

Preparing whole genome libraries using the HiFi prep kit 96

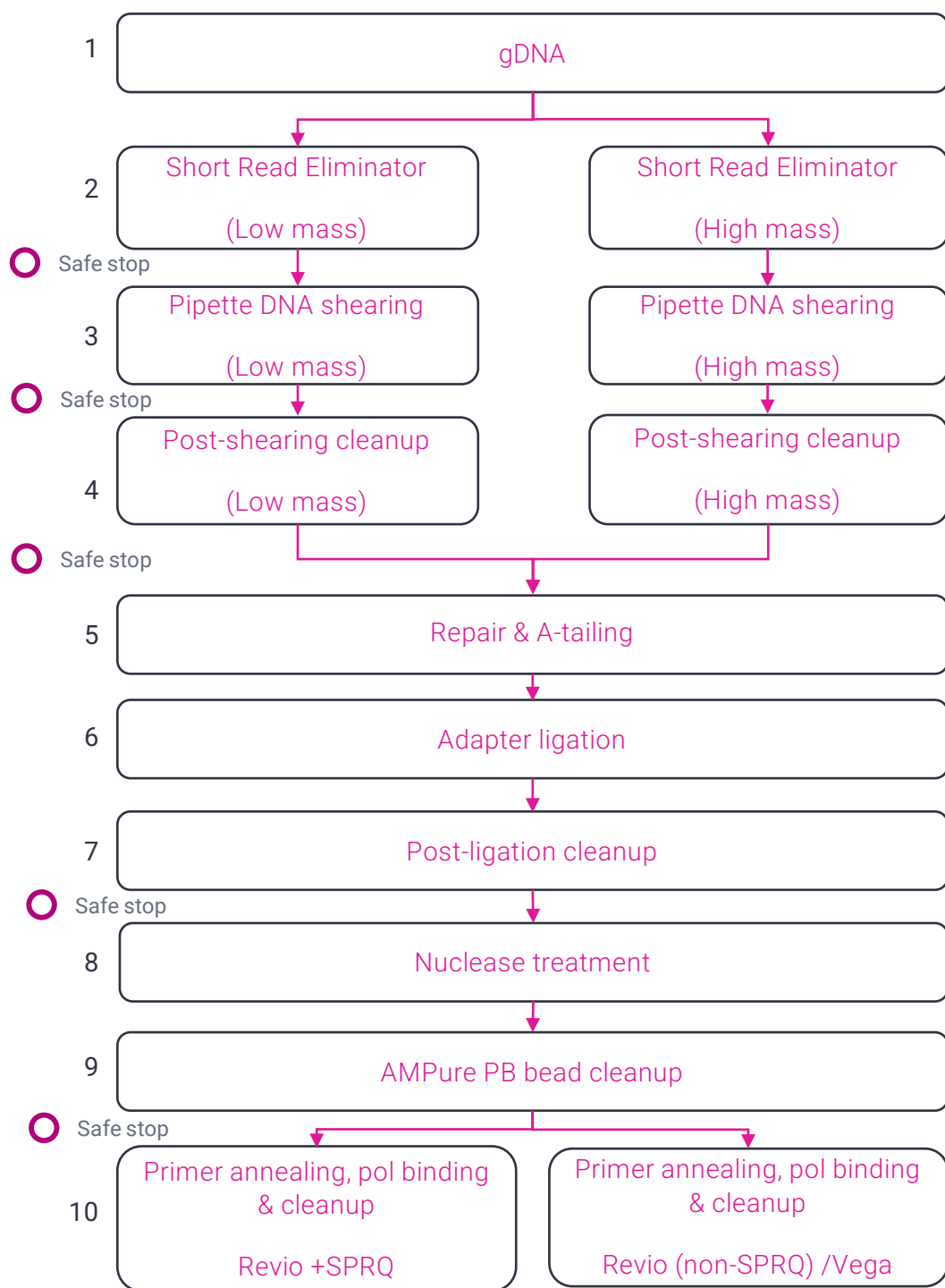
Procedure & checklist

Overview

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 bundle that includes the Revio SPRQ polymerase kit or using the separate HiFi prep kit 96 bundle that doesn't include the polymerase. This workflow is intended as a high-throughput library prep method and has been optimized for use with liquid handler automation but can also be performed manually.

Overview		
Applications	WGS of human, animal, or plant samples	
Samples	24–96 using automation (1–96 when doing manual preps)	
Minimum automated batch size	24	
Maximum automated batch size	96	
Workflow time	Automation time (manual time will vary by user and sample volume).	
	Hamilton NGS STAR MOA	Hamilton Microlab Prep
SRE	3.5 hours for 96 samples	3 hours for 24 samples
Shearing	10 min for 24–96 samples	22 min for 24 samples
Library prep	6.5 hours for 96 samples (start from post-shearing cleanup)	1.5 hours for 24 samples (post-shearing cleanup only)
Anneal, bind, cleanup (ABC)	2.5 hours for 96 samples	N/A
Average total time	13 hours	5 hours
DNA input mass and fragment size recommendations		
gDNA per SMRT [®] Cell	Revio [®] /Vega [™] (non-SPRQ): 2 µg Revio [®] (+SPRQ): 500 ng	
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	

