PacBio

Automated HiFi plex prep 96 for the Hamilton NGS STAR MOA system

Guide & overview

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Introduction

This guide and overview describes the workflow for PacBio® high-throughput semi-automated long read sample prep utilizing the HiFi plex prep kit 96 that is designed for a minimum of 24 and maximum of 96 samples per automated run.

Overview

Overview	
Applications	• Microbial WGS • Metagenome shotgun sequencing • Amplicon sequencing • Long read low pass WGS
Samples	24–96 per kit
Minimum batch size supported	4 x 24
Maximum batch size supported	96
	Hamilton NGS STAR MOA
Preparing master mixes and setting up work deck	1 hour for 96 samples
Shearing	10 min for 24–96 samples
Post-shearing cleanup	1 hour for 96 samples
Repair, ligation, and pooling	2 hours for 96 samples
Manual steps: post-ligation cleanup, nuclease treatment, post-nuclease cleanup	1.5 hours for 96 samples (4 pools of 24 samples)
Average total time	6 hours

DNA input		
	gDNA	Amplicons
Per sample input	50–300 ng	20–200 ng
DNA shearing	Automated pipette-tip shearing	N/A
Target fragment lengths	13–20 kb*	Any size >1 kb

*Smaller fragment lengths can be used when working with lower quality DNA samples (see gDNA quality recommendations)

Workflow overview

Required materials and equipment

Consumables	Catalog Number
Automation	
Hard Shell 96 Well PCR Plate	Bio-Rad, HSP9601
Abgene 96 Well 0.8 mL 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
60 mL Reagent Reservoir Self-Standing	Hamilton, 194051
Heat Sealing Foil	ThermoFisher Scientific, AB-0757
MicroAmp Clear Adhesive Film	ThermoFisher Scientific, 00146104
2 mL Sarstedt Tubes	Sarstedt Inc, 72.694.306
300 mL Reservoir	Agilent, 201244-100
Manual	
0.2 mL 8-tube strips	USA Scientific TempAssure 1402-4708
Magnetic bead rack for PCR tubes or plates	Any major lab supplier (MLS)
Magnetic bead rack for tubes	ThermoFisher Scientific 12321D
Equipment	
Hamilton NGS STAR MOA	Contact Hamilton representative
Vortex Mixer	Any 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
Thermocycler	Any MLS

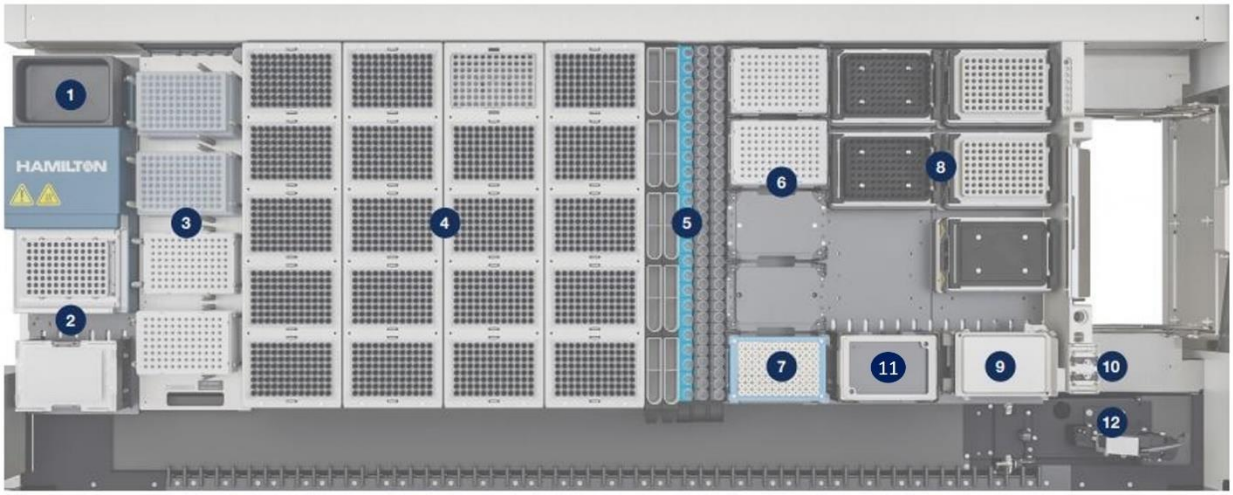
SMRTbell library preparation	Catalog Number
HiFi plex prep kit 96, includes: HiFi plex prep kit 96 SMRTbell® cleanup beads—52 mL Buffer LTE HT Elution buffer	PacBio® 103-381-300
SMRTbell® adapter index plate 96 (A, B, C, and D)	Plate A – PacBio® 102-009-200 Plate B – PacBio® 102-547-800 Plate C – PacBio® 102-547-900 Plate D – PacBio® 102-548-000
Revio® SPRQ™ polymerase kit *or* Vega™ polymerase kit *or* Sequel® II binding kit 3.2*	PacBio® 103-496-900 PacBio® 103-426-500 PacBio® 102-194-100

* Procedure for Sequel II binding kit 3.2 can be found in SMRT® Link Sample Setup.

Other supplies	
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

Hamilton NGS STAR MOA System

Note: Contact your Hamilton representative for installation and deck details



Deck Layout

- Gravity liquid waste for Multi Probe Head (MPH)
- On-Deck Thermal Cycler (ODTC) with lid parking position (Optional)
- Plate stacker
- Tip carriers with MPH tip support adapter
- Reagent carriers
- Plate carrier
- Alpaqua Magnum FLX magnetic plate
- Hamilton Heater Shakers with 96-well PCR plate adapters and flat bottom
- Inheco CPAC with 2mL tube cold block adapter (CPAC 2)
- CO-RE gripper paddles
- Inheco CPAC with 96-well PCR plate adapter (CPAC 1)
- Autoloader with barcode reader

Before you begin

Genomic DNA (gDNA) QC and input amount 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 (FP-1002-0275)
- Dilute samples to 250 pg/µL

Microbial and metagenomics samples may forgo shearing if the DNA is in the fragment length range of 7–12 kb. In such cases, proceed to the 1X SMRTbell cleanup bead step to get the appropriate input amount in the correct volume and buffer.

gDNA and amplicon input amount

It is highly recommended to use a quantification assay specific for double stranded DNA (dsDNA) such as the Qubit dsDNA high sensitivity assays. Please follow manufacturer's instructions for the assay being used.

We *do not* recommend quantification with UV-Vis Spectrophotometers (e.g. NanoDrop).

Remove all RNA from genomic DNA samples prior to beginning.

The following input masses are recommended for library preparation:

gDNA	Amplicons
50 to 300 ng per sample	20 to 200 ng per sample

Important:

- Do not exceed 300 ng of gDNA per sample going into the Repair and A-tailing step.
- At least 24 samples need to be used when using lower input amounts (e.g., 50 ng).
- Samples must be pooled after ligation.

Multiplexing

Important: This procedure requires one of the four available SMRTbell adapter index plates:

- SMRTbell adapter index plate 96A
- SMRTbell adapter index plate 96B
- SMRTbell adapter index plate 96C
- SMRTbell adapter index plate 96D

To balance the number of reads per sample, consider the following:

- Shear all gDNA samples to similar mean fragment sizes and distributions.
- Normalize DNA input across all samples.

To pool more than 96 samples per SMRT® Cell combine multiple HiFi plex preps after the final cleanup. Each prep needs to use a different SMRTbell adapter index plate. A total of 384 samples can be pooled for sequencing on a single SMRT Cell. A total of 1,536 (4 SMRT Cells x 384 samples) can be sequenced on a single Revio run.

