Multiplexed Library Preparation For Structural Variation Detection

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

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

* 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.

### GENOMIC DNA SIZE EVALUATION METHODS AND PROCEDURES

<table>
<thead>
<tr>
<th>METHOD</th>
<th>COMMENTS</th>
<th>PROCEDURE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agilent Femto Pulse System</td>
<td>Highly recommended (200-500 pg)</td>
<td><a href="#">Agilent Femto Pulse Website</a></td>
</tr>
<tr>
<td>Bio-Rad® CHEF Mapper® XA PFGE System</td>
<td>Requires &gt;50 ng</td>
<td><a href="#">Procedure &amp; Checklist - Using the BIO-RAD® CHEF Mapper XA Pulsed Field Electrophoresis System</a></td>
</tr>
<tr>
<td>Sage Science Pippin Pulse System</td>
<td>Requires &gt;50 ng</td>
<td><a href="#">Procedure &amp; Checklist - Using the Sage Science Pippin Pulse Electrophoresis Power Supply System</a></td>
</tr>
</tbody>
</table>
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.

**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
**LIST OF REQUIRED MATERIALS AND EQUIPMENT**

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

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

<table>
<thead>
<tr>
<th>Barcoded Overhang Adapter Kit -8A</th>
<th>Barcoded Overhang Adapter Kit -8B</th>
</tr>
</thead>
<tbody>
<tr>
<td>bc1001</td>
<td>bc1015</td>
</tr>
<tr>
<td>bc1002</td>
<td>bc1016</td>
</tr>
<tr>
<td>bc1003</td>
<td>bc1017</td>
</tr>
<tr>
<td>bc1008</td>
<td>bc1018</td>
</tr>
<tr>
<td>bc1009</td>
<td>bc1019</td>
</tr>
<tr>
<td>bc1010</td>
<td>bc1020</td>
</tr>
<tr>
<td>bc1011</td>
<td>bc1021</td>
</tr>
<tr>
<td>bc1012</td>
<td>bc1022</td>
</tr>
</tbody>
</table>

  - FASTA filename: 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.
DNA SHEARING RECOMMENDATIONS FOR MULTIPLEXED SV LIBRARY CONSTRUCTION (CONT.)

<table>
<thead>
<tr>
<th>Megaruptor System</th>
<th>Recommended Megaruptor Software Setting</th>
<th>Sheared gDNA Target Size Distribution Mode</th>
</tr>
</thead>
<tbody>
<tr>
<td>Megaruptor 1 or 2</td>
<td>30 kb – 35 kb Target Size Setting</td>
<td>20 – 40 kb</td>
</tr>
<tr>
<td>Megaruptor 3</td>
<td>Speed Setting 28 – 30</td>
<td>20 – 40 kb</td>
</tr>
</tbody>
</table>

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

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

- 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

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.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Where Used</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA Prep Additive</td>
<td>Remove single-strand overhangs</td>
</tr>
<tr>
<td>DNA Prep Enzyme</td>
<td>Remove single-strand overhangs</td>
</tr>
<tr>
<td>DNA Damage Repair Mix v2</td>
<td>DNA Damage Repair</td>
</tr>
<tr>
<td>End Prep Mix</td>
<td>End-Repair/A-tailing</td>
</tr>
<tr>
<td>Overhang Adapters v3</td>
<td>Ligation</td>
</tr>
<tr>
<td>Barcoded Overhang Adapters</td>
<td>Ligation</td>
</tr>
<tr>
<td>Ligation Mix</td>
<td>Ligation</td>
</tr>
<tr>
<td>Ligation Additive</td>
<td>Ligation</td>
</tr>
<tr>
<td>Ligation Enhancer</td>
<td>Ligation</td>
</tr>
<tr>
<td>Enzyme A</td>
<td>Nuclease Treatment</td>
</tr>
<tr>
<td>Enzyme B</td>
<td>Nuclease Treatment</td>
</tr>
<tr>
<td>Enzyme C</td>
<td>Nuclease Treatment</td>
</tr>
<tr>
<td>Enzyme D</td>
<td>Nuclease Treatment</td>
</tr>
</tbody>
</table>
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

<table>
<thead>
<tr>
<th>STEP</th>
<th>Pooling</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Use the concentration and average library size* from the Femto Pulse to determine the molarity of each sample.</td>
<td></td>
</tr>
</tbody>
</table>
| 2    | Use the following equation to determine Molarity:  
  \[
  \text{concentration (in ng/\muL)} \times 10^6 = \text{concentration (in nM)} \\
  (650 \text{ g/mol} \times \text{average library size in bp}^*)
  \] |       |
| 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/\muL. 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.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elution Buffer</td>
<td>6.5 mL</td>
<td></td>
</tr>
<tr>
<td>AMPure PB Beads (stock reagent)</td>
<td>3.5 mL</td>
<td></td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td>10.0 mL</td>
<td></td>
</tr>
</tbody>
</table>

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 bead solution for 30 seconds to mix well before use. This solution may be stored at 4°C for 2 months for future use.

