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PacBio SMRTbell Library Preparation Using Kit 3.0 - Ultra Low Input Protocol

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We use this protocol and it's working

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Disclaimer

The duration times presented are estimations only.

Abstract

This is a modified protocol for preparing PacBio SMRTbell libraries with Prep kit 3.0 of ultra low input DNA samples.

This protocol is intended for 80 ng of gDNA in a 50 µL sample volume when going into section "Shearing genomic DNA using Megaruptor 2 system", but you can have up to 100 ng in 46 µL when going into section "Repair and A-tailing".

The protocol is based on Preparing HiFi SMRTbell Libraries from Ultra-Low DNA Input (Version 02 November 2021), but uses SMRTbell Prep Kit 3.0 instead of SMRTbell Express TPK 2.0. Other major change to the original protocol:

- adding $MgCl_2$ and changing incubation times at adapter ligation

Attachments

DOCX

SMRTbell prep kit 3....

27KB

Materials

- PacBio SMRTbell Prep Kit 3.0
- SMRTbell Clean-up beads
- Freshly prepared 80% ethanol
- Pipettes, standard and wide-bore pipette tips
- Microcentrifuge tubes, strips
- $MgCl^2$
- Qubit fluorometer + Qubit 1X dsDNA HS Assay
- Megaruptor 2 system + Long Hydropores - or an equivalent shearing method
- Pippin Pulse Gel Electrophoresis + Quick-Load 1 kb Extend DNA Ladder - or an equivalent method of determining size distribution
- Minicentrifuge
- Vortexer
- Magnetic separation rack
- 5200 Fragment Analyzer System - or an equivalent method of determining size distribution
- Two thermocyclers

Safety warnings

The operator must wear a lab coat, powder-free nitrile gloves and safety specs to perform the laboratory procedures in this protocol.

Waste needs to be collected in a suitable container and disposed of in accordance with local regulations.

Liquid waste needs to be collected in a suitable container and disposed of in accordance with local regulations.

See best practices, thawing and handling instructions, and safety instructions for all the kit reagents, as well as instructions and safety data sheets for other reagents used in this protocol **before starting**.

Input DNA quality control

8h 40m

1 Bring the Qubit 1X dsDNA HS working solution and standards to room temperature. 30m

2 Pulse vortex or pipette mix each sample to homogenize the DNA in solution.

3 Quick spin each sample to collect liquid.

4 Take a 1 µl aliquot from each sample and measure DNA concentration with a Qubit fluorometer using the 1X dsDNA HS kit. 10m

Required

Concentration [ng/µL]

Yield [ng]

5 Resolve DNA size distribution with Pippin Pulse Gel Electrophoresis using 50 - 100 ng of DNA (gDNA) and Quick-Load 1 kb Extend DNA Ladder (size range: 0.5 kb to 48.5 kb). 8h

> Note
>
> Also other suitable methods can be used to determine the size distribution.

6 Proceed to the next section of the protocol if sample quality is acceptable.

SAFE STOPPING POINT - Store at 4°C

Shearing genomic DNA using Megaruptor 2 system + QC

3h

7 If using the Megaruptor 2, ensure that the system is thoroughly washed before shearing. Since this workflow requires amplification, contamination introduced during shearing will be amplified which complicates assembly.

Note

Also other suitable methods can be used to shear the genomic DNA.

7.1 We recommend using 80 ng gDNA in 50 µl for shearing with Megaruptor 2.

Sample volumes between 50 µl and 200 µl can be processed, but please note that there is no clean-up step after shearing in this protocol and you will need 46 µl sample volume to the End repair and A-tailing reaction after the shearing QC.

7.2 Shear gDNA using Long Hydropores and have the Long Hydropore option selected in the Megaruptor software.
- Choose at target shear size of 10 kb in the Megaruptor software setting.
- In the beginning of next section, a single sample is required to have a volume of 46.0 µl.

8 Take a 1 µl aliquot from each sample and measure DNA concentration with a Qubit fluorometer using the 1X dsDNA HS kit.

Required
Concentration [ng/µl]

Yield [ng]

9 Measure DNA size distribution with a 5200 Fragment Analyzer System.

Note

Also other suitable methods can be used to resolve the sample's size distribution.

10 Proceed to the next section of the protocol.

Repair and A-tailing

45m

11 Before starting with the procedure, refer to the table in the Reagent handling section for recommendations surrounding SMRTbell prep kit 3.0 reagent handling. 5m

Use PCR strip tubes throughout this procedure.

