PacBio

Automated HiFi prep 96 and HiFi annealing, binding, and cleanup for the Hamilton NGS Microlab STAR system

Guide & overview

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Introduction

This procedure describes the automated workflow for constructing whole genome sequencing (WGS) libraries from genomic DNA using the Revio™ HiFi prep kit 96 and the Revio polymerase kit 96. The SRE HT kit, HiFi Prep 96 kit and the Revio Polymerase 96 kit are designed for a minimum of 24 and maximum of 96 samples per automated run.

Overview

Overview	
Applications	WGS of human, animal, or plant samples
Samples	24–96 using automation
Minimum automated batch size	24
Maximum automated batch size	96
Workflow time	Automation time
SRE	3.5 hours for 96 samples
Shearing	10 min for 24–96 samples
Library prep	6.5 hours for 96 samples (start from post-shearing cleanup)
Anneal, bind, cleanup (ABC)	2.5 hours for 96 samples
Average total time	13 hours

DNA input and fragment size recommendations	
DNA shearing	Automated pipette-tip shearing
Target fragment lengths	15–20 kb
Size selection	SRE on gDNA, and 3.1X (35% v/v) AMPure® PB on HiFi library
Average library recovery	15% of genomic DNA input

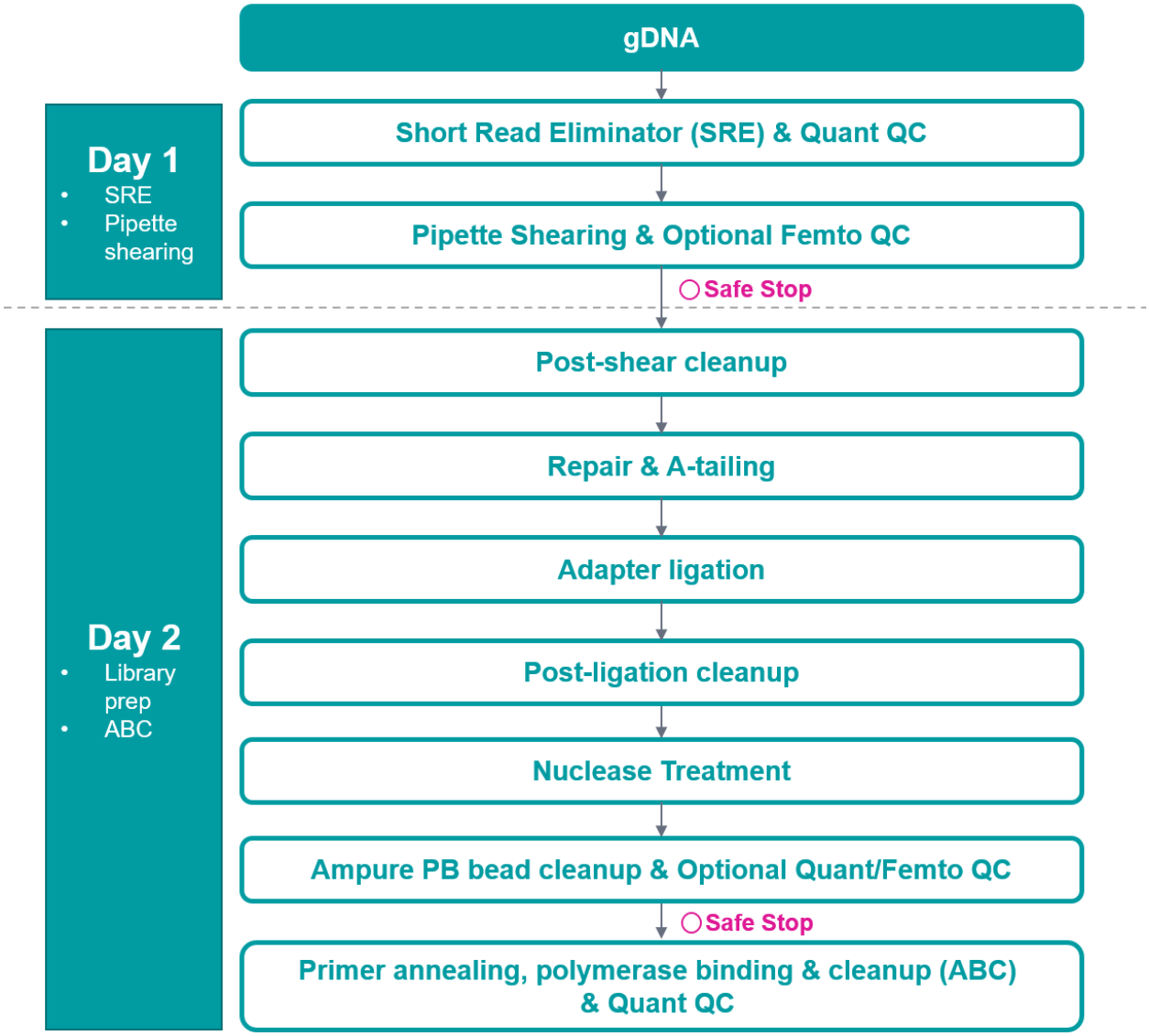
	Expected step recovery	Total recovery	Expected size (Femto Pulse)
Starting Input	100%	100%	GQN 10kb >7.0
Post SRE	75%	75%	GQN 10kb >9.3
Post-shear SMRTbell® bead cleanup	80%	60%	15–20 kb
Post-ligation SMRTbell bead cleanup	80%	48%	
Post-nuclease (pre-cleanup)	40%	19%	
Post-SMRTbell bead cleanup	80%	15%	

DNA input	
SRE DNA input Quantity	2–5 µg (40–100 ng/µL in 50 µL)
DNA Shearing input Quantity	≤3 µg (<10 ng/µL, 300 µL)
DNA size distribution (Femto Pulse system)	50% ≥30 kb & 70% ≥10 kb
Target fragment lengths	15–20 kb

Workflow overview

The recommended automation workflow for the Hamilton NGS Star is shown below.

Following this workflow ensures sufficient volumes from the Revio HiFi prep 96 kit for four 24-sample runs or a single 96-sample run.



Required materials and equipment

Consumables	Catalog Number
Hard Shell 96 PCR Plate	Bio-Rad, HSP9601
Abgene 96 Well 0.8mL Polypropylene Deepwell Storage Plate	ThermoFisher Scientific, AB0859
50 µL CO-RE II Tips (Filtered, Conductive)	Hamilton, 235948
300 µL CO-RE II Tips (Filtered, Conductive)	Hamilton, 235903
1000 µL CO-RE II Tips (Filtered, Conductive)	Hamilton, 235905
60mL Reagent Trough Self-Standing	Hamilton, 194051
Heat Sealing Foil	Thermo Scientific, AB-0757
MicroAmp Clear Adhesive Film	ThermoFisher Scientific, 00146104
2mL Sarstedt Tubes	Sarstedt Inc, 72.694.306
300mL Reservoir	Hamilton, 56669-01
2mL Amber tubes	ThermoFisher Scientific, 03-390-28
Equipment	**Catalog Number**
Hamilton NGS MOA star	Contact Hamilton representative
Vortex Mixer	Any major lab supplier (MLS)
Microcentrifuge	Any MLS
ALPS 50 V-Manual Heat Sealer	ThermoScientific, AB-1443A
Plate Centrifuge with 2250 g force capability	Any MLS
Femto Pulse System	Agilent, M5330AA
Qubit 4 or Qubit Flex Fluorometer	ThermoFisher Scientific, Q33238 (Qubit 4), Q33327 (Qubit Flex)
Varioskan LUX multimode microplate reader	ThermoFisher Scientific, VL0L00D0

Reagents	Catalog Number
Revio HiFi prep kit 96, includes: • SRE HT • HiFi prep kit 96 • SMRTbell® cleanup beads 85 mL • SMRTbell® adapter index plate 96A • AMPure® PB • Elution buffer • Revio polymerase kit 96	PacBio®, 103-382-200
200 Proof ethanol, molecular biology or ACS grade	Any MLS
Nuclease-free water, molecular biology grade	Any MLS
Femto Pulse gDNA 165kb Analysis Kit	Agilent, FP-1002-0275
Qubit 1x dsDNA HS (High Sensitivity) Assay Kit	ThermoFisher Scientific, Q33231
Quant-iT 1X dsDNA HS assay kit (for Varioskan)	ThermoFisher Scientific, Q33232

Genomic DNA (gDNA) QC and input amount recommendations

PacBio Nanobind® DNA extractions kits are recommended to ensure sufficient mass and quality of high molecular weight DNA for this protocol.

gDNA quality QC

The Agilent Femto Pulse system is highly recommended for the accurate sizing of gDNA. Please see the PacBio Technical note for more details.

Recommended guidelines for evaluating gDNA quality for this protocol:

- Use the Femto Pulse gDNA 165 kb analysis kit (Agilent FP-1002-0275)
- Dilute samples to 250 pg/µL
- 70% or more of the DNA should be ≥10 kb for this protocol. This corresponds to a genome quality number (GQN) of 7.0 or higher at 10 kb.

Important:

Because HiFi reads are single molecules of DNA, the total base yield and mean read length of a sequencing run is directly proportional to the quality of the genomic DNA input and the fragment lengths generated after shearing. To maximize yield and genome coverage per SMRT® Cell, start with high quality gDNA containing little to no DNA below 10 kb, and with >50% mass over 30 kb. In general, the better the quality of gDNA going into the protocol, the higher the percent recovery and HiFi sequencing yield.

Please see the Revio spec sheet for more information on yield expectations by insert size.

gDNA input amount

It is highly recommended to use a quantification assay specific for double stranded DNA (dsDNA) such as the Qubit 1X dsDNA high sensitivity assay kit. Alternatively, when a high number of samples will be prepared, we recommend using the Quant-iT 1X dsDNA high sensitivity assay kit with the Varioskan LUX multimode microplate reader. Please follow manufacturer's instructions for the assay being used.

We *do not* recommend quantification with UV-Vis Spectrophotometers (e.g., NanoDrop) that measure all nucleic acids in a sample. For example, measuring all nucleic acid will inflate the true concentration of gDNA in samples.

Table 1. Recommended DNA input amounts by starting gDNA quality

DNA quality	90% >10 kb	80% >10 kb	70% >10 kb
gDNA input into SRE step	2–3 µg	3–4 µg	4–5 µg

The overall recovery is dependent on gDNA quality and size. **The recovery from gDNA to completed SMRTbell library ranges between 10–25% (includes SRE, shearing, and SMRTbell library preparation).**

Starting with 2 µg of genomic DNA will typically provide enough library to load 1 Revio SMRT Cell (Table 2).

Important: The maximum mass tolerated by shearing and library enzymatic reactions is 3 µg.

Table 2. Polymerase-bound library mass necessary for loading on a Revio SMRT Cell.

Mean insert size	Library at 250 pM
15,000 bp	243 ng
18,000 bp	292 ng
21,000 bp	341 ng

If targeting higher insert sizes or working with lower quality DNA (Table 1), start with at least 3 µg of gDNA to ensure adequate library for optimal SMRT Cell loading.

Multiplexing

All libraries constructed using this protocol will include a SMRTbell adapter index. Starting with SMRT® Link v13.1, there will be a pooling calculator in Sample Setup to help determine the appropriate volumes to use for multiplexing libraries.

Prior to pooling HiFi libraries together please consider the following guidelines:

- Each Revio SMRT Cell is expected to yield 90 Gb of HiFi data, on average, when using a mean insert size >15 kb.
- Only pool samples with similar genome sizes to ensure balanced coverage.
- Ensure that the samples to be pooled have a similar mean insert size and insert length size distribution.
- Pool samples in an equal molar concentration for best balanced coverage.

It is recommended to pool HiFi libraries post-ABC for the following reasons:

- Ability to use only the amount of polymerase-bound library needed for that sequencing run and thus preserving un-pooled library for future sequencing runs.
- Ability to quickly pool different libraries together on additional runs to “top off” coverage.
- Prevent an inhibitor in one sample from affecting the polymerase binding of all samples in a pool.

