analyzed on the Femto Pulse system.

- In this example, Sample 1, is not suitable for using the ultra-low DNA input procedure. The gDNA is severely fragmented such that a significant proportion of the fragments are ≤10 kb resulting in preferential amplification of short fragments. We recommend re-extraction of the gDNA to obtain a higher-quality sample for shearing and SMRTbell library construction.
- Sample 2 shows size distribution with the majority of the fragments >20 kb. In this example, the gDNA is appropriate for constructing SMRTbell libraries.

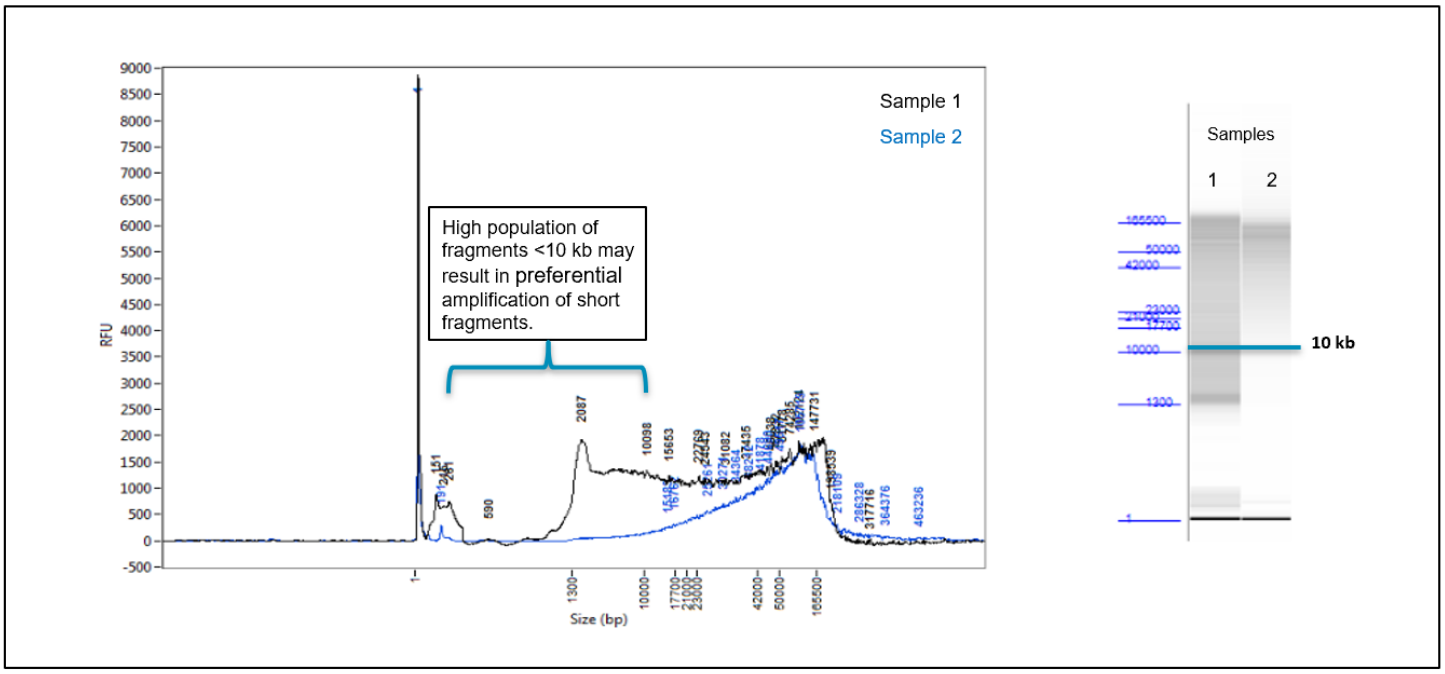

Figure 2: Example Femto Pulse sizing QC analysis of individual insect gDNA samples of varying quality. Sample 1 is lower quality gDNA such that a significant proportion of the fragments are ≤10 and is not suitable constructing SMRTbell libraries using the ultra-low DNA input procedure. Sample 2 show size distribution with the majority of the fragments >20 kb and are appropriate for shearing and constructing SMRTbell libraries.

DNA Shearing is Required

The success of this procedure is highly dependent on the size distribution of the sheared DNA. We recommend shearing to approximately 10 kb size mode using either the g-TUBE by Covaris or Megaruptor by Diagenode (see Figure 3 and 4). Over-shearing gDNA may impact amplification and the yield of the final size selected SMRTbell library. It is, therefore, critical to work with samples where the majority of the starting input genomic DNA is greater than 20 kb and if necessary, optimize the shearing conditions to obtain the optimal DNA size.

For shearing, it is important to note that 5 ng is the minimum starting DNA input. If you have more than 5 ng DNA, we recommend shearing more than 5 ng of input DNA to ensure sufficient sample to perform accurate DNA sizing and quantification.

