# Preparing multiplexed whole genome and amplicon libraries using the HiFi plex prep kit 96

Procedure & checklist

## Overview

This procedure describes the workflow for constructing multiplexed whole genome sequencing (WGS) and amplicon libraries using the HiFi plex prep kit 96 for sequencing on PacBio® systems. Automation is highly recommended for the first half of the protocol, up to and including pooling; however, the entire procedure may be performed manually. Please see instrument-specific protocols for automation details.

| Overview | | |
|---|---|---|
| Applications | • Microbial WGS<br>• Metagenome shotgun sequencing<br>• Amplicon sequencing<br>• Low-pass WGS | |
| Samples | 24–96 per kit | |
| Minimum batch size supported | 4 x 24 | |
| Maximum batch size supported | 96 | |
| Average time with automation | 6 hours (Manual workflow times prior to pooling will vary by user and sample volume) | |
| | **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).


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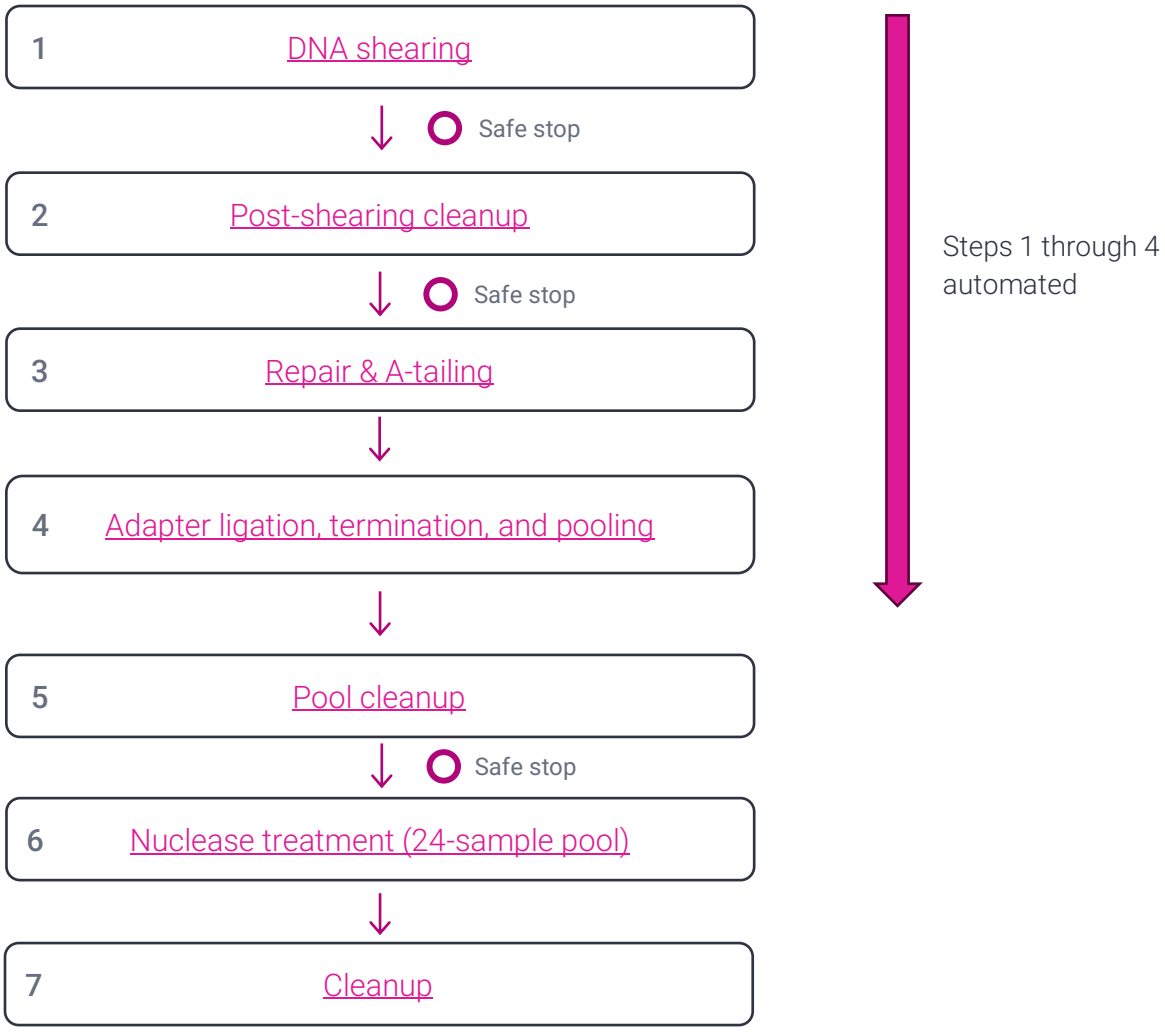
# Workflow



