Template Preparation

FIND MEANING IN COMPLEXITY
Specifics of SMRT® Sequencing Data

Steps of SMRTbell™ Library Creation

- Reagents and Consumables
- Troubleshooting SMRTbell™ Library Performance
- Size Selection Recommended for Large-Insert Libraries
- Where to Find Additional Information
Advantages of SMRTbell™ Templates

Key Advantages:

• Structurally linear
• Topologically circular
• Structural homogeneity of templates
• Provides sequences of both forward and reverse strands in the same trace
Universal SMRTbell™ Template

Standard Sequencing for Continuous Long Reads (CLR)

- Large Insert Sizes
  - Recommended Insert Size: > 3 kb
  - Recommended Movie Collection Time: >1 x 120 min

Circular Consensus Sequencing (CCS)

- Small Insert Sizes
  - Recommended Insert Size: 500 bp – 3 kb
  - Recommended Movie Collection Time: 1 x 55 min
Template Preparation Workflow

1. Fragment DNA and Concentration
2. DNA Damage Repair
3. Repair Ends
4. Ligate Adapters
5. Purify Templates
6. Primer Annealing
7. Bind Polymerase
SMRTbell™ Template Preparation and Binding Steps

- Fragmentation protocol recommends shearing with:
  - Covaris® S2 or LE220 system (500 bp to < 5 kb)
  - Covaris g-TUBE® devices (>6 kb)
  - HydroShear® instrument (>5 kb)

- DNA concentration using AMPure® PB beads
SMRTbell™ Template Preparation and Binding Steps

- Recommended for libraries >3 kb and for PCR products >2 kb
- Reagents are included in the Template Prep Kit
- Repairs abasic sites, nicks, thymine dimers, blocked 3’-ends, oxidized guanines/pyrimidines, deaminated cytosine
SMRTbell™ Template Preparation and Binding Steps

- Polish ends of fragments suitable for ligation
  - 5’ overhangs are filled-in by T4 DNA Polymerase
  - 3’ overhangs are removed by T4 DNA Polymerase
  - T4 PNK phosphorylates the 5’ hydroxyl group
- Repaired fragments can be ligated to blunt adaptors (recommended method)

Fragment DNA and Concentration

DNA Damage Repair

Repair Ends

Ligate Adapters

Purify Templates

Primer Annealing

Bind Polymerase

Fragment DNA and Concentration

5’

DNA Damage Repair

Repair Ends

Ligate Adapters

Purify Templates

Primer Annealing

Bind Polymerase

5’

T4 DNA Polymerase + T4 PNK

5’

P
SMRTbell™ Template Preparation and Binding Steps

- During this step, hairpins are ligated to fragment ends
- Blunt ligation requires 15 minutes
SMRTbell™ Template Preparation and Binding Steps

- Fragment DNA and Concentration
- DNA Damage Repair
- Repair Ends
- Ligate Adapters
- Purify Templates
- Primer Annealing
- Bind Polymerase

- Exonuclease III (from 3’-hydroxyl termini)
- Exonuclease VII (from 5’-termini)
- Template Purification with AMPure® PB beads
SMRTbell™ Template Preparation and Binding Steps

- Sequencing primer annealed to the purified SMRTbell Template
- Binding Calculator is provided
SMRTbell™ Template Preparation and Binding Steps

- Sequencing polymerase is bound to both ends of SMRTbell template
- A Binding Calculator is provided to assist you in preparing your dilutions for binding polymerase
- Adding a control is recommended

1. Fragment DNA and Concentration
2. DNA Damage Repair
3. Repair Ends
4. Ligate Adapters
5. Purify Templates
6. Primer Annealing
7. Bind Polymerase
AMPure® PB Beads Used Throughout Template Preparation

- AMPure PB beads selectively purify the SMRTbell™ templates
- AMPure PB beads are specifically formulated for the SMRT® Sequencing workflow

Note: The process of size selection using AMPure PB beads is controlled by the concentration added to the sample.
Size Selection is Controlled by AMPure® PB Bead Concentration

- Reduced Ampure PB bead concentration enables greater reduction of short fragments.
- Ampure PB beads are specifically formulated for the SMRT® Sequencing workflow.
Sample Input Options