To prepare Reaction mix 1 (RM1) master mix, add the following components (see table) in the order and volume listed to a new tube. Adjust component volumes for the number of samples being prepared, plus 10% overage. For individual preps, add components directly to the 46 µl sheared DNA sample at the specified volumes and skip step 13.

	Kit reagent	Volume / sample	Volume / sample into RM1 master mix
	Repair Buffer	8 µl	8.8 µl
	End repair mix	4 µl	4.4 µl
	DNA repair mix	2 µl	2.2 µl
	Total volume / sample	14 µl	

Reagents for Repair and A-tailing, and recipe for RM1 master mix

12 If you've prepared RM1 master mix: 1m

- Pipette mix RM1.
- Quick spin RM1 to collect liquid.
- Add 14 µl of the RM1 to each sample.

13 The total reaction volume should now be 60 µl. 1m

Pipette-mix 10 times each sample with wide-bore pipette tips and quick-spin the tube strip to collect liquid.

14 Run the Repair and A-tailing thermocycler program 00:36:00 . 37m

	Time	Temperature
	30 min	37°C
	5 min	65°C
	Hold	4°C

Repair and A-tailing thermocycler program

15 Proceed to the next section of the protocol.

Ligate PCR adapters and clean-up

3h 15m

16 To ligate the Amplification adapter to the repaired sample, the Amplification adapters must first be diluted 1:10 with Duplex buffer. You will need 2.5 µl Diluted Amplification adapter per sample. Mix well and quick spin.

2m

	Reagent	Volume for 1-3 samples
	Duplex buffer	9 µl
	Amplification adapter	1 µl
	Total volume	10 µl

Dilution of Amplification adapter

Note

The diluted Amplification adapter should be used immediately, and should not be stored.

17 With the reaction on ice, quick-spin samples in the tube strip to collect liquid and add the components below in the order listed, directly to the repaired and A-tailed sample from step 16.

10m

	Reagent	Volume / sample
	Diluted Amplification Adapter	2.5 µl
	Ligation Mix	24.39 µl
	Ligation enhancer	1 µl
	MgCl2	5.61 µl (final conc. 3 mM)

Reagents for PCR adapter ligation

18 The total reaction volume should now be 93.5 µl. 1m

Pipette mix 10 times with wide-bore pipette tips and quick-spin the tube strip to collect liquid.

19 Run the adapter ligation thermocycler program 2h 32m

	Time	Temperature
	140 min	20°C
	10 min	65°C
	Hold	4°C

Adapter ligation thermocycler program

20 Add 76.7 µl of SMRTbell cleanup beads to the 93.5 µl to each sample and gently pipette mix 10 times. Quick spin the tube strip in a minicentrifuge to collect all liquid from the sides of the tubes. 3m

Note

SMRTbell Clean-up beads must be brought to room temperature for 30 to 60 mins prior to use.

21 Incubate on bench for 00:10:00 at room temperature to allow DNA to bind beads. 10m

22 Place samples on a magnet stand and wait until supernatant is clear. Use a P200 pipette to remove the supernatant. 2m

23 While the strip is on magnet, slowly dispense 200 µl, of freshly prepared 80% ethanol into each tube. After 30 seconds, pipette off the 80% ethanol and discard. 1m

24 Repeat the previous step. 1m

25 Remove residual 80% ethanol: 3m

- Remove tube strip from the magnetic separation rack.
- Quick spin tube strip in a minicentrifuge.
- Place tube strip back in a magnetic separation rack until beads separate fully from the solution.
- Pipette off residual 80% ethanol with a P20 pipette and discard. Do not let the beads to dry out.

26 Remove the tube strip from the magnetic stand. Immediately add 97 µl of Elution buffer and pipette mix 10 times to resuspend. Do not let the beads to dry out. Quick spin to collect all liquid from the sides of the tube. 2m

27 Incubate at room temperature for 00:05:00 to elute the DNA from the beads. 5m

28 Place the tube strip on the magnetic stand to separate the beads from the supernatant. When the supernatant is clear, transfer 97 µl of the purified eluted sample into a to a new tube and set aside on ice until ready to use. 3m

29

Note

For the Library amplification by PCR, the 97 µl of the purified eluted sample will be **divided** and used for **two** reactions (Reaction Mix 3A and Reaction Mix 3B).

Each reaction requires a volume of 48 µl of the purified eluted sample.

Proceed to the next section of the protocol.

SAFE STOPPING POINT - Store at 4°C

Library amplification by PCR and clean-up 4h 13m

30 Divide the sample from previous step to two reactions, 48 µl each (Reaction Mix 3A and Reaction Mix 3B). 1m

In this section, one reaction will be amplified with PCR Master Mix 1 and the other with PCR Master Mix 2.

