PACIFIC BIOSCIENCES®

Multiplexed Library Preparation For Structural Variation Detection

Sequel II System v8.0 / Sequel II Chemistry 2.0 / SMRT Link v8.0

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PN 101-884-000 Version 02 (April 2020)

Multiplexed Library Preparation For Structural Variation Detection

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Multiplexed SV Detection Workflow Overview

MULTIPLEXED STRUCTURAL VARIATION DETECTION SAMPLE PREPARATION PROCEDURE DESCRIPTION

- Procedure & Checklist – Multiplexing SMRTbell Libraries using SMRTbell Express Template Prep Kit 2.0 for Structural Variation Detection protocol document describes how to prepare SMRTbell libraries for structural variation (SV) detection using the **Sequel II System**
- Up to **two genomic DNA samples** can be multiplexed for sequencing on **one SMRT Cell 8M**
- High quality gDNA is sheared using a Megaruptor instrument, constructed to a SMRTbell library using the **SMRTbell Express Template Prep Kit 2.0** and then size-selected
- Size-selection of SMRTbell libraries can be performed using either a **BluePippin System or AMPure PB beads** depending on the desired level of stringency for removing short inserts
- For multiplexed SV samples, **Barcoded Overhang Adapters** are required for the ligation step. Any of the barcoded overhang adapters included with the Barcoded Overhang Adapter Kit 8A or Barcoded Overhang Adapter Kit 8B are suitable for use with this procedure.

DNA QUALITY AND QUANTITY REQUIREMENTS FOR MULTIPLEXED SV LIBRARY CONSTRUCTION (LC) USING SMRTBELL EXPRESS TPK 2.0

SMRTbell Library Type	Recommended Input gDNA Amount	Required Input gDNA Quality	Recommended gDNA Shearing Method*	Sheared gDNA Target Size Distribution Mode	Recommended Sheared gDNA Amount for LC
Multiplexed SV Library for the Sequel II System (Pool up to 2 samples)	7 µg per sample	Majority of gDNA >40 kb	Megaruptor System (Diagenode)	20 – 40 kb	5 µg per sample

* gDNA shearing may optionally be performed using Covaris g-Tubes if a Megaruptor System is unavailable; however, a higher amount of starting input gDNA (≥10 µg) is recommended for this use case.

MULTIPLEXED STRUCTURAL VARIATION DETECTION WORKFLOW OVERVIEW

1. gDNA QC & Shearing

- Qubit dsDNA HS quantitation assay
- CHEF Mapper, Femto Pulse or Pippin Pulse sizing QC
- Megaruptor shearing recommended

2. Multiplexed SMRTbell Express Library Construction

- Single-tube, addition-only reactions with SMRTbell Express TPK 2.0
- Ligation with Barcoded Overhang Adapters
- AMPure PB purification of barcoded SMRTbell libraries prior to pooling

3. Pool Barcoded SMRTbell Express Libraries

- Pool up to two SV samples at equimolar concentration

4. Size Selection Options

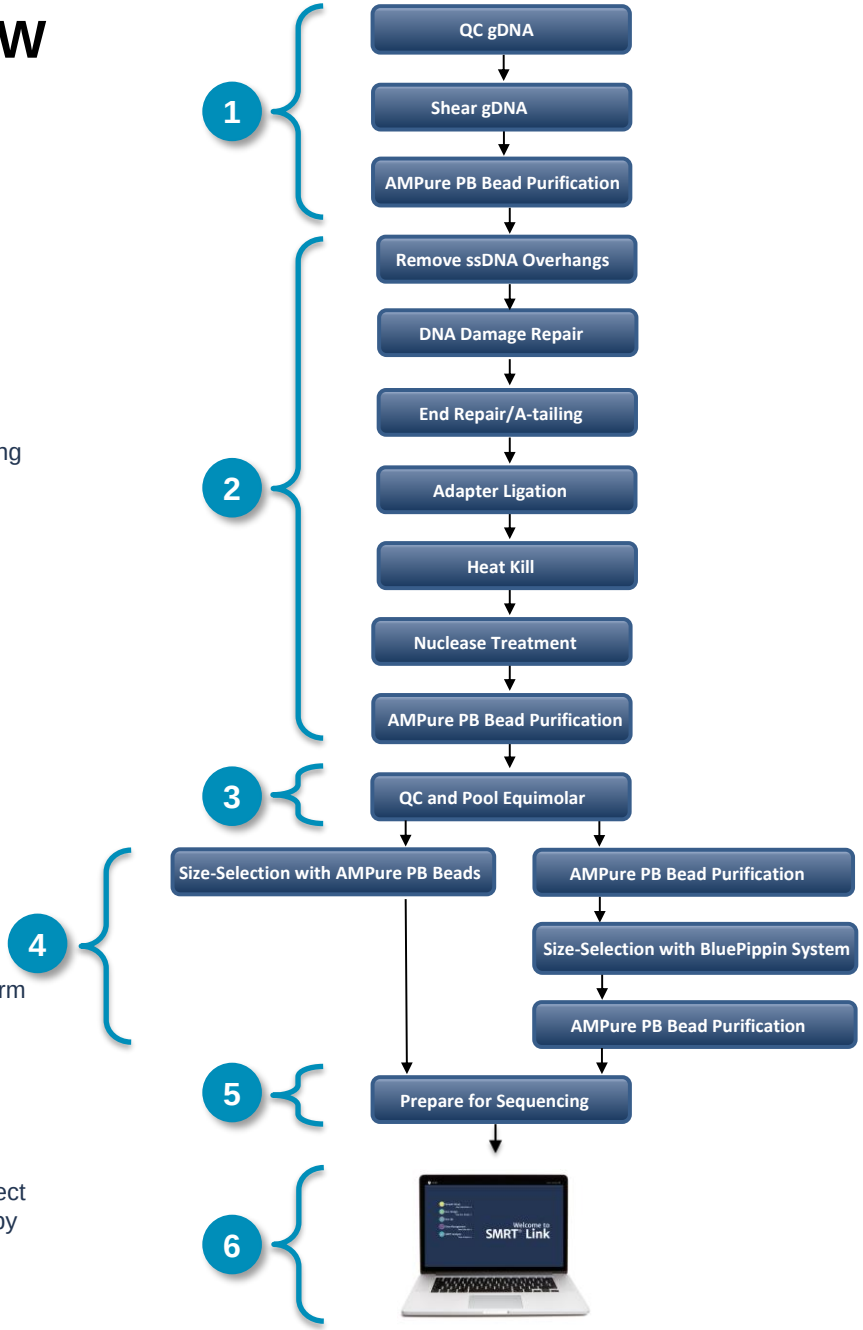
- AMPure PB size selection (removes <3 kb SMRTbell templates); or
- BluePippin size selection (removes <15 kb SMRTbell templates) followed by AMPure PB bead purification of final pooled library

5. Sample A/B/C & Sequence

- Anneal Sequencing Primer V2, Bind Sequel II Polymerase 2.0, perform AMPure PB Complex Cleanup
- Follow QRC for loading recommendations

6. Analyze

- Use SMRT Link Structural Variant Calling analysis application to detect insertions, deletions, duplications, inversions, translocations, and copy number variants
- Output data in standard file formats—BAM and VCF—for seamless integration with downstream analysis tools



Multiplexed SV Library Sample QC Requirements

RECOMMENDED TOOLS FOR GENOMIC DNA QUANTIFICATION AND QUALIFICATION

DNA Quantification

- For quantification of gDNA to be used with the multiplexed SV library preparation workflow, we recommend using the Qubit fluorometer and Qubit High Sensitivity (HS) DNA assay reagents. Measure the gDNA sample concentration as recommended by the manufacturer.

Qubit™ dsDNA HS Assay Kit

Qubit 4 Fluorometer

RECOMMENDED TOOLS FOR GENOMIC DNA QUANTIFICATION AND QUALIFICATION (CONT.)

DNA Sizing

- To accurately determine the size distribution of your gDNA sample, we recommend the use of the **Femto Pulse System (Agilent)** 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 below with links to recommended procedures.

Femto Pulse System

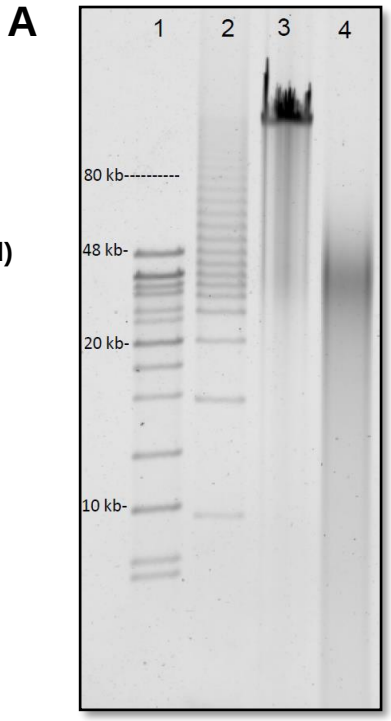
GENOMIC DNA SIZE EVALUATION METHODS AND PROCEDURES

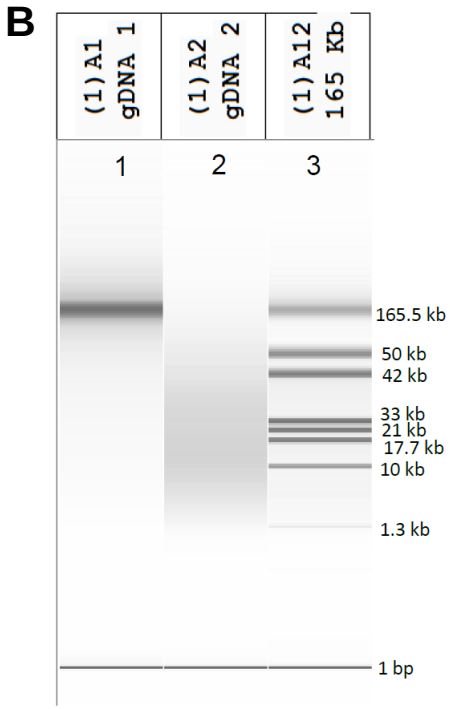
METHOD	COMMENTS	PROCEDURE
Agilent Femto Pulse System	Highly recommended (200-500 pg)	Agilent Femto Pulse Website
Bio-Rad® CHEF Mapper® XA PFGE System	Requires >50 ng	Procedure & Checklist - Using the BIO-RAD® CHEF Mapper XA Pulsed Field Electrophoresis System
Sage Science Pippin Pulse System	Requires >50 ng	Procedure & Checklist - Using the Sage Science Pippin Pulse Electrophoresis Power Supply System

EVALUATION OF GENOMIC DNA FOR MULTIPLEXED SV LIBRARY CONSTRUCTION

This procedure requires high quality, high molecular weight input gDNA with the majority of the DNA fragments >40 kb as determined by pulsed-field gel or capillary electrophoresis.