Reagent handling

Room temperature is defined as any temperature in the range of **18–25°C** for this protocol.

SRE HT kit

Buffer SRE and Buffer LTE are room temperature reagents.

HiFi prep kit 96

Thaw the Repair buffer 96, Nuclease buffer 96, and adapter index plate at room temperature. Once thawed, reaction buffers and adapter index plate may be stored on a cold block, at 4°C, or on-ice prior to making master mix or placing on the liquid handler work deck.

Keep the following temperature-sensitive reagents on ice.

Temperature-sensitive reagents HiFi prep kit 96		
Step used	**Tube**	**Reagent**
Repair and A-tailing	Blue	End repair 96
	Green	DNA repair 96
Adapter ligation	Yellow	Ligation mix 96
	Red	Ligation enhancer 96
Nuclease treatment	Light green	Nuclease mix 96

Bring the following reagents to room temperature 30 minutes prior to use:

- AMPure PB beads
- Elution buffer
- dsDNA quantification reagents

Bring the following reagents to room temperature 1.5 hours prior to use (or the night before if starting protocol in the morning):

- SMRTbell cleanup beads 85 mL

Shake/vortex SMRTbell cleanup beads and AMPure PB beads immediately before use.

Briefly spin down all reagent tubes in a microcentrifuge to collect all liquid at the bottom.

Briefly vortex, then spin down SMRTbell adapter index plate in a centrifuge with a plate adapter to collect all liquid at the bottom of the wells.

Anneal, bind, and cleanup using the Revio polymerase kit 96

Thaw the following reagents at room temperature:

Component	Tube color
Annealing buffer 96	Light blue
Standard sequencing primer 96	Light green
Polymerase buffer 96	Yellow
Loading buffer 96	Green
Dilution buffer 96	Blue

Once thawed, reaction buffers and sequencing primer may be stored on a cold block, at 4°C, or on-ice prior to making master mix or placing on the liquid handler work deck. The loading buffer 96 should be left at room temperature.

Please note that the Loading buffer 96 is light sensitive and should be protected from light when not in use.

Keep the following reagents on a cold block or ice:

- Sequencing polymerase 96
- Sequencing control 96

Bring the following reagents up to room temperature 30 minutes prior to use:

- Loading buffer 96

Bring the following reagents up to room temperature 1.5 hours prior to use (or the night before if starting protocol in the morning):

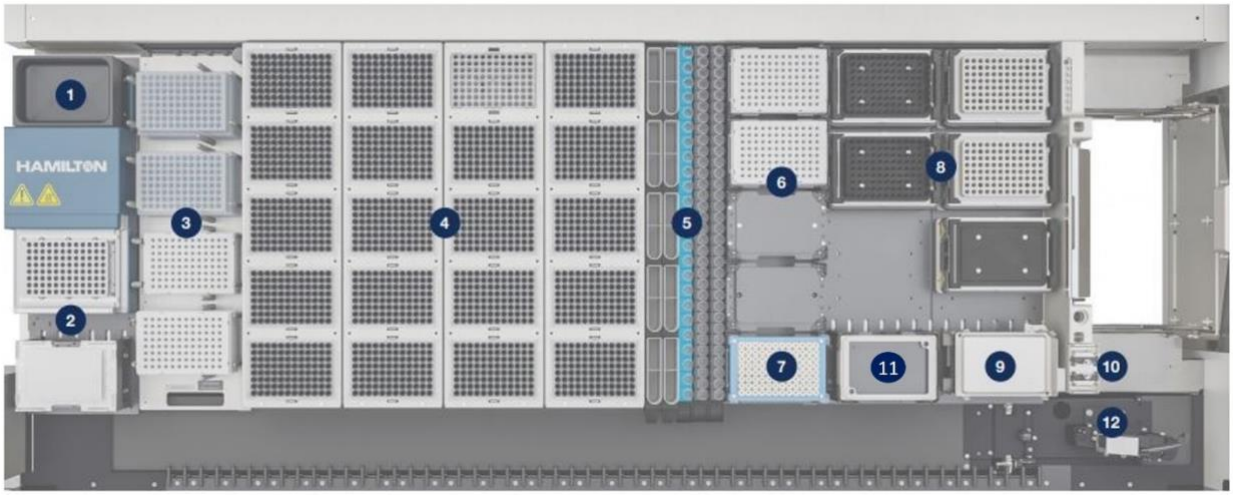
- SMRTbell cleanup beads 85 mL

Polymerase-bound library stability

This protocol brings the entire library through the anneal, bind, and cleanup (ABC) steps. The sequencing polymerase is stable once bound to the HiFi library and can be stored at **4°C for 1 month** or at **-20°C for at least 6 months**. Stored polymerase-bound library shows equivalent loading to freshly prepared bound libraries up to the recommendations listed below.

Please note that the stored polymerase-bound library needs to be protected from light while stored.

Hamilton NGS Star MOA System



Deck Layout

Note: Contact your Hamilton representative for installation and deck details

1. Gravity liquid waste for Multi Probe Head (MPH)
2. On-Deck Thermal Cycler (ODTC) with lid parking position

3. Plate stacker
4. Tip Carriers with MPH tip support adapter
5. Reagent carriers
6. Plate carrier
7. Alpaqua Magnum FLX magnetic plate
8. Hamilton Heater Shakers with 96-well PCR PCR plate adapters and flat bottom
9. Inheco CPAC with 2mL tube cold block adapter (CPAC 2)
10. CO-RE gripper paddles
11. Inheco CPAC with 96-well PCR plate adapter (CPAC 1)
12. Autoloader with barcode reader

Safety precautions

Refer to the Safety Data Sheet (SDS) for information on reagent hazards and protocols for safe handling, use, storage, and disposal.

Workflow steps

Preparation of the Hamilton NGS Star MOA System

- Set CPACs (Cold Plate Air Cooled device) to 4°C before thawing and preparing reagents and consumables.
- Ensure that tip support adapter is empty before starting a run.

1. Hamilton NGS STAR HiFi prep SRE + DNA Shearing Module loading

1. Prepare **gDNA sample plate for SRE.**

   Pipette 50 µL of gDNA (40–100 ng/µL) into a 96-well **PCR 200 µL** plate (Bio-Rad, HSP9601) starting with position A1. Proceed to fill the plate by column in a downward position as shown in the example in Figure 1 below.

Note: Automation SRE module runs in multiples of 8. If sample count is not in multiples of 8, fill the remaining wells in the column with 50 µL of Buffer LTE or water. The method currently supports sample runs in sets of 8, but 96 samples will not be supported in the reagent kit in this configuration. The reagent kit supports 4 sets of 24 sample runs.

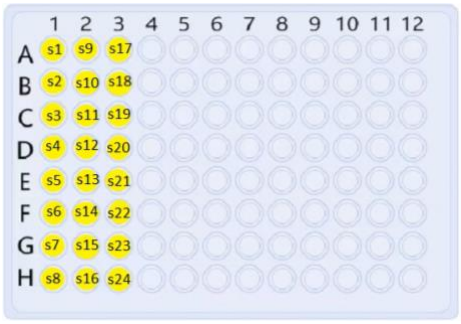


Figure 1. 24 sample input plate example for SRE (s1–s24). Prepare in the 96-well PCR 200 µL plate (Bio-rad, HSP9601).

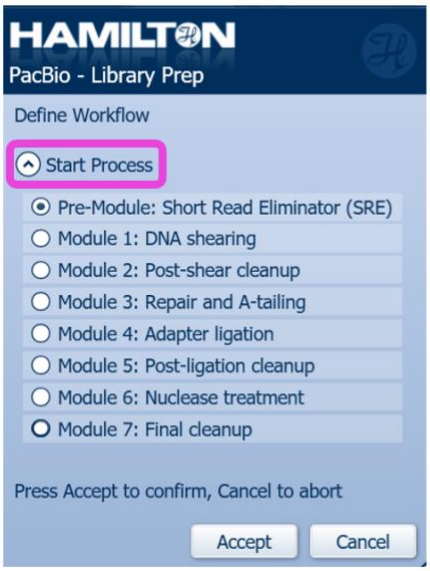
2. **Prepare reagents and consumables for SRE.**
   Gather the following reagents and consumables for SRE. Instrument prompts will provide reagent volumes based on sample count and will indicate where and when to load.

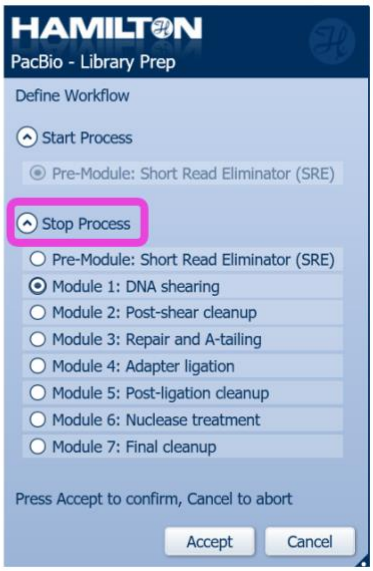
Reagent	Labware
Buffer SRE	2mL tube(s) in kit
Buffer LTE	60 mL trough

3. Start the PacBio HiFi Prep method “PacBio HiFi Prep v4.5.1”.

4. Enter “USER ID” for run. Click “OK”.

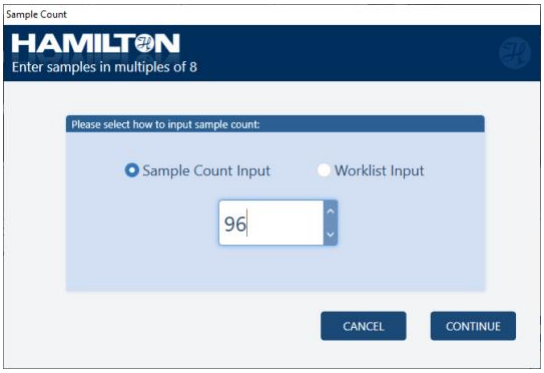
5. **Define Workflow.** Select the start process at: “Pre-Module: Short Read Eliminator (SRE)”. Select the stop process at: “Module 1: DNA Shearing”. Click “Accept”.





Enter sample count.
There are two ways to enter the sample count:
(1) In “Sample Count Input”, type in number that is multiple of 8.
(2) In “Worklist Input”, a worklist input file can be uploaded for sample tracking. Reference Appendix for instruction.

Note: The HiFi prep kit is optimal for 4 sets of 24 sample runs or 1 set of 96 sample run.
Click “CONTINUE”.

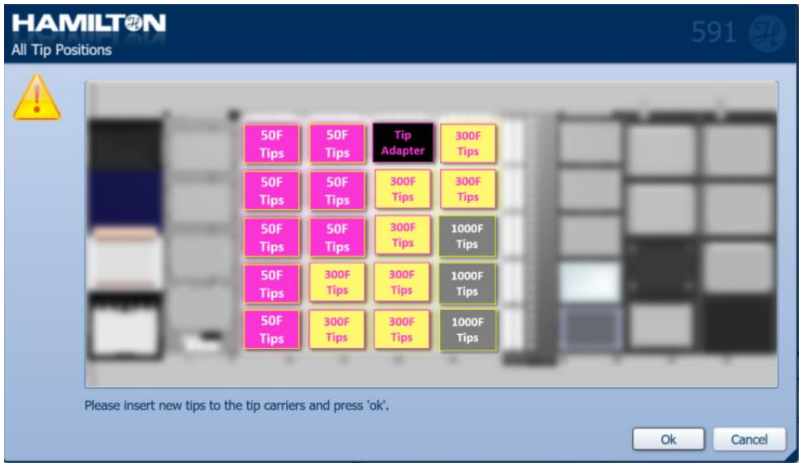


6. **Load the stacker carrier.**
   Load an empty 96-well PCR 200 µL plate (Bio-Rad, HSP9601) in carrier position 4. Click “Ok” to continue.

