Preparing Onso™ libraries from genomic DNA for short-read sequencing

Procedure & checklist

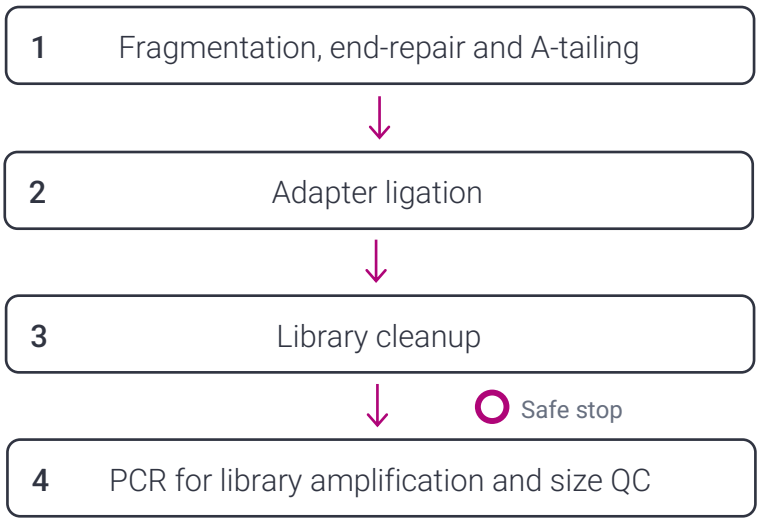
Before you begin

This procedure describes the workflow for constructing Onso libraries from high molecular weight genomic DNA for sequencing on PacBio® short-read sequencing systems. This procedure produces PCR-free and/or PCR-amplified libraries for clustering. After completing this procedure, a qPCR quantification procedure using the Onso library quant kit (PacBio 102-431-800) is advised for accurate cluster generation input.

Overview		
Samples per Onso library prep kit	Up to 96 reactions	
Workflow time	3 hours (library preparation)	1 hour (library size QC)

DNA input	
Quantity	5 - 500 ng
Size selection	Double-sided SPRIselect or AMPure® XP beads

Workflow



PN 103-180-000 REV01 AUG2023

Required materials and equipment

Library preparation	
Onso™ fragmentation DNA library prep kit	PacBio® 102-499-100
Ligation mix	PacBio® 102-554-400
Ligation enhancer	PacBio® 102-554-500
EDTA	PacBio® 102-554-600
Fragmentation buffer	PacBio® 102-554-800
Fragmentation enzyme	PacBio® 102-554-900
Onso™ library amp kit	PacBio® 102-410-800
Primer mix	PacBio® 102-552-600
PCR master mix (2x)	PacBio® 102-552-700
Onso™ indexed adapter plate	PacBio® 102-431-700
0.2 mL 8-tube strips	Any Major Lab Supplier (MLS)
Thermal cycler compatible with 0.2 mL 8-tube strips	Any MLS
Microcentrifuge	Any MLS
Vortex mixer (or bioshaker)	Any MLS
Single-channel or 8-channel pipettes	Any MLS
Magnetic separation rack compatible with 0.2 mL 8-tube strips	Any MLS
Nuclease-free water, molecular biology grade	Any MLS
SPRIselect beads or AMPure XP beads	Beckman Coulter
Low TE buffer (10 mM Tris, 0.1 mM EDTA pH 8.0)	Any MLS
200 Proof ethanol, molecular biology or ACS grade	Any MLS
Nuclease-free, 1.5 mL DNA low-bind tubes	Any MLS
Library sizing instrumentation (one or more of the following)	
2100 Bioanalyzer	Agilent technologies G2939BA
4150 or 4200 TapeStation	Agilent technologies G2992AA or G2991BA
5300 or 5400 Fragment Analyzer	Agilent technologies M5311AA or M5312AA

PN 103-180-000 REV01 AUG2023

General best practices

DNA input

Samples should be of high quality, and high molecular weight. We recommend starting with 200 ng of DNA material to enable downstream workflows. Though not stated as part of this protocol, starting concentrations should be evaluated with a Qubit fluorometer and respective reagents.

