DECLARATION OF MAZHGAN NOVBAKHTIAN

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I, Mozhgan Novbakhtian, declare as follows:

1. I am an employee of Pacific Biosciences of California, Inc. ("Pacific Biosciences"). I have personal knowledge of the facts set forth below.

2. I have worked at Pacific Biosciences since 2009. I have held the role of Director at Pacific Biosciences since 2016, and my duties during that time have included technical publications, technical communications, and compliance review. Prior to 2016, I held the role of Senior Marketing Manager in charge of technical documentation and training at Pacific Biosciences.

3. Pacific Biosciences publishes various documentation, guides, and white papers, which provide information about Pacific Biosciences's products and their applications to customers and other interested members of the public. As part of my duties at Pacific Biosciences, I review certain of these documents to approve their posting, and I am familiar with the practices of Pacific Biosciences related to the storage and maintenance of these hosted documents.

4. Pacific Biosciences maintains hosted documents as files on its servers, and its internal document control system maintains log data that records the date and time that the hosted files were released for publication. When a hosted file is released for publication, it is made available promptly for download by the public within one or two business days. Hosted documents are identified by a product number indicated by "P/N" or "PN" followed by a string of numbers. The hosted

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files and log data are recorded and maintained by Pacific Biosciences as part of its regularly conducted business activity. I have reviewed entries from this log data, and I discuss below what the log data indicates for each of attached Exhibits A and B, each of which is printed from a file hosted on Pacific Biosciences's servers.

5. Attached as Exhibit A is a copy of the Pacific Biosciences Template Preparation and Sequencing Guide. The guide's product number is 000-710-821-13, with the last two digits indicating that it is the 13th version of the document. This guide is hosted on Pacific Biosciences's servers, and it is available for download at https://www.pacb.com/wp-content/uploads/2015/09/Guide-Pacific-Biosciences-Template-Preparation-and-Sequencing.pdf. I reviewed the Pacific Biosciences Template Preparation and Sequencing Guide prior to its release and submitted it into the internal document control system for publication in 2015. Pacific Biosciences's log data confirms that this version of the Pacific Biosciences Template Preparation and Sequencing Guide was released for publication on January 16, 2015, and the same document that was published in 2015 remains publicly available today. Pacific Biosciences revamped its website in 2015, and this document has been available from the address listed above since that revamping in 2015, but it was hosted at a different address prior to then.

6. Attached as Exhibit B is a copy of a Pacific Biosciences white paper entitled "Detecting DNA Base Modifications Using Single Molecule, Real-Time

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Sequencing." The white paper's product number is 001-640-287-02, with the last two digits indicating that it is the second version of the document. This white paper is hosted on Pacific Biosciences's servers, and it is available for download at https://www.pacb.com/wp-content/uploads/2015/09/ WP_Detecting_DNA_Base_Modifications_Using_SMRT_Sequencing.pdf. I reviewed the white paper prior to its release and submitted it into the internal document control system for publication in 2012. Pacific Biosciences's log data confirms that this version of the white paper was released for publication on June 7, 2012, and the same document that was published in 2012 remains publicly available today. Pacific Biosciences revamped its website in 2015, and this document has been available from the address listed above since that revamping in 2015, but it was hosted at a different address prior to then.

7. I declare that all statements made herein of my knowledge are true, and that all statements made on information and belief are believed to be true, and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code.

Dated: September 28, 2023

By: DocuSigned by: Mozhgan Novbakhtian C174E9D3D47B45F...

Mozhgan Novbakhtian

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EXHIBIT A

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PACIFIC BIOSCIENCES®

Template Preparation and Sequencing Guide

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For Research Use Only. Not for use in diagnostic procedures.

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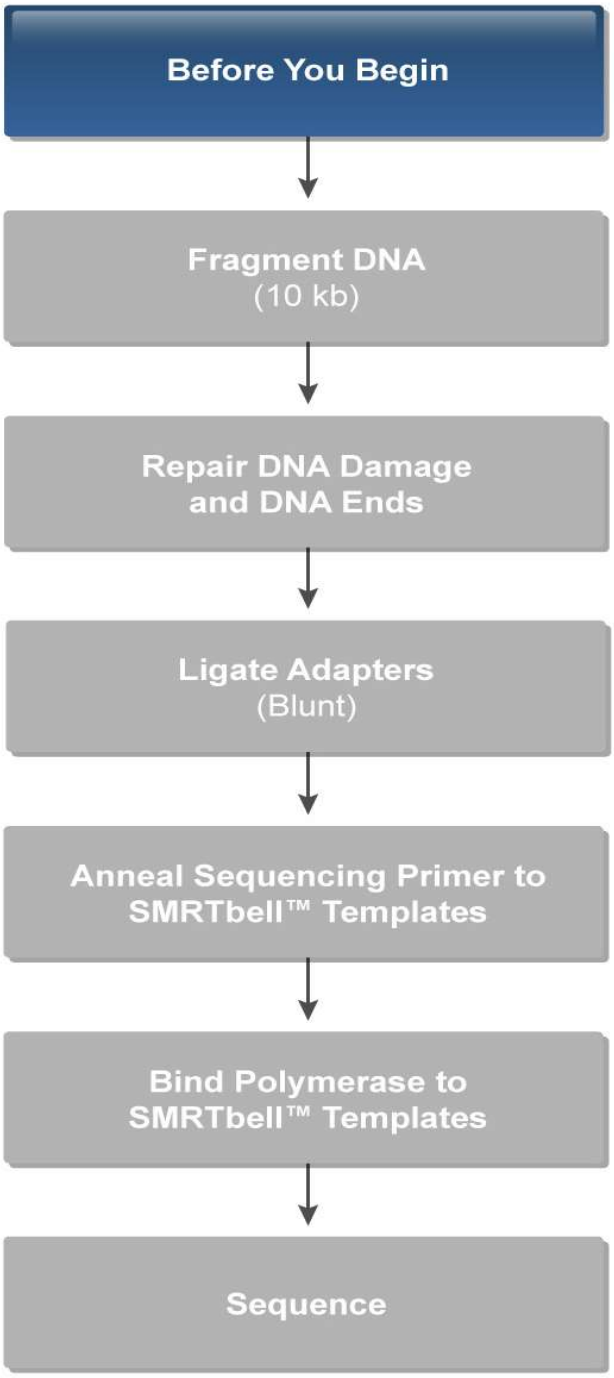
BEFORE YOU BEGIN



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Overview

The PacBio® System includes the Instrument, the accompanying kits needed for DNA template preparation and sequencing on the instrument, and the software used to set up runs and analyze data.

Trained Personnel

Any personnel carrying out the procedures described herein must be trained in proper and safe laboratory practices. Throughout Pacific Biosciences® documentation, the words “you” or “user” refer to and assume properly trained individuals.

Throughout this guide, user attention words and symbols appear that may require a particular level of awareness or action.

User Attention Words and Symbols

Note: Calls attention to an item that may be of interest but is not critical to the process.

Important: Calls attention to an item that is necessary for proper operation of a step.

WARNING! Indicates you should proceed with appropriate caution.

Template Preparation Protocols

This guide describes an optimized procedure for preparing your DNA template for sequencing on the PacBio System. This Blunt-End Ligation protocol, is suitable for all libraries (greater than 250 bp amplicons) and offers a total preparation time of approximately four hours.

SMRTbell™ Templates

A SMRTbell™ template is a double-stranded DNA template capped by hairpin loops at both ends. The SMRTbell template is structurally linear and topologically circular. Some advantages of the SMRTbell structure include the generation of both sense and antisense sequence from a single molecule and the ability to achieve high single-molecule accuracy through circular consensus sequencing approaches.

By avoiding the use of intramolecular ligation, this method of forming circular templates is similarly efficient across a wide range of insert sizes. This allows a single methodology to support all applications.

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In addition to generating circular templates, the hairpin adapters provide two additional benefits. First, they provide a universal primer binding site and initiation sequence. Second, they protect the ends of the DNA fragments. Exonucleases can therefore be used to degrade failed ligation products and templates containing internal nicks, leaving behind only those templates that are suitable for single molecule sequencing.

Template Preparation Process

The SMRTbell template preparation method creates a circularized template for use with multiple sequencing protocols. A single streamlined protocol is used to create different insert size libraries by altering the fragmentation conditions. Amplicons can also be used (in the same size ranges as input DNA).

This template preparation protocol can be used for all modes of on-instrument sequencing. Template preparation can be complete in 3-6 hours with minimal hands on time. Note that the time requirements scale with the number of samples.

The first step in the generation of a SMRTbell library is production of appropriately-sized double-stranded DNA fragments. These fragments can be generated by random shearing of DNA, or by amplification of target regions of interest. The SMRTbell library itself is produced by ligating universal hairpin adapters onto double-stranded DNA fragments.

The hairpin dimers formed during this process are removed at the end of the protocol using a magnetic bead purification step with size-selective conditions. Adapter dimers are also efficiently removed using PacBio's MagBead kit. The final step of the protocol is to remove failed ligation products through the use of exonucleases.

Figure 1 Double-stranded DNA

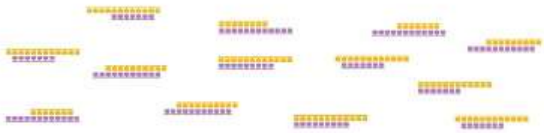

Figure 2 Fragmented DNA

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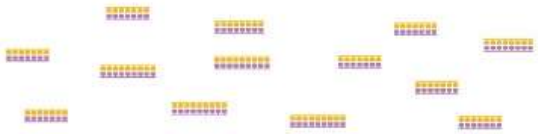

Figure 3 Repaired Ends of Fragmented DNA

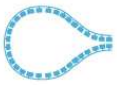

Figure 4 SMRTbell Adapters

5’ – pATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAGAGAT – 3’

Figure 5 Blunt Adapter

5’-AAAAAAAAAAAAAAAAAATTAACGGAGGAGGAGGA-3’

Figure 6 C2 Primer (underlined sequence hybridizes to the hairpin loop and red portion contains 2’-methoxy modifications to increase stability)

After the exonuclease and AMPure PB purification steps, sequencing primer is annealed to the SMRTbell templates, followed by binding of the sequence polymerase to the annealed templates. Finally, the sample is sequenced on the PacBio System.

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DNA Quality

Pacific Biosciences' template preparation process does not use amplification techniques. As a result, input DNA quality will be directly reflected in sequencing results. Any DNA damage (e.g., abasic sites, nicks, interstrand crosslinks) or contaminants (e.g., single-stranded DNA, RNA, proteins, dyes, or salts) present in the input material will impair performance of the system.

Therefore, ensure that your DNA sample:

- Is double-stranded. Single-stranded DNA will not be made into a SMRTbell template in this template preparation process and can interfere with quantitation and polymerase binding
- Has undergone a minimum of freeze-thaw cycles
- Has not been exposed to high temperatures (> 65ºC for 1 hour can cause a detectable decrease in sequence quality)
- Has not been exposed to pH extremes (< 6 or > 9)
- Has an $OD_{260/280}$ ratio of approximately 1.8 to 2.0
- Does not contain insoluble material
- Does not contain RNA
- Has not been exposed to intercalating fluorescent dyes or ultraviolet radiation
- Does not contain chelating agents (e.g., EDTA), divalent metal cations (e.g., Mg^{2+}), denaturants (e.g., guanidinium salts, phenol), or detergents (e.g., SDS, Triton-X100, CTAB)
- Does not contain carryover contamination from the starting organism/tissue (e.g., heme, humic acid, polyphenols)

Assaying the Quality of your Sample

Prior to fragmentation, we recommend one or more of the following quality assessments to ensure that the DNA is pure and of high molecular weight.

Quantitative Assessment

- Spectrophotometry (NanoDrop® Spectrophotometer): For samples of concentration > 10-20ng/µL. We recommend an $OD_{260/280}$ ratio of approximately 1.8 to 2.0
- Fluorimetry: PicoGreen® or Qubit® Fluorimeter

Qualitative Assessment

- Gel electrophoresis and densitometry compared to the appropriate size standards
- Sample DNA should be of high molecular weight and comparable in intensity to a similar mass of control DNA.

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Gel Smear

- A Field-Inversion Gel Electrophoresis system can be used to evaluate the quality of gDNA and determine the sizes of sheared DNA and SMRTbell templates (see figure below). On a field-inversion gel, high-quality gDNA should migrate as a single band of approximately 50 kb (Lane 1). Assaying high-molecular weight DNA is critically important for constructing large insert libraries (e.g. 20 kb libraries).

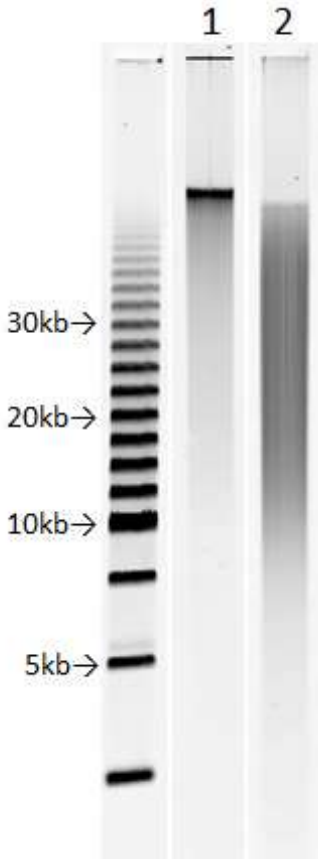

Figure 7 Sizes of Sheared DNA

DNA Input Requirements

The 10 kb procedure described in this guide has been optimized to produce a 10 kb SMRTbell library with an input amount of 5 µg. For all other insert sizes, download the Procedures from the Customer Portal or *www.smrtcommunity.com/SampleNet*.