DNA shearing

This protocol recommends shearing gDNA using automated liquid handler systems to a size between 13 – 20 kb.

For more details, please see the high-throughput DNA shearing Technical note.

Microbial and metagenomic samples often have degraded DNA where the majority is already < 13 kb in length. To better balance the number or reads between samples it may be necessary to shear all samples to a mean size of ~10 kb. For shearing below <13 kb, we recommend the following equipment:

SPEX SamplePrep *1600 MiniG* homogenizer:

- Speed = 1500 RPM
- Time = 3 minutes
- DNA input = 300 ng – 3 µg
- Volume = Up to 300 µL

For more details, see the PacBio Technical note describing experimental conditions for shearing using the 1600 MiniG.

MP Bio FastPrep96*:

- Speed = 1800 RPM
- Time = 60 seconds
- DNA input = 300 ng
- Volume = 50 µL

* Contact MP Bio for the latest shearing protocol for microbial samples. Conditions may need to be adjusted based on DNA input mass and volume.

Reagent and sample handling

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

SMRTbell cleanup beads

Bring SMRTbell cleanup beads to room temperature for at least 1.5 hours prior to use. Alternatively, beads can be left out overnight if being used the next morning.

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 plex prep kit 96

- Thaw the Repair buffer M96, Nuclease buffer M96, SMRTbell adapter index plate, and Stop solution M96 at room temperature. Once thawed, place on ice prior to loading the deck.
- Quick-spin all reagents in a centrifuge to collect liquid at bottom prior to use.
- Keep all temperature-sensitive reagents on ice.

Temperature-sensitive reagents		
Step used	Tube color	Reagent
Repair and A-tailing	Blue	End repair mix M96
	Green	DNA repair mix M96
Adapter ligation	Yellow	Ligation mix M96
	Red	Ligation enhancer M96
Nuclease treatment	Light green	Nuclease mix M96

- Bring 1X dsDNA HS reagents to room temperature for 30 minutes prior to use.
- Samples can be stored at 4°C at all safe stopping points listed in the protocol.
- 80% ethanol should be made fresh for each run.

Polymerase kit

Thaw the following reagents at room temperature:

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

Once thawed, reaction buffers and sequencing primer may be stored on a cold block, at 4°C, or on-ice. The Loading buffer should be left at room temperature.

Note: Loading buffer 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
- Sequencing control

Bring the following reagents up to room temperature:

- Loading buffer
- SMRTbell cleanup beads

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 the tip support adapter is empty before starting a run.

1. Hamilton NGS STAR MOA HiFi plex prep DNA shearing module to pooling module loading procedure

1. Prepare DNA sample plate. If preparing amplicon libraries, shearing can be bypassed. If DNA is already diluted in Elution buffer in a volume of 24.5 µL, sample can be added to a 96 well PCR plate (Bio-Rad, HSP9601) starting with position A1. Proceed to fill the plate by column as shown in Figure 1 below.

   If pipette shearing, adjust volume to 300 µL (≤300 ng gDNA mass, ≤1 ng/µL). Manually pipette diluted DNA into a 96 deep well plate (ThermoFisher Scientific, AB0859) starting with position A1. Proceed to fill the plate by column as shown in Figure 1 below.

Note: This script requires multiples of 8. If sample count is not in multiples of 8, fill the remaining wells in the column with either 24.5 µL of Elution buffer (if bypassing shearing) or 300 µL of Buffer LTE (if pipette shearing). The reagent kit supports 4 sets of 24 sample runs.

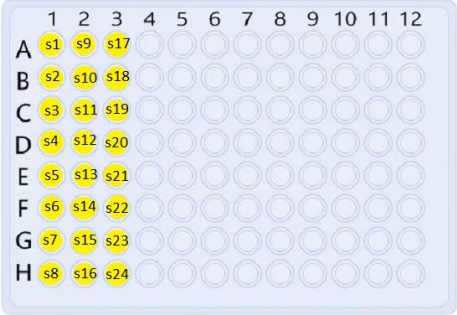


Figure 1: 24 sample input plate example (s1 – s24). Prepare in a 96 well PCR plate (Bio-Rad, HSP9601).

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

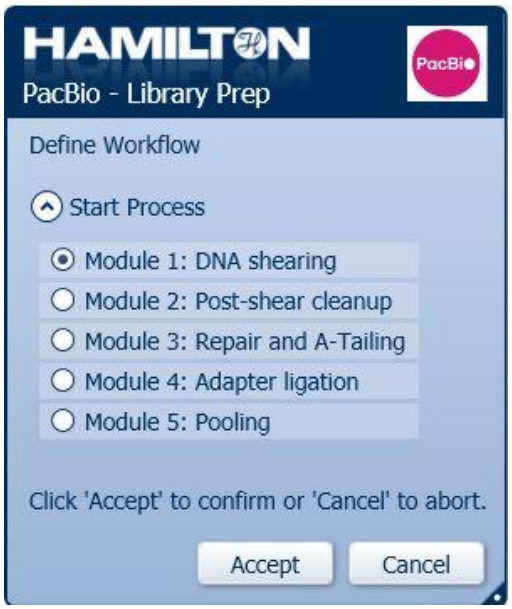
Note: Master mix preparation will be described at step 9.

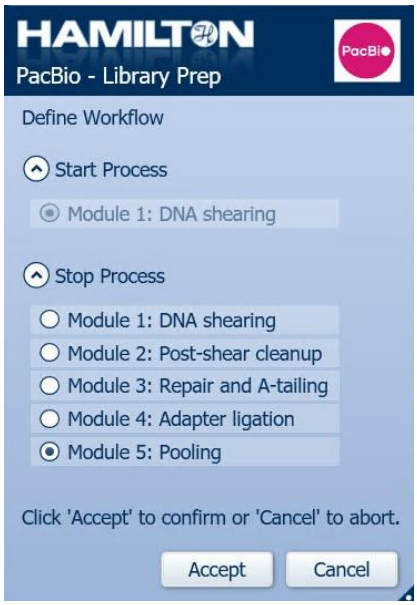
Reagent	Consumables
SMRTbell cleanup beads	60 mL reservoir
Elution buffer	60 mL reservoir
Stop solution M96	60 mL reservoir
80% Ethanol	300 mL reservoir
SMRTbell adapter index plate	96 well PCR plate part of kit
Repair master mix • Repair buffer M96 • End repair mix M96 • DNA repair mix M96	Designated master mix 96 well hard shell plate (Bio-Rad, HSP9601) in column 1

Ligation master mix • Ligation mix M96 • Ligation enhancer M96	Same as above plate (Bio-rad, HSP9601) but in column 2

3. Start the PacBio HiFi plex prep script: “PacBio HiFi Plex Prep v1.0.1”.
4. Enter a “USER ID” for run.

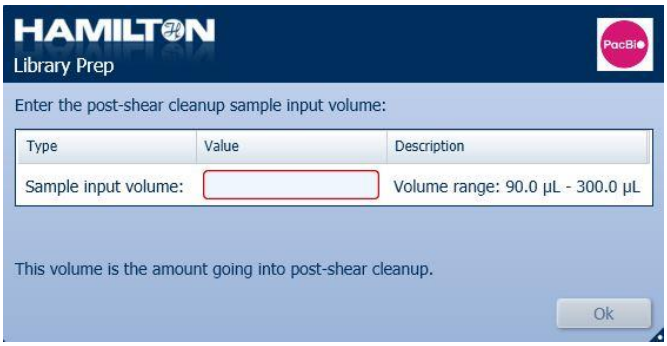
5. Define workflow. Select “Module 1: DNA shearing” for the start process and “Module 5: Pooling” for the stop process.