- **gDNA**
  - 500 bp – 10 kb

- **Fragment DNA & Concentrate**

- **Targeted Enrichment / PCR Products**

- **DNA Damage Repair**

- **Repair Ends**

- **Ligate Adapters**

- **Purify Templates**

- **Primer Annealing**

- **Bind Polymerase**

- **cDNA**
Reagent Consumables
Template Preparation and Instrument Loading

**DNA Template Library Preparation**
- SMRTbell Template Prep Kit (10 Rxn)
- SMRTbell HT Template Prep Kit (96 Rxn)
- AMPure® PB beads

**Polymerase Binding**
- Binding Kit
  - DNA Polymerase Binding Kit (24 Rxn)
- DNA Controls

**Magnetic Beads for Loading**
- MagBead Kit
  - MagBeads
  - MagBead Buffer

**On-instrument DNA Sequencing**
- DNA Sequencing Kit
  - DNA Sequencing Kit (8 Rxn)
- SMRT® Cell 8Pac (8 SMRT Cells)
- SMRT Cell Oil (10 seq runs)

**Storage Times**
- SMRTbell™ library can be stored up to 6 months at -20° C
- Bound Complex can be stored up to 3 days at 4° C
- MagBead-bound Complex can be stored up to 24 hours at 4° C
- Unused SMRT Cells can be stored at room temperature in the original sealable packaging
Troubleshooting SMRTbell™ Library Performance
Factors Affecting SMRTbell™ Library Preparation and Sequencing Performance

- Quality of Starting Material
- Fragmented-DNA Size Distribution
- Short-insert Contaminants
- AMPure® PB Beads
- Inaccurate Quantification
Factors Affecting SMRTbell™ Library Preparation and Sequencing Performance

Quality of Starting Material

- Causes
  - DNA damage (nicked DNA, abasic sites, etc.)
  - Contaminants from gDNA extraction

- Impact on Performance
  - Low SMRTbell template yields
  - Lower sequencing yields
  - Shorter read lengths

- Recommendations
  - Treat your gDNA gently during extraction
  - Always perform DNA damage repair
  - Purify starting DNA material to remove contaminants
Sample Conditions that Lead to Higher-Quality Libraries

- Double-stranded DNA Sample (dsDNA)
- Minimized freeze-thaw cycles
- No exposure to high temp (>65° C)
- No exposure to pH extremes (<6 or >9)
- OD260/280 between 1.8 and 2.0
- OD260/230 between 2.0 and 2.2
- No insoluble material
- No RNA contamination
- No exposure to UV or intercalating fluorescent dyes
- No chelating agents, divalent metal cations, denaturants, or detergents
- No carryover contamination (e.g. polysaccharides) from starting organism
Always assess the quality of the gDNA prior to library construction.

Degraded DNA will adversely affect the quality and fragment distribution of the library.

Lane 2 is an example of degraded gDNA.

Lane 3 is an example of intact gDNA.

1.2% Lonza™ FlashGel® System
Find Shared Protocols for DNA Purification on SampleNet

http://www.smrtcommunity.com/SampleNet
Factors Affecting SMRTbell™ Library Preparation and Sequencing Performance

Quality of Starting Material

Causes
• Insert-size distribution
• Loading bias

Impact on Performance
• Shorter subread lengths
• Preferential loading of shorter templates

Recommendations
• Target larger-insert shear sizes to maximize subread length
• Size selection
• Proper AMPure® sizing
• Proper cleanup of shorter fragments
• Follow recommendations from Covaris or HydroShear® System
• When pooling amplicons, aim for similar sizes

Fragmented-DNA Size Distribution

Short-insert Contaminants

AMPure® Bead Purification

Inaccurate Quantification
Characterization of Fragment Size is Important

- Only ~30 to 40% of sheared DNA is in the desired size range
- Larger fragments do not have any advantage in loading, however, they affect library quantitation
- Small-size fraction within a shear has a higher loading advantage leading to reduced subread length
- Shorter insert sizes contain more individual molecules in a given quantity compared to larger inserts
- Accurate sizing of fragments larger than 17 kb is difficult to achieve on the BioAnalyzer® instrument
- Recommend using pulse-field gel electrophoresis for more accurate sizing
Use PFGE for Accurate Sizing of Fragments >12 Kb