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.

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:

<table>
<thead>
<tr>
<th>Cassette Definition File</th>
<th>If &lt; 3 µg input per lane, use BP Start</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.75% DF Marker S1 high-pass 15 kb – 20 kb</td>
<td>12500 for 15 kb cutoff</td>
</tr>
<tr>
<td></td>
<td>15000 for 20 kb cutoff</td>
</tr>
</tbody>
</table>
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

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

*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:

<table>
<thead>
<tr>
<th>SAMPLE SETUP PARAMETER</th>
<th>PARAMETER VALUE / REAGENT KIT TO USE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sequencing Primer</td>
<td>Sequencing Primer V2</td>
</tr>
<tr>
<td>Sequencing Primer:Template Ratio</td>
<td>20:1</td>
</tr>
<tr>
<td>Polymerase Binding Kit</td>
<td>Sequel II Binding Kit 2.0</td>
</tr>
<tr>
<td>Polymerase:Template Ratio</td>
<td>10:1</td>
</tr>
<tr>
<td>Polymerase Binding Time</td>
<td>4 hours</td>
</tr>
</tbody>
</table>

- 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):

<table>
<thead>
<tr>
<th>SMRT LINK SAMPLE SETUP FIELD</th>
<th>VALUE TO SELECT</th>
<th>PURPOSE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sequencing Primer</td>
<td>“Sequencing Primer V2”</td>
<td>Enables calculation of 10:1 polymerase to template ratio in SMRT Link Sample Setup</td>
</tr>
<tr>
<td>Sequencing Mode</td>
<td>“CCS”</td>
<td>Enables calculation of 10:1 polymerase to template ratio in SMRT Link Sample Setup</td>
</tr>
</tbody>
</table>

- 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 Sequal II System:

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

- 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:


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.
Multiplexed SV Library Example Performance Data
### PRIMARY RUN STATISTICS FOR A BLUEPIPPIN SIZE-SELECTED 36 KB 2-PLEX HUMAN SV LIBRARY

<table>
<thead>
<tr>
<th>Multiplexed SV Library Name</th>
<th>Total Bases (Gb)</th>
<th>Unique Molecular Yield (Gb)</th>
<th>Polymerase RL (bp)</th>
<th>Polymerase RL N50 (bp)</th>
<th>Longest Subread (bp)</th>
<th>Longest Subread N50 (bp)</th>
<th>P0</th>
<th>P1</th>
<th>P2</th>
</tr>
</thead>
<tbody>
<tr>
<td>HG002_BPSS</td>
<td>220.0</td>
<td>89.1</td>
<td>53630</td>
<td>99322</td>
<td>21912</td>
<td>28946</td>
<td>46%</td>
<td>51%</td>
<td>3%</td>
</tr>
</tbody>
</table>

- 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
### Structural Variation Calling Performance Results

#### SV Calling Performance Statistics for a BluePippin Size-Selected 36 kb 2-Plex Human SV Library

<table>
<thead>
<tr>
<th>SAMPLE NAME</th>
<th>SV TYPE</th>
<th>METRIC</th>
<th>PERFORMANCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>HG002_36kb_SV_BC15_BPSS</td>
<td>Deletion</td>
<td>Precision</td>
<td>98%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Recall</td>
<td>86%</td>
</tr>
<tr>
<td></td>
<td>Insertion</td>
<td>Precision</td>
<td>96%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Recall</td>
<td>84%</td>
</tr>
<tr>
<td>HG002_36kb_SV_BC19_BPSS</td>
<td>Deletion</td>
<td>Precision</td>
<td>98%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Recall</td>
<td>90%</td>
</tr>
<tr>
<td></td>
<td>Insertion</td>
<td>Precision</td>
<td>94%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Recall</td>
<td>86%</td>
</tr>
</tbody>
</table>

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

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

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 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
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/](https://www.circulomics.com/)

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


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


*QIAGEN User-Developed Protocol: 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](https://www.qiagen.com/de/resources/resourcedetail?id=cb2ac658-8d66-43f0-968e-7bb0ea2c402a&lang=en)
Insect Tissue