Note that library preparation of varying genomic DNA inputs will require optimization for the fragmentation time and size selection steps within the protocol to achieve the desired final library size. Recommendations made in this protocol are starting guidelines.

Reagent and sample handling

This procedure is performed using a vortex mixer. If using a bioshaker, replace all vortex mixing steps with a bioshaker set to 2200 rpm.

Though the described procedure makes use of strip tubes, higher throughput is possible by using a 96-well plate instead.

Room temperature is defined as any temperature in the range of **18-23°C** for this protocol.

EDTA provided in the Onso fragmentation DNA library prep kit can be aliquoted and stored at room temperature for the duration of library prep.

Quick spin all reagents in a microcentrifuge to collect liquid at the bottom of the tube prior to use.

Keep all temperature-sensitive reagents listed in the table below on wet ice, or cold blocks if wet ice is unavailable.

Temperature-sensitive reagents		
Step used	**Tube**	**Reagent**
Fragmentation, end-repair and A-tailing	Blue	Fragmentation buffer
	Purple	Fragmentation enzyme
Adapter ligation	n/a	Indexed adapter plate
	Yellow	Ligation mix
	Red	Ligation enhancer
Library size QC	Green	PCR master mix (2x)
	Light blue	Primer mix

Fragmentation, end-repair and A-tailing

Thaw the fragmentation buffer at room temperature. If a precipitate is seen in the fragmentation buffer, pipette up and down several times to break it up, and quickly vortex to mix. Place the fragmentation buffer on ice until use.

Vortex the fragmentation enzyme 5-8 seconds prior to use for optimal performance and place on ice until use.

The fragmentation, end-repair and A-tailing reaction is time- and temperature- sensitive. As such, the thermal cycler program should be used as a hot-start: allow the thermal cycler to achieve the temperature listed for step one, pause the thermal cycler program until the reaction tubes are prepared, and only resume once the reaction tubes are placed in the thermal cycler with the lid shut.

Alternatively, an initial hold step set to 37°C can be added (if the particular thermal cycler being used has this option) prior to the thermal cycler program step 1, to allow the thermal cycler to achieve the desired temperature. Users can then advance to the next step once the samples are ready and the reaction tubes are placed in the thermal cycler with the lid shut.

Note that library preparation of varying genomic DNA will require user optimization for the fragmentation time and size selection steps to achieve the desired final library size. Recommendations made in this protocol are starting guidelines.

Adapter ligation

Onso indexed adapter sequences can be found in **Quick reference card – Onso system library preparation adapters**. Thaw the indexed adapter plate at room temperature. Vortex well to mix, centrifuge briefly, and store on ice until use.

Remove the ligation mix and ligation enhancer from storage just prior to use and place on ice. Mix the ligation mix by pipetting up and down several times prior to use.

Library purification/size selection

Bring SPRIselect or AMPure XP beads to room temperature for 30-60 minutes prior to use.

To ensure an even distribution of beads, pipette mix all bead binding and elution steps initially, followed by a brief vortex or bioshake, as described in each relevant step.

Libraries can be stored at 4°C overnight or -20°C for longer times at all safe stopping points listed in the protocol.

Note that library preparation of varying genomic DNA will require user optimization for the fragmentation time and size selection steps. Recommendations made in this protocol are starting guidelines.

Thermal cycler program

Program thermal cycler(s) prior to beginning the protocol for the first time.

1. Fragmentation, end-repair and A-tailing

Fragmentation occurs during incubation at 37°C. The specified time for fragmentation during incubation at 37°C is for desired insert sizes between 150 – 350 bp. It should be noted that the fragmentation time noted here may require adjustment depending on: the desired insert size, the quality of the genomic DNA and for different types of genomic DNA. Sequencing performance with fragmentation sizes above 350 bp have not been assessed.