Workflow overview



Required materials and equipment

DNA sizing

Femto Pulse system	Agilent Technologies, Inc. M5330AA
Femto Pulse gDNA 165kb analysis kit	Agilent Technologies, Inc. FP-1002-0275

DNA quantitation (one or more of the following may be used)

Qubit 4 Fluorometer	ThermoFisher Scientific Q33238
Qubit Flex Fluorometer (cannot be used for quantification of polymerase bound library)	ThermoFisher Scientific Q33327
Varioskan LUX multimode microplate reader	ThermoFisher Scientific VL0L00D0
Quant-iT 1X dsDNA HS assay kit (for Varioskan)	ThermoFisher Scientific Q33232
Qubit 1X dsDNA HS assay kit	ThermoFisher Scientific Q33230

DNA shearing (one of the following)

Hamilton Microlab Prep	PacBio®, 103-283-600
Hamilton assay ready workstation	Contact Hamilton
300 µL CO-RE II Tips (Filtered, Conductive)	Hamilton, 235903

HiFi library preparation for Revio®

Revio® SPRQ™ HiFi prep kit 96, includes:

- SRE HT
- HiFi prep kit 96 (103-122-600)
- SMRTbell® cleanup beads 85 mL (103-294-600)
- SMRTbell® adapter index plate 96A (102-009-200)
- AMPure® PB (100-265-900)
- Elution buffer (101-633-500)
- Revio® SPRQ polymerase kit 96 (103-497-000)*

Revio® SPRQ™ HiFi prep kit 96
PacBio® 103-522-600

Or

HiFi prep kit 96, includes:

- HiFi prep kit 96 (103-122-600)
- Elution buffer (101-633-500)
- SMRTbell® adapter index plate 96A (102-009-200)
- SMRTbell® cleanup beads-85 mL (103-294-600)
- Buffer LTE HT (103-306-100)

HiFi prep kit 96
PacBio® 103-381-200

and

Revio® SPRQ™ polymerase kit
PacBio® 103-520-100

Revio® SPRQ™ polymerase kit, includes:

- Revio® SPRQ™ polymerase kit (103-496-900)
- SMRTbell® cleanup beads (102-158-300)

HiFi library preparation for Vega™

HiFi prep kit 96, includes:

- HiFi prep kit 96 (103-122-600)
- Elution buffer (101-633-500)
- SMRTbell® adapter index plate 96A (102-009-200)
- SMRTbell® cleanup beads-85 mL (103-294-600)
- Buffer LTE HT (103-306-100)

HiFi prep kit 96
PacBio® 103-381-200
and
Vega™ polymerase kit
PacBio® 103-517-600

Vega™ polymerase kit, includes:

- Vega™ polymerase kit (103-426-500)
- SMRTbell® cleanup beads (102-158-300)

Other supplies

200 Proof ethanol, molecular biology or ACS grade	Any major lab supplier (MLS)
Nuclease-free water, molecular biology grade	Any MLS
Magnetic bead rack for PCR tubes or plates	Any MLS
Hard-shell 96-Well PCR Plates, low profile, thin wall, skirted	Bio-Rad HSP9601
Abgene 96 Well 0.8mL Polypropylene Deepwell Plate	ThermoFisher Scientific, AB0859
Thermocycler	Any MLS
1.5 mL DNA LoBind tubes	Eppendorf 022431021
0.2 mL 8-tube strips	USA Scientific TempAssure 1402-4708
Plate Centrifuge with 2,250 x g force capability (if using plate format)	Any MLS
MicroAmp Clear Adhesive Film (if using plate format)	ThermoFisher Scientific 00146104
ALPS 50 V-Manual Heat Sealer (if using plate format)	ThermoFisher Scientific, AB-1443A
Easy Pierce Heat Sealing Foil (if using plate format)	ThermoFisher Scientific, AB-0757

*Note: The Revio® SPRQ™ polymerase kit 96 is only available with the Revio® SPRQ™ HiFi prep kit 96 bundle. The non-SPRQ™ Revio® polymerase kit (102-817-600) is compatible, but SPRQ™ chemistry is preferred.

Before you begin

Automation

The HiFi prep kit 96 and workflow were designed for NGS liquid handling automation. As a result, this protocol is intended to describe the SRE, shearing, library prep enzymatic reactions, and bead cleanups to guide automation method development, or in certain instances manual preparation. Because of differences between automation instruments, modifications not described herein may be needed to adapt the protocol to your specific instrumentation. Please visit the [WGS page](#) or contact your local support team for a list of instruments with a PacBio qualified method.

This protocol was developed using the Hamilton NGS STAR MOA system.

Genomic DNA (gDNA) QC and input mass recommendations

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

gDNA quality QC

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

Recommended guidelines for evaluating gDNA quality for this protocol:

- Use the Femto Pulse gDNA 165 kb analysis kit (Agilent FP-1002-0275)
- Dilute samples to 250 pg/μL
- 70% or more of the DNA should be ≥ 10 kb for this protocol. This corresponds to a genome quality number (GQN) of 7.0 or higher at 10 kb. 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 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. Follow manufacturer's instructions for the assay being used. We *do not* recommend quantification with UV-Vis Spectrophotometers (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 for 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 mass by starting gDNA quality

gDNA quality	Low mass	High mass	Expected SRE recovery (dependent on DNA quality)
70% > 10 kb (recommended)	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).