Lane 1: Starting gDNA sample
Lane 2: 20 kb sheared gDNA
Lane 3: 20 kb SMRTbell™ template before size-selection
Lane 4: Size-selected 20 kb SMRTbell library

Size standard: 2.5 kb Molecular Ruler (Bio-Rad)

Pulse Field Gel Electrophoresis (CHEF Mapper®, Bio-Rad® systems) or Pippin Pulse (Sage Science) are both recommended for sizing >12 kb fragments
Factors Affecting SMRTbell™ Library Preparation and Sequencing Performance

Quality of Starting Material

- Fragmented-DNA Size Distribution
- Short-insert Contaminants
- AMPure® Bead Purification
- Inaccurate Quantification

Causes
- Sub-optimal removal of
  - Adapter dimers
  - Short inserts

Impact on Performance
- Higher sequencing yields
- Shorter subread lengths

Recommendations
- Use MagBeads for loading to remove short inserts and adapter dimers
- Thorough AMPure PB bead purification (3x ethanol washes filling the tube to the rim)
- Ensure correct ratio of adapters to inserts
- If adapter dimer continues to be a problem, A/Tailing ligation is an option
Factors Affecting SMRTbell™ Library Preparation and Sequencing Performance

Quality of Starting Material

Fragmented-DNA Size Distribution

Short-insert Contaminants

AMPure® Bead Purification

Inaccurate Quantification

Causes
- Carry-over contaminants from AMPure XP beads

Impact on Performance
- Lower SMRTbell library yields
- Lower sequencing yields
- Shorter subread lengths
- Sample to sample variability

Recommendations
- Use AMPure PB beads
- Perform additional rounds of SMRTbell purification (2X - 3X) using AMPure PB beads
- Use MagBeads for loading
Factors Affecting SMRTbell™ Library Preparation and Sequencing Performance

- Quality of Starting Material
  - Causes:
    - Variability in methods used
    - Contaminants that inhibit the polymerase
    - Random/systematic errors caused by use of degraded reagents or analytical instrumentation hardware issues
  - Impact on Performance:
    - Inaccurate binding reaction conditions resulting in over/under loading
    - Impact on read length, accuracy, and yield
  - Recommendations:
    - Accurate quantitation methods
    - Use both Qubit® and Nanodrop® systems
    - Avoidance/removal of contaminants, RNA, short inserts, etc.

- Fragmented-DNA Size Distribution

- Short-insert Contaminants

- AMPure® Bead Purification

- Inaccurate Quantification
Common Pitfalls to Avoid During AMPure® Bead Purification

• Incorrect concentration of AMPure PB beads used in purification
  – Will retain undesired short inserts
  – Follow cut-off recommendations listed in the procedures

• Beads not thoroughly washed during purification
  – Will result in retention of short inserts and adapter dimers
  – Wash beads thoroughly by adding 70% ethanol to the rim of the tube

• Over-drying of beads
  – Can result in low yield due to difficulties with bead resuspension during sample elution
  – Do not let beads to dry more than 60 seconds (30-60 seconds recommended in the procedure)
Assessing Sample Quality is Key to Success

What is the source of gDNA?
• Understanding the source of gDNA is critical in upfront QC steps (plants, bacterial, tissues, blood, etc.)

What methods were used in DNA isolation?
• Carry-over contaminants can impact sequencing performance (CTAB, phenol/chloroform, others)

What methods were used in DNA quantification?
• Similar readings from Qubit® and Nanodrop® instruments provide higher confidence in the sample
• Use of intercalating dyes is more accurate (Qubit® instrument)

Have you run gels to assess quality of the gDNA?
• Running gels provides a clearer picture of the quality of the sample (degraded vs. RNA contamination)
• Examples of Case Studies on sample prep and sample QC are covered in the following posters:
  • Importance of Sample QC
  • Sample Quality and Contamination
  • http://www.smrtcommunity.com/Share/Protocol?id=a1q70000000H1fwAAC&strRecordTypeName=Protocol
Size Selection with the BluePippin™ System
Recommended for Large-Insert Libraries
Advantages of Size Selection

