Section 2: NGS library preparation for sequencing

In 1 collection

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ABSTRACT

This protocol details NGS library preparation for sequencing and should be performed after Section 1: Enzymatic DNA Fragmentation (Manually).

ATTACHMENTS

nsr5bn3x7.pdf

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89823

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Section 2: NGS library preparation for sequencing

30m

1 NGS library preparation for sequencing can be done in two ways.

Prepare the ligation master mix

30m 15s

STEP CASE

Manual workflow 90 steps

2 Remove the AMPure XP beads from cold storage and equilibrate to Room temperature for at least 00:30:00 prior use.

3 Thaw On ice End Repair-A Tailing Buffer (yellow cap or bottle, to be used for End Repair-A Tailing master mix) and Ligation Buffer (purple cap or bottle, to be used for Ligation master mix). They may require >20 minutes). In the meantime, proceed to the next steps.

4 Vortex the thawed vial of Ligation Buffer for 00:00:15 at high speed to ensure homogeneity. 15s

Note

The Ligation Buffer used in this step is viscous. Mix thoroughly by vortexing at high speed for 15 seconds before removing an aliquot for use. When combining with other reagents, mix well by pipetting up and down 15– 20 times using a pipette set to at least 80% of the mixture volume or by vortexing at high speed for 10–20 seconds. Use flat top vortex mixers when vortexing strip tubes or plates throughout the protocol. If reagents are mixed by vortexing, visually verify that adequate mixing is occurring.

5 Place T4 DNA Ligase (blue cap) On ice and mix by inversion.

6 Calculate Ligation master mix as on calculations, as on Table 4.

Table 4. Ligation master mix.

	A	B
	Reagent	Volume for 1 reaction
	Ligation Buffer (purple cap or bottle)	23 µl
	T4 DNA Ligase (blue cap)	2 µl
	Total	25 µl

7 Prepare Ligation master mix:

7.1 Slowly pipette the Ligation Buffer into a 1.5-ml Eppendorf tube, ensuring that the full volume is dispensed.

7.2 Slowly add the T4 DNA Ligase, rinsing the enzyme tip with buffer solution after addition.

7.3 Mix well by slowly pipetting up and down 15–20 times or seal the tube and vortex at high speed for 10–20 seconds.

7.4 Spin briefly to collect the liquid.

8 Keep at Room temperature the Ligation master mix for 30–45 minutes before use on step on Sub-section 3 step 2.

Note

If the actual temperature of the lab is far from 20 °C - 25 °C, it is recommended to place the Ligation master mix tube in a Thermo Block set at 20 °C.

Repair and dA-Tail the DNA ends

15s

9 Pre-program a thermal cycler as on Table 5. If required, use a reaction volume setting of 70 µL and lid temperature @ 65 °C.

Table 5. Thermal cycler program for End Repair/dA-Tailing.

	A	B	C
	Step	Temperature (°C)	
	1	20	15
	2	72	15
	3	4	Hold

10 Vortex the thawed vial of End Repair-A Tailing Buffer for 00:00:15 at high speed to ensure homogeneity. Visually inspect the solution; if any solids are observed, continue vortexing until all solids are dissolved. 15s

Note

The End Repair-A Tailing Buffer used in this step must be mixed thoroughly by vortexing at high speed for 15 seconds before removing an aliquot for use. When combining with other reagents, mix well either by pipetting up and down 15–20 times using a pipette set to at least 80% of the mixture volume or by vortexing at high speed for 5–10 seconds.

11 Place End Repair-A Tailing Enzyme Mix (orange cap) On ice and mix by inversion.

12 Calculate End Repair/dA-Tailing master mix as on Table 6.

Table 6. End Repair/dA-Tailing mix.

	A	B
	Reagent	Volume for 1 reaction
	End Repair-A Tailing Buffer (yellow cap or bottle)	16 µl
	End Repair-A Tailing Enzyme Mix (orange cap)	4 µl
	Total	20 µl

13 Prepare End Repair/dA-Tailing master mix:

13.1 Slowly pipette the End Repair-A Tailing Buffer into a 1.5-ml Eppendorf tube, ensuring that the full volume is dispensed.

13.2 Slowly add the End Repair-A Tailing Enzyme Mix, rinsing the enzyme tip with buffer solution after addition.

13.3 Mix well by pipetting up and down 15–20 times or seal the tube and vortex at high speed for 5–10 seconds.

13.4 Spin briefly to collect the liquid and keep On ice.

14 Add 20 µL of the End Repair/dA-Tailing master mix to each sample well containing approximately 50 µL of fragmented DNA.

15 Mix by pipetting up and down 15–20 times using a pipette set to 50 µL or cap the wells and vortex at high speed for 5–10 seconds.

16 Briefly spin the samples.

17 Immediately place the plate or strip tube in the thermal cycler and start the ‘End Repair/dA-Tailing’ program.

18 Once the thermal cycler reaches the 4 °C hold step, centrifuge the samples briefly.

19 Transfer the samples to ice and proceed to **Ligate the molecular-barcoded adaptor**.

Ligate the molecular-barcoded adaptor

20 Pre-program a thermal cycler as on Table 7. If required, use a reaction volume setting of 100 µL and turn the lid temperature OFF.

