PacBio

Automated HiFi prep 96 and HiFi ABC for the Hamilton NGS STAR MOA system

Guide & overview

103-425-700 REV06 DEC2025

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Introduction

This procedure describes the automated workflow for constructing polymerase-bound whole genome sequencing (WGS) libraries from genomic DNA using the Revio® SPRQ™ HiFi prep kit 96 or just the HiFi prep kit 96 (if not using one of the polymerase kits). The Revio SPRQ HiFi prep kit 96 includes the SRE HT kit, HiFi prep kit 96, SMRTbell® adapter index plate 96A, SMRTbell cleanup beads, AMPure® PB beads, and the Revio polymerase kit 96 and is 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 step	**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 shearing and size-selection	
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

Genomic DNA input	Low mass	High mass
SRE DNA input Quantity	**0.5–1.25 µg** (20–50 ng/µL in 25 µL)	**2–4 µg** (40–80 ng/µL in 50 µL)
DNA Shearing input Quantity	≤1 µg (≤5 ng/µL, 200 µL)	≤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

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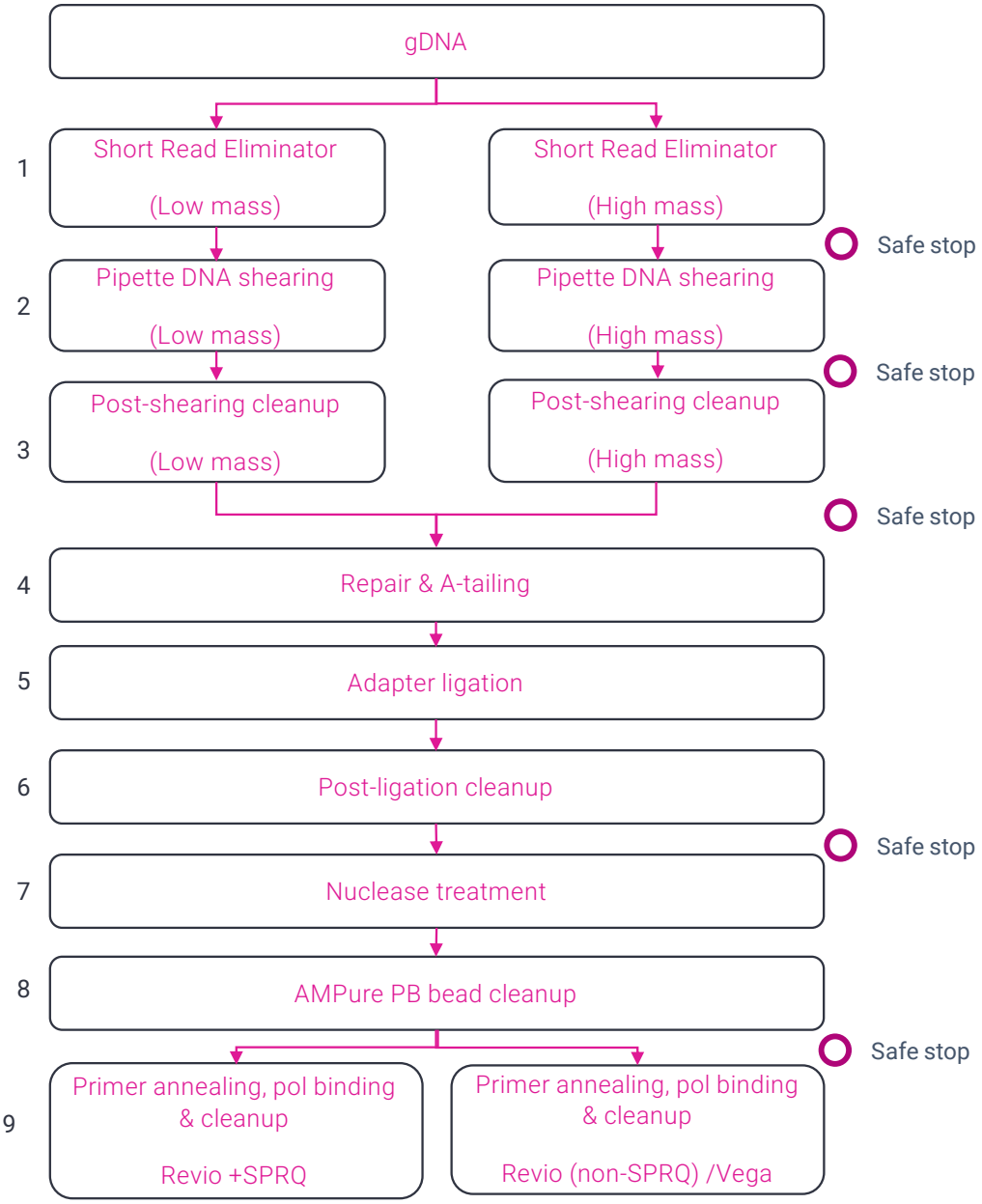
Workflow overview

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



Day 1

- SRE
- Pipette shearing

Day 2

- Library preparation
- ABC

- For steps 1 through 8, use Hamilton script “PacBio_HiFiPrep_v5.1.3”
- For Revio SPRQ ABC, use Hamilton script “PacBio_RevioSPRQABC_v2.2.2”
- For Revio (non-SPRQ) or Vega ABC, use Hamilton script “PacBio_RevioABC_v2.1.3”

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 Reservoir Self-Standing	Hamilton, 194051
Heat Sealing Foil	ThermoFisher Scientific, AB-0757
MicroAmp Clear Adhesive Film	ThermoFisher Scientific, 00146104
2mL Sarstedt Tubes	Sarstedt Inc, 72.694.306
300mL Reservoir	Agilent, 201244-100
2mL Amber tubes	ThermoFisher Scientific, 03-390-28
Equipment	**Catalog Number**
Hamilton NGS STAR MOA	Contact Hamilton representative
Vortex Mixer	Any major lab supplier (MLS)
Microcentrifuge	Any MLS
ALPS 50 V-Manual Heat Sealer	ThermoFisher Scientific, 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® SPRQ 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® SPRQ™ polymerase kit 96	PacBio® 103-522-600 (Revio® SPRQ™ HiFi prep kit 96)
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

Before you begin

Genomic DNA (gDNA) QC and input mass recommendations

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

gDNA quality QC

The Agilent Femto Pulse system is 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. If the GQN at 10 kb is less than 7.0, higher gDNA inputs may be required.
- If the majority of DNA is less than 10 kb, Short read eliminator is not recommended.
- Shearing may be bypassed if the sample is already in the appropriate size-range.

Important:

The HiFi yield and HiFi 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 minimal DNA below 10 kb, and with >50% mass over 30 kb. High quality gDNA will typically have a higher percent library recovery and HiFi sequencing yield.

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

gDNA input

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.

Quantification with UV-Vis Spectrophotometers is not recommended (e.g., NanoDrop).

Low mass vs High mass

Using the Revio system with SPRQ chemistry reduces the gDNA mass required for library preparation by reducing the SMRT Cell loading volume. **While the "High mass" option is still available, there is now a "Low mass" option with updated parameters for SRE and DNA shearing when using 0.5–1.25 µg of gDNA.** Low mass SRE has been optimized to provide comparable recoveries to high mass SRE with the same size selection performance. Low mass shearing has been optimized to maintain the size distribution between 15–20 kb. It is recommended to stay consistent for both SRE and shearing steps (i.e. **use Low or High mass settings for *both* SRE and shearing**).

Note that High mass and Low mass samples cannot be included in a single SRE/shearing automation run. **If 0.5–1.25 µg gDNA is available and Revio SPRQ chemistry is being used, the Low mass workflow will provide enough library to**

load a Revio SMRT Cell (+SPRQ); however, High mass parameters can also be used for Revio SPRQ chemistry if excess library is desired. For Revio non-SPRQ chemistry and Vega™, 2 µg gDNA input is still recommended for loading of 1 SMRT Cell.

Table 1. Recommended DNA input amounts by starting gDNA quality

gDNA quality	Low mass Compatible with Revio (+SPRQ)	High mass	Expected SRE recovery (dependent on DNA quality)
70% >10 kb	0.5–1.25 µg	2–4 µg	60–95%
<70% >10 kb	1–1.75 µg	4–5 µg	40–60%
<10 kb (no SRE)	0.25–1 µg	1–3 µg	Not recommended

Starting with 500 ng and 2 µg of genomic DNA will typically provide enough library to load at least 1 Revio SMRT Cell (+SPRQ) and 1 Revio/Vega SMRT Cell (non-SPRQ), respectively (Table 2, Table 3).

If gDNA mass available is between 1.25 µg and 2 µg, use the "High mass" workflow; however, note that if sequencing on Vega or Revio without SPRQ, there may not be enough final library for loading at an optimal on-plate loading concentration for a single SMRT Cell.

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

Mean insert size	Revio +SPRQ (250 pM)	Revio/Vega (non-SPRQ) (250 pM)
10,000 bp	41 ng	163 ng
15,000 bp	61 ng	244 ng
18,000 bp	73 ng	293 ng
21,000 bp	85 ng	341 ng

HiFi Prep 96 and HiFi ABC protocol stepwise recoveries

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, SMRTbell library preparation, and ABC, see Table 3).

Table 3. Expected stepwise recoveries of DNA and SMRTbell library from the HiFi prep kit 96 protocol. Post-SRE recovery will vary with the quality of the DNA input. The better the quality of DNA, the higer the recovery post-SRE.

Protocol Step	DNA or SMRTbell step recovery	DNA or SMRTbell overall recovery	Expected size (Femto Pulse)
Starting Input	100%	100%	$GQN_{10\ kb}$ >7.0
Post-SRE	65-95%	65-95%	$GQN_{10\ kb}$ >9.3
Post-shear SMRTbell bead cleanup	80-95%*	52-90%	15–20 kb
Post-ligation SMRTbell bead cleanup	80-95%	42-86%	
Post-nuclease (pre-cleanup)	40-50%	17-43%	
Post-3.1x AMPure PB bead cleanup	75-80%	13-34%	
Post-ABC cleanup	75-95%	**10-32%**	

*This can vary based on extraction methods. As low as 60% step recovery has been observed.

Multiplexing

All libraries constructed using this protocol will include a SMRTbell adapter. 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:

- 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.

SMRTbell cleanup beads and AMPure PB beads

Bring SMRTbell cleanup beads and AMPure PB beads to room temperature prior to use.

Vortex or resuspend *immediately* before any addition to sample. Failure to do this will result in low recovery.

Pipette-mix all bead binding and elution steps until beads are distributed evenly in solution.

HiFi prep kit 96

Thaw the Repair buffer 96, Nuclease buffer 96, and SMRTbell 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 mix 96
	Green	DNA repair mix 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 SPRQ 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

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):

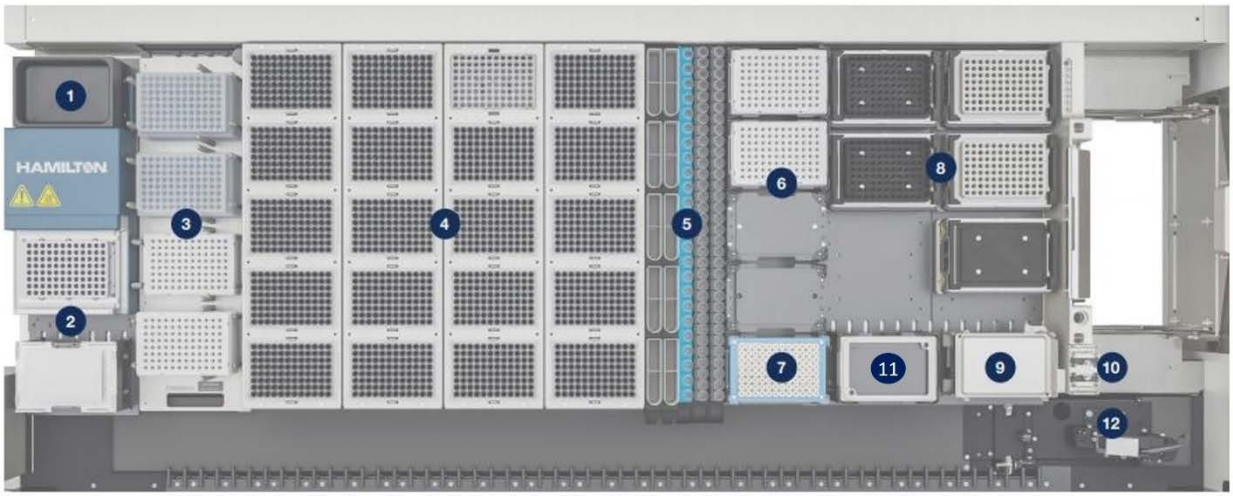
- SMRTbell cleanup beads 85 mL

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 (Optional)
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 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 MOA HiFi prep SRE + DNA Shearing Module loading

1. Prepare **gDNA sample plate for SRE.** Determine whether to use "Low mass" or "High mass" parameters (See Table 1). Pipette gDNA diluted with Buffer LTE according to the table below into a 96-well **PCR** plate (Bio-Rad, HSP9601) starting with position A1. Proceed to fill the plate by column in a downward position as shown in Figure 1.