Note that depending upon the quality of your sample, approximately 20% sample loss is to be expected as a result of the shearing and concentration process. Therefore, be sure to have sufficient amounts of starting DNA in order to have the required amount of starting material for the DNA Damage Repair reaction.

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Scaling Up Reactions

Please note that all reaction volumes are concentration-dependent, and each can be scaled with the input amount of sheared DNA. If preparing larger amounts of DNA, scale all the reaction volumes proportionally. For example, if the input amount is double the amount of DNA, we recommend carrying out the reaction with double of every component and double the total volume.

PacBio® Kits and SMRT® Cells Required

PacBio Kits and SMRT Cell 8Pacs for Sequencing Experiments on the PacBio System

Item	Source
Template Prep Kit	Pacific Biosciences
DNA/Polymerase Binding Kit	Pacific Biosciences
DNA Sequencing Kit	Pacific Biosciences
DNA Internal Control Complex	Pacific Biosciences
MagBead Kit	Pacific Biosciences
MagBead Buffer Kit	Pacific Biosciences
AMPure® PB Kit	Pacific Biosciences
SMRT Cell 8Pac	Pacific Biosciences
SMRT Cell Oil	Pacific Biosciences

Specific Lab Equipment and Related Consumables Required

Required Equipment for Successful Template Preparation

Item	Vendor
Shearing Device: • g-TUBE® microcentrifuge tubes • Covaris® S2 System (1 sample) or Covaris E-Series (96 samples). For Covaris devices: miniTube holders, and clear mini-Tubes will also be needed	Covaris
and/or • Hydroshear® Shearing Device	Hydroshear
Bioanalyzer® Instrument	Agilent Technologies PN 2100
Agilent® 2100 Bioanalyzer DNA 1000, DNA 7500, DNA 12000 DNA Kits, and/or High Sensitivity DNA Kit	Agilent Technologies
NanoDrop® Series (2000, 2000c, 3300, 8000)	Thermo Scientific
or Qubit® Quantitation Platform - Fluorometer (and Quant-iT™ Assay Kits)	Invitrogen PN Q32857

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Item	Vendor
96-well plates, semi-skirted	Bio-Rad HSP9601
Plate Septa	Pacific Biosciences
Tube Septa	Pacific Biosciences
AMPure® PB Beads	Pacific Biosciences

General Lab Supplies

Recommended DNA Isolation Kits

Item	Vendor
Axygen® Sealing Film Roller or Septa Roller (Speedball® Roller)	Axygen PCR-SP-Roller Any Major Laboratory Supplier
Allegra® 6KR Centrifuge	Allegra
Microcentrifuge (1000-16000 RCF)	Eppendorf PN 5415D
Strip-tube Centrifuge	VWR PN 37000-700
Magnetic Particle Concentrator	Invitrogen 123-21D
0.2 mL PCR tubes (can be used for all reactions)	Bio-Rad 0.2 mL flat cap PCR tube (cat# TFI0201) VWR® tube strip with individually attached bubble caps (cat# 82006-634) Molecular BioProducts 0.2 mL PCR tube, flat cap (cat# 3412) Molecular BioProducts 0.2 mL PCR strip tube (cat# 3418)
0.5 mL VWR®/Eppendorf® DNA LoBind tubes (used for all reactions)	Eppendorf PN 80077-236
1.5 mL VWR/Eppendorf DNA LoBind tubes (used for all reactions)	Eppendorf PN 80077-230
Ethanol (absolute)	Sigma-Aldrich
Vortex-Genie® (with plate shaking attachment)	VWR Catalog No. 14005-824
Plate centrifuge	Any Major Laboratory Supplier
Minifuge	Any Major Laboratory Supplier
Aerosol-resistant filter tips	Any Major Laboratory Supplier
Molecular Biology Grade H_20	Any Major Laboratory Supplier

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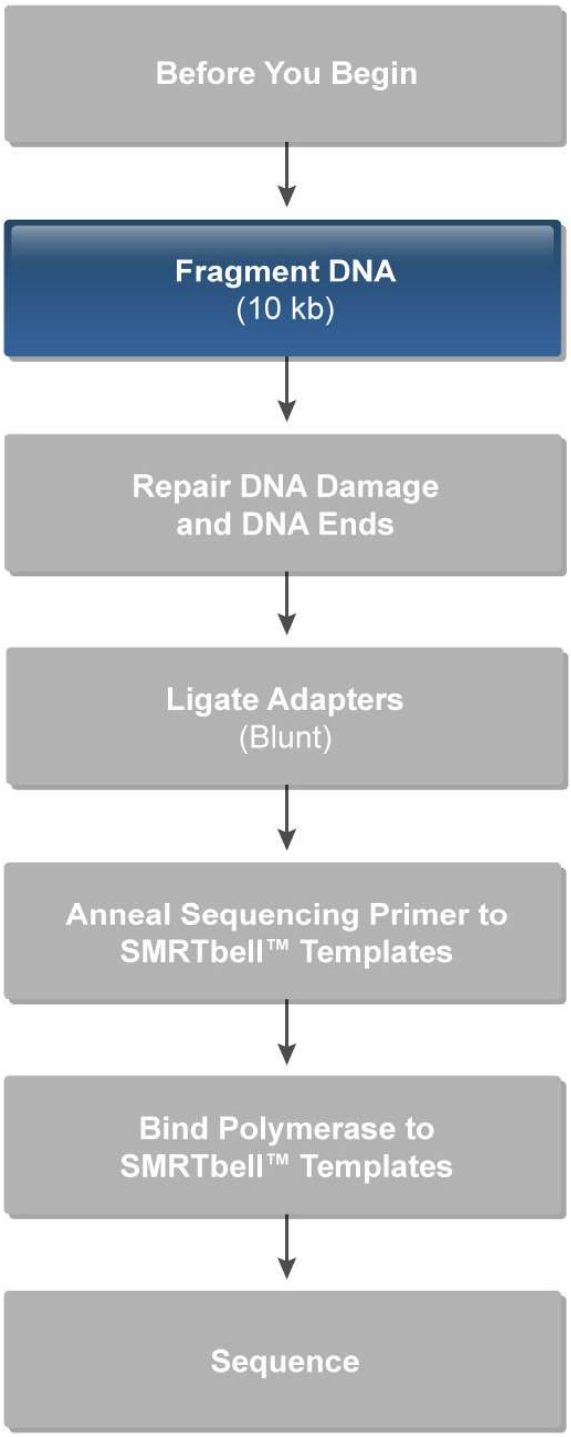
Item	Source
Sample Isolation kits: • Blood and Cell Culture DNA Maxi Kit • Blood and Cell Culture DNA Midi Kit • Qiagen® Large-Construct Kit • QIAquick® PCR Purification Kit	Qiagen • PN 13362 • PN 13343 • PN 12462 • PN 28104

FRAGMENT DNA



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Shearing DNA

Our large insert size protocols have been validated using DNA fragmented with the Covaris® g-TUBE® device. With any system, there will be some variation in the distribution of the sheared fragments. In addition, some DNA will be lost during the shearing process itself.

Depending on the quality of your starting material and the selected method of shearing, you may expect to lose 20% of the starting mass of your DNA sample.

Shearing DNA Using a Covaris® g-TUBE® Shearing Device (> 5 kb Insert Sizes)

The most up-to-date guidance on how to use the g-TUBE device, along with recommended centrifuges and centrifugation speeds, can be found in the g-TUBE device user manual available for download from the Covaris web site.

- After the first centrifuge spin, check the upper chamber for residual liquid. Re-spin if necessary.
- If there is still liquid in the chamber after 2 spins, use a 20 µL pipettor and pipette up and down several times. Then spin the tube down again.

After shearing, determine the approximate size range by loading 30 ng of DNA on to the Bioanalyzer® 12000 chip or by running a low percent (%) agarose gel. Check quantitation on a Nanodrop system. Note that fragments sheared using the g-TUBE device are greatly dependent on gDNA quality and size and may range from 6 kb to 20 kb.

(Optional) Shearing DNA Using a Hydroshear® Shearing Device (10 kb Insert Sizes)

A Hydroshear® Shearing Device can also be used to shear DNA samples. However, because Hydrodynamic shearing performance can vary with each shearing assembly, we recommend optimizing the shearing whenever a new shearing assembly is used.

The sheared DNA can be stored for up to 24 hours at 4ºC or 2 months at -20ºC.

Sheared 10 kb DNA Distribution on a Bioanalyzer® Instrument

The graph below shows an example of fragment size distribution.

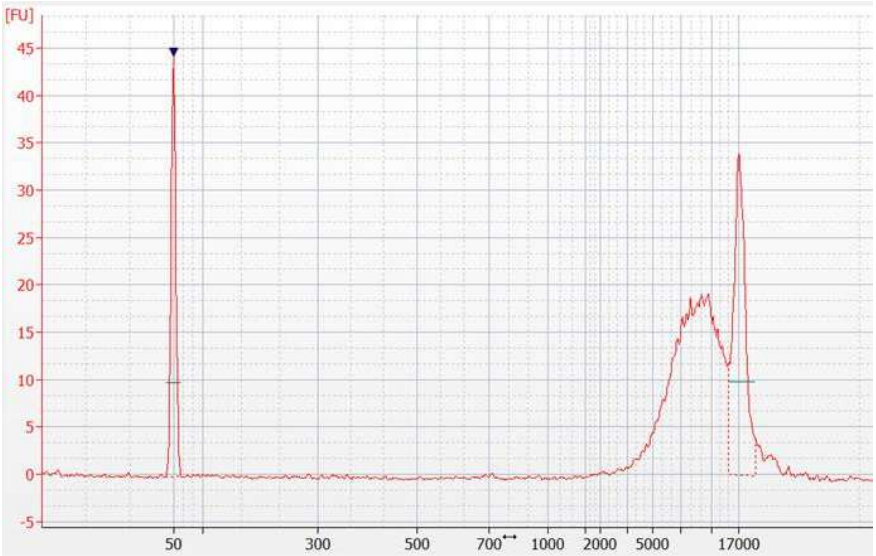

Figure 8 Fragment Size Distribution for 10 kb

AMPure® PB Purification Steps Throughout this Guide

For all **10 kb** purification and concentration steps, you must use **0.45X** AMPure PB beads. For your convenience, the guide details these requirements in each section.

Concentrate DNA

Perform the following steps, at room temperature, to concentrate your DNA sample. Note that you must use low-adhesion (LoBind) microcentrifuge tubes during the entire template preparation process.

1. Add **0.45X** volume of AMPure PB magnetic beads to the sheared DNA.

   Before using, mix the bead reagent well until the solution appears homogenous. Pipette the reagent slowly since the bead mixture is viscous and precise volumes are critical to the purification process.

   Consistent and efficient recovery of your sample is critical to successful SMRTbell library preparation. If using this protocol for the first time, we strongly recommend that you process a control sample first. Using the DNA shearing methods and subsequent AMPure PB bead purification steps described below, you should recover approximately **50% - 80%** of your input DNA (by mass). Typical yields from pre-purified DNA (where smaller fragments are already eliminated) are between 80-100%.

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2. Mix the bead/DNA solution thoroughly. Mix the beads with the DNA by pipetting up and down or inverting the tube until the solution is homogenous.

3. Quickly spin down the tube (for 1 second) to collect the beads.

4. Allow the DNA to bind to beads by shaking in a VWR® vortex mixer at 2000 rpm for 10 minutes at room temperature. Note that the bead/DNA mixing is critical to yield. After vortexing, the bead/DNA mixture should appear homogenous.

   We recommend using a VWR vortex mixer with a foam microtube attachment (see Overview section for part number). If using other instrumentation, ensure that the mixing is equally vigorous. Failure to thoroughly mix the DNA with the bead reagent will result in inefficient DNA binding and reduced sample recoveries.

5. Spin down the tube (for 1 second) to collect beads.

6. Place the tube in a magnetic bead rack until the beads collect to the side of the tube and the solution appears clear. The actual time required to collect the beads to the side depends on the volume of beads added.

7. With the tube still on the magnetic bead rack, slowly pipette off cleared supernatant and save in another tube. Avoid disturbing the bead pellet.

   If the DNA is not recovered at the end of this procedure, you can add equal volumes of AMPure PB beads to the saved supernatant and repeat the AMPure PB bead purification steps to recover the DNA.

8. Wash beads with freshly prepared 70% ethanol.

   Note that 70% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results. Also, 70% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days.

   a. Do not remove the tube from the magnetic bead rack.
   b. Use a sufficient volume of 70% ethanol to fill the tube (1.5 mL for 1.5 mL tube or 2 mL for 2 mL tube). Slowly dispense the 70% ethanol against the side of the tube opposite the beads. Let the tube sit for 30 seconds.
   c. Do not disturb the bead pellet.
   d. After 30 seconds, pipette and discard the 70% ethanol.

9. Repeat step 8 above.

10. Remove residual 70% ethanol and dry the bead pellet.
    a. Remove tube from magnetic bead rack and spin to pellet beads. Both the beads and any residual 70% ethanol will be at the bottom of the tube.
    b. Place the tube back on magnetic bead rack.
    c. Pipette off any remaining 70% ethanol.

11. Check for any remaining droplets in the tube. If droplets are present, repeat step 10.

12. Remove the tube from the magnetic bead rack and allow beads to air-dry (with the tube caps open) for 30 to 60 seconds.

13. Calculate appropriate volume of Elution Buffer.
    a. For 10 kb libraries:
    __ ng X **0.5** / (__ng/µL) = __µL of Elution Buffer needed

    The minimum DNA concentration required to proceed to the next step (End-Repair) is **140 ng/µL** with preferred mass of at least **5 µg**.