QIAGEN User-Developed Protocol: 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](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/](https://www.circulomics.com/)

Fish Tissue

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


QIAGEN User-Developed Protocol: 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](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/](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]


Circulomics Nanobind CBB Big DNA Kit or Nanobind Tissue Big DNA Kit
- [https://www.circulomics.com/](https://www.circulomics.com/)
NEW SAMPLE PREPARATION ONLINE RESOURCE

Literature resource for sample collection and DNA extraction protocol references

ANIMALS
- Invertebrates
  - PacBio 2016 - DNA extraction protocols for whole genome sequencing in marine organisms

- Arthropods
  - Suryawanshi 2015 - The Pacific Blondes, or Not: Sexual Selection of the Mandible in the Cosmopolitan Broadhead Spider, Heteropoda venatoria, 2015
  - Li 2019 - DNA methylation patterns in the social spider, Araneus diadematus
  - Sudarikov 2018 - Draft genome assembly of the leafcutter ant, Atta cephalotes
  - Trefethen 2015 - The draft genome assembly of Drosophila melanogaster supports identification of novel alien isoforms in Drosophila species

- Crustaceans
  - Stickle 2016 - A New Standard for Crustacean Genomes: The Highly Conserved, Annotated Genome Assembly of the Marine Crustacean Drosophila melanogaster and Identifying the Bay Oyster (Crassostrea gigas)

- Mollusks
  - Cornick 2015 - Deep-sea DNA extraction from temperate marine sediments and their communities
  - Cornick 2015 - Phylogenomic analyses place specialized, thermophilic assemblage of the ancient genus Orthoceras in the Pterygotidae family

- Hemichordates
  - Lowes 2018 - Preparing Genomic DNA from Sperm of the Brooding Arrow Worm, Luidia ciliaris

- Cnidarians
  - Cenutyris 2015 - The Draft Genome of an Oncorhynchus mykiss
  - Cenutyris 2015 - The genome of the celtid jellyfish and the evolution of animal complexity
  - Cenutyris 2015 - The anatomy of the giant plumose anemone reveals insights into the early evolution of active predation

- Mammals
  - Cornick 2015 - Deep-sea DNA extraction from temperate marine sediments and their communities

- Worms
  - Sena 2016 - Draft Assembly of the Genome of the Intertidal Polychaete Sabellulopsis kroyeri: Insights into the K-PZ Division (Phylum Echiura: The genome of the sabellulid polychaete Sabellulopsis kroyeri)

- Vertebrates
  - Fish
    - Cegredo 2020 - DNA Extraction protocol for common carp (Cyprinus carpio)
    - Cegredo 2020 - A new method for the extraction of genomic DNA from fish and other aquatic organisms
  - Amphibians
    - UNEP-WCMC 2015 - Genomic-scale assemblies of the mudpuppy, a long-lived amphibian, reveal potential evolutionary mechanisms for longevity
    - Newcomb 2016 - The whole-genome assembly and the evolution of eye tissue regeneration
  - Reptiles
    - Bostro 2015 - The genome of the black lizard, a common reptile, reveals insights into the evolution of physiological mechanisms
    - UHD 2015 - A step-by-step guide to assembling a reptilian genome
  - Birds
    - Clement 2012 - Single-organism sequencing and genome-wide association studies is a powerful tool for understanding avian genome evolution
    - UHD 2015 - A step-by-step guide to assembling a reptilian genome
  - Mammals
    - UHD 2015 - Six new reference-quality bat genomes illuminate the molecular basis and evolution of bat adaptations

- Plants
  - Macrobotswana 2016 - Emergence of high-molecular-weight genomic DNA for long-read sequencing of single molecules
  - Macrobotswana 2016 - Prokaryotes, a separate, independent, high-throughput plant genomic DNA extraction method suitable for genotyping by sequencing
  - Macrobotswana 2016 - Fast and efficient genomic isotopy for optimizing extraction of high-quality DNA from plants inomics samples (review)

- Gymnosperms
  - Conifer 2017 - An improved assembly of the lobolly pine genome using long-read single-molecule sequencing
  - PacBio 2016 - DNA extraction protocols for whole genome sequencing in marine organisms

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.
A. DNA Sizing Characterization

Recommended methods for determining gDNA size distribution:

1. CHEF Mapper XA System (Bio-Rad)
   - Up to 10 Mb

2. Femto Pulse System (Agilent)
   - Up to 165 kb

3. Pippin Pulse System (Sage Science)
   - Up to 80 kb

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
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.