Note

The two reactions will go through two separate thermocycler programs, i.e. you will need to run two thermocyclers

31 On ice, prepare Reaction Mix 3A and Reaction Mix 3B. 4m

When working with multiple samples, prepare enough Master Mix for all reactions, plus 10% of the total reaction mix volume. Pipette mix 10 times with wide-bore pipette tips and then perform a quick spin to collect all liquid from the sides of the tube.

	Reagent	Volume per sample	V/sample into a master mix
	PCR Master Mix 1	50 µl	55 µl
	Amplification PCR primer	2.0 µl	2.2 µl
	Total volume	52.0 µl	

Recipe for Reaction Mix 3A

	Reagent	Volume per sample	V/sample into a master mix
	PCR Master Mix 2	50 µl	55 µl
	Amplification PCR primer	2.0 µl	2.2 µl
	Total volume	52.0 µl	

Recipe form Reaction Mix 3B

32 On ice, add 52 µl of Reaction Mix 3A to one 48 µl reaction of eluted DNA, and 52 µl of Reaction Mix 3B to the other reaction, for a total volume of 100 µl for each reaction. Pipette mix 10 times with wide-bore pipette tips and then perform a quick spin to collect all liquid from the sides of the tube 2m

33 Place the samples in thermocyclers and run the following programs (lid 105°C). The PCR reactions may be left at 4°C overnight. 2h 25m

	Time	Temperature	# Cycles
	45 seconds	98°C	1
	10 seconds	**98°C**	
	15 seconds	**62°C**	**13**
	7 minutes	**72°C**	

	Time	Temperature	# Cycles
	5 minutes	72°C	1
	Hold	4°C	

PCR program for Reaction Mix 3A. The times and temperatures for the part of the program that repeats for 13 cycles are in bold.

	Time	Temperature	# Cycles
	30 seconds	98°C	1
	10 seconds	**98°C**	
	15 seconds	**60°C**	**13**
	10 minutes	**68°C**	
	5 minutes	68°C	1
	Hold	4°C	

PCR program for Reaction Mix 3B. The times and temperatures for the part of the program that repeats for 13 cycles are in bold.

34 Add 82 µl of SMRTbell cleanup beads to the 100 µl PCR Reaction Mix 3A reaction, and to the 100 µl PCR Reaction Mix 3B reaction each, and gently pipette mix 10 times. Perform a quick spin to collect all liquid from the sides of the tube. 3m

Note

SMRTbell cleanup beads must be brought to room temperature for 30 to 60 mins prior to use.

35 Incubate on bench for 00:10:00 at room temperature to allow DNA to bind beads. 10m

36 Place tube strip in a magnetic separation rack until beads separate fully from the solution. Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant. 3m

37 While the strip is on magnet, slowly dispense 200 µl, or enough to cover the beads, of freshly prepared 80% ethanol into each tube. After 30 seconds, pipette off the 80% ethanol and discard. 2m

38 Repeat the previous step. 1m

39 Remove residual 80% ethanol: 2m
- Remove tube strip from the magnetic separation rack.
- Quick spin tube strip in a minicentrifuge.
- Place tube strip back in a magnetic separation rack until beads separate fully from the solution.
- Pipette off residual 80% ethanol with a P20 pipette and discard.
- Do not let the beads to dry out.

40 Remove the tube strip from the magnetic stand. Immediately add 26 µl of Elution buffer and pipette mix 10 times to resuspend. Quick spin to collect all liquid from the sides of the tube. 2m

41 Incubate on bench for 00:05:00 at room temperature to elute the DNA from the beads. 5m

42 Place the tube on the magnetic stand to separate the beads from the supernatant. When the supernatant is clear, transfer 26 µl of eluted amplified DNA reaction to a new tube and set in ice until ready to use. 3m

43 Use 1 µl of sample to quantify with Qubit dsDNA HS kit. 10m

Required
Concentration [ng/µl]

Required
Yield [ng]

If you have **500 ng or more** of purified and amplified DNA, in total after the two amplification reactions, proceed to the next section of the protocol.

If you have less than 500 ng, see next step for recommendations on further amplification.