> **Note**
>
> If possible, keep the lid open while doing the next steps to allow the lid reach Room temperature prior using it.

Table 7. Thermal cycler program for Ligation.

Step	Temperature (°C)	
1	20	30
2	4	Hold

21 Thaw SureSelect XT HS2 Adaptor Oligo Mix (clear cap) On ice.

22 To each end-repaired/dA-tailed DNA sample (approximately 70 µL), add 25 µL of the Ligation master mix that was prepared on section **Prepare the ligation master mix** and kept at Room temperature.

23 Mix by pipetting up and down at least 10 times using a pipette set to 70 µL or cap the wells and vortex at high speed for 5–10 seconds.

24 Briefly spin the samples.

25 Add 5 µL of SureSelect XT HS2 Adaptor Oligo Mix (clear-capped tube) to each sample.

26 Mix by pipetting up and down 15–20 times using a pipette set to 70 µL or cap the wells and vortex at high speed for 5–10 seconds.

Note

Make sure to add the Ligation master mix and the Adaptor Oligo Mix to the samples in separate addition steps as directed above, mixing after each addition.

27 Briefly spin the samples.

28 Immediately place the plate or strip tube in the thermal cycler and start ‘Ligation’ program.

Note

Unique molecular barcode sequences are incorporated into both ends of each library DNA fragment at this step.

1. If you do not continue to the next step, seal the sample wells and store Overnight at either 4 °C or -20 °C.

Purify the sample using AMPure XP beads

44m 30s

29 Verify that the AMPure XP beads were held at Room temperature for at least 00:30:00. 30m

30 Prepare 1000 µL of 70% ethanol per sample.

Note

The freshly prepared 70% ethanol may be used for subsequent purification steps run on the same day. The complete Library Preparation protocol requires 0.8 mL of fresh 70% ethanol per sample.

31 Mix the AMPure XP bead suspension well so that the reagent appears homogeneous and consistent in color.

32 Add 80 µL of homogeneous AMPure XP beads to each DNA sample (approximately 100 µl) in the PCR plate or strip tube.

33 Pipette up and down 15–20 times or cap the wells and vortex at high speed for 5–10 seconds to mix.

34 Incubate samples for 00:05:00 at Room temperature. 5m

35 Put the plate or strip tube into a magnetic separation device.

36 Wait for the solution to clear (approximately 5 to 10 minutes).

37 Keep the plate or strip tube in the magnetic stand. Carefully remove and discard the cleared solution from each well.

Note

Do not touch the beads while removing the solution.

38 Continue to keep the plate or strip tube in the magnetic stand while you dispense 200 µL of freshly prepared 70% ethanol in each sample well.

39 Wait for 00:01:00 to allow any disturbed beads to settle, then remove the ethanol. 1m

40 Repeat ethanol wash by adding another 200 µL of freshly prepared 70% ethanol in each sample well.

41 Wait for 00:01:00 to allow any disturbed beads to settle, then remove the ethanol. 1m

42 Seal the wells with strip caps, then briefly spin the samples to collect the residual ethanol.

43 Return the plate or strip tube to the magnetic stand for 00:00:30. 30s

44 Remove the residual ethanol with a P20 pipette.

Note

If needed, dry the samples by placing the unsealed plate or strip tube on the thermal cycler, set to hold samples at 37°C, until the residual ethanol has just evaporated (typically 1–2 minutes).

Critical note! Do not dry the bead pellet to the point that the pellet appears cracked during any of the bead drying steps in the protocol. Elution efficiency is significantly decreased when the bead pellet is excessively dried.

45 Add 35 µL nuclease-free water to each sample well.

46 Seal the wells with strip caps, then mix well on a vortex mixer and briefly spin the plate or strip tube to collect the liquid.

47 Incubate for 00:02:00 at Room temperature. 2m

48 Put the plate or strip tube in the magnetic stand and leave for approximately 00:05:00, until the solution is clear. 5m

49 Remove the cleared supernatant using a P20 twice (pipette set at 34 µL) to a fresh PCR plate or strip tube sample well and keep On ice. You can discard the beads at this time.

Note

It may not be possible to recover the entire 34-µl supernatant volume at this step; transfer the maximum possible amount of supernatant for further processing. To maximize recovery, transfer the cleared supernatant to a fresh well in two rounds of pipetting, using a P20 pipette set at 17 µL.

Amplify the adaptor-ligated library

5s

50 Determine the appropriate index pair assignment for each sample. See Table 51 through Table 58 in the “Reference” chapter (see original SureSelect XT HS2 DNA System protocol) for sequences of the 8 bp index portion of the primers used to amplify the DNA libraries.

Note

Critical notes!