14. Add Pacific Biosciences® Elution Buffer volume (calculated in step 13 above) to your beads.
    a. Thoroughly resuspend beads by vortexing for 1 minute at 2000 rpm. If the beads appear over-dried or cracked, let the Elution Buffer sit on the beads for 2 to 3 minutes then vortex again.
    b. Spin the tube down to pellet beads, then place the tube back on the magnetic bead rack.
    c. Perform concentration measurements. Verify your DNA concentration using a Nanodrop or Qubit® quantitation platform. If the DNA concentration is estimated to be equal to or below **12 ng/µL**, a Qubit system reading is required. When performing a Qubit system reading, ensure that your sample is within the range of the Qubit kit you are using. For proper concentration calculations, incorporate the dilution factor (used when diluting your sample) to be within range of the Qubit kit and the dilution factor when diluting your sample with the working solution. The latter part of this dilution factor can be calculated automatically by the Qubit system.
    d. Discard the beads.

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15. Perform qualitative and quantitative analysis using a Bioanalyzer instrument. Note that the Bioanalyzer instrument has different kits in its offering and the appropriate kit, based on insert size, should be used.

    Dilute the samples appropriately before loading on the Bioanalyzer chip so that the DNA concentration loaded falls well within the detectable minimum and maximum range of the assay. Refer to Agilent Technologies' guides for specific information on the range of the specific kit you might be using.

    Note that typical yield, at this point of the process (i.e. post-shearing and after one AMPure PB bead purification step), is approximately **50%- 80%**.

16. The sheared DNA can be stored for up to 24 hours at 4ºC or at -20ºC for longer duration.

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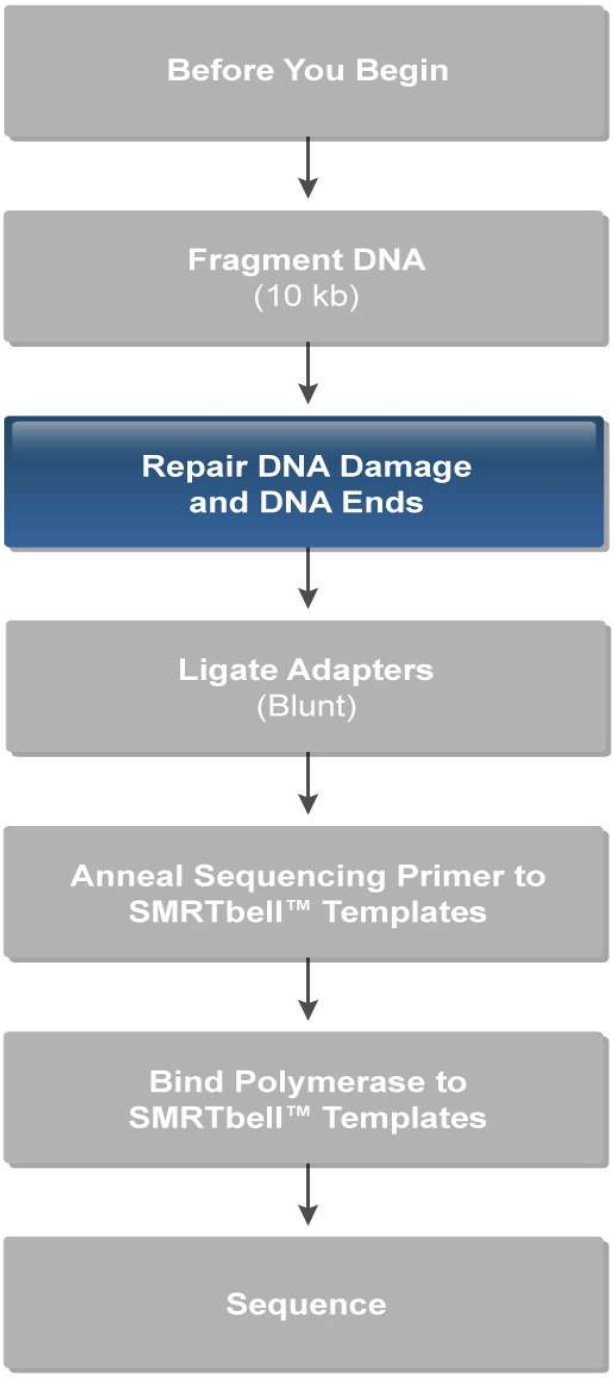
PACIFIC BIOSCIENCES®

REPAIR DNA DAMAGE AND DNA ENDS



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Repair DNA Damage

For sheared DNA libraries and PCR products greater than 2 kb, any DNA damage (generated during DNA extraction and PCR amplifications) must be repaired using the DNA Damage Repair reagents provided by Pacific Biosciences. Common types of damage may include abasic sites, cytosine deamination, and oxidation. Note that DNA damage repair is optional for insert sizes less than 2 kb.

1. Thaw the kit components on ice.
2. In a LoBind microcentrifuge tube, add the following reagents:

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.
Sheared DNA	-	-	5 µg	-
DNA Damage Repair Buffer		10X	5 µL	1X
NAD+		100 X	0.5 µL	1X
ATP Hi		10 mM	5.0 µL	1mM
dNTP		10 mM	0.5 µL	0.1 mM for 10 kb
DNA Damage Repair Mix			2.0 µL	
H_2O		-	to 50 µL	-
Total Volume			50.0 µL	-

If your input amount deviates from the inputs shown in this table, adjust all reagent volumes proportionately. Note that the DNA final concentration cannot exceed 100 ng/µL.

3. Mix the reaction well by pipetting or flicking the tube.
4. Spin down tube contents with a quick spin in a microfuge.
5. Incubate at 37ºC for 20 minutes, then return reaction to 4ºC until ready for purification.

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Repair Ends

The PacBio Template Prep Kit is used to repair the ends of fragmented DNA (or non-phosphorylated 5' ends of PCR products) in preparation for ligation with hairpin adapters. Note that the tube caps are color-coded for your convenience.

Use the following table to prepare your reaction then purify the DNA.

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.
DNA (Damage Repaired)	–		50 µL	–
End Repair Mix		20 X	2.5 µL	1X
Total Volume			52.5 µL	–

1. Mix the reaction well by pipetting or flicking the tube.
2. Spin down contents of tube with a quick spin in a microfuge.
3. Incubate at 25ºC for 5 minutes, return the reaction to 4ºC.

Purify the DNA

Perform the following steps at room temperature. Note that you must use low-adhesion (LoBind) microcentrifuge tubes during the entire template preparation process.

1. Add **0.45X** volume of AMPure PB beads to the End-Repair reaction.

   Before using, mix the bead reagent well until the solution appears homogenous. Pipette the reagent slowly since the bead mixture is viscous and precise volumes are critical to the purification process.
2. Mix the bead/DNA solution thoroughly. Mix the beads with the End-Repair reaction by pipetting up and down or inverting the tube until the solution is homogenous.
3. Quickly spin down the tube (for 1 second) to collect the beads.
4. Allow the DNA to bind to beads by shaking in a VWR® vortex mixer at 2000 rpm for 10 minutes at room temperature. Note that the bead/DNA mixing is critical to yield. After vortexing, the bead/DNA mixture should appear homogenous.

   We recommend using a VWR vortex mixer with a foam microtube attachment (see Overview section with part number). If using other instrumentation, ensure that the mixing is equally vigorous. Failure to thoroughly mix the DNA with the bead reagent will result in inefficient DNA binding and reduced sample recoveries.

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5. Spin down the tube (1 second) to collect beads.
6. Place the tube in a magnetic bead rack until the beads collect to the side of the tube and the solution appears clear. The actual time required to collect the beads to the side depends on the volume of beads added. Slowly pipette off cleared supernatant and discard. Avoid disturbing the bead pellet.

7. With the tube still on the magnetic bead rack, slowly pipette off cleared supernatant and save in another tube. Avoid disturbing the bead pellet.

   If the DNA is not recovered at the end of this Procedure, you can add equal volumes of AMPure PB beads to the saved supernatant and repeat the AMPure PB bead purification steps to recover the DNA.
8. Wash beads with freshly prepared 70% ethanol.

   Note that 70% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results. Also, 70% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days.
   a. Do not remove the tube from the magnetic bead rack.
   b. Use a sufficient volume of 70% ethanol to fill the tube (1.5 mL for 1.5 mL tube or 2 mL for 2 mL tube). Slowly dispense the 70% ethanol against the side of the tube opposite the beads. Let the tube sit for 30 seconds.
   c. Do not disturb the bead pellet.
   d. After 30 seconds, pipette and discard the 70% ethanol.
9. Repeat step 8 above.
10. Remove residual 70% ethanol and dry the bead pellet.
    a. Remove tube from magnetic bead rack and spin to pellet beads. Both the beads and any residual 70% ethanol will be at the bottom of the tube.
    b. Place the tube back on magnetic bead rack.
    c. Pipette off any remaining 70% ethanol.
11. Check for any remaining droplets in the tube. If droplets are present, repeat step 10.

12. Remove the tube from the magnetic bead rack and allow beads to air-dry (with the tube caps open) for 30 to 60 seconds.

13. Elute the DNA off the beads.
    a. Elute the DNA in **30 µL** Elution Buffer.

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b. Thoroughly resuspend beads by vortexing for 1 minute at 2000 rpm. If the beads appear over-dried or cracked, let the Elution Buffer sit on the beads for 2 to 3 minutes then vortex again.

c. Spin the tube down to pellet beads, then place the tube back on the magnetic bead rack

d. Discard beads.

14. Optional: Verify your DNA amount and concentration using Qubit® Nanodrop® or Qubit® quantitation platform, as appropriate.

15. Perform qualitative and quantitative analysis using a Bioanalyzer instrument. Note that the Bioanalyzer instrument has different kits in its offering and the appropriate kit, based on insert size, should be used.

    Dilute the samples appropriately before loading on the Bioanalyzer chip so that the DNA concentration loaded falls well within the detectable minimum and maximum range of the assay. Refer to Agilent's users' guides for specific information on the range of the specific kit you might be using.

    Note that typical yield at this point of the process (following End-Repair and one AMPure PB bead purification step) is approximately between **80-100%** of the total starting material.

16. The end repaired DNA can be stored overnight at 4ºC or at -20ºC for longer duration.

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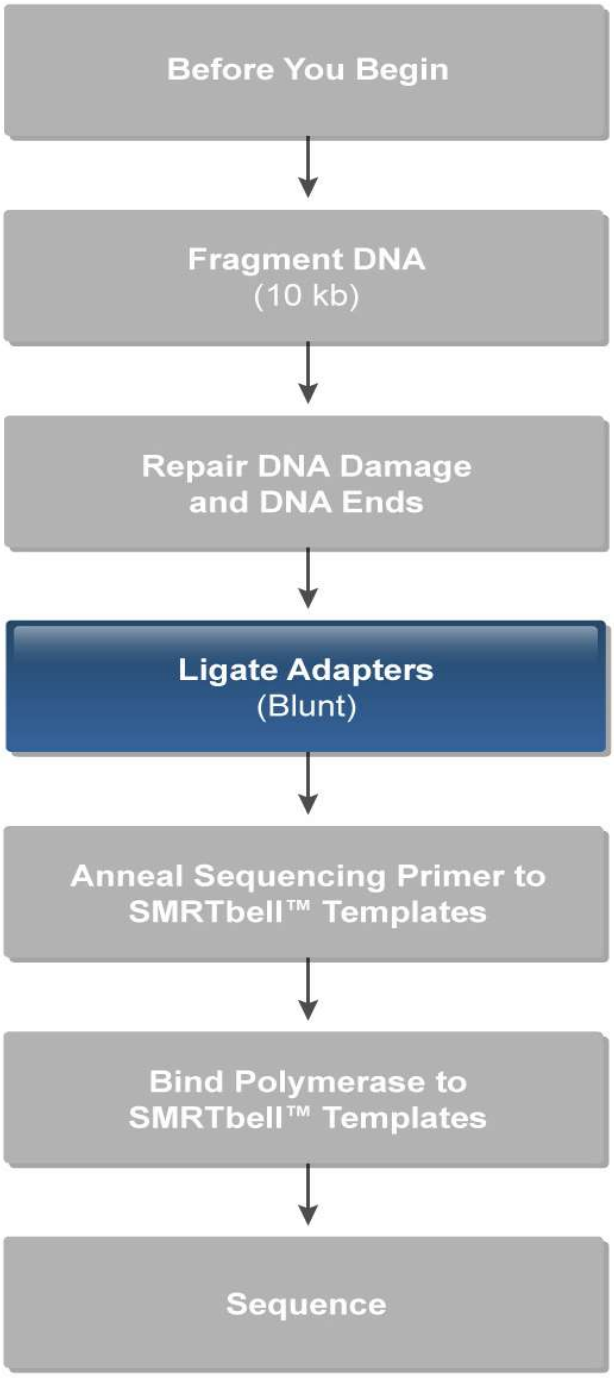
LIGATE ADAPTERS



Blunt-End Ligation of SMRTbell™ Templates

During this step, blunt hairpins are ligated to repaired fragment ends.

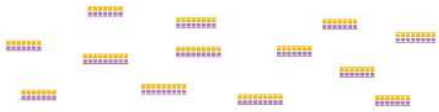


Figure 9 Repaired Fragment Ends

Figure 10 Blunt Hairpin Adapters and Insert DNA Ready for Ligation

To ligate the hairpins (SMRTbell™ templates) to the DNA fragments, you will need BLUNT hairpin adapters. These are shipped as 20 µM oligonucleotide stock and are pre-annealed. This reaction can be scaled for the number of library samples being prepared.

