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Section 2: NGS library preparation for sequencing

In 1 collection

Ester Kalef- Ben Katherine Ezra^{1,2}, Harvey³, Roper³,

Christos Proukakis^{1,2}

¹Department of Clinical and Movement Neurosciences, UCL Queen Square Institute of Neurology, London, UK;

²Aligning Science Across Parkinson's (ASAP) Collaborative Research Network, Chevy Chase, MD, 20815;

³Diagnostics and Genomics Group, Agilent Technologies LDA UK Ltd

ASAP Collaborative Research Network

University College London



Ester Kalef-Ezra
University College London

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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Keywords: ASAPCRN

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

- Remove the AMPure XP beads from cold storage and equilibrate to Room temperature for at least 00:30:00 prior use.
- 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	В
Reagent	Volume for 1 reaction
Ligation Buffer (purple cap or bottle)	23 μΙ
T4 DNA Ligase (blue cap)	2 μΙ
Total	25 μΙ

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.

If the actual temperature of the lab is far from $20 \,^{\circ}\text{C}$ 25 $^{\circ}\text{C}$, it is recommended to place the Ligation master mix tube in a Thermo Block set at $20 \,^{\circ}\text{C}$.

Repair and dA-Tail the DNA ends

15s

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

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

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

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.

Place End Repair-A Tailing Enzyme Mix (orange cap) 8 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	В
Reagent	Volume for 1 reaction
End Repair-A Tailing Buffer (yellow cap or bottle)	16 μΙ
End Repair-A Tailing Enzyme Mix (orange cap)	4 μΙ
Total	20 μΙ

- 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.
- Mix well by pipetting up and down 15–20 times or seal the tube and vortex at high speed for 5–10 seconds.
- Spin briefly to collect the liquid and keep on ice
- Add $\underline{\mathbb{Z}}$ 20 μ L of the End Repair/dA-Tailing master mix to each sample well containing approximately $\underline{\mathbb{Z}}$ 50 μ L of fragmented DNA.
- Mix by pipetting up and down 15–20 times using a pipette set to Δ 50 μ L or cap the wells and vortex at high speed for 5–10 seconds.

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



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

Ligate the molecular-barcoded adaptor

Pre-program a thermal cycler as on Table 7. If required, use a reaction volume setting of Δ 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.

St	tep	Temperature (°C)	
1		20	30
2		4	Hold

Thaw SureSelect XT HS2 Adaptor Oligo Mix (clear cap) 8 On ice

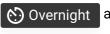
- To each end-repaired/dA-tailed DNA sample (approximately 70 µL), add 25 µL of the Ligation master mix that was prepared on section Prepare the ligation master mix and kept at Room temperature .
- Mix by pipetting up and down at least 10 times using a pipette set to $\boxed{4.70\,\mu\text{L}}$ or cap 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.
- Mix by pipetting up and down 15–20 times using a pipette set to $\frac{1}{2}$ 70 μ L or cap the wells and vortex at high speed for 5–10 seconds.

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.

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 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 \$\mathbb{E}\$ Room temperature for at least \$\mathbb{O}\$ 00:30:00





30m

30 Prepare A 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 A 0.8 mL of fresh 70% ethanol per sample.

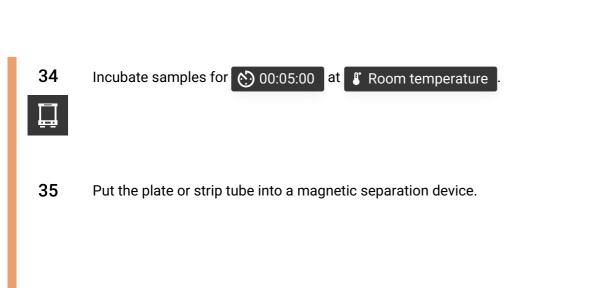
31 Mix the AMPure XP bead suspension well so that the reagent appears homogeneous and consistent in color.



32

Add Δ 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.



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

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

Note

36

Do not touch the beads while removing the solution.

- Continue to keep the plate or strip tube in the magnetic stand while you dispense $\frac{\mathbb{Z}}{200 \, \mu \text{L}}$ of freshly prepared 70% ethanol in each sample well.
- Wait for 00:01:00 to allow any disturbed beads to settle, then remove the ethanol.
- 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.
- 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.

Add \coprod 35 μ L nuclease-free water to each sample well.



- 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

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

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- μ l 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 μ 17 μ .