Shear Genomic DNA Using a Covaris g-TUBE

STEP	✓	Shear DNA	Notes
1		Use 5 -20 ng of gDNA for shearing. Bring the volume of sample in Elution buffer to **50 µL**	
2		Transfer gDNA to the g-TUBE and centrifuge at 1677 x g (5000 rpm in the Eppendorf MiniSpin Plus) for 2-4 minutes to achieve a target mode size of 10 kb.	
3		Repeat spin until the entire gDNA sample has passed through the orifice.	
4		Invert the g-TUBE and centrifuge at 1677 x g (5000 rpm in the Eppendorf MiniSpin Plus) for 2-4 minutes.	
5		Repeat spin until the entire gDNA sample has passed through the orifice.	
6		Examine the sheared DNA on a Femto Pulse to ensure that the sheared DNA has the expected size distribution (10 kb). Load 200-500 picograms of sample for DNA sizing QC by Femto Pulse.	
7		Proceed to the “Remove Single-Strand Overhangs” section.	

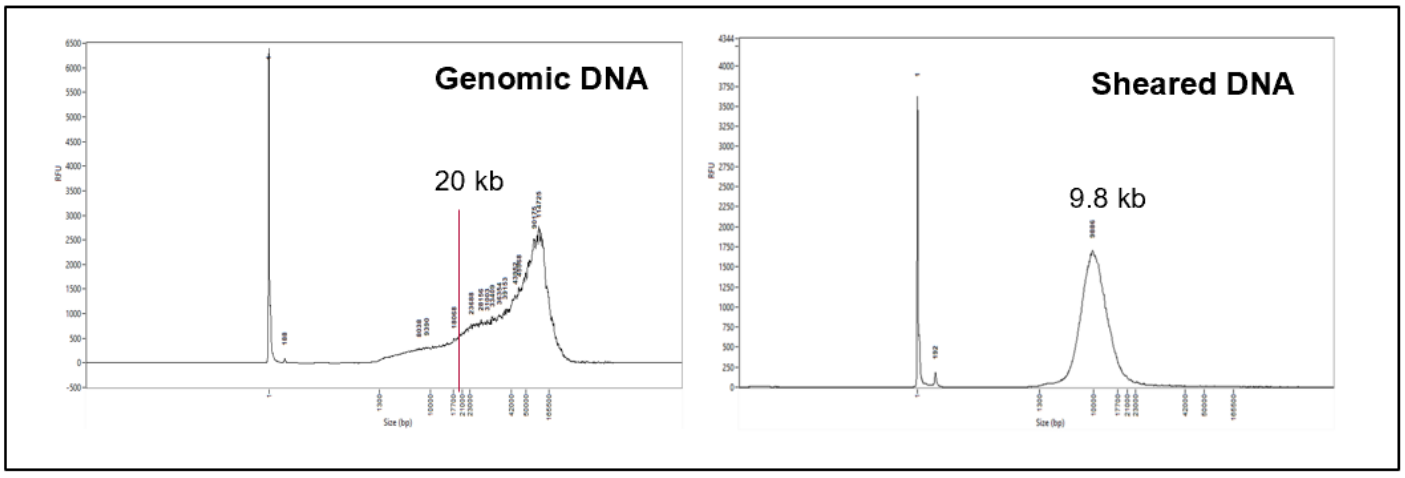

Figure 3: Example of 5 ng of human genomic DNA sheared to approximately 10 kb using the Covaris g-TUBE. Distribution of starting genomic DNA is >20 kb.

Shear Genomic DNA Using Megaruptor

Important: If using the Megaruptor, ensure that the system is thoroughly washed before shearing. Since this workflow requires amplification, contamination introduced during shearing will be amplified which complicates assembly.

STEP	✓	Shear DNA	Notes
1		Use 5 -20 ng of gDNA for shearing. Bring the volume of sample in Elution buffer to **50 µL**.	
2		Shear gDNA using Long Hydropores and have the Long Hydropore option selected in the Megaruptor software.	
3		Choose at target shear size of 10 kb in the Megaruptor software setting.	
4		Do not click “Pre-Load Hydropores” setting. If this option is selected, the sheared sample with be too diluted to go into the first step in the library construction process (removal of single-strand overhangs). A single sample requires a volume of 45.4 µL.	
5		Examine the sheared DNA on a Femto Pulse to ensure that the sheared DNA has the expected size distribution (10 kb). Load 200-500 picograms of sample for DNA sizing QC by Femto Pulse.	
6		Proceed to the “Remove Single-Strand Overhangs” section.	