Blunt-End Ligation Reaction

In a LoBind microcentrifuge tube (on ice), add the following reagents in the order shown (note that you can add water to achieve the desired DNA volume). If preparing a Master Mix, ensure that the adapter is NOT mixed with the ligase prior to introduction of the inserts. Add the adapter to the well with the DNA. All other components, including the ligase, should be added to the Master Mix.

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.
DNA (End Repaired)	-		29µL to 30 µL	
Blunt Adapter (20uM)	●	20 µM	1.0 µL	0.5 µM
Mix before proceeding				
Template Prep Buffer	○	10X	4.0 µL	1X
ATP Lo	●	1 mM	2.0 µL	0.05 mM
Mix before proceeding				
Ligase[a]	●	30 U/µL	1.0 µL	0.75 U/µL
H_2O			to 40.0 µL	
Total Volume	-	-	40 µL	-

a. The Ligase Buffer should remain closed and on ice when not frozen.

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If your insert size or input amount deviates from this table, you can calculate the amount of annealed blunt adapter to be added to the reaction using the following equation. Be sure to keep a 32.5 fold excess of hairpin adapters and adjust the final volume such that the hairpin adapter concentration does not exceed 1 µM.

Total µg of DNA insert *X* * 10^6 * 1/650 X 1/Insert size in bp = ***X*** picomoles of DNA available for ligation

X picomoles of DNA available for ligation X 32.5 = Total excess annealed adapters (***Y***)

Y/20 (20 µM annealed adaptor stock) = ***Z*** total µL of annealed adaptor to be added to the reaction

If scaling of the reaction volume is necessary, keep the buffer and enzyme concentrations proportional to the recommended amounts shown above.

1. Mix the reaction well by pipetting or flicking the tube.
2. Spin down the tube contents with a quick spin in a microfuge.
3. Incubate at 25ºC for 15 minutes. At this point, the ligation can be extended up to 24 hours or cooled to 4ºC (for storage of up to 24 hours).
4. Incubate at 65ºC for 10 minutes to inactivate the ligase, then return the reaction to 4ºC. You must proceed with adding exonuclease after this step.

Add Exonuclease and Incubate

Add exonuclease to remove failed ligation products.

Reagent	Tube Cap Color	Stock Conc.	Volume
Ligated DNA			40 µL
ExoIII	●	100 U/µL	1.0 µL
ExoVII	●	10 U/µL	1.0 µL
Total Volume	-	-	42 µL

1. Spin down the tube contents with a quick spin in a microfuge.
2. Incubate at 37º C for 1 hour, then return the reaction to 4ºC. You must proceed with purification after this step.

Purify SMRTbell™ Templates

In this purification process, there are three (3) distinct and consecutive AMPure PB bead purification steps. The first two (2) steps are performed using **0.45X** volumes of AMPure PB beads and the final step can be performed using either **0.40X** or **0.45X** volumes of AMPure PB beads. Perform all purification steps at room temperature to adequately remove enzymes (exonucleases, ligases, etc.) and ligation products smaller than 0.4 kb (e.g., adapter dimers).

AMPure PB Size-Selection and Purification Step #1:

1. Add **0.45X** volumes of AMPure PB beads to the exonuclease-treated ligation reaction.

   Before using, mix the bead reagent well until the solution appears homogenous. Pipette the reagent slowly (since the bead mixture is viscous and precise volumes are critical to the purification process).
2. Mix the bead/DNA solution thoroughly. Mix the beads with the ligation reaction by pipetting up and down or inverting the tube until the solution is homogenous.
3. Quickly spin down the tube (for 1 second) to collect the beads.
4. Allow the DNA to bind to beads by shaking in a VWR® vortex mixer at 2000 rpm for 10 minutes at room temperature. Note that the bead/DNA mixing is critical to yield. After vortexing, the bead/DNA mixture should appear homogenous.

   We recommend using a VWR vortex mixer with a foam microtube attachment (see Overview section with Catalog part number). If using other instrumentation, ensure that the mixing is equally vigorous. Failure to thoroughly mix the DNA with the bead reagent will result in inefficient DNA binding and reduced sample recoveries.
5. Spin down the tube (for 1 second) to collect beads.
6. Place the tube in a magnetic bead rack until the beads collect to the side of the tube and the solution appears clear. The actual time required to collect the beads to the side depends on the volume of beads added.
7. Slowly pipette off cleared supernatant and save (in another tube). Avoid disturbing the bead pellet.
8. Wash beads with freshly prepared 70% ethanol.

   Note that 70% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results. Also, 70% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days.

   a. Do not remove the tube from the magnetic bead rack.

   b. Use a sufficient volume of 70% ethanol to fill the tube (1.5 mL for 1.5 mL tube or 2 mL for 2 mL tube). Slowly dispense the 70% ethanol against the side of the tube opposite the beads. Let the tube sit for 30 seconds.
   c. Do not disturb the bead pellet.
   d. After 30 seconds, pipette and discard the 70% ethanol.

9. Repeat step 8 above.
10. Remove residual 70% ethanol and dry the bead pellet.
    a. Remove tube from magnetic bead rack and spin to pellet beads. Both the beads and any residual 70% ethanol will be at the bottom of the tube.
    b. Place the tube back on magnetic bead rack.
11. Check for any remaining droplets in the tube. If droplets are present, repeat step 10.
12. Remove the tube from the magnetic bead rack and allow beads to air-dry (with tube caps open) for 30 to 60 seconds.
13. Elute the DNA off the beads in **50 µL** Elution Buffer. Mix until homogenous, then vortex for 1 minute at 2000 rpm.
    a. Thoroughly resuspend beads by vortexing for 1 minute at 2000 rpm. If the beads appear over-dried or cracked, let the Elution Buffer sit on the beads for 2 to 3 minutes then vortex again.
    b. Spin the tube down to pellet beads, then place the tube back on the magnetic bead rack
    c. Discard beads.
14. The eluted DNA, in **50 µL** of Elution Buffer, should be taken into the second **0.45X** AMPure PB bead purification step.

AMPure PB Size-Selection and Purification Step #2:

1. Add **22.5 µL** (**0.45X** volume) of AMPure PB beads to the **50 µL** of eluted DNA from the first AMPure PB bead purification step above.

   Before using, mix the bead reagent well until the solution appears homogenous. Then pipette the reagent slowly (since the bead mixture is viscous and precise volumes are critical to the purification process).
2. Mix the bead/DNA solution thoroughly. Mix the beads with the ligation reaction by pipetting up and down or inverting the tube until the solution is homogenous.
3. Quickly spin down the tube (for 1 second) to collect the beads.

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4. Allow the DNA to bind to beads by shaking in a VWR® vortex mixer at 2000 rpm for 10 minutes at room temperature. Note that the bead/DNA mixing is critical to yield. After vortexing, the bead/DNA mixture should appear homogenous.

   We recommend using a VWR vortex mixer with a foam microtube attachment (see Overview section with Catalog part number). If using other instrumentation, ensure that the mixing is equally vigorous. Failure to thoroughly mix the DNA with the bead reagent will result in inefficient DNA binding and reduced sample recoveries.
5. Spin down the tube (for 1 second) to collect beads.
6. Place the tube in a magnetic bead rack until the beads collect to the side of the tube and the solution appears clear. The actual time required to collect the beads to the side depends on the volume of beads added.
7. Slowly pipette off cleared supernatant and save (in another tube). Avoid disturbing the bead pellet.
8. Wash beads with freshly prepared 70% ethanol.

   Note that 70% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results. Also, 70% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days.
   - Do not remove the tube from the magnetic bead rack.
   - Use a sufficient volume of 70% ethanol to fill the tube (1.5 mL for 1.5 mL tube or 2 mL for 2 mL tube). Slowly dispense the 70% ethanol against the side of the tube opposite the beads. Let the tube sit for 30 seconds.
   - Do not disturb the bead pellet.
   - After 30 seconds, pipette and discard the 70% ethanol.
9. Repeat step 8 above.
10. Remove residual 70% ethanol and dry the bead pellet.
    a. Remove tube from magnetic bead rack and spin to pellet beads. Both the beads and any residual 70% ethanol will be at the bottom of the tube.
    b. Place the tube back on magnetic bead rack.
    c. Pipette off any remaining 70% ethanol.
11. Check for any remaining droplets in the tube. If droplets are present, repeat step 10.
12. Remove the tube from the magnetic bead rack and allow beads to air-dry (with tube caps open) for 30 to 60 seconds.

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13. Elute the DNA off the beads in **100 µL** of Elution Buffer. Vortex for 1 minute at 2000 rpm.
14. Verify your DNA amount and concentration with either a Nanodrop or Qubit quantitation platform reading. If recovery is sufficient to allow for an additional 25% loss in the final AMPure PB purification step (more if the library contains a high number of small fragments), and it is desirable to increase the stringency of size selection, consider using **0.40X** volumes of AMPure PB beads. This will remove most fragments <1.5 kb which will dominate loading, if present. Otherwise, proceed to the third **0.45X** volumes of AMPure PB bead purification step.

    Note that yield from **0.40X** is typically ~ 10% lower than **0.45X** volumes of AMPure PB bead purification.

AMPure PB Size-Selection and Purification Step #3:

1. Add **45 µL** (**0.45X** volume) or **40 µL** (**0.40X** volume) of AMPure PB beads to the **100 µL** of eluted DNA. Note that for **0.40X** volume, it is critical to accurately pipet the desired volume of AMPure PB bead solution; there is a steep drop-off in recovery for concentrations <**0.40X**.
2. Mix the bead/DNA solution thoroughly.
3. Quickly spin down the tube (for 1 second) to collect the beads. Do not pellet beads.
4. Allow the DNA to bind to beads by shaking in a VWR vortex mixer at 2000 rpm for 10 minutes at room temperature.
5. Spin down the tube (for 1 second) to collect beads.
6. Place the tube in a magnetic bead rack to collect the beads to the side of the tube.
7. Slowly pipette off cleared supernatant and save (in another tube). Avoid disturbing the bead pellet.

   Note: It is especially important to save the supernatant for **0.40X** volumes of AMPure PB purification steps, in case of low recovery
8. Wash beads with freshly prepared 70% ethanol.
9. Repeat step 8 above.
10. Remove residual 70% ethanol and dry the bead pellet.
    - Remove tube from magnetic bead rack and spin to pellet beads. Both the beads and any residual 70% ethanol will be at the bottom of the tube.
    - Place the tube back on magnetic bead rack.
    - Pipette off any remaining 70% ethanol.

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11. Check for any remaining droplets in the tube. If droplets are present, repeat step 10.
12. Remove the tube from the magnetic bead rack and allow beads to air-dry (with tube caps open) for 30 to 60 seconds.
13. Elute the DNA off the beads in **10 µL** of Elution Buffer. Vortex for 1 minute at 2000 rpm.
14. Verify your DNA amount and concentration with either a Nanodrop or Qubit quantitation platform reading. For general library yield expect 20% total yield from the Damage Repair input. If your yield concentration is below 12ng/µL, use the Qubit system for quantitation.

    To estimate your final concentration: (____ ng of DNA going into Damage Repair X 0.2) / ___ of Elution Buffer =____ng/µL
15. Perform qualitative and quantitative analysis using a Bioanalyzer instrument. Note that typical DNA yield, at this point of the process (at the end of library preparation) is between approximately **5-20%** of the total starting DNA amount.

SMRTbell™ Library Quality Assessment

Successful sequencing of a SMRTbell library depends on an understanding of template molarity. This requires accurate quantitation and sizing of the final library.

Size distribution can be measured by running 30 ng of the sample using an Agilent Bioanalyzer 12000 chip. Typical library yields will require at least a 1:10 dilution prior to analysis on the Bioanalyzer instrument to ensure reliable quantitation.

The SMRTbell library should be quantitated via fluorescence either in single sample (Qubit system) or plate-based (Quant-iT system) formats. Follow all manufacturer's instructions and ensure that a double-stranded DNA standard is used for the quantitation.

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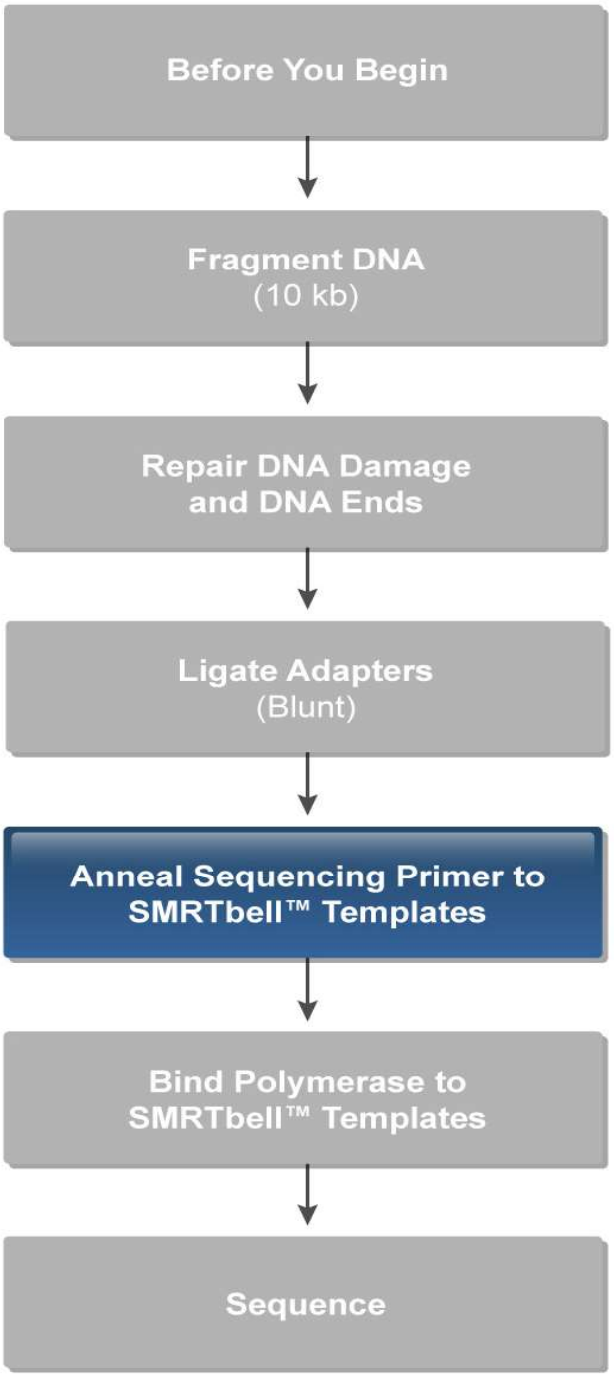
ANNEAL SMRTBELL™ TEMPLATES AND PRIMER



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Primer Annealing and Polymerase Binding

Prior to sequencing, primer must be annealed to the SMRTbell template, and then DNA polymerase is bound to the annealed templates.