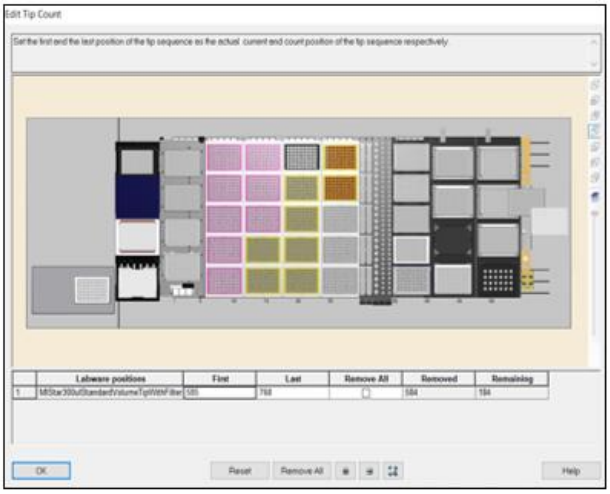
7. **Ensure the tip support for the MPH is empty.**
   CO-RE I instruments require the CO-RE I tip support. CO-RE II instruments require the CO-RE II tip support. Contact your Hamilton Representative to ensure the configuration is set up correctly with your instrument’s CO-RE technology.

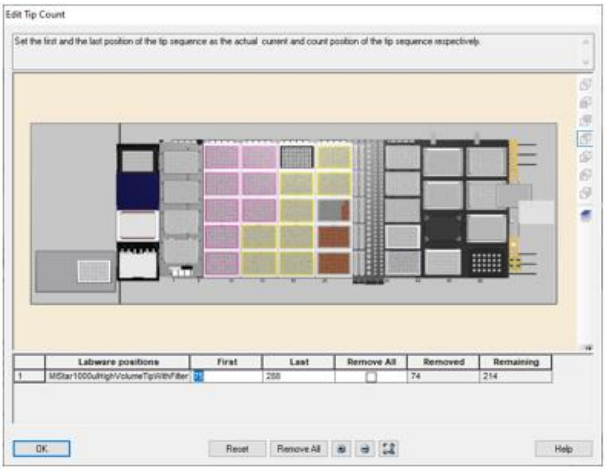
8. **Tip Deck Layout.**

   A prompt displaying the tip positions will appear, including the tip adapter position. There are 4 tip carriers. Refill the tips on deck in the positions for each tip size: 50 µL filtered conductive tips, 300 µL filtered conductive tips and 1000 µL filtered conductive tips.



9. **Select the 300 µL and 1000 µL tips on the instrument.**
   The type of tip will be under “Description” and the number of tips needed will be under “Quantity”. Select ‘OK” to continue to tip deck matching display. To select your tips to match the deck, click and drag so the positions are bolded. Click again to de select. Select “OK” to continue once you have matched tip positions to the deck. Note: It is critical that these selections are accurate and to leave an empty tip rack even if no tips are present (See Appendix for example).



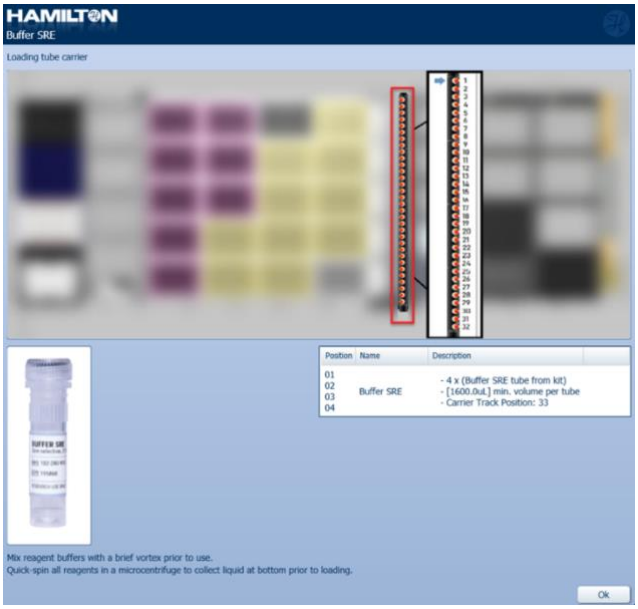


10. **Load 60mL trough in the reagent carrier.**
    Load the appropriate volume of Buffer LTE provided in the SRE HT kit into a 60 mL trough and place it into reagent carrier position 3, track position 30. The table below shows Buffer LTE volumes based on sample count. Select “Ok” to continue. The example prompt below specifies Buffer LTE volume for 96 samples.

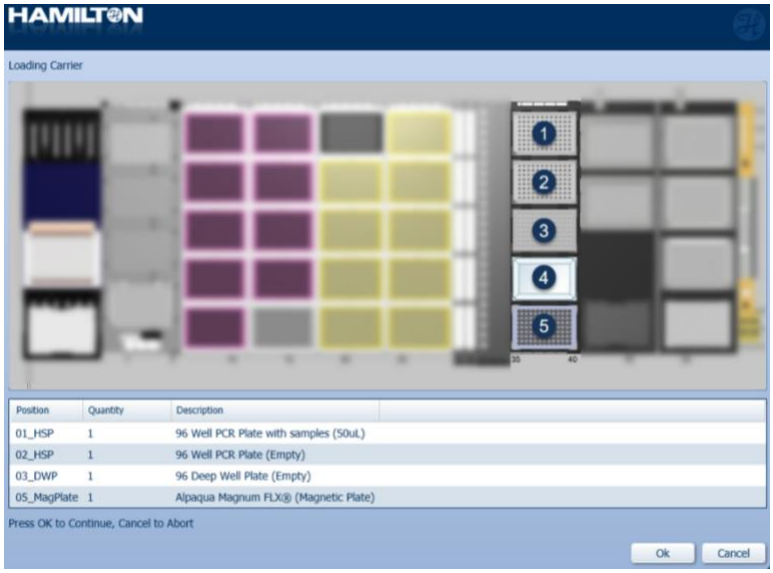
Reagent	Labware	24 Samples	48 Samples	72 Samples	96 Samples
Buffer LTE	60 mL trough	11,200 µL	18,400 µL	25,600 µL	32,800 µL

11. **Load the Buffer SRE tube.**
    Load the appropriate number of Buffer SRE tube(s) directly from the SRE HT kit to the tube carrier in track 33, starting at position 1. The table below shows the Buffer SRE tubes required according to sample count. Spin-down to ensure no bubbles are present in the tube and remove the cap prior to loading. Select “OK” to continue. The example prompt below is the number of Buffer SRE tubes for 96 samples.

Reagent	Labware	24 Samples	48 Samples	72 Samples	96 Samples	
Buffer SRE	2mL tube(s) in kit	1 tube from SRE HT kit (1,600 µL x1)	2 tubes from SRE HT kit (1,600 µL x2)		3 tubes from SRE HT kit (1,600 µL x3)	4 tubes from SRE HT kit (1,600 µL x 4)



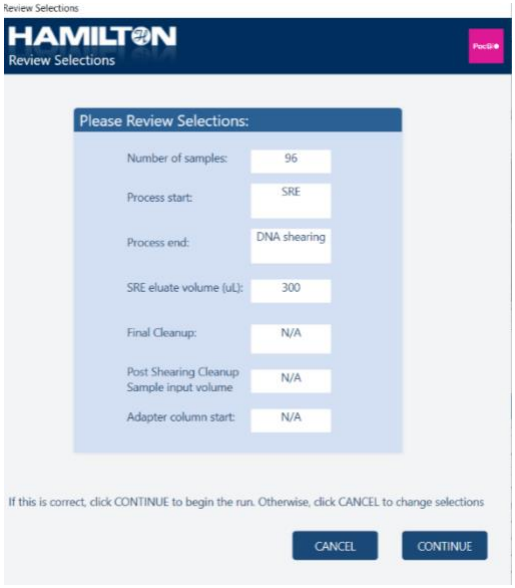
12. **Load plate carrier.**
    Load the following on the plate carrier: Position 1–96-well plate with 50 µL of sample (Bio-Rad, HSP9601); Position 2 – empty 96-well PCR plate (Bio-Rad, HSP9601), Position 3 – empty 96-well Deepwell plate (Thermofisher Scientific, AB0859), which is the SRE output plate, and Position 5 – Alpaqua magnum FLX magnet plate.



13. **Review selections.**
    The prompt is set for 96 samples starting with SRE and ending at the DNA shearing module with a 300 µL SRE eluate volume. The run begins SRE after selecting “CONTINUE”.

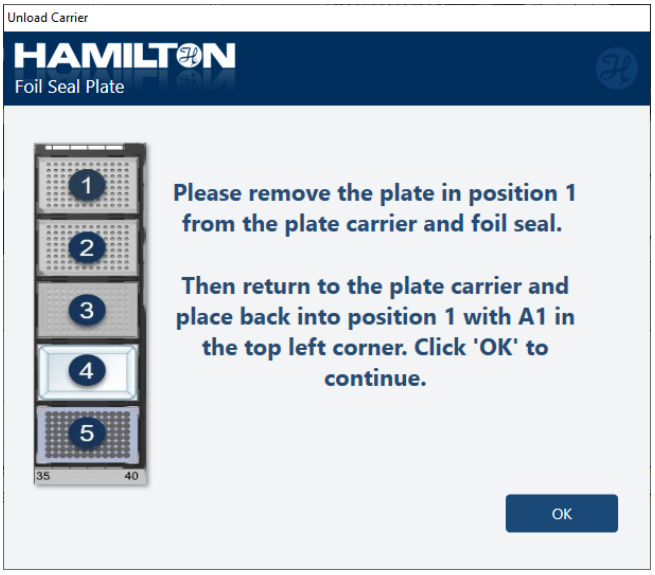
Note: There will be a pause between SRE and DNA shearing to quant after SRE.

Note: For a 96 sample run, the next prompt will display after ~10 minutes.



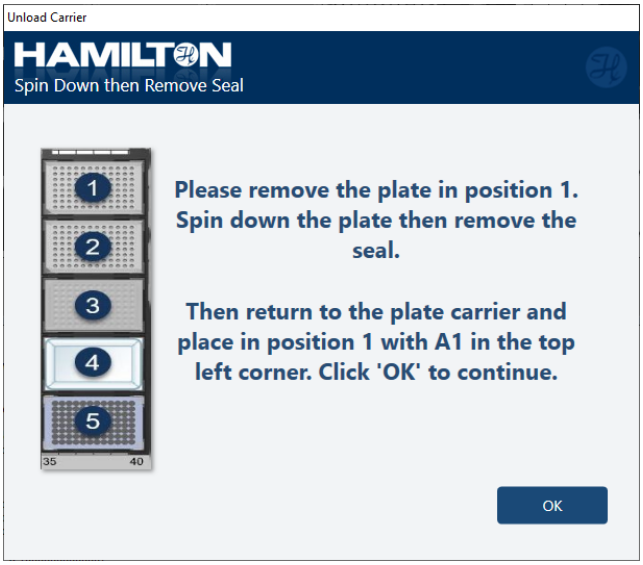
14. **Seal the sample plate for a 1 hour 50ºC incubation.**
    After the instrument adds Buffer SRE to the samples, the plate carrier will unload and prompt the user to heat foil

seal the plate and place back in position 1. Select “OK” to continue. Once “OK” is selected, the instrument pulls the carrier back in and continues the run.



Note: For a 96 sample run, the next prompt will display after ~1 hour.

15. **Remove seal after incubation.**
    Once the incubation is complete, the instrument unloads the plate carrier and prompts the user to carefully spin down the sample plate and remove the seal. Place the sample plate back on the instrument in position 1 then select “OK” for the instrument to pull the plate carrier back in and continue the run.



