

Preparing whole genome and metagenome libraries using SMRTbell® prep kit 3.0

Procedure & checklist

Overview

This procedure describes the workflow for constructing whole genome sequencing (WGS) libraries from genomic and metagenomic DNA using the SMRTbell® prep kit 3.0 for sequencing on PacBio® long-read systems. This procedure may be performed manually or using one of the many qualified automation methods for SMRTbell prep kit 3.0.

Overview

Libraries per SMRTbell prep kit 3.0 1–24

QC and workflow time for 8 samples

- Genomic DNA QC on Femto Pulse 1.5 hours
- SRE to improve gDNA quality 2.5 hours (tube format)
- Library prep with SMRTbell prep kit 3.0 3.5 hours
- SMRTbell library QC on Femto Pulse 1.5 hours

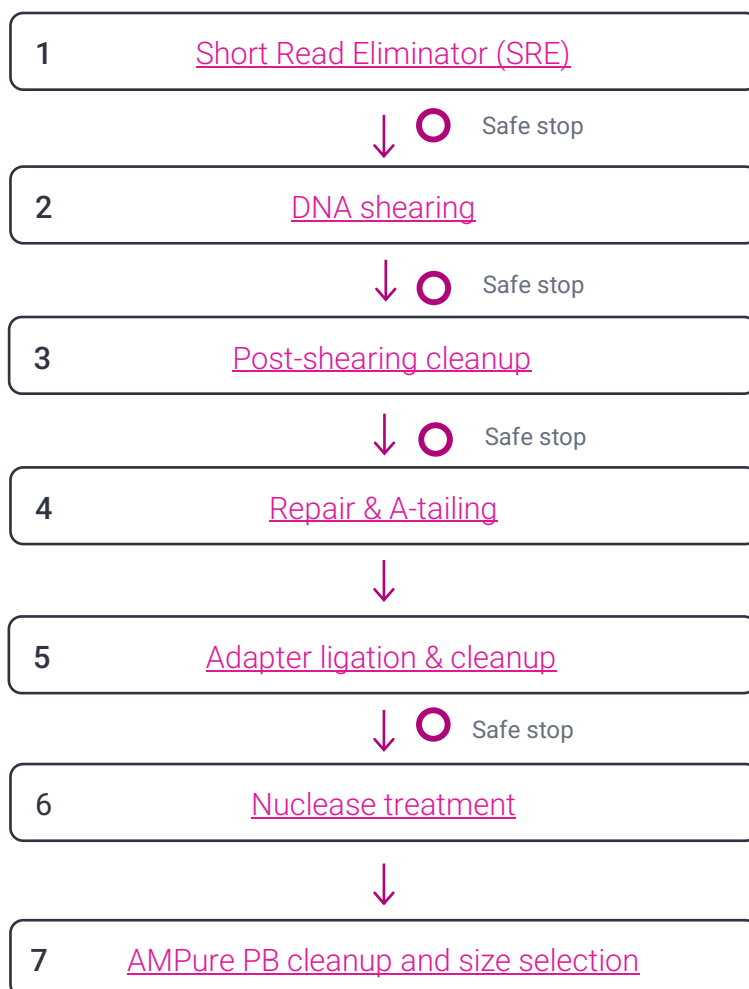
Times may vary by user and available lab equipment

| DNA input into library prep | Sequel II® and Sequel IIe | Revio™ |
|-----------------------------|---------------------------|----------------------------|
| Total DNA per SMRT® Cell | 1 µg per SMRT® Cell 8M | 2 µg per Revio™ SMRT® Cell |
| Multiplex libraries | 300 ng – 1 µg per sample | 300 ng – 2 µg per sample |

| DNA quality recommendation | Femto Pulse genome quality score | |
|--|---|--|
| DNA size distribution (Femto Pulse system) | 70% ≥10 kb (GQN _{10kb} ≥7.0) & 50% ≥30 kb (GQN _{30kb} ≥5.0) | Lower quality DNA may be used with the expectation of lower sequencing yields. |

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

Workflow overview



Required materials and equipment

PCR tube strips are recommended for the enzymatic and bead cleanup steps, but Eppendorf Lo-bind tubes or 0.2 mL 96-well PCR plates are also acceptable. No difference in performance is expected across PCR tube strips, Lo-bind tubes, or plates.