# Required materials and equipment

| | |
|---|---|
| **DNA QC sizing** | |
| Femto Pulse system | Agilent Technologies, Inc. M5330AA |
| Femto Pulse gDNA 165kb analysis kit | Agilent Technologies, Inc. FP-1002-0275 |
| **DNA QC 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 |
| **Library preparation** | |
| HiFi plex prep kit 96, includes:<br>• HiFi plex prep kit 96<br>• SMRTbell cleanup beads—52 mL<br>• Buffer LTE HT<br>• Elution buffer | PacBio – 103-381-300 |
| SMRTbell® adapter index plate 96 (A, B, C, or D) | Plate A – PacBio® 102-009-200<br>Plate B – PacBio® 102-547-800<br>Plate C – PacBio® 102-547-900<br>Plate D – PacBio® 102-548-000 |
| 200 Proof ethanol, molecular biology or ACS grade | Any major lab supplier (MLS) |
| Nuclease-free water, molecular biology grade | Any MLS |
| Thermocycler | Any MLS |
| 0.2 mL 8-tube strips | USA Scientific TempAssure 1402-4708 |
| Magnetic bead rack for PCR tubes or plates | Any MLS |
| 2 mL DNA LoBind tubes | Eppendorf 022431048 |
| 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 |
| Magnetic bead rack | ThermoFisher Scientific 12321D |

# Before you begin

## Automation

The HiFi plex prep kit 96 and its workflow was designed to work with NGS liquid handling automation up to the sample pooling step. Once samples are pooled, the remaining steps are prepared manually. Because of differences between automation instruments, modifications not described herein may be needed to be adapted to the protocol relevant to your specific instrumentation. Please visit the PacBio WGS page or contact your local support team for a list of instruments with qualified PacBio methods.

This protocol was developed using the Hamilton NGS STAR MOA system. Please refer to the Guide & overview for HiFi plex prep kit 96 for step-by-step instructions for running this protocol on the NGS STAR MOA system.

The HiFi plex prep kit 96 contains enough fill volume to support up to 4 x 24 sample automated runs. If setting up fewer than 24 samples, there may not be enough volume to support 96 samples due to automation dead volume requirements.

## Genomic DNA (gDNA) QC and input amount recommendations

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

### gDNA quality QC

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

Recommended guidelines for evaluating gDNA quality for this protocol:

- Use the Femto Pulse gDNA 165 kb analysis kit (Agilent FP-1002-0275)
- Dilute samples to 250 pg/µL
- 70% or more of the DNA should be 10 kb for best results. 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. In general, the better the quality of gDNA going into the protocol, the higher the HiFi sequencing yield.

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

### 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 instructions for the assay being used.

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

Remove all RNA from genomic DNA samples prior to beginning. Residual RNA can inhibit sequencing polymerase binding and can therefore lead to low loading.

| gDNA | Amplicons |
|---|---|
| 50–300 ng per sample | 20–200 ng per sample |

**Important:**

- Do not exceed >300 ng of gDNA per sample going into the Repair and A-tailing steps. Too much DNA may overwhelm enzymatic reactions and lead to poor library recovery.
- At least 24 samples need to be used when using lower input amounts (e.g., 50 ng).
- Samples must be pooled after ligation.

## HiFi plex prep kit 96 stepwise recoveries

Expected DNA and SMRTbell library recovery after each protocol step. The overall yield applies to both an individual sample and to the cumulative mass of all samples being prepared.

| 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% |
| **Overall Recovery** | **9-20%** |

## Multiplexing

**Important:** The procedure requires one of 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, please 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 prep Kits following the final cleanup. Each prep kit 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 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 refer to the Microlab Prep Guide & overview for details on consumables.

