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

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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 sequences 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 still includes an exonuclease treatment to remove transcripts without a cap, but the clean-up relies on RNA-XP beads. cDNA synthesis was done using a shorter TSO sequence. We include a priming reaction and for first strand synthesis we added a Hot start priming reaction and recommend using the Maxima H(-) RT. For PCR amplification we use the SeqAmp polymerase. We recommend using ProNex beads for cDNA and library clean ups.

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.

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)

30C for 60 min in the thermocycler

Cleanup on RNA-XP beads

Add 36 uLs RNA-XP beads

Incubate at 4C for 15 minutes

Magnet 5 minutes, then decant supernatant

Wash pellet on magnet 2X with 200 uLs 80% freshly made ethanol, 30 seconds each wash.

After second wash, quick spin, remagnet, and draw off remaining ethanol with thin pipette tip

Air dry no more than 5 minutes

Elute in 11 uLs Ultra Pure (UP) H2O, 5 minutes at room temperature

Recover 10 uLs eluted exonuclease-treated total RNA.

Proceed directly to priming reaction and cDNA synthesis, do QC analysis later.

Reserve 2.5 uLs for later Qubit and BioAnalyzer QC checks in -80 freezer. Can run BioAnalyzer on mRNA mode to check % rRNA contamination but do this after you have taken the RNA forward to the first strand reaction.

Preparing priming reaction and adding exogenous reference transcripts

Priming reaction: (assemble at 4C)

Amount	Reagent
2 ul	dilute RNAse inhibitor (1 uL RNAse inhibitor + 9.5 uLs UP H2O)
1 ul	dNTPs (10mM from kit)
0.5 ul	dilute SIRV spikes (240 pg/uL working stock)
0.5 ul	UP H2O
2 ul	exonuclease-treated RNA

Prepare first strand cDNA synthesis reaction: (assemble at 4C)

Prepare this reaction just before beginning the priming step in the cycler, but do not add the Maxima RT mix:

4 uLs 5X RT buffer

2 uLs TSO oligo (10 uM stock concentration)

6 uLs UPH2O

Amount	Reagent
4 ul	5X RT buffer
2 ul	TSO oligo (10 uM stock concentration)
6 ul	UP H2O
1 ul	Maxima H(-) reverse transcriptase

IMPORTANT: Add the Maxima H(-) mix until RNA has been primed. When you add the RT, be sure the other components in the mix have been moved to the 50C cycler to come to temperature.

Hot start priming reaction in cycler at 72C, 3 minutes

Ramp down to 50C

Keeping priming reaction tube in the block at 50C, add 1 uL oligo dT(stock conc'n 10nM) and mix with pipet, close tube.

Hold at 50C for 3 minutes while priming.

First strand synthesis:

Transfer the first strand reaction reagents mix tube without the RT to the 50C block while the RNA is priming.

After 1 minute, add the Maxima H (-) mix to the first strand reaction reagents still on the 50C block and mix with pipet. Close the tube.

After the 3 minutes of priming is complete, add 13 uLs of the first strand reaction mix containing the Maxima H(-) to the primed RNA.

Extend at 50C for 90 minutes

Denature at 85C for 5 minutes

4C for ever

Spin down and hold at 4C

PCR amplification reaction:

Amount	Reagent
20 ul	first strand reaction
25 ul	2X reaction buffer'
1 ul	IS primer (10 nM stock concentration)
3 ul	DEPC H2O
1 ul	SeqAmp polymerase

PCR Amplification program:

Step	Temperature	Time
1	95 C	1 min
2	98 C	15 sec
3	65 C	30 sec
4	68 C	13 min
Return to step 2 another 10 times		
5	72 C	10 min
6	4 C	On hold

Clean up PCR reaction on Ampure XP beads:

Add 90 uLs SPRI beads to PCR reaction and mix

Incubate 5 minutes at room temperature

Magnet 5 minutes, then decant supernatant

Wash pellet on magnet 2X 30 seconds with 200 uLs freshly prepared 80% ethanol

After second wash and decant, quick spin and then magnet. Use fine tip to remove residual ethanol.

Air dry no longer than 5 minutes.

Elute in 43 uLs elution buffer (EB) for 5 minutes at room temperature.

Magnet 5 minutes and recover 42 uLs

Reserve 2.5 uLs in freezer for Qubit and BioAnalyzer analysis.

PacBio Template preparation

To achieve a final loading concentration for the Sequel II we suggest a starting amount of 500 ng.

Damage repair reaction:

Amount	Reagent
X ul to make the reaction 57 ul	DEPC H20
7 ul	DNA prep buffer
Up to 40 ul	Amplified library
0.6 ul	NAD
2 ul	DNA damage repair

Thermocycler:

Temperature	Time
37C	30 min
4C	On hold

End repair/A-tail reaction:

Add 3 uLs End Prep mix to 57 uLs of repair reaction

Thermocycler:

Temperature	Time
20C	30 min
65C	20 min

4C	On hold

Adapter Ligation mix:

Amount	Reagent
60 ul	A-tail reaction (Previous reaction)
3 ul	Adapter

Mix with pipet (important!) before adding remaining reagents

Amount	Reagent
30 ul	Ligation mix
1 ul	Ligation enhancer
1 ul	Ligation additive

Thermocycler:

Temperature	Time
20C	60 min
4C	On hold

SMRT Bell cleanup with Pronex beads:

Add 95 uLs Pronex beads

Mix with pipet, brief spin to collect

Incubate 5 minutes in solution

Magnet 5 minutes, then decant

Wash 2X on magnet with 200 uLs of 80% freshly prepared ethanol, 30 seconds each

After second wash and decant, quick spin and then magnet. Use fine tip to remove residual ethanol.

Quick air dry only, very brief.

Elute in 12 uLs elution buffer (EB) 5 minutes at room temperature

Magnet 5 minutes

Recover 11 uLs

Reserve 2 uLs for Qubit and BioAnalyzer.

Reagents, manufacturer and catalog number

Name	Manufacturer	Cat #
RNeasy Mini Kit	Qiagen	74104
Exonuclease kit	Lucigen	TER51020
RNAse Inhibitor	Fisher Scientific	NC9914916
dNTP mix	New England biolabs	N0447S
Maxima H Minus Reverse Transcriptase (200 U/µL)	ThermoScientific	EP0752
ProNex® Size-Selective Purification System	Promega	NG2001
Betaine	Sigma-Aldrich	B0300-1 VL
MgCl2	Any vendor	
Template switching oligos (TSO) – 5′-AAGCAGTGGTATCAACGCAGAGTACrGrG+G-3′		
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 beads	Beckman Coulter	A63880
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 (October 2019).