Binding Calculator

A Binding Calculator is provided to assist with setting up the annealing and binding reactions and setting up the sample plate for sequencing. The Calculator can be used in three different modes:

- **Volume to use**: In this mode, the Calculator uses the entire sample specified to run the maximum number of SMRT Cells possible.
- **# of SMRT Cells**: In this mode, the user specifies how many SMRT Cells to prepare, and the Calculator determines the amount of sample necessary.
- **Loading Titration**: This mode allows the user to set up a loading titration of the bound complex to optimize data yield per SMRT Cell. The Calculator suggests four concentrations around the recommended complex concentration on the sample plate, however, the user can customize the titration range for their sample.

After selecting the appropriate mode, enter the following information (note that tool-tips can be found by placing the cursor on each attribute):

- **Protocol**: Select the loading method (MagBead or Diffusion).
- **Binding Kit**: Select the appropriate sequencing polymerase.
- **Preparation Protocol:** Select the library scale used to prepare the sample.
- **Long Term Storage**: Enables options for complexes to be stored (long-term) at -20ºC.
- **DNA Control Complex**: Allows for DNA Internal Control use.
- **Complex Reuse**: For diffusion loading only. The instrument re-uses the sample for a total of up to three uses.
- **Standard Concentration**: Selecting “No” allows sample calculations with volumes and concentrations that do not meet standard requirements.

Once the sample details are selected, additional parameters may be modified in the Calculator’s “Custom Parameters” section:

- **Concentration on Plate**: Use the default recommendation as a starting point. Note that this can be modified to maximize yield per SMRT Cell
- **DNA Control Complex Ratio to Template**: Use the default recommendation. This is the percentage of DNA Internal Control to add to the sample.
- **Polymerase:Template Ratio**: Use the default recommendation. For applications which may require a ratio other than the default values, contact your FAS.
- **Primer:Template Ratio**: Use the default recommendation.

To Access the Calculator

You can access the Calculator by downloading it from the web at *http://calc.pacb.com* or *http://calc.PacificBiosciences.com*. Be sure to always check for updates.

The Calculator is best viewed using Firefox or Chrome browsers.

Primer Annealing

Overview

In this step, sequencing primer is annealed to both ends of the SMRT-bell template. Primer annealing requirements vary depending on the library insert size and loading method (e.g. Diffusion or MagBead). For example, SMRTbell templates with an average insert size of 500 bp for diffusion loading require less primer and polymerase compared to a 10 kb library using the MagBead loading method.

To achieve efficient loading of SMRTbell templates and to maximize yield per SMRT Cell, PacBio has optimized the ratio of primer to template specific to a library insert size range. Although the ratio can be customized, the recommendation is to use the default settings. See the *Pacific Biosciences Binding Calculator Parameters Quick Reference Card* for a summary of the recommended primer to template ratio.

Reaction Conditions

The Primer (5 µM) and 10X Primer Buffer are included in the Template Prep Kit. Before adding the primer to the SMRTbell template, the primer must go through a melting step at 80ºC. This avoids exposing the sample to heat. The template and primer mix can then be incubated at 20ºC for 30 minutes and cooled to 4ºC indefinitely.

Primer Sequence

The primer is tailed with a poly-A sequence. The poly-A tail is required for MagBead loading but is not required (and does not impact) diffusion loading.

5’-AAAAAAAAAAAAAAAAAATTAACGGAGGAGGAGGA-3’

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SMRTbell molarity calculation

In calculating SMRTbell molarity, we use the following formula:

$$\frac{\text{Insert concentration (ng/µl)}}{\text{Mean insert size (bp)}} * \frac{1{,}000{,}000}{650} = \textbf{Insert concentration in (nM) (or fmol/µL)}$$

The detailed calculation is:

$$\frac{\text{Insert Concentration (ng/µL)}}{\text{Mean insert size (bp)}} * \frac{\text{1 mol basepairs}}{\text{650 g}} * \frac{\text{1 g}}{10^9\text{ ng}} * \frac{10^{15}\text{ fmol}}{\text{1 mol base pairs}} = \frac{\textbf{fmol}}{\textbf{µL}} = \textbf{nM}$$

Note that the Binding Calculator can also be used to convert ng/µl to nM and nM to ng/µl.

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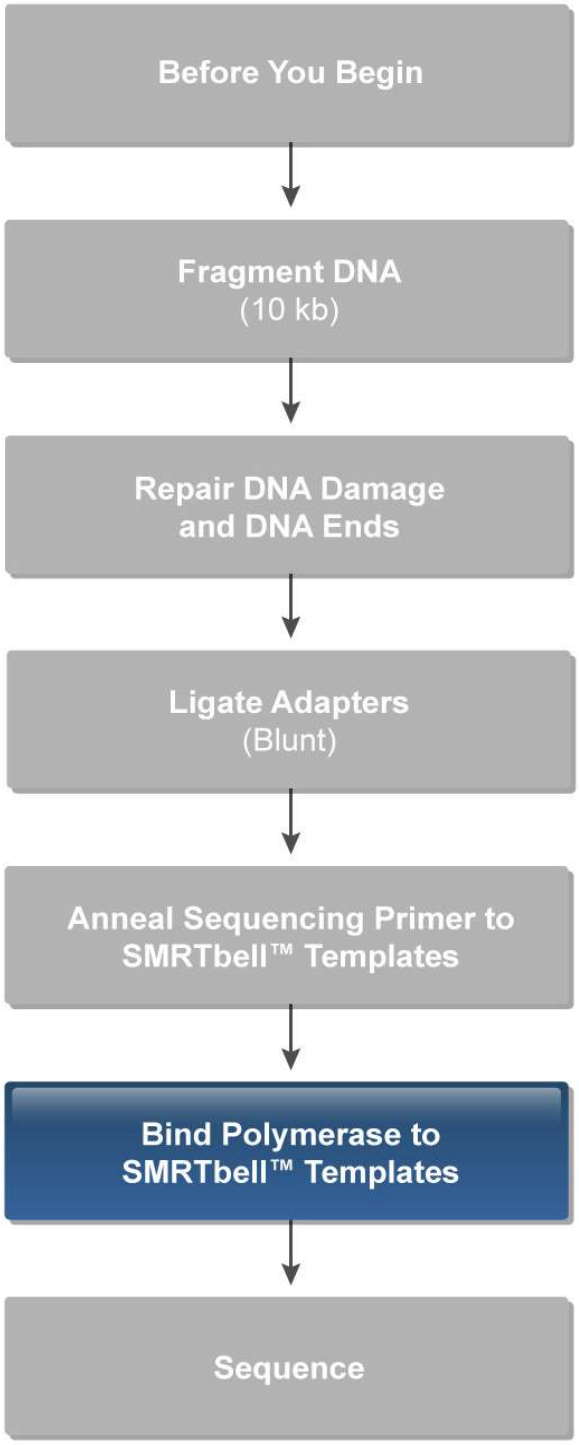
BINDING POLYMERASE TO TEMPLATES



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Binding Reaction

Overview

In the binding reaction step, DNA sequencing polymerases are bound to the primer-annealed SMRTbell templates.

Reaction Conditions

The reaction takes place in the presence of a buffer, DTT and nucleotides to stabilize the complex. For polymerase binding, incubation at 30ºC for 30 minutes is sufficient

Ratio of Polymerase to Template for Binding

The stoichiometric optimum for the polymerase:template ratio is 2 polymerases bound to each template molecule (one to each hairpin adapter). To maximize loading efficiency, binding ratios per library size have been optimized and appear as the default setting in the Binding Calculator. The ratio can be modified in the Optional section of the Calculator, however, it is highly recommended that the default parameters be used. See the *Pacific Biosciences Binding Calculator Parameters Quick Reference Card* for a summary of the recommended ratios of polymerases to templates for each library.

Note that MagBead loading and Diffusion loading require different polymerase to template ratios.

Storage of Polymerase-SMRTbell Complexes

Once the polymerase-SMRTbell template complex is formed, it should either be immediately used or stored at 4ºC for up to 3 days. Yield may be impacted if stored longer than 7 days.

If longer storage time is desired, it is best to store the complex in the Complex Storage Buffer supplied in the Binding Kit. The glycerol-based storage buffer allows the complex to withstand freezing temperature (-20ºC) for more than 30 days, while minimizing the polymerase's loss of activity. The Binding Calculator provides instructions for preparing complexes for long-term storage.

DNA Internal Control Complex: Identity and Amount

The DNA Internal Control Complex (available from Pacific Biosciences) provides a means for independent determination of any problems that may occur during binding and the sequencing run. These controls are SMRTbell templates already bound with the polymerase. They are added to the sample before loading on the instrument. See the *Pacific Biosciences Binding Calculator Parameters Quick Reference Card* for a summary of the DNA Internal Control Complex recommendations.

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The amount of DNA Internal Control Complex to add to experimental templates is determined by the sample insert size and chosen chemistry. The Binding Calculator automatically recommends the amount of DNA Internal Control Complex to add to achieve the total number of reads (between 500-1000 reads per SMRT Cell).

Loading Bias

Loading of SMRTbell templates into ZMWs is size dependent. Small inserts load better than large inserts. This is particularly important when sequencing different PCR amplicon sizes. It is highly recommended to select amplicons of the same size (+/- 10%) to minimize loading bias.

When sequencing large insert libraries (e.g. 20 kb library) for generating long read lengths, it is highly recommended to perform a size-selection step to eliminate short SMRTbell templates that will preferentially load.

The figure below demonstrates loading bias of various insert sizes generated from a 18.5 kb plasmid restriction digest.

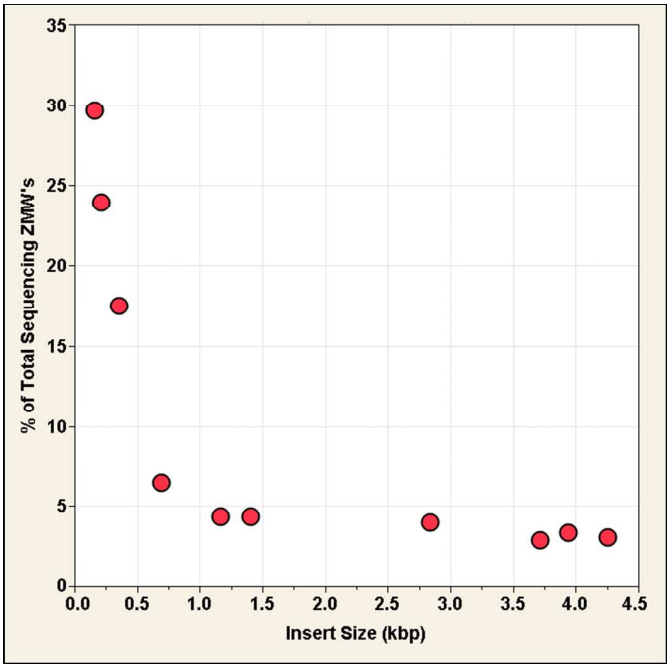

Figure 11 ZMW Loading Bias for Various Insert Sizes

Relative loading (as % of total sequencing ZMWs) versus insert size from a SMRTbell size ladder. A restriction digestion of an 18.5 kb plasmid generated an equimolar distribution of fragments from 160 bp to 4251 bp. The resulting fragments were converted to SMRTbell templates via a modified blunt-end ligation protocol which retains all fragment sizes > 75 bp. The SMRTbell size ladder was sequenced using standard protocols.