Lane 1: 8-48 kb Ladder (Bio-Rad)
Lane 2: 5 kb Ladder (Bio-Rad)
Lane 3: HMW gDNA
Lane 4: Degraded gDNA





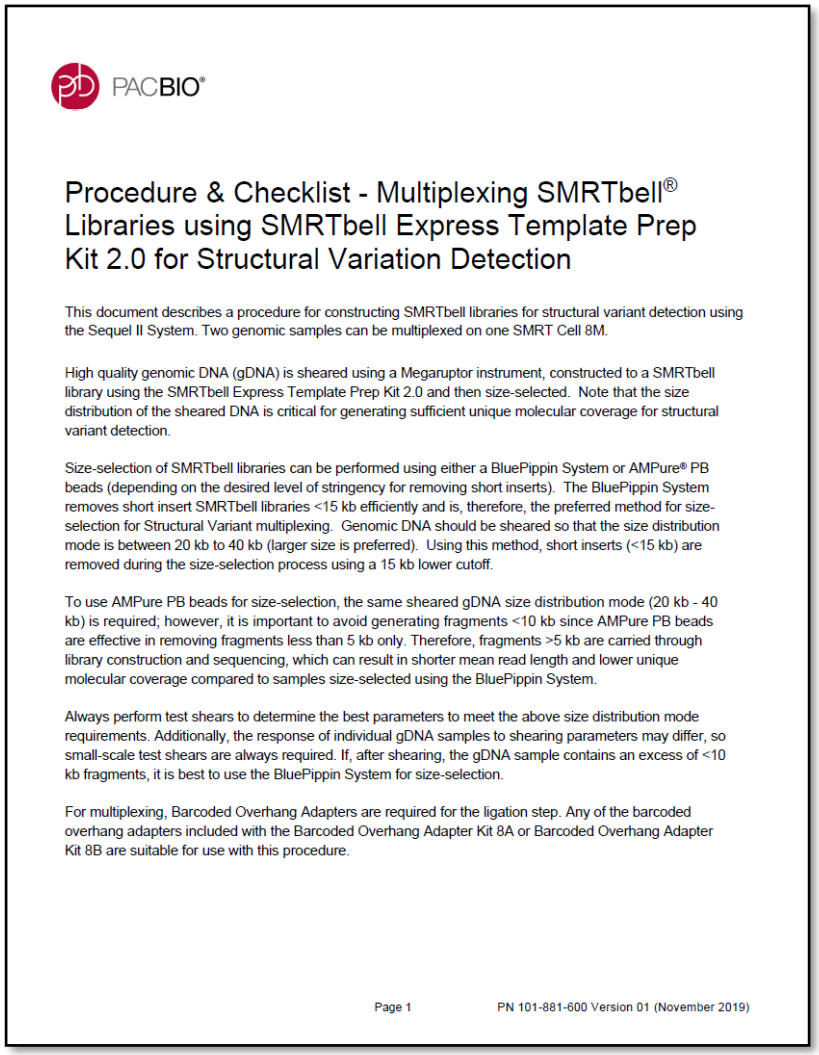
Lane 1: High MW gDNA
Lane 2: Degraded gDNA
Lane 3: 165 kb Ladder

Evaluation of gDNA quality using A) Bio-Rad CHEF Mapper System and B) Femto Pulse System. Lanes 3 A and 1B are examples of high quality, high molecular weight genomic DNA. Lanes 4A and 2B are examples of degraded gDNA that are not suitable for use in this procedure

Multiplexed SV Library Sample Preparation Workflow Details

PROCEDURE & CHECKLIST – MULTIPLEXING SMRTBELL LIBRARIES USING SMRTBELL EXPRESS TEMPLATE PREP KIT 2.0 FOR STRUCTURAL VARIATION DETECTION

- Describes how to prepare multiplexed SMRTbell libraries for structural variation (SV) detection using the Sequel II System
- Up to two genomic DNA samples can be pooled for sequencing on one SMRT Cell 8M
- Protocol document contains:
  1. General laboratory best practices recommendations
  2. Recommendations for gDNA QC and quantification
  3. Guidelines for evaluation of gDNA samples for multiplexed SV library construction
  4. Enzymatic steps for preparation of multiplexed SV SMRTbell libraries
  5. Instructions for size-selection of multiplexed SV libraries using the BluePippin System, and also includes a protocol reference for performing the AMPure BP Size Selection method
  6. Sample setup guidance for preparing multiplexed SV libraries for sequencing on the Sequel II System



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Procedure & Checklist - Multiplexing SMRTbell® Libraries using SMRTbell Express Template Prep Kit 2.0 for Structural Variation Detection

This document describes a procedure for constructing SMRTbell libraries for structural variant detection using the Sequel II System. Two genomic samples can be multiplexed on one SMRT Cell 8M.

High quality genomic DNA (gDNA) is sheared using a Megaruptor instrument, constructed to a SMRTbell library using the SMRTbell Express Template Prep Kit 2.0 and then size-selected. Note that the size distribution of the sheared DNA is critical for generating sufficient unique molecular coverage for structural variant detection.

Size-selection of SMRTbell libraries can be performed using either a BluePippin System or AMPure® PB beads (depending on the desired level of stringency for removing short inserts). The BluePippin System removes short insert SMRTbell libraries <15 kb efficiently and is, therefore, the preferred method for size-selection for Structural Variant multiplexing. Genomic DNA should be sheared so that the size distribution mode is between 20 kb to 40 kb (larger size is preferred). Using this method, short inserts (<15 kb) are removed during the size-selection process using a 15 kb lower cutoff.

To use AMPure PB beads for size-selection, the same sheared gDNA size distribution mode (20 kb - 40 kb) is required; however, it is important to avoid generating fragments <10 kb since AMPure PB beads are effective in removing fragments less than 5 kb only. Therefore, fragments >5 kb are carried through library construction and sequencing, which can result in shorter mean read length and lower unique molecular coverage compared to samples size-selected using the BluePippin System.

Always perform test shears to determine the best parameters to meet the above size distribution mode requirements. Additionally, the response of individual gDNA samples to shearing parameters may differ, so small-scale test shears are always required. If, after shearing, the gDNA sample contains an excess of <10 kb fragments, it is best to use the BluePippin System for size-selection.

For multiplexing, Barcoded Overhang Adapters are required for the ligation step. Any of the barcoded overhang adapters included with the Barcoded Overhang Adapter Kit 8A or Barcoded Overhang Adapter Kit 8B are suitable for use with this procedure.

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LIST OF REQUIRED MATERIALS AND EQUIPMENT

ITEM	VENDOR	PART NUMBER
DNA Sizing QC (One of the following)		
Femto Pulse® System	Agilent Technologies, Inc.	M5330AA
Pippin Pulse Electrophoresis Power Supply	Sage Science	PP10200
Pulsed Field Gel Electrophoresis System: CHEF Mapper XA	Bio-Rad	170-3670
DNA Quantitation		
Qubit™ 4 Fluorometer	ThermoFisher Scientific	Q33226
Qubit™ 1X dsDNA HS Assay Kit	ThermoFisher Scientific	Q33230
DNA Shearing (One of the following)		
Megaruptor 2 (Can shear gDNA to 20 – 40 kb mode size)	Diagenode	B06010002
Long Hydropores	Diagenode	E07010002
Hydrotubes	Diagenode	C30010018
Megaruptor 3 (Can shear gDNA to 20 – 40 kb mode size)	Diagenode	B06010003
Megaruptor 3 Shearing Kit	Diagenode	E07010003
g-TUBE (Can shear gDNA to ~20 kb mode size only)	Covaris	10145
Eppendorf MiniSpin Plus or other equivalent benchtop centrifuge model	Eppendorf	022620100

LIST OF REQUIRED MATERIALS AND EQUIPMENT (CONT.)

ITEM	VENDOR	PART NUMBER
SMRTbell Library Preparation		
SMRTbell Express Template Prep Kit 2.0	PacBio	100-938-900
Barcoded Overhang Adapter Kit 8A (8 adapters)	PacBio	101-628-400
Barcoded Overhang Adapter Kit 8B (8 adapters)	PacBio	101-628-500
AMPure® PB Beads	PacBio	100-265-900
Elution Buffer	PacBio	101-633-500
SMRTbell Enzyme Cleanup Kit	PacBio	101-746-400
Sequencing Primer V2	PacBio	101-847-900
Eppendorf MiniSpin Plus or other equivalent benchtop centrifuge model	Eppendorf	022620100
Wide Orifice Tips (Tips LTS W-O 200 UL Fltr RT-L200WFLR)	Rainin	17014294
100% Ethanol, Molecular Biology Grade	Any MLS	
Rotator	Any MLS	
2.0 mL DNA Lo-Bind Tubes	Any MLS	
Fractionation (If not using AMPure PB size-selection)		
BluePippin System	Sage Science	BLU0001
0.75% Agarose Cassettes, S1 Marker	Sage Science	BLF7510

PACBIO BARCODED OVERHANG ADAPTERS FOR MULTIPLEXED SV LIBRARY CONSTRUCTION

- **PacBio Barcoded Overhang Adapter Kit 8A (PN 101-628-400) or 8B (PN 101-628-500)** are available for multiplexing up to 2 SV samples per SMRT Cell 8M for sequencing on the Sequel II System
  - 8 barcodes in each kit; 6 reactions per tube

Barcoded Overhang Adapter Kit -8A	Barcoded Overhang Adapter Kit -8B
bc1001	bc1015
bc1002	bc1016
bc1003	bc1017
bc1008	bc1018
bc1009	bc1019
bc1010	bc1020
bc1011	bc1021
bc1012	bc1022

- To download the barcode FASTA sequences, visit PacBio's Analytical Software Multiplexing webpage: http://www.pacb.com/products-and-services/analytical-software/smrt-analysis/analysis-applications/multiplexing/
  - FASTA filename: Sequel_16_Barcodes_v3.zip
  - Direct download URL link: https://www.pacb.com/wp-content/uploads/Sequel_16_Barcodes_v3.zip

BEST PRACTICES RECOMMENDATIONS FOR PREPARING MULTIPLEXED SV SMRTBELL LIBRARIES

1. Use wide-bore tips for all mixing steps.
2. Throughout the procedure, 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 genomic DNA samples.
4. Minimize the number of freeze/thaw cycles the gDNA undergoes to reduce DNA damage.
5. Allowing sufficient time for thawing aliquots of DNA, as partially frozen DNA is prone to shearing.
6. Always set your heat blocks or thermocyclers to the appropriate temperature for incubations before proceeding with the procedure.
7. Ensure that the AMPure PB Beads are at **room temperature** prior to performing the purification steps.
8. When performing AMPure PB Bead purification steps, 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.
9. Always follow best practices for DNA quantitation using a Qubit fluorometer system. Use the Qubit dsDNA high sensitivity (HS) reagent kit.

