Procedure & Checklist - Preparing HiFi Libraries from Low DNA Input Using SMRTbell® Express Template Prep Kit 2.0

This document describes preparing HiFi libraries from >150 ng of input genomic DNA (gDNA) for the Sequel® System and from >400 ng of input gDNA for the Sequel II System using SMRTbell Express Template Prep Kit 2.0. Using this procedure, genome assemblies of up to 1 Gb can be achieved.

This procedure also provides recommendations for multiplexing a maximum of 2 small genomes (up to 600 Mb/genome) on the Sequel II System, from >300 ng of gDNA per genome. The two samples are pooled (see Figure 2) after ligation and nuclease-treated.

Table 1 below is a summary of supported workflows described in this document and the required DNA quality and quantity for each.

SMRTbell Library Type	Required Minimum gDNA	Required Quality of Input gDNA	gDNA Shearing Method	Required Size Distribution
Low DNA input for the Sequel System (1 sample)	>150 ng	Majority of gDNA >30 kb	Megaruptor System	12 - 20 kb sheared DNA is optimal
Low DNA input for the Sequel II System (1 sample)	>400 ng	Majority of gDNA >30 kb	Megaruptor System	12 - 20 kb sheared DNA is optimal
Multiplexed low DNA input for the Sequel II System (2 samples up to 600 Mb per genome)	>300 ng per sample	Majority of gDNA >30 kb	Megaruptor System	12 - 20 kb sheared DNA is optimal

Table1: DNA quality and quantity requirements for low DNA input samples run on the Sequel and Sequel II Systems. For DNA shearing, the Megaruptor System is currently recommended (g-TUBEs are under evaluation).

PacBio recommends using the Femto Pulse system for assessing the integrity of the starting gDNA material. The Femto Pulse system requires significantly lower sample amounts (200 - 500 picograms) compared to other sizing analysis systems that require >50 ng of DNA for sizing.

When working with low amounts of gDNA, accurate quantification is necessary. The Qubit High Sensitivity (HS) assay system can be used to obtain accurate dsDNA concentration measurements for low DNA input samples.

Overall, SMRTbell library yields are typically 50% (starting from sheared DNA input)) for the single-sample workflow described in Figure 1 and 30% for the multiplexing workflow described in Figure 2. Depending on the final size of the library, sufficient amounts of SMRTbell template material to run approximately 3 or more SMRT® Cells 1M can be generated for the Sequel System. The Sequel II System requires higher on-plate loading concentrations and, as a result, the amount of SMRTbell library material generated in this procedure is typically sufficient to run only one SMRT Cell 8M.

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 Qualification		
Femto Pulse® Automated Pulsed Field CE Instrument or Pippin Pulse Electrophoresis Power Supply or Pulsed Field Gel Electrophoresis System: CHEF Mapper	Agilent Technologies, Inc Sage Science Bio-Rad	M5330AA PP10200 170-3670
DNA Quantification Qubit™ Fluorometer	ThermoFisher Scientific	Q33226
Qubit™ 1X dsDNA HS Assay Kit	ThermoFisher Scientific	Q33230
DNA Shearing Megaruptor 3 Megaruptor 3 Shearing Kit	Diagenode	 B06010003 E07010003
SMRTbell Library Construction SMRTbell® Express Template Prep Kit 2.0 AMPure® PB beads Sequel® System: Barcoded Overhang Adapter Kit 8A/8B Sequel® II/IIe System: SMRTbell® Barcoded Adapter Plate 3.0 SMRTbell® Enzyme Cleanup Kit Elution Buffer	 PacBio 	 100-938-900 100-265-900 101-628-400/500 102-009-200 101-746-400 101-633-500
DNA Lo Bind microfuge tubes	Eppendorf	022431021
Wide Orifice Tips (Tips LTS W-O 200UL Fltr RT-L200WFLR)	Rainin	17014294
Tube Rotator	VWR	10136-084

Table 2: List of Required Materials and Equipment.

Sequencing One Low DNA Input Sample on the Sequel or Sequel II Systems

To sequence 1 sample per SMRT Cell on the Sequel and Sequel II Systems, >150 ng and >400 ng of input gDNA is required, respectively, and the target DNA shear size distribution is 12 kb - 20 kb. In this procedure, single-strand overhangs are removed before proceeding with DNA Damage Repair and End-Repair/A-tailing. After End-Repair/A-tailing, overhang adapters found in the SMRTbell Express Template Prep Kit 2.0 are ligated (Figure 1).

Following ligation, the SMRTbell library is purified and size-selected using AMPure® PB beads to remove <3 kb templates. Nuclease treatment of the SMRTbell library is not required.

It is important to note that the first step in the library construction process (removal of single-strand overhangs) for a single sample requires a volume of 45.4 µL. Therefore, it is good practice to elute the post- sheared DNA sample in a final volume of 45.4 µL of Elution Buffer during DNA concentration.