| DNA sizing | |
|--|---|
| Femto Pulse system | Agilent Technologies, Inc. M5330AA |
| Femto Pulse gDNA 165kb analysis kit | Agilent Technologies, Inc. FP-1002-0275 |
| DNA quantitation | |
| Qubit fluorometer | ThermoFisher Scientific Q33238 |
| Qubit 1X dsDNA HS assay kit | ThermoFisher Scientific Q33230 |
| DNA shearing | |
| Hamilton Microlab Prep | PacBio® 103-283-600 |
| Hamilton assay ready workstation | Contact Hamilton |
| 300 µL CO-RE II Tips (Filtered, Conductive) | Hamilton, 235903 |
| SMRTbell® library preparation | |
| SMRTbell® prep kit 3.0, includes: <ul style="list-style-type: none"> SMRTbell® prep kit 3.0 SMRTbell® cleanup beads Low TE buffer | PacBio® 102-182-700 |
| SMRTbell® adapter index plate 96A (optional) | PacBio® 102-009-200 |
| 200 Proof ethanol, molecular biology or ACS grade | Any major lab supplier (MLS) |
| Nuclease-free water, molecular biology grade | Any MLS |
| 8-channel pipettes | Any MLS |
| 0.2 mL 8-tube strips | USA Scientific TempAssure 1402-4708 |
| Microcentrifuge | Any MLS |
| Magnetic separation rack compatible with 0.2 mL 8-tube strips | V&P Scientific VP 772F4-1 |
| Thermocycler compatible with 0.2 mL 8-tube strips | Any MLS |
| 1.5 mL DNA LoBind tubes | Eppendorf 022431021 |
| Size selection | |
| SRE | PacBio® 102-208-300 |
| AMPure® PB bead size selection kit | PacBio® 102-182-500 |

Before you begin

The following are general best practices for whole genome sequencing (WGS) using the Sequel[®] II/IIe or Revio systems. Please read carefully prior to beginning library prep.

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 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 at minimum. This corresponds to a genome quality number (GQN) of 7.0 or higher at 10 kb.

Important:

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

Please see the [Revio spec sheet](#) for more information on yield expectations by insert size.

gDNA input amount

It is highly recommended to use a quantification assay specific for double stranded DNA (dsDNA) such as the Qubit 1X dsDNA high sensitivity assay kit. Please follow manufacturer's instructions for the assay being used.

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

Table 1. Recommended DNA input amounts by starting gDNA quality

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

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

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

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

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

If targeting higher insert sizes (> 20 kb) or working with lower quality DNA (Table 1), start with at least 3 µg of gDNA to ensure enough library is available for optimal Revio SMRT Cell loading at 200 to 300 pM.

SMRTbell prep kit 3.0 protocol stepwise recoveries

Table 3. Expected stepwise recoveries of DNA and SMRTbell library from the SMRTbell prep kit 3.0 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 yield |
|-------------------------------------|-----------------------|
| Starting Input | 100% |
| Post-SRE | 65-95% |
| Post-shear SMRTbell bead cleanup | 80-95% |
| Post-ligation SMRTbell bead cleanup | 80-95% |
| Post-nuclease (pre-cleanup) | 40-50% |
| Post-3.1x AMPure PB bead cleanup | 70-80% |
| Overall Recovery | 12-34% |

Metagenomic samples

If preparing metagenomic samples for shotgun sequencing, the following exceptions to the protocol apply.

- Do not perform SRE
- Average insert sizes may be lower than 15 kb.
- Skip shearing if sample QC shows that the average fragment size is < 13 kb.

Because SRE will be skipped, lower DNA input (less than 2 µg) may be used. Please note that a 15 to 30% recovery is expected. Make sure enough DNA is used to meet optimal loading amounts on Sequel II or Revio systems.

Multiplexing

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 libraries together please consider the following guidelines:

- Each Revio SMRT Cell is expected to yield ≥ 90 Gb of HiFi data, on average, when using a mean insert size of >15 kb.
- Only pool samples with similar genome sizes to ensure balanced coverage.

