Guidelines for Preparing 20 kb SMRTbell™ Templates

User Bulletin

This Bulletin provides recommendations and tips for preparing 20 kb SMRTbell templates using the BluePippin™ size-selection method using size-selection cutoffs between 6 kb and 10 kb. Once you have read this User Bulletin, proceed to the *Procedure & Checklist - 20 kb Template Preparation and Sequencing* for preparing your 20 kb SMRTbell templates.

Before You Begin

Materials and Kits needed:

To perform this procedure you must have the following kits, materials, and instrumentation:

Item	Vendor
Template Prep Kit	Pacific Biosciences
DNA/Polymerase Binding Kit (polymerase of choice)	Pacific Biosciences
DNA Sequencing Kit	Pacific Biosciences
MagBead Binding Kit	Pacific Biosciences
SMRT® Cell 8Pac v3	Pacific Biosciences
BluePippin™ system with Software v5.90 or later (see www.sagescience.com for ordering information and links to the latest downloadable software and installation instructions) The BluePippin User Manual may be found at: http://www.sagescience.com/support/	Sage Science (PN BLU0001)
PacBio® SMRTbell cassette definition set (see http://www.sagescience.com/support/ for downloadable file and installation instructions)	Sage Science
0.75% Dye-Free Agarose Gel Cassettes, 1 -10 kb (to order, go to: http://www.sagescience.com/products/cassettes/0-75-agarose/ The Quick Guide for this cassette at: http://www.sagescience.com/wp- content/uploads/2011/10/Quick-Guide-BLF7510-marker-S1.pdf	Sage Science (PN BLF7510)
NanoDrop® Spectrophotometer (Series 2000, 2000c, 3300, or 8000)	Thermo Scientific
Qubit® Fluorometer and Quant-iT™ HS dsDNA reagent	Invitrogen (PN Q32857 or Q32851)
g-TUBE® microcentrifuge tubes	Covaris
AMPure® PB Beads	Pacific Biosciences

Handling High-Molecular-Weight DNA

Proper care of gDNA must be taken to minimize damage.

- Do not vortex gDNA prior to shearing
- Avoid excessive pipetting
- Avoid multiple freeze/thaw cycles
- Avoid exposure to excessive heat

Assess the Quality of Starting DNA

Pure (free from RNA or other organic and inorganic contaminants), high-molecular-weight DNA is required to prepare size-selected ~20 kb SMRTbell templates. Quantification with both a NanoDrop spectrophotometer and a Qubit fluorometer helps characterize DNA quality.

If the gDNA concentration, as determined by the Qubit fluorometer, is significantly less than the concentration determined by the NanoDrop spectrophotometer, this indicates presence of contaminating species which inflate Nanodrop readings (such as RNA contamination). Performing AMPure PB bead purification removes most RNA contaminations. If the gDNA concentration is less than 200 ng/μL and the OD 260/280 is less than 1.8 or greater than 2.0, use 0.45X AMPure PB beads to concentrate and/or purify the DNA before shearing.

To assess the integrity of gDNA, run the sample on gel electrophoresis. Agarose gel electrophoresis, such as 1.2% Lonza™ FlashGel™ with FlashGel DNA marker 100 bp - 4 kb, allows for a quick assessment of genomic DNA.

Visible smears on the gel usually signify DNA degradation (see figure 1). To determine the severity of degradation, running the sample on pulsed-field, field inversion gel electrophoresis such as the CHEF Mapper® XA System (Bio-Rad; P/N 170-3760) or Pippin Pulse power supply (Sage Science; P/N PP10200) is highly recommended. Accurate characterization of fragment distribution, due to degradation, helps determine how to proceed with shearing and which size selection approach to take. Depending on the goals, complexity and size of the project, you may not want to proceed with library construction if the sample is severely degraded. The best solution is to re-extract the genomic DNA using a gentler extraction method.
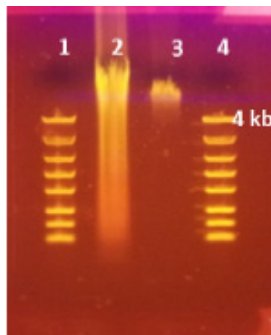

Figure 1: Quality assessment of two samples run on agarose gel electrophoresis (1.2% Lonza Gel)
- Lane 1: FlashGel™ DNA marker 100 bp – 4 kb
- Lane 2: Degraded gDNA
- Lane 3: Partially degraded gDNA
- Lane 4: FlashGel™ DNA marker 100 bp – 4 kb