DNA SHEARING RECOMMENDATIONS FOR MULTIPLEXED SV LIBRARY CONSTRUCTION

For constructing multiplexed SV SMRTbell libraries for sequencing on the Sequel II System, it is necessary to shear the genomic DNA to a target mode size range of 20 kb – 40 kb to allow for detection of the barcodes in the SMRTbell templates

- Shearing to a target size distribution mode between **25 kb - 35 kb is recommended**, followed by size-selection with either the BluePippin System or AMPure PB beads.
- The two size-selection options have different requirements for shearing the DNA:

1. **BluePippin System size-selection** requires starting with sheared gDNA with a size mode of 20 kb – 40 kb. SMRTbell templates <15 kb are efficiently removed using the *0.75% DF Marker S1 high-pass 15 kb – 20 kb* cassette definition. The BluePippin System is the preferred size-selection method.
2. **AMPure PB bead size-selection** also requires starting with sheared gDNA with the same size distribution mode (20 kb – 40 kb) but **it is important to avoid generating fragments <10 kb**. Having fewer <10 kb fragments in the sheared sample will lead to improved data quality since AMPure PB beads can only remove short inserts <5 kb. A high presence of <10 kb inserts will result in shorter subread lengths, and therefore lower unique molecular yield.

DNA SHEARING RECOMMENDATIONS FOR MULTIPLEXED SV LIBRARY CONSTRUCTION (CONT.)

PacBio recommends the Megaruptor System (Diagenode) for shearing genomic DNA because of its ability to generate tight shear distributions and good post-shear recovery efficiencies.

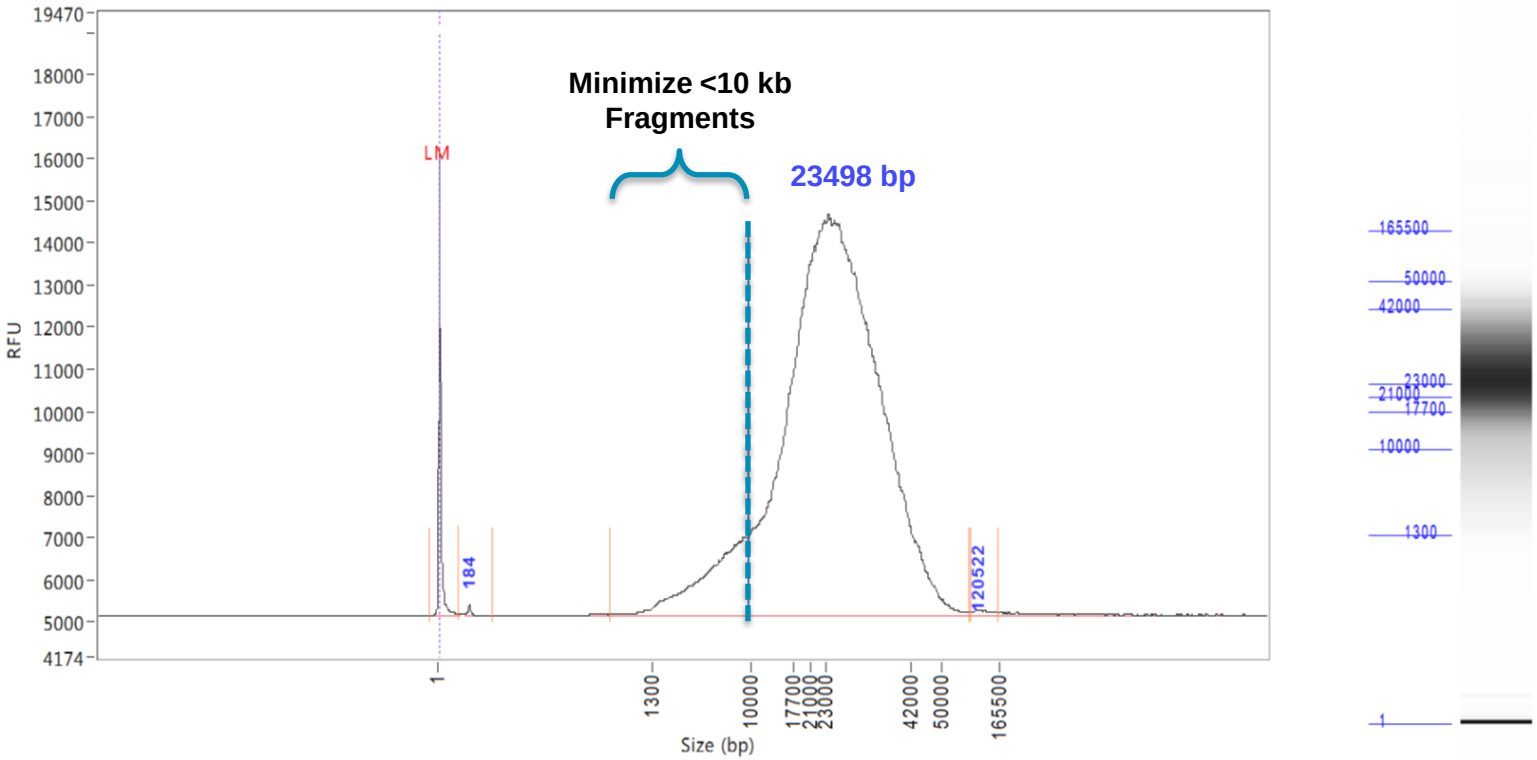
- The Megaruptor System is recommended for shearing gDNA to a **target size distribution mode ~25 – 35 kb**
- A minimum of 5 µg of sheared gDNA for each sample to be multiplexed is required for the subsequent enzymatic steps, so it is recommended to start with **7 µg of input gDNA per sample for shearing**.
- To shear gDNA using the Megaruptor System, generally follow the manufacturer's recommendations.
- It is important to **perform small-scale test shears** (for example, using a 150 µL volume at a DNA concentration of 15 ng/µL) to evaluate the response of each gDNA sample to shearing parameters.
- After shearing the gDNA samples, proceed with the 'Concentrate DNA Using AMPure PB Beads' step and then evaluate the size distribution using a PFGE or capillary electrophoresis system

Megaruptor 2 System

Megaruptor 3 System

DNA SHEARING RECOMMENDATIONS FOR MULTIPLEXED SV LIBRARY CONSTRUCTION (CONT.)

Megaruptor System	Recommended Megaruptor Software Setting	Sheared gDNA Target Size Distribution Mode
Megaruptor 1 or 2	30 kb – 35 kb Target Size Setting	20 – 40 kb
Megaruptor 3	Speed Setting 28 – 30	20 – 40 kb

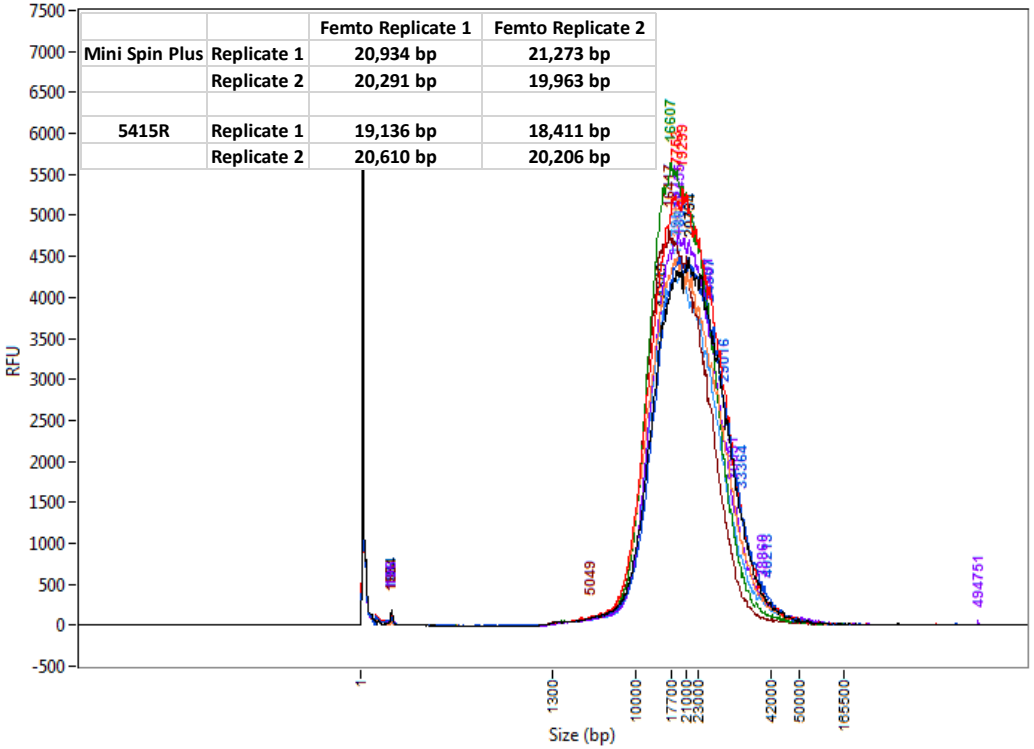


Example Femto Pulse sizing analysis for an ideal sheared gDNA sample suitable for downstream library size selection using either AMPure PB beads or the BluePippin System. With the BluePippin System, short insert SMRTbell templates are removed using a 15 kb lower cutoff. With AMPure PB beads, only SMRTbell templates below 5 kb can be efficiently removed. Therefore, it is important to optimize gDNA shearing conditions so that fragments <10 kb are minimized as shown in the above electropherogram

ALTERNATIVE DNA SHEARING METHOD USING COVARIS G-TUBES IF A MEGARUPTOR SYSTEM IS NOT AVAILABLE

Microcentrifuge Model	Recommended Input gDNA Sample Volume and Concentration	Recommended RCF (RPM) Setting	Recommended Spin Time (# Passes)	Sheared gDNA Target Size Distribution Mode
Eppendorf MiniSpin Plus	50 µL, 100 ng/µL	1700 RCF (5000 RPM)	1 min (6 passes total through orifice)	20 kb
Eppendorf 5415R	50 µL, 100 ng/µL	1500 RCF (4000 RPM)	1 min (6 passes total through orifice)	20 kb

- gDNA shearing may optionally be performed using Covaris g-Tubes if a Megaruptor System is unavailable; however, the maximum sheared gDNA size distribution mode will be limited to **approx. 20 kb**
- If g-Tube is the only available shearing method:
  - Using a higher amount of starting input gDNA (≥10 µg) is recommended
  - BluePippin size-selection is recommended



		Femto Replicate 1	Femto Replicate 2
Mini Spin Plus	Replicate 1	20,934 bp	21,273 bp
	Replicate 2	20,291 bp	19,963 bp
5415R	Replicate 1	19,136 bp	18,411 bp
	Replicate 2	20,610 bp	20,206 bp

Example Femto Pulse sizing analysis of replicate genomic DNA samples sheared with Covaris g-Tubes using an Eppendorf MiniSpin Plus microcentrifuge or a 5415R microcentrifuge.