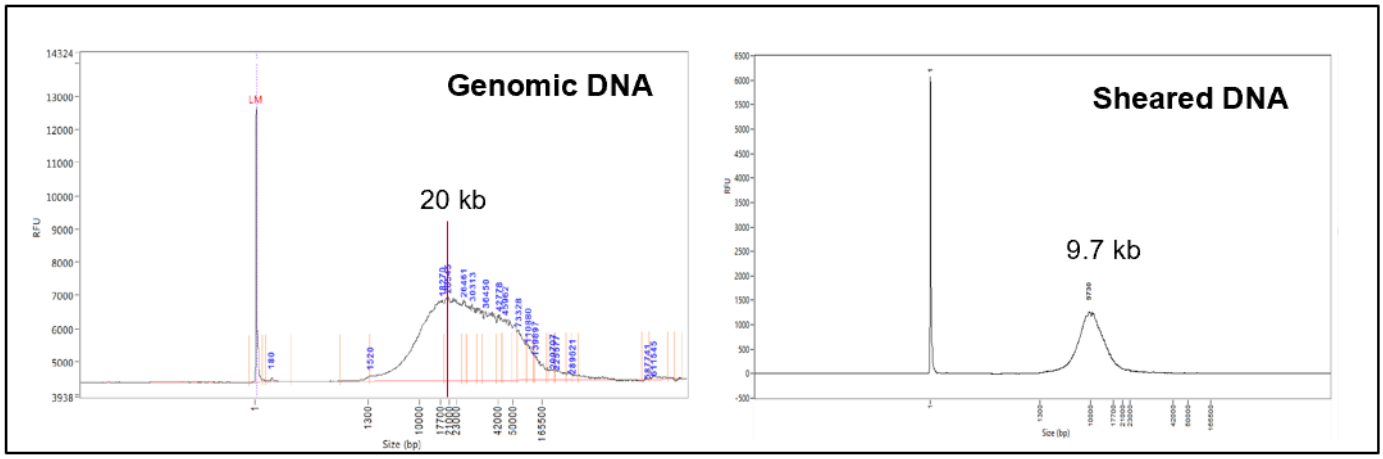

Figure 4: Example of 5 ng of insect genomic DNA sheared to approximately 10 kb using the Megaruptor System. Distribution of starting genomic DNA is >20 kb.

Reagent Handling

Several tubes in the SMRTbell Express TPK 2.0 kit (shown in Table 4 below) are sensitive to temperature and vortexing. We highly recommend to:

- Never leave tubes at room temperature.
- Work on ice at all times when preparing master mixes.
- Finger tap followed by a quick-spin prior to use.

Reagent	Kit	Where Used
Amplification Adapters	SMRTbell gDNA Sample Amplification Kit	Ligation
PCR Master Mix 1	SMRTbell gDNA Sample Amplification Kit	Amplification
PCR Master Mix 2	SMRTbell gDNA Sample Amplification Kit	Amplification
Sample Amplification PCR Primer	SMRTbell gDNA Sample Amplification Kit	Amplification
DNA Prep Additive	SMRTbell Express Template Prep Kit 2.0	Remove single-strand overhangs
DNA Prep Enzyme	SMRTbell Express Template Prep Kit 2.0	Remove single-strand overhangs
DNA Damage Repair Mix v2	SMRTbell Express Template Prep Kit 2.0	DNA Damage Repair
End Prep Mix	SMRTbell Express Template Prep Kit 2.0	End-Repair/A-tailing
Overhang Adapter v3	SMRTbell Express Template Prep Kit 2.0	Ligation
Ligation Mix	SMRTbell Express Template Prep Kit 2.0	Ligation
Ligation Additive	SMRTbell Express Template Prep Kit 2.0	Ligation
Ligation Enhancer	SMRTbell Express Template Prep Kit 2.0	Ligation

Table 4: Temperature sensitive reagents.

Remove Single-Strand Overhangs

Before starting with the procedure, please refer to Table 4 for recommendations surrounding SMRTbell Express TPK 2.0 kit reagent handling. **IMPORTANT**: Use PCR strip tubes throughout this procedure.

Use the following table to set up a reaction to remove single-strand overhangs using up to 20 ng of input sheared gDNA

1. Dilute the DNA Prep Additive with Enzyme Dilution Buffer. Mix well and quick spin.

Reagent	Tube Cap Color	Volume	✓	Notes
Enzyme Dilution Buffer		4.0 µL		
DNA Prep Additive (stock)		1.0 µL		
Total Volume		5.0 µL		

The diluted DNA Prep Additive should be used immediately and should not be stored.

2. For each sample to be processed, add the following components to a single PCR tube of an 8-tube strip on ice:

Reaction Mix 1	Tube Cap Color	Volume	✓	Notes
DNA Prep Buffer		7.0 µL		
Sheared DNA		≤45.4 µL		
NAD		0.6 µL		
Diluted DNA Prep Additive		1.0 µL		
DNA Prep Enzyme		1.0 µL		
H_2O		Up to 55.0 µL		
Total Volume		55.0 µL		

3. Pipette mix 10 times with wide-bore pipette tips.
4. Spin down contents of tube with a quick spin in a microfuge.
5. Place in a thermocycler and run the following program:
   - 15 minutes at 37°C
   - Hold at 4°C
6. Proceed to the next step.

Repair DNA Damage

1. With the reaction on ice, add 2 µL DNA Damage Repair Mix v2 directly to Reaction Mix 1:

Reaction Mix 2	Tube Cap Color	Volume	✓	Notes
Reaction Mix 1		55.0 µL		
DNA Damage Repair Mix v2		2.0 µL		
Total Volume		57.0 µL		

2. Pipette mix 10 times with wide-bore pipette tips.
3. Spin down contents of tube with a quick spin in a microfuge.
4. Place in a thermocycler and run the following program:
    - 30 minutes at 37°C
    - Hold at 4°C
5. Proceed to the next step.

Repair Ends/A-Tailing

1. With the reaction on ice, add 3 µL End Prep Mix directly to Reaction Mix 2:

Reaction Mix 3	Tube Cap Color	Volume	✓	Notes
Reaction Mix 2 (damage-repaired sample)		57.0 µL		
End Prep Mix		3.0 µL		
Total Volume		60.0 µL		

2. Pipette mix 10 times with wide-bore pipette tips.
3. Spin down contents of tube with a quick spin in a microfuge.
4. Place in a thermocycler and run the following program:
    - 30 minutes at 20°C
    - 30 minutes at 65°C
    - Hold at 4°C
5. Proceed to the next step.

