

# Procedure & Checklist - Preparing >15 kb Libraries Using SMRTbell® Express Template Preparation Kit

This document provides recommendations for preparing >15 kb size-selected SMRTbell libraries from 3-5 µg of starting **sheared** genomic DNA. As little as 2 µg of sheared genomic DNA may be used, if gDNA amounts are low and size-selection losses are not limiting.

Only high-quality, high molecular weight gDNA may be used for producing large insert libraries size selected using this Express Template Preparation Procedure. Specifically, a size selection of > 15 kb must be performed. If the sample is predominantly >50 kb, please refer to *Procedure & Checklist - Preparing >30 kb Libraries Using SMRTbell Express Template Preparation Kit*.

In general, when performing SMRT Sequencing using long insert libraries, performance is optimal with the longest inserts possible. For example, if a significant portion of the gDNA migrates below ~40 kb, recovery post size selection with a >15 kb lower cutoff will be negatively impacted (see Figure 2). Additionally, read length and sequencing yield will be compromised.

To ensure success, gDNA size and integrity must be verified by pulsed field gel electrophoresis (PFGE) or equivalent method before beginning library preparation. In addition, conditions for shearing gDNA to a size that can support producing large insert libraries must be determined and verified empirically for each sample.

Overall yields of >15 kb libraries before size selection are typically >50%.

## Required Materials

Item	Vendor	Part Number
<b>Pulsed Field Gel Electrophoresis</b>		
Pulsed Field Gel Electrophoresis System: CHEF Mapper XA	Bio-Rad	170-3670
Pulsed Field Certified Agarose	Bio-Rad	162-0137
CHEF DNA Size Standard 5 kb	Bio-Rad	170-3624
Invitrogen 1 kb DNA extension ladder	Life Technologies	10511-012
<b>Shearing</b>		
g-TUBE	Covaris	010145
<b>SMRTbell Library</b>		
SMRTbell® Express Template Prep Kit	PacBio	101-357-000
<b>Size Selection</b>		
BluePippin Size-Selection System	Sage Science	BLU0001
Marker S1 Reagent kit	Sage Science	
BluePippin Gel Cassettes	Sage Science	BLF7510
<b>Tips</b>		
Wide Orifice Tips (Tips LTS W-O 200UL Fltr RT-L200WFLR)	Rainin	17014294

## SMRTbell Express Workflow



Figure 1: Workflow for Preparing >15 kb SMRTbell Libraries Using the Express Template Preparation Kit

## Best Practice Recommendations

1. Use wide-bore tips for all pipette mixing steps.
2. Never vortex tubes containing high molecular weight DNA.
3. Always follow Qubit best practices:
  - Prepare the Qubit working solution by diluting the Qubit reagent 1:200 in Qubit buffer. Prepare 200  $\mu$ L of working solution for each standard and sample. Always make new standards for each assay.
  - Set up two 190  $\mu$ L assay tubes for the standards and one 199  $\mu$ L assay tube for each sample. Add 10  $\mu$ L standard (from kit) and 1  $\mu$ L 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.
4. Always set your heat blocks or thermocyclers to the appropriate temperature for incubations before proceeding with the procedure.

## Evaluate Genomic DNA Quality

We highly recommend using Bio-Rad<sup>®</sup> CHEF Mapper<sup>®</sup> XA Pulsed Field Electrophoresis system for evaluating gDNA quality. The procedure is available [here](#).