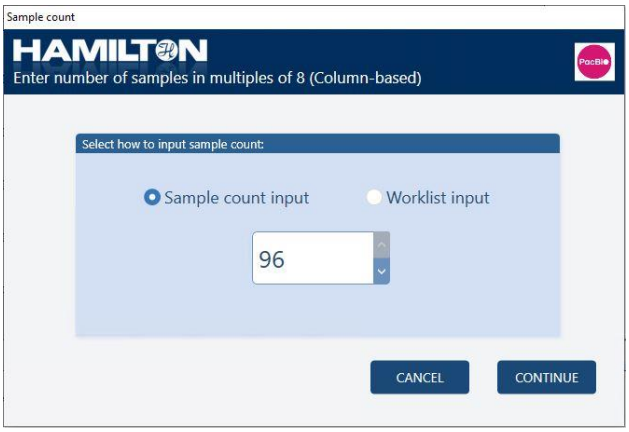


6. Sample input volume. If pipette tip shearing is performed, enter “300” µL as the “Sample input volume” if start process is post-shear cleanup. If an alternative shearing method is performed or amplicons are the input, the “Sample input volume” range is compatible with 90–300 µL.

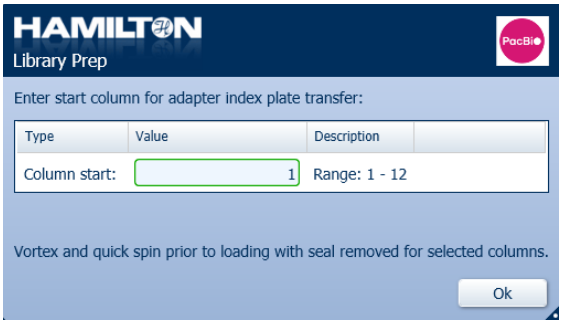
Note: If DNA is already cleaned and in the appropriate volume (24.5 µL), the cleanup can be bypassed.



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



8. Adapter column selection. Enter the start column number (range 1–12) for the SMRTbell adapter index plate transfer. This prompt will only display if the sample count is ≤88 samples.



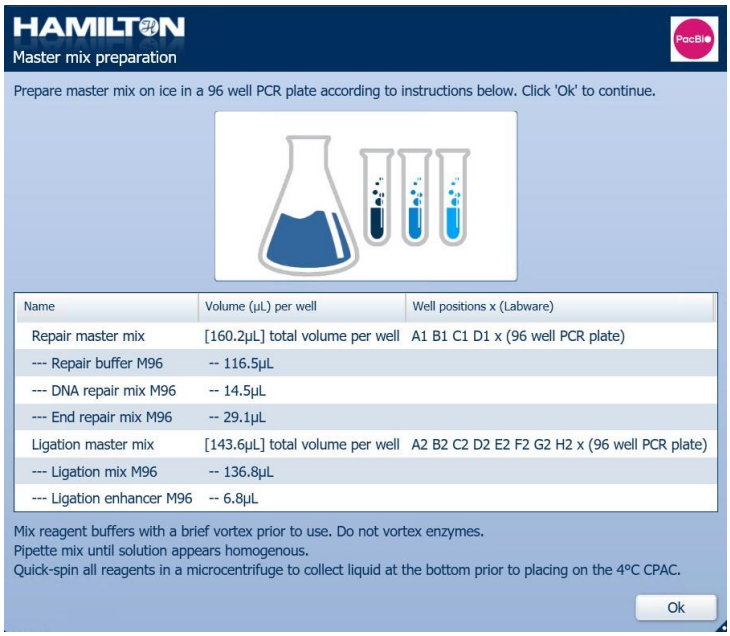
9. Master mix preparation for DNA repair and ligation steps. The table below describes how to prepare the Repair master mix and the Ligation master mix for 24, 48, 72, and 96 samples in a 96-well PCR plate (Bio-Rad, HSP9601). Pipette mix master mixes and spin down the master mix plate to remove any bubbles. Place master mix plate on ice until prompted to load on the deck. The example prompt is for a 96-sample run.

Note: Do not vortex master mix plate.

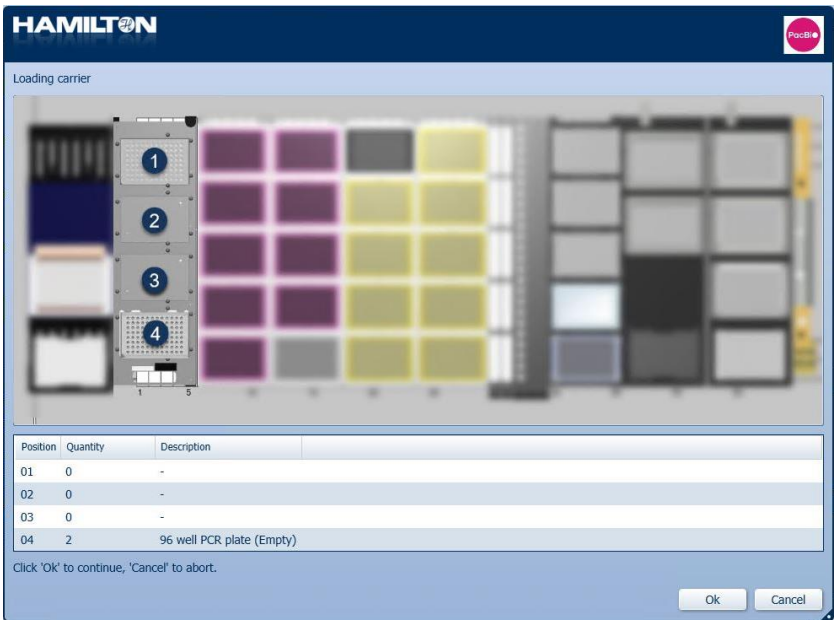
Note: Prepare all master mixes in 24-sample batches (as shown in table below). For example, if running 96 samples, prepare master mix for a 24-sample batch four times. We do not recommend preparing a bulk master mix due to pipetting volume loss.

Repair master mix					
Sample configuration	24 samples	24 samples	48 samples	72 samples	96 samples
# of wells in plate	1	1	2	3	4
Well locations	A1	A1	A1, B1	A1, B1, C1	A1, B1, C1, D1
Reagent name	Reagent volumes (µL)				
Repair buffer 96	116.5 µL	Prepare 24-sample master mix 1-time for each well	Prepare 24-sample master mix 2-times for each well	Prepare 24-sample master mix 3-times for each well	Prepare 24-sample master mix 4-times for each well
DNA repair mix M96	14.5 µL				
End repair mix M96	29.1 µL				
Total volume per well	160.2 µL				

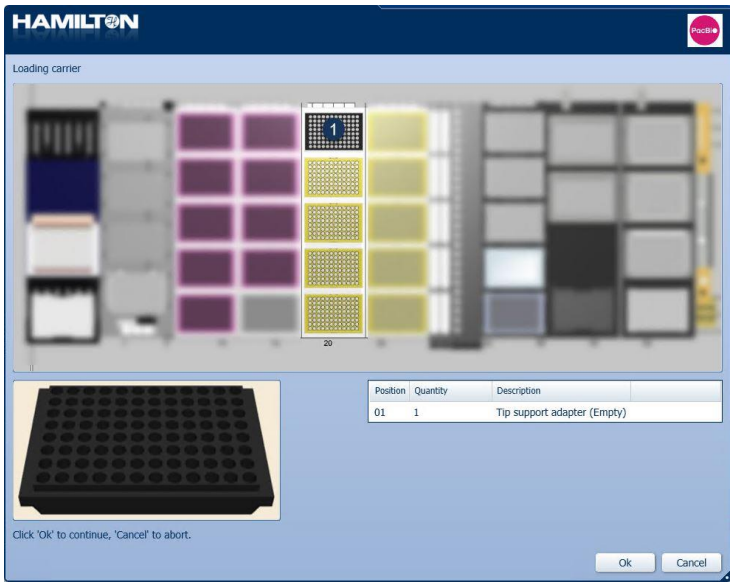
Ligation master mix					
Sample configuration	24 samples	24 samples	48 samples	72 samples	96 samples
# of wells in plate		2	4	6	8
Well locations		A2, B2	A2, B2, C2, D2	A2, B2, C2, D2, E2, F2	A2, B2, C2, D2, E2, F2, G2, H2
Reagent name	Reagent volumes (µL)				
Ligation mix 96	136.8 µL	Prepare 24-sample master mix 2 times for each well	Prepare 24-sample master mix 4 times for each well	Prepare 24-sample master mix 6 times for each well	Prepare 24-sample master mix 8 times for each well
Ligation enhancer 96	6.8 µL				
Total volume per well	143.6 µL				



