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PacBio Ampli-Fi - Amplifying genomic DNA for SMRTbell library preparation and HiFi sequencing

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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.

The protocol is based on Ampli-Fi - Amplifying genomic DNA for SMRTbell library preparation and HiFi sequencing (103-648-000 REV04 JUL2025).

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....

25KB

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
- Magnetic separation rack
- 5200 Fragment Analyzer System - or an equivalent method of 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**.

Input DNA quality control

8h 40m

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

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.

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 step 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 Use 100 ng of gDNA in a 51 µl sample volume for shearing.

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.
- A single sample requires a volume of 51 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. If the DNA size is >11 kb,
repeat step 8.

Note

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

10

Note

It is recommended to confirm gDNA is sheared to the appropriate size range (<11 kb) prior to proceeding. If the DNA is under-sheared, a second shear with the same parameters can be repeated. The same Megaruptor consumables can be used if a secondary shear is required.

11 Proceed to the “Repair & A-tailing” section.
SAFE STOPPING POINT - Store at 4°C

Repair and A-tailing

45m

12 Use PCR strip tubes throughout this procedure.

Use the following table to set up a reaction using around 100 ng of input sheared gDNA. For each sample to be processed, add the following components to a single PCR tube of an 8-tube strip on ice:

	Reagent	Volume
	Sheared DNA	49.0 µl
	Repair Buffer	8.0 µl
	End repair mix	2.0 µl
	DNA repair mix	1.0 µl
	Total volume	60.0 µl

Reagents for Repair and A-tailing

13 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 .

36m

	Time	Temperature
	30 min	37°C

	Time	Temperature
	5 min	65°C
	Hold	4°C

Repair and A-tailing thermocycler program

15 Proceed to the next step of the protocol.

Ligation of amplification adapter and clean-up

3h 15m

16 For individual preps, with the reaction on ice, add the components below in the order listed, directly to the sample in Reaction Mix 1:

	Reagent	Volume / sample
	Twist universal adapter	2 µl
	Ligation Mix	15.02 µl
	Ligation enhancer	1 µl
	MgCl2	4.98 µl (final concentration 3mM)

Reagents for amplification adapter ligation

17 The total reaction volume should now be 83 µl.

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

18 Run the adapter ligation thermocycler program 02:32:00 .

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

Adapter ligation thermocycler program

19 Add 83 µl of SMRTbell cleanup beads to each sample and gently pipette mix 10 times. Perform a quick spin to collect all liquid from the sides of the tube.

Note

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

20 Incubate at 25°C for 00:15:00 . 15m

21 Place on a magnet stand and wait until supernatant is clear. Use a P200 pipette to remove the supernatant.

22 While on magnet, wash two times with 200 µl of freshly prepared 80% ethanol. After removal of second wash of 200 µl of ethanol, spin the tube strip briefly, return to magnetic stand and remove residual ethanol with a P20 pipette. Do not let the beads to dry out.

23 Remove the tube strip from the magnetic stand. Immediately add 25 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. Place at 25°C for 00:07:00 to elute the DNA from the beads. 7m

24 Place the tube on the magnetic stand to separate the beads from the supernatant. When the supernatant is clear, transfer 25 µl of the purified eluted sample into a to a new tube and set aside on ice until ready to use. Discard old tube strip with beads.

25 Take a 1 µl aliquot from each sample and measure DNA concentration with a Qubit fluorometer using the 1X dsDNA HS kit to define number of PCR cycles.

Required
Concentration [ng/µl]

Yield [ng]

	gDNA input	# PCR cycles
	1 ng	14 cycles
	5 ng	12 cycles
	10 ng	11 cycles
	20 ng	10 cycles
	50 ng	8 cycles

Table to decide the number of PCR cycles based on the input amount.

Library amplification by PCR and clean-up

4h 15m

26 To prepare Amplification 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, on ice, add components directly to the sample from the previous step at the specified volumes:

	Reagent	Volume	V / sample into a master mix
	2x Xtreme Buffer	50 µl	55 µl
	dNTP (2 mM each)	20 µl	22 µl
	KOD Xtreme Hot Start DNA polymerase	2 µl	2.2 µl
	Twist UDI Primers	4 µl	4.4 µl
	Total volume / sample	76 µl	

Reagents for amplification by PCR, and recipe for Amplification master mix

27 If you've prepared Amplification master mix:

- Pipette mix Amplification master mix
- Quick spin Amplification master mix to collect liquid.
- On ice, add 76 µl of the Amplification master mix to 24 µl of sample for a total volume of 100 µl.

28 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

29 Place in a thermocycler and run the following program (lid 105°C).
The PCR reactions may be left at 4°C overnight.

	Time	Temperature	# PCR cycles
	2 minutes	94°C	1
	10 seconds	**98°C**	
	30 seconds	**58.8°C**	**8-14**
	10 minutes	**68°C**	
	7 minutes	68°C	1
	Hold	4°C	

PCR program for Library amplification by PCR. The times and temperatures for the part of the program that repeats for 8-14 cycles, based on the input amount, are in bold.

30 Add 100 µl of SMRTbell cleanup beads to the 100 µl of PCR and gently pipette mix 10 times. Perform a quick spin to collect all liquid from the sides of the tube.