	Low mass	High mass
Sample volume	25 µL	50 µL
DNA concentration	20–50 ng/µL	40–80 ng/µL
Recommended max gDNA input	1.25 µg*	4 µg**
Elution volume (Buffer LTE)	200 µL	300 µL
Shearing input limit	≤1 µg	≤3 µg

Note: If working with low quality gDNA with a $GQN_{10\ kb} < 7.0$, input mass and concentration can be increased if the expected recovery (40 – 60%, Table 1) matches the pipette-tip shearing mass limit for each respective workflow. For example, 2 µg of gDNA can be used with the Low mass workflow if expected recovery is 40% (800 ng).

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 25 µL or 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.

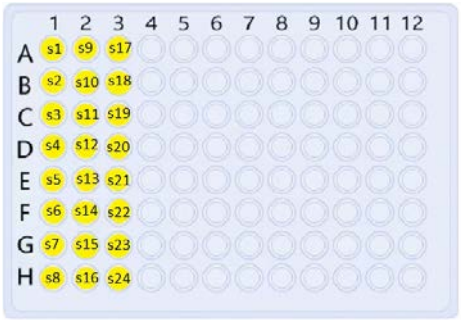


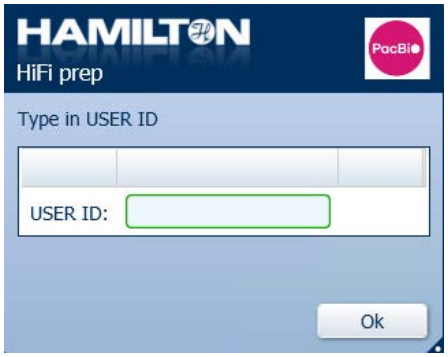
Figure 1. 24 sample input plate example for SRE (s1–s24). Prepare in the 96-well PCR plate (Bio-rad, HSP9601).

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.

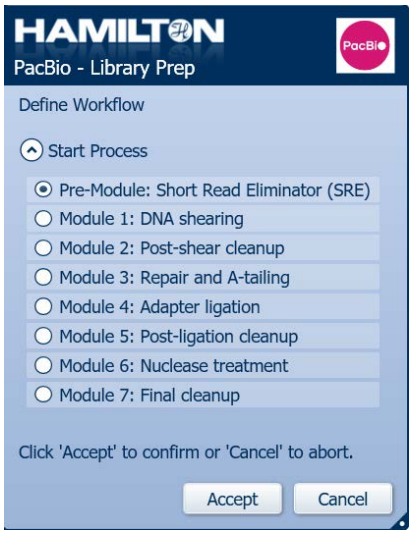
Reagent	Labware
Buffer SRE	2mL tube(s) in kit
Buffer LTE	60 mL reservoir

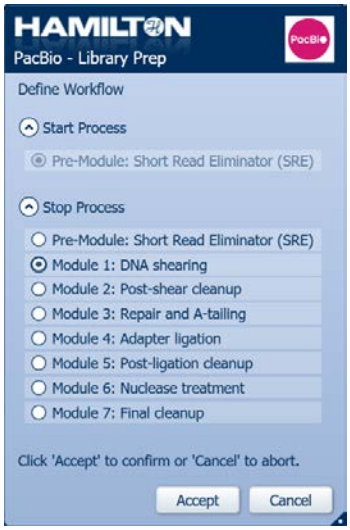
3. Start the PacBio HiFi prep method "**PacBio_HiFiPrep_v5.1.3**".

4. Enter "USER ID" for run.



5. **Define workflow.** Select the start process at "Pre-Module: Short Read Eliminator (SRE)" and the stop process at "Module 1: DNA Shearing".

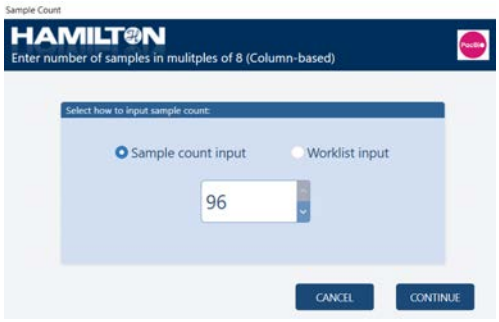




6. **SRE Input.** Select “Low mass” or “High mass” depending on the gDNA mass available for library preparation.

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.

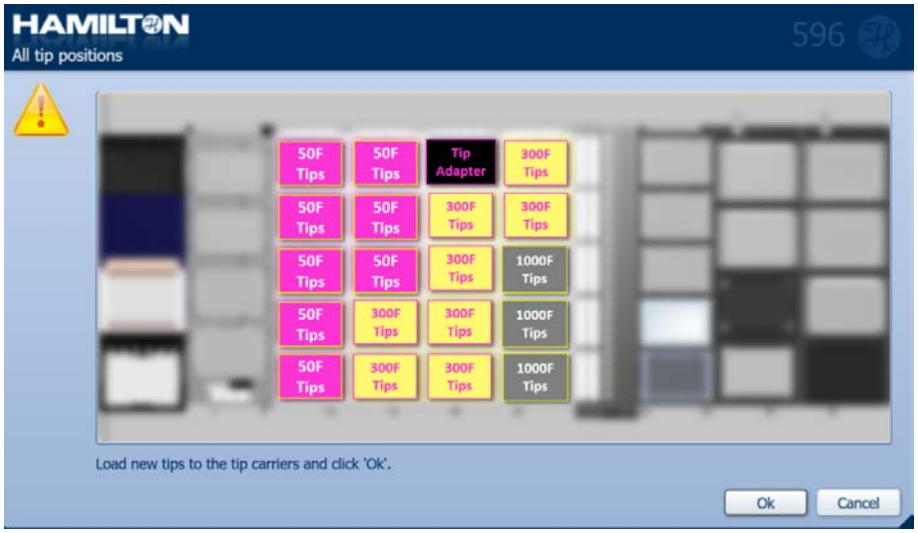
Note: The HiFi prep kit is optimal for 4 sets of 24 sample runs or 1 set of 96 sample run.



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

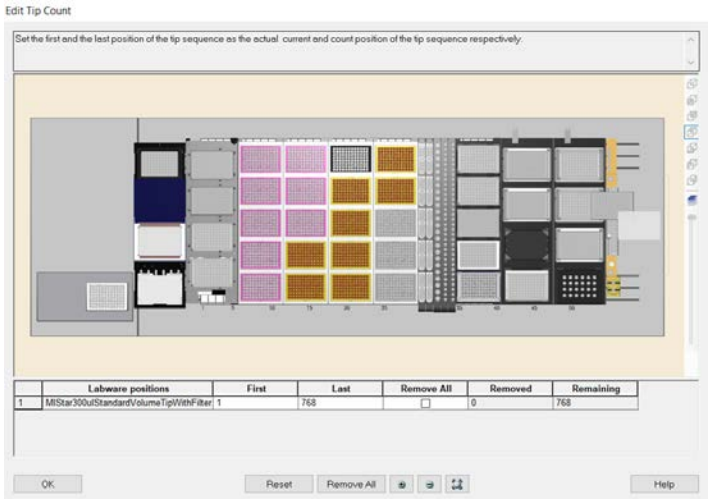
9. **Ensure the tip support adapter for the MPH is empty.** CO-RE I instruments require the CO-RE I tip support. CO-RE II instruments require the CO-RE II tip support. Contact your Hamilton Representative to ensure the configuration is set up correctly with your instrument's CO-RE technology.

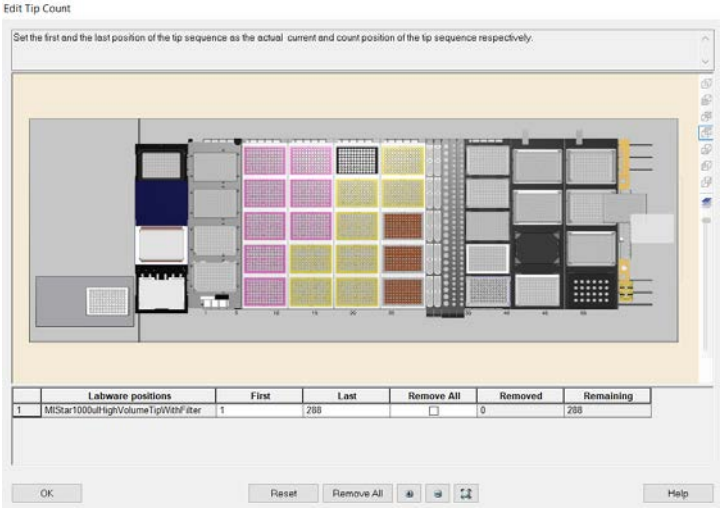
10. **Tip deck layout.** A prompt displaying the tip deck layout will appear, including the tip support adapter position. There are four tip carriers. Refill the tips on deck in the positions for each tip size: 50 µL, 300 µL and 1000 µL filtered conductive tips.



11. **Select the 300 µL and 1000 µL tips on the instrument.**
The tip type is under "Description" and the number of required tips is under "Quantity". Click '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 deselect. Click "Ok" to continue once tip selections match the deck.

Note: It is critical that these selections are accurate and to not leave any empty tip racks (See Appendix for example).



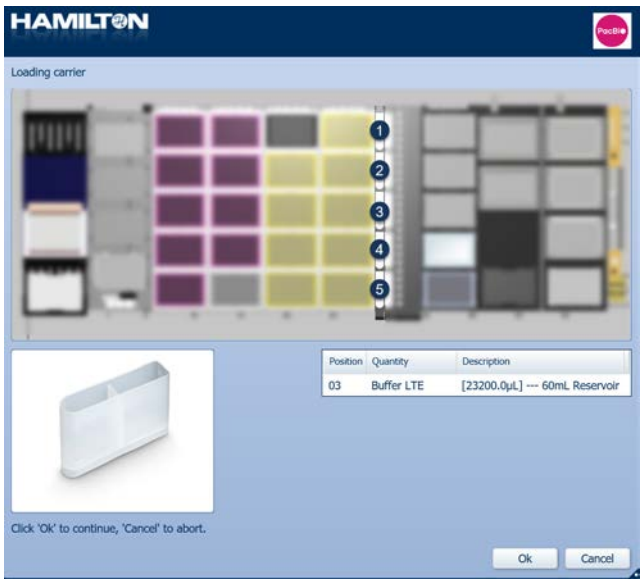


12. Load 60mL reservoir in the reagent carrier.

Load the appropriate volume of Buffer LTE provided in the SRE HT kit into a 60 mL reservoir and place it into reagent carrier position 3, track position 30. The table below shows Buffer LTE volumes based off sample count and mass option.

Note: The example prompt below specifies Buffer LTE volume for 96 samples, High mass SRE.

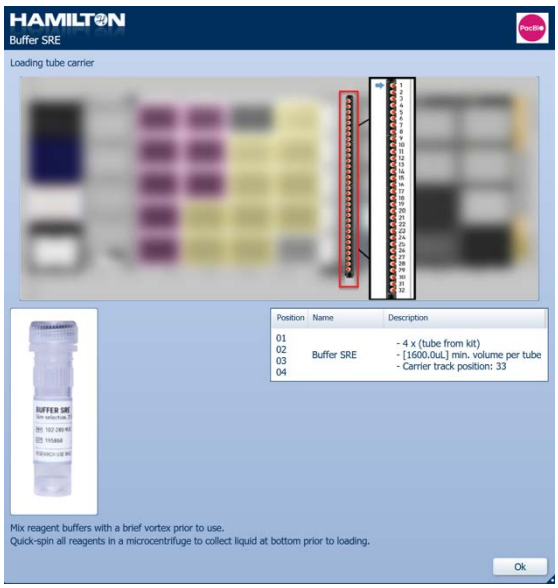
Reagent	Labware	Parameter	24 samples	48 samples	72 samples	96 samples
Buffer LTE	60 mL reservoir	Low mass	8,800 µL	13,600 µL	18,400 µL	23,200 µL
		High mass	11,200 µL	18,400 µL	25,600 µL	32,800 µL



13. **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 number of 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.

    Note: 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)



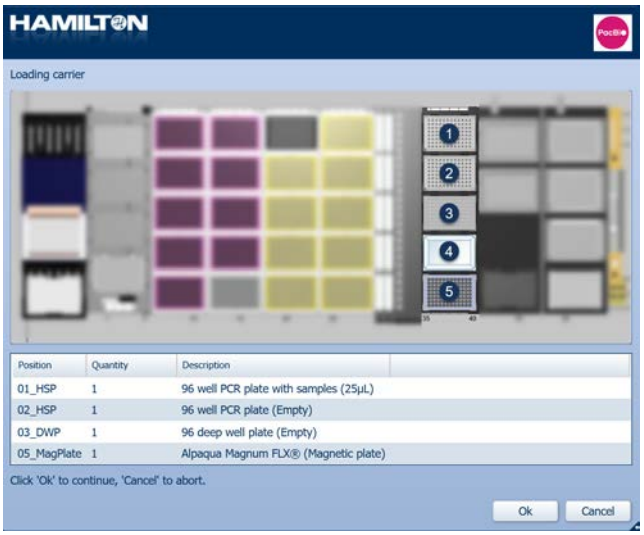
14. Load plate carrier.

