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

This document describes preparing SMRTbell libraries from as low as 150 ng of genomic DNA (gDNA) using SMRTbell Express Template Prep Kit 2.0. The SMRTbell Express Template Prep Kit 2.0 is an additive, single-tube workflow, which minimizes DNA loss during library construction, thereby allowing library construction from low amounts of input DNA.

With the low-input workflow, the size distribution of the starting DNA is critical to generating long subread lengths for successful genome assembly. Since size-selection with BluePippin may not be an option due to low DNA availability, we recommend working with samples where the majority of the DNA is greater than 20 kb (larger is preferred, and a sample with a mean fragment length of 50-60 kb is optimal). Using genomic DNA with significant amounts of fragments less than 20 kb will negatively impact subread lengths and will result in a suboptimal genome assembly.

Figures 2 and 3 demonstrate 4 types of DNA samples with different DNA size distributions. Genomic DNA samples with a mean fragment length above 20 kb perform well (with average subread lengths >7 kb and N50 subread lengths >10 kb), and may be suitable for *de novo* assembly of insect genomes up to 300 Mb. DNA samples with the majority of DNA <20 kb may result in short subread lengths (<5 kb) and a more fragmented genome assembly. For large insects where DNA can be extracted in abundant quantities (>2 µg) from a single individual, we recommend using a workflow that employs size-selection.

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

When working with low amounts of DNA, accurate quantification must be used. The Qubit High Sensitivity (HS) assay system can be used for accurate measurements.

Overall SMRTbell library construction yields in this low-input workflow are typically >50%. Depending on the final size of the library, sufficient amounts of library material to run approximately 4 or more Sequel SMRT® Cells 1M can be generated.

It is important to note that the first step in the library construction process (removal of single stranded overhangs) requires a volume of 45.4 µL of sample containing 150 ng (3.3 ng/ µL) or more DNA. Therefore, it is good practice to elute your DNA sample in a final volume of 45.4 µL of Elution Buffer during DNA extraction. This eliminates the need to concentrate samples due to high volume and reduces potential sample loss.

Required Materials

Item	Vendor	Part Number
Qualification		
FEMTO *Pulse*® Automated Pulsed Field CE Instrument and/or Pippin Pulse Electrophoresis Power Supply and/or Pulsed Field Gel Electrophoresis System: CHEF Mapper	Advanced Analytical Sage Science Bio-Rad	FFv1-CE2 PP10200 170-3670
Quantification Qubit™ Fluorometer	ThermoFisher Scientific	Q33226
Qubit™ dsDNA HS Assay Kit	ThermoFisher Scientific	Q32854
SMRTbell Library Construction SMRTbell® Express Template Prep Kit 2.0 AMPure® PB beads	 PacBio PacBio	 100-938-900 100-265-900
DNA Lo Bind microfuge tubes	Eppendorf	022431021
Wide Orifice Tips (Tips LTS W-O 200UL Fltr RT-L200WFLR)	Rainin	17014294