44 Recommendations for samples with low yield: 1h

- If the total mass < 500 ng (c<19.2 ng/µl) → 1 additional cycles as described in Appendix 1 of the original protocol.
- If the total mass < 275 ng (c<11 ng/µl) → 2 additional cycles as described in Appendix 1 of the original protocol.
- If the total mass <130 ng (<5 ng/µl) → 3 additional cycles as described in Appendix 1 of the original protocol.
- If the total mass < 65 ng (<2.5 ng/µl) → 5 additional cycles as described in Appendix 1 of the original protocol. **Not applicable to Sequel II or Sequel IIe runs for genome size ≤ 1Gb.**

Pool amplified DNA 5m

45 In this section, the amplified DNA from the PCR Reaction Mixes 3A and 3B are pooled together in equal mass quantities. The pooled DNA can then be constructed into a SMRTbell library as a single sample. 5m

Pooling Best Practices:

- Always quantify samples before pooling. Since DNA amounts may be limited at this step, PacBio recommends using the Qubit dsDNA High Sensitivity Assay Kit for concentration measurements.
- To ensure that there is sufficient material for library construction and size selection, the total mass of the pooled DNA **must be 500-1000 ng in 46.0 µl** (e.g., 250 ng of PCR Reaction Mix 3A + 250 ng of PCR Reaction Mix 3B).

45.1 Pool the purified, amplified DNA into a single PCR tube of an 8-tube strip. Mix and spin down the contents of the tube with a quick spin.

45.2 Proceed to the “Repair and A-tailing” section below.

Repair and A-tailing of amplified DNA 40m

46 For each sample to be processed, add the following components in the order listed to a single PCR tube of an 8-tube strip on ice containing 46 µL pooled, amplified DNA: 2m

	Reagent	Volume per sample
	Repair buffer	8 µL
	End repair mix	4 µL

	Reagent	Volume per sample
	DNA repair mix	2 µL
	Total volume / sample	14 µL

Reagents for Repair and A-tailing

If you have many samples, you can also prepare an RM1 master mix as in step 12

go to step #11

47 The total reaction volume should now be 60 µl. 1m

Pipette-mix 10 times each sample with wide-bore pipette tips and quick-spin the tube strip to collect liquid.

48 Run the Repair and A-tailing thermocycler program 00:36:00 37m

	Time	Temperature
	30 min	37°C
	5 min	65°C
	Hold	4°C

Repair and A-tailing thermocycler program

49 Proceed to the next section of the protocol.

Adapter ligation and clean-up 3h 5m

50 Add 4 µL of SMRTbell adapter (non-barcoded) to each sample from the previous section.

51 Add the following components in the order and volume listed below to a new microcentrifuge tube. Adjust component volumes for the number of samples being prepared, plus 10% overage. For individual preps, add components directly to each sample from the previous step in the order and volume listed below, and skip step 53.

	Reagent	Volume per sample	V/sample into a master mix
	Ligation mix	24.3 µl	26.73 µl

	Reagent	Volume per sample	V/sample into a master mix
	Ligation enhancer	1 µl	1.1 µl
	MgCl2	5.7 µl (final concentration 3mM)	6.27 µl
	Total volume/sample	31 µl	

Reagents for Adapter ligation, and recipe for RM2 master mix

52 If you've prepared RM2 master mix: 1m
- Pipette mix RM2.
- Quick spin RM2 to collect liquid.
- Add 31 µl of RM2 to each sample from previous step.

53 The total reaction volume should now be 95 µl. 1m

Pipette mix each sample and quick-spin the tube strip to collect liquid.

54 Run the adapter ligation thermocycler program 02:31:00 2h 32m

	Time	Temperature
	140 min	20°C
	10 min	65°C
	Hold	4°C

Adapter ligation thermocycler program

55 Add 95 µl of resuspended, room-temperature SMRTbell cleanup beads to each sample and pipette mix until evenly distributed. Quick spin the tube strip to collect all liquid from the sides of the tubes. 3m

Note

SMRTbell Clean-up beads must be brought to room temperature for 30 to 60 mins prior to use.

56 Leave at room temperature for 00:10:00 to allow DNA to bind beads. 10m

57 Place tube strip in a magnetic separation rack until beads separate fully from the solution. Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant. 3m

58 While the strip is on magnet, slowly dispense 200 µl, or enough to cover the beads, of freshly prepared 80% ethanol into each tube. After 30 seconds, pipette off the 80% ethanol and discard. 2m

59 Repeat the previous step. 1m

60 Remove residual 80% ethanol: 2m

- Remove tube strip from the magnetic separation rack.
- Quick spin tube strip in a minicentrifuge.
- Place tube strip back in a magnetic separation rack until beads separate fully from the solution.
- Pipette off residual 80% ethanol and discard.

61 Remove tube strip from the magnetic rack. Immediately add 40 µl of Elution buffer to each tube and resuspend the beads. Quick spin the tube strip. 2m

62 Incubate at room temperature for 00:05:00 to elute DNA. 5m

63 Place tube strip in a magnetic separation rack until beads separate fully from the solution. Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a new tube strip. Discard old tube strip with beads. 3m

64 Proceed to the next section of the protocol.

Size selection of the SMRTbell library with Blue Pippin 20h

65 For constructing SMRTbell libraries from ultra-low DNA input, PacBio recommends size-selection using the BluePippin or the PippinHT systems (from Sage Science). In this protocol we describe how we use BluePippin.

