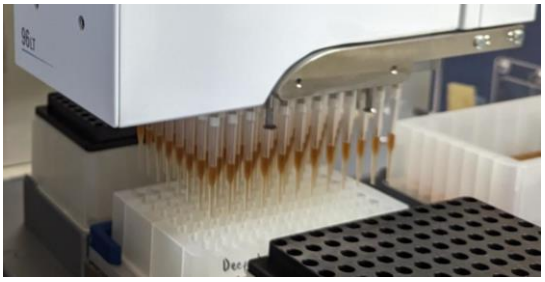
Biology protocol to be shared initially in my folder and then in ASAP Team's Voet folder, please.

Title:

SureSelect XT HS2 DNA to prepare libraries for single-cell whole genome sequencing after single-cell whole genome amplification

Title image:



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Abstract:

We adapted the SureSelect XT HS2 DNA protocol to prepare libraries using as input material samples after single-cell Whole Genome Amplification (scWGA), instead of genomic DNA samples, for library preparation for Illumina single-cell Whole Genome Sequencing (scWGS). This protocol can be employed either manually (option A) or in combination with automation using the Bravo Automated Liquid Handling Platform (option B).

Keywords:

DNA, library, Illumina, short-read, nuclei, human, brain, Single-cell Whole Genome Amplification (scWGA), Single-cell Whole Genome Sequencing (scWGS), Aligning Science Across Parkinson's (ASAP)

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We thank ICH.ZCR UCL Genomics Sequencing and Agilent for technical support.

Guidelines

Disclaimer:

This protocol was adapted with minor modifications from the following Agilent protocols:

- SureSelect Enzymatic Fragmentation Kit
  https://www.agilent.com/cs/library/usermanuals/public/G9702-90050.pdf
- Manual version: SureSelect XT HS2 DNA System
  https://www.agilent.com/cs/library/usermanuals/public/G9985-90000.pdf

- Automated version: SureSelect XT HS2 DNA System Automated using Agilent NGS Bravo Option A https://www.agilent.com/cs/library/usermanuals/public/G9985-90020.pdf

Safety Warnings:

Please follow the Safety Data Sheets (SDS) for all reagents for safe handling and safety hazards.

Critical notes:

- Please follow Good Laboratory Practices.
- To prevent DNA contamination, clean all surfaces and equipment before use with DNA AWAY Surface Decontaminant, separate the lab in pre- and post-PCR areas and use filtered sterile pipette tips.
- For each protocol step that requires removal of tube cap strips, reseal the tubes with a fresh strip of caps.
- In all master mixes to be used manually, include 5-10% excess volume in the calculations.

Materials

Commercial Reagents:

Equipment and consumables for DNA quality control:

1. DNA quantification. Here we used Qubit (Thermo Fisher Scientific):

- Qubit Fluorometer (Q33238)
- Qubit BR or HS dsDNA Assay Kit (Q32853 and Q32854)
- Qubit Assay Tubes (Q32856)

2. Nucleic acid analysis platforms. Here we used TapeStation (Agilent):

- 4150 TapeStation Instrument (G2992AA)
- 8-well tube strips (401428)
- 8-well tube strip caps (401425)
- D1000 Screen Tape (5067-5582) and D1000 Reagents (5067-5583)
- High Sensitivity D1000 Screen Tape (5067-5584) and High Sensitivity D1000 Reagents (5067-5585)

SureSelect XT HS2 performed manually:

- SureSelect Enzymatic Fragmentation Kit (Agilent 5191-4079 or 5191-4080)
- SureSelect XT HS2 DNA Library Preparation Kit with Index Primer Pairs (Choose one of the following: Agilent G9981A (index 1-16), G9985A (index 1-96), G9985B (index 97-192), G9985C (index 193-288) or G9985D (index 289-384))

SureSelect XT HS2 with automation (Bravo):

- SureSelect Enzymatic Fragmentation Kit (Agilent 5191-6764)
- SureSelect XT HS2 DNA Library Preparation Kit with Index Primer Pairs (Choose one of the following: Agilent G9985A (index 1-96), G9985B (index 97-192), G9985C (index 193-288) or G9985D (index 289-384))

Consumables:

- UltraPure™ DNase/RNase-Free Distilled Water (Thermo Fisher 10977049)
- 1X Low TE Buffer (Thermo Fisher Scientific 12090-015)
- 100% Ethanol, molecular grade (Merck 51976-500ML-F)
- Plasticware compatible with the selected thermal cycler. Here we used PCR 8-tube strips w/o caps (VWR 732-1517)
- Low binding filtered tips (sterile)

- Low-binding 1.5ml DNase/RNase-free (sterile). Here we used DNA LoBind Tubes, (Eppendorf 0030108418)
- Powder-free gloves
- DNA Away Surface Decontaminant for surface cleaning (Thermo Scientific 7010PK)
- Cleaning wipes for surface cleaning. Here we used Conti Washcloth Dry Brosch Direct PH5959

Consumables for SureSelect XT HS2 with automation:

- Processing plate: Armadillo PCR Plate, 96-well, clear (Thermo Scientific BC-2396)
- PCR plate: Armadillo 96-Well Plate, Semi-Skirted: Barcoded (VWR 731-0649)
- Deep well plate for AMPure beads and master mixes: 96 DeepWell PP Plate Natural RNase/DNase – Free (Thermo Scientific Nunc 260251)
- Waste Plate: Deep Well Plates (Axygen P-2ML-SQ-C)
- VersiCap Mat, Domed Cap Strips (Thermo Scientific AB-1810)
- 250 µL Filtered (Agilent technologies 19477-022)
- Reservoir, single cavity, polypropylene, 300 mL, 96 pyramids base geometry, 44 mm height (Agilent 201244-100)

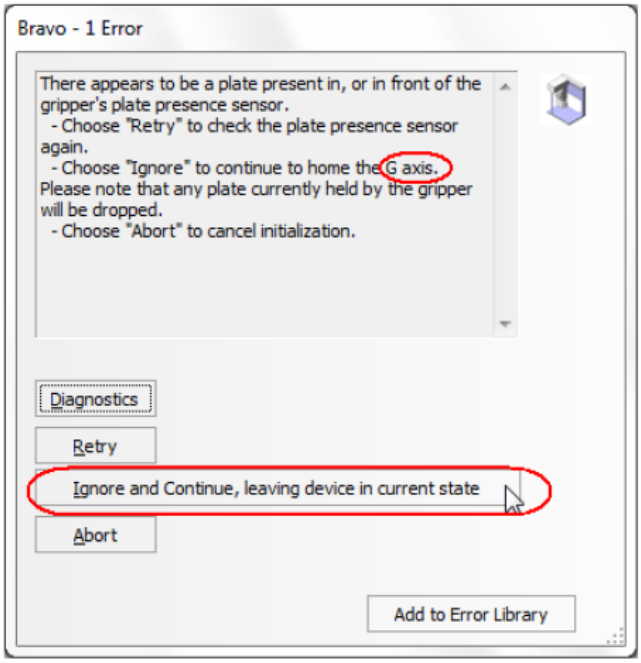
Equipment:

- Thermal Cycler with 96-well, 0.2 ml block. Here we used Multigene Optimax Gradient Thermal cycler with 96 well block (Labnet MB0520)
- Manual version: Magnetic separator compatible with0.2 ml tubes, strips of plates. Here we used either Magnetic Separator - PCR Strip (Takara 635011) or ResolveDNA Dual Volume Strip Tube Magnet (BioSkryB PN100226)
- Plate or strip tube centrifuge. Here we used SciSpin Mini microfuge, blue, 7000rpm (Sciquip SS-6050) and plate centrifuge (Starlab N2631-0008)
- 1.5 ml tube centrifuge. Here we used Mini-Centrifuge (Fisherbrand 16617645)
- PCR cooler. Here we used PCR-Cooler, Blue, Capacity: 0.2 mL (Eppendorf 1019228)
- General lab pipettes (single and multi-channel)
- Nuclease-free filtered pipette tips sterile, if possible, use low binding tips
- 96-well plate mixer. Here we used Vortex mixer. Here we used WhirliMixer (Fisons Scientific 1993-520) and iSwix VT Digital Vortex Mixer (Appleton Woods ST6000)
- Ice bucket
- For automated version: Bravo NGS Option A robot (Agilent G5573A)

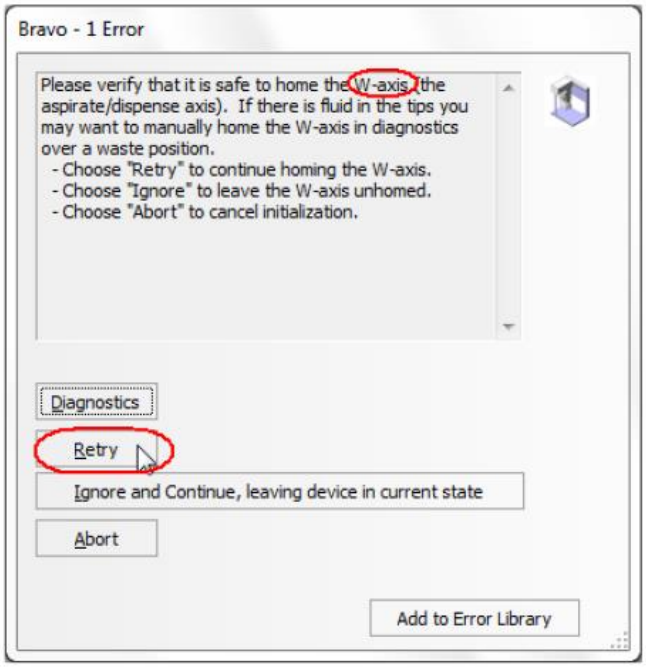
Guidelines

Two error/notification messages are encountered when VWorks Bravo software is initializing:

1. If you encounter the G-axis error message shown below, select Ignore and Continue, leaving device in current state.



2. If you encounter the W-axis error message shown below, select Retry.



Protocol

We have used this protocol for DNA products after single-cell Whole Genome Amplification (scWGA) by either PicoPLEX (Takara), ResolveDNA (BioSkryB) and droplet MDA (Samplix). We recommend to bead purify the PicoPLEX and ResolveDNA products prior library preparation. In all cases, the amplicon quantity and size should be assessed using Qubit and TapeStation or other methods prior to SureSelect XT HS2 DNA library preparation.

Section 1. Enzymatic DNA Fragmentation (Manually)

1. Group the samples scWGA-products of interest for library preparation based on their size length to require the same fragmentation time. The recommended fragmentation times per amplicon are suggested on **Table 1**.

Table 1. Recommended fragmentation time based on amplicon size.

scWGA products peak size (bp)	Fragmentation Time (min)
400-450	3
450-500	4
500-550	5
550-650	6
650-750	7
750-900	8
1000-1100	10
>10.000	15

2. Calculate the amount of input material needed from each scWGA-product to have input of 10-200ng DNA in a total volume of 7 µl.

Note: If possible, use the maximum amount of input DNA available within the recommended range.

3. Thaw the vial of 5X SureSelect Fragmentation Buffer on ice, vortex, then keep on ice.
4. Thaw a PCR plate cooler @RT for 5 min.
5. Pre-label PCR tubes/strips to be used for the enzymatic DNA fragmentation reaction.
6. Place the prelabelled PCR tubes/strips/plate in the PCR plate cooler.
7. Dilute 10-200 ng of each scWGA-product with dH2O up to a final volume of 7 µl in the PCR tubes/strips.

Note: From now on, keep the samples on ice if not stated differently.

8. Pre-program a thermal cycler as on **Table 2**. If required, use a reaction volume setting of 10 µl and lid temperature @60-65^{o}C.

Table 2. Thermal cycler program for Enzymatic Fragmentation (with the heated lid ON).

Step	**Temperature (^{o}C)**	**Time (min)**
1	37	The max need based on Table 1
2	65	5
3	4	hold

9. Prepare the appropriate volume of Fragmentation master mix by combining the reagents as on **Table 3** in 0.2ml or 1.5 ml tube.

Table 3. Fragmentation master mix.

Reagent	**1 reaction**
5X SureSelect Fragmentation Buffer	2 µl
SureSelect Fragmentation Enzyme	1 µl
Total	3 µl

10. Mix well by pipetting up and down 20 times or seal the tube and vortex at high speed for 5–10 sec.
11. Spin briefly to remove any bubbles and keep on ice.
12. Add 3 µl of the Fragmentation master mix to each sample well containing 7 µl of input DNA.
13. Mix well by pipetting up and down 20 times or cap the wells and vortex at high speed for 5–10 seconds.
14. Spin the samples briefly and place on the PCR plate cooler.
15. Place the tubes/strip in the thermal cycler and start the 'Enzymatic Fragmentation' program.

Note: Place the strips in the thermal cycler in an order to perform the recommended enzymatic fragmentation time based on **Table 1**. As an example, place first a strip with samples that require 10 min fragmentation and 2 min later a strip with samples that require 8 min fragmentation.