SMRTbell Express Template 2.0 Preparation Workflow

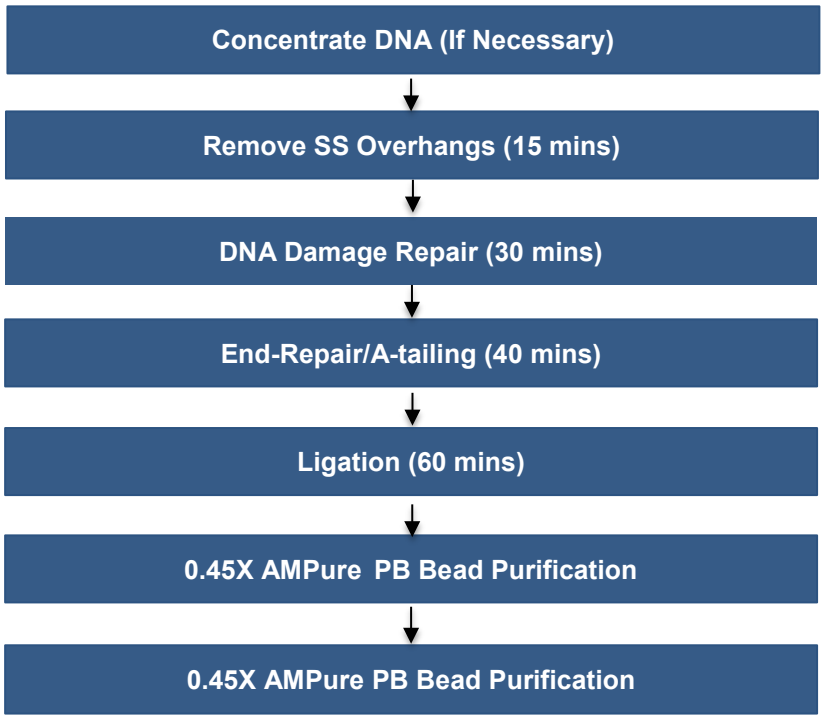


Figure 1: Workflow for Preparing Libraries Using the SMRTbell Express Template Prep Kit 2.0 with low DNA input.

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 the template preparation reagents should be dispensed with a standard pipette tip (i.e., P10 or P20) and then switch over to wide-bore for mixing.
3. Never vortex tubes containing high molecular weight DNA.
4. 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.
5. Always set your heat blocks or thermocyclers to the appropriate temperature for incubations before proceeding with the procedure.

Recommended Tools for gDNA Quantification and Qualification

When working with small amounts of input DNA, accurate sizing and quantification is critical to enable generation of sufficient coverage of long reads 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 accurately determine the size distribution of your gDNA sample, we recommend the use of the FEMTO Pulse System because of its ability to 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 1 below with links to recommended procedures. Note, however, that the CHEF Mapper and Pippin Pulse may not be appropriate options in some cases since both tools require at least 50 ng of DNA sample for analysis.

Method	Comments	Procedure
Advanced Analytical Technologies, Inc. FEMTO *Pulse*	Highly recommended (200-500 picograms)	Advanced Analytical Website
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 1. gDNA Size Evaluation Methods and Procedures.

Evaluation of gDNA for Low-Input Library Construction

Because size-selection may not be an option when working with low amounts of DNA (>150 ng), the size distribution of the initial gDNA is critical to successful SMRTbell template preparation and sequencing.

Evaluate your gDNA samples to see if they are appropriate for library preparation using the low DNA input workflow. Figure 2 below shows examples of mosquito gDNA samples of varying quality run on FEMTO *Pulse*. In this example, Samples 1 and 2 have a majority of gDNA fragments >20 kb with distribution mode sizes of >30 kb suitable for generating long reads for *de novo* assembly. If gDNA is severely fragmented, the average subread lengths will be negatively impacted. For example, samples 3 and 4, when sequenced, generated average subreads lengths of ~4 kb, compared to samples 1 and 2 that generated subreads lengths of ~8 kb and 14 kb, respectively. (see Figure 3).