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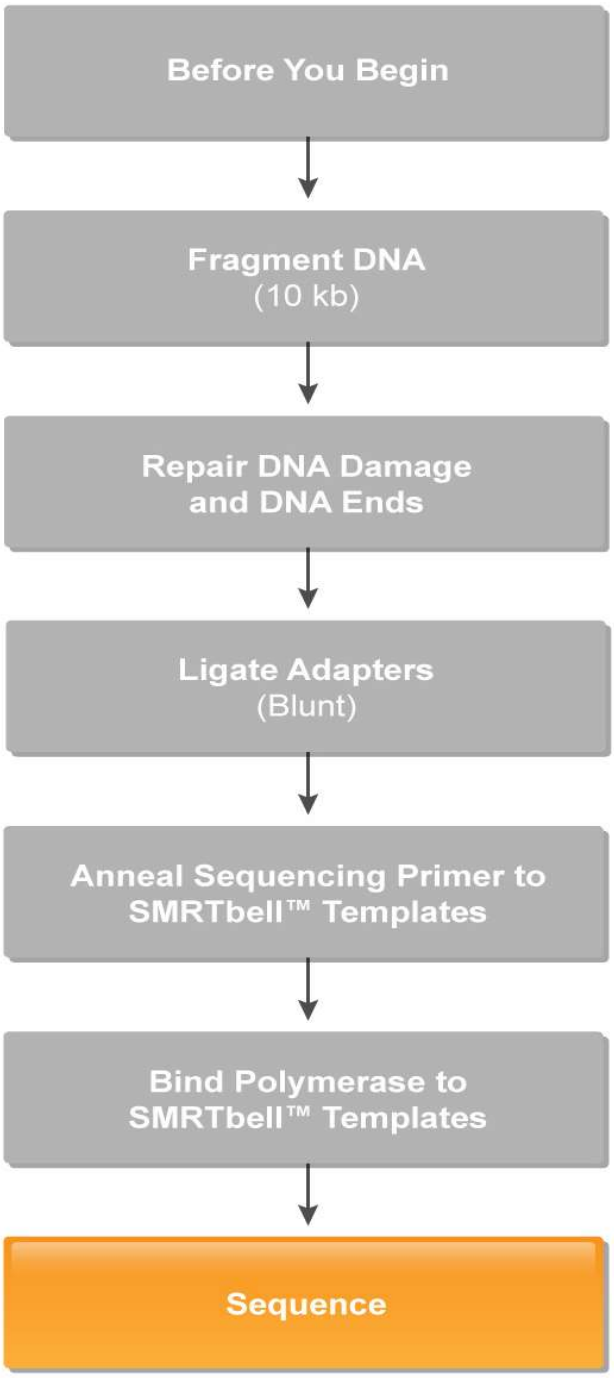
SEQUENCING



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Sequencing

Overview

Prior to sequencing, the template-polymerase complex must be transferred to a 96-well sample plate with concentrations and volumes specified by the Binding Calculator. This section provides background information for preparing the bound complex for sequencing.

Diffusion vs. MagBead Loading

Two options are available for loading. The options are dependent on the library size. Library sizes <1 kb must be loaded using diffusion loading. MagBead loading is highly recommended for libraries >1 kb.

MagBead Loading

During MagBead loading, SMRTbell templates are immobilized to the bottom of the ZMWs by paramagnetic beads.

First, SMRTbell templates are captured by MagBeads through a hybridization process between the primer poly-A tail and the oligo dT (on the magnetic bead surfaces). Then the SMRTbell-bound Magbeads are washed thoroughly with MagBead Binding and MagBead Wash Buffers to remove unwanted molecules such as excess primer and polymerases. The washed SMRTbell-MagBead sample is transferred to a 96-well plate, loaded on the instrument and subsequently transferred to a SMRT Cell for immobilization. MagBead loading is enabled by a built-in MagBead station that moves the MagBeads around the surface of the SMRT Cell. Typically, SMRTbell templates greater than 1 kb are immobilized at the bottom of the ZMWs whereas shorter SMRTbell templates, such as adapter dimers, are not.

In addition to removal of excess polymerase, primer, adapter dimers and short insert SMRTbell templates, MagBead loading offers the additional advantage of requiring significantly lower sample input amounts. This increases the amount of sequencing data that can be achieved from a sample. MagBead loading is recommended for libraries greater than 1 kb.

The Binding Calculator provides concentration recommendations per library size for loading. See the *Pacific Biosciences Binding Calculator Parameters Quick Reference Card* for a summary of the recommended MagBead loading concentrations.

MagBead bound samples are stable for 24 hours. It is highly recommended to use MagBead bound SMRTbell templates immediately following preparation.

For more specific information on preparing your library using the MagBead station, see *Pacific Biosciences Procedure & Checklist - Preparing MagBeads for Sequencing.* The Procedure can be found on our Customer Portal or our website.

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Diffusion Loading

Diffusion loading is a method by which SMRTbell templates are immobilized at the bottom of the ZMWs by the process of diffusion. Polymerase-bound SMRTbell templates are diluted with Complex Dilution Buffer, loaded on a 96-well plate and subsequently transferred to a SMRT Cell for diffusion loading.

The Complex Dilution Buffer and DTT used for dilution, prior to loading in the instrument, are supplied in the DNA/Polymerase Binding kit. The diluted samples should be used as soon as they are prepared.

During a sequencing run, the required diffusion time is 30 minutes for SMRTbell templates up to 3 kb in size and 1 hour for SMRTbell templates greater than 3 kb.

Complex Reuse Feature

Note that this feature is available for diffusion loading only. The bound complex used for immobilization on one SMRT Cell may be reused on several subsequent SMRT Cells, allowing for greater data output for a given amount of input DNA. When **Complex Reuse** is selected on the RS Remote, up to two additional SMRT Cells can be immobilized using the diluted complex from the first SMRT Cell (this is limited by both evaporation and the volume recovery capabilities of the instrument).

Complex reuse is available for all Standard sequencing insert sizes. It is not available for MagBead loading.

Loading Concentration Recommendations

The recommended loading concentration on SMRT Cells is dependent on the preferred loading method and library size. When the library size, concentration, volume and required number of SMRT Cells are entered in the Binding Calculator, an optimal on-plate concentration is recommended by default. See the *Pacific Biosciences Binding Calculator Parameters Quick Reference Card* for a summary of the recommended loading recommendations optimized for library sizes and chemistry.

It is highly recommended to perform loading titrations to achieve maximum performance per SMRT Cell.

Loading Titration

The optimal loading concentration may vary by sample. Slight variations are cumulative in large projects spanning tens or hundreds of SMRT Cells, and a pilot experiment with a loading titration is recommended to maximize overall yields. The data that is generated in the pilot run can be included in any downstream analyses along with larger optimized data sets.

The Binding Calculator facilitates setting up the complex dilutions on the sample plate to titrate loading concentrations. The calculator will generate four dilutions around the recommended concentration. The

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user can make these dilutions, from the same bound complex, and run the four samples in a pilot loading titration run.

After the run has completed and the data processed, the productivity output of primary analysis can be used to select the optimal concentration for running a larger batch of SMRT Cells. Choose the concentration that yields the most data output at an acceptable accuracy. An underloaded SMRT Cell will generate less data (see Figure 12 below). An overloaded SMRT Cell may result in higher data output but with an impact on raw accuracy and read length due to multiple polymerase-template complexes in the same ZMW (see Figure13).

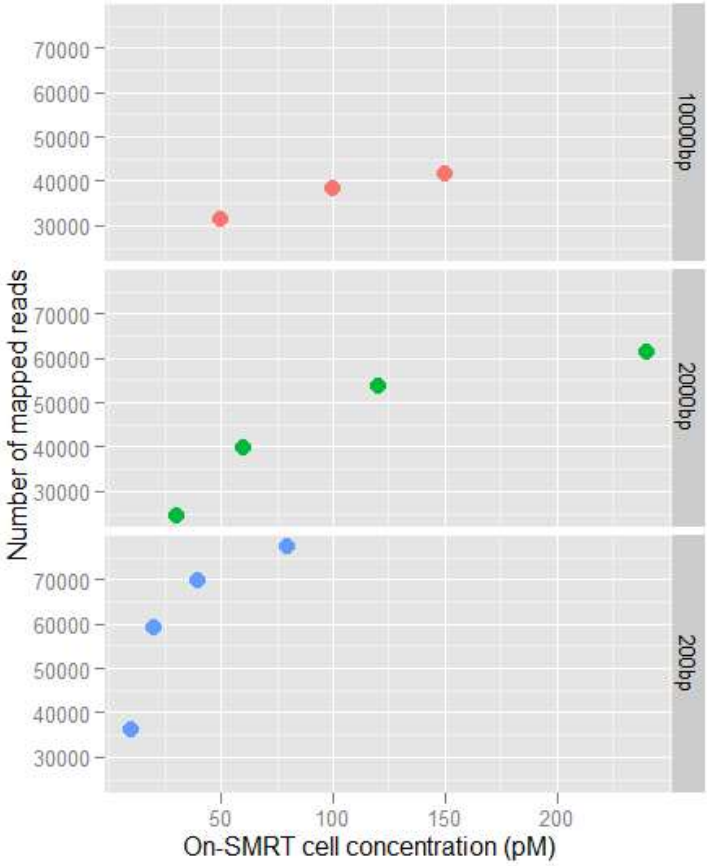

Figure 12 Yield (Mapped Reads) with increasing On-SMRT Cell Loading Concentration. On-SMRT Cell concentration is the final concentration of the diluted sample transferred to the SMRT Cell. On-plate concentration is the concentration of the sample in the 96-well plate and which is the value entered in the Binding Calculator.

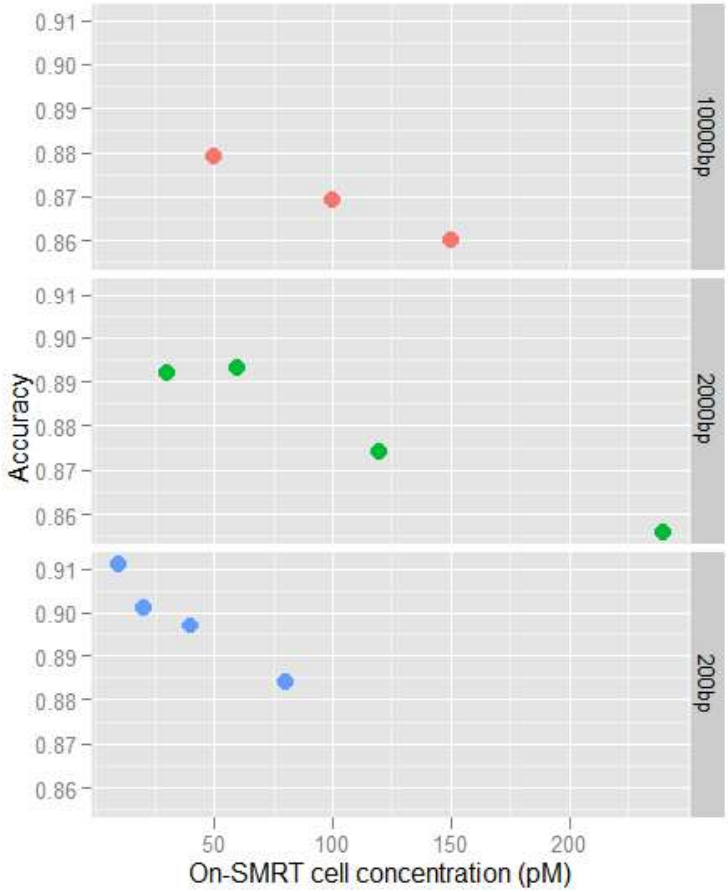

Figure 13 Effects of loading to accuracy with increasing On-SMRT Cell Concentration. Overloading affects raw accuracy and read length.

Required Sample Well Volumes

The sample volume to load in a well of a 96-well sample plate is dependent on the desired number of SMRT Cells, loading concentration and loading method. See the *Pacific Biosciences Binding Calculator Parameters Quick Reference Card* for a summary of required sample well volumes.

During a run, the automated pipettor takes an aliquot of the sample for dilution before delivering it to a SMRT Cell for immobilization. To ensure accurate pipetting, the pipettor requires dead volumes of 5 µL for diffusion loading and 10 µL in the sample plate for MagBead loading. The dead volumes are factored in the required volumes in the *Pacific Biosciences Binding Calculator Parameters Quick Reference Card*.

Prepare Your Reagent Plates

Thaw the following kit components accordingly:

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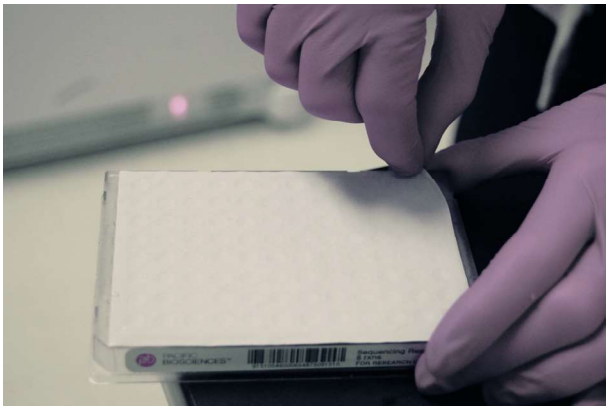
1. OS Enzyme: Remove the OS Enzyme from the reagent package. Keep at -20 ºC at all times. When ready to use, spin down the tube for 5 seconds, replace cap with tube septa, and place on the reagent drawer of the instrument.
2. Reagent plate: The reagent plate can be thawed overnight at 4ºC. Thaw an additional 15 minutes, at room temperature, before using. Note the following precautions which may impact reagent performance:
   - Do not thaw in hot water.
   - Do not remove plate seal until it is ready to be placed on instrument.
   - Do not keep at room temperature after thawing.
   - Do not keep the unsealed plate exposed to air. Place the septa mat on plate immediately.
3. Place the reagent plate on a VWR Microplate Shaker and shake for 1 minute at 1200 rpm.
4. If precipitates are present in any of the filled wells, mix thoroughly by vigorously vortexing until the solution is clear.
5. Spin the plate in a centrifuge briefly at 2000 rpm.
6. Peel the foil seal off the plate as shown:



7. Place the supplied Septa mat on the plate.
8. Using a roller, further press down the Septa mat onto the plate.

Prepare Your Sample Plate

Dilute Your Bound Complex

Load your samples on to a Sample plate.