16. When the program reaches the 4°C Hold step, remove the samples from the thermal cycler.
17. Add 40 µl of nuclease-free water to each sample and place the samples on ice.

Note1. The 50-µl reactions are now ready for NGS sequencing library preparation (section 1).

Note2. This is not a stopping point in the workflow, and analysis of the enzymatically fragmented samples is not required prior to library preparation. Moreover, electrophoretic analysis of the fragmented samples may produce misleading results due to the presence of agents that affect DNA fragment migration.

Section 2. NGS library preparation for sequencing

Option i. Manual workflow

Sub-section 1. Prepare the ligation master mix

1. Remove the AMPure XP beads from cold storage and equilibrate to room temperature for at least 30 minutes prior use.
2. 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.
3. Vortex the thawed vial of Ligation Buffer for 15 seconds at high speed to ensure homogeneity.

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.

4. Place T4 DNA Ligase (blue cap) on ice and mix by inversion.
5. Calculate Ligation master mix as on calculations, as on **Table 4**.

Table 4. Ligation master mix

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

6. Prepare Ligation master mix:
    a. Slowly pipette the Ligation Buffer into a 1.5-ml Eppendorf tube, ensuring that the full volume is dispensed.
    b. Slowly add the T4 DNA Ligase, rinsing the enzyme tip with buffer solution after addition.
    c. Mix well by slowly pipetting up and down 15–20 times or seal the tube and vortex at high speed for 10–20 seconds.
    d. Spin briefly to collect the liquid.

7. Keep at RT 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 20-25, it is recommended to place the Ligation master mix tube in a Thermo Block set at 20C.

Sub-section 2. Repair and dA-Tail the DNA ends

1. 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

Step	Temperature (°C)	Time (min)
1	20	15
2	72	15
3	4	Hold

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

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.

3. Place End Repair-A Tailing Enzyme Mix (orange cap) on ice and mix by inversion.
4. Calculate End Repair/dA-Tailing master mix as on **Table 6**.

Table 6. End Repair/dA-Tailing mix

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

5. Prepare End Repair/dA-Tailing master mix:
    a. Slowly pipette the End Repair-A Tailing Buffer into a 1.5-ml Eppendorf tube, ensuring that the full volume is dispensed.
    b. Slowly add the End Repair-A Tailing Enzyme Mix, rinsing the enzyme tip with buffer solution after addition.
    c. Mix well by pipetting up and down 15–20 times or seal the tube and vortex at high speed for 5–10 seconds.
    d. Spin briefly to collect the liquid and keep on ice.
6. Add 20 µl of the End Repair/dA-Tailing master mix to each sample well containing approximately 50 µl of fragmented DNA.
7. 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.
8. Briefly spin the samples.
9. Immediately place the plate or strip tube in the thermal cycler and start the 'End Repair/dA-Tailing' program.
10. Once the thermal cycler reaches the 4°C hold step, centrifuge the samples briefly.
11. Transfer the samples to ice and proceed to sub-section 3.

Sub-section 3. Ligate the molecular-barcoded adaptor

1. 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 RT prior using it.

Table 7. Thermal cycler program for Ligation

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

2. Thaw SureSelect XT HS2 Adaptor Oligo Mix (clear cap) on ice.
3. To each end-repaired/dA-tailed DNA sample (approximately 70 µl), add 25 µl of the Ligation master mix that was prepared on on Sub-section 1 and kept at RT.
4. 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.
5. Briefly spin the samples.
6. Add 5 µl of SureSelect XT HS2 Adaptor Oligo Mix (clear-capped tube) to each sample.
7. 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.

8. Briefly spin the samples.

9. Immediately place the plate or strip tube in the thermal cycler and start 'Ligation' program.

Note1: Unique molecular barcode sequences are incorporated into both ends of each library DNA fragment at this step.

Note2: If you do not continue to the next step, seal the sample wells and store overnight at either 4°C or –20°C.

Sub-section 4. Purify the sample using AMPure XP beads

1. Verify that the AMPure XP beads were held at room temperature for at least 30 minutes.
2. Prepare 1000 µl of 70% ethanol per sample.

Note1: 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.

3. Mix the AMPure XP bead suspension well so that the reagent appears homogeneous and consistent in color.
4. Add 80 µl of homogeneous AMPure XP beads to each DNA sample (approximately 100 µl) in the PCR plate or strip tube.
5. Pipette up and down 15–20 times or cap the wells and vortex at high speed for 5–10 seconds to mix.
6. Incubate samples for 5 minutes at room temperature.
7. Put the plate or strip tube into a magnetic separation device.
8. Wait for the solution to clear (approximately 5 to 10 minutes).
9. 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.

10. 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.
11. Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
12. Repeat ethanol wash by adding another 200 µl of freshly prepared 70% ethanol in each sample well.
13. Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
14. Seal the wells with strip caps, then briefly spin the samples to collect the residual ethanol.
15. Return the plate or strip tube to the magnetic stand for 30 seconds.
16. 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.

17. Add 35 µl nuclease-free water to each sample well.
18. 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.
19. Incubate for 2 minutes at RT.
20. Put the plate or strip tube in the magnetic stand and leave for approximately 5 minutes, until the solution is clear.
21. 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.

Sub-section 5. Amplify the adaptor-ligated library

1. Determine the appropriate index pair assignment for each sample. See **Table 5**1 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.

Critical note 1: Use a different indexing primer pair for each sample to be sequenced in the same lane.

Critical note 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.

Critical note 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.

2. 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.
3. Mix my pipetting up and down 20 times Herculase II Fusion DNA Polymerase (red cap).
4. Mix by vortexing Herculase II Buffer with dNTPs (clear cap) and SureSelect XT HS2 Index Primer Pairs.
5. Pre-program a thermal cycler as on **Table 8**. If required, use a reaction volume setting of 50 µl.
6. Turn the lid temperature ON @105°C and pre-heat the lid.

Table 8. Thermal cycler program for Amplification PCR

Step	Number of Cycles	Temperature (°C)	Time (min)
1	1	98	2.0
2	5-9*	98	0.5
		60	0.5
		72	1.0
3	1	72	5.0
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.

