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PacBio SMRTbell Library Preparation Using Kit 3.0

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CHIARA NATALI[1], Claudio Ciofi[1]

[1]Department of Biology, University of Florence

Biodiversity Genomics E...

Tuuli Lundbäck-Larva
Department of Immunology, Genetics and Pathology, SciLifeLab...

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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. The protocol is based on Preparing whole genome and metagenome libraries using SMRTbell Prep Kit 3.0 (April 2022).

The main differences are:

- adding $MgCl_2$ and changing incubation times at adapter ligation
- changing reagent amounts and incubation times at nuclease treatment

Attachments

DOCX

SMRTbell prep kit 3....

21KB

Materials

- PacBio SMRTbell Prep Kit 3.0
- SMRTbell Clean-up beads
- Freshly prepared 80% ethanol
- Low TE buffer
- Microcentrifuge tubes, strips
- $MgCl^2$
- Pipettes and pipette tips
- Qubit fluorometer + Qubit 1X dsDNA HS Assay kit
- Megaruptor 2 system - or an equivalent shearing method
- Pippin Pulse Gel Electrophoresis + Quick-Load 1 kb Extend DNA Ladder (size range: 0.5 kb to 48.5 kb) - or an equivalent method for determining fragment size distribution
- Minicentrifuge
- Magnetic separation rack
- 5200 Fragment Analyzer System - or an equivalent method for determining size distribution
- Thermocycler
- Vortexer

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**.

Before start

If using Megaruptor 2, ensure that the system is thoroughly washed before shearing.

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 instruments can be used to determine 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

7 This protocol utilizes the Megaruptor 2 system for shearing.

Note

Also other suitable methods can be used to shear the samples.

7.1 Shear gDNA to an appropriate fragment length to optimize HiFi sequencing yield and read accuracy. Fragments that are too short produce less yield per read, and fragments that are too long may result in lower read accuracy and are less likely to produce HiFi reads.

7.2 Microbial and metagenomics samples may forgo shearing if the DNA is already in the specified fragment length range (7 kb –12 kb). In such cases, proceed to the "Clean-up with 1X SMRTbell Clean-up beads after shearing" to get the appropriate input amount in the correct volume and buffer go to step #10

8 Shear DNA on the Megaruptor 2 system.
- Select fragment size between 20 Kb-25Kb.
- Process sample volumes between 50 µl and 200 µl.
- Target a concentration of 40 ng/µl.
- Recover sheared DNA into a tube strip.

Expected result

Shearing with Megaruptor 2 system will dilute your sample by 45 µl.

9 Proceed to the next section of the protocol.

Clean-up with 1x SMRTbell Clean-up beads after shearing + QC

2h 20m

10 Add 1.0X v/v (volume over volume) of resuspended, room-temperature SMRTbell cleanup beads to each tube of sheared DNA. 1m

11 Pipette mix the beads until evenly distributed. 1m

12 Quick spin the tube strip to collect liquid.

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

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

15 Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant. 1m

16 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

17 Repeat the previous step. 2m

18 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 with a P20 pipette and discard.

19 Remove the tube strip from the magnetic rack. Immediately add 47 µl of low TE buffer to each tube and resuspend the beads by pipetting 10 times or until evenly distributed. Quick spin the tube strip in a minicentrifuge to collect liquid. 2m

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

21 Place tube strip in a magnetic separation rack until beads separate fully from the solution. 2m

22 Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a new tube strip. Discard old tube strip with beads.

23 Recommended: Evaluate sample quality (concentration and size distribution). 1h 50m

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

Required
Concentration [µg/µl]

Yield [ng]

23.2 Take a 1 µl aliquot from each sample and measure DNA size distribution with a 5200 Fragment Analyzer System.

After taking the aliquot, add 1 µl Low TE buffer to the sample, so that the sample volume is 46 µl when proceeding to next step.

Note

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

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

SAFE STOPPING POINT - Store at 4°C

Repair and A-tailing

45m

25 To prepare Reaction mix 1 (RM1) master mix, add the following components (see table) in the order and volume listed 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 the sample from the previous step at the specified volumes and skip RM1 step 26.