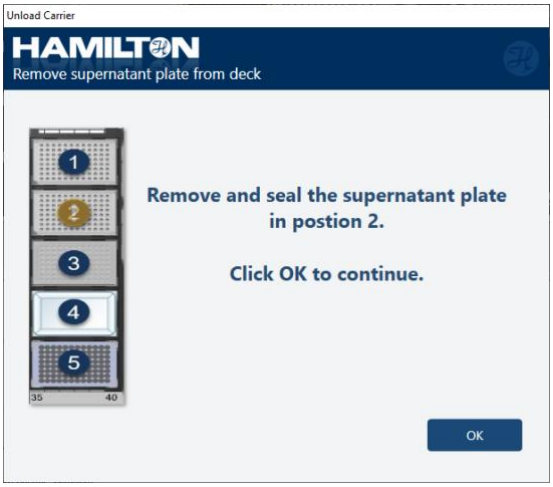
16. **Centrifugation.**
    The instrument unloads the plate carrier and prompts the user to seal the plate with an adhesive seal. The user will then take the deep well sample plate in position 3 to the plate centrifuge. Centrifuge at >2250 rcf (max 3220

rcf) for 60 minutes at room temperature. If the centrifuge has a cooling function, set temperature to 29°C. After centrifugation, immediately remove the seal and place the sample plate back into position 3. Select “OK” to continue. The instrument will pull the plate carrier back in.

Note: Adhesive seal is required due to heat sealing melting the plate.

Note: For a 96 sample run, the next prompt will display after ~1 hour.

17. **SRE method complete and DNA shearing start.**
    At the end of SRE, the instrument prompts the user to remove and seal the **supernatant plate in position 2**. This plate will only be needed to recover samples if a sample dropout occurs (see troubleshooting appendix).



The deep well plate with post-SRE samples is in position 3. Quant post-SRE samples with a Qubit 1x dsDNA HS assay. Adjust DNA concentration to ≤10 ng/µL, if necessary (e.g. if more than 3 µg of gDNA was recovered from SRE). Use Buffer LTE to dilute samples if necessary. Spin down and return the deep well post-SRE sample plate to deck position 3 and click “OK” to begin DNA shearing.

Note: For a 96 sample run, the next prompt will display after ~30 minutes.

18. **DNA shearing complete.** The deep well plate with sheared DNA is in position 3. Recommended: Further dilute a subset of the samples to 250 pg/µL with Femto Pulse dilution buffer. Check the sheared DNA distribution with a Femto Pulse system to ensure efficient shearing. Store plate with an adhesive seal in the 4ºC until ready to proceed with library preparation.

2. Hamilton NGS STAR HiFi prep loading (Post-shear cleanup – 3.1x diluted Ampure PB final cleanup)

1. **Prepare reagents and consumables for library preparation.** Gather the following reagents and consumables as shown in the table below. Instrument prompts will guide when to load and prepare each reagent.

Note: Master mix preparation will be described at step 9.

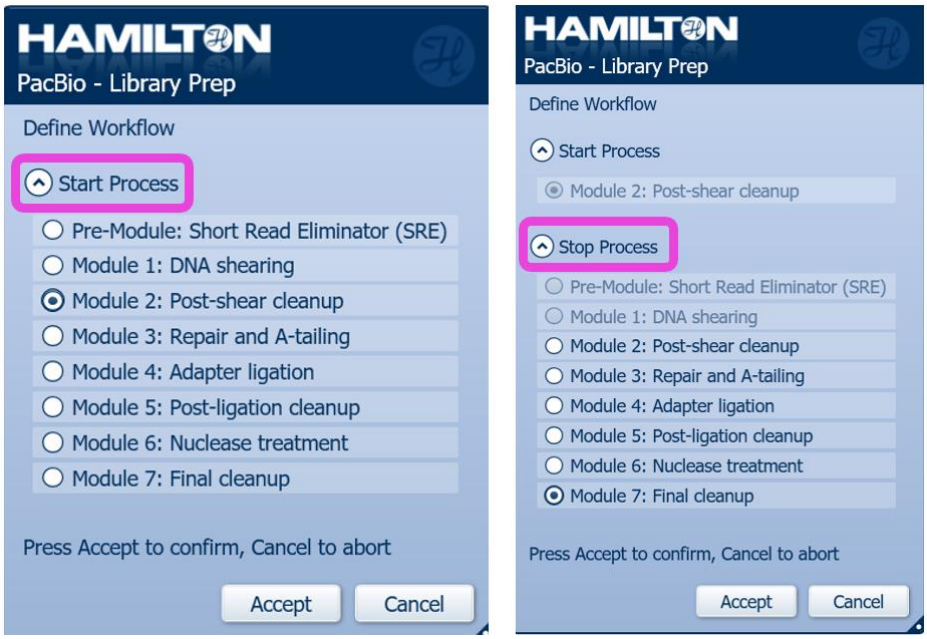
Reagent	Consumables
SMRTbell cleanup beads	60 mL trough
Elution Buffer	60 mL trough
80% Ethanol	300 mL reservoir
SMRTbell Adapter Plate	96 Well PCR Plate part of kit
End Repair Master Mix (Repair Buffer 96, End Repair 96, and DNA Repair 96)	2mL Sarstedt tube(s) – for master mix prep only
Ligation Master Mix (Ligation mix 96 and Ligation Enhancer 96)	2mL Sarstedt tube(s) – for master mix prep only
Nuclease Master Mix (Nuclease buffer 96, Nuclease mix 96)	2mL Sarstedt tube(s) – for master mix prep only

2. Start the **Hamilton library prep script:** “PacBio HiFi Prep v4.5.1”.

3. Enter “USER ID” for run. Click “Ok”.

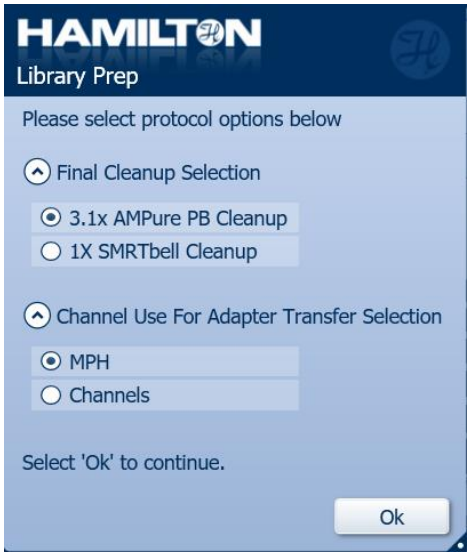
4. Define Workflow

Select start process at “Module 2: Post-shear cleanup” and stop process at “Module 7: Final Cleanup”. Click “Accept”.

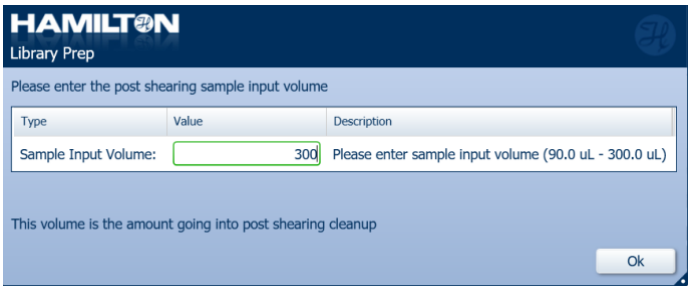


5. **Final Cleanup Selection.** Select “**3.1x AMPure PB Cleanup**” for the final cleanup. 3.1x AMPure PB will remove contaminants and deplete DNA <3kb. 1X SMRTbell Cleanup is only recommended if doing an alternative size selection method. Select “MPH” or “Channels” for adapter addition. MPH = CO-RE 96 channel Mulit-Probe Head. Channels = 8 channels. Click “Ok”.

Note: MPH is highly recommended.

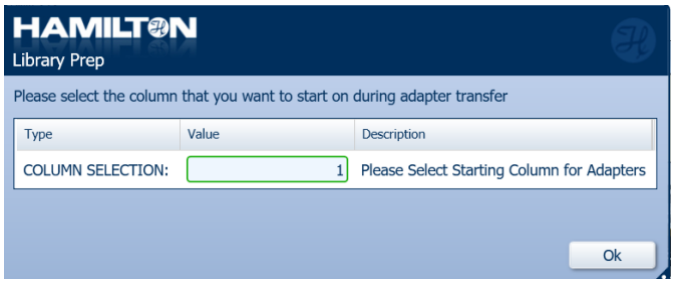


6. **Sample Input Volume.** If pipette tip shearing is performed, enter “300” µL as “Sample Input Volume” for post-shear cleanup. If an alternative shearing method is performed, the “Sample Input Volume” range is compatible with 90–300 µL. Click “Ok” to continue.



7. **Sample Count Input.** There are two ways to enter the sample count:
(1) In “Sample Count Input”, type in number that is multiple of 8.
(2) In “Worklist Input”, a worklist input file can be uploaded for sample tracking. Reference Appendix for instruction.
Click “CONTINUE”.

8. **Adapter Column Selection.** Enter start column number (range 1–12) for adapter plate transfer. This prompt will only show up if sample count is ≤88 samples.



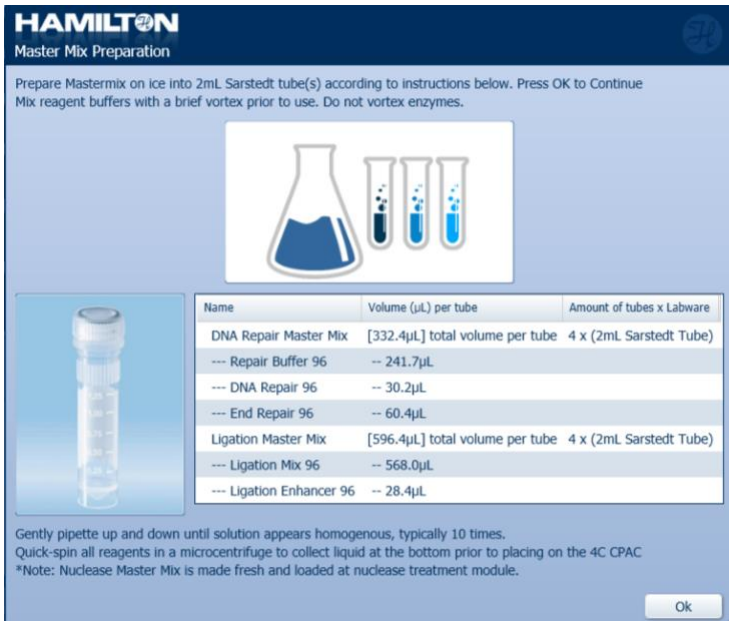
9. **Master mix preparation for DNA repair and ligation.** The table below describes how to prepare the DNA repair master mix and the ligation master mix for 24, 48, 72, 96 samples in 2 mL Sarstedt tubes. The prompt is an example for a 96-sample run. Gently pipette mix master mixes and spin down tubes to remove any bubbles. Place master mix tubes on ice until prompted to load on deck.

Note: Nuclease master mix must be prepared fresh at the nuclease treatment step for optimal performance. The prompt for this preparation is at step 20.

Note: Ligation mix 96 is very viscous, pipette slowly.