Consider using an FIGE system to evaluate the presence of high-molecular weight DNA and determine the sizes of sheared DNA or SMRTbell templates (see figure 2 below). On a field-inversion gel, high-quality gDNA should migrate as a single band of approximately 50 kb (Lane 2). Run approximately 200 ng of gDNA on a field-inversion gel.
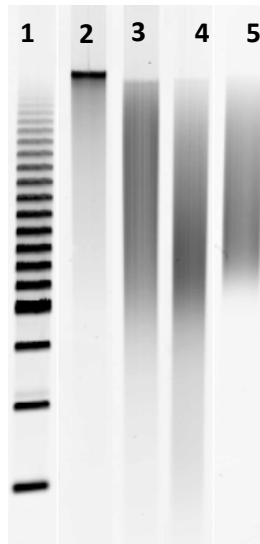

Figure 2. FIGE was performed using a CHEF Mapper (Bio-Rad) according to the manufacturer's recommendations, with a low-range target of 1 kb and a high range target of 50 kb. 100-200 ng of each sample was loaded per lane.

- Lane 1: 2.5 kb Molecular Ruler (Bio-Rad; P/N 170-8205)
- Lane 2: starting gDNA sample
- Lane 3: 20 kb sheared DNA
- Lane 4: 20 kb SMRTbell template before size selection
- Lane 5; BluePippin size-selected 20 kb SMRTbell template

A comparison between constant voltage and field inversion gel electrophoresis is described in this section. Because constant voltage gel electrophoresis does not resolve high-molecular-weight fragments, what appears to be high-molecular-weight DNA may not be accurate. Figures 3a and 3b below show the same set of samples run on both constant voltage electrophoresis and field inversion gel electrophoresis (FIGE), respectively.
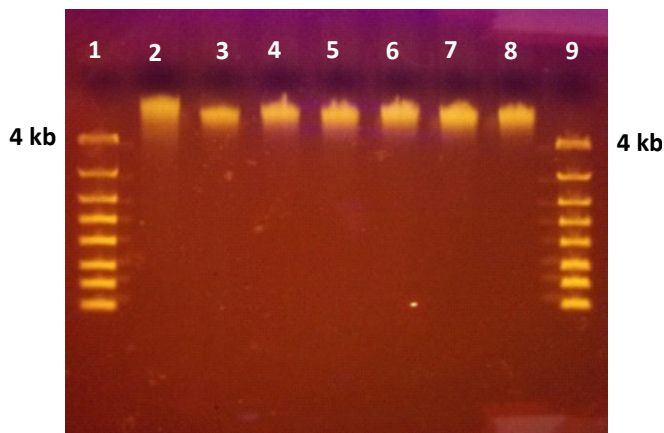

Figure 3a: Samples run on 1.2% Lonza Gel. Lanes 3-8 appear to be high molecular weight using constant voltage.

Lane 1: FlashGel™ DNA marker 100 bp – 4 kb
Lane 2: Sample 1
Lane 3: Sample 2
Lane 4: Sample 3
Lane 5: Sample 4
Lane 6: Sample 5
Lane 7: Sample 6
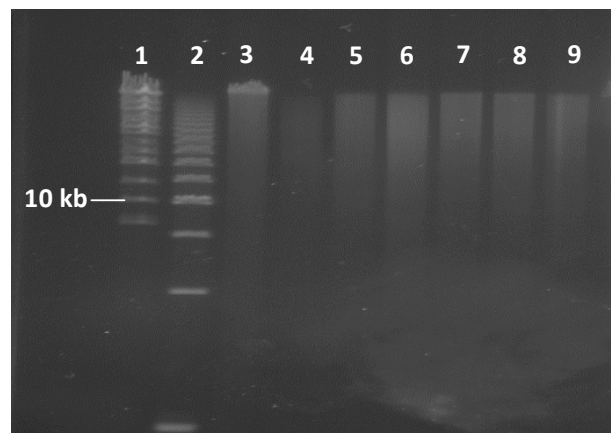Lane 8: Sample 7
Lane 9: FlashGel™ DNA marker 100 bp – 4 kb

Figure 3b: The same set of samples run on FIGE using a Pippin Pulse Power Supply shows degradation.