Position 1– 96 well PCR plate with 25 µL or 50 µL of sample (Bio-Rad, HSP9601)

Position 2 – Empty 96 well PCR plate (Bio-Rad, HSP9601)

Position 3 – Empty 96 deep well plate (ThermoFisher Scientific, AB0859), which is the SRE output plate

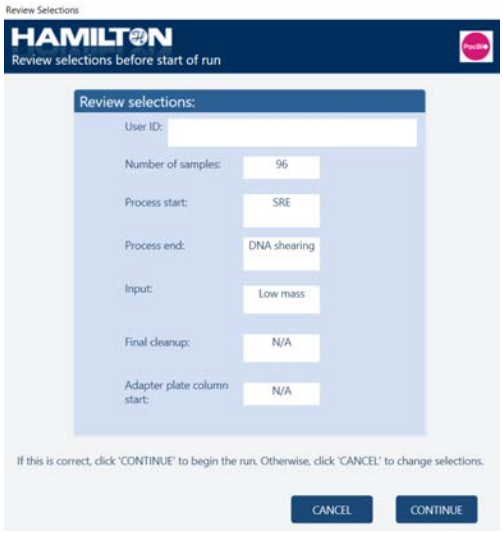
Position 5 – Alpaqua magnum FLX magnet plate



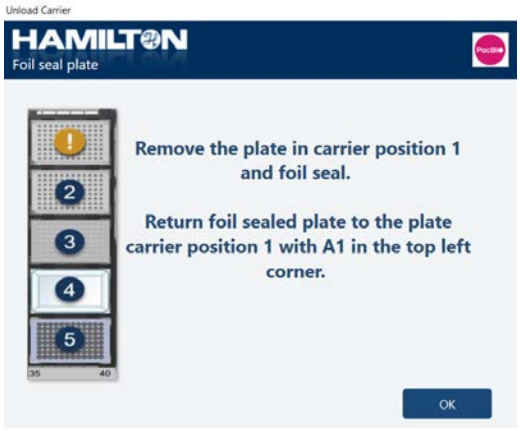
15. Review selections. The example prompt is set for 96 samples starting with “Low mass” SRE and ending at “Low mass” DNA shearing (recommended workflow for day 1, see Workflow Overview). Click “CONTINUE” to begin SRE.

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.

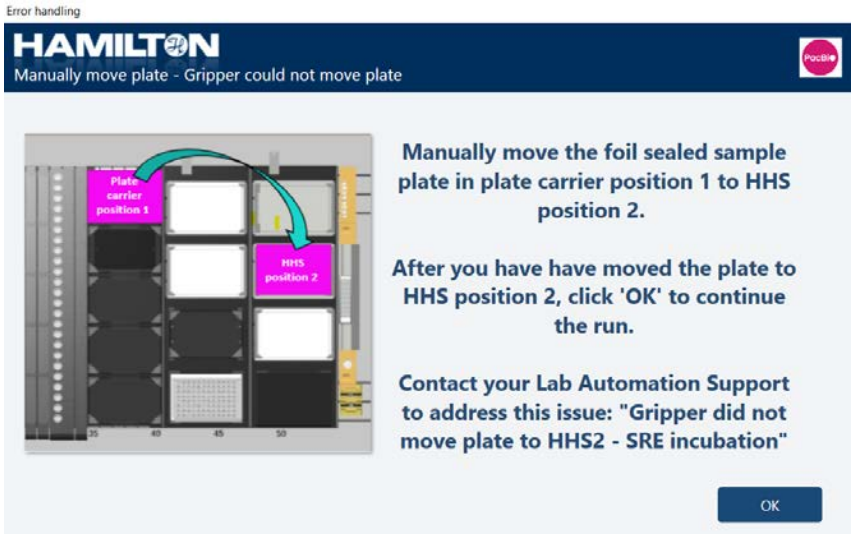


16. **Seal sample plate for 1 hour 50ºC incubation.** After the instrument adds buffer SRE to the samples, the plate carrier will unload and prompt the user to manually heat foil seal the plate and place it back in plate carrier position 1. Click “OK” to continue. The instrument pulls the carrier back in and continues the run.

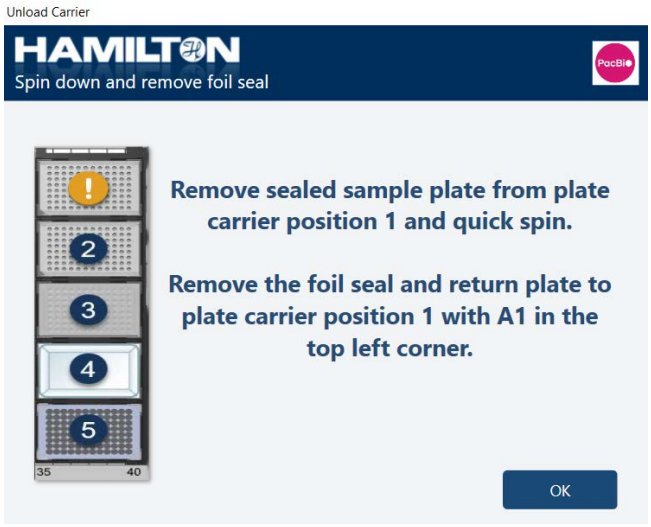


Note: For a 96 sample run, the next prompt will display after ~1 hour.

Note: If the gripper fails to move the plate, the following prompt will appear to provide guidance on manually moving the plate.

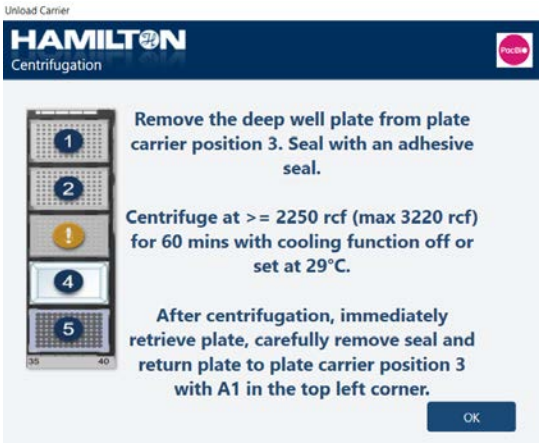


17. **Remove seal after Buffer SRE incubation.** Once the incubation is complete, the instrument unloads the plate carrier and prompts the user to spin down the sample plate and carefully remove the seal. Return the sample plate to plate carrier position 1. Click “OK” for the instrument to pull the plate carrier back in and continue the run.



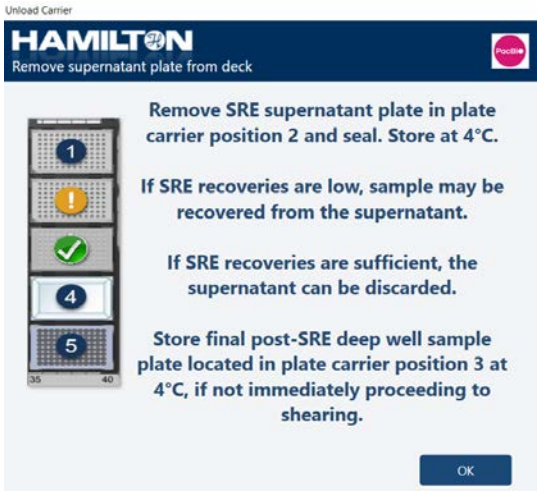
18. **Centrifugation.** The instrument unloads the plate carrier and prompts the user to seal the plate with an adhesive seal and centrifuge the sample plate in position 3 at ≥ 2250 rcf (max 3220 rcf) for 60 minutes at room temperature. If the centrifuge has a cooling function, set temperature to 29°C. After centrifugation, immediately remove the seal and place the sample plate back into position 3. Click "OK" to continue. The instrument pulls 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.

19. **SRE complete.** 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.



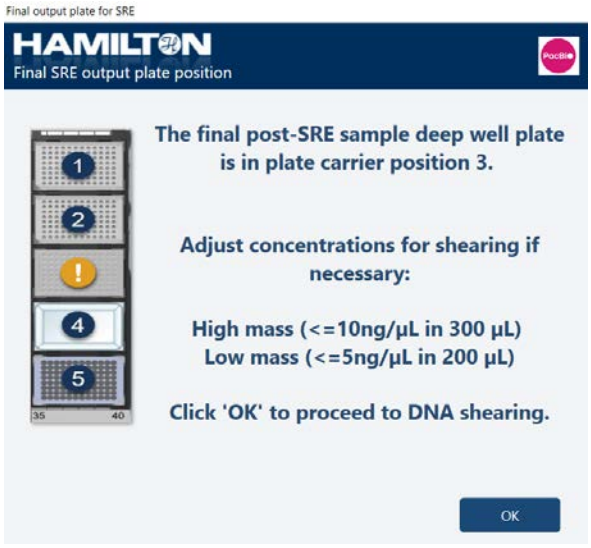
If not proceeding immediately to DNA shearing, the following “Method completed” prompt will appear.

20. DNA shearing

Adjust DNA concentration to ≤5 ng/µL in 200 µL or ≤10 ng/µL in 300 µL, if necessary (e.g., if more than 1 µg or 3 µg of gDNA was recovered from SRE). Use Buffer LTE to dilute samples in a 96 deep well plate (ThermoFisher Scientific, AB0859).

Parameter	Low mass shearing	High mass shearing
DNA concentration	≤5 ng/µL	≤10 ng/µL
Volume of Buffer LTE	200 µL	300 µL

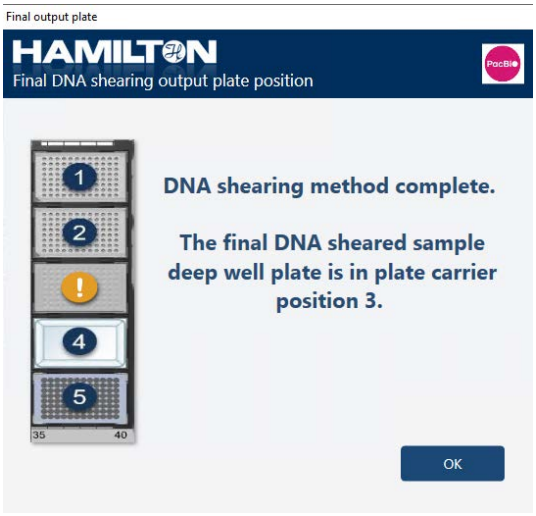
Spin down and return the deep well post-SRE sample plate to deck position 3 and click “OK” to begin DNA shearing.



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

21. **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 MOA HiFi prep loading (Post-shear cleanup to 3.1X diluted AMPure PB final cleanup)

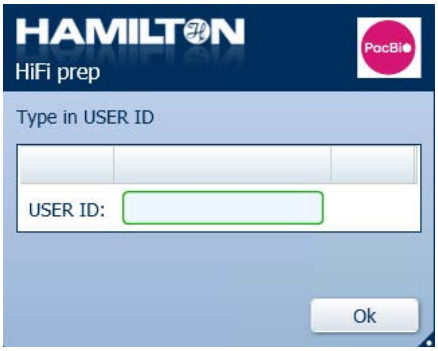
1. **Prepare reagents and consumables for library preparation.** Gather the following reagents and consumables as shown in the table below. Instrument prompts will guide when to load and prepare each reagent.

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

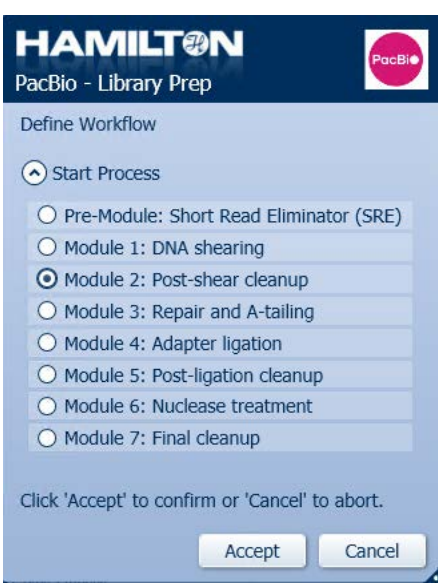
Reagent	Consumables
SMRTbell cleanup beads	60 mL reservoir
Elution buffer	60 mL reservoir
80% ethanol	300 mL reservoir
SMRTbell adapter index plate	96 well PCR plate part of kit
Repair master mix (End repair mix 96, DNA repair mix 96, Repair buffer 96)	2 mL Sarstedt tube(s) – for master mix prep only
Ligation master mix (Ligation mix 96, Ligation enhancer 96)	2 mL Sarstedt tube(s) – for master mix prep only
Nuclease master mix (Nuclease mix 96, Nuclease buffer 96)	2 mL Sarstedt tube(s) – for master mix prep only
AMPure PB beads	96 deep well plate

2. Start the PacBio HiFi prep script**:** "**PacBio HiFi Prep v5.1.3**".

3. Enter "USER ID" for run.



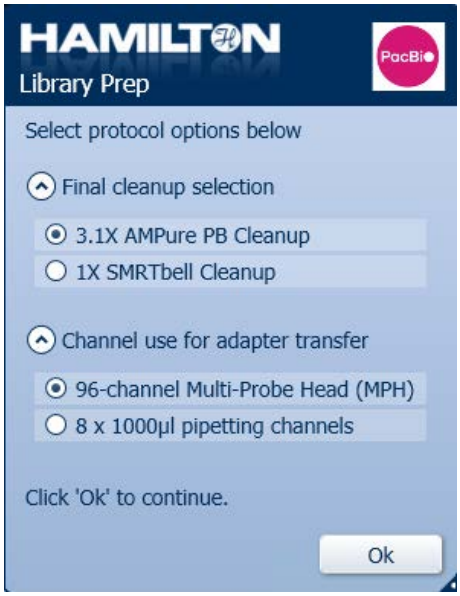
4. **Define workflow.** Select start process at “Module 2: Post-shear cleanup” and stop process at “Module 7: Final Cleanup”.