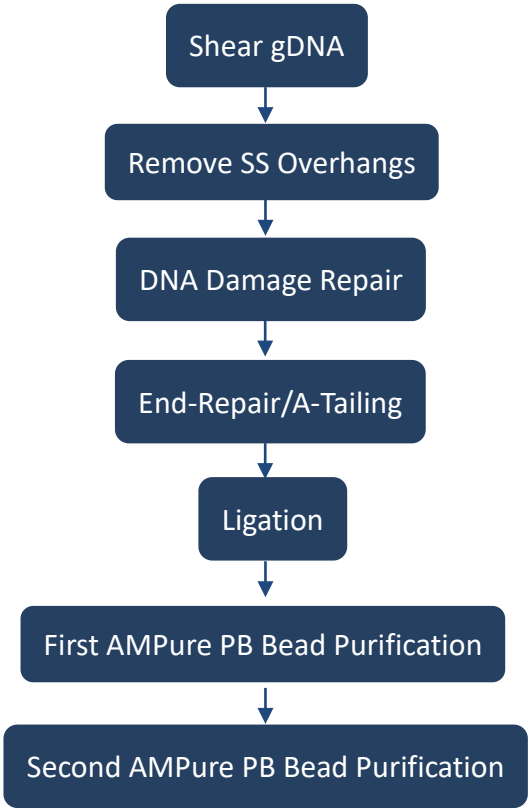

Figure 1: Workflow for preparing single-sample (non-multiplexed) HiFi libraries using the SMRTbell Express Template Prep Kit 2.0 with low DNA input for sequencing on the Sequel and Sequel II Systems.

Multiplexing Two Low DNA Input Samples on the Sequel II System

To multiplex two samples per SMRT Cell 8M for sequencing on the Sequel II System, >300 ng of input gDNA per sample is required and the target DNA shear size distribution is 12 kb - 20 kb.

In this workflow, individual gDNA samples are sheared and single-strand overhangs are removed before independently going through DNA Damage Repair and End-Repair/A-tailing. After End-Repair/A-tailing, barcoded overhang adapters (Barcoded Overhang Adapter Kit 8A or 8B) are ligated to each sample separately. Following ligation, the two SMRTbell libraries are treated with nucleases to remove any damaged or partial SMRTbell templates prior to pooling (Figure 2).

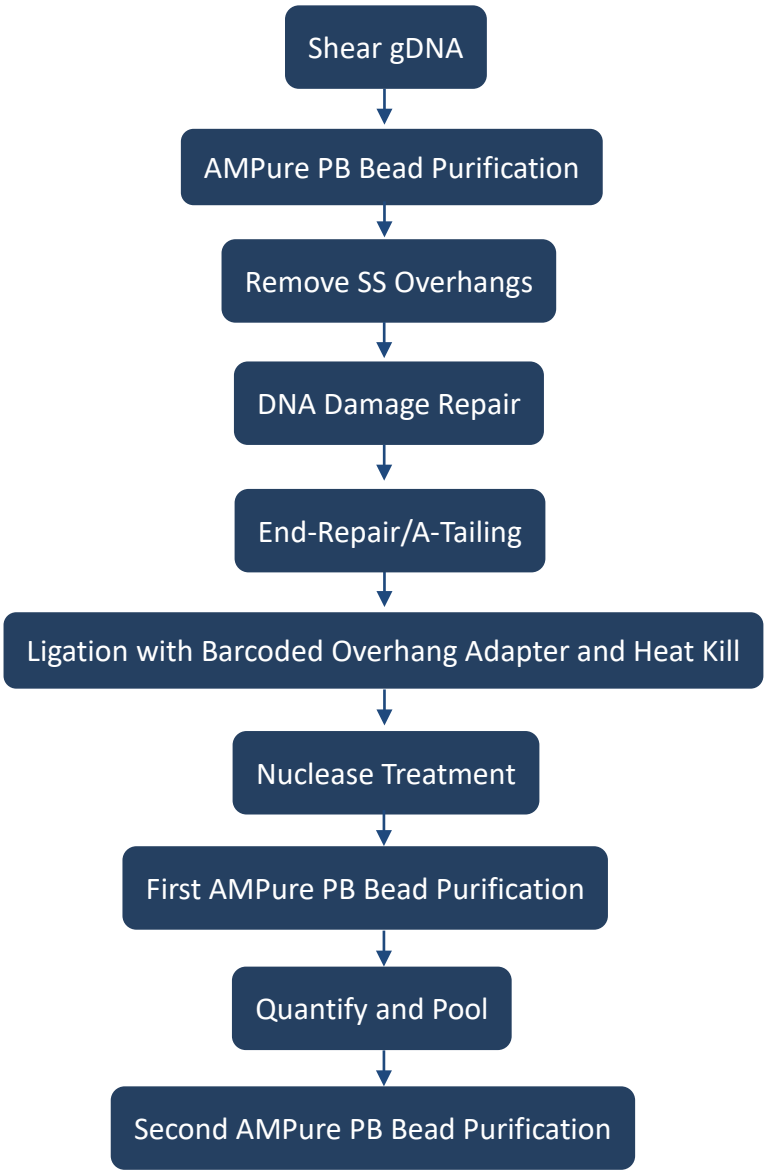

Figure 2: Workflow for preparing multiplexed HiFi libraries using the SMRTbell Express Template Prep Kit 2.0 with low DNA input for sequencing on the Sequel II System.