Place the white Septa mat on the plate and press down with a roller to ensure a tight fit:

- Spin your sample plate down for 30 seconds at 2000rpm
- Place on the instrument

Load Your Reagent, Mixing and Sample Plates

At the instrument, open the **Reagents/Samples** drawer. You can either press the illuminated **Open** button on the instrument Reagents/Samples drawer or the **Open** button on the touchscreen User Interface.

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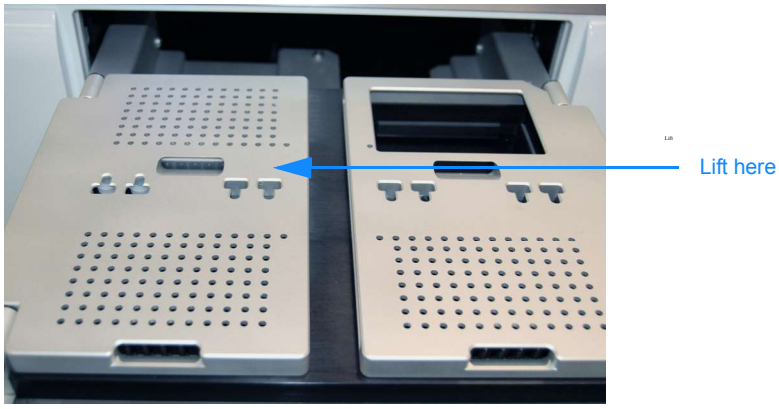
Load your Reagents on to the Instrument:

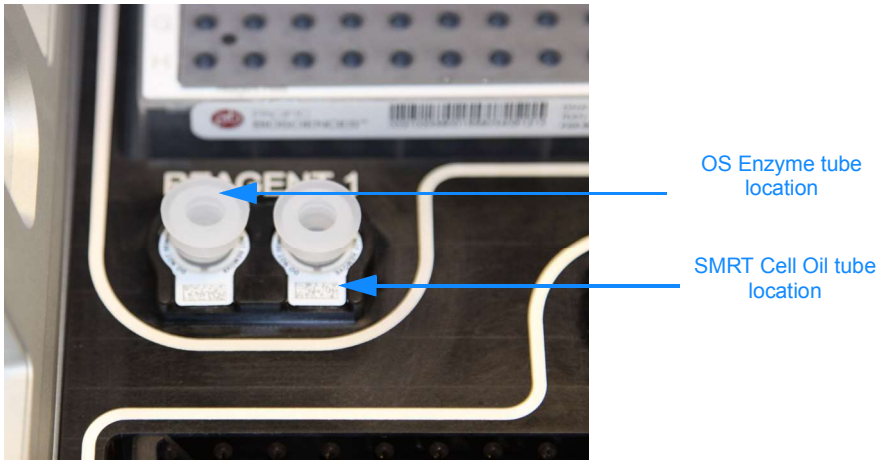
1. When the drawer opens, lift up the metal door covering the Reagents slots.



2. Place your reagent plate in the Reagent slot. Note that there are 2 slots, if you have only one plate, you can place it in either slot. The Barcode Scanner will locate the plate.

3. Remove the OS Enzyme from the refrigerator or ice.
4. Replace the cap with a tube septa. Be careful not to remove the barcode collar, the Machine Vision System will read the barcode.

5. Place the tube in the left slot encircled by your reagent plate location.
6. Remove the blue cap from the SMRT Cell Oil tube.
7. Replace the cap with a tube septa. Place the SMRT Cell Oil tube in the right hand reagent slot. Note that the SMRT Cell Oil tube is slightly larger and will fit only in that slot.



8. Gently close the metal door.

Load your samples and mixing plate on to the Instrument:

9. Lift up the metal door covering the Sample and Mixing slots.

10. Place your sample plate in the slot labelled **Sample**.

11. Place a 384-well mixing plate (Eppendorf LoBind Deepwell plates) in the slot labeled **Mixing**.

12. Gently close the metal door.
13. Press the **Close** button on the touchscreen User Interface (optionally, press the Close button on the instrument).

Load the SMRT® Cells and Tips

Open the **SMRT Cells/Tips** drawer. You can either press the illuminated **Open** button on the instrument **SMRT Cells/Tips** drawer or the **Open** button on the touchscreen User Interface.

Load your SMRT Cells/Tips on to the Instrument:

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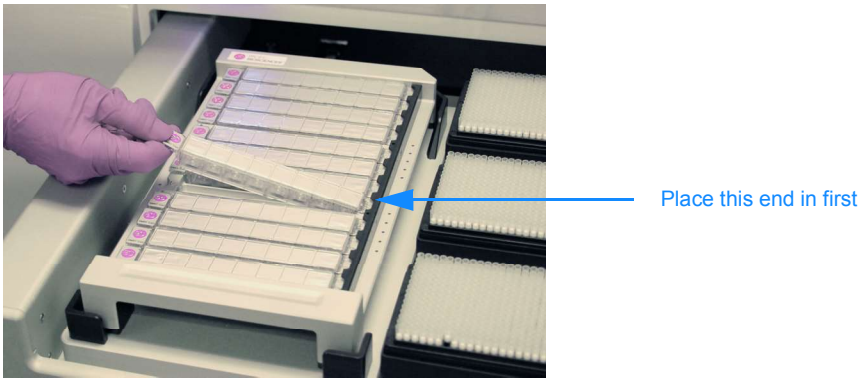
1. When the drawer opens, place the appropriate number of SMRT Cell 8Pacs (determined previously during project planning) into the SMRT Cells tray.



2. Place tip boxes in the slots. Be sure to remove the lid and tape from the tip boxes.

3. Gently close the drawer door.
4. Press the **Close** button on the touchscreen User Interface (optionally, press the Close button on the instrument).

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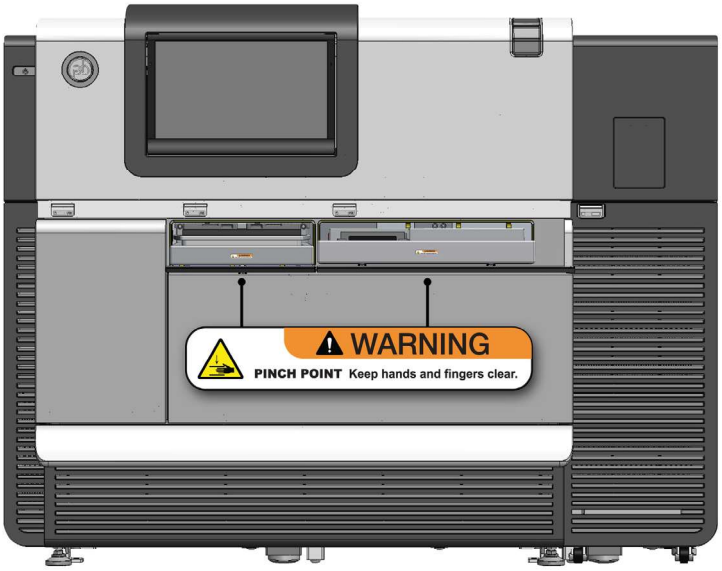
WARNING! Pinch Point. Do not place fingers inside drawers as they are closing.



Front View

Start Your Run

Before starting your run, press **Scan** (on the RS touch screen) in order for the instrument to scan the contents of the drawers. Once the contents have been scanned, the **Start** button will be enabled. After pressing **Start**, the UI will prompt you to verify that certain steps have been performed prior to sequencing:

- Waste has been emptied. Verify that the Waste bin is less than half full.
- Nitrogen supply is within range. Locate your facility's N2 tank supply gauge and verify that the supply is > 80 psi (preferably > 100 ~ 120 psi).

Refer to the RS Remote online help systems for more information.

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Troubleshooting Low Library Yields

When Adapter Dimers May Be Cause of Low Yield

If you suspect that the low yield is due to adapter dimers, then you can try re-purifying the library:

- Use MagBead loading.
- Increase the current annealed template volume to 50 µL using 1X Primer Buffer (dilute the Primer Buffer 1:10 in water).
- Then perform an additional AMPure® PB bead purification step using (e.g., for a 10 kb library use 0.45X AMPure PB beads). However, do not shake in a VWR vortex mixer at 2000 rpm (room temperature) for more than 10 minutes as adapter-dimers may bind to the beads.
- After the ethanol wash, resuspend the beads in 1X Primer Buffer.
- Calculate the appropriate volume of Elution Buffer and elute the DNA off the beads.
- Perform an $OD_{260/280}$ calculation to recalculate molarity before setting up the Annealing and Binding reaction. Then repeat the Primer Annealing steps using the Sample Preparation Calculator (see the Anneal and Binding sections of this Guide).

When DNA Quality is Suspected to be Sub-Optimal

- Ensure that the concentration of AMPure PB beads is appropriate for the size of the library being prepared. For example, 250 bp libraries should be purified with 1.8X volume of AMPure PB beads; if less than this amount is used then library yield will suffer.
- The ends of the DNA insert fragments should not be labeled in order to allow the hairpin adapter -insert DNA ligation reactions to proceed. For instance, 5'FAM labeled PCR products can not be used as the input DNA for making SMRTbell templates. A failure at the ligation step will lead to little or no library after the exonuclease treatment, as partially ligated products are degraded.
- The PacBio Template Prep Kit should be properly handled with all reagents and enzymes stored at -20°C. The Template Prep Kit uses a number of different enzymes and improper storage of these enzymes can lead to reductions in their catalytic efficiency and drops in either library recovery or eventual sequencing yield. For example, using inactive ligase will compromise the ligation step and lead to little or no library recovery.

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When the Sheared Library is the Wrong Size

- Make sure to optimize shearing conditions. While this guide makes certain recommendations about shearing conditions, it is also important to verify conditions effective for shearing your particular DNA sample on your shearing device.
- Verify that the input DNA used for shearing is high molecular weight DNA at the appropriate concentration for your shearing device. If the input DNA is damaged, this can lead to smaller insert sizes and difficulty in generating large insert size libraries. Additionally, the concentration of the input DNA can also alter the shear-size.

When Library Yield is High but Sequencing Yield from the Library is Still Low

- Properly quantify SMRTbell libraries before annealing sequencing primer. Run a Bioanalyzer gel (from Agilent) following manufacturer's loading recommendations. Sheared libraries are comprised of a distribution of fragment sizes around a targeted size, and molarities are best approximated using the Bioanalyzer software.
- Check the level of adapter dimer contamination. Because of an intrinsic loading bias that favors the immobilization of smaller DNA template sizes in the SMRT Cells, the amount of hairpin dimers in your sample should be kept as low as possible (ideally < 1%; adapter dimer levels approaching 2% or higher will significantly decrease sequencing yields). We strongly encourage performing at least a double-AMPure PB bead purification step prior to performing the primer-template annealing reactions (when automating the library preparation procedure using a robotic platform, a triple purification process may be necessary).

Internal Control Complex Sequence

TCCTCCTCCTCCGTTGTTGTTGTTGAGAGAGAGAAGCGCTCGCACACCACATCAAGGCTGGTGCGG
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CTCTCTCTCTTT

Insert sequence (lowercase, no color)
Hairpin stem sequence
Hairpin loop sequence (primer binding site in **bold/underline**)
Site of first incorporation

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EXHIBIT B

DocuSign Envelope ID: E6ADB88D-E82A-43AE-91B5-1ED3CA14C4B2

PACIFIC BIOSCIENCES®

Detecting DNA Base Modifications Using Single Molecule, Real-Time Sequencing

Introduction

Base modifications are important to the understanding of biological processes such as gene expression, host-pathogen interactions, DNA damage, and DNA repair[1]. Single Molecule, Real-Time (SMRT®) sequencing has the potential to revolutionize the study of base modifications through direct detection of unamplified source material.

Traditionally, it has been a challenge to study the wide variety of base modifications that are seen in nature. Most high-throughput techniques focus only on cytosine methylation and involve both bisulfite sequencing to convert unmethylated cytosine nucleotides to uracil nucleotides, and comparison of sequence reads from bisulfite-treated and untreated samples[2]. SMRT sequencing, in contrast, does not require base conversion within the source material in order to detect base modifications. Instead, the kinetics of base addition is measured during the normal course of sequencing. These kinetic measurements present characteristic patterns in response to a wide variety of base modifications – researchers at PacBio have been able to observe unique kinetic characteristics for over 25 base modifications[3].

As a result of this breakthrough methodology to detect base modifications, it is now possible to sequence modifications other than 5-methylcytosine (5-mC). Bacterial modifications such as 6-methyladenine (6-mA), 4-methylcytosine (4-mC), or more recently identified eukaryotic modifications such as 5-hydroxymethylcytosine[4] (5-hmC), are also accessible to study using a single sequencing method on the PacBio® *RS* system.

As our understanding of kinetic information grows, the analysis of base modifications using SMRT technology will continue to become easier and faster.

Types of Base Modification in Biology

DNA base modifications have a variety of functional roles which include:

- Epigenetic markers for influencing gene expression such as 5-mC, 5-hmC, 5-formylcytosine (5-fC), and 5-carboxylcytosine (5-caC).
- Bacterial identity markers for affecting host-pathogen interactions such as 6-mA, 4-mC, and 5-mC.
- Bacterial epigenetic markers for regulating DNA replication and repair and transcription regulation such as 6-mA.
- Products of DNA damage such as 8-oxoguanine (8-oxoG) and 8-oxoadenine (8-oxoA).