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



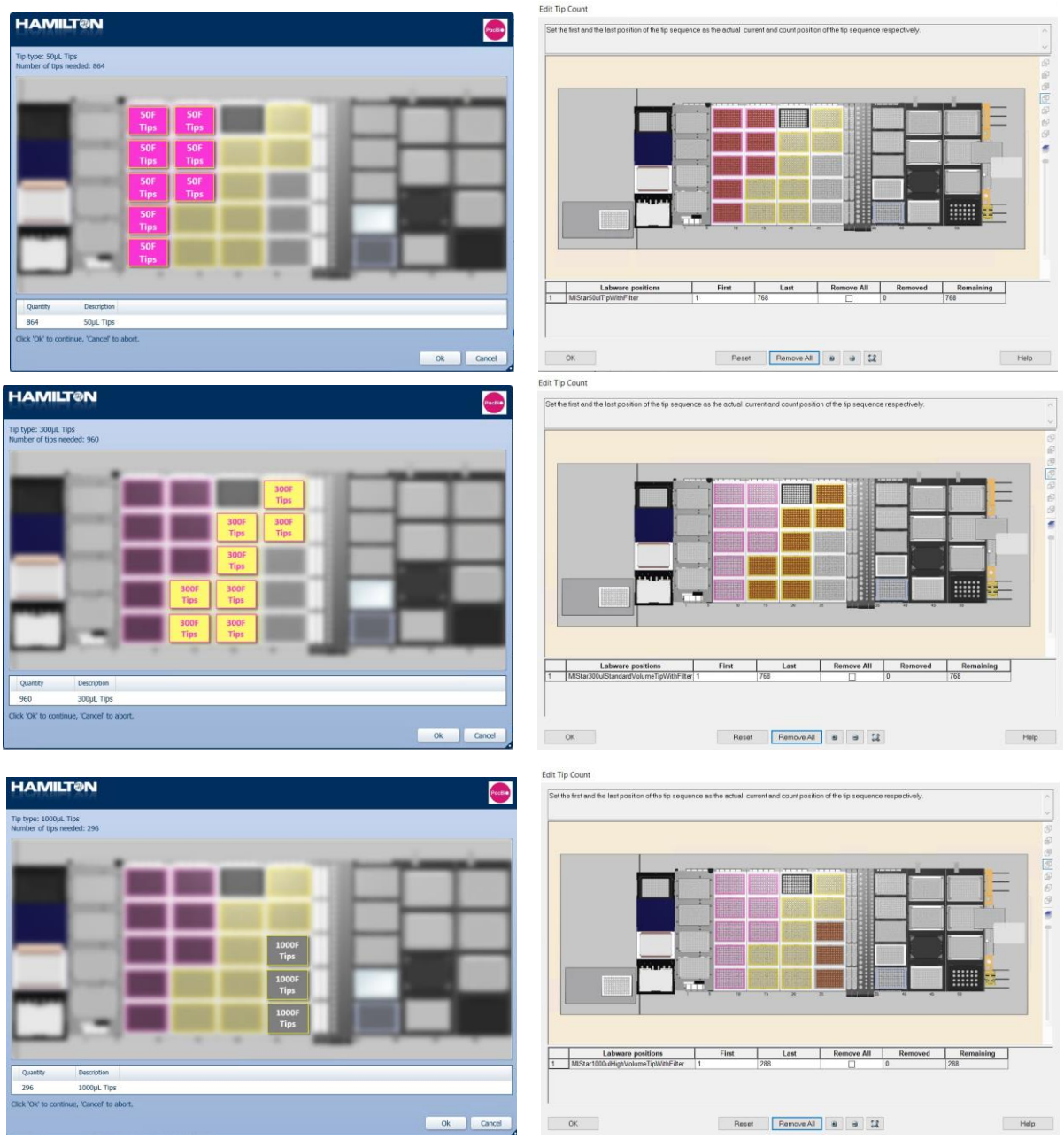
11. Ensure the tip support adapter for the MPH is empty. CO-RE I instruments require the CO-RE I tip support. CO-RE II instruments require the CO-RE II tip support. Contact your Hamilton Representative to ensure the configuration is set up correctly with your instrument's CO-RE technology.



12. 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.

13. Select the 50 µL, 300 µL, and 1000 µL tips on the instrument. The pipette tip type is under “Description” and the number of tips required is indicated in “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.

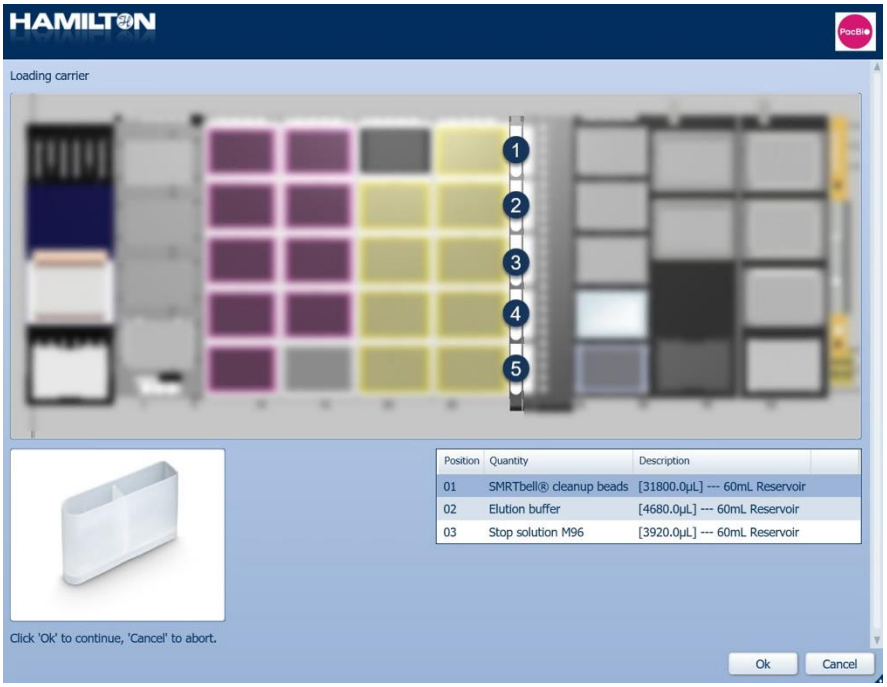
Note: It is critical that these tip selections are accurate and to not leave any empty tip racks (See Appendix for example).



14. Load 60 mL reservoir reagent carrier. Load the SMRTbell cleanup beads, Elution buffer, and Stop solution M96 into the 60 mL reservoirs. Place reservoirs into the reagent carrier in track position 30. Reagent carrier position 1 is for SMRTbell cleanup beads, position 2 is for Elution buffer, and position 3 is for Stop solution M96. The table below shows the volumes for each reagent based on sample count when starting at DNA shearing and stopping at pooling. The example prompt is for 96-samples.