SMRTBELL EXPRESS TEMPLATE PREP KIT 2.0 REAGENT HANDLING RECOMMENDATIONS

- Several tubes in the kits are sensitive to temperature and vortexing
- PacBio highly recommends:
  - 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

LIST OF TEMPERATURE-SENSITIVE REAGENTS INCLUDED IN SMRTBELL EXPRESS TPK 2.0.

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

SAMPLE POOLING BEST PRACTICES RECOMMENDATIONS FOR MULTIPLEXED SV LIBRARY CONSTRUCTION

- Always quantify libraries before pooling. PacBio recommends using the **Qubit dsDNA High Sensitivity (HS) Assay Kit** for performing DNA concentration measurements.
- **Equal-molar pooling** of barcoded libraries is recommended in order to generate even sequencing read coverage for each SV sample.
- After pooling, perform BluePippin size-selection to remove SMRTbell templates <15 kb or AMPure PB size-selection to remove SMRTbell templates <3 kb

STEP	✓	Pooling	Notes
1		Use the concentration and average library size* from the Femto Pulse to determine the molarity of each sample.	
2		Use the following equation to determine Molarity: $\frac{\text{concentration (in ng/ µL) X } 10^6}{\text{(650 g/mol x average library size in bp*)}}$ = concentration (in nM)	
3		If you are using the BluePippin for size-selection, go to the next section "Purify SMRTbell Library Using 0.45X AMPure PB Beads".	
4		If you are using AMPure PB beads for size-selection, go to the Procedure & Checklist – Using AMPure PB Beads for Size-Selection here. – Use 3.1X of 35% AMPure PB beads for size-selection of <5 kb SMRTbell templates – The AMPure PB bead size-selection requires sample concentration **0.5–10 ng/µL.** Make sure to adjust the concentration with Elution Buffer to meet this requirement.	

AMPURE PB SIZE SELECTION FOR MULTIPLEXED SV LIBRARY CONSTRUCTION

- If you are using AMPure PB beads for size-selection, refer to the **Procedure & Checklist – Using AMPure PB Beads for Size-Selection** protocol document (https://www.pacb.com/support/documentation/)
- AMPure PB size-selection purification step removes SMRTbell templates <3 kb
- AMPure PB bead stock solution is first diluted to 35% (vol./vol.) with Elution Buffer and subsequently used for purification
- **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 35% (v/v) AMPure PB bead working solution in EB

Dilute AMPure PB Beads with Elution Buffer

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 35% (v/v) AMPure PB bead solution in Elution Buffer.

Reagent	Volume	✓	Notes
Elution Buffer	6.5 mL		
AMPure PB Beads (stock reagent)	3.5 mL		
Total Volume	10.0 mL		

1. Bring the AMPure PB bead stock to room temperature.
2. Vortex the stock solution for 30 seconds to mix well.
3. Using a P1000 pipette, transfer 6.5 mL of Elution Buffer into a 15 mL conical tube.
4. Add 3.5 mL of the stock AMPure PB beads to the Elution Buffer. When pipetting the viscous AMPure PB bead solution, pipette slowly to ensure that the volume aspirated is as precise as possible. Large residual AMPure PB beads adhering outside of the tip should be removed prior to adding to the Elution Buffer.
5. Vortex the diluted AMPure PB beads solution for 30 seconds to mix well before use. This solution may be stored at 4°C for 2 months for future use.

https://www.pacb.com/wp-content/uploads/Procedure-Checklist-%E2%80%93-Using-AMPure-PB-Beads-for-Size-Selection.pdf

BLUEPIPPIN SIZE SELECTION FOR MULTIPLEXED SV LIBRARY CONSTRUCTION

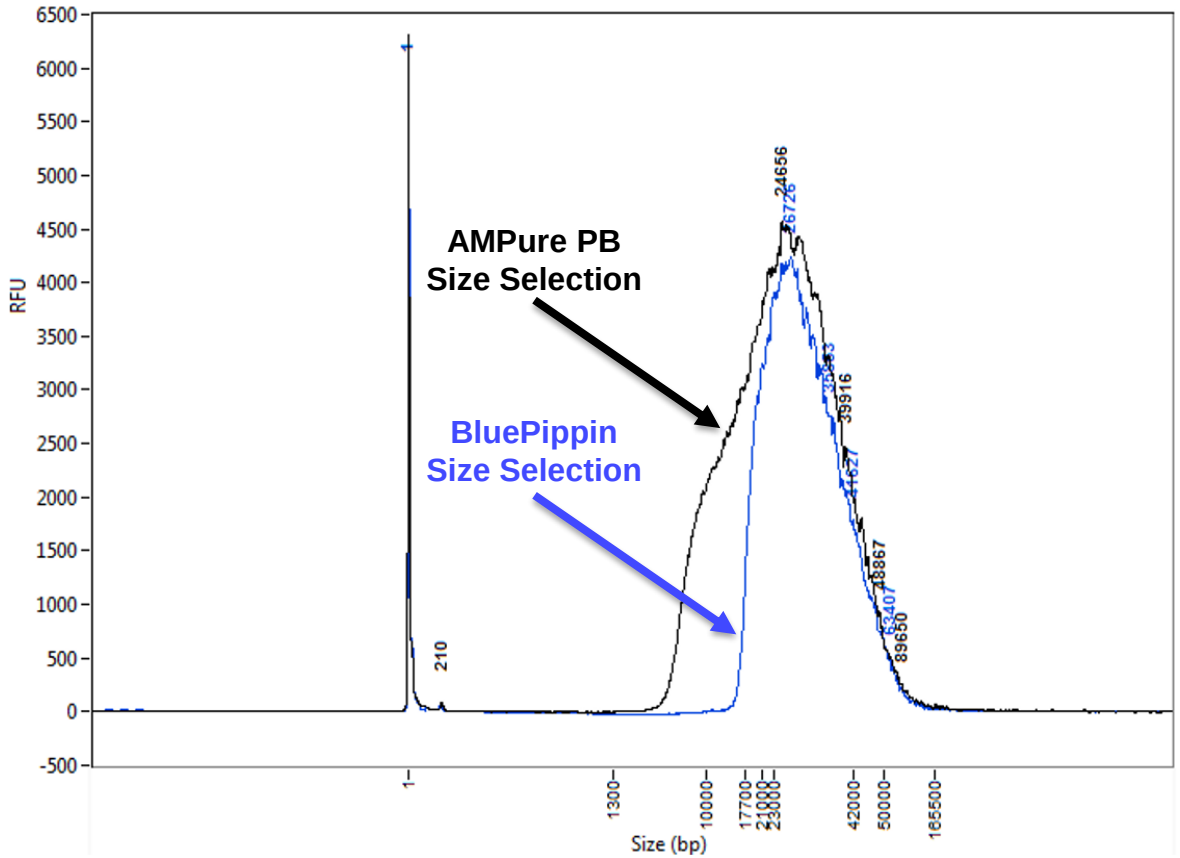
- When constructing SMRTbell libraries for SV detection, it is beneficial to remove small insert SMRTbell templates by performing size selection with the BluePippin System
- With the BluePippin System, the BP Start (cut-off) value may be adjusted depending on the size distribution and total available mass of the SMRTbell library sample

Target lower cut-off threshold (BP Start)	Cassette Definition File	Min. Input DNA per Lane	Marker	BP End (bp)	Run Time
15 kb - 20 kb	0.75% DF Marker S1 high-pass 15 kb – 20 kb	>3.0 µg	S1	50000	5.5 hrs

- Visit Sage's website (http://www.sagescience.com) to verify that your BluePippin software is up-to-date. (Current version as of December 2019 is v6.31)
- Perform size-selection using the *0.75% DF Marker S1 high-pass 15 kb -20 kb* run protocol and S1 marker. Enter the BPstart values indicated in the table above based on the size-distribution and quantity of your pooled sample. Use **Lane 4** for Size Markers.
- **Note:** When using the *0.75% DF Marker S1 high-pass 15 kb -20 kb* cassette definition file, sample lanes containing <3 µg of SMRTbell library material will run faster during electrophoresis. In such cases, PacBio recommends adjusting the BP Start values as follows:

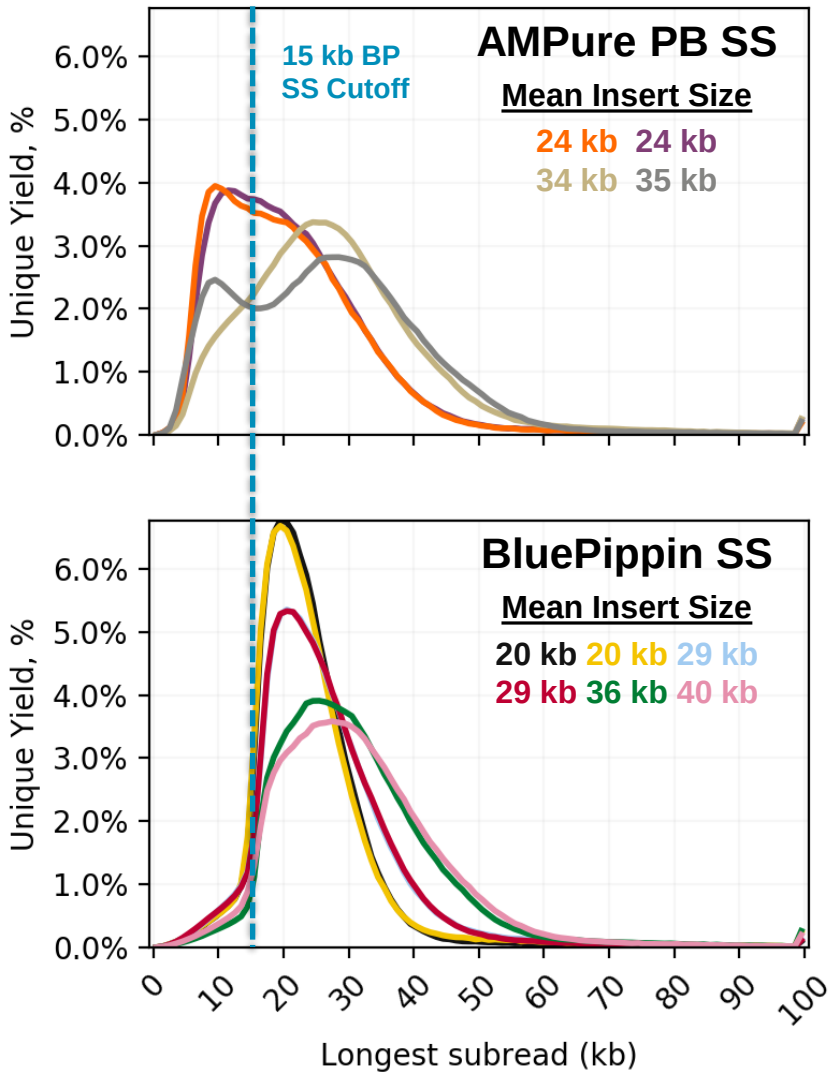
Cassette Definition File	If < 3 µg input per lane, use BP Start
0.75% DF Marker S1 high-pass 15 kb – 20 kb	12500 for 15 kb cutoff
	15000 for 20 kb cutoff

SIZING QC OVERLAY COMPARISON OF FINAL SMRTBELL LIBRARIES SIZE-SELECTED WITH AMPURE PB BEADS AND THE BLUEPIPPIN SYSTEM



Overlay of Femto Pulse sizing profiles for SMRTbell libraries size-selected using AMPure PB beads (black) and the BluePippin System (Blue). The BluePippin System removes short insert SMRTbell templates more efficiently than AMPure PB beads, which can result in improved unique molecular coverage during sequencing. While using AMPure PB beads is an alternative option for library size selection, ensure that the sheared gDNA sample does not contain excess levels of fragments <10 kb prior to starting SMRTbell library construction.

COMPARISON OF SUBREAD READ LENGTH DISTRIBUTIONS FOR BLUEPIPPIN SIZE-SELECTED *VS* AMPURE PB SIZE-SELECTED SV LIBRARIES (SEQUEL II SYSTEM CHEMISTRY 2.0)



- BluePippin size selection eliminates shorter subread lengths <15 kb
  - Enables higher total unique molecular yield and thus higher unique molecular coverage per sample during sequencing
- Yield of Barcoded Reads is in the range of ~50 – 60% for both BluePippin size-selected and AMPure SS SV libraries

ESTIMATED RECOVERY YIELDS FOR MULTIPLEXED SV LIBRARY CONSTRUCTION WORKFLOW STEPS

LIBRARY CONSTRUCTION WORKFLOW STEP	ESTIMATED YIELD
Average Post-Shearing Yield (Starting from input gDNA)	**~70 %**
Average SMRTbell Yield (Starting from sheared gDNA input into first enzymatic reaction [Remove ssDNA Overhangs step])	**~50 %**
Average Yield of Size-Selected Library (starting from DNA input into BluePippin System or AMPure PB size-selection)	**~ 40% – 60%***

***** SV library yield after size selection is highly dependent on the final SMRTbell library size distribution. Size-selection recovery yield range shown in the Table above was obtained for gDNA samples sheared to >25 kb.

Multiplexed SV Library Sequencing Workflow Details

SAMPLE SETUP RECOMMENDATIONS FOR MULTIPLEXED SV LIBRARIES – SEQUEL II SYSTEM (CHEMISTRY 2.0)

- Sample setup requirements for sequencing multiplexed SV libraries on the **Sequel II System**:

SAMPLE SETUP PARAMETER	PARAMETER VALUE / REAGENT KIT TO USE
Sequencing Primer	Sequencing Primer V2
Sequencing Primer:Template Ratio	20:1
Polymerase Binding Kit	Sequel II Binding Kit 2.0
Polymerase:Template Ratio	10:1
Polymerase Binding Time	4 hours

- To enable preparation of binding reactions with a **10:1 polymerase-to-template ratio**, enter the following parameter information below in **SMRT Link Sample Setup (v8.0 or higher)**:

SMRT LINK SAMPLE SETUP FIELD	VALUE TO SELECT	PURPOSE
Sequencing Primer	“Sequencing Primer V2”	Enables calculation of 10:1 polymerase to template ratio in SMRT Link Sample Setup
Sequencing Mode	“CCS”	Enables calculation of 10:1 polymerase to template ratio in SMRT Link Sample Setup

- After entering all the required parameter information, follow the instructions in SMRT Link Sample Setup to anneal the multiplexed SV samples with **Sequencing Primer V2** and to bind the samples with **Sequel II Binding Kit 2.0**

SEQUENCING SETUP RECOMMENDATIONS FOR MULTIPLEXED SV LIBRARIES – SEQUEL II SYSTEM (CHEMISTRY 2.0)

- Sequencing conditions and SMRT Link Run Design (v8.0 and higher) settings for sequencing multiplexed SV libraries on the **Sequel II System**:

SEQUENCING SETUP PARAMETER	PARAMETER VALUE TO USE
Sequencing Kit	Sequel II Sequencing Plate 2.0
Movie Collection Time	15 hours
Pre-Extension Time	2 hours for ≤20 kb mean library insert size
	4 hours for >20 kb mean library insert size
On-Plate Loading Concentration	70 pM – 100 pM
Target %P1	50% – 70%
Sequencing Mode (SMRT Link Run Design)	CLR

- Refer to the latest recommendations provided in the Quick Reference Card – Loading and Pre-Extension Time Recommendations for the Sequel II System for setting up BluePippin and AMPure PB size-selected SV samples for sequencing.

IMPORTING THE BARCODE FASTA FILE INTO SMRT LINK V8.0 FOR AUTOMATED DEMULTIPLEXING OF POOLED SV LIBRARY SAMPLES

- The following steps describe how to import the file containing the barcode sequences for the barcoded adapters provided in the PacBio Barcoded Overhang Adapter Kit 8A and 8B into SMRT Link for use in automated demultiplexing:

1. Download the FASTA file containing the barcode sequences using the following URL link:

   https://www.pacb.com/wp-content/uploads/Sequel_16_Barcodes_v3.zip

2. Import the Sequel_16_Barcodes_v3.zip file into SMRT Link.

   i. On the Home Page, select **Data Management**.

   ii. Click **Import Data**.

   iii. Specify whether to import data from the **SMRT Link Server**, or from a **Local File System**. (**Note:** Only references and barcodes are available if you select Local File System.)

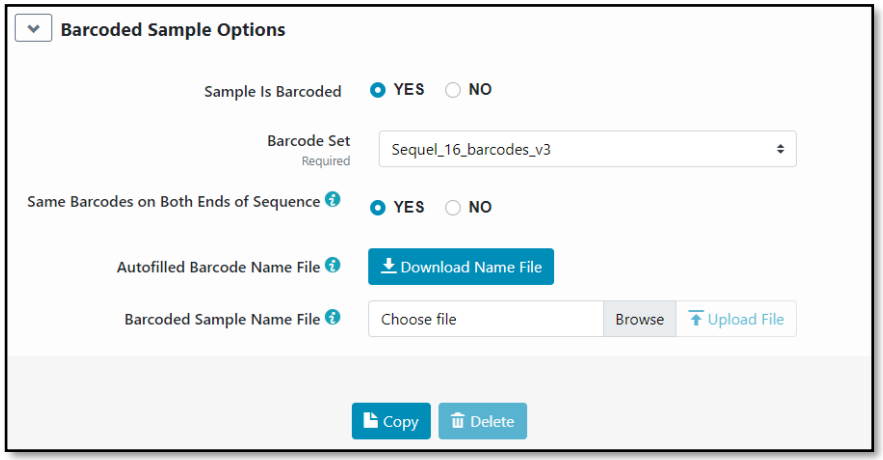
   iv. Select the data type to import: **Barcodes** – FASTA (.fa or .fasta), XML (.barcodeset.xml), or ZIP files containing barcodes.

   v. Navigate to the appropriate file and click **Import**. The sequence data, reference, or barcodes are imported and becomes available in SMRT Link.

SMRT LINK RUN DESIGN SETUP PROCEDURE FOR AUTOMATED DEMULTIPLEXING OF POOLED SV LIBRARY SAMPLES

- Open the Run Design module in SMRT Link. Click **New Run Design**. Fill in the Sample Information section, then click the small arrow to open **Barcoded Sample Options**. Specify the following options:

1. Sample is Barcoded: **Yes**
2. Barcode Set: **Sequel_16_Barcodes_v3**
3. Same Barcodes on Both Ends of Sequence: **Yes**
4. Autofilled Barcode Name File: Click **Download Data**
5. Barcoded Sample Name File: In the downloaded Autofilled Barcode Name File, fill in the desired values for Bio Sample Name, as shown here for the first 2 barcodes. Save the file and upload it using **Browse** and **Upload File**
6. Save the Run Design and launch the sequencing run.