Adapter Ligation

1. To ligate the amplification adapter to the repaired sample, the Amplification Adapters must first be diluted with Duplex Buffer. Mix well and quick spin.

Reagent	Tube Cap Color	Volume	✓	Notes
Duplex Buffer		9.0 µL		
Amplification Adapters		1.0 µL		
Total Volume		10.0 µL		

The diluted Amplification Adapters should be used immediately and should not be stored.

2. Prepare the reaction below. With the reaction on ice, add the components below in the order listed, directly to Reaction Mix 3:

Reaction Mix 4	Tube Cap Color	Volume	✓	Notes
Reaction Mix 3 (A-tail sample)		60.0 µL		
Diluted Amplification Adapters		2.5 µL		
Ligation Mix		30.0 µL		
Ligation Additive		1.0 µL		
Ligation Enhancer		1.0 µL		
Total Volume		94.5 µL		

3. Pipette mix 10 times with wide-bore pipette tips.
4. Spin down contents of tube with a quick spin in a microfuge.
5. Place in a thermocycler and run the following program:
   - 60 minutes at 20°C
   - Hold at 4°C
6. Proceed to the next step “Purification of SMRTbell Library”.

Purification of SMRTbell Library

STEP	✓	Purification with ProNex Beads	Notes
1		ProNex Beads must be brought to room temperature for 30 to 60 mins prior to use.	
2		Add **77.5 µL** of ProNex beads to the 94.5 µL Reaction Mix 4 and gently pipette mix 10 times. Perform a quick spin to collect all liquid from the sides of the tube.	
3		Incubate on bench for 5 minutes at room temperature.	
4		Place on a magnet stand and wait until supernatant is clear. Use a P200 pipette to remove the supernatant.	
5		While on magnet, wash two times with 200 µL of freshly prepared 80% ethanol. After removal of second wash of 200 µL of ethanol, spin the tube strip briefly, return to magnetic stand and remove residual ethanol with a P20 pipette. Do not let the beads to dry out.	
6		Remove the tube strip from the magnetic stand. **Immediately** add **97 µL** of EB and pipette mix 10 times to resuspend. Do not let the beads to dry out. Quick spin to collect all liquid from the sides of the tube. Place at 37°C for 10 minutes to elute the DNA from the beads.	
7		Place the tube on the magnetic stand to separate the beads from the supernatant. When the supernatant is clear, transfer **97 µL** of the purified eluted sample into a to a new tube and set aside on ice until ready to use. Proceed to “Library Amplification by PCR”. **IMPORTANT**: For the Library Amplification by PCR, the **97 µL** of the purified eluted sample will be divided and used for two reactions (Reaction Mix 5A and Reaction Mix 5B). Each reaction requires a volume of 48 µL of the purified eluted sample.	

Library Amplification by PCR

Divide the sample (48 µL each) and amplify with PCR Master Mix 1 and PCR Master Mix 2.

1. On ice, prepare Reaction Mix 5A and Reaction Mix 5B. When working with multiple samples, prepare enough Master Mix for all reactions, plus 10% of the total reaction mix volume. Pipette mix 10 times with wide-bore pipette tips and then perform a quick spin to collect all liquid from the sides of the tube.

Reaction Mix 5A	Tube Cap Color	Volume	✓	Notes
PCR Master Mix 1		50.0 µL		
Sample Amplification PCR Primer		2.0 µL		
Total Volume		52.0 µL		

Reaction Mix 5B	Tube Cap Color	Volume	✓	Notes
PCR Master Mix 2		50.0 µL		
Sample Amplification PCR Primer		2.0 µL		
Total Volume		52.0 µL		

2. On ice, add 52 µL of Reaction Mix 5A and Reaction Mix 5B to the 48 µL eluted DNA for a total volume of 100 µL each reaction.
3. Pipette mix 10 times with wide-bore pipette tips and then perform a quick spin to collect all liquid from the sides of the tube.
4. Place in a thermocycler and run the following program (lid 105°C). The PCR reactions may be left at 4°C overnight.

PCR Program for Reaction Mix 5A	
45 seconds at 98°C	**1 cycle**
10 seconds at 98°C	**13 cycles**
15 seconds at 62°C	
7 minutes at 72°C	
5 minutes at 72°C	**1 cycle**
Hold at 4°C	

PCR Program for Reaction Mix 5B	
30 seconds at 98°C	**1 cycle**
10 seconds at 98°C	**13 cycles**
15 seconds at 60°C	
10 minutes at 68°C	
5 minutes at 68°C	**1 cycle**
Hold at 4°C	