Microbial and metagenomic samples often have degraded DNA where the majority of fragments are already <15 kb in length. To better balance the number of reads between samples it may be necessary to shear all samples to a mean size of ~10 kb. For shearing below <15 kb, we recommend the following equipment:

SPEX SamplePrep *1600 MiniG* homogenizer:

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

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

FastPrep96 (contact MP Bio for latest protocol on shearing for microbial samples). PacBio recommendations below. Conditions may need to be adjusted based on DNA input and volume used.

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

## Reagent and sample handling

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

Thaw the repair buffer M96, nuclease buffer M96, index plate, and stop solution M96 at room temperature. Once thawed, place on ice.

Quick-spin all reagents in a microcentrifuge 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.

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

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

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

## 1. Automated DNA shearing for WGS using Hamilton automation

If starting with amplicon DNA, skip this step and proceed to the post-shear cleanup step (if cleanup is required) or the Repair and A-tailing step.

This protocol utilizes the Hamilton Microlab Prep or Hamilton NGS STAR MOA system for shearing. For more details, please refer to the Microlab Prep Guide & overview for details on consumables

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.

**Important:** Please follow the exact shearing conditions outlined below for the automated DNA shearing method using Hamilton automation. Deviating from these conditions may result in undersheared or unsheared DNA.

Estimated time for this step is: 10 minutes for up to 96 samples using the Hamilton NGS STAR MOA system or 22 minutes for 24 samples using the Microlab Prep system.

<table>
<tr><th>✔</th><th>Step</th><th>Instructions</th></tr>
<tr><td></td><td>1.1</td><td>Use Buffer LTE HT to bring all samples up to 300 µL total volume in a 0.8 mL, 96 DeepWell plate (Thermo Fisher Scientific AB0859). The concentration of each sample must be <10 ng/µL.</td></tr>
<tr><td></td><td>1.2</td><td>Parameters for shearing on the Microlab Prep, or Hamilton assay-ready workstations. These parameters should already be part of the installed method on the instrument.<br><table><tr><th>Parameter</th><th>Setting</th></tr><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></table></td></tr>
<tr><td></td><td>1.3</td><td>Place the plate on the appropriate work deck position and start the shearing procedure.</td></tr>
<tr><td></td><td>1.4</td><td>Optional: measure DNA concentration with a Qubit fluorometer using the 1x dsDNA HS kit once shearing procedure is complete.<br><br>Recommended: Further dilute each aliquot to 250 pg/µL with Femto Pulse dilution buffer. Measure the final SMRTbell library size distribution with a Femto Pulse system to ensure efficient shearing.</td></tr>
<tr><td></td><td>1.5</td><td>Proceed to the 1X SMRTbell cleanup bead procedure to concentrate samples for library preparation.</td></tr>
</table>

## 2. Post-shearing cleanup

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

| ✔ | Step | Instructions for SMRTbell cleanup bead step |
|---|---|---|
| | 2.1 | **Sheared genomic DNA**: add 300 µL (1.0X) of resuspended, room-temperature SMRTbell cleanup beads to each sample.<br>Amplicon DNA: if clean-up or concentration is required for amplicons, add 1.0X (amplicons >3 kb) or 1.3X (amplicons <3 kb) (v/v) concentration of SMRTbell cleanup beads. |
| | 2.2 | Pipette-mix the sample until the beads are evenly distributed. If necessary, quick-spin the samples to collect liquid. |
| | 2.3 | Leave at **room temperature** for **10 minutes** to allow DNA to bind beads. |
| | 2.4 | 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. |
| | 2.5 | Slowly pipette off the supernatant without disturbing the beads. Discard the supernatant. |
| | 2.6 | Slowly dispense **200 µL**, or enough to cover the beads, of **freshly prepared 80% ethanol** to each sample. After **30 seconds**, pipette off the 80% ethanol and discard. |
| | 2.7 | Repeat the previous step. |
| | 2.8 | Remove residual 80% ethanol:<br>• Remove the samples from the magnet and quick-spin to collect liquid.<br>• Place samples back on the magnet and wait until beads separate fully from the solution.<br>• Carefully pipette-off the residual 80% ethanol without disturbing the bead pellet and discard. |
| | 2.9 | Remove samples from the magnet and immediately add **24.5 µL of elution buffer.** Resuspend by pipette mixing until beads are evenly distributed in the solution. Quick-spin samples if necessary to collect liquid. |
| | 2.10 | Leave samples at **room temperature** for **5 minutes** to elute DNA off beads. |
| | 2.11 | 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. |
| | 2.12 | Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a new plate or tube strip. Discard old plate or tube with beads. |