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 non-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 SMRT Cell

Mean insert size	Revio + SPRQ (250 pM)	Revio/Vega (non-SPRQ)
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 kit 96 workflow stepwise expected 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 higher 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 index. Starting with SMRT® Link v13.1, there will be a pooling calculator in Sample Setup to help determine the appropriate volumes to use for multiplexing libraries.

Prior to pooling HiFi libraries, 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 (annealing, binding, cleanup) 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 *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 adapter index plate at room temperature. Once thawed, reaction buffers and adapter index plate may be stored on a cold block, at 4°C, or on-ice prior to making master mix or placing on the liquid handler work deck.

Keep the following temperature-sensitive reagents on ice.

Temperature-sensitive reagents HiFi prep kit 96		
Step used	Tube	Reagent
Repair and A-tailing	Blue	End repair 96
	Green	DNA repair 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 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.

Note: The Loading buffer 96 is light sensitive and should be protected from light when not in use.

Keep the following reagents on a cold block or ice:

- Sequencing polymerase 96
- Sequencing control 96

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

- Loading buffer 96

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

- SMRTbell cleanup beads 85 mL

Polymerase-bound library stability

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

Safety precautions

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

Procedure and checklist

Prior to beginning, it is recommended to evaluate the quantity and size distribution of input DNA to determine whether it is suitable for this protocol. See recommendations above.

1. Short Read Eliminator

Short read eliminator (SRE) will progressively deplete fragments up to 25 kb from genomic DNA samples. **SRE should not be done on gDNA samples that are <10 kb.**

Important: Use SRE on genomic DNA only. Attempting to use SRE on sheared DNA or HiFi libraries (post-library construction) will result in poor recoveries.

Please refer to the [NGS STAR MOA Guide & overview for the HiFi prep kit 96](#) or the [Microlab Prep Guide & overview](#) for details on consumables for automation.

Estimated automated time for this step (plate format): 3.5 hours. Estimated manual time for this step (tube format): 2.5 hours

✓	Step	Instructions for SRE on gDNA																		
	1.1	<p>Dilute gDNA ($GQN_{10\text{ kb}} > 7.0$) to the appropriate concentration in Buffer LTE according to the table below.</p> <table border="1"> <thead> <tr> <th></th> <th>Low mass</th> <th>High mass</th> </tr> </thead> <tbody> <tr> <td>Sample volume</td> <td>25 μL</td> <td>50 μL</td> </tr> <tr> <td>DNA concentration</td> <td>20–50 ng/μL</td> <td>40–80 ng/μL</td> </tr> <tr> <td>Recommended max gDNA mass</td> <td>1.25 μg</td> <td>4 μg</td> </tr> <tr> <td>Elution volume (Buffer LTE)</td> <td>200 μL</td> <td>300 μL</td> </tr> <tr> <td>Shearing mass limit</td> <td>≤ 1 μg</td> <td>≤ 3 μg</td> </tr> </tbody> </table> <p>Note: If working with low quality gDNA with a $GQN_{10\text{ kb}} < 7.0$, 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).</p>		Low mass	High mass	Sample volume	25 μL	50 μL	DNA concentration	20–50 ng/ μL	40–80 ng/ μL	Recommended max gDNA mass	1.25 μg	4 μg	Elution volume (Buffer LTE)	200 μL	300 μL	Shearing mass limit	≤ 1 μg	≤ 3 μg
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	1.2	<p>Add Buffer SRE to each sample:</p> <table border="1"> <thead> <tr> <th></th> <th>Low mass</th> <th>High mass</th> </tr> </thead> <tbody> <tr> <td>Buffer SRE volume</td> <td>25 μL</td> <td>50 μL</td> </tr> </tbody> </table> <p>If working in a plate format, heat seal with foil. Vortex/shake to mix for 5 seconds at max speed.</p>		Low mass	High mass	Buffer SRE volume	25 μL	50 μL												
	Low mass	High mass																		
Buffer SRE volume	25 μL	50 μL																		
	1.3	<p>Incubate the sample for 1 hour at 50°C in a heat block or thermal cycler. After incubation, if using a plate format, ensure that it is compatible with a 300 μL elution. If not, transfer to the appropriate deep well plate after incubation and seal with an adhesive seal.</p>																		

- 1.4 Load plate or tube (with the hinge facing toward the outside of the rotor) into centrifuge.

Tube: centrifuge at 10,000 rcf for 30 minutes.
Plate: centrifuge at >2250 rcf (max 3220 rcf) for 1 hour.

- 1.5 **Important:** if using a centrifuge with temperature control (i.e., cooling function), turn this function off by specifying a target temperature set point higher than ambient room temperature (e.g., 29°C or 30°C).

Carefully remove supernatant without disturbing the pellet.

- 1.6
 - Leaving up to 5 µL and 10 µL supernatant with the low and High mass SRE workflow, respectively, is acceptable to ensure the pellet is not aspirated.