Best Practice Recommendations

1. Use wide-bore tips for all pipette mixing steps.
2. Throughout the procedure, do not flick the tubes to mix. Flicking induces damage to DNA. Instead, use gentle pipetting with wide-bore tips to mix reagents. Note that template preparation reagents should first be dispensed with a standard pipette tip (i.e., P10 or P20) and then a wide-bore tip should be used for pipette mixing.
3. Never vortex tubes containing high-molecular weight DNA.
4. Always follow Qubit best practices:
   - 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.
5. Always set your heat blocks or thermocyclers to the appropriate temperature for incubations before proceeding with the procedure.
6. Always allow AMPure PB beads to equilibrate to room temperature before use.

Recommended Tools for gDNA Quantification and Qualification

When working with small amounts of DNA, accurate sizing and quantification are important for generating sufficient coverage to produce a high-quality genome assembly.

For quantification of gDNA to be used with the low DNA input library preparation workflow, we recommend using the Qubit fluorometer and Qubit HS DNA assay reagents. Measure the gDNA sample concentration as recommended by the manufacturer.

To determine the size distribution, we recommend the Femto Pulse system because of its ability to rapidly evaluate size distributions using only ~200 – 500 picograms of DNA. Three commercially available systems that may be used to evaluate gDNA size distribution are listed in Table 3 below with links to recommended procedures. Note, however, that the CHEF Mapper and Pippin Pulse systems require at least 50 ng of DNA for analysis and are recommended only if there is sufficient DNA sample available.

DNA Sizing QC Method	Comments	Procedure
Femto Pulse	Highly recommended (Requires 200-500 picograms)	Agilent Technologies, Inc.
Bio-Rad® CHEF Mapper® XA Pulsed Field Electrophoresis System	Requires >50 ng	Procedure & Checklist - Using the BIO-RAD® CHEF Mapper® XA Pulsed Field Electrophoresis System
Sage Science Pippin Pulse	Requires >50 ng	Procedure & Checklist - Using the Sage Science Pippin Pulse Electrophoresis Power Supply System

Table 3. gDNA Size Evaluation Methods and Procedures.

Evaluation of gDNA Samples for Low-Input Library Construction

The size distribution of the starting input gDNA sample is critical to successful HiFi SMRTbell library construction and sequencing on the Sequel or 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 >30 kb. Figure 3 below shows examples of individual mosquito gDNA samples of varying quality analyzed on the Femto Pulse system.

- In this example, Samples 1 and 2 show size distributions with the majority of the fragments >30 kb. Both samples are appropriate for shearing and constructing HiFi SMRTbell libraries for *de novo* assembly.
- If the gDNA is severely fragmented such that a significant proportion of the fragments are ≤10 kb (for example samples 3 and 4 in Figure 3), we recommend re-extraction of the gDNA to obtain a higher-quality sample for HiFi SMRTbell library construction.
- If the sample is moderately fragmented with a size distribution of ~10 – 20 kb with relatively few <10 kb fragments, a HiFi SMRTbell library may optionally be constructed directly without shearing (with the caveats noted below).

Note: PacBio has found that SMRTbell libraries constructed from low-quality, fragmented gDNA samples tend to generate shorter read lengths compared to libraries constructed from high-quality, high-molecular weight gDNA samples. This may be caused by sequencing termination events occurring at damaged sites introduced during DNA extraction that were not repairable by DNA Damage repair enzymes. Additionally, fragmented DNA usually contains excess levels of short fragments (<10 kb) that cannot be removed even with the most aggressive AMPure PB bead size-selection procedure. Such types of low-quality DNA samples typically lead to the generation of short subread lengths and – consequently – a more fragmented *de novo* genome assembly.