5. **Post-shear cleanup input**. Select “Low mass” if Low mass shearing parameters were used or select “High mass” if High mass shearing parameters were used.

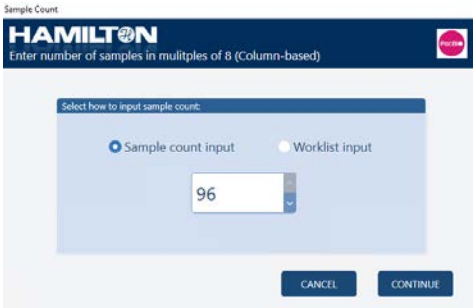
6. **Final cleanup selection.** Select "**3.1X AMPure PB Cleanup**" for the final cleanup. 3.1X AMPure PB will remove contaminants and deplete DNA <3 kb. 1X SMRTbell cleanup is only recommended if doing an alternative size selection method. Select "MPH" or "Channels" for adapter addition. MPH = CO-RE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

Note: MPH is highly recommended.

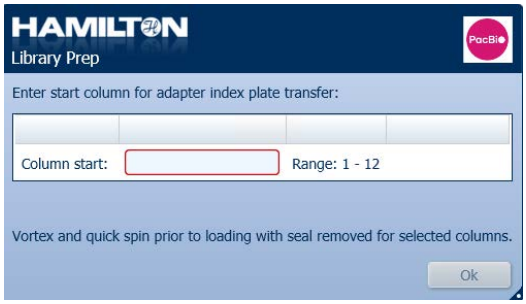


7. **Sample count input.** There are two ways to enter the sample count:
(1) In "Sample count input", type in number that is multiple of 8.
(2) In "Worklist input", a worklist input file can be uploaded for sample tracking. Reference Appendix for instructions.

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



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.



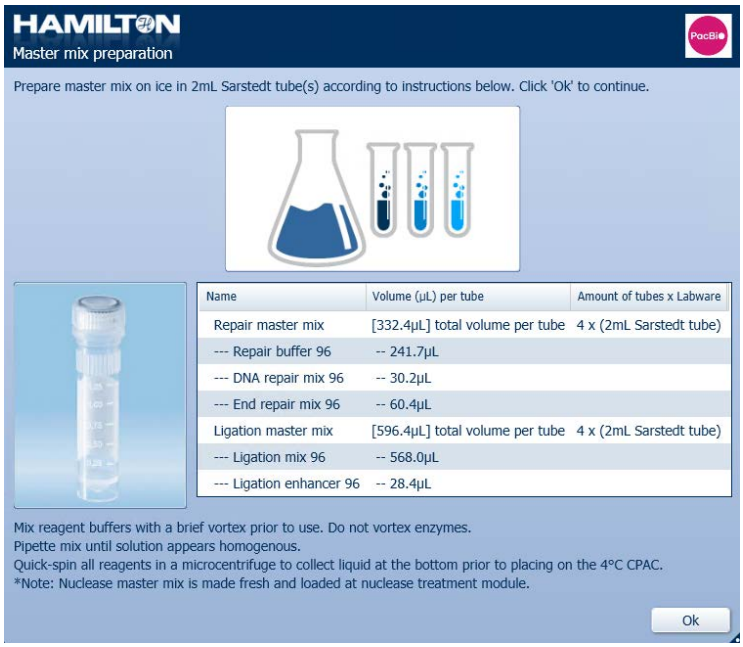
9. **Master mix preparation for Repair, A-tailing and Ligation.** The table below describes how to prepare the Repair master mix and the Ligation master mix for 24, 48, 72, and 96 samples in 2 mL Sarstedt tubes. Pipette mix master mixes and spin down tubes to remove any bubbles. Place master mix tubes on ice until prompted to load on deck. The prompt below is for 96 samples.

Note: Nuclease master mix must be prepared fresh at the nuclease treatment step for optimal performance. The prompt for this preparation is at step 2.21.

Note: Ligation mix 96 is viscous – pipette slowly.

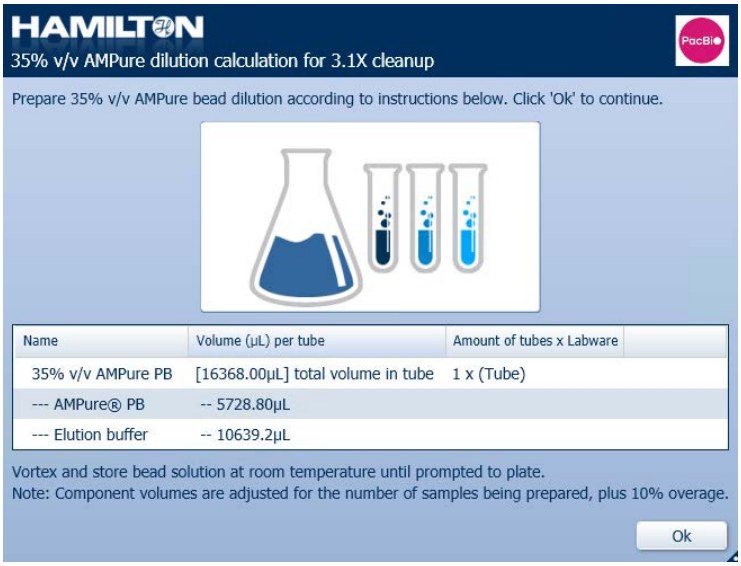
Repair master mix				
Sample configuration	**24 samples**	**48 samples**	**72 samples**	**96 samples**
# 2 mL Sarstedt Tubes	1	2	3	4
Reagent name	Reagent volumes (µL)			
Repair buffer 96	241.7 µL	Prepare 24-sample master mix 2-times for each tube	Prepare 24-sample master mix 3-times for each tube	Prepare 24-sample master mix 4-times for each tube
DNA repair mix 96	30.2 µL			
End repair mix 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			

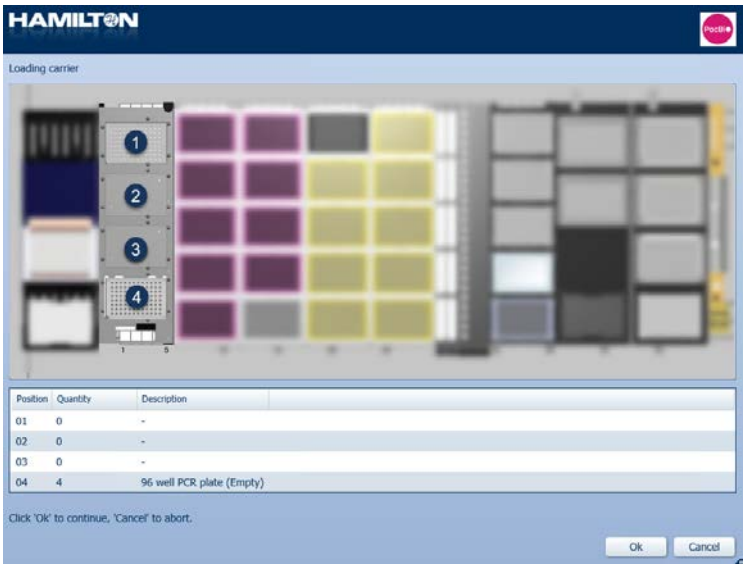


10. **Prepare 35% v/v AMPure PB bead dilution for 3.1X cleanup.** Prepare 35% v/v dilution of room temperature AMPure PB beads in a tube as described in the table below based off sample count. Accurate pipetting is critical at this step. Failure to make a 35% AMPure solution will result in sample loss. 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 shown below is for 96 samples.

Reagent	24 samples	48 samples	72 samples	96 samples
AMPure PB beads	1,432.2 µL	2,864.4 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	8,184 µL	12,276 µL	16,368 µL

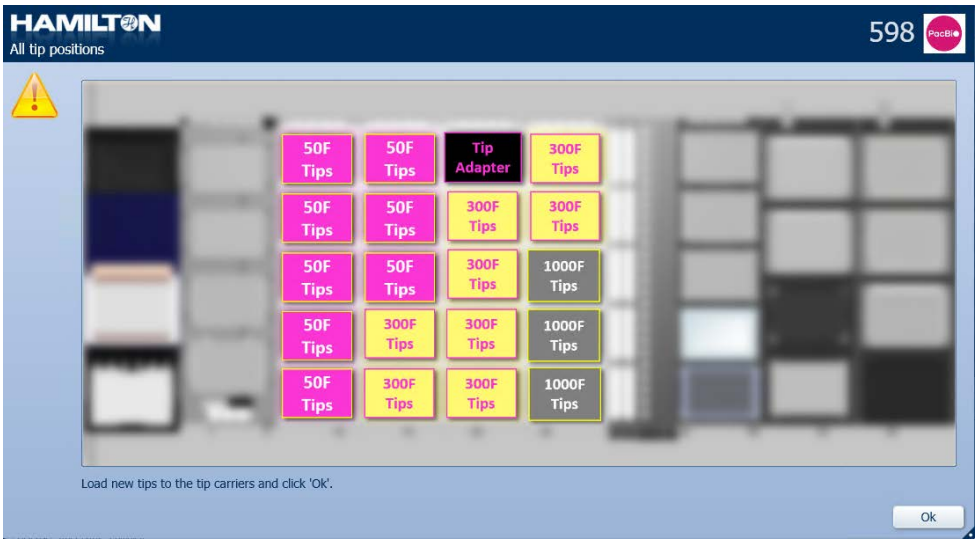


11. **Load plate stacker carrier.** Load the plate stacker carrier with four 96 well PCR plates stacked (Bio-Rad, HSP9601) in position 4.



12. **Ensure tip support adapter 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.

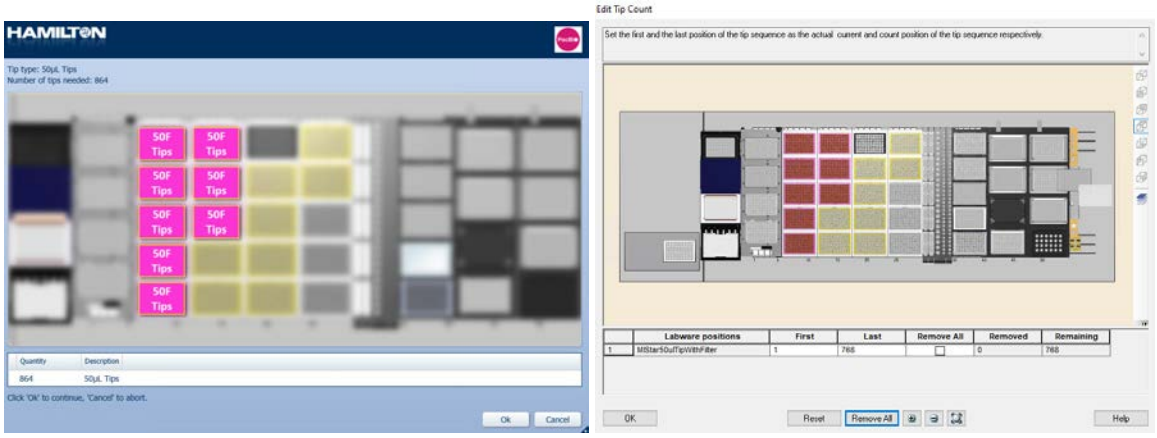
13. **Tip deck layout.** A prompt displaying the tip positions will appear, including the tip support adapter position. There are four tip carriers. Refill the tips on deck in the positions for each tip size: 50µL filtered conductive tips, 300µL filtered conductive tips and 1000 µL filtered conductive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

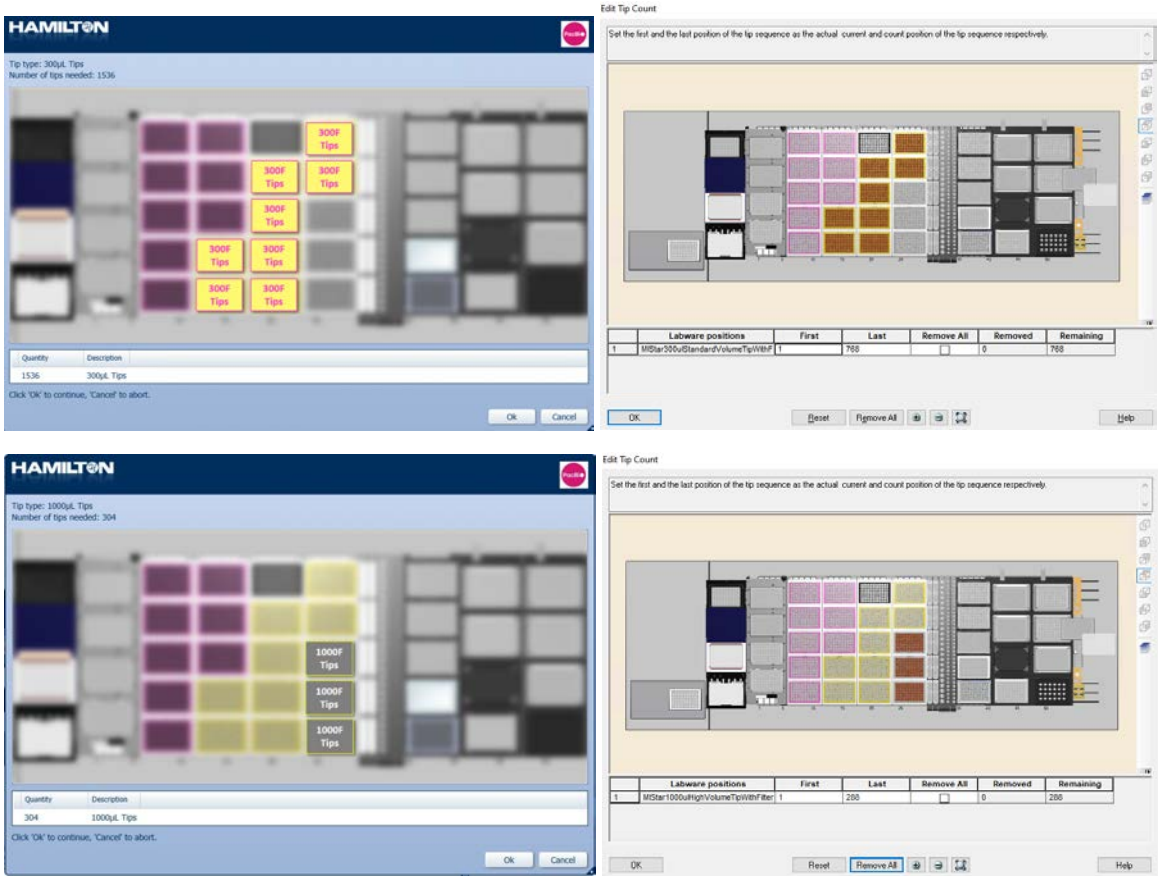


14. Select 50 µL, 300 µL, and 1000 µL tips on the instrument.

The tip type is under “Description” and the number of required tips is under “Quantity”. Click ‘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 deselect. Click “Ok” to continue once tip selections match the deck.