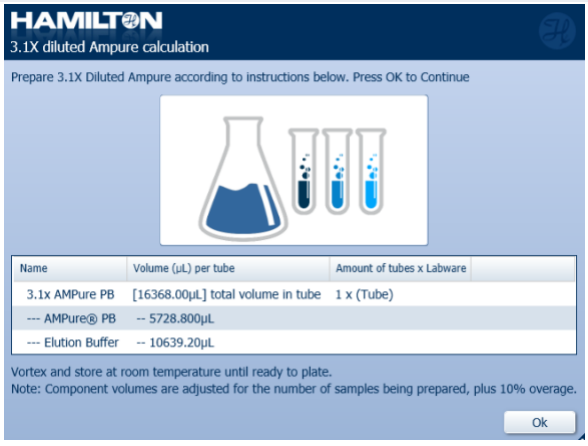
Master mix for library preparation DNA Repair Master Mix				
Sample Configuration	24-samples	48-samples	72-samples	96-samples
# 2 mL Sarstedt Tubes	1	2	3	4
Reagent Name	Reagent Volumes (µL)			
Repair Buffer 96	241.7 µL	Prepare 24-sample master mix 2-times for each tube	Prepare 24-sample master mix 3-times for each tube	Prepare 24-sample master mix 4-times for each tube
DNA Repair 96	30.2 µL			
End Repair 96	60.4 µL			
Total volume per 2mL tube	332.4 µL			

Ligation Master Mix				
Sample Configuration	24-samples	48-samples	72-samples	96-samples
# 2mL Sarstedt Tubes	1	2	3	4
Reagent Name	Reagent Volumes (µL)			
Ligation Mix 96	568.0 µL	Prepare 24-sample master mix 2 times for each tube	Prepare 24-sample master mix 3 times for each tube	Prepare 24-sample master mix 4 times for each tube
Ligation Enhancer 96	28.4 µL			
Total Volume per 2 mL tube	596.4 µL			

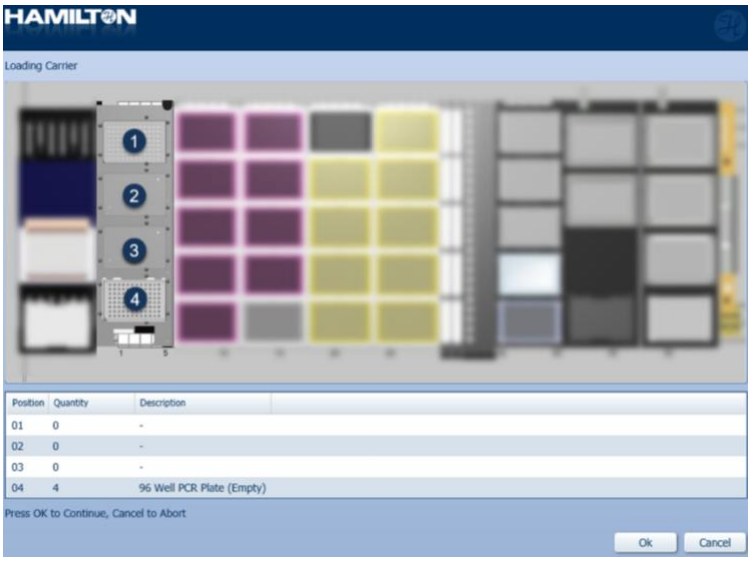


10. **3.1x diluted AMPure PB calculation.** Prepare 35% v/v dilution of room temperature AMPure PB beads in a tube as described in the table below based off sample count. Accurate pipetting is critical at this step. Failure to make a 35% AMPure solution will result in sample loss. Vortex AMPure PB beads before making the dilution. After making the 35% v/v diluted beads, leave at room temperature until prompted to load. Do not plate AMPure PB beads until prompted to prevent evaporation. The prompt is an example for a 96-sample run.

Reagent	24-sample	48-sample	72-sample	96-sample
AMPure PB beads	1,432.2 µL	2,758.8 µL	4,296.6 µL	5,728.8 µL
Elution Buffer	2,659.8 µL	5,319.6 µL	7,979.4 µL	10,639.2 µL
Total Volume	4,092 µL	8,078.4 µL	12,276 µL	16,368 µL



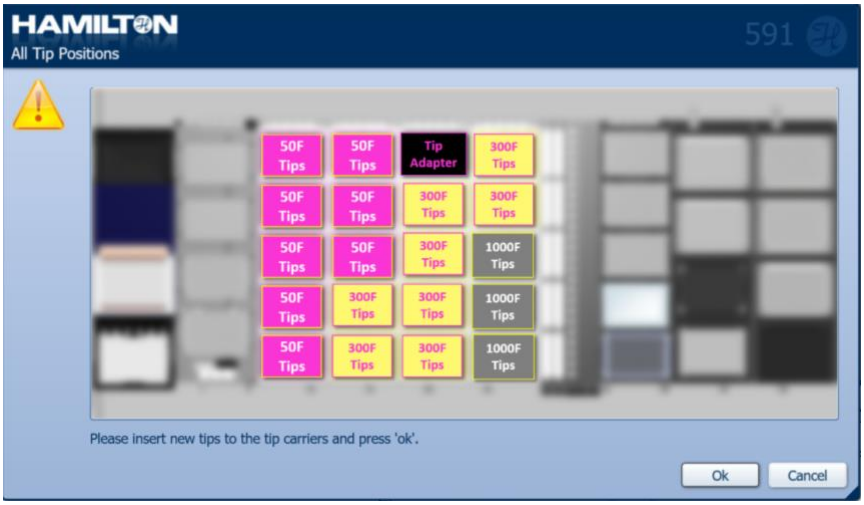
11. **Load the stacker carrier.** Load the stacker plate carrier with four 96-well PCR plates stacked (Bio-Rad, HSP9601) in position 4.



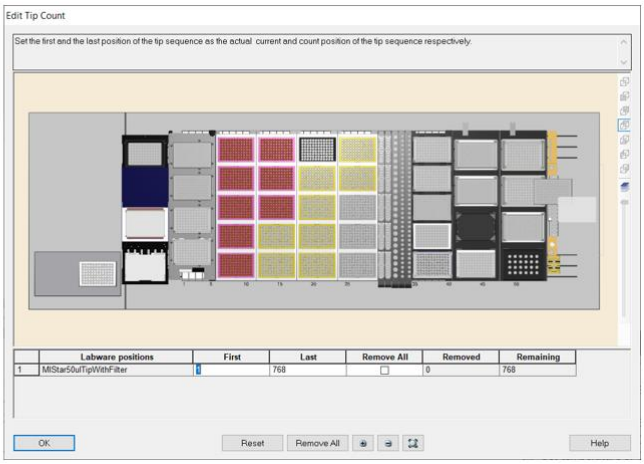
12. **Ensure the tip support for the MPH is empty.**

    CO-RE I instruments require the CO-RE I tip support. CO-RE II instruments require the CO-RE II tip support. Contact your Hamilton Representative to ensure the configuration is set up correctly with your instrument's CO-RE technology.

13. **Tip Deck Layout.** A prompt displaying the tip positions will appear, including the tip adapter position. There are 4 tip carriers. Refill the tips on deck in the positions for each tip size: 50µL filtered conductive tips, 300µL filtered conductive tips and 1000 µL filtered conductive tips.



14. **Select the 50 µL, 300 µL, and 1000 µL tips on the instrument.**
The type of tip will be under “Description” and the number of tips needed will be under “Quantity”. Select ‘OK” to continue to tip deck matching display. To select your tips to match the deck, click and drag so the positions are bolded. Click again to de select. Select “OK” to continue once you have matched tip positions to the deck.
Note: It is critical that these tip selections are accurate and to leave an empty tip rack even if no tips are present (See Appendix for example).



15. **Load 60mL trough in reagent carrier**. Load the SMRTbell cleanup beads and Elution Buffer into the 60 mL reagent troughs. Place troughs into the 60mL reagent carrier in track position 30. Reagent carrier position 1 for SMRTbell cleanup beads and position 3 for Elution Buffer. The table below shows SMRTbell cleanup beads and elution buffer volume amounts based off sample count. The example prompt is for 96-samples. Click “OK.”

Volume of SMRTbell cleanup beads and elution buffer required when starting at post-shear cleanup and ending at 3.1x AMPure PB final cleanup.

Reagent	Consumables	24 Samples	48 Samples	72 Samples	96-Samples
SMRTbell cleanup beads	60 mL trough	11,740 µL	20,980 µL	30,220 µL	39,460 µL
Elution Buffer	60 mL trough	5,880 µL	8,760 µL	11,640 µL	14,520 µL

16. **Load plate carrier.** Load the following on the plate carrier: Position 1 – empty 96 well plate (Bio-Rad, HSP9601), Position 2 - unsealed SMRTbell barcode adapter plate, Position 3 - 96 deep well plate with samples (ThermoFisher Scientific, AB0859), Position 4 – reagent trough (Hamilton, 56669-01) with fresh 80% ethanol (see table for volumes based off sample count) and Position 5 – Alpaqua magnum FLX magnet plate.

Note: Briefly vortex, spin down and remove the seal only for columns selected for SMRTbell barcode adapter plate. Do not let instrument tips pierce the foil seal to prevent the risk of instrument crash or improper aspiration and dispense of adapters. Only remove the seal for the columns used, otherwise the adapters may evaporate.

Reagent	Consumables	24 Samples	48 Samples	72 Samples	96 Samples
80% Ethanol	300 mL reservoir	64,000 µL	88,000 µL	112,000 µL	136,000 µL

17. **Load Master Mix Tubes on Cold Block.** Follow prompt to load the **DNA repair** and **ligation** master mix tubes in the designated positions on the cold block 2mL tube adapter (CPAC 2) at 4°C. The prompt below is for 96 samples. Click “OK”.

If bubbles present in master mix tubes, spin down to remove any bubbles prior to loading on cold block.

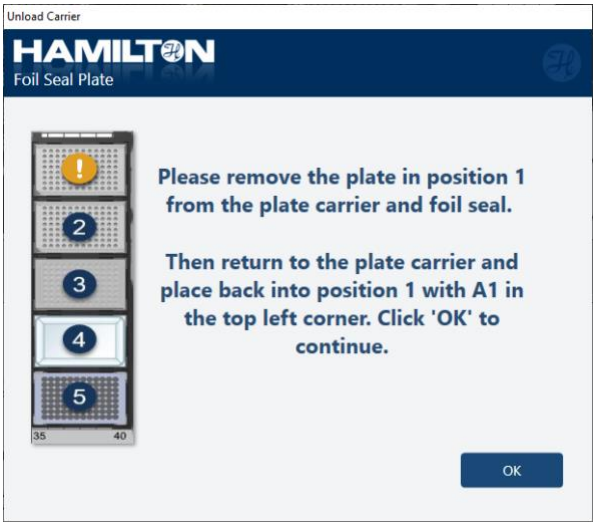
18. **Review selections.** The prompt is set for 96 samples starting at the post-shear cleanup module and stopping at the 3.1x AMPure PB cleanup with a 300 µL sheared sample input volume using adapter column 1. The run begins post-shear cleanup after clicking “Continue”.

Note: For a 96 sample run, after ~40 minutes a prompt will appear to reload 1 mL tips. After reloading tips, the next prompt will display for sealing the plate after ~30 minutes.

19. **Seal the plate for incubation during end repair.**
    The instrument will prompt the user to unload the sample plate from the carrier position 1. Manually heat foil seal the 96-well PCR sample plate for incubation. Return plate to position 1. Click “OK”. After incubation, the instrument will prompt the user to spin down the plate and remove the seal prior to loading back onto the carrier.

Note: Carefully remove the seal to prevent contamination. The instrument will pull the carrier back.

Note: For a 96 sample run, the next prompt will display after ~40 minutes.



20. **Unseal the plate after incubation.** The instrument will prompt the user to retrieve sample plate in position 1 to spin down and remove the seal off deck prior to loading back onto the same position carrier. Click “OK”. The instrument proceeds to adapter ligation and post-ligation cleanup.