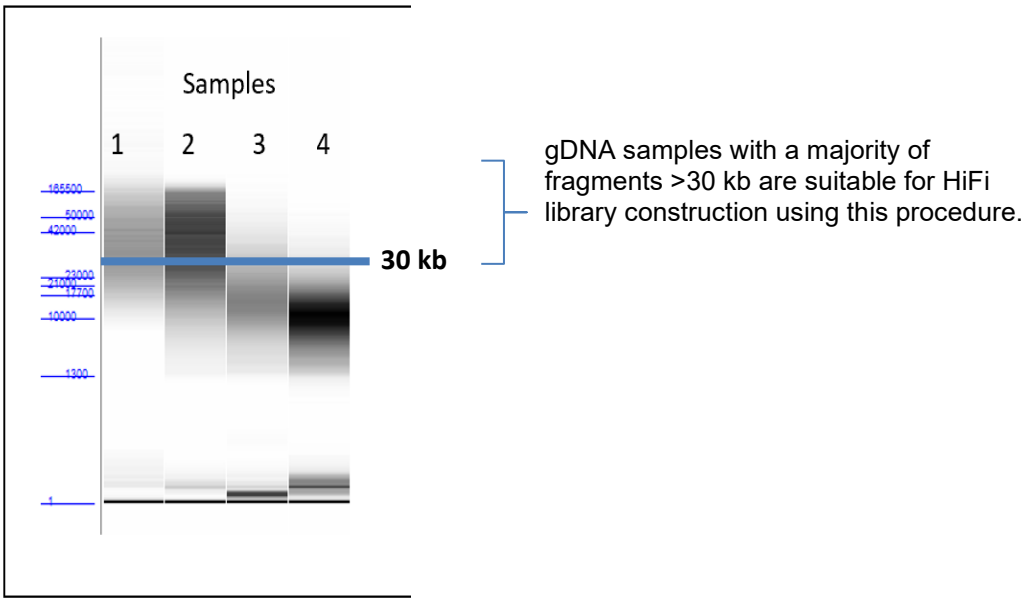

Figure 3: Example Femto Pulse sizing QC analysis of individual mosquito gDNA samples of varying quality. Samples 1 and 2 show size distributions with the majority of the fragments >30 kb and are appropriate for shearing and constructing HiFi SMRTbell libraries using the low DNA input procedure. Samples 3 and 4 are lower-quality samples such that a significant proportion of the fragments are ≤10 and are not suitable constructing HiFi SMRTbell libraries using the low DNA input procedure.

Multiplexing on the Sequel II System (not recommended for the Sequel System)

For constructing multiplexed HiFi SMRTbell libraries for the Sequel II System, PacBio recommends the following:

- It is necessary to shear the gDNA so that the fragment size distribution is 12 kb - 20 kb. This insert size range maximizes the detection of barcodes during the demultiplexing analysis step.
- Samples for a multiplex experiment must have the same average size and distribution to avoid biased read representation. Therefore, it is highly recommended to work with samples that contain mostly high-molecular weight DNA so that they can be sheared to achieve a similar target size and distribution for HiFi SMRTbell library construction.

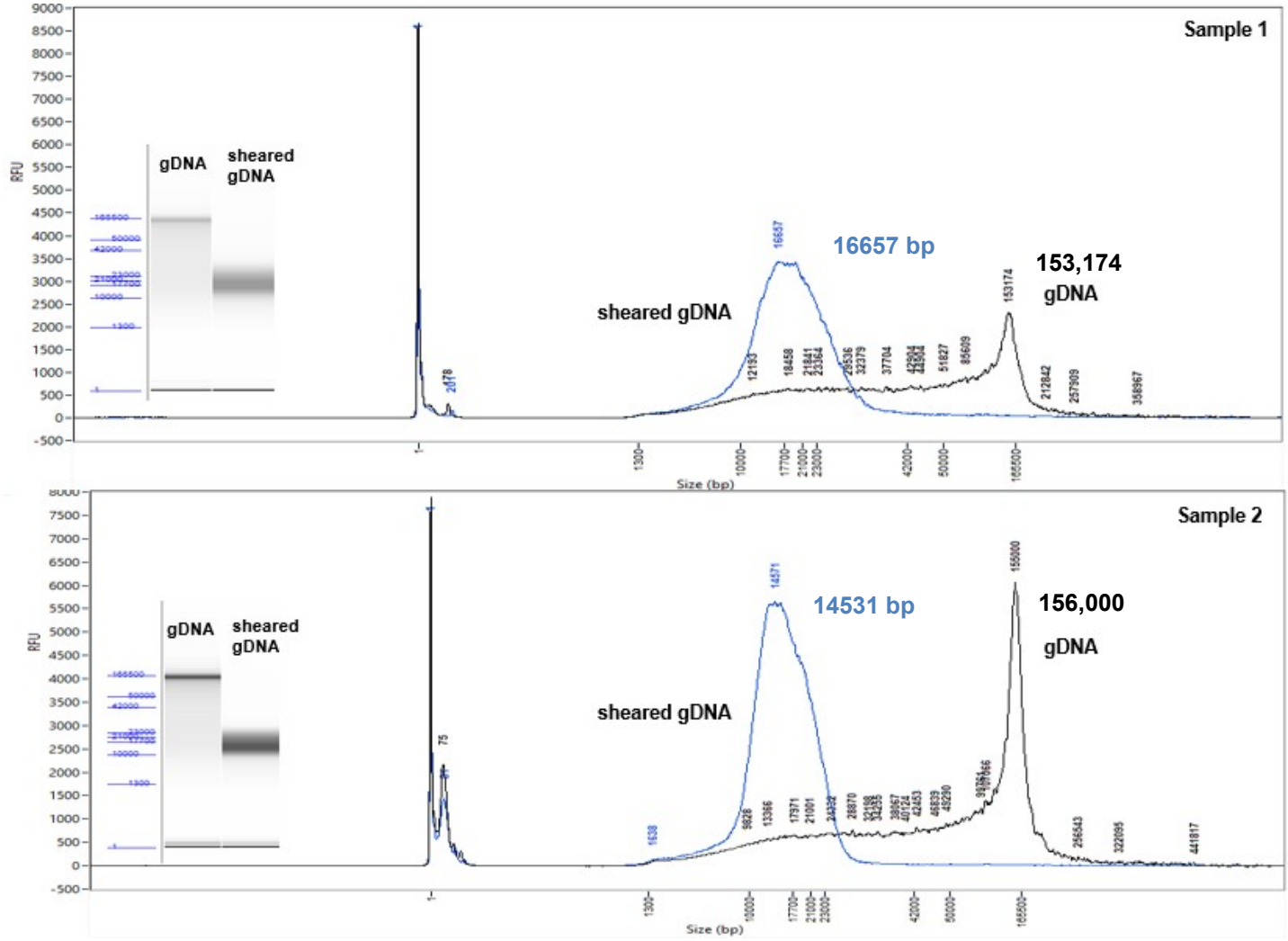

Figure 4: Example Femto Pulse sizing QC analysis of input gDNA and sheared gDNA samples. Samples 1 and 2 are examples of gDNA suitable for library construction using the low DNA input procedure for multiplexing. Both samples were sheared using a Megaruptor 3 system with speed setting 33. Sample 1 sheared DNA mode size is ~16 kb and Sample 2 sheared DNA mode size is ~14 kb.