Add **Buffer LTE** to the tube and incubate at room temperature for 10 minutes.

1.7

Shearing option	Low mass	High mass
Pipette shearing	200 µL	300 µL

- 1.8 After incubation, pipette-mix 20 times and vortex/shake the tube/sealed plate for 15s to ensure that the DNA is properly re-suspended and mixed.

- 1.9 Quantify the resuspension to measure DNA recovery. If the recovery is lower than 50%, repeat pipette-mixing 20 times and vortex/shake. If the recovery is greater than 50%, proceed to next step (DNA shearing).

- 1.10 Proceed to automated DNA shearing. It is recommended to proceed to DNA shearing within 2 weeks of performing SRE.

SAFE STOPPING POINT - Store at 4°C

2. Automated DNA shearing for WGS using Hamilton robots

This section describes the procedure for DNA shearing with the Hamilton Microlab Prep or Hamilton assay ready workstations (NGS STAR MOA, STARlet, and STAR V). It may be possible to shear DNA using other NGS liquid handler systems. Please check with your local PacBio support team for updated information on all qualified DNA shearing methods. Estimated time for the shearing step is 10 minutes for 96 samples on the NGS STAR MOA system, or 22 minutes for 24 samples on the Microlab Prep.

Important: A mean fragment size between 15 to 20 kb with a narrow distribution (typically ~10 – 35 kb) is recommended for this protocol. **If gDNA is within these ranges or lower, the DNA shearing step can be bypassed.** Deviating from the concentration and automation settings is not recommended and will result in undersheared DNA.

These shearing parameters are specific for the Hamilton Microlab Prep, or assay ready workstations (Hamilton NGS STAR, STARlet, and STAR V systems).

✓	Step	Instructions for automated DNA shearing on Hamilton systems																					
	2.1	Adjust DNA concentration to ≤ 5 ng/ μ L in 200 μ L (Low mass) or ≤ 10 ng/ μ L in 300 μ L (High mass), if necessary (e.g., if more than 1 μ g or 3 μ g of gDNA was recovered from SRE). Use the Low TE buffer provided with the kit (or Buffer LTE from the SRE kit) to dilute samples in a 0.8 mL, 96 DeepWell plate (Thermo Fisher Scientific AB0859). Parameters for shearing on the Microlab Prep, or Hamilton assay-ready workstations are listed below. These parameters should already be part of the installed method on the instrument.																					
	2.2	<table border="1"> <thead> <tr> <th>Parameter</th> <th>Low mass</th> <th>High mass</th> </tr> </thead> <tbody> <tr> <td>DNA concentration</td> <td>≤ 5 ng/μL</td> <td>≤ 10 ng/μL</td> </tr> <tr> <td>Volume of Buffer LTE</td> <td>200 μL</td> <td>300 μL</td> </tr> <tr> <td>Number of mixes</td> <td>300 cycles</td> <td>300 cycles</td> </tr> <tr> <td>Pipette mixing speed</td> <td>400 μL/sec</td> <td>500 μL/sec</td> </tr> <tr> <td>Liquid following</td> <td>83% volume</td> <td>83% volume</td> </tr> <tr> <td>Pipette tip</td> <td>300 μL CO-RE II tips (filtered, black, non-sterile)</td> <td>300 μL CO-RE II tips (filtered, black, non-sterile)</td> </tr> </tbody> </table>	Parameter	Low mass	High mass	DNA concentration	≤ 5 ng/ μ L	≤ 10 ng/ μ L	Volume of Buffer LTE	200 μ L	300 μ L	Number of mixes	300 cycles	300 cycles	Pipette mixing speed	400 μL/sec	500 μL/sec	Liquid following	83% volume	83% volume	Pipette tip	300 μ L CO-RE II tips (filtered, black, non-sterile)	300 μ L CO-RE II tips (filtered, black, non-sterile)
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	2.3	Place the plate on the appropriate work deck position and start the shearing procedure.																					
	2.4	Optional: measure DNA concentration with a Qubit fluorometer using the 1x dsDNA HS kit once the shearing procedure is complete. Recommended: Further dilute each aliquot to 250 pg/μL with Femto Pulse dilution buffer. Measure the sheared DNA size distribution with a Femto Pulse system to ensure efficient shearing.																					
	2.5	Proceed to the next step of the protocol.																					

SAFE STOPPING POINT - Store at 4°C

3. Post-shearing cleanup

Estimated time for this step is 1.5 hours when processing 96 samples using the NGS STAR MOA system or 24 samples on the Microlab Prep. Times will vary when preparing samples manually. This step concentrates the DNA for library prep.

Important: Allow the SMRTbell cleanup beads to equilibrate to room for at least 1.5 hours prior to beginning. If performing the cleanup in the morning, beads may be left out at room temperature overnight.