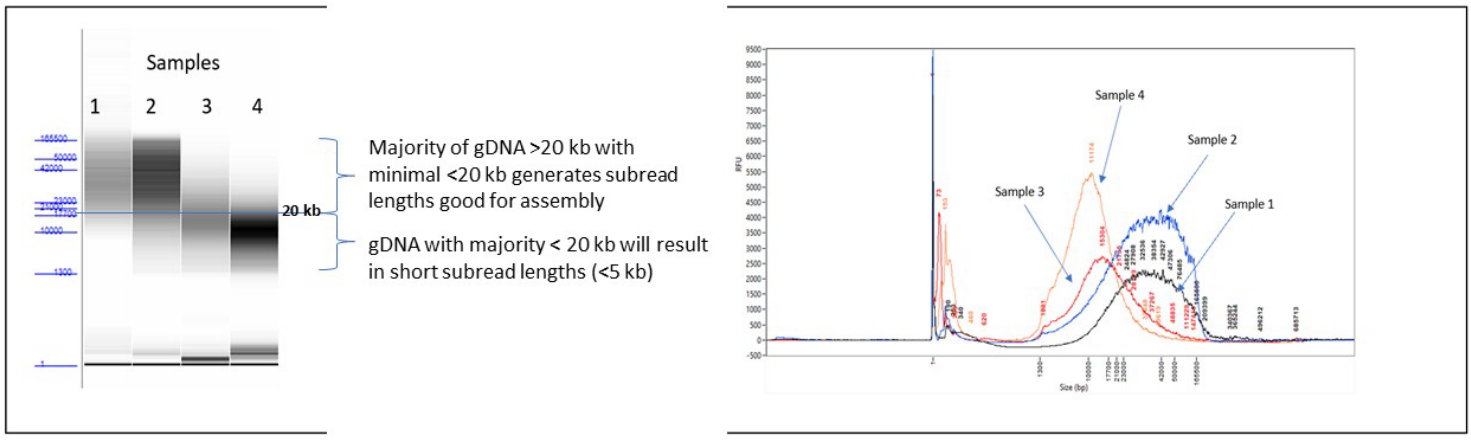
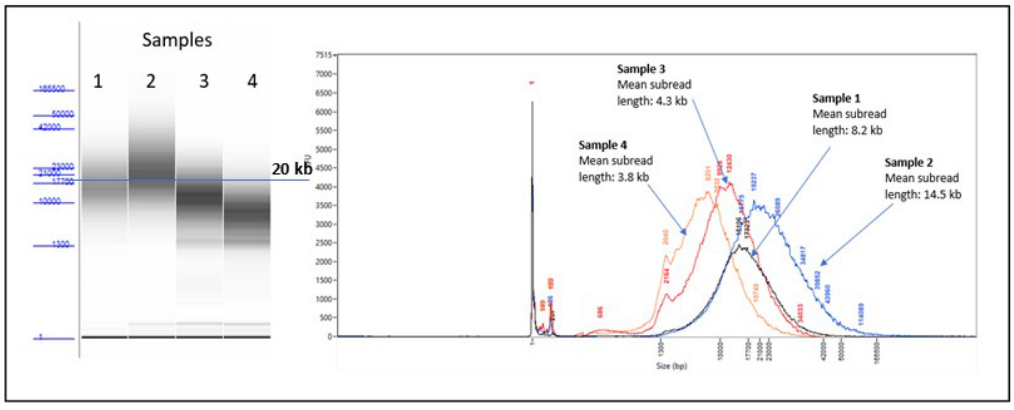


Figure 2: Evaluation of gDNA quality using FEMTO *Pulse*. Samples 1 and 2 are examples of gDNA suitable for library construction using the low DNA input procedure. Both samples contain a majority of gDNA >20 kb and a minimal amount of <20 kb fragments. Samples 3 and 4 are examples of degraded gDNA and should not be used for library preparation with workflow described in this procedure.



Sample	Subread length	N50 Subread length
1	8.2 kb	12 kb
2	14.5 kb	13 kb
3	4.3 kb	7 kb
4	3.8 kb	6 kb

Figure 3: Final SMRTbell libraries from gDNA samples in Figure 2. Libraries 1 and 2 generated subread lengths of ~8 kb and 14 kb, respectively, which can facilitate a high-quality genome assembly. Libraries 3 and 4 both generated shorter subread lengths of ~4 kb, which would result in morefragmented genome assemblies.