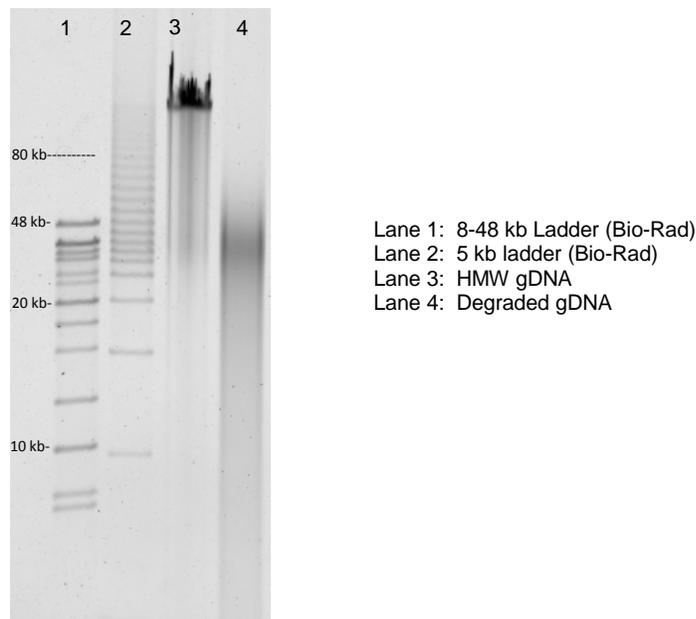


Figure 2: Evaluation of gDNA quality using Bio-Rad CHEF Mapper. Lane 3 is an example of a high quality, high molecular weight genomic DNA. Lane 4 is an example of degraded gDNA and should not be used for production of a large insert library size selected using >15 kb cutoff.

Additionally, there are other commercially available systems capable of resolving DNA fragments and smears up to ~50 kb. Recommendations for using Sage Science's Pippin Pulse Electrophoresis Power Supply are available [here](#). Alternatively, you can use the Advanced Analytical Technologies, Inc. FEMTO Pulse (link [here](#)) automated pulsed-field capillary electrophoresis instrument for evaluating the integrity of genomic DNA with a run time of approximately 1.5 hours.

## Optimize Shearing Conditions

To ensure sufficient yields of final >15 kb libraries, input gDNA must be sheared carefully so that the average size of fragmented DNA remains well above the desired size selection cut-off. The response of individual gDNA samples to recommended shearing parameters may differ and must be determined empirically and evaluated by PFGE. Test shears are highly recommended.

Note that for preparing >15 kb libraries, gDNA may be sheared by g-TUBE. Here we provide initial starting parameters methods as well as strategies for optimization of gDNA shearing.

## Fragment and Concentrate DNA

Use a Covaris® g-TUBE® device to shear the DNA sample. General recommendations for g-TUBE usage can be found in the g-TUBE user manual available on the Covaris website (see <http://covarisinc.com/products/g-tube/>). Note that although Covaris provides recommendations for shearing gDNA to 20 kb, these parameters result in sheared gDNA with average insert sizes of less than 20 kb and are, therefore, **NOT** suitable for use in this procedure. To generate sheared gDNA with average insert sizes >15 kb, you **MUST** follow the specific instructions provided here:

1. Dilute the DNA to 200-300 ng/μL in Elution Buffer (EB). The sample volume may range from 20-100 μL.
2. Shear at 5500 rpm (2029 x g) for 2 minutes in an Eppendorf® MiniSpin plus.
3. Check for any residual sample remaining in the upper chamber. If present, re-spin for another 2 minutes. Continue spinning until all the sample has passed through the orifice.
4. Invert and spin at 5500 rpm (2029 x g) until all the sample has passed through the orifice.
5. Recover the sample into a 1.5 mL LoBind microcentrifuge tube.

Depending upon the quality of the sample, approximately 20% sample loss is to be expected (as a result of the shearing and concentration process). Therefore, be sure to have sufficient amounts of starting DNA in order to have at least 3 μg of sheared and concentrated DNA for the subsequent repair steps.

## Concentrate DNA Using AMPure® PB Beads (If Necessary)

STEP	✓	Concentrate DNA	Notes
1		<p>Add <b>0.60X</b> volume of AMPure PB magnetic beads to the sheared gDNA</p> <p>_____ μL of sample X <b>0.60X</b> = _____ μL of beads</p> <p>Note that the beads must be brought to room temperature and all AMPure PB bead purification steps should be performed at room temperature.</p> <p>Before using, mix the bead reagent well until the solution appears homogenous. Pipette the reagent slowly since the bead mixture is viscous and precise volumes are critical to the purification process.</p>	
2		Pipette mix 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		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 until the beads collect to the side of the tube and the solution appears clear. The actual time required to collect the beads to the side depends on the volume of beads added.	
7		<p>With the tube still on the magnetic bead rack, slowly pipette off cleared supernatant and save in another tube. Avoid disturbing the beads.</p> <p>If the DNA is not recovered at the end of this procedure, you can add equal volumes of AMPure PB beads to the saved supernatant and repeat the AMPure PB bead purification steps to recover the DNA.</p>	
8		<p>Wash beads with freshly prepared 80% ethanol.</p> <p>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.</p> <ul style="list-style-type: none"> <li>– Do not remove the tube from the magnetic rack.</li> <li>– 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.</li> <li>– Do not disturb the beads.</li> <li>– After 30 seconds, pipette and discard the 80% ethanol.</li> </ul>	
9		Repeat <a href="#">step 8</a> .	
10		<p>Remove residual 80% ethanol.</p> <ul style="list-style-type: none"> <li>– Remove tube from magnetic bead rack and spin. Both the beads and any residual 80% ethanol will be at the bottom of the tube.</li> <li>– Place the tube back on magnetic bead rack.</li> <li>– Pipette off any remaining 80% ethanol.</li> </ul>	
11		Check for any remaining droplets in the tube. If droplets are present, repeat <a href="#">step 10</a> .	