7. 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

Reagent	Volume for 1 reaction
5× Herculase II Buffer with dNTPs (clear cap)	10 µl
Herculase II Fusion DNA Polymerase (red cap)	1 µl
Total	11 µl

8. Add 11 µl of the PCR reaction mixture to each purified DNA library sample (¬34 µl) in the PCR plate wells.
9. Add 5 µl of the appropriate SureSelect XT HS2 Index Primer Pair to each reaction.
10. Cap the wells then vortex at high speed for 5 seconds.
11. Spin the plate or strip tube briefly to collect the liquid and release any bubbles.
12. Immediately place the plate or strip tube in the thermal cycler and start 'Amplification PCR' program.
    Caution: The lid of the thermal cycler is hot and can cause burns. Use caution when working near the lid.

Sub-section 6. Purify the sample using AMPure XP beads

1. Verify that the AMPure XP beads were held at room temperature for at least 30 minutes.
2. Prepare 400 µl of 70% ethanol per sample, if not have done already on the same day.
3. Mix the AMPure XP bead suspension well so that the reagent appears homogeneous and consistent in color.
4. Add 50 µl of homogeneous AMPure XP beads to each amplification reaction sample (approximately 50 µl) in the PCR plate or strip tube.
5. Pipette up and down 15–20 times or cap the wells and vortex at high speed for 5–10 seconds to mix.
6. Incubate samples for 5 minutes at room temperature.
7. Put the plate or strip tube into a magnetic separation device.
8. Wait for the solution to clear (approximately 5 minutes).

9. 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.

10. 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.
11. Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
12. Repeat ethanol wash by adding another 200 µl of freshly prepared 70% ethanol in each sample well.
13. Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
14. Seal the wells with strip caps, then briefly spin the samples to collect the residual ethanol.
15. Return the plate or strip tube to the magnetic stand for 30 seconds.
16. 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).

17. Add 15 µl nuclease-free water to each sample well.
18. 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.
19. Incubate for 2 minutes at RT.
20. Put the plate or strip tube in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.
21. 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.

22. 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.

Option ii. Automation using Agilent Bravo NGS Option A

Sub-section 1. Preparation of AMPure XP Bead Plates

AMPure XP bead plates are needed for 2 sub-sections in this protocol:

A. Sub-section 3. Purify adaptor-ligated DNA using AMPure XP beads. 02 Cleanup_LibPrep_XT_HS2_ILM protocol requires a bead plate containing 80 µL of beads in each well. Use the 'AMPureXP_Aliquot_LibPrep' protocol to prepare the bead plate needed for library preparation.
B. Sub-section 5. Purify amplified DNA using AMPure XP beads. 04a Cleanup_Pre-CapPCR_SP_XT_HS2_ILM require a bead plate containing 50 µL of beads in each well. Use the 'AMPureXP_Aliquot_PreCap' protocol to prepare the bead plate needed for purification of pre-capture PCR products.

Critical note: If you are running the workflow over multiple days, AMPure XP bead plates should only be prepared for the same day. Do not prepare AMPure XP bead plates more than 12 hours in advance of when they are needed.

In each case, follow these instructions:

1. Clear the Bravo deck of all plates and tip boxes, then wipe it down with DNA Away.
2. Switch on Bravo computer, VWorks software, Bravo robot, Inheco Multi TEC control and Thermo Cube.
3. Place AMPure XP beads for at least 30 minutes @RT, mix well and verify that they are in suspension.
4. On the VWorks software SureSelect setup form, under Select protocol to execute, select the 'AMPureXP_Aliquot_LibPrep' or 'AMPureXP_Aliquot_PreCap' protocol and label a deep well plate accordingly.
5. Select the number of columns of samples to be processed.
6. Click Display Initial Bravo Deck Setup.
7. Pour the AMPure XP beads in suspension into the Reservoir and place the reservoir with the beads at Bravo deck 6 position as on **Figure 1**.
8. Place on Bravo a new tip box without the cap (deck 2), empty tip box (deck 8) and empty deep well plate (deck 5) as on **Figure1**.
9. Verify that the Bravo deck has been set up as displayed on the right side of the form.

10. Verify that the Current Tip State indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively.
11. When verification is complete, click Run Selected Protocol.

Note: Running the 'AMPureXP_Aliquot_LibPrep' or 'AMPureXP_Aliquot_PreCap' protocols takes approximately 5 minutes.

12. When verification is complete, click Run Selected Protocol.
13. When the protocol is complete, remove the Agilent Deep Well plate containing the AMPure XP bead suspension from position 5 of the Bravo deck.

Note1: If needed, seal the plate, keep it @4°C, and use it within the next 12 hours.
Note2: If desired, pour any unused bead suspension still present in the Agilent deep well reservoir back into the original stock bottle.

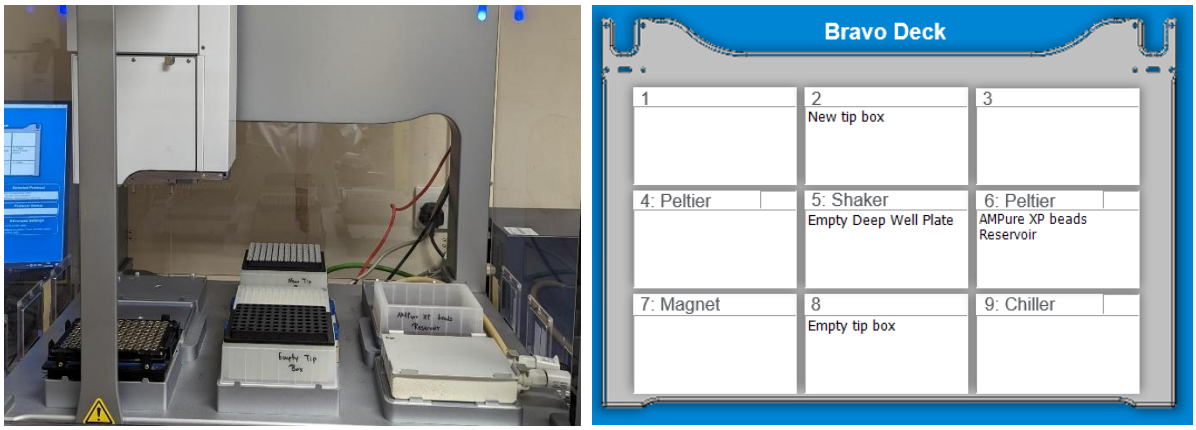


Figure 1. Example of the Bravo deck preparation and computer screen for 'AMPureXP_Aliquot_LibPrep' and 'AMPureXP_Aliquot_LibPrep' protocols.