- Ensure that the samples to be pooled have a similar mean insert size and insert length size distribution.
- Pool samples in an equal molar concentration for best balanced coverage.

It is recommended to pool HiFi libraries post-ABC (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 unpooled 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 and sample handling

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

SRE

SRE buffer and buffer LTE are stored at room temperature.

SMRTbell prep kit 3.0

Thaw the repair buffer, nuclease buffer, SMRTbell adapter and elution buffer at room temperature.

Mix reagent buffers with a brief vortex prior to use. Do not vortex enzymes.

Quick-spin all reagents in microcentrifuge to collect liquid at bottom prior to use.

Keep all temperature-sensitive reagents on ice.

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

Pipette-mix all bead binding and elution steps until beads are distributed evenly in solution. It is recommended to pipette-mix all SMRTbell prep reactions by pipetting up and down 10 times.

Samples can be stored at 4°C at all safe stopping points listed in the protocol.

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. Please follow manufacturer instructions as appropriate for the QC instruments being used. See [recommendations](#) above.

1. Short read eliminator

Short read eliminator (SRE) will progressively deplete fragments up to 25 kb from genomic DNA samples. This means that depletion decreases as the fragment size approaches 25kb. The depletion occurs in an unbiased manner and results in improved sample quality for HiFi sequencing with most fragments under 10 kb removed.

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

If automating this step, please refer to the [Microlab Prep Guide & overview](#) for details on consumables.

If performing SRE manually, please see the [SRE protocol](#) for additional details.

This step is optional. If not performing SRE, then please proceed to the DNA shearing step.

| ✓ | Step | Instructions |
|---|------|---|
| | 1.1 | Bring the DNA samples to a concentration between 40 to 100 ng/μL in a total volume of 50 μL using Buffer LTE in a 1.5 ml Lobind tube or hard-shell plate. The DNA input requirement for shearing is <3 μg so gDNA input into SRE will depend on expected recovery. |
| | 1.2 | Add 50 μL of Buffer SRE to each sample. If working in a plate format, heat seal with foil. Vortex/shake to mix for 5 seconds at max speed. |
| | 1.3 | 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. |
| | 1.4 | Load plate or tube (with the hinge facing toward the outside of the rotor) into the centrifuge. |
| | 1.5 | Centrifuge a tube at 10,000 rcf for 30 minutes Centrifuge a plate at >2250 rcf (max 3220 rcf) for 1 hour <ul style="list-style-type: none"> • 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). |
| | 1.6 | Carefully remove supernatant without disturbing the pellet. <ul style="list-style-type: none"> • Leaving up to 10 μL is acceptable to be sure the pellet is not disturbed. |
| | 1.7 | Add 300 μL of Buffer LTE to the tube and incubate at room temperature for 10 minutes. |
| | 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. DNA shearing for WGS using automation

Please see [Appendix](#) for instructions on shearing with the Megaruptor 3 system.

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.

Important: A mean fragment size between 15 to 20 kb is recommended for this protocol. In addition, the distribution of fragment sizes should be narrow and generally between 10 to 30 kb. Fragments that are too short produce less yield per read, and fragments that are too long may result in lower read accuracy and are less likely to produce HiFi reads. **Deviating from the concentration and automation settings is not recommended and will result in undersheared DNA.**

These shearing parameters are not universal and are specific for only the Hamilton Microlab Prep, or assay ready workstations like the NGS STAR, STARlet, and STAR V systems