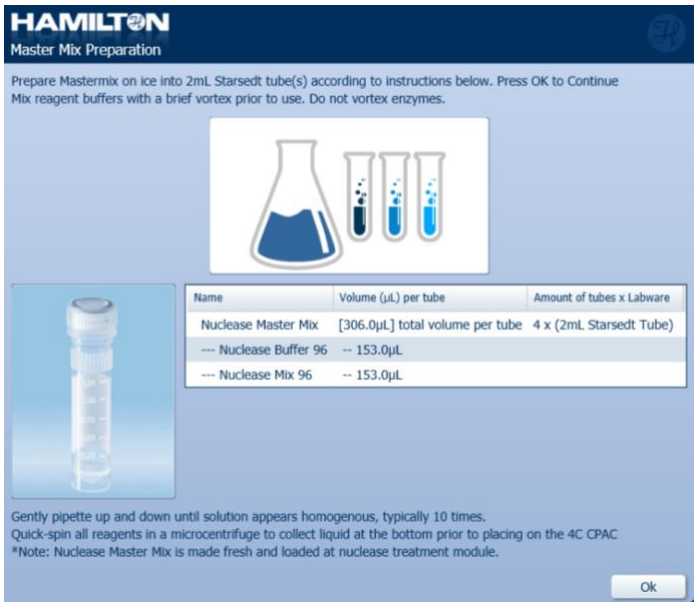
Note: Carefully remove the seal to prevent contamination. The instrument will pull the carrier back.

Note: For a 96 sample run, after 1 hour, 15 minutes a prompt will appear to reload the 50 µL and 300 µL tips. After reloading the tips, the next prompt will display the nuclease master mix preparation after ~25 minutes.

21. **Master mix preparation for nuclease treatment.** The table below describes how to prepare the **nuclease** master mix for 24, 48, 72, 96 samples in 2 mL Sarstedt tubes. The prompt is an example for a 96-sample run. Gently pipette mix master mixes and spin down tubes to remove any bubbles. Place master mix tubes on ice until prompted to load on deck.

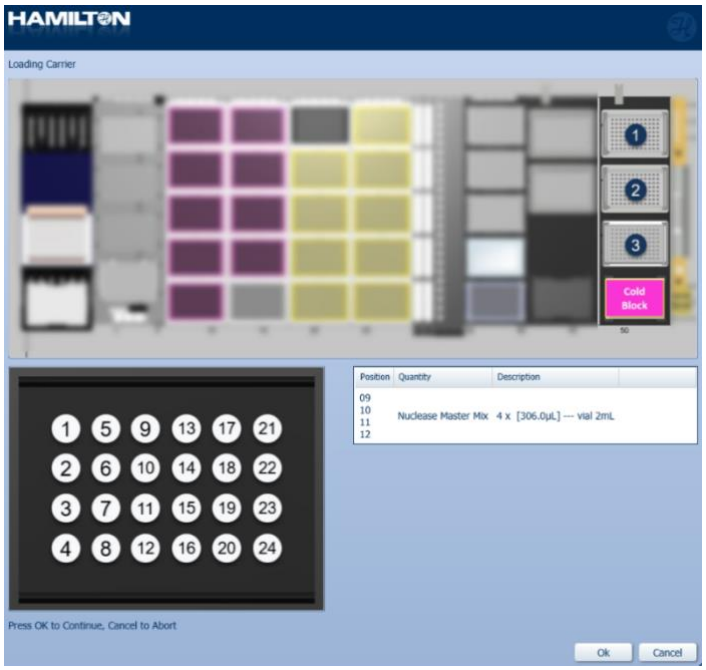
Note: It is mandatory to prepare and load the Nuclease Master mix when prompted.

Nuclease Master Mix				
Sample Configuration	24-samples	48-samples	72-samples	96-samples
# of 2mL Sarstedt Tubes	1	2	3	4
Reagent Name	Reagent Volumes (µL)			
Nuclease buffer 96	153 µL			
Nuclease mix M96	153 µL	Prepare 24-sample master mix 2 times for each tube	Prepare 24-sample master mix 3 times for each tube	Prepare 24-sample master mix 4 times for each tube
Total volume per 2 mL tube	306 µL			

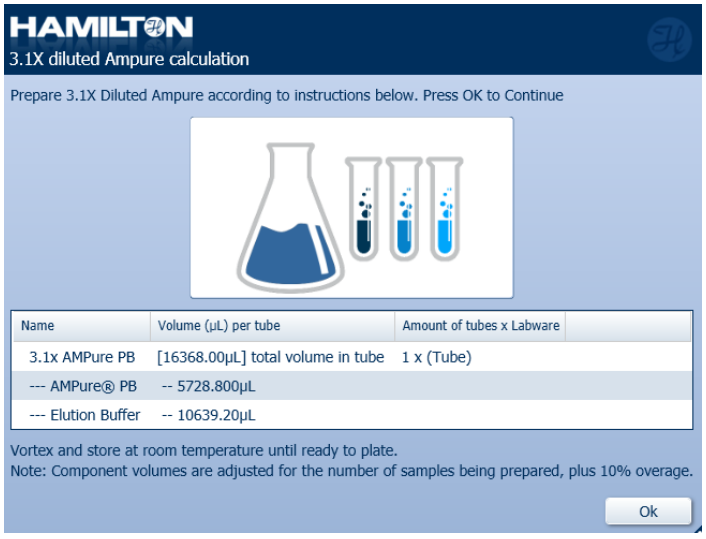


22. Load **Nuclease Master Mix Tube(s) on Cold Block.** Follow prompt to load the **nuclease** master mix tube(s) in the designated positions on the cold block 2mL tube adapter (CPAC 2) at 4ºC. The prompt below is for 96 samples. Click “OK”.

Note: If bubbles present in master mix tubes, spin down to remove any bubbles prior to loading on cold block.



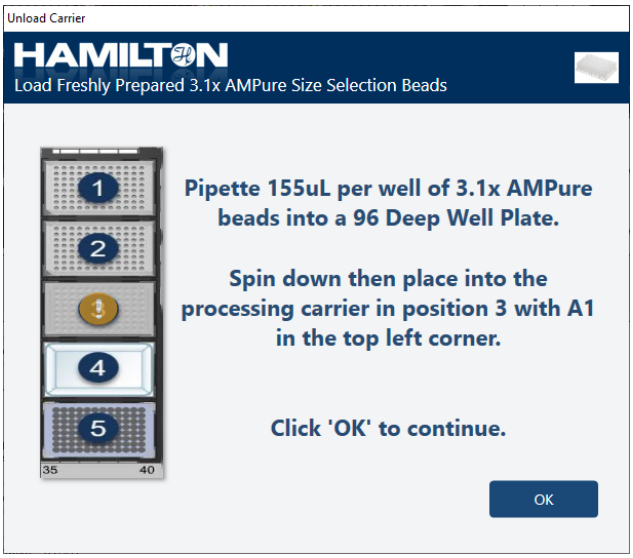
23. **Prepare 35% v/v AMPure PB beads.** In 3.1x AMPure PB final cleanup, the instrument will again display the 35% diluted AMPure PB calculation (same as step 29). If not already prepared, return to step 10 for best preparation practices and make 35% diluted AMPure PB beads in a tube. Click “OK”.

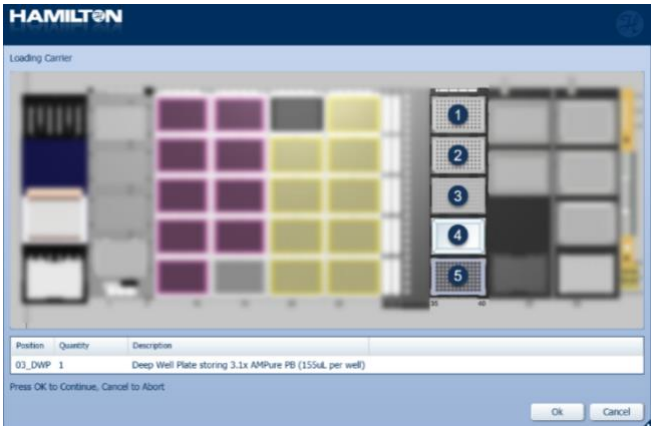


24. **Prepare a deep well plate with 35% v/v AMPure PB beads.** In a 96 deep well plate (Thermofisher Scientific, AB0859), manually pipette 155uL of 35% diluted AMPure PB beads for the number of samples. Click “OK” after loading the 96 deep well 35% diluted AMPure PB plate onto the carrier in position 3.

Note: Vortex diluted AMPure PB beads rigorously before adding to 96 deep well plate.

Note: Use a reservoir to pour diluted AMPure PB beads and use multichannel pipette to aliquot 155µL.It is best practice to change tips after each dispense.

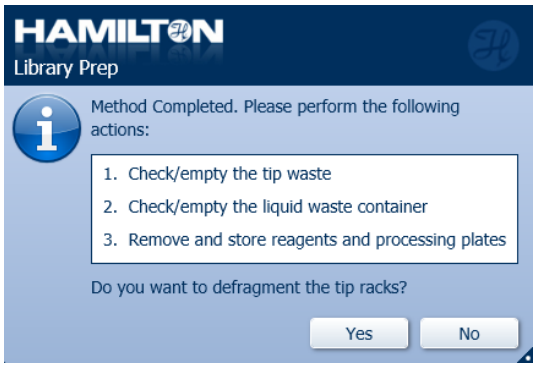




25. **Library preparation complete.** When the run is complete, remove the final library output plate (Biorad, HSP9601) in plate carrier position 1. The final library volume is 25 µL. Seal and store the supernatant plate from the 3.1x Ampure PB bead cleanup in the plate carrier at position 2. This plate will only be needed to recover samples if a sample dropout occurs (see troubleshooting appendix).

    Optional Quant QC: Take a 1 µL aliquot from each tube and dilute with 9 µL of elution buffer or water. Measure DNA concentration with a Qubit fluorometer using the 1x dsDNA HS kit. DNA concentration must be less than 60 ng/µL to proceed to ABC; however, libraries typically are at <40 ng/µL after the SMRTbell library preparation process.

    Optional library size QC: Further dilute each aliquot to 250 pg/µL with Femto Pulse dilution buffer. Measure final SMRTbell library size distribution with a Femto Pulse system. Perform an optional quant QC with Qubit 1x dsDNA HS assay and library size QC with the Femto pulse. Continue with HiFi Prep ABC with this sample output plate.

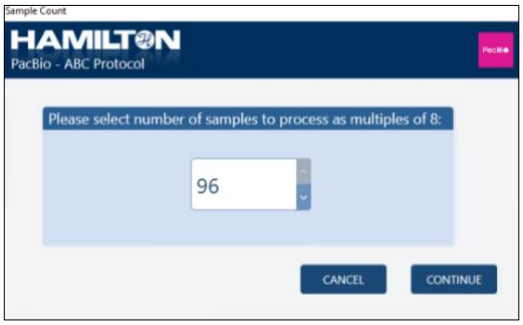


3. Hamilton NGS STAR HiFi Prep annealing, binding, and cleanup (ABC) loading procedure

1. **Prepare reagents and consumables for ABC.** Gather the following reagents and consumables as shown in the table below. Instrument prompts will guide when to load and prepare each reagent.

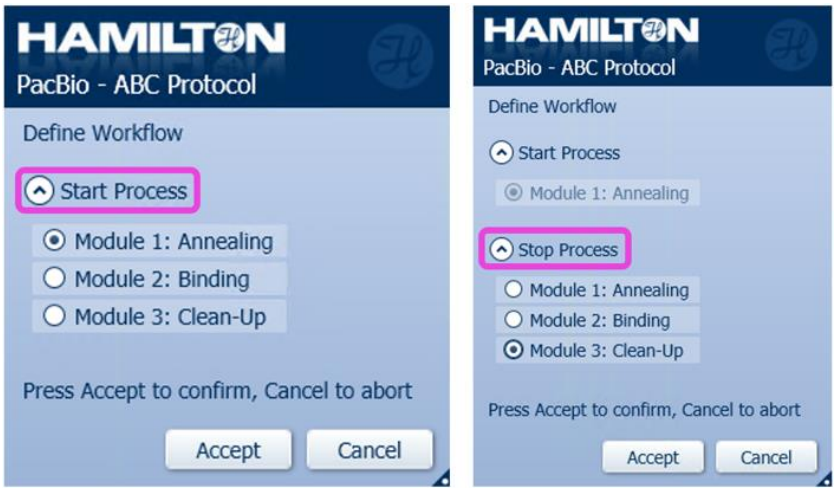
Reagent	Consumables
SMRTbell cleanup beads	60 mL trough
Loading Buffer 96	2mL Amber tubes
Annealing Master Mix (Annealing Buffer 96, Standard Sequencing Primer 96)	2mL Sarstedt tube(s) – for master mix prep only
Binding Master Mix (Polymerase Buffer 96, Sequencing Polymerase 96)	2mL Sarstedt tube(s) – for master mix prep only

2. Start the HiFi **Prep ABC method: "PacBio HiFi Prep ABC v2.1.1".**
3. **Enter sample count.** Enter the number of samples to process. Make sure sample count is a multiple of 8 for column-based processing.