Note: It is critical that these tip selections are accurate and to not leave any empty tip racks. (See Appendix for example).





15. **Load 60mL reservoir in reagent carrier**. Load the SMRTbell cleanup beads and Elution buffer into the 60 mL reservoirs. Place reservoirs into the reagent carrier in track position 30. Reagent carrier position 1 is for SMRTbell cleanup beads and position 2 is for Elution buffer. The table below shows the volumes for each reagent based off sample count when starting at post-shear cleanup and stopping at 3.1X AMPure PB final cleanup.

Note: The example prompt is for 96-samples.

Reagent	Consumables	Parameter	24 samples	48 samples	72 samples	96 samples
SMRTbell cleanup beads	60 mL reservoir	Low mass	9,340 µL	16,180 µL	23,020 µL	29,860 µL
		High mass	11,740 µL	20,980 µL	30,220 µL	39,460 µL
Elution buffer	60 mL reservoir	Low/High mass	5,880 µL	8,760 µL	11,640 µL	14,520 µL

16. Load plate carrier.

Position 1 – Empty 96 well PCR plate (Bio-Rad, HSP9601)

Position 2 – Unsealed SMRTbell adapter index plate

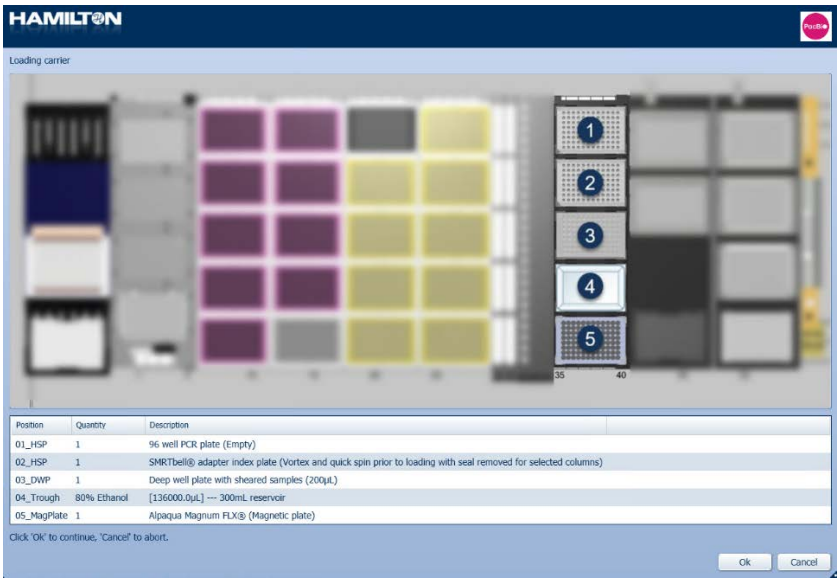
Position 3 – 96 deep well plate with sheared samples (ThermoFisher Scientific, AB0859)

Position 4 – 300mL reservoir (Hamilton, 56669-01) with fresh 80% ethanol (see table for volumes)

Position 5 – Alpaqua magnum FLX magnet plate.

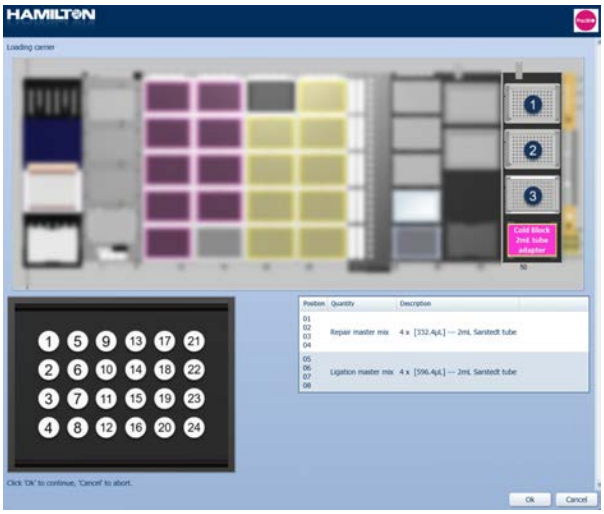
Note: Briefly vortex, spin down and remove the seal only for columns selected for SMRTbell adapter index plate. Do not let instrument tips pierce the foil seal to prevent the risk of instrument crash or improper aspiration and dispense of adapters. Only remove the seal for the columns used.

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



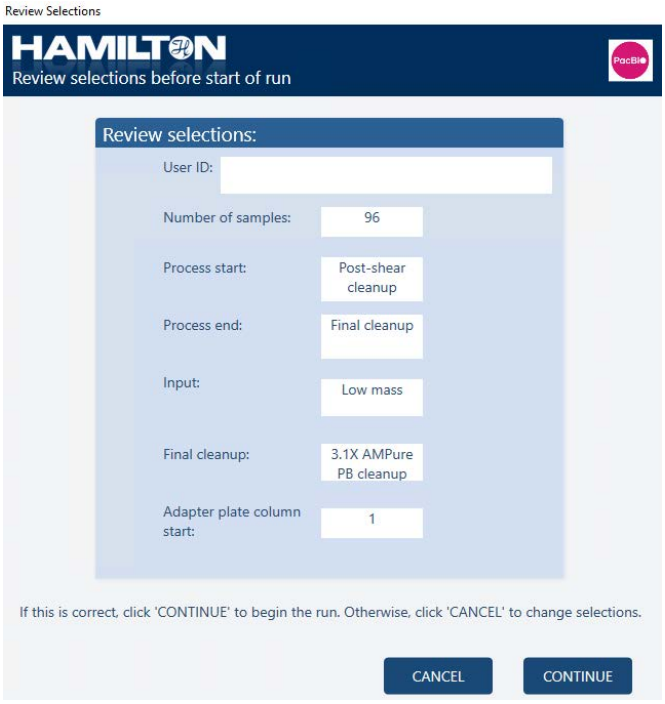
17. **Load master mix tubes on cold block.** Follow prompt to load the **Repair** and **Ligation master mix tubes** in the designated positions on the cold block 2mL tube adapter (CPAC 2) at 4°C. The prompt below is for 96 samples.

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



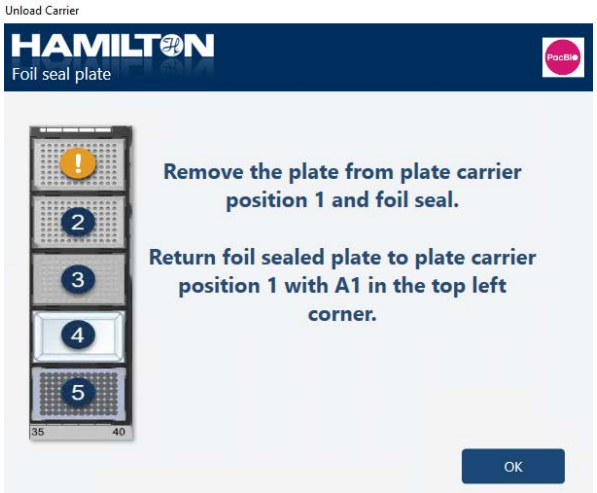
18. **Review selections.** The example prompt is set for 96 samples starting at the post-shear cleanup and stopping at the 3.1x AMPure PB cleanup with a 200 µL sheared sample input volume (Low mass parameter) starting from adapter column 1. Click “CONTINUE” to begin post-shear bead cleanup.

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.

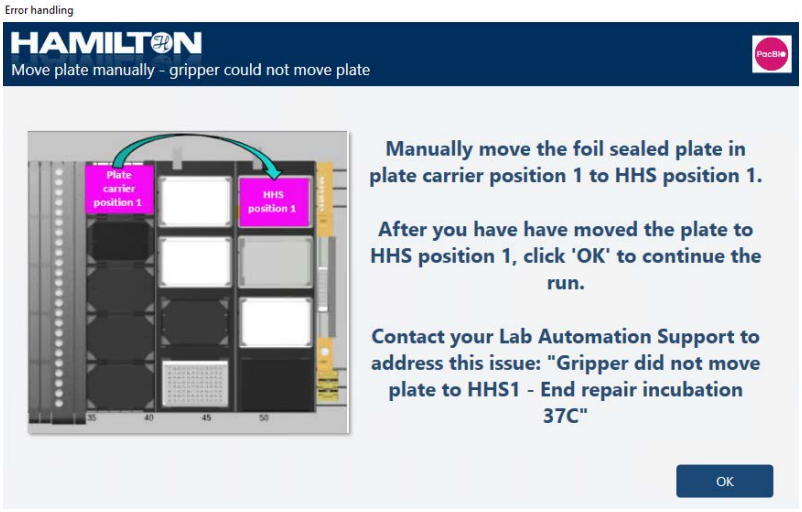


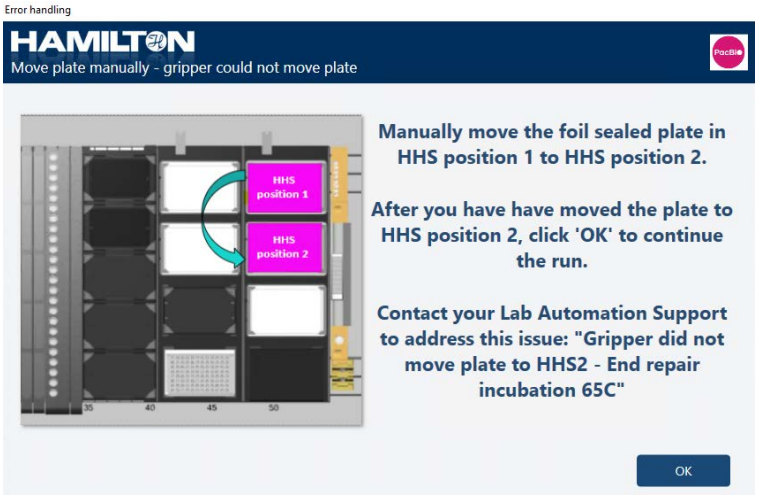
19. **Seal sample plate for incubation during End repair and A-tailing.** The plate carrier will unload and prompt the user to manually heat foil plate from plate carrier position 1. Return the foiled sealed plate to the same location. Click 'Ok' for the instrument to pull the carrier back.

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



Note: If gripper fails during the subsequent steps, the following prompts will appear for the Repair and/or A-tailing step.

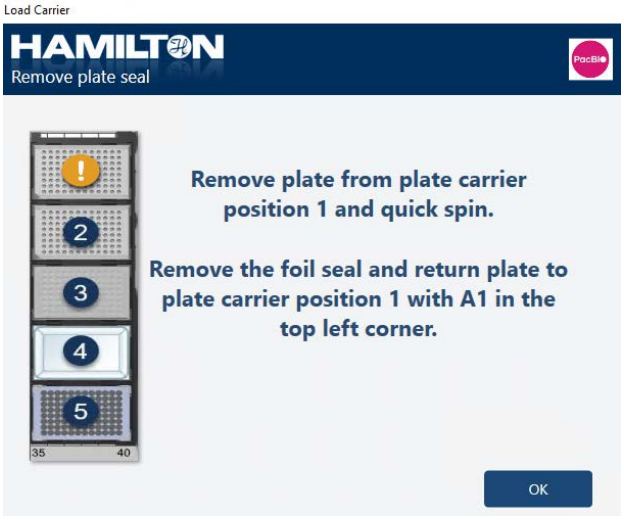




20. **Remove sample plate seal after incubation.** The plate carrier will unload and prompt the user to remove sample plate in carrier position 1 and quick spin. Remove the foil seal and return plate to the same location. Click 'Ok' for the instrument to pull the carrier back and continue to adapter ligation and post-ligation bead cleanup.

Note: Carefully remove the seal to prevent contamination.