The fragmentation, end-repair and A-tailing reaction is time- and temperature-sensitive. As such, the thermal cycler program should be used as a hot-start: allow the thermal cycler to achieve the temperature listed for step one, pause the thermal cycler program until reaction tubes are prepared, and only resume once the reaction tubes are placed in the thermal cycler with the lid shut.

Alternatively, an initial hold step set to 37°C can be added (if the particular thermal cycler being used has this option) prior to the thermal cycler program step 1, to allow the thermal cycler to achieve the desired temperature. Users can then advance to the next step once the samples are ready and the reaction tubes are placed in the thermal cycler with the lid shut.

Set the thermal cycler to run with lid temperature set to 75°C with default ramp rate.

Step	Time	Temperature
1	15 min	37°C
2	5 min	65°C
3	Hold	4°C

2. Adapter ligation

Set the thermal cycler to run with no heated lid and with default ramp rate.

Step	Time	Temperature
1	15 min	20°C
2	Hold	4°C

3. PCR for library amp and size QC

The number of cycles for step 2 in the thermal cycler PCR program varies depending on the amount of input DNA (ng) that is used at the start of the library prep. Refer to the table below for the appropriate number of cycles in step 2. If using reduced quality DNA samples, then additional PCR cycles may be necessary.

Input DNA (ng)	Number of cycles for PCR program Step 2	
	1 µL of PCR-free library (PCR-free library QC)	15 µL of PCR-free library (PCR-amp library generation, and QC)
5	14	9
10	13	8
25	12	7
50	11	6
100	10	5
200	9	4
500	7	2

Set the thermal cycler to run with lid temperature set to 100°C with default ramp rate.

Step	Time	Temperature	Cycles
1	30 sec	98°C	1
2	10 sec	98°C	Variable - see table above
	30 sec	65°C	
	30 sec	72°C	
3	5 min	72°C	1
4	Hold	10°C	1

Workflow steps

1. Fragmentation, end-repair and A-tailing

Thaw the fragmentation buffer at room temperature, ensuring it is thawed prior to use. If a precipitate is seen in the fragmentation buffer, pipette up and down several times to break it up, and quickly vortex to mix, followed by a brief spin down in a microcentrifuge. Once thawed and mixed, place the fragmentation buffer on ice or cold block until ready to use.

Vortex the fragmentation enzyme 5-8 seconds prior to use for optimal performance and place on ice until use.

The fragmentation, end-repair and A-tailing reaction is time- and temperature-sensitive. As such, the thermal cycler program should be used as a hot-start: allow the thermal cycler to achieve the temperature listed for step one, pause the thermal cycler program until reaction tubes are prepared, and only resume once the reaction tubes are placed in the thermal cycler with the lid shut.

Alternatively, an initial hold step set to 37°C can be added (if the particular thermal cycler being used has this option) prior to the thermal cycler program step 1, to allow the thermal cycler to achieve the desired temperature. Users can then advance to the next step once the samples are ready and the reaction tubes are placed in the thermal cycler with the lid shut.

✔	Step	Instructions
	1.1	Bring genomic DNA (gDNA) to a final volume of **24 µL** with **nuclease-free water** in a 0.2 mL PCR strip tube. ⚠ For genomic DNA stocks that are at a starting concentration >100 ng/µL, first dilute the gDNA down to **50 ng/µL** for more accurate pipetting. Then proceed to bring up the volume of the desired mass to **24 µL** as described above.
	1.2	Start the **Fragmentation, end-repair and A-tailing** thermal cycler program, allow the program to reach 37°C, and immediately pause the program.
	1.2	Add the following components in the order and volume listed below to a new microcentrifuge tube at room temperature. Adjust component volumes for the number of samples prepared, plus 10% overage. For individual preps, add components directly to the sample from the previous step at the specified volumes and skip **RM1** steps (1.4 to 1.6).