Lane 1: 8 kb - 48 kb DNA Size Standard from Bio-Rad
Lane 2: 2.5 kb Molecular Ruler from Bio-Rad
Lane 3: Sample 1
Lane 4: Sample 2
Lane 5: Sample 3
Lane 6: Sample 4
Lane 7: Sample 5
Lane 8: Sample 6
Lane 9: Sample 7

The FIGE run provides a more accurate picture of the genomic DNA integrity than that of a constant gel electrophoresis run. Depending on the severity of degradation and fragment distribution, you may still be able to generate a SMRTbell library, however, yield and read length may be compromised. If the majority of the degraded sample is < 10 kb, and there is no higher-quality sample available, you may prepare a library without shearing. Follow the standard 10 kb SMRTbell library protocol. Be aware that the resulting subread lengths will be less than ideal. If the goal of the project is to generate long reads using PacBio's latest chemistries and long movie collection times, consider re-extracting the gDNA using a gentler DNA extraction method that preserves the integrity of the DNA.

An example of subread length comparison from a degraded and high quality sample is shown in Figure 4. Degraded gDNA impacts the average subread length of a library. PacBio continues to provide solutions for increasing read lengths through advances in enzymology and chemistry and DNA quality and integrity are of utmost importance.
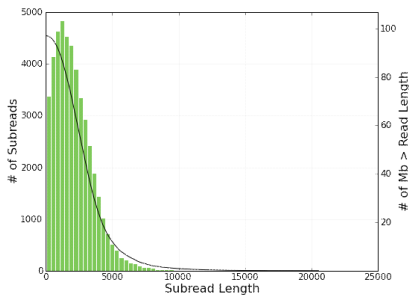

Figure 4a: Subread-length Distribution from a degraded genomic DNA.
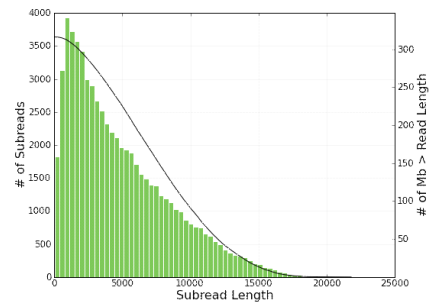
Figure 4b: Subread-length Distribution from high-quality genomic DNA

Once you have assessed the quality of your gDNA, you can proceed by following the workflow below.

QC

QC Input gDNA

↓

Optional 0.45X AMPure® Purification

- -

Shearing

Shear

↓

0.45X AMPure® Purification

↓

QC Sheared DNA

- -

Library Construction

ExoVII Treatment/Damage Repair

↓

DNA End Repair

↓

0.45X AMPure® Purification

↓

Ligation and Exo Digest

↓

0.45X AMPure® Purification

- -

BluePippin™ System Size Selection   0.40X AMPure® Purification

↓

1.0X AMPure® Purification

↓

Anneal and Bind SMRTbell™ Templates

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Prepare for MagBead Station Loading

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Sequence

Shear and Purify DNA

Note that prior to shearing, gentle mixing is recommended. This recommendation will hold for larger insert sizes, however, for 20 kb libraries no damage has been observed as a result of vortexing. Also, typical yields after shearing and AMPure purification are 50-70%, depending on the quality and purity of the input gDNA. We recommend starting with at least 7.5 µg to 10 µg input gDNA in order to produce 5 µg sheared and concentrated DNA for subsequent steps.

Use a Covaris$^®$ g-TUBE$^®$ device to shear > 5 µg DNA sample. The most up-to-date guidance on how to use the g-TUBE device, along with recommended centrifuges and centrifugation speeds, can be found in the g-TUBE device user manual available for download from the Covaris website or the Shared Protocols page of SampleNet, with the following recommendations:

1. Dilute your DNA concentration to 200-300 ng/µL in Elution Buffer (EB). The sample volume may range from 25-100 µL.
2. Shear at 5500 rpm 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 a very small volume remains. This can be removed by doing a final spin for 1 -2 minutes at 8000 - 10000 rmp.
4. Invert and spin at 5500 until all samples have passed through the orifice.
5. Recover your sample into a 1.5 or 2.0 mL LoBind microcentrifuge tube. Add EB if necessary to adjust volume to at least 100 µL.

BluePippin™ Size Selection

With BluePippin size selection, the lower and upper limits of the selected range can be adjusted. We recommend keeping the upper size limit (BP end) at 50000 (50 kb). The lower size limit (BP start) may be varied, depending on several factors:

- Size distribution in the library
- Amount of SMRTbell DNA in the library
- Target size for sequencing reads for the project
- Number of Mbases required for the project

Current size-selection recommendations, using the BluePippin System are summarized below.