Recommended DNA Shearing System

PacBio recommends the Megaruptor system for shearing gDNA because of its ability to generate tight shear distributions with good recovery post-shearing.

Table 4 below shows recommended parameter settings for shearing gDNA samples to a size of 12 kb - 20 kb using Diagenode's Megaruptor 3 system. To minimize biased representation in the sequencing read data, DNA samples for multiplexing must have a similar shear size distribution (+/- 15%) as shown in Figure 5.

To shear gDNA using Diagenode's Megaruptor 3 system, generally follow the manufacturer's recommendations. After shearing the DNA samples, evaluate the size distribution by using a Femto Pulse system. If the size distribution mode of the sheared DNA is within 12 kb - 20 kb, proceed with the "Concentrate DNA Using AMPure PB Beads" step below.

Required gDNA Input Amount	Sample Volume	Speed Setting	Target Shear Size
≥150 ng for Sequel ≥400 ng for Sequel II	80-100 µL	30-33	12 kb - 20 kb

Table 4: Recommended shearing parameters for the Megaruptor 3 system.

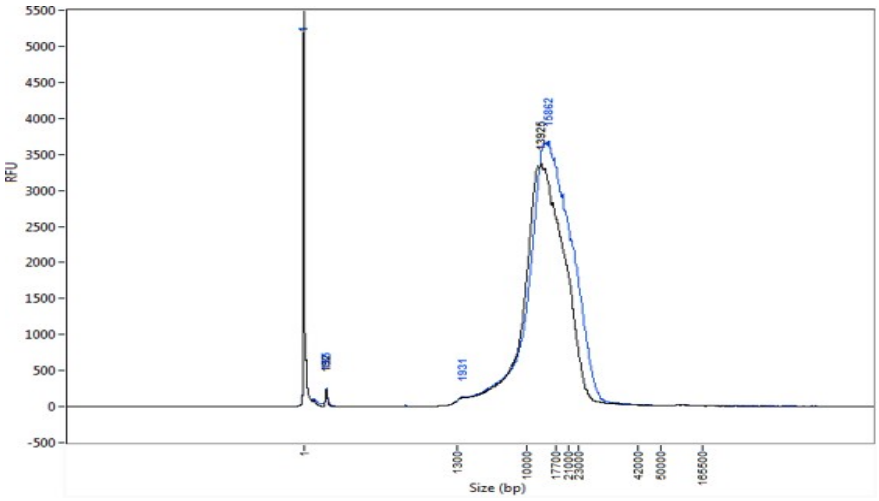

Figure 5: Example Femto Pulse sizing QC smear analysis of two mosquito samples sheared to an average size of 14 kb and 16 kb using speed 33 on the Megaruptor 3 system.

Concentrate DNA Using AMPure PB Beads (if necessary)

STEP	✓	Concentrate DNA	Notes
1		Bring the volume of the sample to **100 µL** with Elution Buffer. Add **1.8X** volume of AMPure PB beads to the DNA sample.	
2		Mix the bead/DNA solution thoroughly by pipette mixing 15 times with wide-bore pipette tips. Do not flick the tube.	
3		Quickly spin down the tube (for 1 second) to collect the beads.	
4		Incubate the mix on a benchtop for 5 minutes at room temperature.	
5		Spin down the tube (for 1 second) to collect the beads.	
6		Place the tube in a magnetic bead rack to collect the beads to the side of the tube.	
7		Slowly pipette off the cleared supernatant and save (in another tube). Avoid disturbing the beads.	
8		Wash the beads with freshly prepared 80% ethanol. Note that 80% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results. – Do not remove the tube from the magnetic rack. – Use a sufficient volume of 80% ethanol to fill the tube (i.e., 1.5 mL for a 1.5 mL tube or 2 mL for a 2 mL tube). Slowly dispense the 80% ethanol against the side of the tube opposite the beads. – Do not disturb the beads. – After 30 seconds, pipette and discard the 80% ethanol.	
9		Repeat step 8.	
10		Remove residual 80% ethanol. – Remove the tube from the magnetic bead rack and perform a quick spin. Both the beads and any residual 80% ethanol will be at the bottom of the tube. – Place the tube back on the magnetic bead rack. – Pipette off any remaining 80% ethanol.	
11		Check for any remaining droplets in the tube. If droplets are present, repeat step 10.	
12		Add **45.4 µL** of Elution Buffer to the beads (this is the volume required to go into the Remove Single-Strand Overhangs reaction). Pipette mix 15 times with wide-bore pipette tips. Do not flick the tube. – Place at 37°C for 15 minutes to elute the DNA from the beads. – Spin the tube down, then place the tube back on the magnetic bead rack. – Let the beads separate fully. Then without disturbing the beads, transfer the supernatant to a new 1.5 ml Lo-Bind tube. – Discard the beads.	
13		Verify the recovered DNA amount and concentration using a Qubit quantitation platform. – Use 1 µL of the eluted sample to measure the concentration using a Qubit fluorometer and the Qubit dsDNA HS Assay kit according to the manufacturer's recommendations.	
14		Proceed to library construction. If necessary, the concentrated DNA sample can also be stored for up to 2 weeks at 4°C or at -20°C for longer duration. Avoid freeze/thaw cycles.	