1. Use a different indexing primer pair for each sample to be sequenced in the same lane.
2. The SureSelect XT HS2 Index Primer Pairs are provided in single-use aliquots. To avoid cross-contamination of libraries, do not retain and re-use any residual volume in wells for subsequent experiment.
3. To avoid cross-contaminating libraries, set up PCR reactions (all components except the library DNA) in a dedicated clean area or PCR hood with UV sterilization and positive air flow.

51 Thaw the following reagents for PCR amplification On ice:

- Herculase II Fusion DNA Polymerase (red cap)
- 5× Herculase II Buffer with dNTPs (clear cap)
- SureSelect XT HS2 Index Primer Pairs

52 Mix my pipetting up and down 20 times Herculase II Fusion DNA Polymerase (red cap).

53 Mix by vortexing Herculase II Buffer with dNTPs (clear cap) and SureSelect XT HS2 Index Primer

Pairs.

54 Pre-program a thermal cycler as on Table 8. If required, use a reaction volume setting of 50 µL.

55 Turn the lid temperature ON @ 105 °C and pre-heat the lid.

Table 8. Thermal cycler program for Amplification PCR.

	A	B	C	D
	Step	Number of Cycles	Temperature (°C)	Time (min)
	1	1	98	2
	2	5-9*	98	0.5
			60	0.5
			72	1
	3	1	72	5
	4	1	4	Hold

***Note**: We use 5-9 cycles. However, we suggest the users to assess this initially in a small scale as it is based on the input material and magnet used. Examples of library preparations are presented in Figure 8. As an example, too little cycles may result in very low concentration of the libraries and too many cycles in over-amplified libraries (Figure 8C). In that case, we recommend excluding the over-amplified libraries from further analysis.

56 Prepare the appropriate volume of Pre-Capture PCR reaction mix as on Table 9.

Note

This is an amplification PCR step, but in our case it is not followed by a capture step.

Table 9. Amplification PCR mix.

	A	B
	Reagent	Volume for 1 reaction

	A	B
	5× Herculase II Buffer with dNTPs (clear cap)	10 µl
	Herculase II Fusion DNA Polymerase (red cap)	1 µl
	Total	11 µl

57 Add 11 µL of the PCR reaction mixture to each purified DNA library sample (~ 34 µL) in the PCR plate wells.

58 Add 5 µL of the appropriate SureSelect XT HS2 Index Primer Pair to each reaction.

59 Cap the wells then vortex at high speed for 00:00:05. 5s

60 Spin the plate or strip tube briefly to collect the liquid and release any bubbles.

61 Immediately place the plate or strip tube in the thermal cycler and start 'Amplification PCR' program.

Safety information

Caution: The lid of the thermal cycler is hot and can cause burns. Use caution when working near the lid.

Purify the sample using AMPure XP beads 46m 30s

62 Verify that the AMPure XP beads were held at Room temperature for at least 00:30:00. 30m

63 Prepare 400 µL of 70% ethanol per sample, if not have done already on the same day.

64 
Mix the AMPure XP bead suspension well so that the reagent appears homogeneous and consistent in color.

65 
Add 50 µL of homogeneous AMPure XP beads to each amplification reaction sample (approximately 50 µL) in the PCR plate or strip tube.

66 
Pipette up and down 15–20 times or cap the wells and vortex at high speed for 5–10 seconds to mix.

67 
Incubate samples for 00:05:00 at Room temperature. 5m

68 Put the plate or strip tube into a magnetic separation device.

69 Wait for the solution to clear (approximately 00:05:00). 5m

70 Keep the plate or strip tube in the magnetic stand. Carefully remove and discard the cleared solution from each well.

Note

Do not touch the beads while removing the solution.

71 Continue to keep the plate or strip tube in the magnetic stand while you dispense 200 µL of freshly prepared 70% ethanol in each sample well.

72 Wait for 00:01:00 to allow any disturbed beads to settle, then remove the ethanol. 1m

73 Repeat ethanol wash by adding another 200 µL of freshly prepared 70% ethanol in each sample well.

74 Wait for 00:01:00 to allow any disturbed beads to settle, then remove the ethanol. 1m

75 Seal the wells with strip caps, then briefly spin the samples to collect the residual ethanol.

76 Return the plate or strip tube to the magnetic stand for 00:00:30. 30s

77 Remove the residual ethanol with a P20 pipette.

Note

If needed, dry the samples by placing the unsealed plate or strip tube on the thermal cycler, set to hold samples at 37 °C, until the residual ethanol has just evaporated (typically 1–2 minutes).

78 Add 15 µL nuclease-free water to each sample well.

79 Seal the wells with strip caps, then mix well on a vortex mixer and briefly spin the plate or strip tube to collect the liquid.

80 Incubate for 00:02:00 at Room temperature. 2m

81 Put the plate or strip tube in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.

82 Remove the cleared supernatant (approximately 15 µL) to a fresh PCR plate or strip tube sample well and keep On ice. You can discard the beads at this time.

Note

It may not be possible to recover the entire 15-µl supernatant volume at this step; transfer the maximum possible amount of supernatant for further processing.

83 If you do not continue to the next step, seal the sample wells and store at 4 °C Overnight or at -20 °C for prolonged storage. 2m