Sub-section 2. Prepare adaptor-ligated libraries

1. Clear the Bravo deck of all plates and tip boxes, then wipe it down with DNA Away.
14. Switch on Bravo computer, VWorks software, Bravo robot, Inheco Multi TEC control and Thermo Cube.
15. Set Thermo Cube to 0°C at position 9 of the Bravo deck. Add 300 ml 25% EtOH to Thermo Cube if necessary (this will be indicated on Thermo Cube screen).
16. Select protocol for Bravo to execute '01 LibraryPrep_XT_HS2_ILM' program.
17. Verify that the Processing Plate selection is set to the correct plate type.
18. Select the number of columns of samples to be processed.
19. Click Display Initial Bravo Deck Setup.
20. Pre-set the temperature of Bravo deck position 4 (CPAC 2 position 1) to 79°C and deck position 6 to 20°C (CPAC 2 position 2) using the Inheco Multi TEC control touchscreen.
21. Thaw on ice End Repair-A Tailing Buffer and Ligation Buffer (purple cap or bottle, to be used for Ligation master mix). They may require >20 minutes). Then vortex at high speed for 15 sec them to mix well.
22. Place on ice and mix by inversion End Repair-A Tailing Enzyme Mix (orange cap), T4 DNA Ligase (blue cap).
23. Place on ice and mix by vortexing SureSelect XT HS2 Adaptor Oligo Mix (white cap).
24. Prepare the DNA End-Repair/dA-Tailing master mix (**Table 10**), Ligation master mix (**Table 11**) and Adaptor Oligo Mix (**Table 12**) based on the number of columns needed. Store on ice until use.

Note1: For End Repair-A Tailing Buffer and Ligation Buffer, if any solids are observed, continue vortexing until all solids are dissolved. Pipette these buffers slowly, as they are viscous.
Note2: When preparing master mixes, mix them well by pipetting up and down 15–20 times using a pipette set to at least 80% of the mixture volume. For large volumes, instead vortex at medium speed for 25–30 seconds to ensure homogeneity.) Spin briefly to collect the liquid and keep on ice.

Table 10. Preparation of End Repair/dA-Tailing master mix.

Reagent	Volume for 1 Library	Volume (µL) Based on Number of Columns of Samples (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
End Repair-A Tailing Buffer (yellow cap or bottle)	16 µL	204.0	340.0	476.0	612.0	748.0	884.0	1042.7	1201.3	1360.0	1518.7	1677.3	1836.0
End Repair-A Tailing Enzyme Mix (orange cap)	4 µL	51.0	85.0	119.0	153.0	187.0	221.0	260.7	300.3	340.0	379.7	419.3	459.0
Total Volume	**20 µL**	**255.0**	**425.0**	**595.0**	**765.0**	**935.0**	**1105.0**	**1303.4**	**1501.6**	**1700.0**	**1898.4**	**2096.6**	**2295.0**

Table 11. Preparation of Ligation master mix.

Reagent	Volume for 1 Library	Volume (µL) Based on Number of Columns of Samples (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
Ligation Buffer (purple cap or bottle)	23 µL	293.3	488.8	684.3	879.8	1075.3	1270.8	1515.1	1759.5	2003.9	2248.3	2492.6	2737.0
T4 DNA Ligase (blue cap)	2 µL	25.5	42.5	59.5	76.5	93.5	110.5	127.5	153.0	174.3	195.5	216.8	238.0
Total Volume	**25 µL**	**318.8**	**531.3**	**743.8**	**956.3**	**1168.8**	**1381.3**	**1642.6**	**1912.5**	**2178.2**	**2443.8**	**2709.4**	**2975.0**

Table 12. Preparation of Adaptor Oligo Mix dilution.

Reagent	Volume for 1 Library	Volume (µL) Based on Number of Columns of Samples (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
Nuclease-free water	2.5 µL	42.5	63.8	85.0	106.3	127.5	148.8	170.0	191.3	212.5	233.8	255.0	276.3
SureSelect XT HS2 Adaptor Oligo Mix (white cap)	5 µL	85.0	127.5	170.0	212.5	255.0	297.5	340.0	382.5	425.0	467.5	510.0	552.5
Total Volume	**7.5 µL**	**127.5**	**191.3**	**255.0**	**318.8**	**382.5**	**446.3**	**510.0**	**573.8**	**637.5**	**701.3**	**765.0**	**828.8**

25. Prepare the master mix source plate containing the mixtures prepared on step 4 as shown on **Table 13** based on the number of columns needed. Distribute them as on **Figure 2**.

Table 13. Preparation of the master mix source plate for 01 LibraryPrep_XT_HS2_ILM protocol.

Master Mix Solution	Position on Source Plate	Volume (µL) of Master Mix added per Well of Agilent Deep Well Source Plate Based on Number of Columns in the Run (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
End Repair-dA Tailing master mix	Column 1 (A1-H1)	31.0	52.0	73.0	94.0	115.0	136.0	158.8	182.0	205.8	230.0	254.8	280.0
Ligation master mix	Column 2 (A2-H2)	36.0	62.0	88.0	114.0	140.0	166.0	195.7	226.5	258.3	291.1	325.1	360.0
Adaptor Oligo Mix dilution	Column 3 (A3-H3)	15.0	22.5	30.0	37.5	45.0	52.5	60.6	68.8	76.9	85.0	93.1	101.3

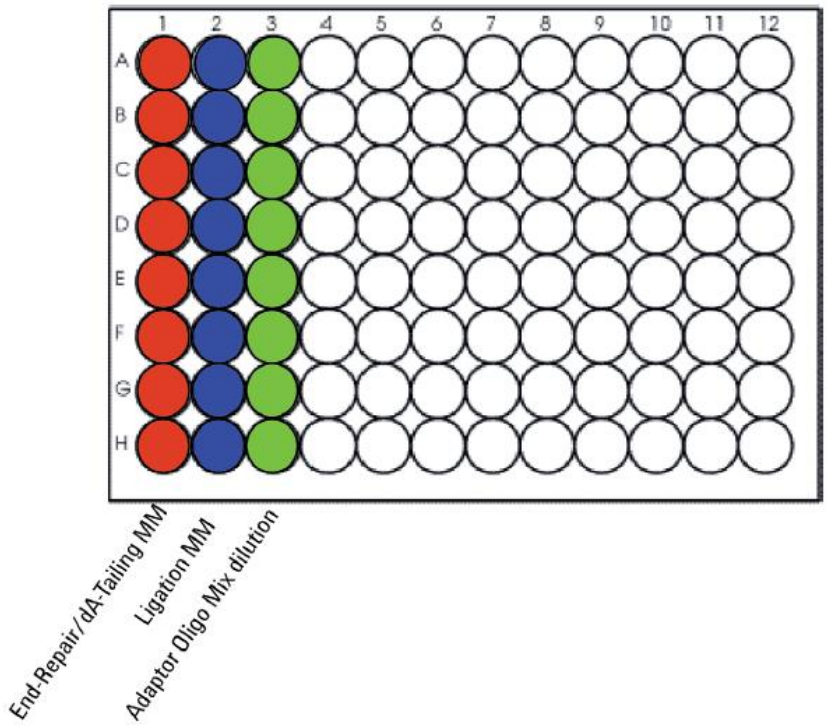


Figure 2. Configuration of the Agilent Deep Well master mix source plate for protocol 01 LibraryPrep_XT_HS2_ILM.