Reagent Handling

Several reagents required in this procedure for SMRTbell library construction are sensitive to temperature and vortexing (see Table 5). We highly recommend to:

- Never leave these reagents at room temperature.
- Always work on ice when preparing master mixes.
- Finger tap to mix followed by a quick-spin prior to use.

Reagent	Where Used
DNA Prep Additive	Remove single-strand overhangs
DNA Prep Enzyme	Remove single-strand overhangs
DNA Damage Repair Mix v2	DNA Damage Repair
End Prep Mix	End-Repair/A-tailing
Overhang Adapters v3	Ligation
Barcoded Overhang Adapters	Ligation
Ligation Mix	Ligation
Ligation Additive	Ligation
Ligation Enhancer	Ligation
Enzyme A	Nuclease Treatment
Enzyme B	Nuclease Treatment
Enzyme C	Nuclease Treatment
Enzyme D	Nuclease Treatment

Table 5: Temperature sensitive reagents.

Remove Single-Strand Overhangs

Before starting with the procedure below, refer to Table 5 for recommendations surrounding handling of reagents required for SMRTbell library construction.

Table 6 is a summary of the minimum amount of sheared DNA required to proceed with the first enzymatic reaction step “Remove Single-Strand Overhangs.”

Low DNA Input SMRTbell Library Type	Minimum Amount of Sheared DNA Required for Library Construction
Low DNA Input Library for the Sequel System (1 sample)	>100 ng
Low DNA Input Library for the Sequel II System (1 sample)	>300 ng
Low DNA Input Multiplexed Library for the Sequel II System (2 samples)	200 ng per sample

Table 6. Minimum sheared DNA amounts required for the “Remove Single-Strand Overhangs” reaction step.

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		

2. For each sample to be processed, prepare the following reaction. Refer to Table 6 for sheared DNA input requirements.

Reagent	Tube Cap Color	Volume	✓	Notes
DNA Prep Buffer		7.0 µL		
DNA		≤ 45.4 µL		
Water		Up to 55 µL		
NAD		0.6 µL		
Diluted DNA Prep Additive		1.0 µL		
DNA Prep Enzyme		1.0 µL		
Total Volume		55.0 µL		

3. Pipette mix 10 times with wide-bore pipette tips. Do not flick the tube.
4. Spin down the contents of the tube with a quick spin in a microfuge.
5. Incubate at 37°C for 15 minutes, then return the reaction to 4°C. Proceed to the next step.

Repair DNA Damage

For each sample to be processed, use the following table to prepare the reaction.

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

1. Pipette mix 10 times with wide-bore pipette tips. Do not flick the tube.
2. Spin down the contents of the tube with a quick spin in a microfuge.
3. Incubate at 37°C for 30 minutes, then return the reaction to 4°C.
4. Proceed to the next step.

Repair Ends/A-Tailing

For each sample to be processed, use the following table to prepare the reaction.

Reagent	Tube Cap Color	Volume	✓	Notes
DNA (Damage Repaired)		57.0 µL		
End Prep Mix		3.0 µL		
Total Volume		60.0 µL		

1. Pipette mix 10 times with wide-bore pipette tips. Do not flick the tube.
2. Spin down the contents of the tube with a quick spin in a microfuge.
3. Incubate at 20°C for 10 minutes
4. Incubate at 65°C for 30 minutes and return the reaction to 4°C. Proceed to the next step.

Adapter Ligation

For each sample to be processed, use the following table to prepare the reaction, adding the components below in the order listed.

For preparation of multiplexed libraries, use Barcoded Overhang Adapters (see Table 2) in place of the Overhang Adapters v3 reagent listed below.

Reagent	Tube Cap Color	Volume	✓	Notes
DNA (End-Repaired)		60.0 µL		
Overhang Adapter v3 (or Barcoded Overhang Adapter if preparing a multiplexed library)		5.0 µL		
Ligation Mix		30.0 µL		
Ligation Additive		1.0 µL		
Ligation Enhancer		1.0 µL		
Total Volume		97.0 µL		

1. Pipette mix 10 times with wide-bore pipette tips. Do not flick the tube.
2. Spin down the contents of the tube with a quick spin in a microfuge.
3. Incubate at 20°C for 60 minutes, then return the reaction to 4°C. (Overnight ligation at 20°C is optional.)
4. Proceed to the next steps as instructed below:
   - **For non-multiplexed libraries for the Sequel and Sequel II Systems**, proceed to "Purify SMRTbell Templates."
   - **For multiplexed libraries for the Sequel II System**, incubate at 65°C for 10 minutes to inactivate the ligase, then return the reaction to 4°C. Proceed to "Nuclease Treatment of SMRTbell Libraries."