Reaction Mix 1 (RM1)

✔	Tube	Component	Volume
	Blue	Fragmentation buffer	8 µL
	Purple	Fragmentation enzyme	3 µL
	Total volume		**11 µL**

	1.3	Vortex **RM1** briefly to ensure a homogeneous suspension.
	1.4	Quick spin **RM1** in a microcentrifuge to collect liquid. Return the microcentrifuge tube to ice or cold block.
	1.5	Add **11 µL** of **RM1** to each sample on ice or cold block. Total reaction volume should be **35 µL**.
	1.6	Vortex briefly to ensure a homogeneous suspension.
	1.7	Quick spin the strip tube in a microcentrifuge to collect liquid. Return the strip tubes to ice or cold block. ⚠ Visually check for air bubbles in sample tubes. If bubbles are present in the bottom of sample tubes, re-spin the strip tube to try to remove them.
	1.8	Remove strip tubes from ice or cold block and immediately add the reaction tubes to the thermal cycler. Close the thermal cycler lid and resume the **Fragmentation, end-repair and A-tailing** thermal cycler program.
	1.9	Quick spin sample tubes after thermal cycler program completes and proceed to the next step of the protocol.

2. Adapter ligation

Thaw the indexed adapter plate at room temperature. Vortex well to mix, centrifuge briefly, and store on ice until use.

Indexed adapter plate wells are intended for single use.

Remove the ligation mix and ligation enhancer from storage just prior to use and place on ice or cold block.

✔	Step	Instructions
	2.1	Manually pierce foil seal of indexed adapter plate wells to be used with fresh pipette tips, each. Discard pipette tips used to manually pierce the plate foil seal. Use a fresh tip to add **2.5 µL** of adapter from the **Indexed adapter plate** to each sample from step 1.9.
	2.2	Add the following components in the order and volume listed below to a new microcentrifuge tube on ice or cold block. Adjust component volumes for the number of samples being prepared, plus 10% overage. For individual preps, add components directly to each sample from step 1.9, in the order and volume listed below, then skip **RM2** steps (2.3 to 2.5). ⚠ The ligation mix reagent is very viscous and should be slowly aspirated and dispensed. Mix the ligation mix by pipetting up and down several times prior to use.
	2.3	Vortex **RM2** briefly to ensure a homogeneous suspension.
	2.4	Quick spin **RM2** in a microcentrifuge to collect liquid.
	2.5	Add **31 µL** of **RM2** to each sample. Total reaction volume should be **68.5 µL**.
	2.6	Vortex briefly to ensure a homogeneous suspension.
	2.7	Quick spin the strip tube in a microcentrifuge to collect liquid. ⚠ Visually check for air bubbles in sample tubes. If bubbles are present in the bottom of sample tubes, re-spin the strip tube to try to remove them.
	2.8	Run the **Adapter ligation** thermal cycler program.
	2.9	**Immediately** proceed to the next step of the protocol.

Reaction Mix 2 (RM2)

✔	Tube	Component	Volume
	Yellow	Ligation mix	30 µL
	Red	Ligation enhancer	1.0 µL
		Total volume	**31 µL**

3. Library cleanup

Recommended bead-to-sample volume ratios are provided within the instructions to achieve specific library sizes with a double-sided size selection.

Addition of EDTA to beads in both steps helps quench the ligation reaction, and improve adapter dimer removal. Bead cleanup can be performed with either SPRIselect beads or AMPure XP beads, as they provide comparable performance.

Bring SPRIselect or AMPure XP beads to room temperature for 30-60 minutes prior to use.

To ensure an even distribution of beads, pipette mix all bead binding and elution steps initially, followed by a brief vortex or bioshake, as described in each relevant step.