Purification of Amplified DNA

<table>
<tr><th>STEP</th><th>✓</th><th>Purification with ProNex Beads</th><th>Notes</th></tr>
<tr><td>1</td><td></td><td>ProNex Beads must be brought to room temperature for 30 to 60 mins prior to use.</td><td></td></tr>
<tr><td>2</td><td></td><td>Add 82 µL of ProNex beads to the 100 µL Reaction Mix 5A and to the 100 µL Reaction Mix 5B and gently pipette mix 10 times. Perform a quick spin to collect all liquid from the sides of the tube.</td><td></td></tr>
<tr><td>3</td><td></td><td>Incubate on bench for 5 minutes at room temperature.</td><td></td></tr>
<tr><td>4</td><td></td><td>Place on a magnet stand and wait until supernatant is clear. Use a P200 pipette to remove the supernatant.</td><td></td></tr>
<tr><td>5</td><td></td><td>While on magnet, wash two times with 200 µL of freshly prepared 80% ethanol. After removal of second wash of 200 µL of ethanol, spin the tube strip briefly, return to magnetic stand and remove residual ethanol with a P20 pipette. Do not let the beads to dry out.</td><td></td></tr>
<tr><td>6</td><td></td><td>Remove the tube strip from the magnetic stand. Immediately add 26 µL of EB and pipette mix 10 times to resuspend. Do not let the beads to dry out. Quick spin to collect all liquid from the sides of the tube. Incubate on bench for 5 minutes at room temperature to elute the DNA from the beads.</td><td></td></tr>
<tr><td>7</td><td></td><td>Place the tube on the magnetic stand to separate the beads from the supernatant. When the supernatant is clear, transfer 26 µL of eluted amplified DNA reaction to a new tube and set it aside in ice until ready to use.</td><td></td></tr>
<tr><td>8</td><td></td><td>Use 1 µL of sample to quantify with Qubit dsDNA HS kit.

IMPORTANT: You must have the required mass of purified amplified DNA per reaction to proceed with “Pool Amplified DNA”. See guidelines below:
<table>
<tr><th colspan="2">Recommendations for Samples with Low Yield
(Reaction Mix 5A and 5B)</th></tr>
<tr><td>If the total mass < 275 ng (<11 ng/µL)</td><td>2 additional cycles as described in Appendix 1</td></tr>
<tr><td>If the total mass <130 ng (<5 ng/µL)</td><td>3 additional cycles as described in Appendix 1</td></tr>
<tr><td>If the total mass < 65 ng (<2.5 ng/µL)</td><td>5 additional cycles as described in Appendix 1</td></tr>
</table></td><td></td></tr>
<tr><td>9</td><td></td><td>Use 1 µL of amplified DNA for DNA sizing QC by Femto Pulse automated pulsed-field capillary electrophoresis to ensure that the DNA has the expected size distribution (8-10kb size mode). Load 200-500 picograms of sample for DNA sizing QC by Femto Pulse.

The amplified DNA can be stored at 4°C or at -20°C for future use.</td><td></td></tr>
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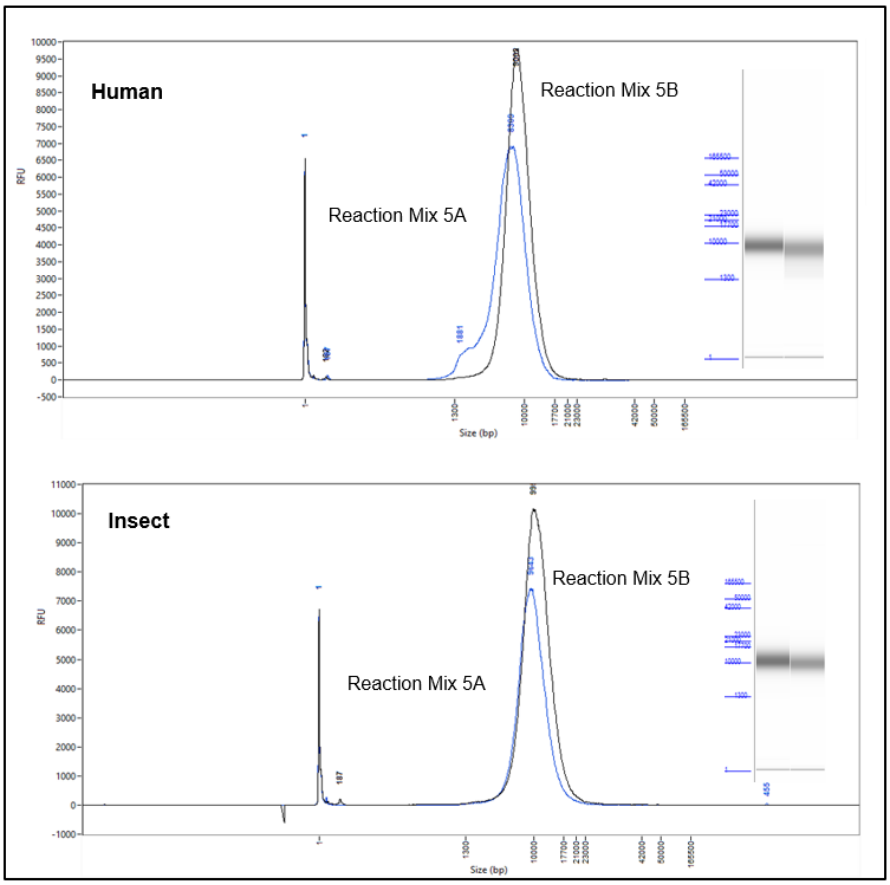

Figure 5: Examples of human and insect samples amplified with Reaction Mix 5A and 5B. Size distribution of amplified products are approximately 10 kb and appropriate to proceed to SMRTbell library construction.