✓	Step	Instructions for SMRTbell cleanup bead step
3.1		Add 200 µL (Low mass) or 300 µL (High mass) (1.0X v/v) of resuspended, room-temperature SMRTbell cleanup beads to each sample. Note: If using less than 200 µL, add 1.0X (v/v) concentration of SMRTbell cleanup beads.
3.2		Pipette-mix the sample until the beads are evenly distributed.
3.3		Incubate at room temperature for 10 minutes to allow DNA to bind to the beads.
3.4		Place samples on an appropriate magnet and allow beads to separate fully from the solution. The solution should be clear and beads pelleted to the magnet.
3.5		Slowly remove the supernatant without disturbing the beads. Discard the supernatant.
3.6		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.
3.7		Repeat the previous step.
3.8		Remove residual 80% ethanol: <ul style="list-style-type: none"> Remove the samples from the magnet Quick-spin to collect liquid at the bottom of the tube or well. Place the tube or plate back in a magnetic separation rack until beads separate fully from the solution. Remove residual 80% ethanol and discard. Alternatively, air dry samples for 1 minute to allow residue ethanol to evaporate. Do not let the bead pellet completely dry out.
3.9		Remove samples from the magnet and immediately resuspend the beads with 49 µL of elution buffer .
3.10		Resuspend by pipetting mixing until beads are evenly distributed in solution.
3.11		Incubate samples at room temperature for 5 minutes to elute DNA off beads.
3.12		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 aspirating the supernatant.
3.13		Slowly aspirate the cleared eluate without disturbing the beads. Transfer eluate to a new tube strip or plate. Discard old tube strip or plate with beads.

SAFE STOPPING POINT - Store at 4°C

4. Repair and A-tailing

This step repairs sites of DNA damage and prepares the sheared DNA for ligation to the SMRTbell adapter.

✓	Step	Instructions for repair and A-tailing step		
		Prepare the appropriate volume of master mix with 15% overage using the per reaction volumes listed below.		
		Repair master mix		
4.1	✓	Tube	Component	Per rxn vol.
		Purple	Repair buffer 96	8 μ L
		Blue	End repair mix 96	2 μ L
		Green	DNA repair mix 96	1 μ L
		Total volume		11 μL
4.2		<i>Slowly</i> pipette mix the Repair master mix and quick-spin to collect liquid at the bottom of the tube. If bubbles form during mixing, pulse spin to remove.		
4.3		Add 11 μL of the Repair master mix to each sample. Total reaction volume should be 60 μL .		
4.4		Pipette-mix the reactions and quick-spin to collect liquid at the bottom of the well/tube. If using a plate format, seal with a heated foil seal.		
		Run the Repair and A-tailing thermocycler program. Set lid temperature to $\geq 37^{\circ}\text{C}$ if programmable.		
4.5		Step	Time	Temperature
		1	30 min	37°C
		2	5 min	65°C
		3	Hold	4°C
4.6		Proceed to the next step of the protocol.		

5. Adapter ligation

This step ligates the SMRTbell adapter to the ends of each DNA fragment.

✓	Step	Instructions for adapter ligation																				
	5.1	<p>Add 4 μL of indexed adapter to each sample from the previous step. Any of the adapters from the four SMRTbell adapter index plates can be used (e.g. 96A, 96B, 96C, or 96D).</p> <table border="1"> <thead> <tr> <th>Component</th> <th>Per rxn vol.</th> </tr> </thead> <tbody> <tr> <td>SMRTbell adapter index plate 96(A, B, C, or D)</td> <td>4 μL</td> </tr> </tbody> </table>	Component	Per rxn vol.	SMRTbell adapter index plate 96(A, B, C, or D)	4 μ L																
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	5.2	<p>Prepare the appropriate volume of master mix with 10% overage using the per reaction volumes listed below.</p> <table border="1"> <thead> <tr> <th colspan="4">Ligation master mix</th> </tr> <tr> <th>✓</th> <th>Tube</th> <th>Component</th> <th>Volume</th> </tr> </thead> <tbody> <tr> <td></td> <td>Yellow</td> <td>Ligation mix 96</td> <td>20 μL</td> </tr> <tr> <td></td> <td>Red</td> <td>Ligation enhancer 96</td> <td>1 μL</td> </tr> <tr> <td colspan="3">Total volume</td> <td>21 μL</td> </tr> </tbody> </table>	Ligation master mix				✓	Tube	Component	Volume		Yellow	Ligation mix 96	20 μ L		Red	Ligation enhancer 96	1 μ L	Total volume			21 μL
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	Yellow	Ligation mix 96	20 μ L																			
	Red	Ligation enhancer 96	1 μ L																			
Total volume			21 μL																			
	5.3	Pipette mix the Ligation master mix and quick spin to collect liquid.																				
	5.4	Add 21 μL of Ligation master mix to each sample. Total reaction volume should be 85 μL .																				
	5.5	Pipette mix each reaction to ensure reagents are thoroughly mixed. Quick spin plate if necessary to collect liquid at the bottom of the well.																				
	5.6	<p>Run the Adapter ligation thermocycler program. Set lid temperature to $\geq 30^{\circ}\text{C}$ if programmable.</p> <table border="1"> <thead> <tr> <th>Step</th> <th>Time</th> <th>Temperature</th> </tr> </thead> <tbody> <tr> <td>1</td> <td>30 min</td> <td>20$^{\circ}$C</td> </tr> <tr> <td>2</td> <td>Hold</td> <td>4$^{\circ}$C</td> </tr> </tbody> </table>	Step	Time	Temperature	1	30 min	20 $^{\circ}$ C	2	Hold	4 $^{\circ}$ C											
Step	Time	Temperature																				
1	30 min	20 $^{\circ}$ C																				
2	Hold	4 $^{\circ}$ C																				
	5.7	Proceed to post-ligation cleanup step.																				