<table>
<tr><th>✔</th><th>Step</th><th>Instructions</th></tr>
<tr><td colspan="3">EDTA/Bead Mix preparation</td></tr>
<tr><td></td><td>3.1</td><td>Prepare EDTA /Bead Mix at the volumes listed below to a new microcentrifuge tube at room temperature. Adjust component volumes for the number of samples being prepared.
<table>
<tr><th colspan="4">EDTA/Bead Mix</th></tr>
<tr><th>✔</th><th>Tube</th><th>Component</th><th>Volume</th></tr>
<tr><td></td><td>n/a</td><td>Beads</td><td>125 µL</td></tr>
<tr><td></td><td>n/a</td><td>EDTA</td><td>25 µL</td></tr>
<tr><td></td><td></td><td>Total volume</td><td>150 µL</td></tr>
</table></td></tr>
<tr><td></td><td>3.2</td><td>Mix the EDTA/Bead Mix by first pipetting up and down 10 times, followed by a pulse vortex, to achieve a homogeneous solution.</td></tr>
<tr><td></td><td>3.3</td><td>Quick spin EDTA/Bead Mix in a microcentrifuge to collect liquid.</td></tr>
<tr><td colspan="3">Bead binding for double-sided size selection</td></tr>
<tr><td></td><td></td><td>Select the 1st and 2nd bead addition ratios for the library size desired.
<table>
<tr><th colspan="3">Double-sided bead-to-sample volume ratio selection for library sizes</th></tr>
<tr><td>Desired insert size distribution</td><td>150-250 bp</td><td>300-350 bp</td></tr>
<tr><td>Final library size (insert + adapter)</td><td>300-400 bp</td><td>450-500 bp</td></tr>
<tr><td>1st Bead Addition</td><td>0.50X</td><td>0.35X</td></tr>
<tr><td>2nd Bead Addition</td><td>0.25X</td><td>0.25X</td></tr>
</table></td></tr>
<tr><td></td><td>3.4</td><td>Add (selected 1st bead addition ratio x 68.5 µL) = X µL of resuspended EDTA/Bead Mix to each ligation reaction to achieve the desired ratio of EDTA/Bead Mix volume to ligation reaction volume.
For example, for a 0.35X selected ratio, add (0.35 x 68.5 µL) = 24.1 µL resuspended EDTA/Bead Mix.</td></tr>
<tr><td></td><td>3.5</td><td>Pipette up and down 10 times to mix, then vortex briefly to resuspend the beads. Check that the contents are well mixed.</td></tr>
<tr><td></td><td>3.6</td><td>Quick spin the samples in a microcentrifuge to collect liquid.</td></tr>
<tr><td></td><td>3.7</td><td>Incubate samples for 5 minutes at room temperature on the bench. If incubating on a bioshaker, quick spin the samples afterwards in a microcentrifuge to collect liquid.</td></tr>
<tr><td></td><td>3.8</td><td>Place tubes in a magnetic separation rack for 5 minutes, allowing the beads to separate fully from the solution.</td></tr>
<tr><td></td><td>3.9</td><td>Transfer each sample's supernatant to a new strip tube without disturbing beads and discard beads.</td></tr>
<tr><td></td><td>3.10</td><td>Add (selected 2nd bead addition ratio x 68.5 µL) = X µL of resuspended EDTA/Bead Mix to each sample to achieve the desired ratio of EDTA/Bead Mix volume to ligation reaction volume.
For example, for a 0.25X selected ratio, add (0.25 x 68.5 µL) = 17 µL resuspended EDTA/Bead Mix.</td></tr>
<tr><td></td><td>3.11</td><td>Pipette up and down 10 times to mix., then vortex briefly to resuspend the beads. Check that the contents are well mixed.</td></tr>
<tr><td></td><td>3.12</td><td>Quick spin the samples in a microcentrifuge to collect liquid.</td></tr>
<tr><td></td><td>3.13</td><td>Incubate samples for 5 minutes at room temperature on the bench. If incubating on the bioshaker, quick spin the samples afterwards in a microcentrifuge to collect liquid.</td></tr>
<tr><td></td><td>3.14</td><td>Place tubes in a magnetic separation rack for 5 minutes, allowing the beads to separate fully from the solution.</td></tr>
<tr><td colspan="3">Bead washing and sample elution for double-sided size selection</td></tr>
<tr><td></td><td>3.15</td><td>Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant.</td></tr>
</table>