Barcode Name	Bio Sample Name
bc1001_BAK8A--bc1001_BAK8A	DNA Sample 1
bc1002_BAK8A--bc1002_BAK8A	DNA Sample 2

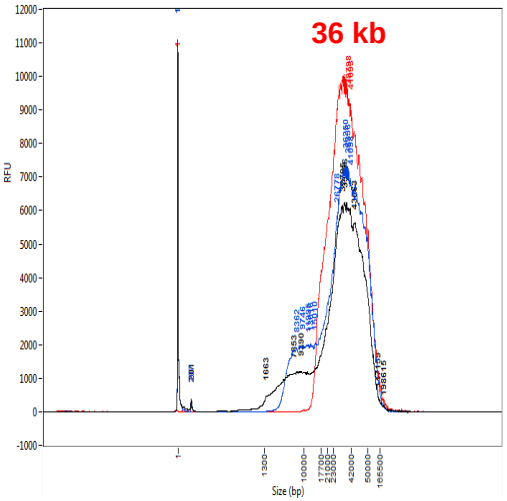
Multiplexed SV Library Example Performance Data

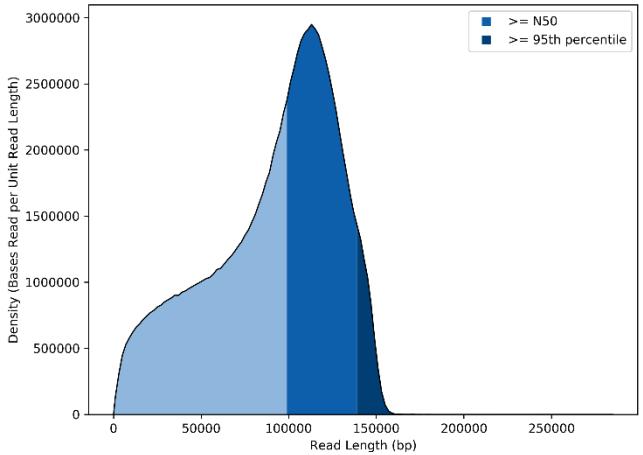
MULTIPLEXED HUMAN SV LIBRARY EXAMPLE PERFORMANCE (SEQUEL II SYSTEM CHEMISTRY 2.0)

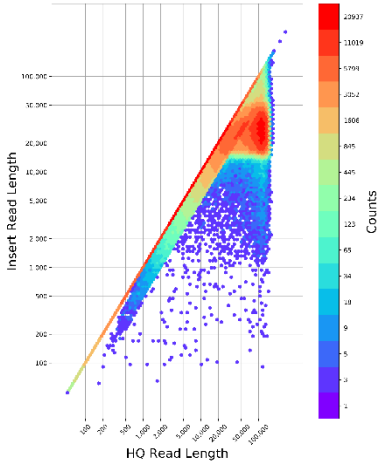
Primary Sequencing Performance Results

PRIMARY RUN STATISTICS FOR A BLUEPIPPIN SIZE-SELECTED 36 KB 2-PLEX HUMAN SV LIBRARY

Multiplexed SV Library Name	Total Bases (Gb)	Unique Molecular Yield (Gb)	Polymerase RL (bp)	Polymerase RL N50 (bp)	Longest Subread (bp)	Longest Subread N50 (bp)	P0	P1	P2
HG002_BPSS	220.0	89.1	53630	99322	21912	28946	46%	51%	3%







- Multiplexed library containing two human SV samples
- Pooled library was size-selected using BluePippin System (36 kb mean insert size)
- Yield of Barcoded Reads = 2.6 Million (64%)
- At P1= 51%, >10-fold unique molecular coverage (UMC) was achieved per sample

MULTIPLEXED HUMAN SV LIBRARY EXAMPLE PERFORMANCE (SEQUEL II SYSTEM CHEMISTRY 2.0) (CONT.)

Structural Variation Calling Performance Results

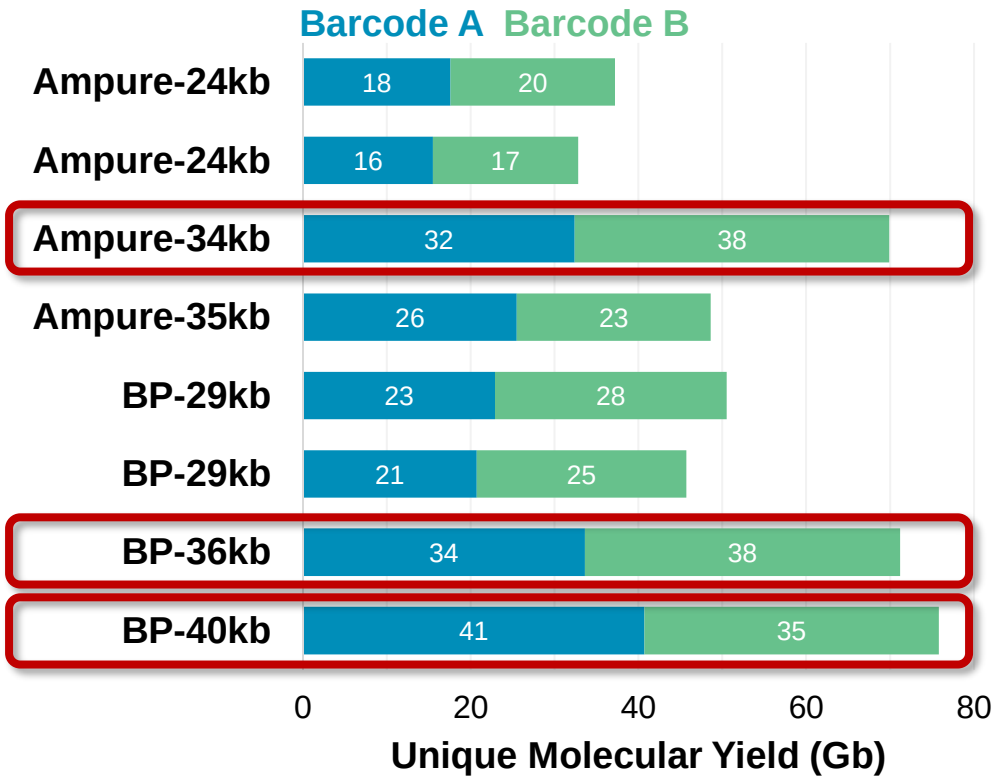
SV CALLING PERFORMANCE STATISTICS FOR A BLUEPIPPIN SIZE-SELECTED 36 KB 2-PLEX HUMAN SV LIBRARY

SAMPLE NAME	SV TYPE	METRIC	PERFORMANCE
HG002_36kb_SV_BC15_BPSS	Deletion	Precision	98%
		Recall	86%
	Insertion	Precision	96%
		Recall	84%
HG002_36kb_SV_BC19_BPSS	Deletion	Precision	98%
		Recall	90%
	Insertion	Precision	94%
		Recall	86%

For each sample, 10-fold unique molecular coverage was used for SV data analysis using SMRT Link v8.0.

COMPARISON OF UNIQUE MOLECULAR YIELDS FOR BLUEPIPPIN SIZE-SELECTED *VS* AMPURE PB SIZE-SELECTED SV LIBRARIES (SEQUEL II SYSTEM CHEMISTRY 2.0)

- Unique molecular coverage (UMC) recommendations for SV detection:
  - ≥5-fold UMC for human population genetics
  - ≥10-fold UMC for human disease research
- To achieve ≥5-fold UMC per sample for human population genetics research:
  - ≥15 Gb unique molecular yield (UMY) per sample is required
- To achieve ≥10-fold UMC per sample for human disease research
  - ≥30 Gb UMY per sample is required
  - Constructing larger SV libraries (≥ 30 kb with BluePippin size selection) is recommended for this use case



Technical Documentation & Applications Support Resources

BEST PRACTICES: STRUCTURAL VARIANT DETECTION USING WHOLE GENOME SEQUENCING (SEQUEL II CHEMISTRY 2.0)

LIBRARY PREP

SMRTbell Template Preparation

- Prepare >15 kb library using SMRTbell Express Template Preparation Kit 2.0
- Start with high-quality input genomic DNA (≥5 µg) and shear to a target mean fragment size ~20 kb – 40 kb
- Multiplex samples with barcoded SMRTbell adapters
- Enrich for longest inserts with AMPure PB size-selection or BluePippin size-selection

SMRT SEQUENCING

Sequence on the Sequel II System (CLR Sequencing Mode)

- Recommended unique molecular coverage (UMC) based on study goals*
  - 5- to 10-fold UMC: Population genetics studies – sensitivity limited per individual, but high for variants shared in the population using joint calling
  - 10- to 20-fold UMC: Rare undiagnosed disease studies – sensitivity high per individual allowing discovery of pathogenic SVs
  - 25-fold: Saturate discovery in an individual, recommended for detection of de novo SVs
- For human population genetics studies, run ~1,000 samples/year by multiplexing 2 samples / SMRT Cell at a cost of ~$670/sample†

DATA ANALYSIS

Data Analysis Solutions with the PacBio Analytical Portfolio

- Detect the broadest range of SV types including insertions, deletions, duplications, inversions, translocations, and copy number variants
- Discover SVs with the highest precision and recall
- Identify common SVs across multiple samples with joint calling
- Resolve breakpoints to sequence level
- Limit costly validation efforts with a low false discovery rate of <5%
- Output data in standard file formats – BAM and VCF – for seamless integration with downstream analysis tools
- Confirm SV calls visually with IGV and GenomeRibbon

** Read lengths, reads/data per SMRT Cell 8M and other sequencing performance results vary based on sample quality/type and insert size.*

† Prices, listed in USD, are approximate and may vary by region. Pricing includes library and sequencing reagents run on a Sequel II System and does not include instrument amortization or other reagents.