6. Post-ligation cleanup

Please ensure SMRTbell cleanup beads have been brought up to room temperature before proceeding to the cleanup steps.

✓	Step	Instructions for SMRTbell cleanup bead step
	6.1	Add 85 μL (1.0X) of resuspended, room-temperature SMRTbell cleanup beads to each sample.
	6.2	Pipette mix the sample until the beads are evenly distributed.
	6.3	Incubate at room temperature for 10 minutes to allow DNA to bind to the beads.
	6.4	Place samples on an appropriate magnet and allow beads to separate fully from the solution. The solution should be clear and beads pelleted to the magnet.
	6.5	Slowly remove the supernatant without disturbing the beads. Discard the supernatant.
	6.6	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.
	6.7	Repeat the previous step.
		Remove residual 80% ethanol:
	6.8	<ul style="list-style-type: none"> • Remove the sample from the magnet. • Quick-spin to collect liquid at the bottom. • Place sample back on the magnet and allow beads separate fully from the solution. • Remove residual 80% ethanol and discard.
	6.9	Remove samples from the magnet and immediately resuspend the beads with 40 μL of elution buffer .
	6.10	Resuspend by pipetting mixing until beads are evenly distributed in solution.
	6.11	Incubate samples at room temperature for 5 minutes to elute DNA off beads.
	6.12	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 aspirating the supernatant.
	6.13	Slowly aspirate the cleared eluate without disturbing the beads. Transfer eluate to a new tube strip or plate. Discard old tube strip or plate with beads.

SAFE STOPPING POINT - Store at 4°C

7. Nuclease treatment

This step removes unligated DNA fragments and SMRTbell adapter from the sample.

✓	Step	Instructions																				
		Prepare the appropriate volume of master mix with 10% overage using the per reaction volumes listed below. Prepare the master mix immediately before use to ensure optimal activity.																				
		<table border="1"> <thead> <tr> <th colspan="4">Nuclease master mix</th> </tr> <tr> <th>✓</th> <th>Tube</th> <th>Component</th> <th>Volume</th> </tr> </thead> <tbody> <tr> <td></td> <td>Light purple</td> <td>Nuclease buffer 96</td> <td>5 μL</td> </tr> <tr> <td></td> <td>Light green</td> <td>Nuclease mix 96</td> <td>5 μL</td> </tr> <tr> <td colspan="3">Total volume</td> <td>10 μL</td> </tr> </tbody> </table>	Nuclease master mix				✓	Tube	Component	Volume		Light purple	Nuclease buffer 96	5 μ L		Light green	Nuclease mix 96	5 μ L	Total volume			10 μ L
Nuclease master mix																						
✓	Tube	Component	Volume																			
	Light purple	Nuclease buffer 96	5 μ L																			
	Light green	Nuclease mix 96	5 μ L																			
Total volume			10 μ L																			
7.1																						
7.2		Pipette-mix Nuclease master mix and quick spin to collect liquid.																				
7.3		Add 10 μL of Nuclease master mix to each sample. Total volume should equal 50 μL .																				
7.4		Pipette-mix each sample and quick-spin to collect liquid.																				
		Run the Nuclease treatment thermocycler program. Set lid temperature to $\geq 47^{\circ}\text{C}$ if programmable.																				
7.5		<table border="1"> <thead> <tr> <th>Step</th> <th>Time</th> <th>Temperature</th> </tr> </thead> <tbody> <tr> <td>1</td> <td>15 min</td> <td>37°C</td> </tr> <tr> <td>2</td> <td>Hold</td> <td>4°C</td> </tr> </tbody> </table>	Step	Time	Temperature	1	15 min	37°C	2	Hold	4°C											
Step	Time	Temperature																				
1	15 min	37°C																				
2	Hold	4°C																				
7.6		Proceed to the next step of the protocol.																				

8. Diluted AMPure PB cleanup and size selection

The AMPure PB bead cleanup removes potential contaminants and progressively depletes DNA fragments up to 5 kb. Size selection performance is sensitive to bead concentrations. **Ensure accurate pipetting volumes when diluting the beads and adding them to the library.**

✓	Step	Instructions for AMPure PB bead cleanup
8.1		Make a 35% v/v dilution of AMPure PB beads by adding 1.75 mL of resuspended AMPure PB beads to 3.25 mL of elution buffer. The 35% dilution can be stored at 4°C for 30 days. Note: The AMPure PB dilution may be scaled as appropriate for smaller/larger scale projects.
8.2		Add 3.1X v/v (155 μ L) of resuspended, room-temperature 35% AMPure PB beads to each sample from the previous step.
8.3		Pipette-mix the beads until evenly distributed.