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. Pipette mix master mixes and spin down tubes to remove any bubbles. Place master mix tubes on ice until prompted to load on deck. The prompt below is for 96 samples.

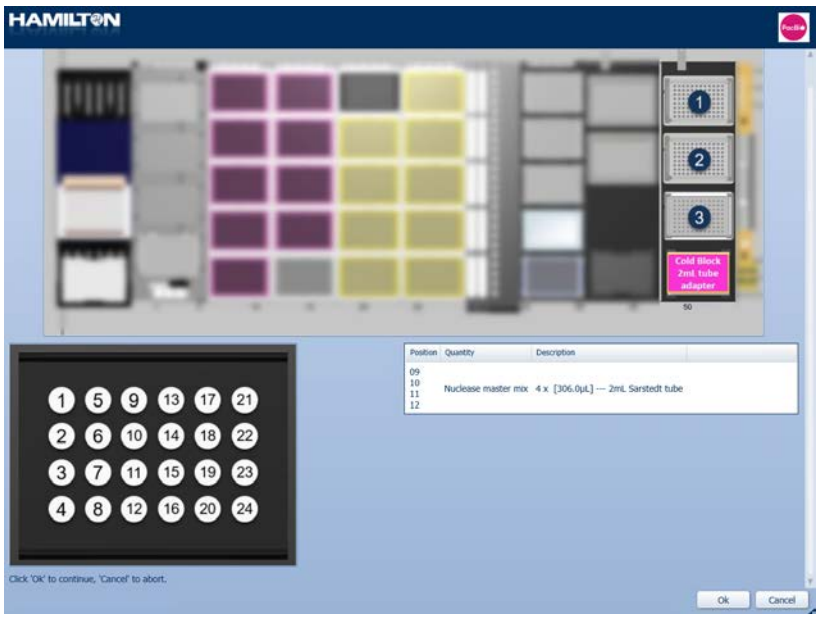
Note: Nuclease master mix must be made immediately prior to use.

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	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
Nuclease mix 96	153 µL			
Total volume per 2 mL tube	306 µL			

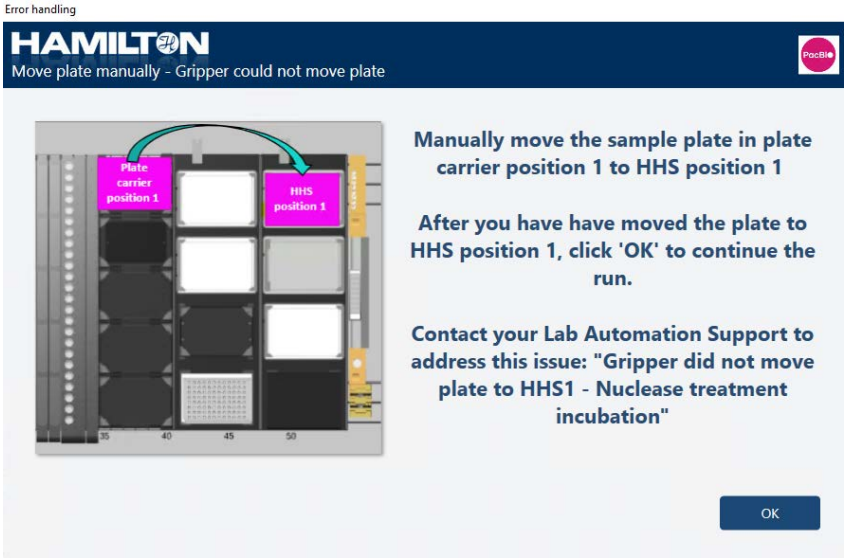
22. Load **nuclease master mix tube(s) on cold block.** Follow prompt to load the Nuclease master mix tube(s) in the designated positions on the cold block 2mL tube adapter (CPAC 2) at 4ºC. Click "Ok" to proceed to nuclease treatment. The prompt below is for 96 samples.

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

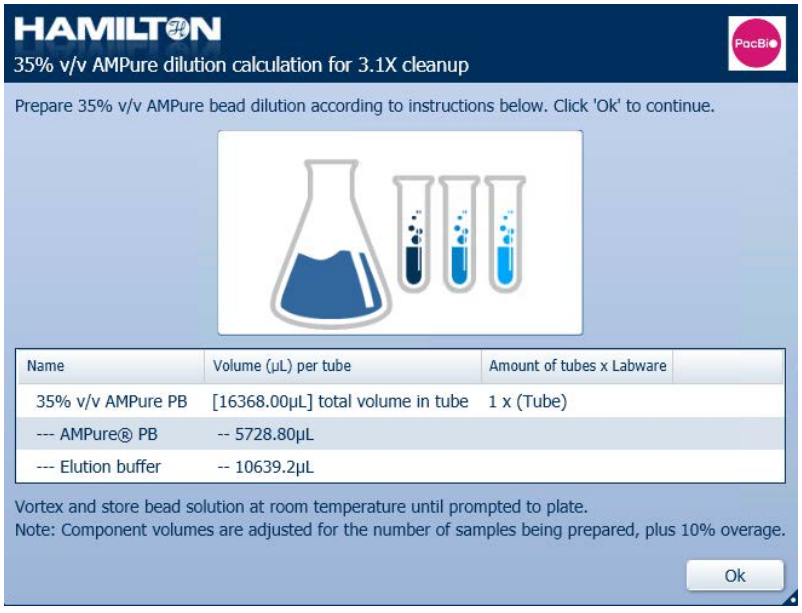
Note: For a 96-sample run, the next prompt will display the 35% v/v AMPure PB bead dilution after ~30 minutes.



Note: If gripper fails during the subsequent step, the following prompt will appear.



23. **Prepare 35% v/v AMPure PB bead dilution for 3.1x cleanup.** The instrument will again display the 35% v/v AMPure PB dilution calculation (same as Section 2, Step 10). If not already prepared, return to Section 2, Step 10 for best preparation practices and make the bead dilution in a tube. The prompt below is for 96 samples.

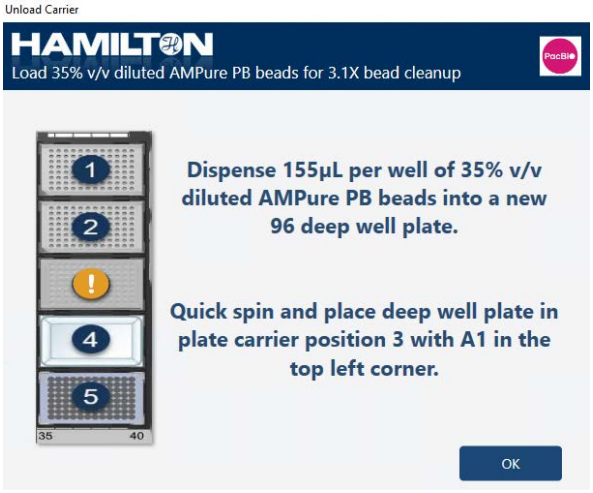


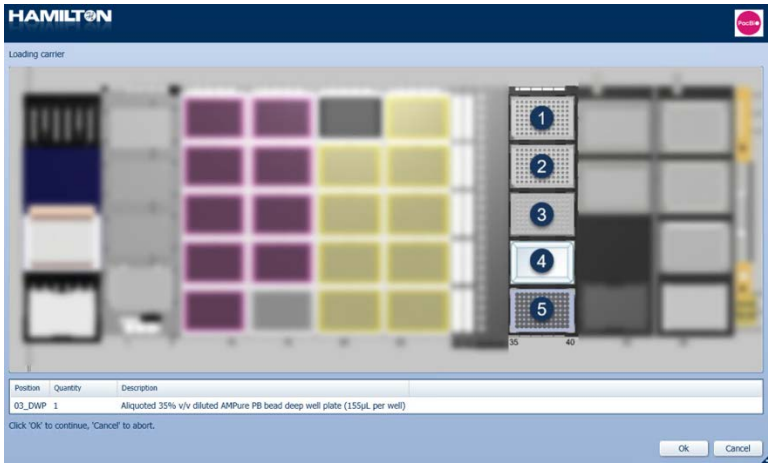
24. **Prepare deep well plate with diluted AMPure PB beads.** In a 96 deep well plate, manually pipette 155µL of 35% diluted AMPure PB beads for the number of samples. Click “OK” after loading the 96 deep well plate of diluted AMPure PB beads onto plate carrier position 3.

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

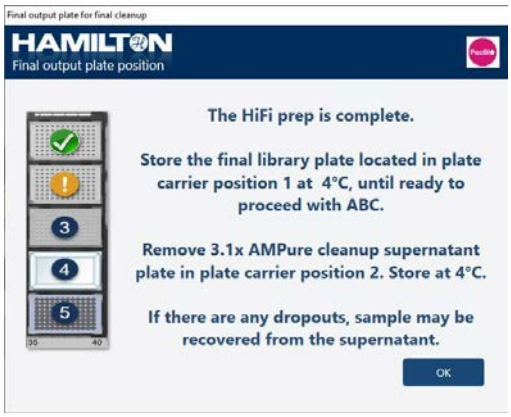
Note: Use a reservoir to pour diluted AMPure PB beads and use a multichannel pipette to aliquot 155µL.

Note: For a 96-sample run, a prompt will appear to reload the 50 µL tips after ~40 minutes.





25. Library preparation complete. When the run is complete, remove the final library output plate 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 plate carrier 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. Continue with HiFi prep ABC or SPRQ ABC with this sample output plate.

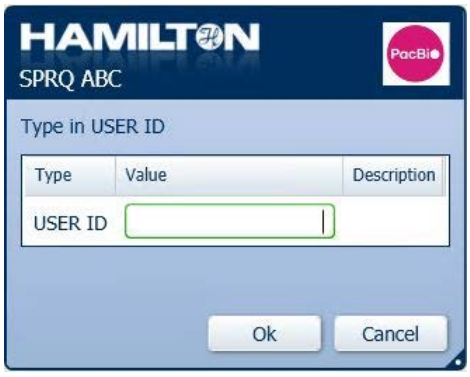
3. Hamilton NGS STAR MOA HiFi prep annealing, binding, and cleanup (ABC) loading procedure

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 reservoir
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
Polymerase dilution (Polymerase buffer 96, Sequencing polymerase 96)	2mL Sarstedt tube(s) – for master mix prep only

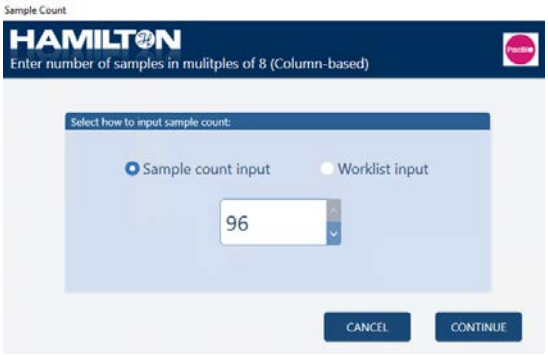
2. Select a HiFi prep ABC method to run:
    - Revio +SPRQ ABC**: PacBio_RevioSPRQABC_v2.2.2**
    - Revio non-SPRQ**: PacBio_RevioABC_v2.1.3**

3. Enter “USER ID” for run.



4. **Define workflow.** Select the start process at “Module 1: Annealing” and the stop process at “Module 3: Cleanup”.

5. **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 instructions.

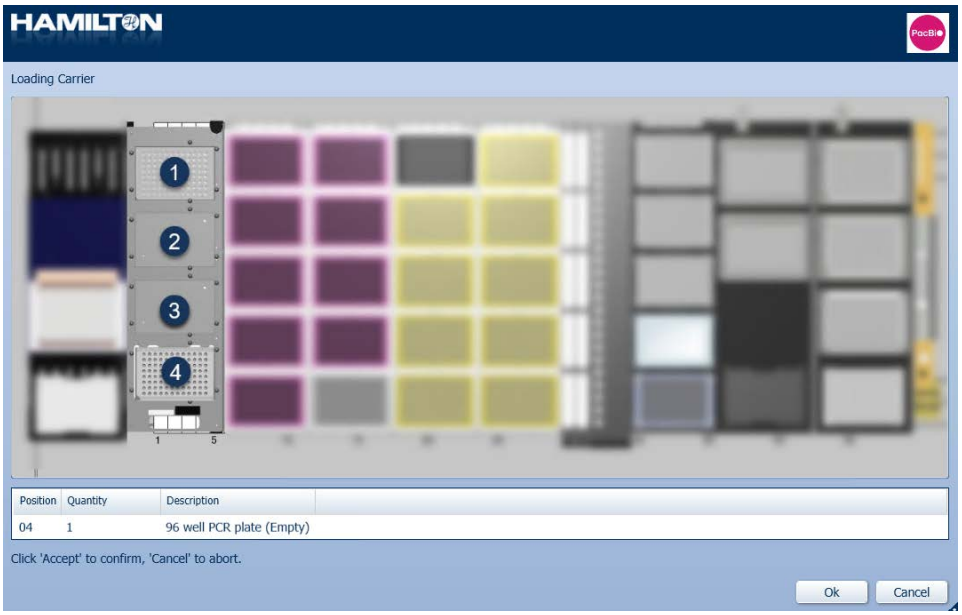


6. **Master mix preparation for ABC.** The table below describes how to prepare the Annealing master mix and the Polymerase dilution for 24, 48, 72, 96 samples in 2 mL Sarstedt tubes. Gently pipette mix master mixes and spin down tubes to remove any bubbles. Place master mix tubes on ice until prompted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