3.16	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.
3.17	Repeat the previous step.
3.18	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.
3.19	Remove the tubes containing beads from the magnetic separation rack. **Immediately** add **50 µL** of **Low TE** to each tube and resuspend the beads by pipetting 10 times or until evenly distributed.
3.20	Quick spin the samples in a microcentrifuge to collect liquid.
3.21	Incubate the samples by vortexing or bioshaking at **2300 rpm** for **1 minute** at **room temperature**.
3.22	Quick spin the samples in a microcentrifuge to collect liquid.
	Final bead cleanup
3.23	Add **60 µL** of resuspended **EDTA/Bead Mix** to each sample to achieve a 1.2:1 ratio of EDTA/Bead Mix volume to sample volume.
3.24	Pipette up and down 10 times to mix., then vortex briefly to resuspend the beads. Check to ensure contents are well mixed.
3.25	Quick spin the samples in a microcentrifuge to collect liquid.
3.26	Incubate samples for **5 minutes** at **room temperature** on the bench. If incubating on the bioshaker, quick spin the samples afterwards in a microcentrifuge to collect liquid.
3.27	Place tubes in a magnetic separation rack for **5 minutes**, allowing beads to bind and separate fully from the solution.
3.28	Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant.
3.29	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.
3.30	Repeat the previous step.
3.31	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.
3.32	Remove the tubes containing beads from the magnetic separation rack. **Immediately** add **20 µL** of **Low TE** to each tube and resuspend the beads off the sides of the tubes by pipetting 10 times or until evenly distributed.
3.33	Vortex briefly to ensure beads are resuspended.
3.34	Quick spin the samples in a microcentrifuge to collect liquid.
3.35	Place tubes in a magnetic separation rack for **2 minutes**, allowing beads to collect at the magnet side, and separate fully from the solution.
3.36	Slowly pipette off **18 µL** of the cleared supernatant without disturbing the beads. Transfer the supernatant to a new tube strip. Discard the old tube strip with beads.
3.37	Proceed to the next step of the protocol for library size QC and/or amplification.

SAFE STOPPING POINT - Store at 4°C overnight or -20°C for longer time

⚠ This is PCR-free library content and should be stored or used for PCR-free applications. Proceed to section 4 “PCR for library amp and size QC” to conduct the required amplification to appropriately QC this library.

4. PCR for library amp and size QC

PCR **must** be performed for accurate library sizing measurement, regardless of the intended use, be it PCR-free or PCR-amplified library applications.

The product from section 3 is used for PCR-free applications. To perform library size QC, a small volume of this PCR-free product must be PCR-amplified. Once QC is complete, the PCR-free library (product of section 3) should be used for qPCR quantification and subsequent clustering. Refer to **section 4a**.

For PCR-amplified library applications, amplified product is generated in **section 4b**, using 15 µL of the PCR-free contents from section 3. The resulting library should be used for qPCR quantification and subsequent clustering.

✔	Step	4a. Library size QC for PCR-free library applications
	4a.1	Add the following components in the order and volume listed below to a new microcentrifuge tube at room temperature. Adjust component volumes for the number of samples prepared, plus 10% overage.

Reaction Mix 3 (RM3)

Tube	Component	Volume
n/a	Nuclease-free water	21.5 µL
Green	PCR master mix (2x)	25
Light blue	Primer mix	2.5 µL
	Total volume	**49 µL**