STEP	Concentrate DNA		Notes
12	The volume to use for elution is <b>48 <math>\mu</math>L</b> (the volume to go into Exo VII).		
13	<p>Add the Elution Buffer volume to your beads. Pipette mix 15 times with wide-bore pipette tips. It is important to mix well.</p> <ul style="list-style-type: none"> <li>- Place at 37°C for 15 minutes to elute the DNA from the beads.</li> <li>- Spin the tube down, then place the tube back on the magnetic bead rack.</li> <li>- Let beads separate fully. Then without disturbing the beads, transfer supernatant to a new 1.5 ml Lo-Bind tube.</li> <li>- Discard the beads.</li> </ul>		
14	<p>Verify your DNA amount and concentration using a Qubit quantitation platform.</p> <ul style="list-style-type: none"> <li>- Measure the DNA concentration using a Qubit fluorometer.</li> <li>- Using 1 <math>\mu</math>L of the eluted sample, make a 1:10 dilution in EB.</li> <li>- Use 1 <math>\mu</math>L of this 1:10 dilution to measure the DNA concentration using a Qubit dsDNA BR Assay kit and the dsDNA HS Assay kit according to the manufacturer's recommendations.</li> </ul> <p>The remaining 9 <math>\mu</math>L of 1:10 diluted sample may be used for QC by pulsed field gel electrophoresis.</p>		
15	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.		
16	Actual recovery per $\mu$ L and total available sample material: _____		

## Exo VII Pre-treatment of DNA

Use the following table to set up a reaction to remove single-stranded ends from 3 - 5 µg of sheared gDNA. As little as 2 µg of sheared genomic DNA may be used, if gDNA amounts are low and size-selection losses are not limiting. If starting with more than 5 µg of sheared gDNA, scale reaction volumes proportionally (i.e., for a mass between 5-10 µg of DNA scale the total volume to 110 µL).

Reagent	Volume	✓	Notes
Sheared DNA (3 µg)	≤ 48.0 µL		
10X DNA Damage Repair Buffer v2	6.0 µL		
Exo VII v2	1.0 µL		
Water	Up to 55 µL		
Total Volume	55.0 µL		

1. Pipette mix 15 times with wide-bore pipette tips. It is important to mix well.
2. Spin down contents of tube with a quick spin in a microfuge.
3. 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 your reaction.

Reagent	Volume	✓	Notes
DNA (Exo VII treated)	55.0 µL		
DNA Damage Repair Mix	2.0 µL		
Total Volume	57.0 µL		

1. Pipette mix 15 times with wide-bore pipette tips. It is important to mix well.
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 your reaction.

Reagent	Volume	✓	Notes
DNA (Damage Repaired)	57.0 µL		
End Repair Mix v2	2.0 µL		
End Repair Additive	1.0 µL		
Total Volume	60 µL		

1. Pipette mix 15 times with wide-bore pipette tips. It is important to mix well.
2. Spin down contents of tube with a quick spin in a microfuge.
3. Incubate at 20°C for 5 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 your reaction, adding the components below in the order listed.

Reagent	Volume	✓	Notes
DNA (End-Repaired)	60.0 $\mu$ L		
5X Ligation Buffer	22.0 $\mu$ L		
Water	17.0 $\mu$ L		
Overhang Adapter v3	5.0 $\mu$ L		
Pipette mix 15 times with wide-bore tips.			
Ligation Additive	1.0 $\mu$ L		
Ligase v2	5.0 $\mu$ L		
Total Volume	110.0 $\mu$ L		

1. Pipette mix 15 times with wide-bore pipette tips. It is important to mix well.
2. Spin down contents of tube with a quick spin in a microfuge.
3. Incubate at 20°C for 30 minutes, then return the reaction to 4°C.
4. Proceed to the next step.