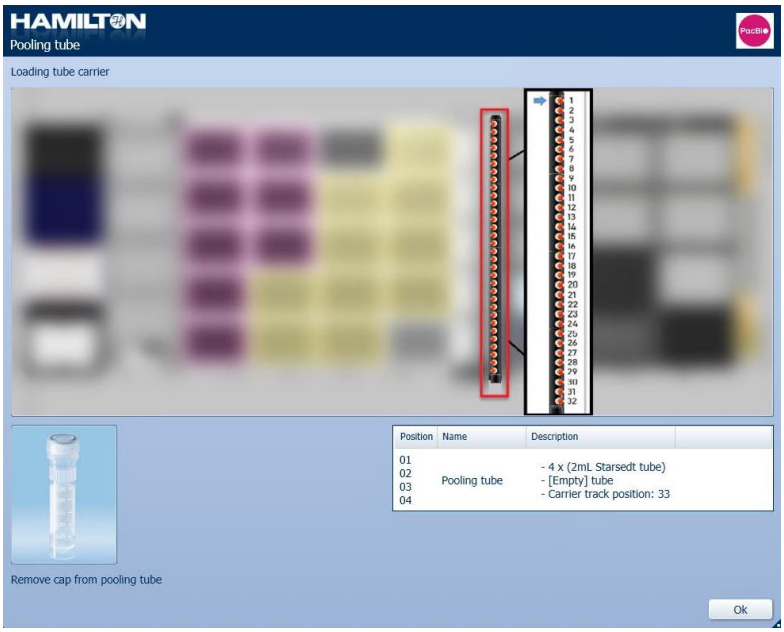
Note: Note: SMRTbell cleanup bead volumes in the table assume the max sheared DNA input volume, 300 µL.

Reagent	Consumables	24 samples	48 samples	72 samples	96 samples
SMRTbell cleanup beads	60 mL reservoir	10,200 µL	17,400 µL	24,600 µL	31,800 µL
Elution buffer	60 mL reservoir	2,520 µL	3,240 µL	3,960 µL	4,680 µL
Stop solution M96	60 mL reservoir	2,480 µL	2,960 µL	3,440 µL	3,920 µL



15. Load the pooling tube(s). Load the appropriate number of pooling tubes (Sarstedt Inc, 72.694.306) to the tube carrier in track 33, starting at position 1. The table below shows the number of pooling tubes required according to sample count. Uncap prior to loading. The example prompt below is the number of pooling tubes for 96 samples.

Labware	24 samples	48 samples	72 samples	96 samples
2mL Sarstedt tube	1 tube	2 tubes	3 tubes	4 tubes



16. Load plate carrier.

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

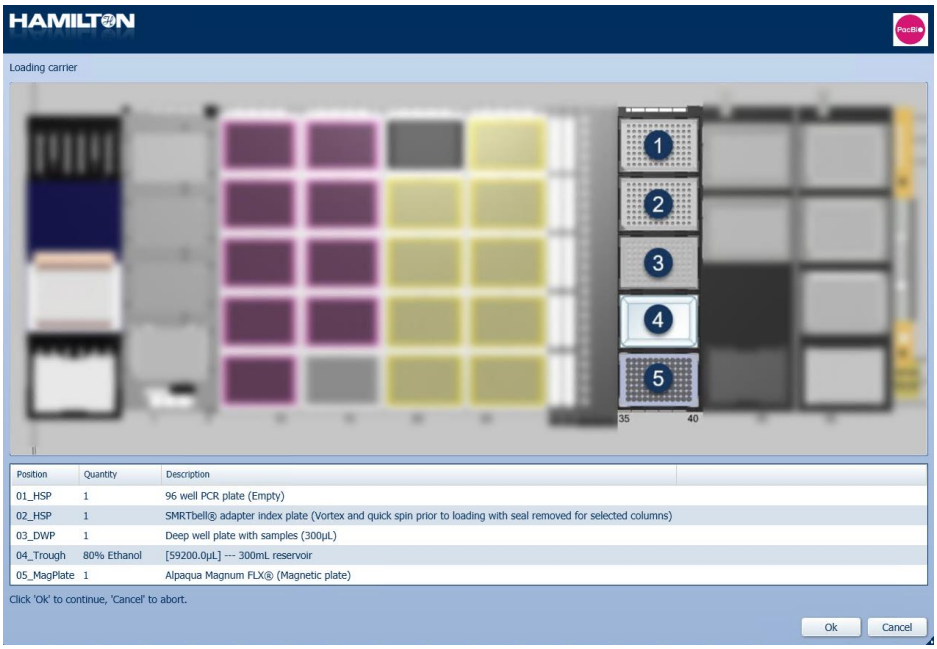
    Position 2 – Unsealed SMRTbell adapter index plate

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

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

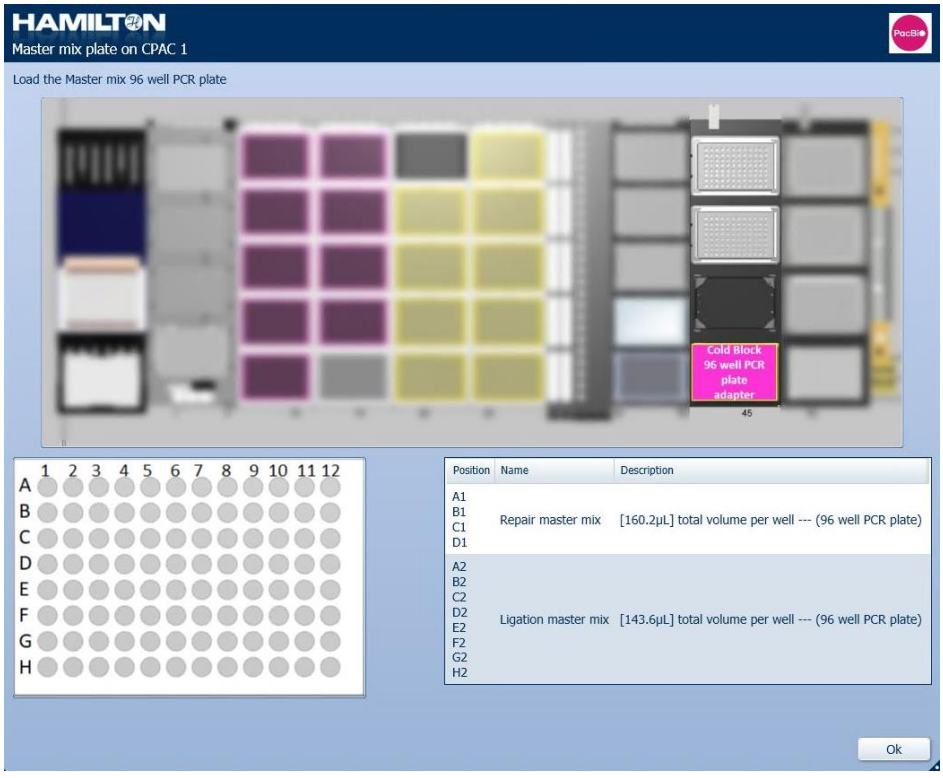
    Position 5 – Alpaqua magnum FLX magnet plate

Reagent	Labware	24 samples	48 samples	72 samples	96 samples
80% Ethanol	300 mL reservoir	44,800 µL	49,600 µL	54,400 µL	59,200 µL



