Protocol to build non-size selected cDNA libraries for Pacific Biosciences long-read sequencing

Version 2.0 (March 4, 2020)

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Overview

This protocol describes an optimized method for preparing long read cDNA libraries using the SMRTbell™ Express Template Prep Kit 2.0 that are then sequenced on the PacBio-Sequel II.

The product of this protocol is called a SMRTbell™ template, which is characterized by a double-stranded DNA template capped by a hairpin loop (blunt adapters) on each end. This structure allows generation of sense and antisense sequence from a single molecule of cDNA, which also facilitates error correction using circular consensus sequencing approaches.

The ends of poly-A selected cDNA are repaired first in order for the cDNA molecule to be suitable for ligation of SMRTbell™ adapters. Once the ligation step is done, a sequencing primer is annealed to the adapters. The sequencing primers allow for the binding of the polymerase during sequencing.

This protocol includes an exonuclease treatment to remove transcripts without a cap. This should increase our certainty about the completeness of the transcripts being used to build the library. Since the exonuclease will decrease the starting RNA material, we added a clean-up and concentration step using a Zymo kit, that also helps to remove any EDTA leftovers from previous steps. We use the Superscript IV™ from ThermoFisher Scientific as the reverse transcriptase enzyme in this protocol.

Considerations

A modified Smart-Seq2 Protocol from Picelli et al. 2014 was used for cDNA synthesis (Refer to https://www.nature.com/articles/nprot.2014.006)

The SMRTbell™ Template Express Prep Kit 2.0 was used for library building. All the reagents and samples should be kept on ice during preparation of the libraries, especially the enzymes included in the kit.

The amount of starting material is of high relevance. If the concentration of DNA goes higher than the recommendation (500 ng), a high proportion of chimeric template molecules may form, making the sequencing run unsuccessful.

Ampure® PB Insert Beads are free of reagents that can interfere with polymerase binding.

For the bead clean up steps, we highly recommend saving the first supernatant in case the bead binding does not work properly. This will let you repeat the step, applying the proper corrections.

When using the PacBio calculator, you can increase the concentration on the plate if the template concentration after library prep is too low. The numbers in this protocol are what we usually use in the lab.

RNA extraction

The RNeasy Plus Mini Kit was used for RNA extraction following the manufacturer instructions (Refer to:https://www.qiagen.com/us/resources/resourcedetail?id=c8cdc6bf-5bbf-4e3b-a0f4-476da9215012&lang=en).

Accurate RNA quantification is done using Qubit® and analysis of the integrity with Bioanalyzer® are essential to make sure the generation of high-quality libraries in future steps.

***OPTIONAL STOPPING POINT** store RNA at -80°C only if necessary!!!!! We recommend not stopping until the synthesis of cDNA step because it is more stable than RNA.

Exonuclease treatment

Once the RNA concentration has been determined, we suggest using at least 1500 ng of total RNA for exonuclease and follow the protocol as recommended. Buffer A and the enzyme are included in the kit.

Amount	Reagent
X	Sample (~1500 ng RNA)
2 uL	Buffer A
0.5 uL	RNAse inhibitor
1 uL	Exonuclease
X	DEPC water (Total volume: 20uL)

Adding exogenous reference transcripts

In order to track sequence mapping performance, we include the Lexogen SIRV set 3 reference transcripts to the sample. The SIRV set 3 solution is stored as a 1:10 dilution with a final concentration of 3.03 ng/ul. A further 1:10 dilution of the 1:10 dilution may be needed to keep the VSIRV under 1 ul (making it a 1:100 dilution overall, and 0.303 ng/ul). Since 3% of RNA in a sample are messenger RNA, we want to target 1% of that, therefore the formula to calculate the amount is:

MSIRV= (total RNA ng) * 0.0003

Then to calculate the volume, we do:

VSIRV= MSIRV / (SIRV concentration)

Add the volume to the RNA sample

Hybridization of oligo-dT_{30}

Volume	Reagent
1 uL	10 uM oligo-dT primer
1 uL	dNTP mix
0.1 uL	RNase inhibitor

For hybridization of oligo-dT_{30} to total RNA, pipet 2 µL of total RNA into PCR tube/strip, then add 2.1 µL of a master mix (below)

Quick spin and then incubate at 72°C for 3 min. Immediately place the tube on ice.

Reverse transcription

Quick spin and collect samples on ice.

Prepare the reverse transcription buffer (RTB, below) and add 5.7 µL of RTB to the previous step (If working with multiple samples make a master mix for # of samples + 1).

Volume	Reagent
0.5 µL	Superscript IV (200 U ul^{-1})
0.25 µL	RNase Inhibitor (40 U ul^{-1})
2 µL	Superscript IV first-strand buffer (5x)
0.5 µL	DTT (100 mM)
2 µL	Betaine (5 M)
0.06 µL	$MgCl_2$ (1 M)

0.10 µL	TSO (100 mM)
0.29 µL	DEPC water

(Based on Picelli et al. 2014)

Mix gently by pipetting, quick spin, and run the next PCR protocol.