	4a.2	Vortex **RM3** briefly to ensure a homogeneous suspension.
	4a.3	Quick spin **RM3** in a microcentrifuge to collect liquid.
	4a.4	Add **49 µL** of **RM3** to a 0.2 mL PCR strip tube for each sample.
	4a.5	Add **1 µL** of **sample library from step 3.36** to the strip tube with **RM3**. Total reaction volume should be **50 µL.**
	4a.6	Vortex briefly to ensure a homogeneous suspension.
	4a.7	Quick spin the strip tube in a microcentrifuge to collect liquid.
	4a.8	Run the **PCR for library amp and size QC** thermal cycler program, based on the amount of DNA input used in the library prep, and volume of PCR-free library used in the PCR reaction above.
	4a.9	For each sample, measure the library size distribution using the product of step 4a.8 with the recommended sizing technology (Agilent 2100 Bioanalyzer, 4150 or 4200 TapeStation, or 5300 or 5400 Fragment Analyzer) following the manufacturer's instructions.
	4a.10	For each sample, take 2 µL of the PCR-free library from step 3.37 for quantification purposes: accurate assessment of library quantity by qPCR must next be performed following procedure "**qPCR Quantification of Onso™ libraries**" using **Onso Library quant kit** (PacBio 102-431-800). This will ensure optimal cluster density can be achieved during cluster generation. **Note:** Step 4a.10 can be conducted simultaneously with step 4a.9.
	4a.11	Once complete, use the PCR-free libraries from step 3.37 for subsequent clustering and sequencing efforts.

PROTOCOL COMPLETE

OR (see next page)

✔	Step	4b. Library amplification and QC for PCR-amplified library applications
	4b.1	Add the following components in the order and volume listed below to a new microcentrifuge tube at room temperature. Adjust component volumes for the number of samples prepared, plus 10% overage.

Reaction Mix 3 (RM3)

✔	Tube	Component	Volume
	n/a	Nuclease-free water	7.5 µL
	Green	PCR master mix (2x)	25
	Light blue	Primer mix	2.5 µL
		Total volume	**35 µL**

4b.2	Vortex **RM3** briefly to ensure a homogeneous suspension.
4b.3	Quick spin **RM3** in a microcentrifuge to collect liquid.
4b.4	For PCR-amplified library applications, add **35 µL** of **RM3** to a 0.2 mL PCR strip tube for each sample.
4b.5	Add **15 µL** of **sample library** to the strip tube with **RM3**. Total reaction volume should be **50 µL.**
4b.6	Vortex briefly to ensure a homogeneous suspension.
4b.7	Quick spin the strip tube in a microcentrifuge to collect liquid.
4b.8	Run the **PCR for library amp and size QC** thermal cycler program, based on the amount of DNA input used in the library prep and volume of PCR-free library used in the PCR reaction above.
4b.9	Add **50 µL** of SPRIselect or Ampure beads to each sample for a 1X cleanup.
4b.10	Repeat steps 3.24 to 3.31 to conduct bead binding, separation, and ethanol clean up steps.
4b.11	Remove the tubes containing beads from the magnetic separation rack. **Immediately** add **50 µL** of **Low TE** to each tube and resuspend the beads off the sides of the tubes by pipetting 10 times or until evenly distributed.
4b.12	Vortex briefly to ensure beads are resuspended.
4b.13	Quick spin the samples in a microcentrifuge to collect liquid.
4b.14	Place tubes in a magnetic separation rack for **2 minutes**, allowing beads to collect at the magnet side, and separate fully from the solution.
4b.15	Slowly pipette off **50 µL** of the cleared supernatant without disturbing the beads. Transfer supernatant to a new tube strip. Discard the old tube strip with beads.
4b.16	For each sample, measure the library size distribution using the PCR-amplified library product of step 4b.15 with the recommended sizing technology (Agilent 2100 Bioanalyzer, 4150 or 4200 TapeStation, or 5300 or 5400 Fragment Analyzer) following the manufacturer's instructions.
4b.17	For each sample, take **2 µL** of the PCR-amplified library from step 4b.15 for quantification purposes: accurate assessment of library quantity by qPCR must next be performed following procedure "**qPCR Quantification of Onso™ libraries**" using **Onso Library quant kit** (PacBio 102-431-800). This will ensure optimal cluster density can be achieved during cluster generation. **Note:** Step 4b.17 can be conducted simultaneously with step 4a.16.
4b.18	Once complete, use the PCR-amplified library products from step 4b.15 for subsequent clustering and sequencing efforts.

PROTOCOL COMPLETE

Revision history (description)	Version	Date
Initial release	01	AUG 2023

PN 103-180-000 REV01 AUG2023