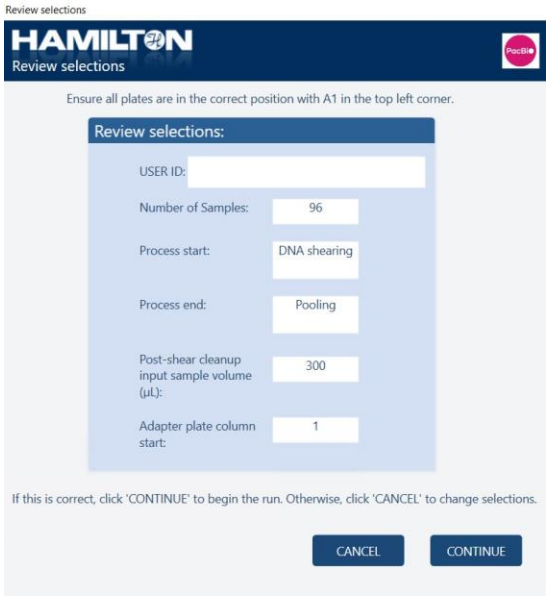
17. Load master mix plate on cold block. Follow the prompt to load the master mix plate (containing Repair and Ligation master mixes) on the cold block 96-well PCR plate adapter (CPAC 1) at 4ºC. The prompt below is for 96 samples.

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



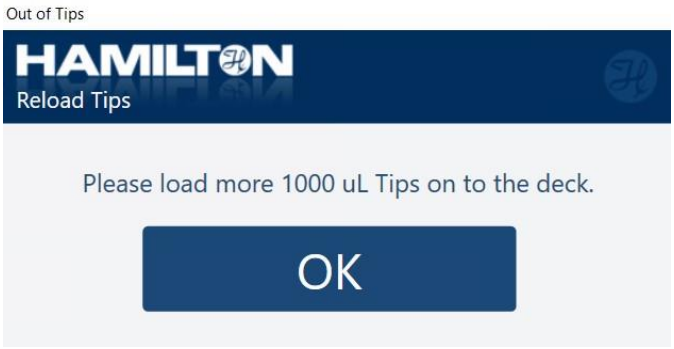
18. Review selections. The example prompt below is set for 96 samples starting at “DNA shearing” and ending at “Pooling” with the post-shear cleanup input sample volume at 300 µL using adapter plate column 1. Click “CONTINUE” to begin DNA shearing.

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



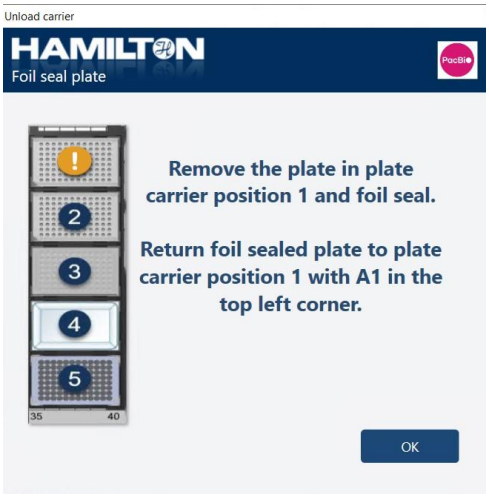
Post DNA Shearing Optional QC. Pause the instrument after shearing completion and measure the concentration and size distribution of the sheared DNA.

Note: For a 96-sample run, refill the 1000 µL tips after ~30 minutes (during supernatant removal of post-shear cleanup).

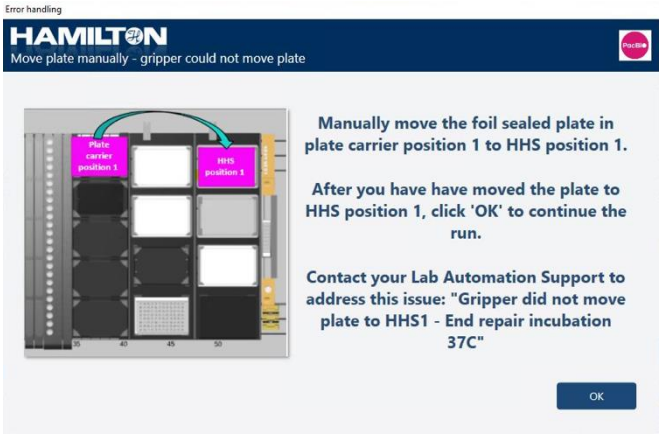


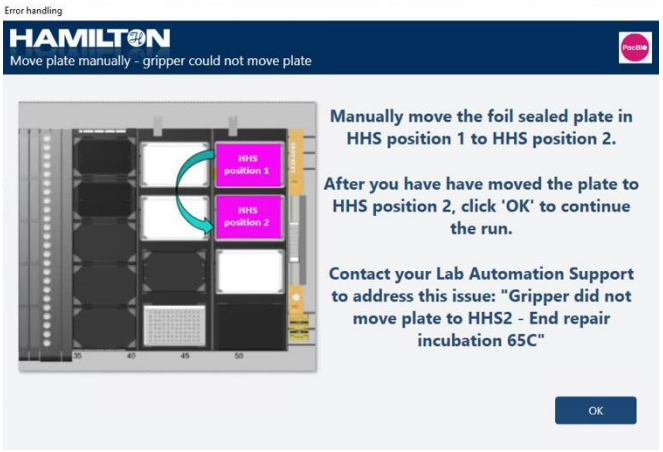
19. Seal sample plate for incubation during End repair and A-tailing step. The plate carrier will unload and prompt the user to manually heat foil plate from plate carrier position 1. Return the foiled sealed plate to the same location. Click ‘Ok’ for the instrument to pull the plate carrier back.

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



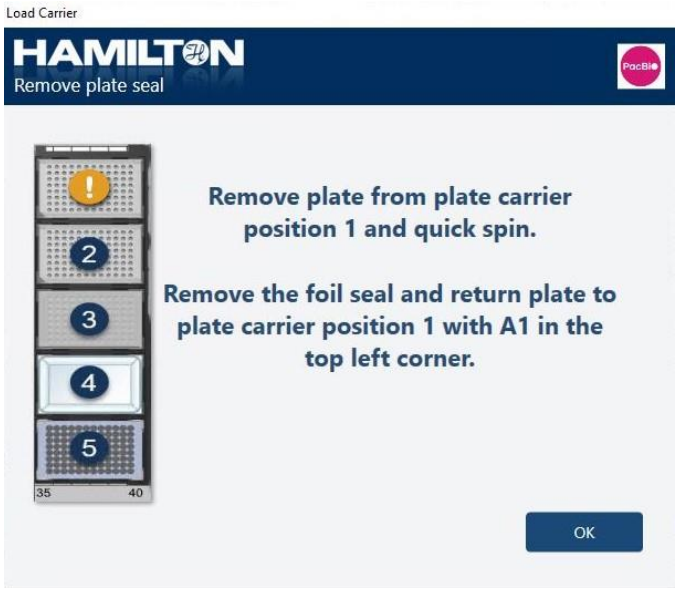
Note: If the gripper fails to move the plate, the following prompts will appear to provide guidance on manually moving the plate for the repair and/or A-tailing incubations.