## Termination Reaction

1. Dilute the Terminase with Termination Buffer.

Reagent	Volume	✓	Notes
Termination Buffer	9.0 $\mu$ L		
Terminase (stock)	1.0 $\mu$ L		
Total Volume	10 $\mu$ L		

2. Use the following table to prepare the Terminase reaction.

Reagent	Volume	✓	Notes
Ligated DNA	110 $\mu$ L		
Diluted Terminase	2.0 $\mu$ L		
Total Volume	112 $\mu$ L		

- a. Pipette mix 15 times with wide-bore pipette tips. It is important to mix well.
- b. Spin down contents of tube with a quick spin in a microfuge.
- c. Incubate at 37°C for 15 minutes, then return the reaction to 4°C. Proceed to the next step.

## Purify SMRTbell Templates

STEP	Purify DNA	Notes
1	Add <b>0.60X</b> volume of AMPure PB beads to the Terminase-treated DNA.	
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	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	<p>Wash beads with freshly prepared 80% ethanol.</p> <p>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.</p> <ul style="list-style-type: none"> <li>– Do not remove the tube from the magnetic rack.</li> <li>– 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.</li> <li>– Do not disturb the beads.</li> <li>– After 30 seconds, pipette and discard the 80% ethanol.</li> </ul>	
9	Repeat <a href="#">step 8</a> .	
10	<p>Remove residual 80% ethanol.</p> <ul style="list-style-type: none"> <li>– Remove tube from magnetic bead rack and spin. Both the beads and any residual 80% ethanol will be at the bottom of the tube.</li> <li>– Place the tube back on magnetic bead rack.</li> <li>– Pipette off any remaining 80% ethanol.</li> </ul>	
11	Check for any remaining droplets in the tube. If droplets are present, repeat <a href="#">step 10</a> .	
12	<p>Immediately add <b>31 <math>\mu</math>L</b> of Elution Buffer volume to your beads. Pipette mix 15 times with wide-bore pipette tips. It is important to mix well.</p> <ul style="list-style-type: none"> <li>– Elute the DNA by letting the mix incubate at 37 °C for 15 minutes. This is important to maximize recovery of high molecular weight DNA.</li> <li>– Spin the tube down, then place the tube back on the magnetic bead rack.</li> <li>– Let beads separate fully. Then without disturbing the beads, transfer supernatant to a new 1.5 ml Lo-Bind tube.</li> <li>– Discard the beads.</li> </ul>	

13	Verify your DNA amount and concentration using a Qubit quantitation platform. <ul style="list-style-type: none"> <li>– Measure the DNA concentration using a Qubit fluorometer.</li> <li>– Using 1 <math>\mu</math>L of the eluted sample, make a 1:10 dilution in EB.</li> <li>– Use 1 <math>\mu</math>L of this 1:10 dilution to measure the DNA concentration using a Qubit fluorometer and the dsDNA HS Assay kit according to the manufacturer's recommendations.</li> </ul>	
14	It is highly recommended to perform qualitative and quantitative analysis using Pulse Field Gel Electrophoresis before size selection. This allows to choose appropriate Blue Pippin cut off for size selection. Choosing aggressive BP cutoff without knowing size distribution of SMRTbell Templates might lead to significant sample loss.	
15	Proceed with size-selection after AMPure PB Bead purification of libraries. If the library is not ready for size selection, it can be stored for up to 2 weeks at 4°C or at -20°C for longer duration. Do not freeze/thaw.	
16	Actual recovery per $\mu$ L and total available sample material: _____	

## Size-Selection Using the BluePippin™ System

Follow the instructions in the BluePippin User Manual and User Guides (see [www.sagescience.com](http://www.sagescience.com)), and the specific recommendations below, for >15 kb.

Note that you must use BluePippin Software **v6.20** (or higher) and the “0.75% DF Marker S1 high-pass 15 kb -20kb” run protocol for this procedure. Use the S1 marker for this protocol.