Cycle	Temperature	Time
1	50	10 min
2	80	10 min
3	4	Hold

***OPTIONAL STOPPING POINT** store cDNA at -20°C.

PCR preamplification

To the previous reaction (10 µL), add 15 µL of the following mix (If working with multiple samples make a mix for # of samples + 1):

Reagent	Volume
KAPA HiFI HotStart ReadyMix	12.5 µL
IS PCR primers 10 M (Picelli *et al.*, 2014)	2 µL
Nuclease-free water	0.5 µL

Vortex tubes to mix. Quick spin and run the next PCR protocol .

The input amount of total RNA is important to choose the number of cycles. Our lab suggests the following:

Cycle	Denature	Anneal	Extend	Hold
1	95°C, 2 min	-	-	-
2-19	98°C, 20 s	65°C, 15 s	74°C, 10 min	-
20			72°C, 5 min	-
21			∞	4°C

Amount of input RNA (ng)	# of PCR cycles for preamp
0.1	18
1	16
10	12
100	9

PCR purification

Before library construction, it is crucial to remove excess primers, nucleotides, salts, and enzymes using Ampure® XP beads. This product binds DNA fragments 100 bp and larger to paramagnetic beads to facilitate DNA purification and cleanup.

Add 25 µL of Ampure® XP beads previously equilibrated at room temperature to the preamplified material (1:1 ratio) and mix by pipetting until solution is homogeneous. Transfer to a new 1.5 mL tube for easy use in the magnetic stand in future steps. Incubate at room temperature for 10 min to allow the binding of DNA to the beads. Then place the tube on the magnetic stand for 2 min or until the solution is clear. Remove the supernatant carefully so the beads are not disturbed and save it in a new tube (Saving the supernatant can be helpful. If the bead binding doesn't work, you still have your initial material).

Wash the beads on the magnetic stand with 200 µL of 80% ethanol solution and incubate for 30s, then remove the ethanol and repeat the wash. Remove any trace of ethanol, move the tube to a rack and allow them to dry with the cap open for 1 min. Don't let the beads dry too much (formation of cracks in the bead pellet) because this will make the dilution step difficult.

Resuspend the beads in 30 µL of EB buffer. Place the tube on the magnetic stand and move 27ul of supernatant into a clean tube. A Qubit® quantification is necessary, because in future steps the amount of input material is key to a successful library prep.

You can also run the sample on Bioanalyzer to assess the quality of the material you will use as input ***OPTIONAL STOPPING POINT** store at -20°C.

The full-length cDNA from the previous step needs in end to have enough input for library construction (500 ng).

PacBio Template preparation

To achieve a final loading concentration for the Sequel II we suggest a starting amount of 1000 ng.

Remove Single-Strand Overhangs

Dilute the DNA prep additive with enzyme dilution buffer. Mix well and quick spin.

Volume	Reagent
4 µL	Enzyme Dilution Buffer
1 µL	DNA Prep Additive (stock)

For each sample to be processed, prepare the following reaction (minimum input DNA 300 ng).

Volume	Reagent
7 µL	DNA Prep Buffer
<= 45.4 µL	DNA
Up to 55 µL	Water
0.6 µL	NAD
1 µL	Diluted DNA Prep Additive
1 µL	DNA Prep Enzyme

Once the mix is done, pipette mix 10 times. Do not flick the tube.
Spin down contents of tube with a quick spin in a microfuge.
Incubate at 37°C for 15 min, then return the reaction to 4°C. Proceed to the next step.

Repair DNA Damage

For each sample to be processed, use the following table to prepare the reaction:

Volume	Reagent
57 µL	DNA (Previous Rx)
2 µL	DNA Damage Repair Mix v2

Pipette mix 10 times. Do not flick the tube.
Spin down contents of tube with a quick spin in a microfuge
Incubate at 37°C for 30 minutes, return the reaction to 4°C.
Proceed to the next step

Repair Ends/A-Tailing

For each sample to be processed, use the following table to prepare the reaction.

Volume	Reagent
57 µL	DNA (Damage repaired)
3 µL	End Prep Mix

Pipette mix 10 times. Do not flick the tube.
Spin down contents of tube with a quick spin in a microfuge.
Incubate at 20°C for 10 minutes.
Incubate at 65°C for 30 minutes, return the reaction to 4°C.
Proceed to the next step.

Adapter Ligation

For each sample to be processed, use the following table to prepare the reaction, adding the components below in the order listed.