TECHNICAL DOCUMENTATION AND APPLICATIONS SUPPORT RESOURCES FOR MULTIPLEXED SV LIBRARY PREPARATION

- Procedure & Checklist – Multiplexing SMRTbell Libraries using SMRTbell Express Template Prep Kit 2.0 for Structural Variation Detection (PN 101-881-600)
- Quick Reference Card – Loading and Pre-Extension Recommendations for the Sequel II System (PN 101-769-100)
- Overview – Sequel Systems Application Options and Sequencing Recommendations (PN 101-851-300)
- Application Brief: Structural variant detection using whole genome sequencing – Best Practices (PN BP104-092319)

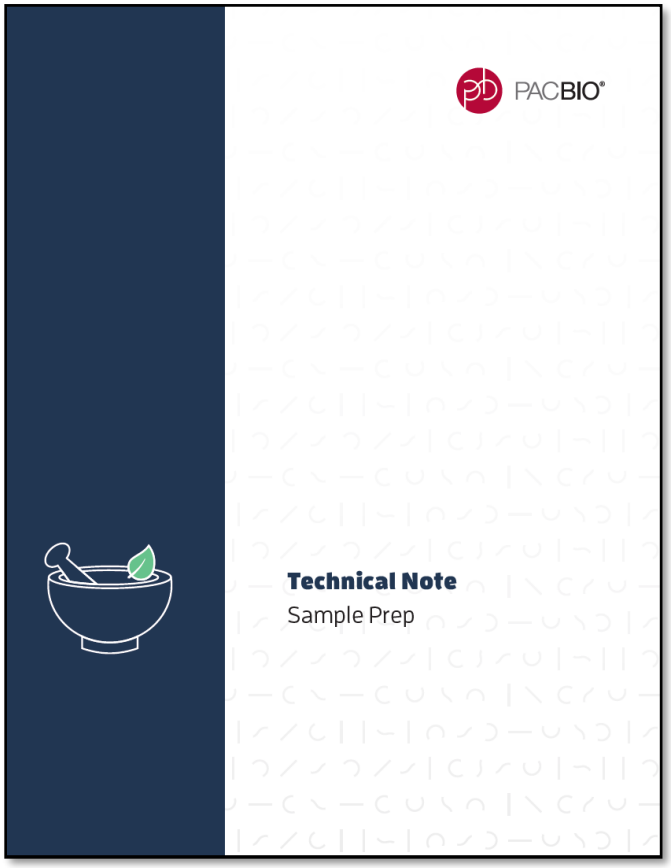
Appendix: General Recommendations for High-Molecular Weight gDNA QC and Handling for SMRTbell Library Construction

SAMPLE COLLECTION, PREPARATION, AND STORAGE FOR SMRT SEQUENCING WHOLE GENOME PROJECTS

To obtain the highest quality genomic DNA, it is important to start with sample types compatible with HMW DNA extraction methods

- This Technical Note provides general guidance on biological sample collection, preparation, and storage across a range of commonly encountered sample types used for SMRT Sequencing whole genome projects
- Includes sample and storage recommendations for:
  - Vertebrates - mammals, birds, fish, amphibians, reptiles
  - Invertebrates - marine, terrestrial
  - Arthropods - insects, crustaceans
  - Fungi - microorganisms, mushrooms, algae*
  - Plants - broad leaf plants, grasses

  *Algae is included with fungi due to similar growth and storage conditions
- Includes additional considerations for planning HMW DNA isolation



Technical Note TN100-040518: Preparing samples for PacBio whole genome sequencing for *de novo* assembly – Collection and storage

GENERAL RECOMMENDATIONS FOR ISOLATING HIGH-MOLECULAR WEIGHT GENOMIC DNA

- If gel purification is required, avoid using ethidium/UV based visualization methods. One alternative is to use SYBR® Safe (Invitrogen) and visualize with blue light
- To help resuspend the DNA, carefully invert the tube several times after adding buffer and/or tap the tube gently. Avoid vortexing genomic DNA when possible as vortexing can cause shearing of the DNA. It is also recommended to use wide bore tips in sample handling
- Alternatively, allow the DNA to stand in buffer overnight at 25°C to resuspend
- Overheating can introduce DNA damage. Inactivate DNAase as recommended by the vendor kit. It is best to avoid heat inactivation when possible
- DNA storage conditions: 4 °C (short-term); -20°C / -80°C (long-term)

EXAMPLE THIRD-PARTY HIGH-MOLECULAR WEIGHT GENOMIC DNA ISOLATION PROTOCOL AND KIT SOLUTIONS

Note: The products below have not been extensively tested or validated by PacBio R&D but are listed here as examples of third-party kits or methods used by other PacBio customers for isolating genomic DNA for SMRTbell library preparation

Plant Tissue

QIAGEN Genomic-tip 20/100/500/G Kit

Circulomics Nanobind Plant Nuclei Big DNA Kit

- https://www.circulomics.com/

Unsupported Protocol – Switchgrass (Panicum virgatum) DNA isolation [USDA]

- http://www.pacb.com/wp-content/uploads/2015/09/Switchgrass-DNA-isolation.pdf

Unsupported Protocol – DNA extraction of Chlamydomonas using CTAB [JGI]

- http://www.pacb.com/wp-content/uploads/2015/09/DNA-extraction-chlamy-CTAB-JGI.pdf

QIAGEN User-Developed Prototocol: Isolation of genomic DNA from plants and filamentous fungi using the QIAGEN Genomic-tip Kit

- https://www.qiagen.com/de/resources/resourcedetail?id=cb2ac658-8d66-43f0-968e-7bb0ea2c402a&lang=en

Insect Tissue

QIAGEN User-Developed Prototocol: Isolation of genomic DNA from mosquitoes or other insects using the QIAGEN Genomic-tip Kit

- https://www.qiagen.com/ca/resources/resourcedetail?id=b45c3cc3-7f2b-4f4a-aa37-21d814ed3730&lang=en

Circulomics Nanobind Tissue Big DNA Kit

- https://www.circulomics.com/

Fish Tissue

QIAGEN Genomic-tip 20/100/500/G Kit

- Reference: Chromosomal-Level Assembly of the Asian Seabass Genome Using Long Sequence Reads and Multi-layered Scaffolding. *PLOS Genetics* 12(4): e1005954. (2016) doi: 10.1371/journal.pgen.1005954 http://journals.plos.org/plosgenetics/article?id=10.1371%2Fjournal.pgen.1005954

QIAGEN User-Developed Prototocol: Purification of archive-quality DNA from 10–20 mg fish tissue using the Gentra® Puregene® Tissue Kit or Gentra Puregene Mouse Tail Kit

- https://www.qiagen.com/ca/resources/resourcedetail?id=948e5a5e-57c4-482f-a852-43ec3da97fce&lang=en

Circulomics Nanobind Tissue Big DNA Kit

- https://www.circulomics.com/

Human Tissue

QIAGEN Genomic-tip 20/100/500/G Kit / QIAGEN Gentra Puregene Cell Kit

Unsupported Protocol – Gentra Puregene Cell Kit (Qiagen) DNA Isolation [Univ. Washington]

- http://www.pacb.com/wp-content/uploads/2015/09/Gentra-Puregene-Qiagen-DNA-Isolation.pdf

  Reference: Resolving the complexity of the human genome using single-molecule sequencing. *Nature* **517**, 608-611 (29 January 2015) doi:10.1038/nature13907 http://www.nature.com/nature/journal/v517/n7536/full/nature13907.html

Circulomics Nanobind CBB Big DNA Kit or Nanobind Tissue Big DNA Kit

- https://www.circulomics.com/

NEW SAMPLE PREPARATION ONLINE RESOURCE

Literature resource for sample collection and DNA extraction protocol references

ANIMALS

- **Invertebrates**
  - Panova2016 - DNA extraction protocols for whole-genome sequencing in marine organisms
  - *arthropods*
    - arachnids
      - Guerrero2019 - The Pacific Biosciences de novo assembled genome dataset from a parthenogenetic New Zealand wild population of the longhorned tick, *Haemaphysalis longicornis* Neumann, 1901
      - Liu2019 - DNA Methylation Patterns in the Social Spider, *Stegodyphus dumicola*
      - Burgess2018 - Draft genome assembly of the sheep scab mite, *Psoroptes ovis*
      - Randall2018 - The draft genome assembly of *Dermatophagoides pteronyssinus* supports identification of novel allergen isoforms in *Dermatophagoides* species
      - Cramaro2017 - Genome scaffolding and annotation for the pathogen vector *Ixodes ricinus* by ultra-long single molecule sequencing
    - crustaceans
      - Baldwin-Brown2018 - A New Standard for Crustacean Genomes: The Highly Contiguous, Annotated Genome Assembly of *Eulimnadia texana* Reveals HOX Gene Order and Identifies the Sex Chromosome
      - Gonçalves Athanasio2016 - Optimisation of DNA extraction from the crustacean *Daphnia*
    - insects
      - Kingan2019 - A High-Quality De novo Genome Assembly from a Single Mosquito Using PacBio Sequencing
      - Oppert2019 - Optimized Extraction of Insect Genomic DNA for Long-Read Sequencing
  - *hemichordates*
    - Lowe2018 - Preparing Genomic DNA from Sperm of the Brokenhearted Acorn Worm (*Schizocardium californicum*)
  - *cnidarians*
    - Jeon2019 - The Draft Genome of an Octocoral, *Dendronephthya gigantea*
    - Gold2018 - The genome of the jellyfish *Aurelia* and the evolution of animal complexity
    - Kim2018 - The genome of the giant Nomura's jellyfish sheds light on the early evolution of active predation
  - *mollusks*
    - Zagrodzka2019 - PacBio protocol *Littorina saxatilis*
    - Arseneau2017 - Modified low-salt CTAB extraction of high-quality DNA from contaminant-rich tissues
  - *worms*
    - Serra2019 - Hybrid Assembly of the Genome of the Entomopathogenic Nematode *Steinernema carpocapsae* Identifies the X-Ch
    - Grohme2018 - The genome of *Schmidtea mediterranea* and the evolution of core cellular mechanisms
    - Grohme2018 - Small- and Large-Scale High Molecular Weight Genomic DNA Extraction from Planarians
    - Rödelsperger2017 - Single-Molecule Sequencing Reveals the Chromosome-Scale Genomic Architecture of the Nematode Mo *Pristionchus pacificus*
- **Vertebrates**
  - *fish*
    - Zagrodska2020 - DNA Extraction protocol for corkwing wrasse (*Symphodus melops*; September 2016 updated January 2020)
    - Chen2019 - The sequencing and de novo assembly of the Larimichthys crocea genome using PacBio and Hi-C technologies
    - Zhou2019 - Chromosome genome assembly and annotation of the yellowbelly pufferfish with PacBio and Hi-C sequencing d
  - *amphibians*
    - Li2019 - Chromosome-level assembly of the mustache toad genome using third-generation DNA sequencing and Hi-C analy
    - Edwards2018 - Draft genome assembly of the invasive cane toad, *Rhinella marina*
    - Nowoshilow2018 - The axolotl genome and the evolution of key tissue formation regulators
  - *reptiles*
    - Roscito2018 - The genome of the tegu lizard *Salvator merianae* - combining Illumina, PacBio, and optical mapping data to generate a highly contiguous assembly
    - Ullate-Agote2017 - A Step-by-Step Guide to Assemble a Reptilian Genome
  - *birds*
    - Formenti2019 - SMRT long-read sequencing and Direct Label and Stain optical maps allow the generation of a high-quality genome assembly for the European barn swallow (*Hirundo rustica rustica*)
  - *mammals*
    - Jebb2019 - Six new reference-quality bat genomes illuminate the molecular basis and evolution of bat adaptations

PLANTS

- Mayjonade2016 - Extraction of high-molecular-weight genomic DNA for long-read sequencing of single molecules
- **Angiosperms**
  - Anderson2018 - Protocol- a versatile, inexpensive, high-throughput plant genomic DNA extraction method suitable for genotyping-by-sequencing
  - Inglis2018 - Fast and inexpensive protocols for consistent extraction of high quality DNA and RNA from challenging plant and fungal samples for high throughput SNP genotyping and sequencing applications
- **Gymnosperms**
  - Zimin2017 - An improved assembly of the loblolly pine mega-genome using long-read single-molecule sequencing
- **Other**
  - Panova2016 - DNA extraction protocols for whole-genome sequencing in marine organisms (algae)

Beet Rhizosphere Mycoparasite *Pythium oligandrum*
ols for *Ganoderma boninense*, *G. miniatocinctum* and *G. tornatu*
DNA from fungal rust spores for long read sequencing
cryopreservation and DNA extraction from anaerobic gut fungi
tion landscape of the button mushroom *Agaricus bisporus var. bisporus*

uttalii reveals mammalian host-related molecular signatures and a novel octapeptide-repeat

diatom *Fragilariopsis cylindrus*

Methods

Sample collection

A female yellowbelly pufferfish (Fig. 2), reared in the fish breeding centre of Fujian Normal University in Fuzhou City of Fujian Province was used for genome sequencing and assembly. Fresh white muscle, eye, skin, gonad, gut, liver, kidney, blood, gall bladder and air bladder tissues were collected and quickly frozen in liquid nitrogen for one hour. White muscle tissues were used for DNA sequencing for genome assembly, while all tissues were used for transcriptome sequencing.