26. Optional: Seal the plate and centrifuge for 30 sec. Then remove the plate sealing. Alternatively, inspect visually and remove the bubbles with a pipette tip.

Note: The presence of bubbles in source plate solutions may cause inaccurate volume transfer by the Bravo liquid handling platform. Ensure that the source plate is sealed and centrifuged prior to use in a run.

1. Place the source plate on Deck position 9.
2. Place all materials as on the right side of the Bravo form as on **Figure 3**.

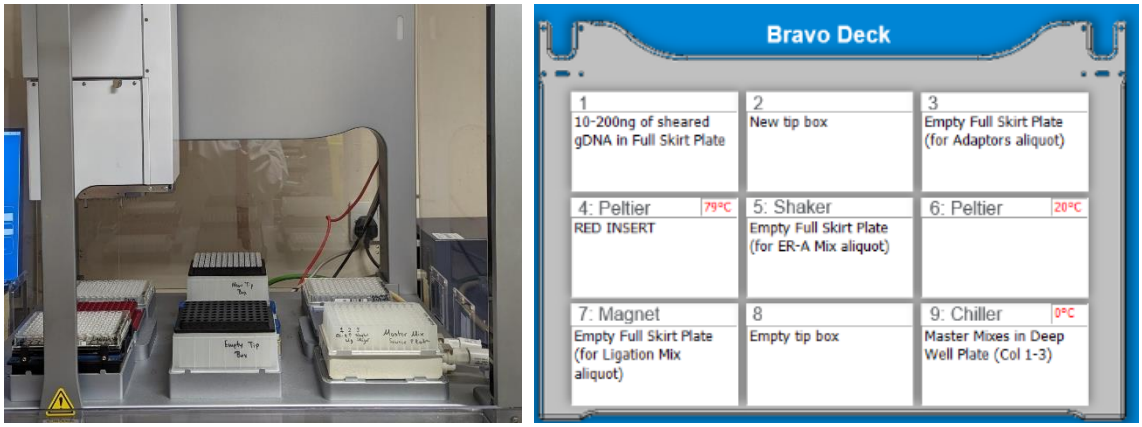


Figure 3. Example of the Bravo deck preparation and computer screen for protocol 01 LibraryPrep_XT_HS2_ILM.

3. Verify that the Bravo deck has been set up as displayed on the right side of the form.
4. Verify that the Current Tip State indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively.
5. When verification is complete, click Run Selected Protocol.

Running the 01 LibraryPrep_XT_HS2_ILM protocol takes approximately 1.5 hours. Once complete, the adaptor-ligated DNA samples are located in the selected processing plate at position 9 of the Bravo deck.

Note1: You must be present during the run to complete tip box replacement, if necessary.

Note2: Optional stopping point - If you do not continue to the next step, seal the DNA sample plate and store at 4°C overnight or at –20°C for prolonged storage.

Sub-section 3. Purify adaptor-ligated DNA using AMPure XP beads

1. Clear the Bravo deck of all plates and tip boxes, then wipe it down with DNA Away.
2. If not already, switch on Bravo computer, VWorks software, Bravo robot and Inheco Multi TEC control.
3. Switch OFF Thermo Cube.
4. Select protocol for Bravo to execute '02 Cleanup_LibPrep_XT_HS2_ILM' program.
5. Pre-set the temperature of Bravo deck position 4 (CPAC 2 position 1) to 45°C and deck position 6 to 20°C (CPAC 2 position 2) using the Inheco Multi TEC control touchscreen.
6. Prepare 2 Agilent deep well reservoirs containing: 30 mL of nuclease-free water and with 50 ml 70% EtOH (freshly made).

Note: Make sure that the reservoirs do not contain bubbles. If bubbles are present, dislodge them with a clean pipette tip.

7. Select the number of columns of samples to be processed.
8. Verify that the Processing Plate selection is set to the correct plate type. Here we use Thermo Scientific BC-2396.
9. Click Display Initial Bravo Deck Setup and place all plates and tips needed.
10. Verify that the Bravo deck has been set up as displayed on the right side of the form, as on **Figure 4**.

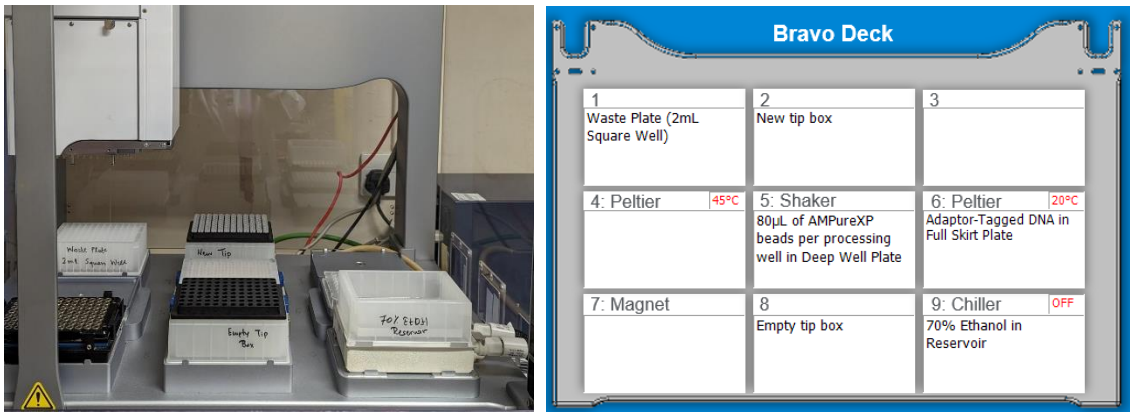


Figure 4. Example of the Bravo deck preparation and computer screen for 02 Cleanup_LibPrep_XT_HS2_ILM.

11. Verify that the Current Tip State indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively.
12. When setup and verification is complete, click Run Selected Protocol.