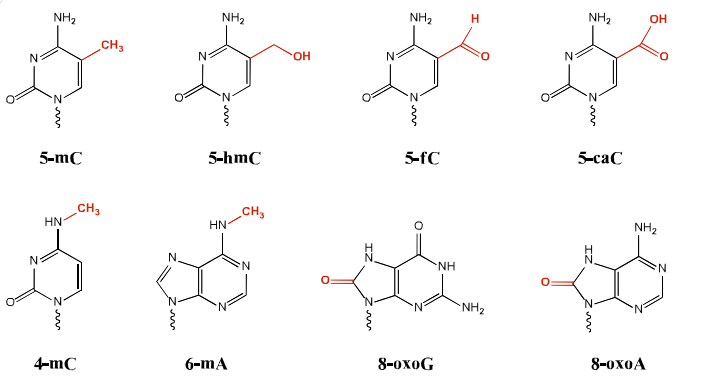

Figure 1. Molecular structures of base modifications including 5-mC, 5-hmC, 5-fC, 5-caC, 4-mC, 6-mA, 8-oxoG, and 8-oxoA.

Today, the most commonly studied base modification is 5-mC, commonly referred to as "methylation" despite the fact that other bases are also naturally modified by methyl groups.

Methylation has gathered interest from researchers in a variety of disciplines including early developmental biology, cancer biology, and neurological disorders. Methylation and de-methylation help regulate gene expression and have been linked to several human diseases through mechanisms such as deactivating tumor suppressors or activating oncogenes[5].

Other modifications, such as 6-mA in bacteria, have been studied with lower resolution methods – such as chromatography or through methylation's protective effect against restriction endonucleases – because they are not easily accessible with standard sequencing techniques. This modification is associated with basic functions such as DNA replication and repair[6]. It is also common in protists and plants, and some studies suggest that it may also be present in mammalian DNA[7].

SMRT sequencing is capable of detecting 6-mA as well as other common bacterial base modifications. As a result, the technology is expected to increase our understanding of a broad array of biological processes.

The potential benefits of detecting base modification, using SMRT sequencing, include:

- Single-base resolution detection of a wide variety of base modifications (including those in Figure 1 and more).
- Single-molecule resolution over long-read distances.
- Unamplified double-stranded input DNA, which means that strand-specific modifications, such as hemimethylation, are detectable.
- Hypothesis-free base detection which allows discovery of unknown or unexpected modifications through the effects on sequencing kinetics (as described below).

Studying Polymerase Kinetics with SMRT® Sequencing

SMRT Sequencing allows the observation of single DNA polymerases reading individual molecules of DNA in real time. Therefore, the kinetic characteristics of DNA polymerization are observable on a single-molecule basis. The kinetic characteristics, such as the time duration between two successive base incorporations, are altered by the presence of a modified base in the DNA template[3]. This is observable as an increased space between fluorescence pulses, which is called the interpulse duration (IPD), as shown in Figure 2.

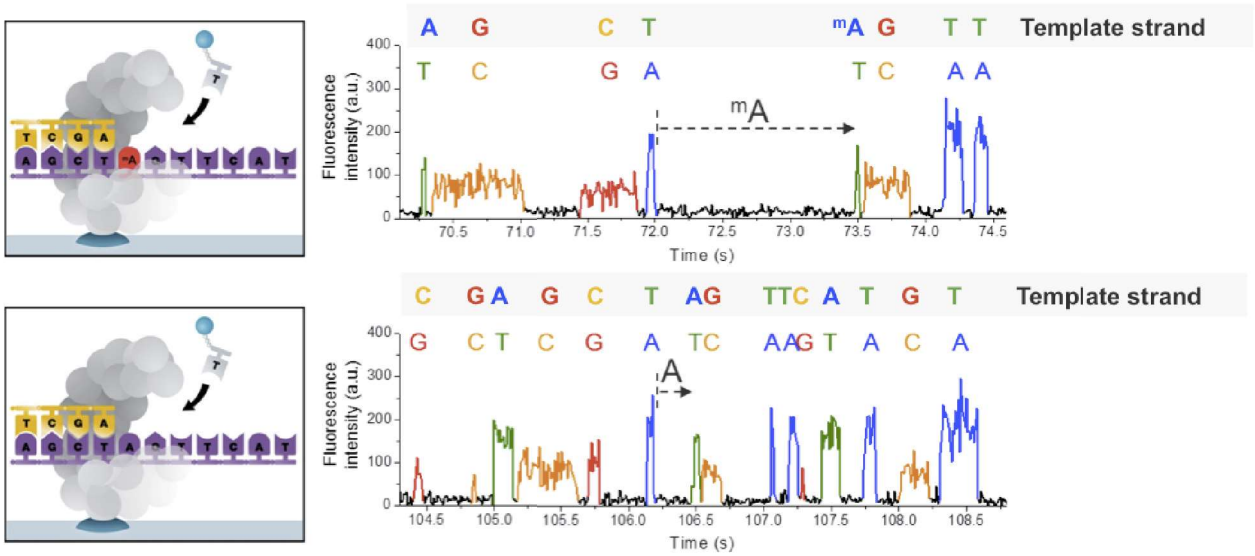

Figure 2. Principle of detecting modified DNA bases during SMRT sequencing. The presence of the modified base in the DNA template (top), shown here for 6-mA, results in a delayed incorporation of the corresponding T nucleotide, i.e. longer interpulse duration (IPD), compared to a control DNA template lacking the modification (bottom).[3]

These changes in the DNA polymerase speed, relative to an *amplified control* template lacking modified bases, can be measured for each template position to indicate the presence of modified bases in the DNA template. In order to quantify the change in IPD distributions between a control sample and a native sample, we define the *IPD ratio* as the ratio of the mean IPD at a site in the native sample to the mean IPD at the same site in the amplified control.

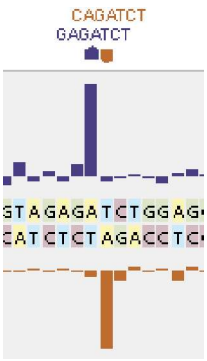

Figure 3. Image from SMRT® View software using the *in silico* control showing detection of 6-mA at GATC motifs using IPD ratios. For each template position (x-axis), the ratio (y-axis, purple bar up for forward strand and orange bar down for reverse strand) of average interpulse durations (IPDs) for each strand of the native DNA to the control is plotted. Excursions from the baseline indicate the presence of a modified base (6-mA, in this example, marked with indicators at the top along with a seven-base context in 5' -> 3' orientation), slowing down the polymerase at the position of the modification[9].

An alternative method calculates IPD ratios based on a computational model rather than using an unmodified control sample. This model is referred to as the "*in silico* control". The model must be trained to recognize the kinetics of a specific sequencing chemistry. The *in silico* control has obvious benefits in reducing the time required to perform an initial experiment and avoids using an amplified control.

The local sequence context of unmodified DNA influences polymerase dynamics. As such, the *in silico* control uses a predicted value of the mean IPD at each reference position that is based on the local sequence context.

Kinetic effects are not necessarily limited to just the nucleotide incorporation opposite the modified base position in the DNA template. This is because the DNA polymerase is in intimate contact with the DNA over an extended region of approximately twelve bases, and the modified base can impart effects on the polymerase dynamics at several positions over this region. This results in kinetic "signatures" which can aid in identifying the type of base modification.

For example, for the three common bacterial identity markers[8] (see Figure 4):

- 5-mC has typical characteristic kinetic signals two and six bases downstream of the methylated position
- 4-mC just at the position of the modification
- 6-mA at the modified position and often five bases downstream

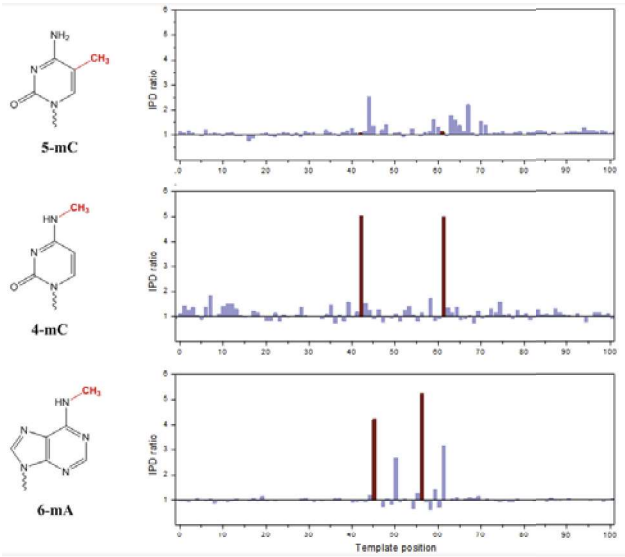

Figure 4. Kinetic signatures for the three common bacterial identity markers 5-mC, 4-mC, and 6-mA. The methylated template positions are highlighted in red. Note that the kinetic signatures vary both in magnitude as well as in the length of the region over which the polymerase dynamics are affected.

Different signal magnitudes translate to different amounts of sequencing-fold coverage required to obtain similar confidence levels for modification detection.

The current *in silico* control algorithms have proven reliable for studying 6-mA in bacteria, for example in methylome motif analysis. However, when studying subtle kinetic effects such as those for native 5-mC, using an amplified control is more accurate than

using the *in silico* control. Future commercial products are expected to improve use of the sequence context to better identify and interpret modification signals.

Based on the measurement and analysis principle outlined above, a typical experiment for detecting DNA base modifications can be designed as follows:

- Obtain native DNA of interest and prepare a SMRTbell™ library for SMRT sequencing (see the Pacific Biosciences Sample Preparation and Sequencing Guide).
- If using an amplified control design, use a small aliquot of the original DNA sample and perform a whole-genome amplification (WGA) reaction to obtain the control DNA sample lacking any base modifications. Then prepare a SMRTbell library for this sample.
- Perform adequate SMRT sequencing (for both samples when using an amplified control) to obtain the necessary coverage needed for the modifications under study, and sufficient overall coverage to characterize the genome (or portion of the genome) of interest.
- Use the bioinformatics tools to perform kinetic analysis for base modification[9].

Examples of Current Applications

Direct DNA sequencing of base modifications requires that the following general considerations be fulfilled during the experimental design:

1. Unamplified DNA is necessary. Amplification of DNA through PCR, WGA, or other techniques, will result in the loss of base modifications. Therefore, enough native DNA must be available to make a sequencing library. With version 2 sequencing reagents ("C2 chemistry"), the sample requirement for preparing a ~500 bp SMRT sequencing library is 250 ng of input DNA.

2. As DNA input requirements are reduced over time, a smaller or targeted region of a genome is expected to be sufficient for studies without amplification requirements.

3. The different magnitudes of kinetic signal, for the different base modifications, translate to different amounts of sequencing fold coverage required to obtain high confidence levels of detection.

We recommend the minimum sequencing fold coverage *per strand* as follows[9]:

4-methylcytosine	25x
5-methylcytosine	250x
5-hydroxymethylcytosine	250x
glucosylated 5-hydroxymethylcytosine[1]	25x
hydroxymethylcytosine enriched with the Hydroxymethyl Collector™ Kit [2]	5x
6-methyladenine	25x
8-oxoguanine	25x

4. The genome, or portion of a genome, to be interrogated also has to be compatible with the current throughput performance of the PacBio *RS*. With C2 Chemistry, the base throughput of a two hour run on the PacBio *RS* is approximately 100 Mb. Studying methyladenine in *E. coli* requires 4 to 6 SMRT Cells per sample,which can be run in one afternoon. Over time, we expect SMRT technology throughput to increase, making larger genome sizes more practical to sequence for base modifications.

Applications particularly suitable for SMRT sequencing based on the criteria above include:

- Sequencing of *E. coli* to find the sites of 5-mC, 4-mC, and 6-mA modification when studying virulence, gene expression, or pathogen-host interactions. See the *Pacific Biosciences Technical Note - Detecting DNA Base Modifications* for more detailed information on strategies for experimental design and motif analysis in bacteria.
- Isolating and purifying mitochondrial, chloroplast or other small genomes, containing known or novel DNA base modifications.
- Enriching portions of a larger genome (e.g., isolation of DNA regions modified with 5-hmC,

[1] Generated by T4 Phage β-glucosyltransferase (Josse, J. and Kornberg, A. (1962) J. Biol.Chem., 237, 1968-1976)
[2] Available from Active Motif (http://www.activemotif.com/catalog/775/hydroxymethyl-collector-trade)

and enriched by specific biotinylation chemistries of 5-hmC and subsequent streptavidin pulldown). Normalizing IPDs with the *in silico* control is particularly useful for this type of experiment, where generating a completely overlapping set of amplified control sequence data can be a challenge.

Conclusion

SMRT sequencing is the only commercially available technology capable of measuring the kinetics of base incorporation during a sequencing run. This kinetics information can be used to identify sites in the target DNA that have been chemically modified in a variety of ways (including methylation, formylation, carboxylation, and more). These base modifications are associated with several biological processes including gene expression and DNA oxidative damage.

Most of these modifications have not been extensively studied due to difficulties in adapting experimental techniques to a higher throughput sequencing method – we expect that these modifications will be possible to study with SMRT sequencing. Examples of novel studies that may be performed on the PacBio *RS* today include full-genome bacterial modification studies and hydroxymethylcytosine studies in mammalian genome regions enriched for that modification.

We anticipate that over time the range of target genomes that will be addressable by SMRT sequencing will improve and ultimately address regions as small as single genes as well as larger whole genomes. We further expect that the advances afforded by SMRT sequencing will enhance genetic studies into gene regulation, DNA damage and repair, bacterial virulence, and other important biological pathways. Novel DNA modifications could potentially be discovered, expanding the utility of sequencing to even broader areas of study.

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9. SMRT Analysis version 1.3.1 or higher, and/or "DNA Modification Detection with SMRT Sequencing using R" found at https://github.com/PacificBiosciences/R-kinetics.

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