| ✓ | Step | Instructions | | | | | | | | | | | | | | |
|----------------------|--|--|-----------|---------|-------------------|-----------------------|----------------------|-------------|-----------------|------------|----------------------|-----------------|------------------|------------|-------------|--|
| | 2.1 | Adjust DNA concentration to ≤ 10 ng/ μ L, if necessary (e.g., if more than 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. Bring all samples up to 300 μ L 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>Setting</th> </tr> </thead> <tbody> <tr> <td>DNA concentration</td> <td>≤ 10 ng/μL</td> </tr> <tr> <td>Volume of Buffer LTE</td> <td>300 μL</td> </tr> <tr> <td>Number of mixes</td> <td>300 cycles</td> </tr> <tr> <td>Pipette mixing speed</td> <td>500 μL/sec</td> </tr> <tr> <td>Liquid following</td> <td>83% volume</td> </tr> <tr> <td>Pipette tip</td> <td>300 μL CO-RE II tips (filtered, black, non-sterile)</td> </tr> </tbody> </table> | Parameter | Setting | DNA concentration | ≤ 10 ng/ μ L | Volume of Buffer LTE | 300 μ L | Number of mixes | 300 cycles | Pipette mixing speed | 500 μ L/sec | Liquid following | 83% volume | Pipette tip | 300 μ L CO-RE II tips (filtered, black, non-sterile) |
| Parameter | Setting | | | | | | | | | | | | | | | |
| DNA concentration | ≤ 10 ng/ μ L | | | | | | | | | | | | | | | |
| Volume of Buffer LTE | 300 μ L | | | | | | | | | | | | | | | |
| Number of mixes | 300 cycles | | | | | | | | | | | | | | | |
| Pipette mixing speed | 500 μ L/sec | | | | | | | | | | | | | | | |
| Liquid following | 83% volume | | | | | | | | | | | | | | | |
| Pipette tip | 300 μ L CO-RE II tips (filtered, black, non-sterile) | | | | | | | | | | | | | | | |
| | 2.3 | Place the plate on the appropriate work deck position and start the shearing procedure. | | | | | | | | | | | | | | |
| | | Optional: measure DNA concentration with a Qubit fluorometer using the 1x dsDNA HS kit once the shearing procedure is complete. | | | | | | | | | | | | | | |
| | 2.4 | Recommended: Further dilute each aliquot to 250 pg/μL with the Femto Pulse dilution buffer. Measure the final SMRTbell library size distribution with a Femto Pulse system to ensure efficient shearing. | | | | | | | | | | | | | | |

- 2.5 Proceed to the 1X SMRTbell cleanup bead procedure to concentration samples for library preparation.

3. Post-shearing cleanup

This step concentrates the sheared DNA for the Repair and A-tailing step. Before beginning, ensure the SMRTbell cleanup beads are at room temperature.

| ✓ | Step | Instructions post-shear cleanup |
|------|------|--|
| 3.1 | | Add 1.0X v/v (volume over volume) of resuspended, room-temperature SMRTbell cleanup beads to each tube of sheared DNA. <ul style="list-style-type: none"> Automated pipette shearing = 300 μL Megaruptor 3 shearing = 100–130 μL |
| 3.2 | | Pipette-mix the beads until evenly distributed. |
| 3.3 | | Quick-spin the tube strip in a microcentrifuge to collect liquid. |
| 3.4 | | Leave at room temperature for 10 minutes to allow DNA to bind beads. |
| 3.5 | | Place the tube strip in a magnetic separation rack until beads separate fully from the solution. |
| 3.6 | | Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant. |
| 3.7 | | Slowly dispense 200 μL , or enough to cover the beads, of freshly prepared 80% ethanol into each tube. After 30 seconds , pipette off the 80% ethanol and discard. |
| 3.8 | | Repeat the previous step. |
| 3.9 | | Remove residual 80% ethanol: <ul style="list-style-type: none"> Remove the tube strip from the magnetic separation rack. Quick-spin the tube strip in a microcentrifuge. Place the tube strip back in a magnetic separation rack until beads separate fully from the solution. Pipette off residual 80% ethanol and discard. |
| 3.10 | | Remove the tube strip from the magnetic rack. Immediately add 47 μL of low TE buffer to each tube and resuspend the beads by pipetting 10 times or until evenly distributed. |
| 3.11 | | Quick-spin the tube strip in a microcentrifuge to collect liquid. |
| 3.12 | | Leave at room temperature for 5 minutes to elute DNA. |
| 3.13 | | Place the tube strip in a magnetic separation rack until beads separate fully from the solution. |
| 3.14 | | Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a new tube strip . Discard old tube strip with beads. |

Recommended: Evaluate sample quality (concentration and size distribution).

- Take a **1 μL** aliquot from each tube and dilute with **9 μL** of **elution buffer or water**.
- 3.15**
- Measure DNA concentration with a Qubit fluorometer using the 1x dsDNA HS kit.
 - Dilute each aliquot to **250 $\text{pg}/\mu\text{L}$** in Femto Pulse dilution buffer.
 - Measure DNA size distribution with a Femto Pulse system.
- 3.16** Proceed to the next step of the protocol if sample quality is acceptable.