Nuclease Treatment of SMRTbell Libraries

Use the following table to set up a reaction to remove damaged SMRTbell templates after the adapter ligation step. Enzymes A through D are found in the SMRTbell Enzyme Cleanup Kit.

1. To the remove damaged SMRTbell templates, prepare a Nuclease Treatment Master Mix:

Reagent	Tube Cap Color	Volume	✓	Notes
Enzyme A		4.0 µL		
Enzyme B		1.0 µL		
Enzyme C		1.0 µL		
Enzyme D		2.0 µL		
Total Volume		8.0 µL		

2. For each sample to be processed, add 4.0 µL Nuclease Treatment Master Mix to the ligated SMRTbell template.

Reagent	Volume	✓	Notes
SMRTbell library	97.0 µL		
Nuclease Treatment Master Mix	4.0 µL		
Total Volume	101.0 µL		

3. Mix the reaction well by pipetting up and down 10 times. It is important to mix well.
4. Incubate at 37°C for 1 hour, then return the reaction to 4°C.
5. Proceed immediately to “Purify SMRTbell Templates.”

Purify SMRTbell Templates

STEP	✓	Purify SMRTbell Templates	Notes
1		**For non-multiplexed libraries for the Sequel and Sequel II Systems**, first bring the sample volume to 100 µL by adding Elution Buffer to the Adapter Ligation reaction and then add **45 µL (0.45X)** volume of AMPure PB beads. **For multiplexed libraries for the Sequel II System**, add **81 µL (0.80X)** of AMPure PB beads to each nuclease-treated SMRTbell library sample.	
2		Mix the bead/DNA solution thoroughly by pipette mixing 15 times with wide-bore pipette tips. Do not flick the tube.	
3		Quickly spin down the tube (for 1 second) to collect the beads.	
4		Incubate samples on a benchtop for 5 minutes at room temperature.	
5		Spin down the tube (or 1 second) to collect beads.	
6		Place the tube in a magnetic bead rack to collect the beads to the side of the tube.	
7		Slowly pipette off the cleared supernatant and save (in another tube). Avoid disturbing beads.	
8		Wash the beads with freshly prepared 80% ethanol. Note that 80% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results. Also, 80% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days. – Do not remove the tube from the magnetic rack. – Use a sufficient volume of 80% ethanol to fill the tube (i.e., 1.5 mL for a 1.5 mL tube or 2 mL for a 2 mL tube). Slowly dispense the 80% ethanol against the side of the tube opposite the beads. – Do not disturb the beads. – After 30 seconds, pipette and discard the 80% ethanol.	
9		Repeat step 8.	
10		Remove residual 80% ethanol. – Remove the tube from the magnetic bead rack and perform a quick spin. Both the beads and any residual 80% ethanol will be at the bottom of the tube. – Place the tube back on the magnetic bead rack. – Pipette off any remaining 80% ethanol.	
11		Check for any remaining droplets in the tube. If droplets are present, repeat step 10.	
12		Immediately add **the appropriate volume** (see below) of Elution Buffer volume to the beads to elute the DNA. **Elution Volume** Non-multiplexed libraries for the Sequel and Sequel II Systems: 100 µL Multiplexed libraries for the Sequel II System: 20 µL – Pipette mix 15 times with wide-bore pipette tips. Do not flick the tube. – Elute the DNA by letting the mix incubate at 37 ºC for 15 minutes. – Spin the tube down, then place the tube back on the magnetic bead rack. – Let beads separate fully. Then without disturbing the beads, transfer the supernatant to a new 1.5 ml Lo-Bind tube.	
13		**For non-multiplexed libraries for the Sequel and Sequel II System,** proceed to "Size-Selection with AMPure PB Beads to remove <3 kb SMRTbell Templates." **For multiplexed libraries for the Sequel II System**, verify your DNA amount and concentration using a Qubit quantitation platform. – Measure the DNA concentration using a Qubit fluorometer. – Using 1 µL of the eluted sample to measure the DNA concentration using a Qubit fluorometer and the Qubit dsDNA HS Assay kit according to the manufacturer's recommendations. – Proceed to the next section "Pooling for Multiplexed Libraries".	

Pooling for Multiplexed Libraries

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.
- Equal mass pooling is recommended for samples that have a similar size distribution (+/- 15%)
  - The total mass of the pooled library must be >150 ng (e.g., 75 ng of Sample 1 + 75 ng of Sample 2)
  - If the total mass of the two samples is <150 ng, use the remaining sample to adjust the total combined mass to 150 ng. This is to ensure that there is sufficient library material to load at least one Sequel II SMRT Cell 8M. Note that in such cases, the resulting number of reads generated per sample may be slightly imbalanced.

STEP	✓	Pooling	Notes
1		Pool barcoded libraries into a single 1.5 mL DNA Lo-Bind tube.	
2		Mix and spin down the contents of the tube with a quick spin in a microfuge.	
3		Bring the total volume of the pooled sample to 100 µL with Elution Buffer.	
4		Proceed to the **"Size-Selection with AMPure PB Beads to remove <3 kb SMRTbell Templates**" section below.	