- 8.4 Incubate at **room temperature** for **20 minutes** to allow DNA to bind beads.
- 8.5 Place sample on an appropriate magnet and allow beads separate fully from the solution.
- 8.6 Slowly remove the cleared supernatant without disturbing the beads.
- 8.7 Slowly dispense **200 μL** , or enough to cover the beads, of **freshly prepared 80% ethanol** into each sample. After **30 seconds**, remove the 80% ethanol and discard.
- 8.8 Repeat the previous step.
- Remove residual 80% ethanol:
- Remove the sample from the magnet.
 - Quick-spin to collect liquid at the bottom.
 - Place sample back on the magnet and allow beads separate fully from the solution.
 - Remove residual 80% ethanol and discard.
- 8.9
- 8.10 Remove samples from the magnet and **immediately** add **26 μL** of **elution buffer** to each sample.
- 8.11 Pipette-mix the beads until evenly distributed.
- 8.12 Incubate at **room temperature** for **5 minutes** to elute DNA of the beads.
- 8.13 Place samples on the magnet and allow the beads separate fully from the solution.
- 8.14 Slowly remove the cleared eluate without disturbing the beads. Transfer eluate to a new tube.
- Recommended:** 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. Calculate the total mass. The final overall recovery should be 10–25% as measured from gDNA mass to completed SMRTbell library (includes SRE, shearing, and library prep).
- 8.15 **Optional:** Further dilute each aliquot to **250 $\text{pg}/\mu\text{L}$** with Femto Pulse dilution buffer. Measure final SMRTbell library size distribution with a Femto Pulse system.
- DNA concentration must be less than 60 $\text{ng}/\mu\text{L}$ to proceed to ABC;** however, libraries typically are <40 $\text{ng}/\mu\text{L}$ after the SMRTbell library preparation process.
- 8.16 Proceed to Section 9 to prepare library for sequencing on Revio (with or without SPRQ) and Vega.

SAFE STOPPING POINT – Can be stored at 4°C for up to 1 month or at -20°C for at least 6 months

9. Annealing, binding, and cleanup (ABC)

This step is for preparing the libraries (25 μ L) for sequencing on Revio and Vega systems. The Polymerase kit will be specific to the sequencing chemistry/sequencer used.

Instrument	Compatible kits	Kit PN
Revio	Revio SPRQ polymerase kit 96	103-497-000
	Revio SPRQ polymerase kit	103-520-100
Vega	Vega polymerase kit	103-517-600

✓ Step	Instructions																				
	Prepare the appropriate volume of master mix with 10% overage using the per reaction volumes listed below.																				
9.1	<table border="1"> <thead> <tr> <th colspan="4">Annealing mix</th> </tr> <tr> <th>✓ Tube</th> <th>Component</th> <th colspan="2">Volume</th> </tr> </thead> <tbody> <tr> <td>Light blue</td> <td>Annealing buffer 96</td> <td colspan="2">12.5 μL</td> </tr> <tr> <td>Light green</td> <td>Standard sequencing primer 96</td> <td colspan="2">12.5 μL</td> </tr> <tr> <td colspan="3">Total volume</td> <td>25 μL</td> </tr> </tbody> </table>	Annealing mix				✓ Tube	Component	Volume		Light blue	Annealing buffer 96	12.5 μ L		Light green	Standard sequencing primer 96	12.5 μ L		Total volume			25 μ L
Annealing mix																					
✓ Tube	Component	Volume																			
Light blue	Annealing buffer 96	12.5 μ L																			
Light green	Standard sequencing primer 96	12.5 μ L																			
Total volume			25 μ L																		
9.2	Pipette-mix the Annealing mix and quick spin to collect liquid.																				
9.3	Add 25 μL of the Annealing mix to each library. Total volume should equal 50 μL .																				
9.4	Pipette-mix each sample and quick spin to collect liquid.																				
9.5	Incubate at room temperature for 15 minutes .																				
9.6	During primer incubation, prepare the polymerase dilution (see below) and store on ice.																				
9.7	<p>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.</p> <table border="1"> <thead> <tr> <th colspan="4">Polymerase dilution</th> </tr> <tr> <th>✓ Tube</th> <th>Component</th> <th colspan="2">Volume</th> </tr> </thead> <tbody> <tr> <td>Yellow</td> <td>Polymerase buffer 96</td> <td colspan="2">47 μL</td> </tr> <tr> <td>Purple</td> <td>Sequencing polymerase 96</td> <td colspan="2">3 μL</td> </tr> <tr> <td colspan="3">Total volume</td> <td>50 μL</td> </tr> </tbody> </table>	Polymerase dilution				✓ Tube	Component	Volume		Yellow	Polymerase buffer 96	47 μ L		Purple	Sequencing polymerase 96	3 μ L		Total volume			50 μ L
Polymerase dilution																					
✓ Tube	Component	Volume																			
Yellow	Polymerase buffer 96	47 μ L																			
Purple	Sequencing polymerase 96	3 μ L																			
Total volume			50 μ L																		
9.8	Pipette mix the Polymerase dilution and quick-spin to collect liquid.																				
9.9	Add 50 μL of Polymerase dilution to the primer annealed sample for a total volume of 100 μL .																				
9.10	Pipette-mix each sample and quick-spin to collect liquid.																				