Volume	Reagent
60 µL	DNA (End-Repaired)
5 µL	Overhang Adapter v3
30 µL	Ligation Mix
1 µL	Ligation Additive
1 µL	Ligation Enhancer

Pipette mix 10 times. Do not flick the tube.
Spin down contents of tube with a quick spin in a microfuge.
Incubate at 20°C for 60 minutes, return the reaction to 4°C. (Overnight ligation is optional).
Proceed to Purify SMRTbell Templates.

Purify DNA

Bring the volume to 100 µL by adding Elution Buffer to the Adapter Ligation reaction and add 45 µL (0.45X) volume of AMPure PB beads.
Mix the bead/DNA solution by pipette mixing 15 times. Do not flick the tube.
Quick spin down the tube for 1 second to collect beads.
Incubate samples on bench top for 5 minutes at room temperature.
Place the tube in a magnetic bead rack to collect the beads to the side of the tube.

Wash the beads on the magnetic stand freshly prepared 80% ethanol solution and incubate for 30 s, then remove the ethanol and repeat the wash, repeat this step one more time (2 washes).

Remove any trace of ethanol. If necessary, do a quick spin, so both beads and ethanol are at the bottom of the tube, place it at the magnetic rack and pipette the remaining ethanol. Check for droplets and remove them.

Immediately add 100 uL of elution buffer to the beads. Pipette mix 15 times. Do not flick the tube.
Elute the DNA by letting the mix incubate at 37°C for 15 min. Spin down the tube, then place the tube back on the magnetic bead rack.
Let beads separate fully. Then without disturbing the beads. Transfer supernatant to a new 1.5 mL loBind tube.

Second round of purification
Add 45 µL (0.45X) volume of AMPure PB beads.
Mix the bead/DNA solution by pipette mixing 15 times. Do not flick the tube.
Quick spin down the tube for 1 second to collect beads.
Incubate samples on bench top for 5 minutes at room temperature.
Place the tube in a magnetic bead rack to collect the beads to the side of the tube.

Wash the beads on the magnetic stand freshly prepared 80% ethanol solution and incubate for 30 s, then remove the ethanol and repeat the wash, repeat this step one more time (2 washes).

Remove any trace of ethanol. If necessary, do a quick spin, so both beads and ethanol are at the bottom of the tube, place it at the magnetic rack and pipette the remaining ethanol. Check for droplets and remove them.

Add 10 uL of elution buffer to the beads. Pipette mix 15 times. Do not flick the tube.
Elute the DNA by letting the mix incubate at 37°C for 15 min. Spin down the tube, then place the tube back on the magnetic bead rack.
Let beads separate fully. Then without disturbing the beads. Transfer supernatant to a new 1.5 mL loBind tube. Discard the beads and proceed to quality check.

Verify your DNA amount and concentration using a Qubit quantitation platform. – Using 1 µL of the purified sample, make a 1:1 dilution in EB. – Use 1 µL of this 1:1 dilution to measure the DNA concentration using a Qubit fluorometer and the dsDNA HS Assay kit according to the manufacturer's recommendations.
Use the other 1 µL of 1:1 diluted sample for DNA sizing QC in Bioanalyzer.
AMPure PB bead purified SMRTbell libraries may be stored at -20°C until ready for sequencing.

Reagents, manufacturer and catalog number

Name	Manufacturer	Cat #
RNeasy Mini Kit	Qiagen	74104
Exonuclease kit	Lucigen	TER51020
RNA concentration and clean up	Zymo	11-325
RNAse Inhibitor	Fisher Scientific	NC9914916
dNTP mix	New England biolabs	N0447S
SuperScriptIV reverse transcriptase	Thermo scientific	18090050
SuperScript IV first-strand buffer	Thermo scientific	Included with SuperScript II
Betaine	Sigma-Aldrich	B0300-1 VL
$MgCL_2$	Any vendor	
Template switching oligos (TSO)	Exiqon	
DEPC water	Life technologies	750023
KAPA HiFi HotStart Ready Mix	Kapa Biosystems	KK2601
Nuclease-free water	Hypure	16750
SMRTbellTM Express template prep kit Template Prep Kit 2.0	Pacific Biosciences	101-737-500
Ampure® XP	Beckman Coulter	A63880
Ampure® PB Insert	Pacific Biosciences	100-265-900
SIRV set3	Lexogen	

References

Smrt-Seq2: Simone Picelli, Åsa K Björklund, Omid R Faridani, Sven Sagasser, Gösta Winberg & Rickard Sandberg, 2014, Full-length RNA-seq from single cells using Smart-seq2, Nature Protocols 9, 171–181.

RNeasy protocol: https://www.qiagen.com/us/resources/resourcedetail?id=c8cdc6bf-5bbf-4e3b-a0f4-476da9215012&lang=en

PacBio protocol version: Part Number 101-730-400 Version 02 (April 2019).

https://www.pacb.com/wp-content/uploads/Procedure-Checklist-Preparing-SMRTbell-Libraries-Using-Express-Template-Prep-Kit-2.0-With-Low-DNA-Input.pdf