Fig. 2

A picture of the yellowbelly pufferfish used in the genome sequencing and assembly.

DNA and RNA sequencing

Genomic DNA from white muscle tissue was extrac

www.ExtractDNAforPacBio.com

- PacBio does not assume responsibilities/guarantees for these external publications/protocols, but we are happy to help as best as we can to guide/connect. Please contact ExtractDNA@pacb.com for more discussions around your particular species & sequencing project!

GENERAL RECOMMENDATIONS TO HELP CLEANUP HIGH MOLECULAR WEIGHT GENOMIC DNA AND SMRTBELL LIBRARIES

High Molecular Weight gDNA and SMRTbell Library Cleanup

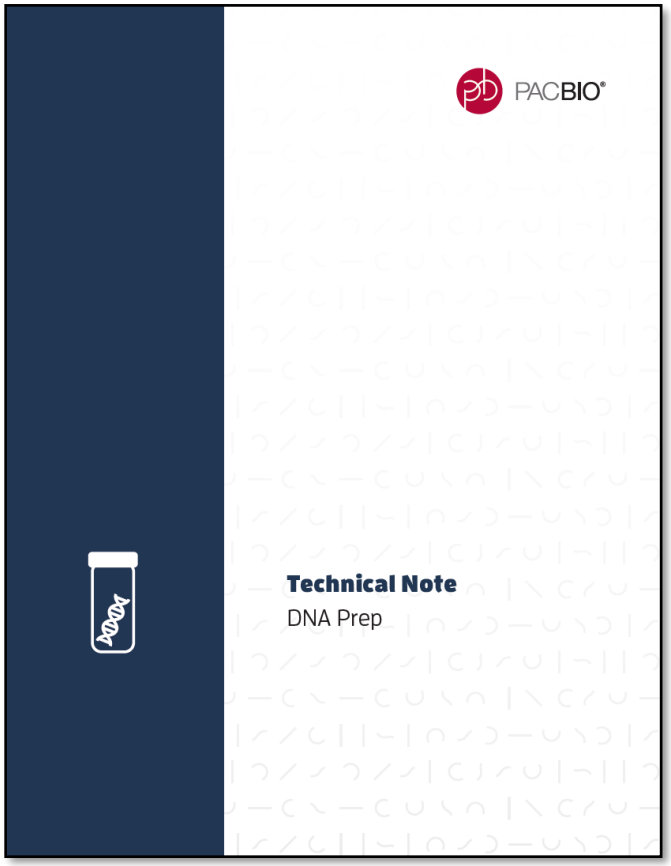
AMPure PB Bead Wash

- Refer to the appropriate Procedure & Checklist protocol document for specific recommendations for AMPure PB bead purification of different SMRTbell library insert sizes

METHODS FOR EVALUATION OF GENOMIC DNA QUALITY

Starting with high-quality, high molecular weight (HMW) genomic DNA will result in longer libraries and better *de novo* assembly performance

- Input genomic DNA must be carefully QC'd to assess integrity
  - PFGE/FIGE or Femto Pulse sizing tool is highly recommended
  - High molecular weight DNA → long read lengths
  - Degraded DNA → short read lengths, low library recovery yields (dependent on BluePippin size selection parameters)
  - DNA purity can be determined by using a NanoDrop instrument or other spectrophotometer device
  - PacBio highly recommends using the Qubit High Sensitivity fluorometric assay for accurate dsDNA quantitation
- This Technical Note provides recommendations, tips and tricks for assessing and preserving the quality and size of your gDNA sample, shearing methods, and size selection procedures for samples intended to be used with whole genome sequencing for *de novo* assembly.



Technical Note TN101-040518: Preparing DNA for PacBio Whole Genome Sequencing for *de novo* Assembly: Quality Control and Shearing

A. DNA Sizing Characterization

Recommended methods for determining gDNA size distribution:

1. CHEF Mapper XA System (Bio-Rad)

➢ **Up to 10 Mb**

http://www.bio-rad.com/en-us/category/pulsed-field-gel-electrophoresis-systems

2. Femto Pulse System (Agilent)

➢ **Up to 165 kb**

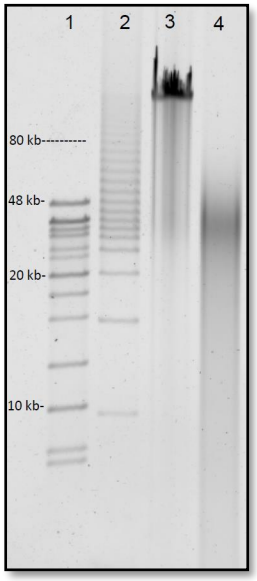
https://www.agilent.com/en/product/automated-electrophoresis/femto-pulse-systems

3. Pippin Pulse System (Sage Science)

➢ **Up to 80 kb**

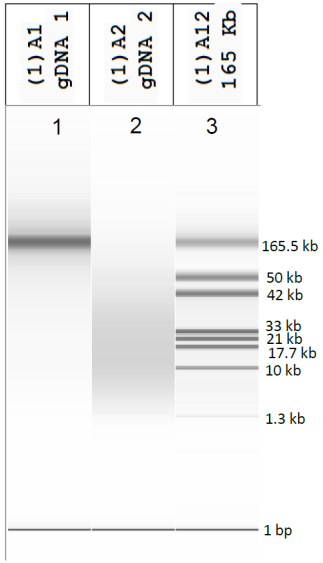
http://www.sagescience.com/products/pippin-pulse/

A



Lane 1: 8-48 kb Ladder (Bio-Rad)
Lane 2: 5 kb Ladder (Bio-Rad)
Lane 3: HMW gDNA
Lane 4: Degraded gDNA

B



Lane 1: High MW gDNA
Lane 2: Degraded gDNA
Lane 3: 165 kb Ladder

Evaluation of gDNA quality using A) Bio-Rad CHEF Mapper System and B) Femto Pulse System. Lanes 3 A and 1B are examples of high quality, high molecular weight genomic DNA. Lanes 4A and 2B are examples of degraded gDNA.

B. DNA Purity Determination

- DNA purity can be determined by using a **NanoDrop®** instrument or other spectrophotometers
- For ultrapure gDNA, A260/280 ratio is typically between ~1.8 - 2.0 and A260/230 ratio is ≥2.0
- If A260/280 and A260/230 readings are out of the range specified above, PacBio recommends performing an AMPure® purification step followed by re-assessment of quantity and purity of the gDNA sample

260/280 Ratio

- A low A260/A280 ratio may indicate the presence of protein, phenol, or other contaminants that absorb strongly at or near 280 nm. Sometimes it may be caused by a very low concentration of nucleic acid.
- High 260/280 ratios are not indicative of an issue

260/230 Ratio

- A low A260/A230 ratio may be the result of:
  - Carbohydrate carryover (often a problem with plants)
  - Residual phenol from nucleic acid extraction
  - Residual guanidine (often used in column-based kits)
  - Glycogen used for precipitation
- A high A260/A230 ratio may be the result of:
  - Making a blank measurement on a dirty pedestal of a Nanodrop instrument
  - Using an inappropriate solution for the blank measurement

C. DNA Quantification

- Accurate quantitation of DNA concentration is critical for PacBio template preparation procedures.
  - Specifically, it is critical to determine the concentration of the double-stranded DNA, since only double-stranded DNA will be converted into sequencing templates.
- PacBio highly recommends using a **Qubit® fluorometer** tool and **Qubit ds DNA High Sensitivity (HS) Assay Kit** for routine DNA quantitation during SMRTbell library construction.
- When assessing gDNA QC, PacBio recommends using both fluorometric and spectrophotometric methods – for example, using both the Qubit and NanoDrop instruments
  - If the sample is pure gDNA, free of any RNA contaminants and other small molecules, the two methods should converge to similar DNA concentration measurement values
- If the measured NanoDrop concentration is significantly different (>50%) from the Qubit measurement, PacBio recommends doing an AMPure purification step (as specified by your chosen library preparation protocol), followed by a re-measurement with both methods. Typically, a single AMPure purification step resolves the discrepancy.
  - If the concentration measurement discrepancy after one AMPure purification step is not reduced, we recommend trying another cleanup approach before a re-measurement with both methods.

SPECIAL HANDLING RECOMMENDATIONS FOR PREPARING >15 KB AND >30 KB SMRTBELL LIBRARIES

1. For small numbers of samples, use DNA Lo-Bind 1.5 mL microcentrifuge tubes for all enzymatic and AMPure PB bead purification steps.
2. Multi-channel pipettes and 96-well plates/PCR strip tubes may be used to efficiently process large numbers of samples.
3. Use wide-bore tips for all pipette mixing steps when preparing >15 kb and >30 kb size-selected libraries.
4. Never vortex tubes containing high-molecular weight DNA samples. Mix by gentle pipetting unless stated otherwise.
5. Always follow best practices for DNA quantitation using a Qubit fluorometer system:
   - Use the Qubit dsDNA High Sensitivity (HS) Assay reagent kit.

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