9.11 Incubate at **room temperature for 15 minutes**.

9.12 Proceed immediately to the next step of the protocol to remove excess polymerase.

Post-binding cleanup with 1X SMRTbell cleanup beads

9.13 Add **100 μ L** of resuspended, room-temperature SMRTbell cleanup beads to each sample

9.14 Pipette-mix the beads until evenly distributed and quick-spin if necessary to collect all liquid from the sides of the tube.

9.15 Incubate at **room temperature for 10 minutes** to allow DNA to bind beads

9.16 Place sample on an appropriate magnet and allow beads to separate fully from the solution

9.17 Slowly remove the cleared supernatant without disturbing the beads. Discard the supernatant. **DO NOT USE EtOH**. Proceed immediately to the elution. It is important not to let the beads dry out.

Remove sample from the magnet and **immediately** add **Loading Buffer 96** to each tube and resuspend the beads by pipette mixing.

9.18

	Revio SPRQ polymerase kit 96	Revio polymerase kit 96/Vega polymerase kit (non-SPRQ)
Loading buffer 96	25 μ L	50 μ L

9.19 Quick-spin the samples to collect any liquid from the sides of the tube.

9.20 Incubate at **room temperature for 15 minutes** to elute DNA

9.21 Place sample on magnet and allow beads to separate fully from the solution.

9.22 Slowly aspirate the cleared eluate without disturbing the beads. **Transfer eluate to a new tube.** Discard the old tube with beads

Use **1 μ L** of sample to measure DNA concentration with a Qubit fluorometer using the 1x dsDNA HS kit.

9.23

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

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

PROTOCOL COMPLETE

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

Troubleshooting FAQs

- 1. The “Low mass” workflow specifies an input mass of 0.5 – 1.25 µg and the “High mass” workflow specifies an input mass of 2 – 4 µg gDNA. What should I use if I have 1.25 – 2 µg of gDNA?**
 - If you have 1.25 – 2 µg of gDNA input mass for SRE, you can use the “High mass” workflow. This corresponds to a concentration range of 25 ng/µL – 40 ng/µL in a 50 µL volume. Please note that if sequencing on Vega or Revio non-SPRQ, you may not have enough final library for loading at an optimal on-plate loading concentration for a single SMRT Cell.
- 2. How should gDNA be quanted to ensure that the concentration is accurate?**
 - Use a quantification method specific to dsDNA, such as Qubit. High molecular weight (HMW) DNA will be inhomogenous and will therefore give inaccurate results depending on where the sample was pulled from in the tube. Vigorously vortexing gDNA prior to quantifying will improve quant accuracy. The vortexing will not adversely affect the DNA.
- 3. During the SRE step, what is the result of leaving a high volume of residual supernatant prior to elution?**
 - During the centrifugation step, the larger DNA is pelleted while the shorter DNA is left in the supernatant. If too much residual supernatant is left during the removal step, there will be excess short DNA going forward into library prep. It is recommended to leave <5 (Low mass) or <10 µL (High mass) of supernatant behind to ensure that the pellet containing the HMW DNA is not aspirated during supernatant removal.
- 4. What are possible error modes if low SRE recovery is observed?**
 - If Buffer SRE is not appropriately mixed with the sample prior to incubation and centrifugation, recovery will be low. The DNA will remain in the supernatant and not efficiently pellet. It is critical that the Buffer SRE and the sample are well mixed by vortexing. In some cases, pipette mixing prior to vortexing improves performance.
 - Try to mix your final eluted sample again. If the final pellet is not properly resuspended, your quant reading will be low. Try pipette mixing and vortexing followed by a re-quant.
 - You may have aspirated the DNA pellet during supernatant removal. If you still have the supernatant, you can re-vortex/mix, incubate at 55°C for 1 hour, and re-centrifuge to re-pellet the sample and follow the procedure to remove the supernatant and re-suspend the pellet.
 - Was the sample immediately retrieved after centrifugation? With time, the pellet may detach or diffuse and will therefore be aspirated during supernatant removal.
- 5. What happens if the library is not diluted to the recommended concentration going into ABC?**
 - The primer and polymerase concentration remains constant, regardless of sample input concentration. The recommended concentrations keep the SMRTbell libraries below a certain molarity so that there is an appropriate ratio of primer and polymerase to library. If a concentration above the recommended range is used, there will be a higher proportion of library without a primer/polymerase, which will result in a lower P1 value.

6. What should I do if I made a mistake during ABC and my whole library was prepped?

- Run a 1x SMRTbell bead cleanup (50 μ L SMRTbell cleanup beads in 50 μ L of bound library, for example) according to standard procedures (with EtOH) and elute in 25 μ L of EB. Repeat ABC according to standard procedures.

Revision history (description)	Version	Date
Initial release	01	March 2024
Updated to correct reference to the Hamilton NGS STAR MOA system and include stepwise recovery table	02	April 2024
Updated for SPRQ chemistry and the Vega system	03	December 2024
Corrected part number for Revio SPRQ polymerase kit 96 and deleted duplicated table from step 2.2	04	April 2025
Clarify kit options for use of HiFi prep kit 96 with SPRQ chemistry	05	June 2025

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