Note: Running the 02 Cleanup_LibPrep_XT_HS2_ILM protocol takes approximately 40 minutes. You must be present during the run to complete tip box replacement and to transfer the water reservoir to the Bravo deck, when directed by the VWorks software, as on **Figure 5**.

Figure 5. VWorks replace reservoir notification.

Sub-section 4. Amplify adaptor-ligated libraries

1. Clear the Bravo deck of all plates and tip boxes, then wipe it down with DNA Away.
2. Switch ON Thermo Cube. Be sure that the chiller reservoir contains at least 300 mL of 25% ethanol.
3. Select protocol for Bravo to execute '03 Pre-CapPCR_XT_HS2_ILM' program.
4. Pre-set the temperature of Bravo deck position 6 to 4°C (CPAC 2 position 2) using the Inheco Multi TEC control touchscreen.
5. Select the number of columns of samples to be processed.
6. Verify that the Processing Plate selection is set to the correct plate type. Here we use Thermo Scientific BC-2396.
7. Click Display Initial Bravo Deck Setup.
8. Thaw on ice and mix by vortexing 5× Herculase II Reaction Buffer (clear cap) and SureSelect XT HS2 Index Primer Pairs to be used.

Note1: Indexing primer pairs are provided in a 96-well plate.

Note2. Select carefully and record the indexing primer pairs you use. Use a different indexing primer for each library you would like to pool together for sequencing.

9. Thaw on ice and mix by pipetting up and down 15-20 times Herculase II Fusion DNA Polymerase (red cap).
10. Centrifuge briefly 5× Herculase II Reaction Buffer (clear cap), SureSelect XT HS2 Index Primer Pairs and Herculase II Fusion DNA Polymerase (red cap).
11. Pre-program a thermal cycler as on **Table 14**. If required, use a reaction volume setting of 50 µl and lid temperature @105°C.

Table 14. Thermal cycler program for Pre-Capture PCR

Step	Temperature (°C)	Time (min)
1	98	2.0
2	98	0.5
3	60	0.5
4	72	1.0
5	Return back to step 2 until 8 cycles in total	
6	72	5.0
7	4	Hold

12. Using a multichannel pipette, transfer 5 µL of each SureSelect XT HS2 Index Primer Pair from the 96-well plate in which the primer pairs are provided into the PCR plate to be used for the pre-capture PCR thermal cycling.

Note1: Make sure to maintain the same well location for each primer pair when transferring to the PCR plate.
Note2: Keep the PCR plate on ice until ready and then transfer it to the red insert at Bravo position deck 6.

13. Prepare the appropriate volume of pre-capture PCR master mix, according to **Table 15**. Vortex at medium speed for 15–20 seconds and keep on ice.

Table 15. Preparation of the Pre-Capture master mix for 03 Pre-CapPCR_XT_HS2_ILM protocol.

Reagent	Volume for 1 Library	Volume (µL) Based on Number of Columns of Samples (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
5× Herculase II Buffer with dNTPs (clear cap)	10 µL	170	255	340	425	510.0	574	656.0	738.0	820.0	902.0	984.0	1066
Herculase II Fusion DNA Polymerase (red cap)	1 µL	17	25.5	34	42.5	51.0	57.4	65.6	73.8	82.0	90.2	98.4	106.6
Total Volume	**11 µL**	**187**	**280.5**	**374**	**467.5**	**561.0**	**631.4**	**721.6**	**811.8**	**902.0**	**992.2**	**1082.4**	**1172.6**

14. Distribute the Pre-Capture master mix in Column 2 of a full skirt source plate (Eppendorf twin.tec or Armadillo plate) as on **Table 16**.

Table 16. Volumes for Pre-Capture master mix source plate for 03 Pre-CapPCR_XT_HS2_ILM protocol.

Master Mix Solution	Position on Source Plate	Volume (µL) of Master Mix added per Well of Selected Processing Source Plate Based on Number of Columns in the Run (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
Pre-Capture PCR Master Mix	Column 2 (A2-H2)	22	33	44	55	66	77	88	99	110	121	132	143

15. Cap the Pre-Capture master mix source plate and centrifuge for 30 min to minimize bubbles.
16. Uncap the plate and place it on Bravo position deck 9.
17. Place a full tip box, empty tip box and waste plate as needed.
18. Verify that the Bravo deck has been set up as displayed on the right side of the form, as on **Figure 6**.

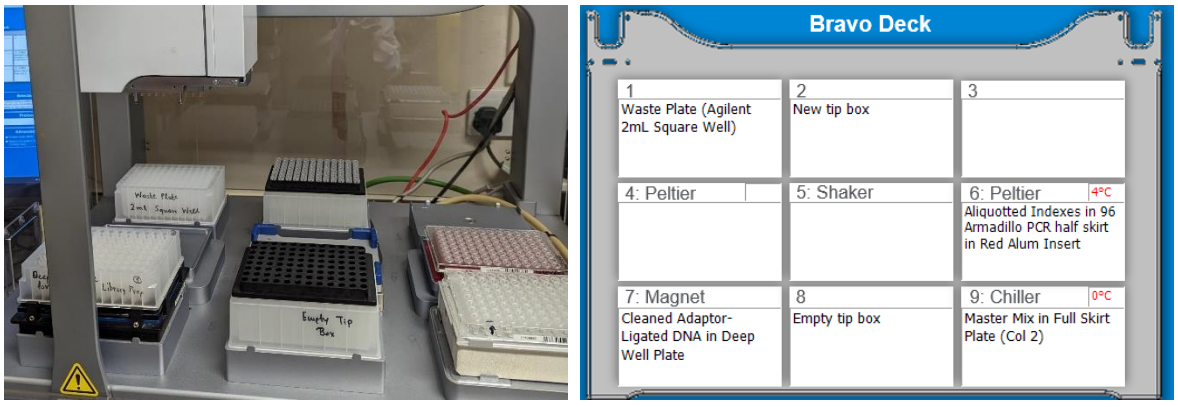


Figure 6. Example of the Bravo deck preparation and computer screen for 03 Pre-CapPCR_XT_HS2_ILM.

19. Verify that the Bravo deck has been set up as displayed on the right side of the form, as on **Figure 6**.
20. Verify that the Current Tip State indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively.
21. When verification is complete, click Run Selected Protocol.

Note: Running the '03 Pre-CapPCR_XT_HS2_ILM'protocol takes approximately 10 minutes. Once complete, the PCR-ready samples, containing prepped DNA and PCR master mix, are located in the PCR plate at position 6 of the Bravo deck.