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 DNA damage and end repair

Add the following components in the order and volume listed below to a new microcentrifuge tube. Adjust component volumes for the number of libraries being prepared, plus 15% overage. For individual preps, add components directly to the sample from the previous step at the specified volumes and skip steps (4.2 to 4.4).

| Repair mix | | | | | |
|------------|--------|---------------------|------------------------------------|--------------------------------------|---------------------------------------|
| 4.1 | ✓ Tube | Component | Volume | | |
| | | | Per library | 4 libraries | 8 libraries |
| | Purple | Repair buffer | 8 μL | 36.8 μL | 73.6 μL |
| | Blue | End repair mix | 4 μL | 18.4 μL | 36.8 μL |
| | Green | DNA repair mix | 2 μL | 9.2 μL | 18.4 μL |
| | | Total volume | 14 μL | 64.4 μL | 128.8 μL |

4.2 Pipette-mix the **Repair mix**.

4.3 Quick-spin the **Repair mix** in a microcentrifuge to collect liquid.

4.4 Add **14 μL** of the **Repair mix** to each sample. Total reaction volume should be **60 μL** .

4.5 Pipette-mix each sample.

4.6 Quick-spin the tube strip in a microcentrifuge to collect liquid.

Run the **repair and A-tailing** thermocycler program. Set the lid temperature to 75°C if programmable.

4.7

| Step | Time | Temperature |
|------|--------|-------------|
| 1 | 30 min | 37°C |
| 2 | 5 min | 65°C |
| 3 | Hold | 4°C |

4.8 Proceed to the next step of the protocol.

5. Adapter ligation and cleanup

This step ligates the SMRTbell adapter to the ends of each DNA fragment. Please ensure SMRTbell cleanup beads have been brought up to room temperature before proceeding to the cleanup steps.

✓ Step Instructions for SMRTbell adapter ligation and reaction cleanup

SMRTbell adapter ligation

5.1

(Optional) If using an adapter index: add **4 µL** of barcoded adapters from the **SMRTbell adapter index plate 96A** to each respective sample from the previous step and exclude the SMRTbell adapter from the ligation mix.

Skip this step if not using an adapter index.

Add the following components in the order and volume listed below to a new microcentrifuge tube. Adjust component volumes for the number of libraries being prepared, plus 10% overage. For individual preps, add components directly to each sample from the previous step in the order and volume listed below, then skip steps (5.3 to 5.5).

5.2

| Ligation mix | | | | | |
|---------------------|--------|-------------------|--------------|---------------|---------------|
| ✓ | Tube | Component | Volume | | |
| | | | Per library | 4 libraries | 8 libraries |
| | Orange | SMRTbell adapter* | 4 µL | 17.6 µL | 35.2 µL |
| | Yellow | Ligation mix | 30 µL | 132 µL | 264 µL |
| | Red | Ligation enhancer | 1 µL | 4.4 µL | 8.8 µL |
| Total volume | | | 35 µL | 154 µL | 308 µL |