Note

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

31 Incubate at 25°C for 00:15:00 . 15m

32 Place on a magnet stand and wait until supernatant is clear. Use a P200 pipette to remove supernatant While on magnet, wash two times with 200 µl of freshly prepared 80% ethanol. After removal of second wash of 200 µl of ethanol, spin the tube strip briefly, return to magnetic stand and remove residual ethanol with a P20 pipette. Do not let the beads to dry out.

33 Remove the tube strip from the magnetic stand. Immediately add 51 µl of elution buffer (preheated at 37°C) and pipette mix 10 times to resuspend. Do not let the beads to dry 7m

out. Quick spin to collect all liquid from the sides of the tube. Incubate at 25°C for 00:07:00 to elute the DNA from the beads.

34 Place the tube on the magnetic stand to separate the beads from the supernatant. When the supernatant is clear, transfer 51 µl of eluted amplified DNA reaction to a new tube and set in ice until ready to use.

35 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]

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

Note

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

37 Proceed to the next step of the protocol if sample quality and mass is acceptable.
SAFE STOPPING POINT - Store at 4°C

Repair and A-tailing of amplified DNA

40m

38 Add the following components from the SMRTbell prep kit 3.0 to a microcentrifuge tube. Adjust
component volumes for the number of libraries being prepared, plus 15% overage.

For individual preps, add components directly to the 49 µl amplified DNA sample from the previous section:

	Reagent	Volume per sample	V / sample into a master mix
	Repair buffer	8 µl	9.2 µl
	End repair mix	2 µl	2.3 µl
	DNA repair mix	1 µl	1.15 µl
	Total volume / sample	11 µl	

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

39 The total reaction volume should now be 60 µl.
Pipette mix, and run the Repair and A-tailing thermocycler program 00:37:00 .

37m

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

Repair and A-tailing thermocycler program

40 Proceed to the next step of the protocol.

Adapter ligation and clean-up

3h 5m

41 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 section in the order and volume listed below and skip step 43.

	Reagent	Volume per sample	V/sample into a master mix
	SMRTbell adapter	4 µl	4.4 µl
	Ligation mix	14.9 µl	16.39 µl
	Ligation enhancer	1 µl	1.1 µl

	Reagent	Volume per sample	V/sample into a master mix
	MgCl2	5.1 µl (final concentration 3mM)	4.64 µl
	Total volume/sample	25 µl	

Reagents for Adapter ligation, and recipe for RM2 master mix

42 If you've prepared a master mix:
- Pipette mix the master mix.
- Quick spin the master mix to collect liquid.
- Add 25 µl of master mix to each sample from previous step.

43 Total volume should be 85 µl.

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

44 Run the adapter ligation thermocycler program 02:31:00 .

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

Adapter ligation thermocycler program

45 Add 85 µl of resuspended, room-temperature SMRTbell cleanup beads to each sample.

46 Pipette mix the beads until evenly distributed.

47 Quick spin the tube strip in a microcentrifuge to collect all liquid from the sides of the tubes.

48 Incubate at 25°C for 00:15:00 . 15m

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

50 Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant.

51 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.

52 Repeat the previous step.

53 Remove residual 80% ethanol:
• 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.

54 Remove tube strip from the magnetic rack. Immediately add 26 µl of elution buffer to each tube and resuspend the beads.

55 Quick spin the tube strip.

56 Incubate at 25°C for 00:07:00 to elute the DNA from the beads.. 7m

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

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

59 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]

60 Proceed to the next step of the protocol.
SAFE STOPPING POINT - Store at 4°C

Nuclease treatment of SMRTbell library

15m

61 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 section, then skip RM3 step 63.

	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 Nuclease master mix

62 If you've prepared Nuclease master mix:

- Pipette mix Nuclease master mix.
- Quick spin Nuclease master mix to collect liquid.
- Add 5 µl of Nuclease master mix to each sample.

63 The total reaction volume should now be 31 µl.

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

64 Run the nuclease treatment thermocycler program 00:08:00 .

	Time	Temperature
	7 min	37°C
	Hold	4°C

Nuclease treatment thermocycler program

65 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]

66 Proceed to the next step of the protocol.

Size selection of SMRTbell library

20h

67 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.

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

69 Bring the Loading solution to room temperature and then, add 10 µl of Loading solution to each 30 µl sample remaining from the previous step above. Mix well by gentle pipetting and spin briefly to collect the contents at the bottom of the tube.

70 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.

71 Load S1 marker and samples into the BluePippin gel cassette and start the run. Run time is approximately 4.0 hours.

72 Collect Eluate. 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. 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%.

73 Measure the volume of your size-selected sample (eluate plus wash). 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]

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

Note

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

75 Proceed to the next section if sample quality is acceptable.

Purification of size-selected SMRTbell library 35m

76 Add 1X SMRTbell cleanup beads to each sample from the previous section.

77 Pipette mix the beads until evenly distributed.

78 Quick spin the tube strip in a microcentrifuge to collect all liquid.

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

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

81 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.

82 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.

83 Repeat the previous step.

84 Remove residual 80% ethanol:
• 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.

85 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.

86 Quick spin the tube strip in a microcentrifuge to collect liquid.

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

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

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

90 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]

Required
Yield [ng]

91 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

The protocol is based on Ampli-Fi - Amplifying genomic DNA for SMRTbell library preparation and HiFi sequencing (PN 103-648-000 REV04 JUL2025).