4. Enter a "USER ID" for run. Click "OK."

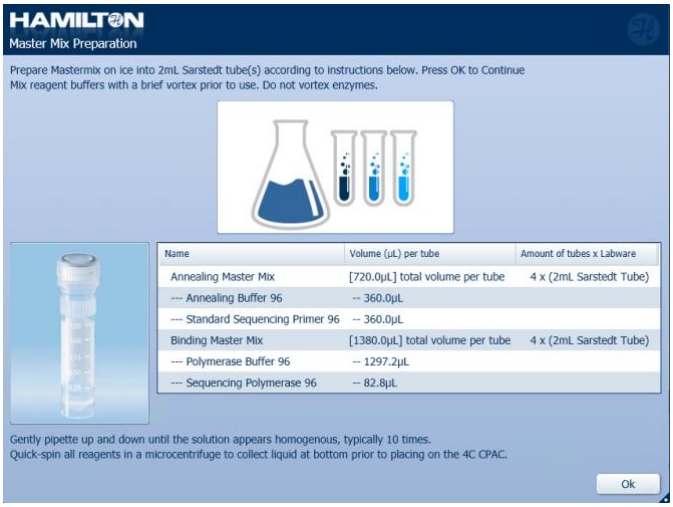
5. **Define Workflow.** Select the start process at: “Module 1: Annealing”. Select the stop process at: “Module 3: Clean-Up”. Click “Accept”.



6. **Master mix preparation for ABC.** The table below describes how to prepare the annealing master mix and the binding master mix for 24, 48, 72, 96 samples in 2 mL Sarstedt tubes. The prompt is an example for a 96-sample run. Gently pipette mix master mixes and spin down tubes to remove any bubbles. Place master mix tubes on ice until prompted to load on deck.

Master Mix for ABC				
Annealing Master Mix				
Sample Configuration	24-samples	48-samples	72-samples	96-samples
# 2mL Sarstedt Tubes	1	2	3	4
Reagent Name	Reagent Volumes (µL)			
Annealing Buffer 96	360 µL	Prepare 24-sample master mix 2-times for each tube	Prepare 24-sample master mix 3-times for each tube	Prepare 24-sample master mix 4-times for each tube
Standard Sequencing Primer 96	360 µL			
Total Volume per 2mL tube	720 µL			

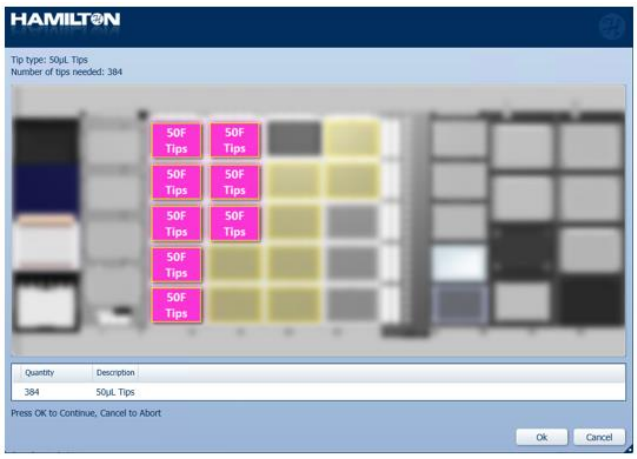
Binding Master Mix				
Sample Configuration	24-samples	48-samples	72-samples	96-samples
# 2mL Sarstedt Tubes	1	2	3	4
Reagent Name	Reagent Volumes (µL)			
Polymerase Buffer 96	1297.2 µL	Prepare 24-sample master mix 2-times for each tube	Prepare 24-sample master mix 3-times for each tube	Prepare 24-sample master mix 4-times for each tube
Sequencing Polymerase 96	82.8 µL			
Total Volume per 2mL tube	1380 µL			

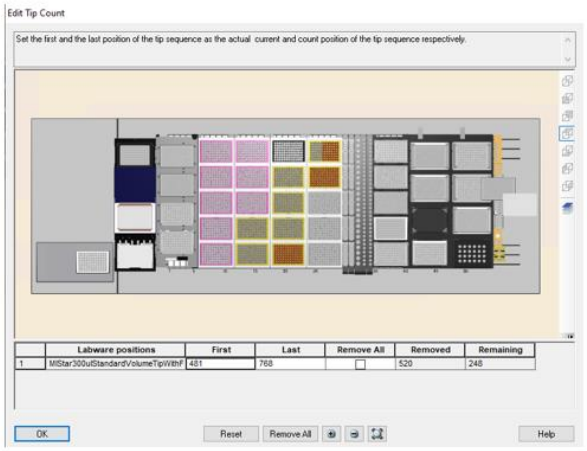


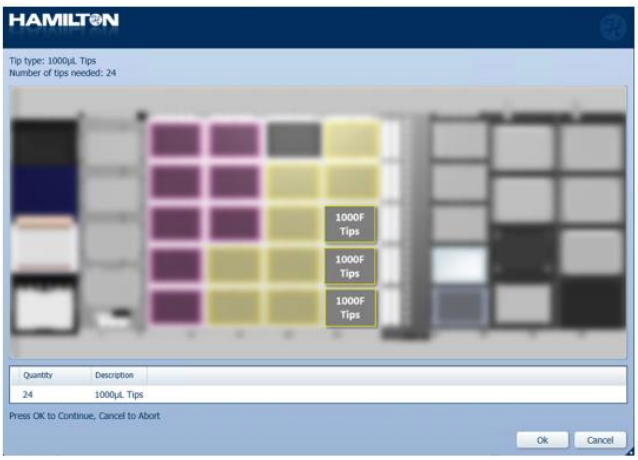
7. **Load stacker carrier.** Load an empty 96 well PCR plate (Bio-Rad, HSP9601) into the stacker carrier in position 4.

8. **Ensure the tip support for the MPH is empty.**
   CO-RE I instruments require the CO-RE I tip support. CO-RE II instruments require the CO-RE II tip support. Contact your Hamilton Representative to ensure the configuration is set up correctly with your instrument's CO-RE technology.

9. **Select the 50 µL, 300 µL, and 1000 µL tips on the instrument.**
   The type of tip will be under "Description" and the number of tips needed will be under "Quantity". Select 'OK" to continue to tip deck matching display. To select your tips to match the deck, click and drag so the positions are bolded. Click again to de select. Select "OK" to continue once you have matched tip positions to the deck.
   Note: It is critical that these tip selections are accurate and to leave an empty tip rack even if no tips are present (See Appendix for example).

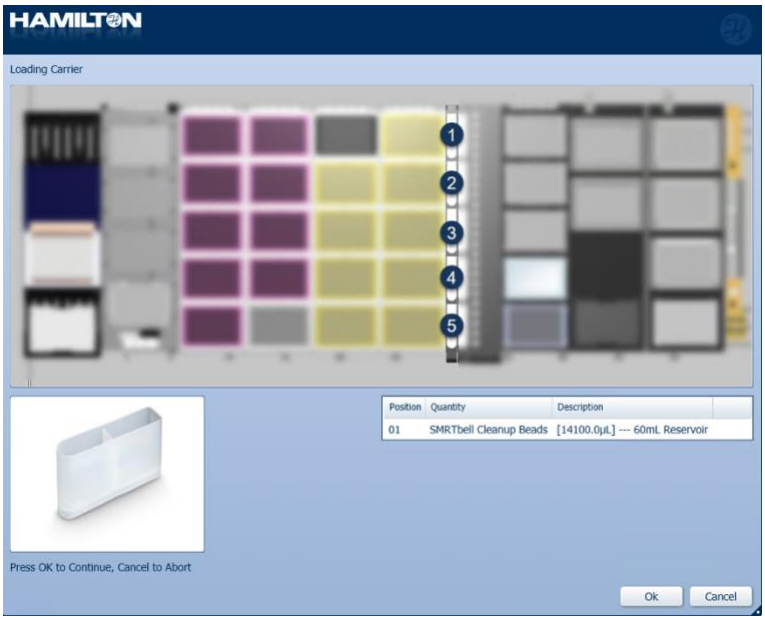






10. **Load 60 mL trough in reagent carrier**.
    Fill a 60 mL trough with the appropriate amount of SMRTbell beads based off sample count shown in table below. Load the 60 mL trough to reagent carrier track position 30 in position 1.

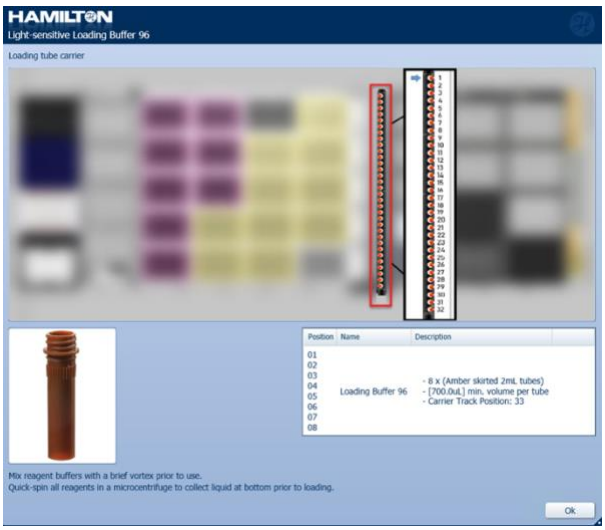
Reagent	Labware	24 Sample	48 Sample	72 Sample	96-Sample
SMRTbell cleanup beads	60 mL trough	6,900 µL	9,300 µL	11,700 µL	14,100 µL



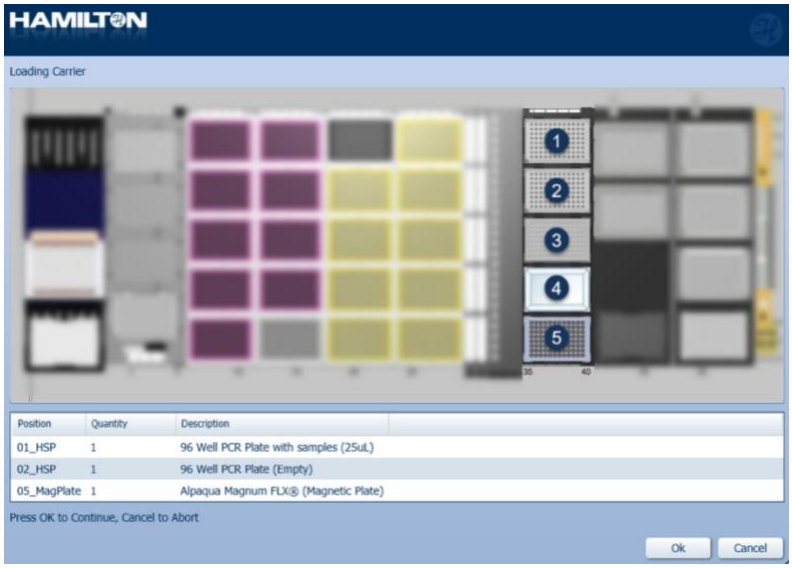
11. **Load Loading Buffer 96 tube.** Load the Loading Buffer 96 in 2mL amber tubes into the tube carrier located in track position 33 with the 700uL of reagent starting with position 1. Spin down the tubes to ensure no bubbles are present. The number of tubes needed is based off the selected sample count as shown in table below. The prompt is an example for a 96-run. Click “OK”.