5m

	Kit reagent	V per sample	V/sample into a 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

26 If you've prepared RM1 master mix: 1m
- Pipette mix RM1.
- Quick spin RM1 in a minicentrifuge to collect liquid.
- Add 14 µl of the RM1 to each sample.

27 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 in a minicentrifuge to collect liquid.

28 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

29 Proceed to the next section of the protocol.

Adapter ligation and clean-up 3h 15m

30 Add 4 µl of SMRTbell adapter (non-barcoded) or SMRTbell barcoded adapter 3.0 to each sample from the previous step. 1m

31 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 32. 8m

	Reagent	Volume per sample	V/sample into a master mix
	Ligation mix	24.3 µl	26.73 µl
	Ligation enhancer	1 µl	1.1 µl

	Reagent	Volume per sample	V/sample into a master mix
	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

32 If you've prepared RM2 master mix: 1m

- Pipette mix RM2.
- Quick spin RM2 in a microcentrifuge to collect liquid.
- Add 31 µl of RM2 to each sample from previous step.

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

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

34 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

35 Add 95 µl of resuspended, room-temperature SMRTbell cleanup beads to each sample and pipette mix until evenly distributed. 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.

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

37 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

38 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

39 Repeat the previous step. 1m

40 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 and discard.

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

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

43 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

44 Proceed to the next section of the protocol.

SAFE STOPPING POINT - Store at 4°C

Nuclease treatment 15m

45 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, then skip RM3 step 46. 3m

	Reagent	Volume per sample	V/sample into a master mix
	Nuclease buffer	2.5 µl	2.75 µl
	Nuclease mix	2.5 µl	2.75 µl
	Total volume/sample	5 µl	

Reagents for Nuclease treatment, and recipe for RM3 master mix

46 If you've prepared RM3 master mix: 2m

- Pipette mix RM3.
- Quick spin RM3 in a microcentrifuge to collect liquid.
- Add 5 µl of RM3 to each sample.

47 The total reaction volume should now be 45 µl. 1m

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

48 Run the nuclease treatment thermocycler program 00:08:00 . 9m

	Time	Temperature
	7 min	37°C
	Hold	4°C

Nuclease treatment thermocycler program

49 Proceed to the next step of the protocol.

Size-selection of the SMRTbell library with Blue Pippin 20h

50

Note

Also other size-selection methods can be used.

51 Prepare ~1.5 µg of SMRTbell library in a final volume of 30.0 µL (in elution buffer) for each BluePippin (Sage Science) lane.

52 Bring the Loading solution to room temperature, and then add 10 µL of the Loading solution to the 30 µL DNA sample and mix well. For loading multiple lanes with the same sample, scale up the volumes proportionally. The loading solution is viscous, so pipette slowly to ensure complete transfer into the DNA sample. Spin briefly to collect the contents at the bottom of the 1.5 mL tube.

53 Follow the manufacturer's recommendations to set up a run protocol.
- Select the "0.75% DF Marker S1 High-Pass 6–10kb vs3" Cassette Definition File.
- Using the "Range" selection mode, enter a desired "BPstart" value of 10000 and a "BPend" value of 50000.
- Be sure to assign a marker lane.

54 Load the S1 marker and samples into the gel cassette and start the run. Run time is approximately 4 hrs 30 mins, but to maximize recovery of eluted DNA unselect "End Run when Eluition is Completed" and wait overnight
after the run is finished before removing the sample from the elution chamber.

55
- Collect the eluate (~40 µl) into a new 1.5 mL tube.
- Wash the elution well with 40 µL PippinHT 0.1% Tween20 (supplied with the cassettes).
- Wait 1 minute and add the recovered wash liquid to the eluted sample.

Expected result

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

56 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]

57 Measure DNA size distribution with a 5200 Fragment Analyzer System

Note

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

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

Purification of the size-selected SMRTbell library

35m

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

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

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

62 Place tube strip in a magnetic separation rack until beads separate fully from the solution. 2m

63 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. 1m

64 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. 1m

65 Repeat the previous step. 1m

66 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.

67 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

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

69 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. 2m

70 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]

71 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 whole genome and metagenome libraries using SMRTbell prep kit 3.0" (April 2022 PN 102-166-600).