*Exclude the SMRTbell adapter if using the SMRTbell adapter index plate 96A

5.3 Pipette-mix the **Ligation mix**.5.4 Quick-spin the **Ligation mix** in a microcentrifuge to collect liquid.

| ✓ | Step | Instructions for SMRTbell adapter ligation and reaction cleanup | | | | | | | | | |
|------|--------|--|------|------|-------------|---|--------|------|---|------|-----|
| | | Indexed samples: add 31 µL of the Ligation mix to each sample from the previous step. | | | | | | | | | |
| 5.5 | | Non-indexed samples: add 35 µL of the Ligation mix containing the SMRTbell adapter to each sample from the previous step. The total volume per sample should be 95 µL . | | | | | | | | | |
| 5.6 | | Pipette-mix each sample. | | | | | | | | | |
| 5.7 | | Quick-spin the tube strip in a microcentrifuge to collect liquid. | | | | | | | | | |
| 5.8 | | Run the adapter ligation thermocycler program. Set the lid temperature to 75°C if programmable. | | | | | | | | | |
| | | <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°C</td> </tr> <tr> <td>2</td> <td>Hold</td> <td>4°C</td> </tr> </tbody> </table> | Step | Time | Temperature | 1 | 30 min | 20°C | 2 | Hold | 4°C |
| Step | Time | Temperature | | | | | | | | | |
| 1 | 30 min | 20°C | | | | | | | | | |
| 2 | Hold | 4°C | | | | | | | | | |
| | | Cleanup with 1X SMRTbell cleanup beads | | | | | | | | | |
| 5.9 | | Add 95 µL of resuspended, room-temperature SMRTbell cleanup beads to each sample. | | | | | | | | | |
| 5.10 | | Pipette-mix the beads until evenly distributed. | | | | | | | | | |
| 5.11 | | Quick-spin the tube strip in a microcentrifuge to collect all liquid from the sides of the tubes. | | | | | | | | | |
| 5.12 | | Leave at room temperature for 10 minutes to allow DNA to bind beads. | | | | | | | | | |
| 5.13 | | Place the tube strip in a magnetic separation rack until beads separate fully from the solution. | | | | | | | | | |
| 5.14 | | Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant. | | | | | | | | | |
| 5.15 | | Slowly dispense 200 µL , or enough to cover the beads, of freshly prepared 80% ethanol into each tube. After 30 seconds , pipette off the 80% ethanol and discard. | | | | | | | | | |
| 5.16 | | Repeat the previous step. | | | | | | | | | |
| 5.17 | | Remove residual 80% ethanol: <ul style="list-style-type: none"> • Remove the tube strip from the magnetic separation rack. • Quick-spin the tube strip in a microcentrifuge. • Place the tube strip back in a magnetic separation rack until beads separate fully from the solution. • Pipette off residual 80% ethanol and discard. | | | | | | | | | |
| 5.18 | | Remove the tube strip from the magnetic rack. Immediately add 40 µL of elution buffer to each tube and resuspend the beads. | | | | | | | | | |
| 5.19 | | Quick-spin the tube strip in a microcentrifuge. | | | | | | | | | |
| 5.20 | | Leave at room temperature for 5 minutes to elute DNA. | | | | | | | | | |
| 5.21 | | Place the tube strip in a magnetic separation rack until beads separate fully from the solution. | | | | | | | | | |

| ✓ Step | Instructions for SMRTbell adapter ligation and reaction cleanup |
|--------|---|
| 5.22 | Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a new tube strip . Discard old tube strip with beads. |
| 5.23 | Proceed to the next step of the protocol. |

SAFE STOPPING POINT - Store at 4°C

6. Nuclease treatment

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

| ✓ Step | Instructions for nuclease treatment | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|---------------------|---|--------------|--------------|--------------|--|--------------|------|-------------|---|--------|------|--------|-----------|-------------|-------------|-------------|--|--------------|-----------------|------|-------|-------|--|-------------|--------------|------|-------|-------|--|---------------------|--|--------------|--------------|--------------|--|
| | Add the following components in the order and volume listed below to a new microcentrifuge tube. Adjust component volumes for the number of libraries being prepared, plus 10% overage. For individual preps, add components directly to each sample from the previous step in the order and volume listed below, then skip steps (6.2 to 6.4). | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 6.1 | <table border="1"> <thead> <tr> <th colspan="6">Nuclease mix</th> </tr> <tr> <th>✓ Tube</th> <th>Component</th> <th>Per library</th> <th>4 libraries</th> <th>8 libraries</th> <th></th> </tr> </thead> <tbody> <tr> <td>Light purple</td> <td>Nuclease buffer</td> <td>5 µL</td> <td>22 µL</td> <td>44 µL</td> <td></td> </tr> <tr> <td>Light green</td> <td>Nuclease mix</td> <td>5 µL</td> <td>22 µL</td> <td>44 µL</td> <td></td> </tr> <tr> <td colspan="2">Total volume</td> <td>10 µL</td> <td>44 µL</td> <td>88 µL</td> <td></td> </tr> </tbody> </table> | | | | | Nuclease mix | | | | | | ✓ Tube | Component | Per library | 4 libraries | 8 libraries | | Light purple | Nuclease buffer | 5 µL | 22 µL | 44 µL | | Light green | Nuclease mix | 5 µL | 22 µL | 44 µL | | Total volume | | 10 µL | 44 µL | 88 µL | |
| Nuclease mix | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| ✓ Tube | Component | Per library | 4 libraries | 8 libraries | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Light purple | Nuclease buffer | 5 µL | 22 µL | 44 µL | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Light green | Nuclease mix | 5 µL | 22 µL | 44 µL | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Total volume | | 10 µL | 44 µL | 88 µL | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 6.2 | Pipette-mix Nuclease mix . | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 6.3 | Quick-spin the Nuclease mix in a microcentrifuge to collect liquid. | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 6.4 | Add 10 µL of Nuclease mix to each sample. Total volume should equal 50 µL . | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 6.5 | Pipette-mix each sample. | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 6.6 | Quick-spin the tube strip in a microcentrifuge to collect liquid. | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 6.7 | Run the nuclease treatment thermocycler program. Set the lid temperature to 75°C if programmable. | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | <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 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |

Proceed to the next step of the protocol.

- 6.8 **Note:** It is necessary to remove the nucleases using either AMPure PB size selection or SMRTbell cleanup beads prior to safely storing the library or libraries.

7. Diluted AMPure PB bead cleanup and size selection

The AMPure PB bead size selection step will clean the library and deplete DNA fragments shorter than 5 kb. Please see the [Technical note – HiFi WGS Performance with AMPure PB bead size selection](#) for more information on performance of this method.

If performing gel-based size selection, skip the AMPure PB cleanup and perform a cleanup using the 1X SMRTbell cleanup beads described in the [Appendix](#).

Size selection performance is sensitive to bead concentrations; therefore, ensure accurate pipetting volumes when diluting the beads and adding them to the library.

| ✓ | Step | Instructions for AMPure PB bead size selection |
|------|------|--|
| 7.1 | | 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. |
| 7.2 | | Add 3.1X v/v (155 µL) of resuspended, room-temperature 35% AMPure PB beads to each sample from the previous step. |
| 7.3 | | Pipette-mix the beads until evenly distributed. |
| 7.4 | | Leave at room temperature for 20 minutes to allow DNA to bind beads. |
| 7.5 | | Place sample on an appropriate magnet and allow beads separate fully from the solution. |
| 7.6 | | Slowly pipette off the cleared supernatant without disturbing the beads. |
| 7.7 | | Slowly dispense 200 µL , or enough to cover the beads, of freshly prepared 80% ethanol into each sample. After 30 seconds , pipette off the 80% ethanol and discard. |
| 7.8 | | Repeat the previous step. |
| 7.9 | | Remove residual 80% ethanol: <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. Pipette off residual 80% ethanol and discard. |
| 7.10 | | Remove samples from the magnet and immediately add 15 µL of elution buffer to each sample. |
| 7.11 | | Pipette-mix the beads until evenly distributed. |
| 7.12 | | Leave at room temperature for 5 minutes to elute DNA of the beads. |

7.13 Place samples on the magnet and allow the beads to separate fully from the solution.

7.14 Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a new tube.

7.15 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 input to completed SMRTbell library (includes SRE, shearing, and library prep). DNA concentration must be less than 60 ng/ μl to proceed to ABC.

Recommended: Further dilute each aliquot to **250 pg/ μL** with Femto Pulse dilution buffer. Measure final SMRTbell library size distribution with a Femto Pulse system.

Proceed to SMRT Link Sample Setup for preparing samples for sequencing. Alternatively, libraries can be stored at 4°C if sequencing within 1 month. Store long-term at -20°C.

7.16

SMRTbell libraries are expected to degrade over time at the same rate as any appropriate buffered pure DNA sample. Minimize freeze/thaw cycles and do not expose to direct sunlight or UV radiation.