Several tubes in the kits (shown in Table 4 below) are sensitive to temperature and vortexing. We highly recommend:

- Never leaving tubes at room temperature.
- Working on ice at all times when preparing master mixes.
- Finger tapping 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
Barcoded Overhang Adapters	Ligation
Ligation Mix	Ligation
Ligation Additive	Ligation
Ligation Enhancer	Ligation

Table 2: Temperature sensitive reagents

Concentrate DNA Using AMPure PB Beads (If Necessary)

STEP	✓	Concentrate DNA	Notes
1		Add **1.0X** volume of AMPure PB beads to the sheared gDNA.	
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 bench top for 5 minutes at room temperature.	
5		Spin down the tube (for 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 cleared supernatant and save (in another tube). Avoid disturbing the beads.	
8		Wash 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 tube from 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 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		The volume to use for the elution is **45.4 µL** (the volume to go into Remove Single-Stranded Overhang reaction).	
13		Add the Elution Buffer volume to the beads. 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 beads separate fully. Then without disturbing the beads, transfer supernatant to a new 1.5 ml Lo-Bind tube. – Discard the beads.	
14		The sheared DNA can be stored for up to 2 weeks at 4°C or at -20°C for longer duration. Do not freeze/thaw.	

Remove Single-Stranded Overhangs

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		5.0µL		

2. Use the following table to set up a reaction to remove single-stranded ends with >150 ng of sheared gDNA.

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 contents of 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

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 contents of tube with a quick spin in a microfuge.
3. Incubate at 37°C for 30 minutes, return the reaction to 4°C.
4. Proceed to the next step.

Repair Ends/A-Tailing

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 contents of 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

Use the following table to prepare the reaction, adding the components below in the order listed.

Reagent	Tube Cap Color	Volume	✓	Notes
DNA (End-Repaired)		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		

1. Pipette mix 10 times with wide-bore pipette tips. Do not flick the tube.
2. Spin down contents of tube with a quick spin in a microfuge.
3. Incubate at 20°C for 60 minutes, then return the reaction to 4°C.
4. Proceed to the next step.

Purify SMRTbell Templates

There are 2 purification steps requiring 0.45X volumes of AMpure PB beads.

STEP	✓	Purify SMRTbell Templates – First Purification	Notes
1		Bring volume to 100 ul by adding 3 µL of Elution Buffer to the Adapter Ligation reaction and then add **0.45X** volume of AMPure PB beads.	
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 bench top for 5 minutes at room temperature.	
5		Spin down the tube (for 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 cleared supernatant and save (in another tube). Avoid disturbing the beads.	
8		Wash 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 (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 tube from 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 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 **100 µL** of Elution Buffer volume to the beads. 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. This is important to maximize recovery of high molecular weight DNA. – Spin the tube down, then place the tube back on the magnetic bead rack. – Let beads separate fully. Then without disturbing the beads, transfer supernatant to a new 1.5 ml Lo-Bind tube. – Discard the beads.	

Purify SMRTbell Templates

STEP	✓	Purify SMRTbell Templates – Second Purification	Notes
1		Add **0.45X** volume of AMPure PB beads to the SMRTbell library.	
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 bench top for 5 minutes at room temperature.	
5		Spin down the tube (for 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 cleared supernatant and save (in another tube). Avoid disturbing the beads.	
8		Wash 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 tube from 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 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 the **10 µL** Elution Buffer volume to the beads. 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 beads separate fully. Then without disturbing the beads, transfer supernatant to a new 1.5 ml Lo-Bind tube. – Discard the beads.	
13		Verify the DNA amount and concentration using a Qubit quantitation platform. – Using 1 µL of the purified sample, make a 1:1 dilution in EB. – Use 1 µL of this 1:1 dilution to measure the DNA concentration using a Qubit fluorometer and the dsDNA HS Assay kit according to the manufacturer's recommendations. – Use the other 1 µL of 1:1 diluted sample for QC by FEMTO *Pulse,* automated pulsed-field capillary electrophoresis.	
14		AMPure PB bead purified, libraries may be stored at -20ºC.	

Anneal and Bind SMRTbell Templates

Use the Sample Setup Calculator for instructions for primer annealing and polymerase binding.

For primer annealing, use **Sequencing Primer v4**. This primer is for diffusion loading only.

It is not appropriate for MagBead loading.

Prepare for Sequencing

We recommend collecting 10-hour movies, with 120-minute pre-extension.

It is highly recommended to purify the complex using AMPure PB bead purification to remove excess primer and polymerase prior to sequencing.

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