Pool Amplified DNA

The amplified DNA from the PCR Reaction Mix 5A and 5B are pooled together in equal mass quantities. The pooled DNA can then be constructed into a SMRTbell library as a single sample.

Pooling Best Practices:

- Always quantify samples before pooling. Since DNA amounts may be limited at this step, PacBio recommends using the Qubit dsDNA High Sensitivity Assay Kit for concentration measurements.
- The total mass of the pooled DNA must be **≥500 ng** in 47.4 µL (e.g., 250 ng of Reaction Mix 5A + 250 ng of Reaction Mix 5B)
    - If the total mass of the two PCR reactions is <500 ng, use any remaining sample to adjust the total combined mass to 500 ng. This is to ensure that there is sufficient material for library construction and size selection.

STEP	✓	Pooling	Notes
1		Pool amplified DNA into a single PCR tube of an 8-tube strip.	
2		Mix and spin down the contents of the tube with a quick spin in a microfuge.	
3		Proceed to the **“**Repair DNA Damage” section below.	

Repair DNA Damage

1. For each sample to be processed, add the following components to a single PCR tube of an 8-tube strip on ice.

Reaction Mix 6	Tube Cap Color	Volume	✓	Notes
DNA Prep Buffer		7.0 µL		
Pooled Amplified DNA		≤47.4 µL		
NAD		0.6 µL		
DNA Damage Repair Mix v2		2.0 µL		
H_2O		Up to 57.0 µL		
Total Volume		57.0 µL		

2. Pipette mix 10 times with wide-bore pipette tips.
3. Spin down contents of tube with a quick spin in a microfuge.
4. Place in a thermocycler and run the following program:
    - 30 minutes at 37°C
    - Hold at 4°C
5. Proceed to the next step.

Repair Ends/A-Tailing

1. With the reaction on ice, add 3 µL End Prep Mix directly to Reaction Mix 6:

Reaction Mix 7	Tube Cap Color	Volume	✓	Notes
Reaction Mix 6		57.0 µL		
End Prep Mix		3.0 µL		
Total Volume		60.0 µL		

2. Pipette mix 10 times with wide-bore pipette tips.
3. Spin down contents of tube with a quick spin in a microfuge.
4. Place in a thermocycler and run the following program:
    - 30 minutes at 20°C
    - 30 minutes at 65°C
    - Hold at 4°C
5. Proceed to the next step.

Adapter Ligation

1. With the reaction on ice, add the components below in the order listed, directly to Reaction Mix 7:

Reaction Mix 8	Tube Cap Color	Volume	✓	Notes
Reaction Mix 7		60.0 µL		
Overhang Adapter v3		5.0 µL		
Ligation Mix		30.0 µL		
Ligation Additive		1.0 µL		
Ligation Enhancer		1.0 µL		
Total Volume		97.0 µL		

2. Pipette mix 10 times with wide-bore pipette tips.
3. Spin down contents of tube with a quick spin in a microfuge.
4. Place in a thermocycler and run the following program:
    - 60 minutes at 20°C
    - Hold at 4°C
5. Proceed to “Purification of SMRTbell Library”.

Purification of SMRTbell Library

STEP	✓	Purification with ProNex Beads	Notes
1		Add **97 µL** of resuspended, room-temperature ProNex beads to the 97 µL Reaction Mix 8. Pipette mix 10 times. Perform a quick spin to collect all liquid from the sides of the tube.	
2		Incubate sample on bench top for 5 minutes at room temperature.	
3		Place the tube on a magnetic stand to separate the beads from the supernatant. Use a P200 pipettor to remove supernatant.	
4		Wash 2 times with 200 µL of freshly prepared 80% ethanol. After removal of the second wash of 200 µL of ethanol, spin the tube strip briefly, return to magnetic stand and remove residual ethanol with a P20 pipette. Do not let the beads to dry out.	
5		Remove the tube from the magnetic stand. **Immediately** add **32 µL** of EB and pipette mix to resuspend. Perform a quick spin to collect all liquid from the sides of the tube. Place at room temperature for 5 minutes to elute the DNA from the beads.	
6		Place the tube on a magnetic stand to separate the beads from the supernatant. Transfer the eluted DNA samples to a new tube.	
7		Use 1 µL of SMRTbell library to quantify with Qubit dsDNA HS kit.	
8		Use 1 µL of SMRTbell library for DNA sizing QC by Femto Pulse automated pulsed-field capillary electrophoresis. Load 200-500 picograms of sample for DNA sizing QC by Femto Pulse.	
9		Proceed with “BluePippin Size-Selection of SMRTbell Library” or stored at 4°C or at -20°C for future use.	

BluePippin Size-Selection of SMRTbell Library

For constructing SMRTbell libraries from ultra-low DNA input, PacBio highly recommends size-selection using the BluePippin system. The BluePippin system size-selection requires starting with a SMRTbell library size mode of approximately 8 kb - 10 kb. SMRTbell templates <8 kb are efficiently removed using *0.75% DF 3-10 kb Marker S1- Improved Recovery* cassette definition. High presence of fragments <8 kb may result in high abundance of short reads which may impact assembly.