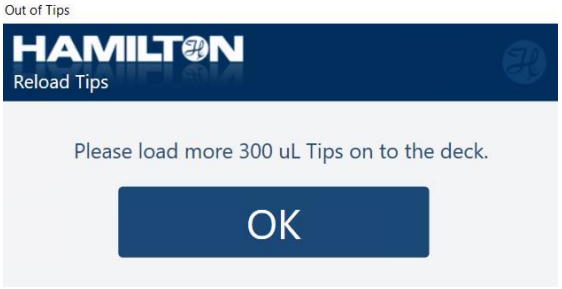


20. Remove sample plate seal after incubation. The plate carrier will unload and prompt the user to remove sample plate in carrier position 1 and quick spin. Remove the foil seal and return the plate to the same location. Click ‘Ok’ for the instrument to pull the carrier back and continue to adapter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

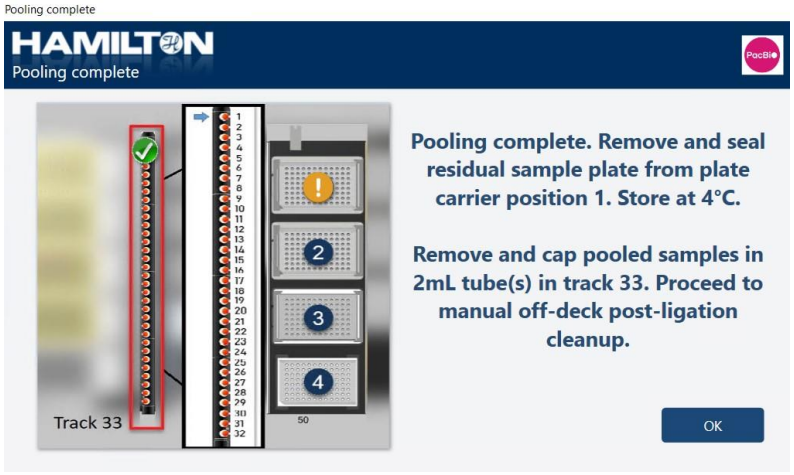
Note: Carefully remove the seal to prevent contamination.



Note: For a 96-sample run, refill the 300 µL tips after ~1.5 hours.



21. Run complete. Once the automated run is complete, a prompt displays. Gather the 2 mL pooling tubes from the tube carrier in track 33 and continue library prep off-deck. Cap and spin down tubes to remove bubbles. Proceed to 1X SMRTbell cleanup bead purification of pooled libraries manually off deck. The residual sample plate in plate carrier position 1 should be sealed and stored at 4ºC.



2. Manual off-deck post-ligation cleanup, nuclease treatment, and final SMRTbell cleanup procedure

Post-ligation SMRTbell cleanup bead purification of pooled libraries

Bring SMRTbell cleanup beads to room temperature prior to the purification step.

1. Add 960 µL (1.0X) of resuspended, room-temperature SMRTbell cleanup beads to each pool.

Note: If using less than 960 µL, add 1.0X (v/v) concentration of SMRTbell cleanup beads.

2. Pipette mix or invert the sample until the beads are evenly distributed. Quick-spin the samples to collect liquid.
3. Incubate at room temperature for 10 minutes to allow DNA to bind beads.
4. Place samples on a magnet and allow beads to separate fully from the solution. The solution should be clear and beads pelleted to the magnet.
5. Slowly remove the supernatant without disturbing the beads. Discard the supernatant.
6. Slowly dispense 2000 µL, or enough to cover the beads, of freshly prepared 80% ethanol to each sample. After 30 seconds, remove the 80% ethanol and discard.
7. Repeat the previous step.

8. Remove residual 80% ethanol:

   - Remove the samples from the magnet and quick spin to collect liquid.
   - Place samples back on the magnetic and wait until beads separate fully from the solution.
   - Carefully remove the residual 80% ethanol without disturbing the bead pellet and discard.

9. Remove samples from the magnet and immediately add 40 µL of elution buffer.

   Resuspend by pipetting mixing until beads are evenly distributed in solution. Quick spin samples if necessary to collect liquid.

10. Incubate samples at room temperature for 5 minutes to elute DNA off beads.
11. Place samples back on the magnet and allow beads to separate fully from the solution. The solution should be clear, and beads pelleted to the magnet before proceeding.
12. Slowly aspirate the cleared eluate without disturbing the beads. Transfer eluate to a new tube strip. Discard old tube with beads.
13. Proceed to the next step (nuclease treatment), or store samples at 4°C.

SAFE STOPPING POINT - Store at 4°C

Nuclease treatment

1. Add the following components in the order and volume listed below to each of the pools from the previous step.

Nuclease mix		
Tube	Component	Volume
Light purple	Nuclease buffer M96	5 µL
Light green	Nuclease mix M96	5 µL
	Total volume	10 µL

2. Pipette-mix and spin down to collect liquid. Total volume should equal 50 µL.
3. Run the Nuclease treatment thermocycler program. Set lid temperature to 75°C if programmable.

Step	Time	Temperature
1	15 min	37°C
2	Hold	4°C

3. Pooling and SMRTbell cleanup bead purification

Bring the SMRTbell cleanup beads to room temperature prior to the purification step.

Pool the completed nuclease reactions based on the number of SMRT Cells to be run. For example, if 96 samples will be sequenced on a single SMRT Cell, pool four 24-sample nuclease reactions prior to SMRTbell cleanup.

Nuclease reactions pooled	Total volume	SMRTbell cleanup bead volume for 1.0x	SMRTbell cleanup bead volume for 1.3x	Elution volume
1	50 µL	50 µL	65 µL	25 µL
2	100 µL	100 µL	130 µL	25 µL
3	150 µL	150 µL	195 µL	25 µL
4	200 µL	200 µL	260 µL	25 µL

SMRTbell cleanup bead purification

Bring SMRTbell cleanup beads to room temperature prior to the purification step.

1. For DNA >3kb, add 50–200 µL (1.0X) of resuspended, room-temperature SMRTbell cleanup beads to each sample. For DNA <3kb, add 65–260 µL (1.3x) of resuspended, room-temperature SMRTbell cleanup beads to the sample.
2. Pipette-mix the sample until the beads are evenly distributed. Quick-spin the samples to collect liquid.
3. Incubate at room temperature for 10 minutes to allow DNA to bind beads.
4. Place samples on a magnet and allow beads to separate fully from the solution. The solution should be clear and beads pelleted to the magnet.
5. Slowly remove the supernatant without disturbing the beads. Discard the supernatant.
6. Slowly dispense 200 µL, or enough to cover the beads, of freshly prepared 80% ethanol to each sample. After 30 seconds, remove the 80% ethanol and discard.
7. Repeat the previous step.
8. Remove residual 80% ethanol:

    - Remove the samples from the magnet and quick spin to collect liquid.
    - Place samples back on the magnetic and wait until beads separate fully from the solution.
    - Carefully remove the residual 80% ethanol without disturbing the bead pellet and discard.

9. Remove samples from the magnet and immediately add 26 µL of Elution buffer.

    Resuspend by pipetting mixing until beads are evenly distributed in solution. Quick-spin samples if necessary to collect liquid.

10. Incubate samples at room temperature for 5 minutes to elute DNA off beads.

11. Place samples back on the magnet and allow beads to separate fully from the solution. The solution should be clear, and beads pelleted to the magnet before proceeding.
12. Slowly aspirate the cleared eluate without disturbing the beads. Transfer eluate to a new tube strip. Discard tube with beads.
13. Recommended: quality control checkpoint. Use 1 µL from each pool to measure the concentration and size distribution of the completed library.
14. Proceed to Section 8 to prepare library for sequencing on Vega or Revio (+SPRQ chemistry). If necessary, dilute the 25 µL SMRTbell library to the concentrations indicated below. Failure to normalize libraries to the appropriate concentration prior to ABC may result in low sequencing yield.

    Or

    Proceed to SMRT Link Sample Setup for preparing samples for sequencing with Revio (non-SPRQ) or Sequel II/e.

SMRTbell library size	Concentration (ng/µL)
>10 kb	<60 ng/µL
3–10 kb	<20 ng/µL
<3 kb	<10 ng/µL

SAFE STOPPING POINT - Store at 4°C

4. Annealing, binding, and cleanup (ABC)

This step is for preparing the libraries (25 µL) for sequencing on Revio or Vega systems. The polymerase kit used will depend on which sequencer or chemistry is being used (see below).