Long, continuous read lengths are essential for applications such as de novo assembly

• Span long repeats and complex regions
• Improve mappability
• Close gaps or scaffold shorter contigs

Large-insert libraries (compared to short-insert libraries) generate longer subread lengths

Size selection removes the shorter SMRTbell™ templates, allowing the longer SMRTbell templates to load
Advantages of Size Selection

Size selection removes short inserts that preferentially load.
Size Selection using the BluePippin™ System from Sage Science
Long Subread Lengths with the BluePippin™ System

In collaboration with Jeff Rogers, Muthuswamy Raveendran at Baylor College of Medicine
Mouse lemur samples provided by Anne Yoder, Duke Lemur Center

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Where to Find Additional Information
Where to Find Procedures and Other Important Documents
Recommended Template Preparation Documentation

These documents are available through the Customer Portal or SampleNet:

- Guide - Pacific Biosciences Template Preparation and Sequencing
- Procedure & Checklist - 250 bp Amplicon Library Preparation and Sequencing
- Procedure & Checklist - 500 bp Template Preparation and Sequencing
- Procedure & Checklist - 1 kb Template Preparation and Sequencing
- Procedure & Checklist - 2 kb Template Preparation and Sequencing
- Procedure & Checklist - 5 kb Template Preparation and Sequencing
- Procedure & Checklist - 10 kb Template Preparation and Sequencing (with Low-Input DNA)
- Procedure & Checklist - Greater Than 10 kb Template Preparation Using AMPure® PB Beads
- Procedure & Checklist - Large Scale 2 kb Template Preparation and Sequencing
- Procedure & Checklist – Low Input 10 kb Library Preparation and Sequencing
- Procedure & Checklist - 10 kb to 20 kb Template Preparation and Sequencing
- Procedure & Checklist - 20 kb Template Preparation Using BluePippin™ Size-Selection
- User Bulletin - Guidelines for Preparing Size-Selected ~20 kb SMRTbell Templates
- Shared Protocol - Isoform Sequencing (Iso-Seq™) using the Clontech® SMARTer® PCR cDNA Synthesis Kit and Manual Agarose-gel Size Selection (SampleNet)
- Shared Protocol - Isoform Sequencing (Iso-Seq™) using the Clontech® SMARTer® PCR cDNA Synthesis Kit and the BluePippin™ Size Selection System (SampleNet)
- Sample Quality and QC Posters from User Group Meeting (SampleNet)
Welcome to the PacBio® Sample Network!

PacBio SampleNet is the central resource for information and discussion on sample prep and sequencing with the PacBio RS System. In addition to downloading the latest PacBio sample prep documents, you can find out what other users are thinking and doing, share your protocols, ask questions and join in the discussion!

PacBio SMRT® Sample Prep
Download Pacific Biosciences’ latest template prep and sequencing guides, procedures & checklists, and quick reference cards.

Sample Prep Discussion Forums
Have a comment or question about a sample prep? We welcome your input in any area of sample prep, including input DNA purification, amplification, target enrichment, fragmentation, SMRTbell™ template preparation, polymerase binding and storage of polymerase-template complex, library quantification and QC, instrument loading and sequencing, and automation of template prep.

Shared Protocols
Have you modified a PacBio template prep protocol, or developed your own protocol for preparing samples for the RS? Post your version of any steps in the sample prep process, including shearing, amplification, target enrichment, SMRTbell prep, polymerase binding, and automation.

Ideas
Are you (thinking of) sequencing an alternate type of template? Would you like to use the RS for a new or different application? Do you have a suggestion for improving the SampleNet site? Make a suggestion, and see if others have thought of it, too. Then you can vote on the ideas you’d like to see implemented.

http://www.smrtcommunity.com/SampleNet
Summary of Key Points

• Key advantages of SMRTbell™ template library

• SMRTbell library creation workflow

• During Sample Preparation, pay attention to:
  – Quality of starting material
  – Size distribution of fragmented DNA
  – Short-insert contaminants
  – Proper AMPure PB purification steps
  – Quantification

• Additional information available on SampleNet