PROTOCOL COMPLETE

Appendix

A1. DNA shearing with the Megaruptor 3 system

| DNA shearing | |
|---------------------------|---------------------|
| Megaruptor 3 system | Diagenode B06010003 |
| Megaruptor 3 shearing kit | Diagenode E07010003 |

This step describes the procedure for shearing DNA using the Megaruptor 3 system.

| ✓ | Step | Instructions DNA shearing and cleanup | | | | | | | | | |
|-------------------------|-------------|--|--------|-------------|----------------------|-------------------------|----|----------|---------|----|---------|
| | | DNA shearing | | | | | | | | | |
| A1.1 | | Bring DNA up to a final volume of 100–130 µL with low TE buffer . Target a concentration of 30 ng/µL (range: 3–39 ng/µL). | | | | | | | | | |
| A1.2 | | Shear DNA on the Megaruptor 3 system. Recommended settings are below. | | | | | | | | | |
| | | <table border="1"> <thead> <tr> <th>Genome</th> <th>Shear speed</th> <th>Target insert length</th> </tr> </thead> <tbody> <tr> <td>Human, plant, or animal</td> <td>31</td> <td>15–18 kb</td> </tr> <tr> <td>Microbe</td> <td>40</td> <td>7–10 kb</td> </tr> </tbody> </table> | Genome | Shear speed | Target insert length | Human, plant, or animal | 31 | 15–18 kb | Microbe | 40 | 7–10 kb |
| Genome | Shear speed | Target insert length | | | | | | | | | |
| Human, plant, or animal | 31 | 15–18 kb | | | | | | | | | |
| Microbe | 40 | 7–10 kb | | | | | | | | | |
| A1.3 | | Transfer sheared DNA into a tube strip or other appropriate tube for the 1x SMRTbell cleanup bead step. Typical volume loss during shearing is between 5–10 µL. | | | | | | | | | |
| A1.4 | | Proceed to the post-shearing cleanup step (section 3) using 1X SMRTbell cleanup beads. | | | | | | | | | |

A2. 1X cleanup for performing gel-based size selection.

This step describes the cleanup prior to performing gel-based size selection. Please see the [Technical Note - Alternative size selection methods for SMRTbell prep kit 3.0](#) for procedural details.

| ✓ | Step | Instructions for bead binding, washing, and sample elution |
|------|------|--|
| A2.1 | | Add 50 µL of SMRTbell cleanup beads to each nuclease treated library. |
| A2.2 | | Pipette-mix the beads until evenly distributed. |
| A2.3 | | Quick-spin the tube strip in a microcentrifuge to collect all liquid. |
| A2.4 | | Leave at room temperature for 10 minutes to allow DNA to bind beads. |

- A2.5** Place tube strip in a magnetic separation rack until beads separate fully from the solution.
- A2.6** Slowly pipette off the cleared supernatant without disturbing the beads. It is recommended to save the supernatant in another tube strip in case of poor DNA recovery.
- A2.7** Slowly dispense **200 μL** , or enough to cover the beads, of **freshly prepared 80% ethanol** into each tube. After **30 seconds**, pipette off the 80% ethanol and discard.
- A2.8** Repeat the previous step.
- Remove residual 80% ethanol:
- Remove tube strip from the magnetic separation rack.
 - Quick-spin tube strip in a microcentrifuge.
 - Place tube strip back in a magnetic separation rack until beads separate fully from the solution.
 - Pipette off residual 80% ethanol and discard.
- A2.9**
- A2.10** Remove tube strip from the magnetic rack. **Immediately** add **21 μL** of **elution buffer** to each tube and resuspend the beads by pipetting 10 times or until evenly distributed.
- A2.11** Quick-spin the tube strip in a microcentrifuge to collect liquid.
- A2.12** Leave at **room temperature** for **5 minutes** to elute DNA.
- A2.13** Place tube strip in a magnetic separation rack until beads separate fully from the solution.
- A2.14** Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a **new tube strip**. Discard old tube strip with beads.
- A2.15** 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.
- Recommended:** 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.
- Store libraries at 4°C if sequencing within 1 month. Store long-term at -20°C.
- A2.16** SMRTbell libraries are expected to degrade over time at the same rate as any appropriate buffered pure DNA sample. Minimize freeze/thaw cycles and do not expose to direct sunlight or UV radiation.

| Revision history (description) | Version | Date |
|---|---------|---------------|
| Initial release | 01 | April 2022 |
| Updated to include new information for the Revio system | 02 | February 2023 |
| Updated with new recommendations for WGS library prep | 03 | March 2024 |
| Updated with stepwise recovery table | 04 | April 2024 |

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