## 3. Repair and A-tailing

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

| ✔ | Step | Instructions |
|---|---|---|

**3.1** Prepare the appropriate volume of master mix with 15% overage using the per reaction volumes listed below.

**Repair mix**

| ✔ | Tube | Component | Volume |
|---|---|---|---|
| | Purple | Repair buffer M96 | 4 µL |
| | Blue | End repair mix M96 | 1 µL |
| | Green | DNA repair mix M96 | 0.5 µL |
| | | **Total volume** | **5.5 µL** |

**3.2** Slowly pipette-mix the **repair mix** and quick spin to collect liquid at the bottom of the tube. If bubbles form during mixing, pulse-spin to remove bubbles.

**3.3** Add **5.5 µL** of the repair mix to each sample. The total reaction volume should be **30 µL**.

**3.4** Pipette-mix the reactions and quick-spin to collect liquid at the bottom of the well.

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

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

**3.6** Proceed to the next step of the protocol.

# 4. Adapter ligation, termination, and pooling

This step ligates the adapter index, stops the ligation reaction, and pools samples together.

| ✔ | Step | Instructions for adapter index ligation |
|---|---|---|
| | 4.1 | Add **4 µL** of indexed adapter to each sample from the previous step. |

| Component | Volume |
|---|---|
| SMRTbell adapter index plate 96 (A, B, C, or D) | 4 µL |

| ✔ | Step | Instructions for adapter index ligation |
|---|---|---|
| | 4.2 | Prepare the appropriate volume of master mix using the per reaction volumes listed below.<br>Add 10% overage if preparing the reactions manually. |

| Ligation mix | | | |
|---|---|---|---|
| ✔ | Tube | Component | Volume |
| | Yellow | Ligation mix M96 | 10 µL |
| | Red | Ligation enhancer M96 | 0.5 µL |
| | | **Total volume** | **10.5 µL** |

| ✔ | Step | Instructions for adapter index ligation |
|---|---|---|
| | 4.3 | Pipette-mix the ligation mix and quick-spin to collect liquid. |
| | 4.4 | Add **10.5 µL** of ligation mix to each sample from previous step. The total volume should be **44.5 µL.** |
| | 4.5 | Pipette-mix each sample thoroughly to ensure the mix is evenly distributed. |
| | 4.6 | Quick-spin to collect liquid. |
| | 4.7 | Run the **Adapter ligation** thermocycler program. Set the lid temperature to 75°C if programmable. |

| Step | Time | Temperature |
|---|---|---|
| 1 | 30 min | 20°C |
| 2 | Hold | 4°C |

| ✔ | Step | Instructions for adapter index ligation |
|---|---|---|
| | | Proceed to the next step to terminate the ligation reaction prior to pooling. |
| | 4.8 | Terminate the ligation reaction by adding **15.5 µL** of **Stop Solution M96** to each sample. Pipette mix thoroughly. |
| | 4.9 | Pool **40 µL** from up to 24 reactions into a **2 mL LoBind tube**. The total pooled volume for 24 samples should be **960 µL**. If processing 96 samples, use four 2 mL LoBind tubes. |
| | 4.10 | Proceed to the next step of the protocol. |