The average size distribution of the final size-selected library is approximately 10 kb - 11 kb. Typical recovery yields after size-selection are 20% to 30% (from input of purified SMRTbell library) and are highly dependent on the size distribution of the starting SMRTbell library. See Figure 6 for an example of SMRTbell library size-selected using the BluePippin system.

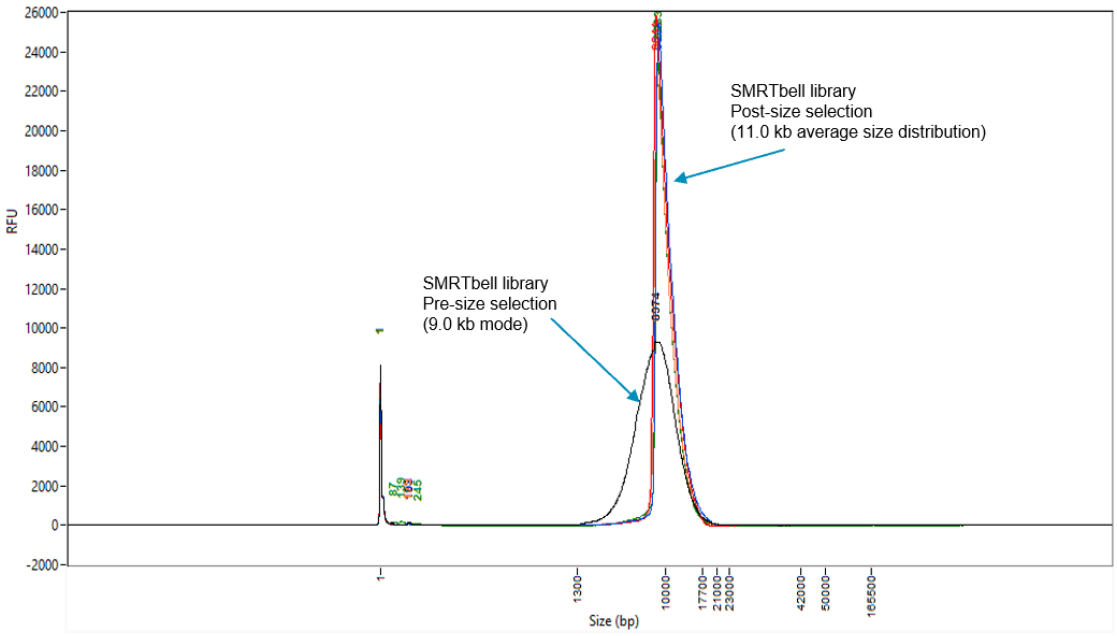

Figure 6: Example of SMRTbell library size-selected using Sage Science's BluePippin System. After size-selection, 11.0 kb insert size library was generated.

Perform size-selection using Sage Sciences' BluePippin system according to the manufacturer's recommendations. For the latest BluePippin User Manual and guidance on the size-selection protocol, please contact Sage Science (www.sagescience.com).

STEP	✓	BluePippin Size Selection	Notes
1		Add **10 µL** of Loading Solution to each **30 µL** **(containing ≥400 ng of purified SMRTbell library)** sample remaining from the previous step above. Mix well by gentle pipetting and spin briefly to collect the contents at the bottom of the tube.	
2		Follow the manufacturer's recommendations to set up a run protocol. • Select the "**0.75% DF 3-10 kb Marker S1- Improved Recovery**" Cassette Definition File for your sample. • Using the "Range" selection mode, enter a desired "BP Start" value of 8000 and a "BP End" value of 17000. **Note**: We do not recommend running lanes with **<400 ng** of SMRTbell library material. • Be sure to assign a marker lane.	
3		Load S1 marker and samples into the BluePippin gel cassette and start the run. Run time is approximately 4.0 hours.	
4		**Collect Eluate.** To maximize recovery of eluted DNA, wait at least 20 minutes after the run terminates before removing the sample from the elution chamber. Collect the eluate into a single PCR tube of an 8-tube strip.	
5		After collecting the eluate, wash the eluate chamber with 40 µL of Sage Science's 0.1% Tween-20 Wash Solution and then combine the recovered wash solution with the eluted sample. Washing the elution well may further increase recovery yields by approximately 10-20%.	
6		Measure the volume of your size-selected sample (eluate plus wash). Proceed to the "Purification of the Size-Selected SMRTbell Library" step below.	