1. Prepare up to 5  $\mu$ g SMRTbell templates in a final volume of 30  $\mu$ L Elution Buffer for each lane. Size selection using this protocol can be aggressive and if not cautious, recovery may be impacted.
2. Bring the Loading Solution to room temperature, then add 10  $\mu$ L of the Loading Solution to the 30  $\mu$ L DNA sample. For multiple lanes, scale volumes proportionally. The Loading Solution is viscous so pipet slowly to ensure complete transfer into the DNA sample.
  - a. Pipette mix using wide-bore pipette tips to mix.
  - b. Spin briefly to collect the contents at the bottom of the tube.
3. Follow the manufacturer's recommendations to set up a run protocol.
  - a. When setting up the run protocol, select the “0.75% DF Marker S1 high-pass 15-20kb” cassette definition file.
  - b. Using the “Range” selection mode, enter the desired “BPstart” value of 15000 or 20000 bp. A “BP End” value of 50000 bp should automatically appear.  
**Note: If using < 3  $\mu$ g per lane, use BP start = 12500 for >15 kb size selection and BP start = 15000 for >20 kb size selection.**
  - c. Be sure to assign a marker lane.
4. Load samples and be sure to load the S1 marker. Start the run. Typical run times are ~5.5 hours.
5. To maximize recovery of eluted DNA, wait at least 30 minutes after the run terminates before removing the sample from the elution chamber.
  - a. Collect the eluate into a 1.5 mL DNA LoBind tube.
  - b. Wash elution well with 40  $\mu$ L of Sage Science's 0.1% Tween-20 Wash Solution, and add wash to eluted sample. Washing the elution well may increase yield 10-20%.

## Purify Size-Selected SMRTbell Library with 1X AMPure PB Beads

STEP	Purify DNA	Notes
1	Add <b>1X</b> volume of AMPure PB beads to the size-selected library.	
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	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	<p>Wash beads with freshly prepared 80% ethanol.</p> <p>Note that 80% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results.</p> <ul style="list-style-type: none"> <li>– Do not remove the tube from the magnetic rack.</li> <li>– 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.</li> <li>– Do not disturb the beads.</li> <li>– After 30 seconds, pipette and discard the 80% ethanol.</li> </ul>	
9	Repeat <a href="#">step 8</a> .	
10	<p>Remove residual 80% ethanol.</p> <ul style="list-style-type: none"> <li>– Remove tube from magnetic bead rack and spin. Both the beads and any residual 80% ethanol will be at the bottom of the tube.</li> <li>– Place the tube back on magnetic bead rack.</li> <li>– Pipette off any remaining 80% ethanol.</li> </ul>	
11	Check for any remaining droplets in the tube. If droplets are present, repeat <a href="#">step 10</a> .	
12	<p>Add the <b>10 <math>\mu</math>L</b> Elution Buffer volume to your beads. Pipette mix 15 times with wide-bore pipette tips. It is important to mix well.</p> <ul style="list-style-type: none"> <li>– Place at 37°C for 15 minutes to elute the DNA from the beads.</li> <li>– Spin the tube down, then place the tube back on the magnetic bead rack.</li> <li>– Let beads separate fully. Then without disturbing the beads, transfer supernatant to a new 1.5 ml Lo-Bind tube.</li> <li>– Discard the beads.</li> </ul>	
13	<p>Verify your DNA amount and concentration using a Qubit quantitation platform.</p> <ul style="list-style-type: none"> <li>– Using 1 <math>\mu</math>L of the purified sample, make a 1:10 dilution in EB.</li> <li>– Use 1 <math>\mu</math>L of this 1:10 dilution to measure the DNA concentration using a Qubit fluorometer and the dsDNA HS Assay kit according to the manufacturer's recommendations.</li> <li>– The remaining 9 <math>\mu</math>L of 1:10 diluted sample may be used for QC by pulsed field gel electrophoresis.</li> </ul>	
14	AMPure PB bead purified, size-selected libraries may be stored at -20°C.	

## Anneal and Bind BluePippin™ Size-Selected SMRTbell Templates

Use SMRT Link Sample Setup for instructions for primer annealing and polymerase binding. For primer annealing, use **Sequencing Primer v4** (found in the SMRTbell Express Template Prep Kit). This primer is for diffusion loading only. It is not appropriate for MagBead loading.

### Prepare for Sequencing

For the Sequel System, Diffusion loading is appropriate for loading large insert libraries prepared by the SMRTbell Express Template Prep Kit.

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

See the *Procedure & Checklist - AMPure PB Purification of Polymerase Bound SMRTbell Complexes for Diffusion Loading*.

Revision History (Description)	Version	Date
Initial release.	01	November 2017
Fixed typo on page 8, Adapter Ligation table, Ligase v2 said "50 µL". It's supposed to be "5.0 µL".	02	December 2017
On page 12, corrected kit name to say SMRTbell "Express" Template Prep Kit for location of Sequencing Primer v4. Removed on-plate loading recommendations.	03	January 2018

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