Note: Can use the tubes from the kit provided.

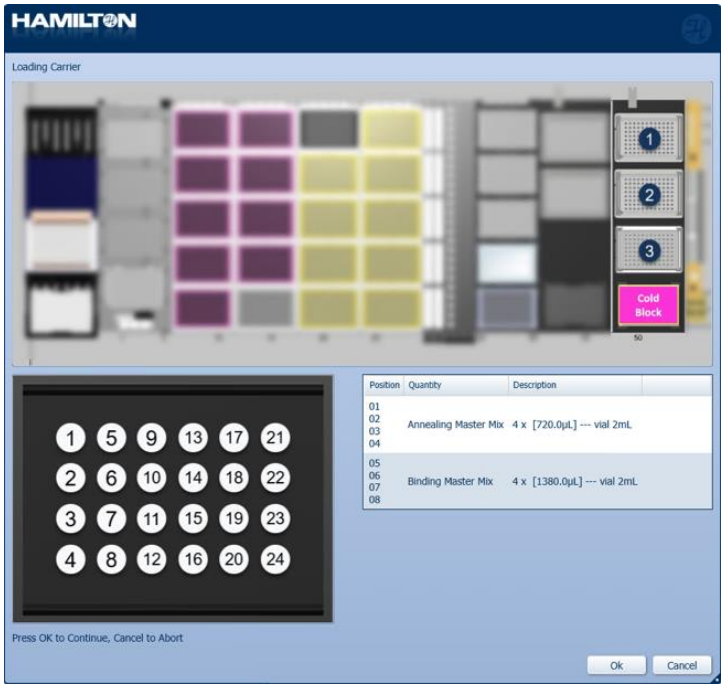
Reagent	Labware	24 Sample	48 Sample	72 Sample	96-Sample
Loading Buffer 96	2mL Amber tube	2 tubes of 700 µL	4 tubes of 700 µL	6 tubes of 700 µL	8 tubes of 700 µL



12. **Load the plate carrier.** Load the plate carrier with an empty 96 well PCR plate (Bio-Rad, HSP 9601) in position 2. The sample plate is loaded into position 01. Ensure the magnet plate is in position 5. Click “OK” to continue.



13. **Load the Master Mix tubes on cold block.** into the designated positions on the 4°C cold block adapter. The prompt is set for 96 samples. Click “OK” to continue.

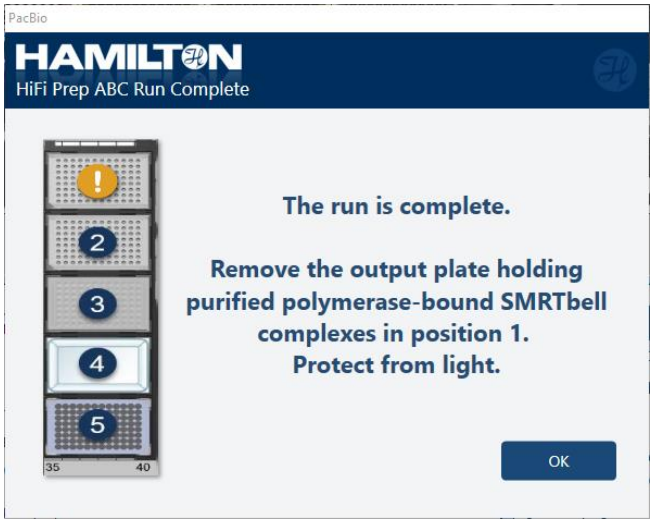


14. **Review selections.** The prompt is set for 96 samples starting at “Annealing” and ending at “Cleanup” with a 25 µL library plate. Click “Continue” to begin ABC.

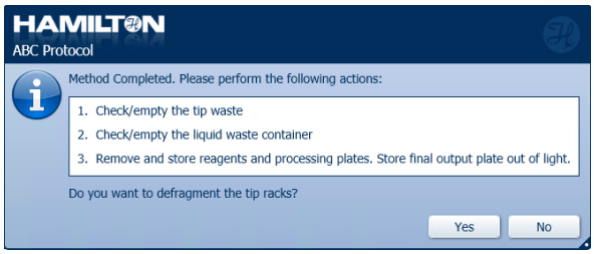
15. **ABC complete.** Once the run is complete, the final polymerase-bound library plate is in position 1. Quant 1 µL of sample to measure concentration with a Qubit fluorometer using the 1x dsDNA HS kit. Click “OK”.

Note: The Qubit Flex instrument is not compatible with measuring polymerase-bound library in Loading Buffer 96. Concentration readings will not be accurate.

Note: Polymerase-bound libraries can be stored at 4°C for 1 month, or at -20°C for >6 months prior to sequencing. Polymerase-bound libraries can withstand >4 freeze-thaw cycles without affecting sequencing performance.



16. Method completion prompt displays.



Proceed to the Loading Calculator in SMRT Link v13.1 or higher to calculate the final dilution for adding the sample to the Revio sequencing plate. The prompt below shows the SMRT Link 'Sample Setup' page.

Note: SMRT Link → Sample Setup → Add Calculation pull down menu → Revio polymerase kit 96

Appendix

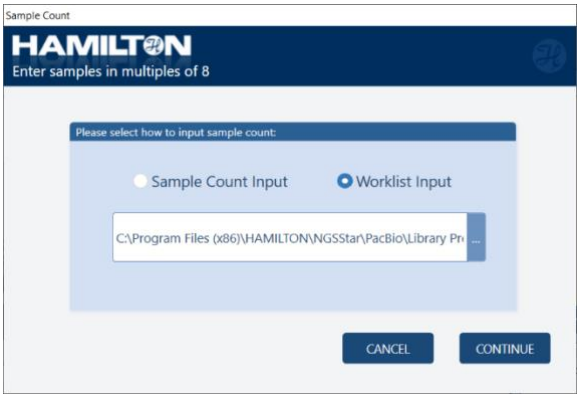
Input file:

Users can enter a .csv file containing sample tracking information. This file can be found in the path installed with instrument C:\Program Files (x86)\HAMILTON\NGSStar\PacBio\Library Prep\Files\Example Worklists. Download an example and edit the .csv file. Save to a known location.

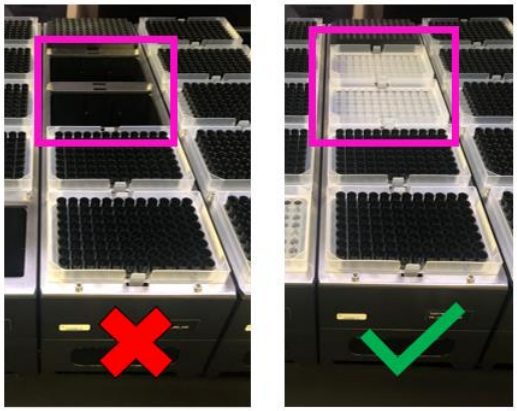
Note: When editing the file ensure sample count is a multiple of 8.

	A	B	C	D
1	SampleID	Barcode	WellPosition	Comment
2	Sample 1	Barcode01	A1	
3	Sample 2	Barcode02	B1	
4	Sample 3	Barcode03	C1	
5	Sample 4	Barcode04	D1	

When sample count is prompted at start-up, navigate to edited file with updated sample information and select.



Empty tip racks: It is possible to have empty tip racks during tip selection. If leaving a tip rack empty, to prevent possible instrument crashes or incomplete liquid transfers for the MPH, place only the tip wafer in the tip carriers. An example of what not to-do (red x) and what to-do (green check mark) for empty tip rack selection is shown below.



Troubleshooting

Bead carryover note

Beads transferred into sample plate eluate does not adversely affect the procedure, although can interfere with quanting accuracy. When measuring concentrations, place sample plate on a magnetic plate to avoid beads.

QC pauses

QC pauses can be toggled on and off to create pauses at end of SRE, post shearing cleanup, post-ligation cleanup and post nuclease treatment. To turn on toggle, enter the Dev Toggles grouping under the main method. Change t_blnQCPausesActivated from False to True.

Note: It is important to switch back the toggle to False after quality checking to ensure the method runs to completion without pauses.

SRE

Issue	Possible Cause	Corrective Action
Low to no recovery at end of run	1. Supernatant Removal	1. Position teaching positions vary per instrument. Adjust teaching positions to leave behind 8 – 10 uL of supernatant in the wells while not disturbing the DNA pellet.
	2. Tip pinching	2. Position teaching positions vary per instrument. To prevent pinching that can leave behind varying volumes in wells, raise the z coordinate slightly in the pipetting position
	3. Incorrect centrifuge settings	3. Ensure plate centrifuge is set at > 2250 rcf (max 3220 rcf) at 29ºC or room temperature for 60 minutes. Maintain a balance with 100 µL per well that matches sample count.
	4. Plate to plate transfer leaves behind volume	4. Check the z coordinates in the pipetting position 1 and adjust to make sure it leaves no volume behind
	5. Quantification method	5. It is essential that DNA concentration is determined by Qubit or PicoGreen assay. Using concentrations derived solely from UV-Vis measurements will often result in low recovery as the DNA concentration will be overestimated due

		to RNA that may also be present in the sample
	6. Highly fragmented gDNA	6. Recovery will be low if DNA is not HMW. Please refer to Genomic DNA (gDNA) QC and input amount recommendations of this protocol.
	7. Sample lost in supernatant	7. DNA pellet aspirated in the tip during supernatant removal. Quant the supernatant plate. If sample is present in supernatant plate, the sample can be recovered by transferring ~100 µL in a 1.5 mL DNA Lo-Bind tube and recentrifuging as outlined in the HiFi prep kit 96 protocol steps 1.5–1.10.
Instrument error of insufficient volume	1. Not enough volume in the consumable	1. Fill the consumable to the recommended amount in the loading dialog
	2. No blanks were inserted into the remaining wells of the last column. Liquid level detection is used.	2. Insert a blank of water or TE buffer into the wells to fill out the column
<10 kb fragments not depleted as expected	1. Not heating effectively	1. Ensure plate sealing before 50°C incubation Ensure HHS records 50ºC and working correctly
	2. Sample input volume not accurate	2. Sample input volume in buffer LTE must be 50 µL per well.
	3. Eluate salt content	3. The DNA sample should be Buffer LTE. If the sample buffer differs significantly or contains high levels of salt, the size selection properties and recoveries may be affected.
	4. Jelly-like, inhomogeneous sample	4. Recovery efficiency and size selection performance of the Short Read Eliminator Kits depends on the input DNA

		being homogeneous and fully in solution. Pipette mix sample at end of run until homogenized. Sample homogeneity can be evaluated by performing triplicate concentration measurements and verifying that the concentration CV is <20%.

DNA Shearing:

Issue	Possible Cause	Corrective Action
Shearing distribution not as expected	1. Concentration per well is more than 10 ng/µL	1. For shearing to be in the desired size distribution the concentration must be 10 ng/µL or less.
	2. Volume per well is not 300 µL	The volume must be 300 µL per sample.
	3. Quantification method	3. Be sure to use fluorescence for an accurate quant.

Library Prep:

Issue	Possible Cause	Corrective Action
Sample dropout in final SMRTbell library plate.	1. Sample lost in supernatant during final 3.1x AMPure PB bead cleanup.	1. Quant supernatant plate to check if sample in present. Recover sample by manually transferring supernatant to a tube. Perform 1x SMRTbell cleanup, elute in 50 µL of elution buffer. Then, manually re-run 3.1x AMPure PB cleanup, elute in 25 µL elution buffer.
Low to no recovery at end of run	1. Supernatant removal during bead cleanups	1. Position teaching positions vary per instrument. Adjust teaching positions to remove all supernatant in the wells while not disturbing the ring bead pellet.

Revision history (description)	Version	Date
Initial release	01	March 2024

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