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			

Polymerase dilution				
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			

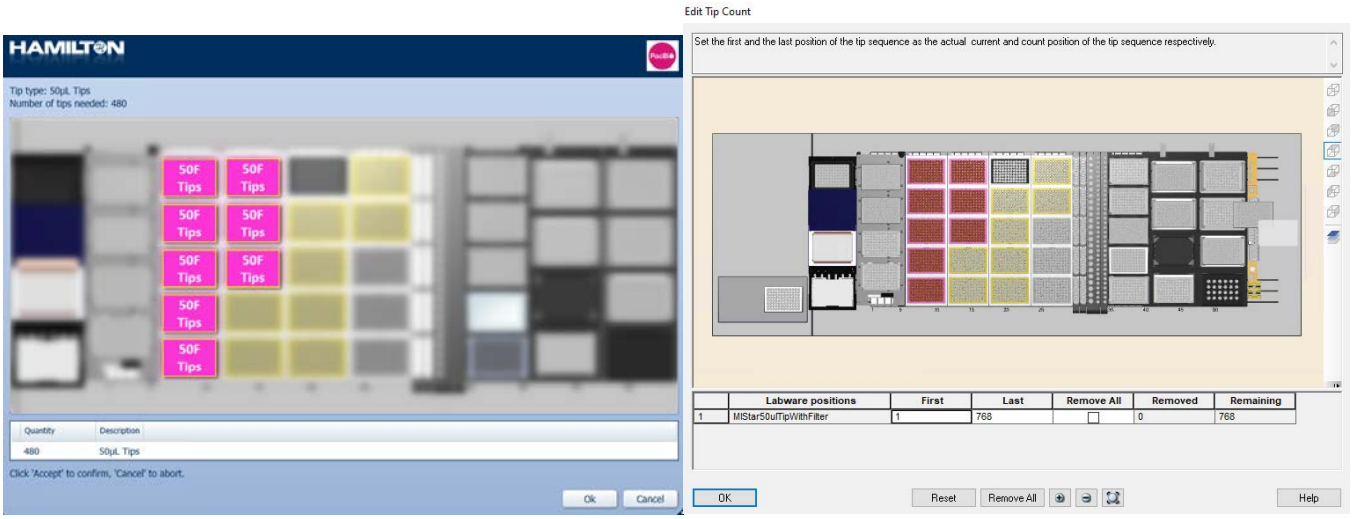
7. **Load plate stacker carrier.** Load an empty 96 well PCR plate (Bio-Rad, HSP9601) in stacker carrier position 4.

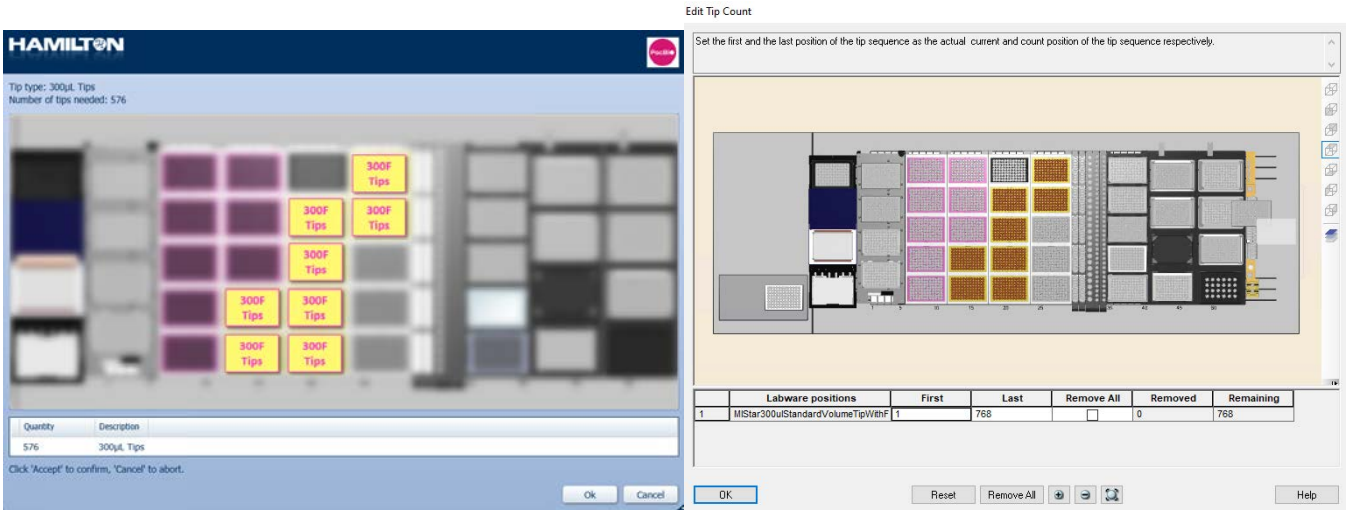


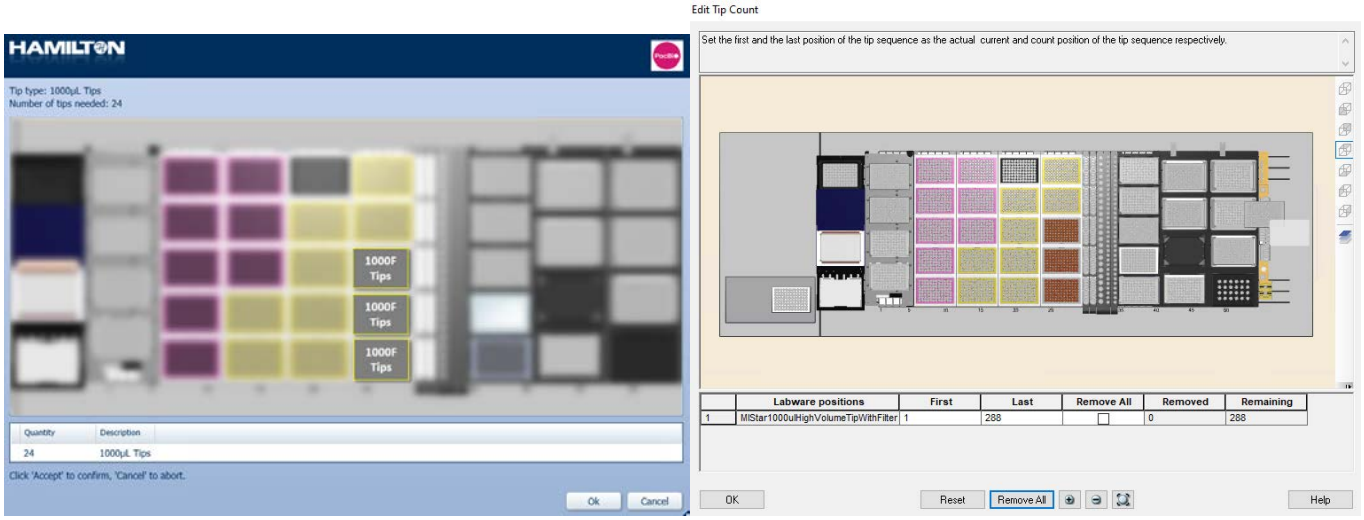
8. **Ensure the tip support adapter for the MPH is empty.** CO-RE I instruments require the CO-RE I tip support. CO-RE II instruments require the CO-RE II tip support. Contact your Hamilton Representative to ensure the configuration is set up correctly with your instrument's CO-RE technology.

9. **Select the 50 µL, 300 µL, and 1000 µL tips on the instrument.** The tip type is under “Description” and the number of tips required is under “Quantity”. Click ‘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 deselect. Click “OK” to continue once tip selection match the deck.

Note: It is critical that these tip selections are accurate and to not leave any empty tip racks. (See Appendix for example).





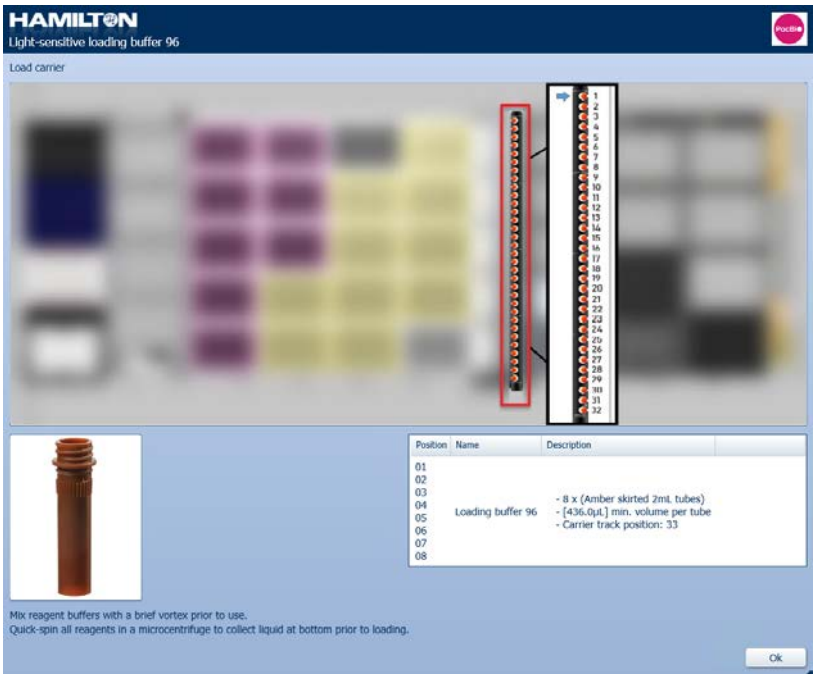


10. **Load 60 mL reservoir in reagent carrier**. Fill a 60 mL reservoir with the appropriate amount of SMRTbell beads based off sample count shown in table below. Load the 60 mL reservoir to reagent carrier track position 30 in position 1.

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

11. **Load Loading buffer tube(s).** Load the Loading buffer 96 in 2mL amber tubes from the corresponding kit shown in the table below into the tube carrier located in track position 33 starting at position 1. Briefly vortex and spin down the tubes to ensure no bubbles are present. The number of tubes required is based off the selected sample count. Loading buffers from Revio +SPRQ and Revio non-SPRQ/Vega polymerase kits are not interchangeable. The prompt below is an example for a 96-run Revio SPRQ.

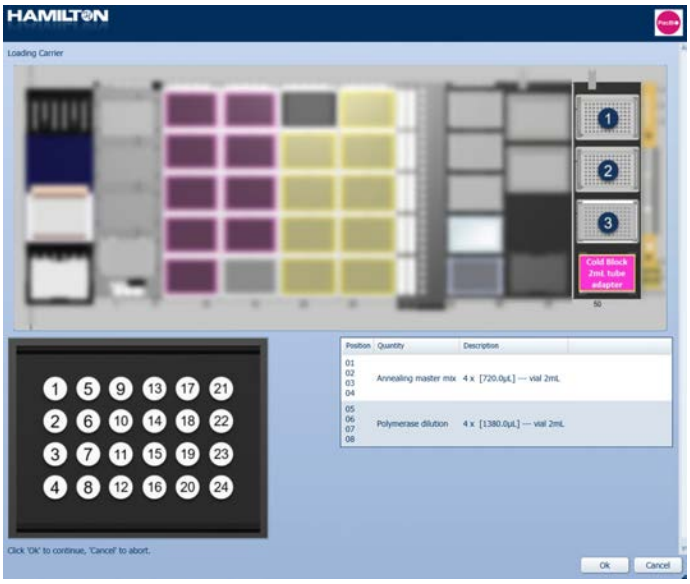
Kit	Reagent	Labware	24 samples	48 samples	72 samples	96 samples
Revio SPRQ polymerase kit 96	Loading buffer 96	2mL amber tube	2 tubes of 436 µL	4 tubes of 436 µL	6 tubes of 436 µL	8 tubes of 436 µL
Revio polymerase kit 96	Loading buffer 96	2mL amber tube	2 tubes of 700 µL	4 tubes of 700 µL	6 tubes of 700 µL	8 tubes of 700 µL



12. **Load plate carrier.** Load the plate carrier with the sample plate in position 1 and empty 96 well PCR plate (Bio-Rad, HSP 9601) in position 2. Ensure the magnet plate is in position 5.

13. **Load master mix tubes on cold block.** Follow prompt to load the **Annealing master mix** and **Polymerase dilution** in the designated positions on the cold block 2mL adapter (CPAC 2) at 4°C. The prompt below if for 96 samples.

Note: The prompt is set for a 96-sample run.