Mass of SMRTbell Library	Size Selection Cut-off Requirement	Recommended Cut-off (bp)		Cassette Definition File	Ver	Marker	Kit Part Number
		BP Start	BP End				
< 2 µg	4,000 kb to 5,000 kb	4,000	50,000	0.75%DF Marker S1 high-pass 4-10kb v2	v2	S1	PAC20KB or BLF7510
		5,000	50,000	0.75%DF Marker S1 high-pass 4-10kb v2	v2	S1	PAC20KB or BLF7510
> 600 ng	6,000 kb to 10,000 kb	6,000	50,000	0.75%DF Marker S1 high-pass 6-10kb v3	v3	S1	PAC20KB or BLF7510
		7,000	50,000	0.75%DF Marker S1 high-pass 6-10kb v3	v3	S1	PAC20KB or BLF7510
		8,000	50,000	0.75%DF Marker S1 high-pass 6-10kb v3	v3	S1	PAC20KB or BLF7510
		9,000	50,000	0.75%DF Marker S1 high-pass 6-10kb v3	v3	S1	PAC20KB or BLF7510
		10,000	50,000	0.75%DF Marker S1 high-pass 6-10kb v3	v3	S1	PAC20KB or BLF7510
> 5 µg	15,000 kb to 20,000 kb	15,000	50,000	0.75%DF Marker S1 high-pass 15-20kb	0	S1	PAC20KB or BLF7510
		16,000	50,000	0.75%DF Marker S1 high-pass 15-20kb	0	S1	PAC20KB or BLF7510
		17,000	50,000	0.75%DF Marker S1 high-pass 15-20kb	0	S1	PAC20KB or BLF7510
		18,000	50,000	0.75%DF Marker S1 high-pass 15-20kb	0	S1	PAC20KB or BLF7510
		19,000	50,000	0.75%DF Marker S1 high-pass 15-20kb	0	S1	PAC20KB or BLF7510
		20,000	50,000	0.75%DF Marker S1 high-pass 15-20kb	0	S1	PAC20KB or BLF7510

If the library contains < 0.5 µg, or if there is a broad size distribution to < 4 kb, then a 0.40X AMPure purification step is recommended instead of the BluePippin size selection system to avoid losing the library.

Alternative Removal of Short Fragments with 0.40X AMPure® PB Beads

If the library is < 0.5 µg, or if there is a broad size distribution of < 4 kb, recovery from gel sizing will likely be very low, and a lower X AMPure PB bead purification may be preferred. This will remove or minimize fragments in the 1 – 2 kb range.

Note that it is critical to accurately pipet the desired volume of AMPure PB bead solution; there is a steep drop-off in recovery for concentrations < 0.40X. To facilitate accurate pipetting, carry out 0.40X AMPure PB bead purification separations with a minimum 100 µL sample and 40 µL AMPure PB beads; dilute sample in EB, if necessary. Follow the protocol on page 12 (except for the volume of AMPure PB beads in Step 1). Recovery is typically 60-90% from 0.40X AMPure PB Beads. If a higher stringency size selection is desired, AMPure PB concentration may be reduced to as low as 0.35X. For < 0.40X AMPure purifications, recovery is often in the range of 5-25%.

For < 0.40X AMPure purifications, be sure to save the supernatant from the binding step until after you have verified the eluted library. If the final yield is too low, add 0.6X AMPure PB beads to the saved supernatant and re-bind to recover lost the sample. Continue from above.

Anneal and Bind BluePippin™ Size-Selected SMRTbell™ Templates

Use the Binding Calculator to anneal sequencing primer to the SMRTbell template.

Before adding the primer to the SMRTbell template, the primer must go through a melting step at 80ºC. This avoids exposing the sample to heat. The template and primer mix can then be incubated at 20ºC for 30 minutes.

For polymerase binding, incubation at 30ºC for 30 minutes is sufficient. Instructions for polymerase binding are provided by the calculator.

For more information about using the Binding Calculator, see the *Pacific Biosciences Template Preparation and Sequencing Guide* and *QRC - Annealing and Binding Recommendations*.

On-plate Loading Recommendations

There are differences in loading requirements between BluePippin size-selected libraries and AMPure only/non-size selected libraries. BluePippin size-selected libraries require higher loading concentrations with approximately 125 pM the Plate. However, if the library is purified using AMPure PB beads and no size-selection (using the BluePippin system) is done, then 15 pM to 50 pM on the Plate is required.

Even with the most aggressive AMPure PB bead concentrations (0.375X -0.40X), short insert SMRTbell templates are not eliminated and will readily load. A 125 pM on Plate loading is required for the BluePippin size-selected libraries but will result in overloading for non-size selected libraries. Always perform titration runs to maximize the yield per SMRT Cell.