Purification of Size-Selected SMRTbell Library

STEP	✓	Purification with ProNex Beads	Notes
1		ProNex Beads must be brought to room temperature for 30 to 60 mins prior to use.	
2		Add **1X volume** of resuspended, room-temperature ProNex beads to the size-selected SMRTbell library. Pipette mix 10 times. Perform a quick spin to collect all liquid from the sides of the tube.	
3		Incubate on bench for 5 minutes at room temperature.	
4		Place on a magnet stand and wait until supernatant is clear. Use a P200 pipette to remove the supernatant.	
5		While on magnet, wash two times with 200 µL of freshly prepared 80% ethanol. After removal of second wash of 200 µL of ethanol, spin the tube strip briefly, return to magnetic stand and remove residual ethanol with a P20 pipette. Do not let the beads to dry out.	
6		Remove the tube strip from the magnetic stand. **Immediately** add **12 µL** of EB and pipette mix 10 times to resuspend. Do not let the beads to dry out. Quick spin to collect all liquid from the sides of the tube. Place at room temperature for 5 minutes to elute the DNA from the beads.	
7		Place the tube on the magnetic stand to separate the beads from the supernatant. When the supernatant is clear, transfer **12 µL** of eluted size selected SMRTbell library to a new tube and set it aside in ice until ready to use.	
8		Use 1 µL of the size selected SMRTbell library to quantify with Qubit dsDNA HS kit.	
9		Use 1 µL of the size-selected SMRTbell library for DNA sizing QC by Femto Pulse automated pulsed-field capillary electrophoresis. Load 200-500 picograms of sample for DNA sizing QC by Femto Pulse.	
10		Proceed with “Anneal and Bind SMRTbell Templates” or stored at 4°C or at -20°C for future use.	

Prepare for Sequencing

Follow the instructions in SMRT® Link Sample Setup v10.2 or higher. Select “HiFi Reads” from the application dropdown.

Appendix 1 – Recommendations for Additional DNA Amplification by PCR for Samples with a Lower Yield

If there is not enough DNA to proceed with library construction, this section describes a workflow for additional DNA amplification by PCR.

1. On ice, prepare the following reaction. Combine in the order shown. Pipette mix 10 times with wide-bore pipette tips and then perform a quick spin to collect all liquid from the sides of the tube.

PCR Amplification Reaction 1	Tube Cap Color	Volume	✓	Notes
PCR Master Mix 1		50.0 µL		
Sample Amplification PCR Primer		2.0 µL		
H20		24.0 µL		
Purified, Amplified DNA **(Reaction Mix 5A)**		24.0 µL		
Total Volume		100.0 µL		

PCR Amplification Reaction 2	Tube Cap Color	Volume	✓	Notes
PCR Master Mix 2		50.0 µL		
Sample Amplification PCR Primer		2.0 µL		
H20		24.0 µL		
Purified, Amplified DNA **(Reaction Mix 5B)**		24.0 µL		
Total Volume		100.0 µL		

2. Place in a thermocycler and run the following program (lid 105°C). The PCR reactions may be left at 4°C overnight.

   The number of cycles to use depends on the available mass used for amplification. See Table 5 for recommendations.

PCR Program for PCR Amplification Reaction 1	
45 seconds at 98°C	1 cycle
10 seconds at 98°C	N* cycles (see below)
15 seconds at 62°C	
7 minutes at 72°C	
5 minutes at 72°C	1 cycle
Hold at 4°C	

* Use Table 5 for guidelines determining the number of cycles.

PCR Program for PCR Amplification Reaction 2	
30 seconds at 98°C	1 cycle
10 seconds at 98°C	N* cycles (see below)
15 seconds at 60°C	
10 minutes at 68°C	
5 minutes at 68°C	1 cycle
Hold at 4°C	

* Use Table 5 for guidelines to determine the number of cycles.

Additional # of Cycles	Condition
2	If the total mass <275 ng (< 11 ng/µL)
3	If the total mass <130 ng (< 5 ng/µL)
5	If the total mass <65 ng (< 2.5 ng/µL)

Table 5: Use the following guidelines to determine the number of cycles for PCR Amplification Reaction 1 and PCR Amplification Reaction 2.

Purification of Reamplified Samples

STEP	✓	Purification with ProNex Beads	Notes
1		For low yield reamplified samples, add **82 µL** of resuspended, room-temperature ProNex beads to the 100 µL PCR Amplification Reaction 1 and to the 100 µL PCR Amplification Reaction 2. Pipette mix 10 times. Quick spin to collect liquid from the sides of the tube.	
2		Incubate sample on bench top for 5 minutes at room temperature.	
3		Place the tube on a magnetic stand to separate the beads from the supernatant. Use a P200 pipette to remove supernatant.	
4		Wash 2 times with 200 µL of freshly prepared 80% ethanol. After removal of second wash of 200 µL of ethanol, spin the tube strip, return to magnetic stand and remove residual ethanol with a P20 pipette. Do not let the beads to dry out.	
5		Remove the tube from the magnetic stand. **Immediately** add **26 µL** of EB and pipette mix to resuspend. Quick spin to collect liquid from the sides of the tube. Incubate at room temperature for 5 minutes to elute the DNA from the beads.	
6		Place the tube on a magnetic stand to separate the beads from the supernatant. Transfer the eluted DNA samples to a new tube.	
7		Use 1 µL of reamplified samples to quantify with Qubit dsDNA HS kit. • If the concentration is < 10 ng/µL, the library may not have enough mass after size-selection for proper loading. Additional PCR cycles can be performed. • If the concentration >10 ng/µL, return to "Pool Amplified DNA" section.	
8		Use 1 µL of reamplified samples for DNA sizing QC by Femto Pulse automated pulsed- field capillary electrophoresis. Load 200-500 picograms of sample for DNA sizing QC by Femto Pulse. The reamplified DNA can be stored at 4°C or at -20°C for future use.	

Revision History (Description)	Version	Date
Initial Release	01	August 2020
Direct to SMRT Link for instructions on preparing for sequencing	02	November 2021