## 5. SMRTbell cleanup bead purification of pooled libraries

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

| ✔ | Step | Instructions |
|---|---|---|
| | 5.1 | Add **960 µL** (1.0X) of resuspended, room-temperature SMRTbell cleanup beads to each pool.<br>If using less than 960 µL, add 1.0X (v/v) concentration of SMRTbell cleanup beads. |
| | 5.2 | Pipette-mix or invert the sample until the beads are evenly distributed. Quick-spin the samples to collect liquid. |
| | 5.3 | Leave at **room temperature** for **10 minutes** to allow DNA to bind beads. |
| | 5.4 | 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. |
| | 5.5 | Slowly pipette off the supernatant without disturbing the beads. Discard the supernatant. |
| | 5.6 | Slowly dispense 2000 µL, or enough to cover the beads, of freshly prepared 80% ethanol to each sample. After 30 seconds, pipette off the 80% ethanol and discard. |
| | 5.7 | Repeat the previous step. |
| | 5.8 | Remove residual 80% ethanol:<br>• Remove the samples from the magnet and quick-spin to collect liquid.<br>• Place samples back on the magnet and wait until beads separate fully from the solution.<br>• Carefully pipette off the residual 80% ethanol without disturbing the bead pellet and discard. |
| | 5.9 | Remove samples from the magnet and immediately **add 40 µL of elution buffer.**<br>Resuspend by pipetting mixing until beads are evenly distributed in solution. Quick-spin samples if necessary to collect liquid. |
| | 5.10 | Leave samples at **room temperature** for **5 minutes** to elute DNA off beads. |
| | 5.11 | 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. |
| | 5.12 | Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a new tube strip. Discard the old tube with beads. |
| | 5.13 | Proceed to the next step (nuclease treatment), or store samples at 4°C. |

**SAFE STOPPING POINT - Store at 4°C**

## 6. Nuclease treatment

This step removes unligated or damaged DNA from the library to improve sequencing performance.

| ✔ | Step | Instructions for nuclease treatment |
|---|---|---|
| | 6.1 | 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** |

| | | |
|---|---|---|
| 6.2 | Pipette-mix and spin down to collect liquid. The total volume should equal **50 µL.** |
| 6.3 | 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 |

## 7. SMRTbell cleanup bead purification

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

| ✔ | Step | Instructions |
|---|---|---|
| | 7.1 | For **DNA >3 kb, add 50 µL (1.0X)** of resuspended, room-temperature SMRTbell cleanup beads to each sample.<br><br>**For DNA <3kb, add 65 µL (1.3x)** of resuspended, room-temperature SMRTbell cleanup beads to the sample. |
| | 7.2 | Pipette-mix the sample until the beads are evenly distributed. Quick spin the samples to collect liquid. |
| | 7.3 | Leave at **room temperature** for **10 minutes** to allow DNA to bind beads. |
| | 7.4 | 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. |
| | 7.5 | Slowly pipette off the supernatant without disturbing the beads. Discard the supernatant. |
| | 7.6 | Slowly dispense 200 µL, or enough to cover the beads, of freshly prepared 80% ethanol to each sample. After 30 seconds, pipette off the 80% ethanol and discard. |

| | |
|---|---|
| 7.7 | Repeat the previous step. |
| 7.8 | Remove residual 80% ethanol:<br>• Remove the samples from the magnet and quick-spin to collect liquid.<br>• Place samples back on the magnet and wait until beads separate fully from the solution.<br>• Carefully pipette off the residual 80% ethanol without disturbing the bead pellet and discard. |
| 7.9 | Remove samples from the magnet and immediately **add 25 µL of elution buffer.**<br><br>Resuspend by pipette mixing until beads are evenly distributed in solution. Quick-spin samples if necessary to collect liquid. |
| 7.10 | Leave samples at **room temperature** for **5 minutes** to elute DNA off beads. |
| 7.11 | 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. |
| 7.12 | Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a new tube strip. Discard tube with beads. |
| 7.13 | **Recommended:** quality control checkpoint. Use 1 µL from each pool to measure the concentration and size distribution of the completed library. |
| 7.14 | Please note: elutions from the bead cleanup can be pooled together at this stage prior to proceeding to ABC in SMRT® Link Sample Setup.<br>The input library concentration into ABC must be <60 ng/µL for >10 kb libraries, <20 ng/µL for 3–10 kb libraries, and <10 ng/µL for <3 kb libraries. Failure to dilute library prior to ABC may result in low loading. |

**PROTOCOL COMPLETE**

| Revision history (description) | Version | Date |
|---|---|---|
| Initial release | 01 | March 2024 |
| Updated to correct reference to the Hamilton NGS STAR MOA system, workflow time in overview table, ethanol volume in section 5 for post-ligation cleanup, and to include stepwise recovery table | 02 | April 2024 |