22. When the Bravo program is complete, collect the PCR plate at position 6 of the Bravo deck.
23. Cap the plate, centrifuge briefly and initiate Pre-Capture PCR.

Sub-section 5. Purify amplified DNA using AMPure XP beads

1. Clear the Bravo deck of all plates and tip boxes, then wipe it down with DNA Away.
2. If not already, switch on Bravo computer, VWorks software, Bravo robot and Inheco Multi TEC control.
3. Switch OFF Thermo Cube.
4. Select protocol for Bravo to execute 04a Cleanup_Pre-CapPCR_SP_XT_HS2_ILM.
5. Pre-set the temperature of Bravo deck position 4 (CPAC 2 position 1) to 45°C and deck position 6 to 20°C (CPAC 2 position 2) using the Inheco Multi TEC control touchscreen.

13. Prepare 2 Agilent deep well reservoirs containing: 30 mL of nuclease-free water and with 50 ml 70% EtOH.

Note1: Make sure that the reservoirs do not contain bubbles. If bubbles are present, dislodge them with a clean pipette tip.

Note2: You can re-use the same reservoir that contains water from Sub-section 3, but you will need to add more 70% EtoH on the reservoir if you use more than 6 columns and re-using the reservoir from Sub-section 3.

14. Select the number of columns of samples to be processed.
15. Verify that the Processing Plate selection is set to the correct plate type. Here we use Thermo Scientific BC-2396.
16. Click Display Initial Bravo Deck Setup and place all plates and tips needed.
17. Verify that the Bravo deck has been set up as displayed on the right side of the form, as on **Figure 7**.

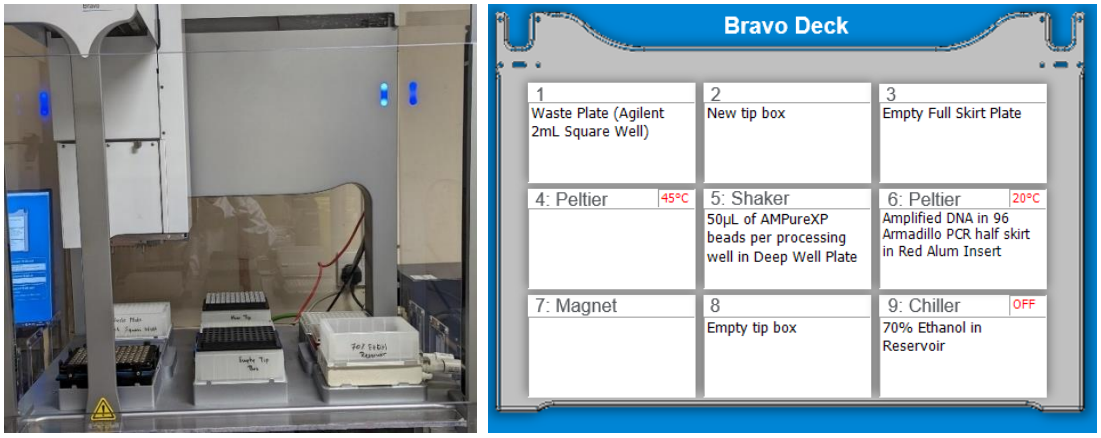


Figure 7. Example of the Bravo deck preparation and computer screen for 04a Cleanup_Pre-CapPCR_SP_XT_HS2_ILM.

18. Verify that the Current Tip State indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively.
19. When setup and verification is complete, click Run Selected Protocol.

Note: Running the 04a Cleanup_Pre-CapPCR_SP_XT_HS2_ILM protocol takes approximately 40 minutes. You must be present during the run to complete tip box replacement and to transfer the water reservoir to the Bravo deck, when directed by the VWorks software, as on **Figure 5**.

20. Cap and centrifuge briefly the purified libraries.

Note: The libraries can be stored 4°C overnight or at @–20°C for prolonged storage.

Section 3: Libraries quality control (QC)

1. Thaw the libraries on ice.
2. Centrifuge briefly.

3. Quantify the libraries using BR or HS Qubit (depending on the starting material and the amplification cycles).
4. Analyse libraries using TapeStation with HS D1000 or D1000 Screen tapes and reagents (depending on the starting material and the amplification cycles), according to manufacturer guidelines. Examples of these are presented in **Figure 8**. Other methods can be used such as Agilent 2100 Bioanalyzer system with DNA 1000 kit.
5. Dilute the libraries to concentration needed and pool them together. The QC the pooled library using both HS Qubit and HS D1000 tapes.

Note1: We dilute the libraries to ¬ 4nM (aim for 2-10nM) and calculate the molarity using the following formula: x (nM)= [Qubit concentration (ng/µl)*1000000]/[l196*peak size (bp)]

Note2: In our hands, we observe library DNA fragment size peak position is approximately 280-450 bp and in concentration of approximately 10-80 ng/µl for input 20ng and 200-350 ng/µl for input 200ng. The library yield depends on the number of amplification cycles. Moreover, we observed slightly higher DNA concentration when the library preparation steps were performed by automation compared to when prepared manually.

Note3: We occasionally observe a low molecular weight peak, in addition to the expected library fragment peak which indicates the presence of adaptor- dimers (50-180 bp) in the library (**Figure 8B**). However, in most cases they consist of less than 2% of the total libraries of the total library.

Note4: The libraries can be stored 4°C overnight or at @–20°C for prolonged storage.

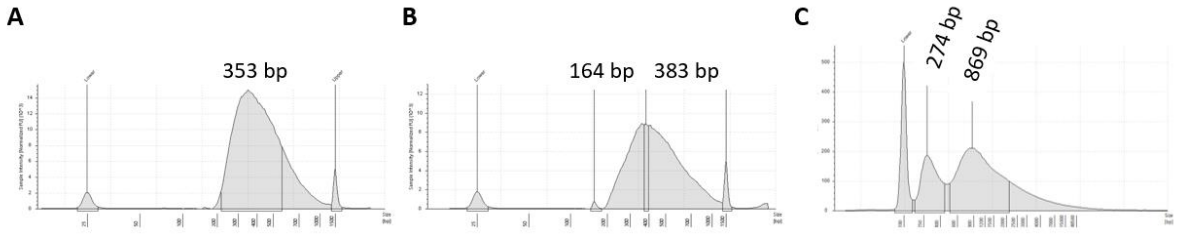


Figure 8. Examples of libraries from a single-cell whole genome amplified sample after analysis by TapeStation. A. properly amplified, B. library with a small amount of primer adapters and C. over-amplified library. A-B. were generated using D1000 DNA tapes and C. with Genomic tape.