The average size distribution of the final size-selected library is approximately 10 kb - 11 kb. Typical recovery yields after size-selection are 20% to 30% (from input of purified

SMRTbell library) and are highly dependent on the size distribution of the starting SMRTbell library.

66 Prepare SMRTbell libraries in a final volume of 30 µl of elution buffer for each BluePippin lane.

67 Bring the Loading solution to room temperature and then, add 10 µl of Loading solution to each 30 µl DNA sample from the previous section. The loading solution is viscous, so pipette slowly to ensure complete transfer into the DNA sample. Mix well by gentle pipetting and spin briefly to collect the contents at the bottom of the tube.

68 Follow the manufacturer's recommendations to set up a run protocol.

- Select the "0.75% DF 3-10 kb Marker S1 - Improved Recovery" Cassette Definition File for your sample.
- Using the "Range" selection mode, enter a desired "BP Start" value of 7000 and a "BP End" value of 17000.
- Be sure to assign a marker lane.

Note

We do not recommend running lanes with <400 ng of SMRTbell library material.

69 Load S1 marker and samples into the BluePippin gel cassette and start the run. Run time is approximately 4.0 hours, but to maximize recovery of eluted DNA, unselect "End Run when Elution is Completed" and wait overnight after the run is finished before removing the sample from the elution chamber.

70 After collecting the eluate, wash the eluate chamber with 40 µl of Sage Science's 0.1% Tween-20 Wash Solution and then combine the recovered wash solution with the eluted sample. Washing the elution well may further increase recovery yields by approximately 10-20%.

Expected result

The total volume after pooling the recovered wash with the original eluted sample is ~80 µL.

71 Measure the volume of your size-selected sample (eluate plus wash) by taking a 1 µl aliquot from each sample and measure DNA concentration with a Qubit fluorometer using the 1X dsDNA HS kit.

Required
Concentration [ng/µl]

Yield [ng]

72 Measure DNA size distribution with a 5200 Fragment Analyzer System.

Note

Also other suitable instruments can be used to measure DNA size distribution.

73 Proceed to the “Purification of the size-selected SMRTbell library” step.

Purification of size-selected SMRTbell library

35m

74 Add 1.0X v/v (volume over volume) of resuspended, room-temperature SMRTbell cleanup beads to each sample from the previous step. 1m

75 Pipette mix the beads until evenly distributed and quick spin to collect all liquid.

76 Leave at room temperature for 00:10:00 to allow DNA to bind beads. 10m

77 Place tube strip in a magnetic separation rack until beads separate fully from the solution. 3m

78 Slowly pipette off the cleared supernatant without disturbing the beads. It is recommended to save the supernatant in another tube strip in case of poor DNA recovery.

79 Slowly dispense 200 µL, or enough to cover the beads, of freshly prepared 80% ethanol into each tube. After 30 seconds, pipette off the 80% ethanol and discard. 2m

80 Repeat the previous step. 2m

81 Remove residual 80% ethanol: 1m
• Remove tube strip from the magnetic separation rack.
• Quick spin tube strip in a microcentrifuge.
• Place tube strip back in a magnetic separation rack until beads separate fully from the solution.
• Pipette off residual 80% ethanol and discard.

82 Remove tube strip from the magnetic rack. Immediately add 15 µL of elution buffer to each tube and resuspend the beads by pipetting 10 times or until evenly distributed. Quick spin the tube strip to collect liquid. 1m

83 Leave at room temperature for 00:05:00 to elute DNA. 5m

84 Place tube strip in a magnetic separation rack until beads separate fully from the solution. Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a new tube strip. Discard old tube strip with beads.

85 Take a 1 µl aliquot from each tube and dilute with 9 µl of elution buffer or water. Measure DNA concentration with a Qubit fluorometer using the 1x dsDNA HS kit. Calculate the total mass. 10m

Required
Concentration [ng/µl]

Required
Yield [ng]

86 Proceed to SMRT Link Sample Setup to prepare the SMRTbell library for sequencing.

Note

Store SMRTbell libraries at 4°C if sequencing within the week. Long-term storage should be at -20°C. Minimize freeze-thaw cycles when handling SMRTbell libraries.

Protocol references

This protocol is based on Preparing HiFi SMRTbell Libraries from Ultra-Low DNA Input (Version 02 November 2021).