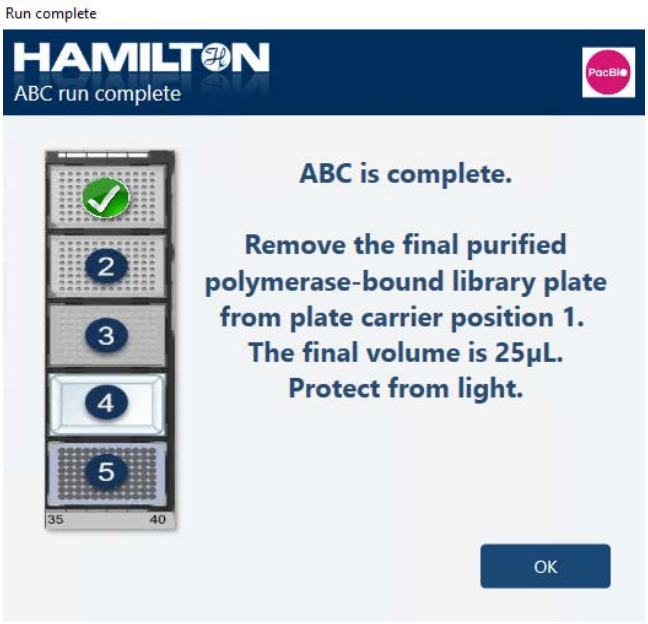


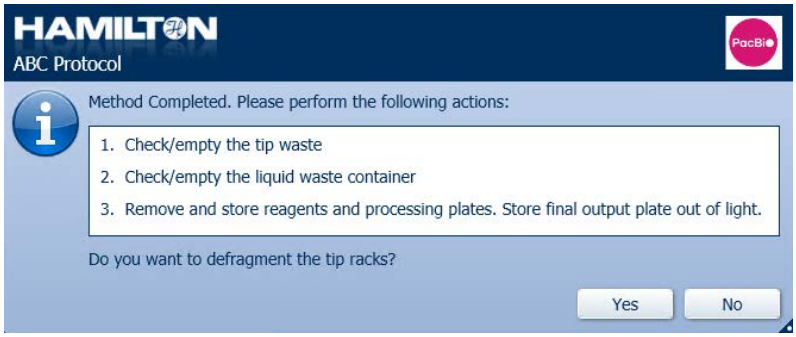
14. **Review selections.** The example prompt is set for 96 samples starting at “Annealing” and stopping at “Cleanup”. Click “CONTINUE” to begin ABC.

15. **ABC 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. The prompt below is for Revio +SPRQ ABC, the Revio non-SPRQ final volume is 50 µL.

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.





Proceed to the Loading Calculator in SMRT Link v13.3 or higher to calculate the final dilution for adding the sample to the sequencing plate.

Note: SMRT Link (>13.3) → Sample Setup → "Add Calculation" pull down menu → "Loading calculator"

Appendix

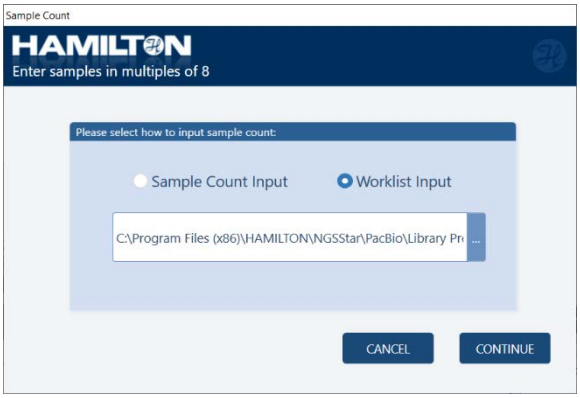
Input file:

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.

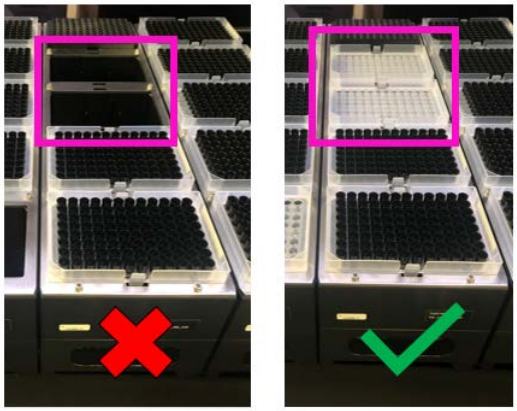
Note: When editing the file ensure sample count is a multiple of 8.

	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 FAQs

General automation

How many automation runs can a HiFi Prep 96 support?

- Four 24-sample runs are supported with the HiFi prep 96 kit. Processing samples in cohorts of less than 24 samples is not supported.

What should be evaluated if the instrument shows errors due to insufficient volume?

- Ensure that the entire column has solutions. If sample is not required, use water as a blank.
- If the instrument shows errors and there is sufficient volume, you can select “bottom” to force the instrument to pipette regardless of liquid level detection.

Can instrument pauses be inserted during validation of the script?

- QC pauses can be toggled on and off to create pauses at the end of the SRE step, post shearing cleanup, post-ligation cleanup and post nuclease treatment. To turn on the 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.

General library preparation

How should gDNA be quantified to ensure that the concentration is accurate?

- Use a quantification method specific to dsDNA, such as the Qubit system. Some high molecular weight (HMW) DNA will be in-homogenous and will therefore give inaccurate results depending on where the sample is pulled from in the tube. Vigorously vortexing gDNA prior to quantifying will improve quantification accuracy. The vortexing will not adversely affect the DNA.

Is there a control DNA you recommend prior to using actual sample?

- Full length Lambda DNA (Lambda DNA | NEB) can be used prior to testing gDNA to ensure that SRE, shearing and library preparation is working properly.

Is bead carryover problematic?

- The presence of some bead carryover should not adversely affect subsequent reactions.

To ensure everything is working properly, what steps should be quantified?

- Post-SRE
- Post-shearing (there should be no change in recovery from post-SRE; however, quantifying is often more accurate after shearing since the DNA is less viscous).
- Post-shear cleanup
- Post-ligation cleanup
- Post-nuclease treatment, pre-cleanup. During this step, un-ligated DNA and damaged DNA are digested by the nuclease. Therefore, if recovery is lower than expected, this means DNA repair, end-repair, or ligation efficiency is low. If recovery is higher than expected, it indicates that the nuclease treatment was ineffective.
- Post-3.1x AMPure PB bead cleanup
- Overall recovery

Note: Qubit is recommended for quantification. If Qubit is not standard procedure, it is still recommended to use it to validate that all steps are working as expected.

Protocol Step	DNA or SMRTbell step recovery	DNA or SMRTbell overall recovery	Expected size (Femto Pulse)
Starting Input	100%	100%	$GQN_{10\ kb}$ >7.0
Post-SRE	65-95%	65-95%	$GQN_{10\ kb}$ >9.3
Post-shear SMRTbell bead cleanup	80-95%*	52-90%	15–20 kb
Post-ligation SMRTbell bead cleanup	80-95%	42-86%	
Post-nuclease (pre-cleanup)	40-50%	17-43%	
Post-3.1x AMPure PB bead cleanup	75-80%	13-34%	
Post-ABC cleanup	75-95%	**10-32%**	

*This can vary based on extraction methods. As low as 60% step recovery has been observed.

If less recovery is observed after bead cleanups, what should be evaluated?

- **Are the beads mixed appropriately prior to adding to sample?** Beads should always be mixed immediately before addition to sample.
- **Are the beads mixed with sample?** After bead addition to sample, the mixture should look homogenous.
- **Are the beads appropriately magnetized after incubation on the magnet?** The supernatant should appear clear.
- **Are beads aspirated when the supernatant is removed?** A slow aspiration speed (Hamilton NGS Star, 20 µl/aspiration) with an appropriate Z-height is required to ensure that beads (with sample bound) are not aspirated when supernatant is removed.
- **Is all EtOH removed?** <1 µL of residual EtOH should remain after removal. Note that bead aspiration is less frequent with EtOH as compared to supernatant, but a slow aspiration speed (Hamilton NGS Star, 20 µl/aspiration) with an appropriate Z-height should still be used. Note that beads do not need to be dry before proceeding. In the manual protocol, beads are immediately resuspended in elution buffer after EtOH removal. In the automated protocol, we recommend waiting 1 minute after EtOH removal and Elution buffer addition.
- **Are the beads appropriately mixed with the elution buffer?** The mixture should appear homogenous.

- **Is all elution buffer transferred to a new plate?** A slow aspiration speed (Hamilton NGS Star, 10 µl/s for final elution after 3.1x AMPure PB bead cleanup) should be used at this step to ensure that minimal bead carryover occurs. However, some bead carryover will not hurt downstream enzymatic reactions. Ensure that all eluate is transferred.

If less recovery is observed for the nuclease step, what steps should be evaluated?

- **Repair/A-tailing**. Is the appropriate volume of mastermix added? The NGS Star uses an aspiration of mastermix at 10 µL/s, and dispenses into the sample at 20 µL/s. After addition of the correct volume, is the sample appropriately mixed? Pipette mixing at this step is sufficient (30x mix, mix volume = 80%, liquid following, 50 µL/s mix speed).
- **Index adapter addition**. Is the appropriate volume of adapter added to the sample? If adapter is not added to the sample, you can expect close to 0% recovery after the nuclease step. The Hamilton NGS Star aspirates adapter at 45 µL/s and dispense with a blowout and a settling time of 5 sec.
- **Ligation mix**. Is the appropriate volume of mastermix added? The NGS Stars aspirates ligation mastermix at 10 µL/s and dispenses into the sample at 20 µL/s, with a settling time of 4 s. After addition of the correct volume, is the sample appropriately mixed? Due to the high viscosity of the ligation mix, mixing is critical at this step. Inefficient mixing will result in low nuclease recovery. Shaking is sufficient (1400 rpm, 1 min).
- Note that if pipette mixing is used, ensure that volume loss does not occur during this step.

What are possible issues if DNA is not recovered after the final 3.1x AMPure PB elution?

- It is possible that the 35% dilution was made incorrectly or that the AMPure PB:sample ratio is off. Exactly 155 µL of 35% AMPure PB dilution should be added to 50 µL of nuclease reaction. If library is not recovered in the elution, the library should be in the supernatant. To recover library from the supernatant, perform a 1x SMRTbell bead cleanup on the supernatant and elute in 50 µL of Elution buffer. Repeat the 3.1x AMPure PB step according to the procedure.

Short read eliminator (SRE)

What volume transfer steps should be carefully evaluated for the Short Read Eliminator (SRE) method?

- **The Buffer SRE:sample ratio should be exactly 1:1.** If less Buffer SRE is added relative to the sample, there is a chance of low recovery. If more Buffer SRE is added, size-selection will be less effective. The Hamilton NGS Star script aspirates buffer SRE at 80 µL/s and dispenses into the sample at 30 µL/s. To mix, it shakes at 1400 rpm for 1 minute (after foil seal).
- **Removal of supernatant after centrifugation.** There should be ≤10 µL of supernatant remaining for the High Mass setting and ≤5 µL supernatant remaining for the Low Mass setting, after the supernatant removal step. If more than the recommended volume remains, there will be a larger quantity of short DNA fragments. However, it is recommended to leave residual volume to ensure that the DNA pellet is not aspirated.

If pipetting coordinates all look correct but low recovery is still observed from SRE, what could be the issue?

- Thorough mixing of Buffer SRE with sample is critical. If sample and Buffer SRE and not efficiently mixed, the DNA will fail to precipitate and pellet and will remain in the supernatant. The Hamilton NGS Star has a 1 min shake at 1400 rpm along with pipette mixing.
- Remove the plate immediately after centrifugation to avoid dispersion of the pellet.

Is there a way to recover the sample if the pellet is aspirated during supernatant removal?

- The sample should be in the supernatant. Re-centrifuge and follow subsequent steps according to SOP.

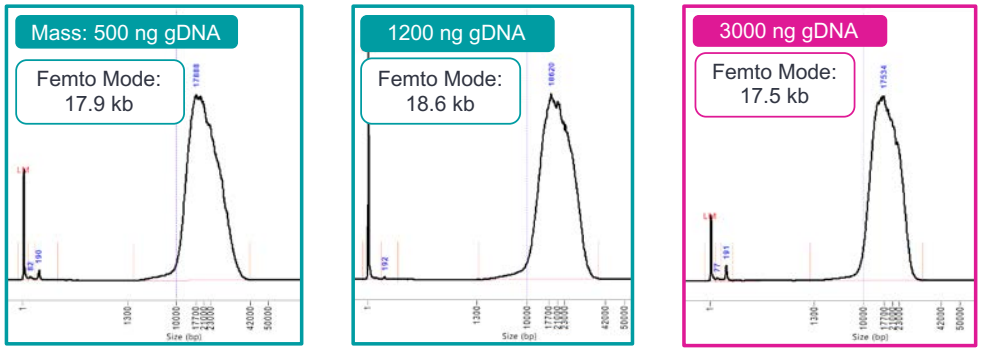
Pipette shearing

DNA was not sheared. What are possible issues?

- Genomic DNA was inaccurately quantified. It is recommended to vortex gDNA prior to quantifying to ensure a homogenous solution and accurate quantification. Use a quantification method specific for double-stranded DNA.
- DNA is too concentrated. If the DNA concentration is higher than the recommended parameters, shearing will be ineffective.

Why do I observe variability in post-shear size distribution? Is the same shear size expected for Low mass vs High mass shearing parameters?

- Variability in the final size distribution will depend on the DNA source/extraction method. Higher quality DNA typically generates a post-shearing mode >15 kb. Lower quality DNA may have a slightly lower mode (13 – 15 kb).
- Low mass and High mass shearing parameters typically generate similar size distributions for the same input gDNA. Within the High mass or Low mass parameters, shifting to more dilute gDNA may result in a slightly shorter product:



Revision history (description)	Version	Date
Initial release	01	March 2024
Updated to correct reference to the Hamilton NGS STAR MOA system	02	April 2024
Updated for SPRQ chemistry and the Vega system	03	December 2024
Updated PDF for quality management, no changes to content	04	December 2024
Added FAQ section at end. Ohter minor updates throughout.	05	May 2025
Corrected AMPure PB bead volume for 3.1X cleanup	06	December 2025

103-425-700 REV06 DEC2025