Kit	PN
Revio SPRQ polymerase kit	103-496-900
Vega polymerase kit	103-426-500

1. Prepare the appropriate volume of master mix with 10% overage using the per reaction volumes listed below.

Annealing mix			
✔	Tube	Component	Volume
	Light blue	Annealing buffer	12.5 µL
	Light green	Standard sequencing primer	12.5 µL
		Total volume	25 µL

2. Pipette-mix the Annealing mix and quick spin to collect liquid.
3. Add 25 µL of the Annealing mix to each library. Total volume should equal 50 µL.
4. Pipette-mix each sample and quick spin to collect liquid.
5. Incubate at room temperature for 15 minutes.
6. During primer incubation, prepare the polymerase dilution (see below) and store on ice.
7. To prepare the polymerase, add the following components to a new microcentrifuge tube on ice. Adjust component volumes for the number of samples being prepared, plus 10% overage.

Polymerase dilution		
Tube	Component	Volume
Yellow	Polymerase buffer	47 µL
Purple	Sequencing polymerase	3 µL
	Total volume	50 µL

8. Pipette-mix the Polymerase dilution and quick-spin to collect liquid.
9. Add 50 µL of Polymerase dilution to primer annealed sample. Total volume should equal 100 µL.
10. Pipette-mix each sample and quick-spin to collect liquid.
11. Incubate at room temperature for 15 minutes.
12. Proceed immediately to the next step of the protocol to remove excess polymerase.

Post-binding cleanup with SMRTbell cleanup beads

13. For DNA >3 kb, add 100 µl (1.0x v/v) of resuspended, room-temperature SMRTbell cleanup beads to each sample.

    For DNA <3 kb, add 130 µL (1.3x v/v) of resuspended, room-temperature SMRTbell cleanup beads to each sample.

14. Pipette-mix the beads until evenly distributed and quick-spin if necessary to collect all liquid from the sides of the tube.
15. Incubate at room temperature for 10 minutes to allow DNA to bind beads
16. Place sample on an appropriate magnet and allow beads to separate fully from the solution
17. Slowly remove the cleared supernatant without disturbing the beads. Discard the supernatant.

    DO NOT USE ETHANOL. Proceed immediately to the elution. It is important not to let the beads dry out.

18. Remove sample from the magnet and immediately add Loading buffer to each tube and resuspend the beads by pipette mixing.

	Revio (+SPRQ)	Vega
Loading buffer	25 µL	50 µL

19. Quick-spin the samples to collect any liquid from the sides of the tube.
20. Incubate at room temperature for 15 minutes to elute DNA.
21. Place sample on magnet and allow beads to separate fully from the solution.
22. Slowly remove the cleared eluate without disturbing the beads. Transfer eluate to a new tube. Discard the old tube with beads
23. Use 1 µL of sample to measure DNA concentration with a Qubit fluorometer using the 1x dsDNA HS kit.

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

24. Proceed to the Loading Calculator in SMRT Link v13.3 or higher to calculate the final dilution for adding the sample to the Revio/Vega sequencing plate. The recommended loading concentration is 200–300 pM.

PROTOTCOL COMPLETE

Important: Polymerase-bound libraries can be stored at 4°C for up to 1 month, or at -20°C for up to 6 months prior to sequencing. Polymerase-bound libraries can withstand up to 4 freeze-thaw cycles. Note that the Loading buffer is light sensitive.

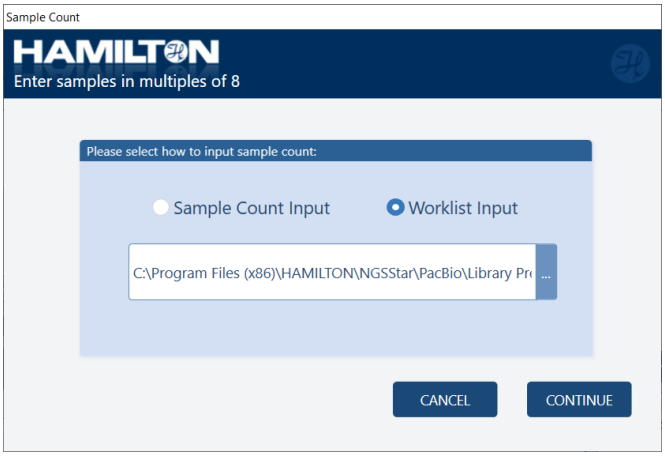
Appendix

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.

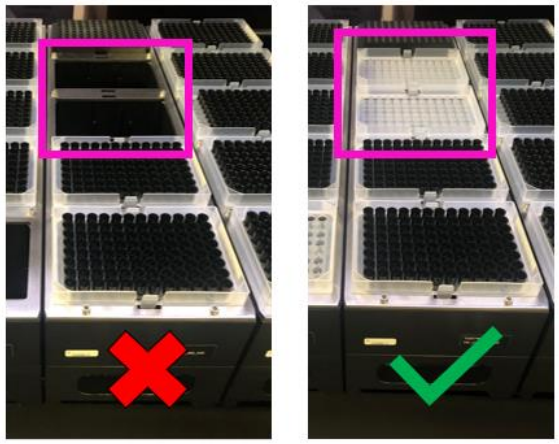
Note: When editing the file, ensure sample count is a multiple of 8.

	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

1. What should be evaluated if the instrument 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 errors and there is sufficient volume, you can select “bottom” to force the instrument to pipette regardless of liquid level detection.

2. 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 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.

General library preparation

1. 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.

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

- Use a quantification method specific to dsDNA, such as Qubit. Some high molecular weight (HMW) DNA will be non-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 quant accuracy. The vortexing will not adversely affect the DNA.

3. Is bead carryover problematic?

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

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

- 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 termination pooling
- Post-ligation SMRTbell bead 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-1x SMRTbell 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 recovery
Starting Input	100%
Post-shear SMRTbell bead cleanup	80–95%
Post ligation termination pooling (40/60 µL pooled)	67%
Post-ligation SMRTbell bead cleanup	80–95%*
Post-nuclease (pre-cleanup)	25–35%
Post-nuclease SMRTbell bead cleanup recovery	80–95%
Post-ABC SMRTbell bead cleanup recovery	80–95%
Overall Recovery	**7–19%**

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

5. 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) 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.

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

- **Repair/A-tailing**. Is the appropriate volume of mastermix added? We use aspiration of mastermix at 10 µL/s, and dispense into sample at 20 µL/s. After addition of the correct volume, is the sample appropriately mixed? We have found pipette mixing at this step to be effective (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 Hamilton NGS Star aspirates ligation mastermix at 10 µL/s and dispenses into 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. We have found shaking to be effective (1400 rpm, 1 min).
- Note that if pipette mixing is used, ensure that volume loss does not occur during this step.

7. What happens if you fail to add termination solution prior to the pooling step?
    - The termination solution inhibits ligase activity. This prevents sample/barcode cross contamination when the samples are pooled.

8. Can the pooling strategy be modified so that <24 reactions are pooled?
    - The HiFi Plex Prep kit supports enough nuclease mix and nuclease buffer in the HiFi Plex Prep Kit 96 for 4 nuclease reactions.

Pipette shearing

1. 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.

Revision history (description)	Version	Date
Initial release	01	March 2024
Updated to correct reference to the Hamilton NGS STAR MOA system, automated workflow time, and ethanol volume for the post-ligation cleanup.	02	April 2024
Updated for SPRQ chemistry and the Vega system	03	December 2024
Expanded Troubleshooting FAQ section and other minor edits	04	May 2025