Amplify the adaptor-ligated library

5s

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



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

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- Pre-program a thermal cycler as on Table 8. If required, use a reaction volume setting of $\ \ \, \underline{\ \ \, }$ 50 μL .
- Turn the lid temperature ON @ \$\ 105 \cdot C \ and pre-heat the lid.

Table 8. Thermal cycler program for Amplification PCR.

A	В	С	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.

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	В
Reagent	Volume for 1 reaction

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

Add \triangle 11 μ L of the PCR reaction mixture to each purified DNA library sample (\sim \triangle 34 μ L) in the PCR plate wells.





Cap the wells then vortex at high speed for 00:00:05

Ea

- Spin the plate or strip tube briefly to collect the liquid and release any bubbles.
- Immediately place the plate or strip tube in the thermal cycler and start 'Amplification PCR' program.

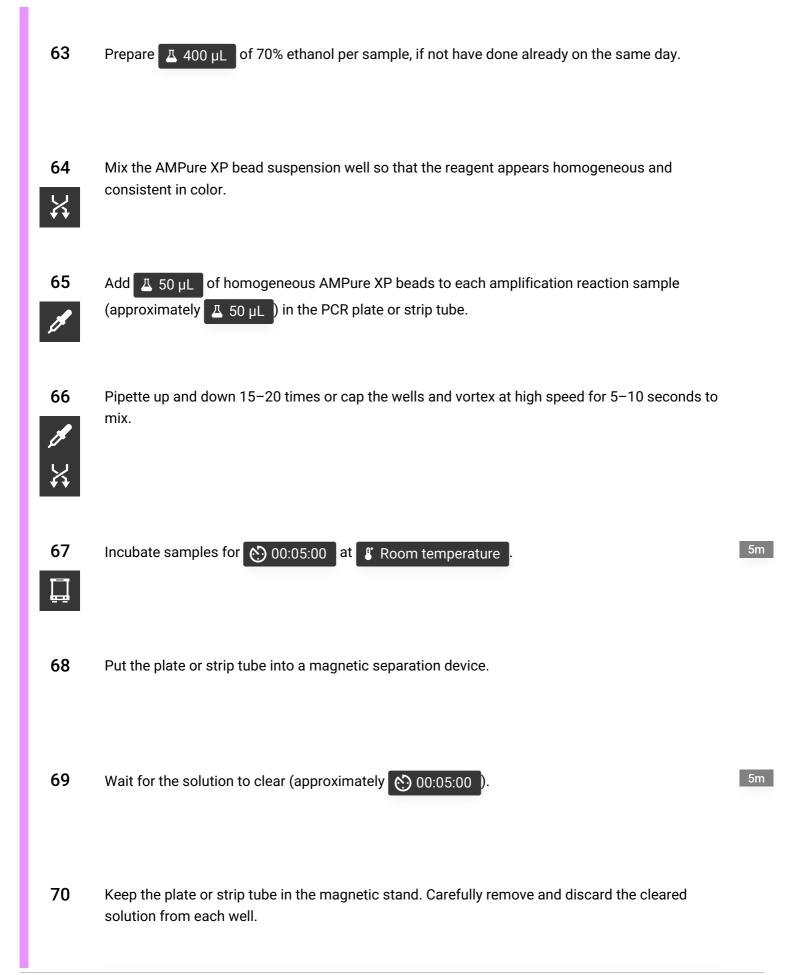


Safety information

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

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



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 $200 \, \mu$ of freshly prepared 70% ethanol in each sample well.
- Wait for 00:01:00 to allow any disturbed beads to settle, then remove the ethanol.
- Repeat ethanol wash by adding another Δ 200 μL of freshly prepared 70% ethanol in each
- Repeat ethanol wash by adding another 200 µL of freshly prepared 70% ethanol in each sample well.
 - Wait for 00:01:00 to allow any disturbed beads to settle, then remove the ethanol.
 - 75 Seal the wells with strip caps, then briefly spin the samples to collect the residual ethanol.
 - Return the plate or strip tube to the magnetic stand for 00:00:30
 - Remove the residual ethanol with a P20 pipette.

1m

If needed, dry the samples by placing the unsealed plate or strip tube on the thermal cycler, set to hold samples at \$\mathbb{g}^* 37 \cdot^{\chick}\$, until the residual ethanol has just evaporated (typically 1-2 minutes).

78 Add $\underline{\mathbb{Z}}$ 15 μL nuclease-free water to each sample well.



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.



Incubate for (5) 00:02:00 at [5] Room temperature





80

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

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

Note

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

If you do not continue to the next step, seal the sample wells and store at 4 °C

Overnight or at 4 °C

for prolonged storage.

2m