Size-Selection with AMPure PB Beads to remove <3 kb SMRTbell Templates

The final AMPure PB purification step in this procedure removes short SMRTbell templates <3 kb. The AMPure PB bead stock solution is first diluted (40% volume/volume) with Elution Buffer and subsequently used for purification as described below. When diluting AMPure PB beads, accurate measurement is of utmost importance.

Dilute AMPure PB Beads with Elution Buffer to 40% (v/v)

The final AMPure PB bead concentration is critical to the success of this procedure. Therefore, accurate pipetting is of utmost importance to achieve a final 40% (v/v) AMPure PB bead working solution in Elution Buffer.

Reagent	Volume	✓	Notes
Elution Buffer	3.0 mL		
AMPure PB Bead (stock reagent, thoroughly mixed)	2.0 mL		
Total Volume	5.0 mL		

1. Bring the AMPure PB bead stock solution to room temperature.
2. Vortex the stock solution for 30 seconds to mix well.
3. Using a P1000 pipette, transfer 3.0 mL of Elution Buffer into a 15 mL conical tube.
4. Add 2.0 mL of the AMPure PB bead stock solution to the 3.0 mL of Elution Buffer. When pipetting the viscous AMPure PB bead stock solution, pipette slowly to ensure that the volume aspirated is as precise as possible. Large residual AMPure solution adhering to the tip should be removed prior to addition to the 3.0 mL of Elution Buffer.
5. Vortex the diluted AMPure PB bead solution for 30 seconds to mix well before use. This solution may be stored at 4°C for 2 weeks for future use.

Size-Selection with AMPure PB Beads

STEP	✓	Purify SMRTbell Templates	Notes
1		Add **2.2X** volume of **diluted** AMPure PB Bead Solution (40% v/v) to the 100 µL of DNA sample. **It is critical to mix precise volumes of both the sample and the diluted AMPure PB bead solution to achieve successful removal of short-insert SMRTbell templates.**	
2		Mix the bead/DNA solution thoroughly by pipette mixing 15 times with wide-bore pipette tips. It is important to mix well.	
3		Quickly spin down the tube (for 1 second) to collect the beads.	
4		Allow the DNA to bind to beads by gentle rotation at room temperature for 30 minutes.	
5		Spin down the tube (for 1 second) to collect the beads.	
6		Place the tube in a magnetic bead rack to collect the beads to the side of the tube.	
7		Slowly pipette off cleared supernatant and save (in another tube). Avoid disturbing the beads.	
8		Wash the beads with freshly prepared 80% ethanol. Note that 80% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results. – Do not remove the tube from the magnetic rack. – Use a sufficient volume of 80% ethanol to fill the tube (1.5 mL for 1.5 mL tube or 2 mL for 2 mL tube). Slowly dispense the 80% ethanol against the side of the tube opposite the beads. – Do not disturb the beads. – After 30 seconds, pipette and discard the 80% ethanol.	
9		Repeat step 8.	
10		Remove residual 80% ethanol. – Remove the tube from the magnetic bead rack and spin. Both the beads and any residual 80% ethanol will be at the bottom of the tube. – Place the tube back on the magnetic bead rack. Beads will be on the side of the tube. – Pipette off any remaining 80% ethanol.	
11		Check for any remaining droplets in the tube. If droplets are present, repeat step 10.	
12		Add **10 µL** of Elution Buffer volume to the beads. When adding the 10 µL of EB, dispense the volume directly to the beads. Do not let the beads dry. Pipette- mix 15 times with wide-bore pipette tips. – Elute the DNA by letting the mix incubate at 37 ºC for 15 minutes. – Spin the tube down, then place the tube back on the magnetic bead rack. – Let the beads separate fully. – Without disturbing the bead pellet, transfer the supernatant to a new 1.5 mL Lo-Bind tube. – Discard the beads.	
13		Verify the recovered DNA amount and concentration using a Qubit quantitation platform. – Using 1 µL of the purified sample, make a 1:1 dilution in Elution Buffer. – Use 1 µL of this 1:1 dilution to measure the DNA concentration using a Qubit fluorometer and the Qubit dsDNA HS Assay kit according to the manufacturer's recommendations. Use the other 1 µL of 1:1 diluted sample for DNA sizing QC by using a Femto Pulse system.	
14		AMPure PB bead purified SMRTbell libraries may be stored for up to 2 weeks at 4°C or at -20°C for longer duration until ready for sequencing.	

Preparation for Sequencing

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

Revision History (Description)	Version	Date
Initial Release (Internal Only).	01	February 2019
Updated kit name terminology, added recommended tools, provided tables showing reagents and recommended handling. Other similar updates and clarifications.	02	April 2019
Updated to include recommendations for the Sequel II System.	03	September 2019
Updated to include multiplex recommendations for the Sequel II System.	04	November 2019
Updated with instructions on how to construct HiFi libraries (instead of CLR libraries).	05	June 2020
In Primer Annealing and Polymerase Binding table on page 17, changed 30:1 to 20:1 for Primer-to-Template ratio and 20:1 to 30:1 for Polymerase-to-Template ratio.	06	June 2020
Direct to SMRT Link for instructions on preparing for sequencing. Reference SMRTbell Barcoded Adapter Plate 3.0.	07	November 2021