

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

Procedure & checklist

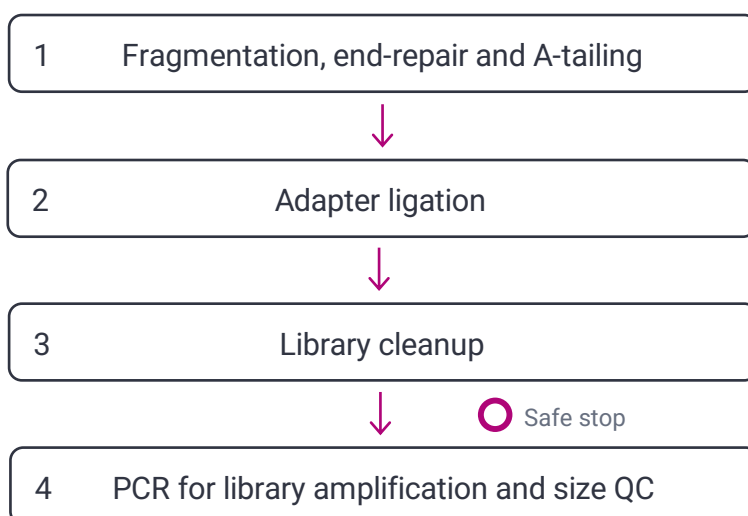
Before you begin

This procedure describes the workflow for constructing libraries from high molecular weight genomic DNA for sequencing on the PacBio® Onso system. This procedure produces PCR-free 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 recommended for accurate input onto the cluster generator.

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 (≥200 ng for PCR-free or <200 ng for PCR-amplified)
Size selection	Double-sided SPRIselect or AMPure® XP beads

Workflow



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

General best practices

DNA input

Samples should be of high quality, and high molecular weight. For PCR-free libraries, we recommend starting with **200 ng of DNA** material to enable downstream workflows. Depending on library recovery, starting with 200 ng is sufficient for one flowcell. Starting concentrations should be evaluated with a Qubit fluorometer and respective reagents. If starting with < 200 ng, library yield may not be sufficient to support one flowcell. As such, we recommend an amplification step to generate more library.

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 to avoid interruptions during the procedure.

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. Library Amplification

For size QC and PCR-amplified library generation.

Program the thermal cycler using the following parameters and name the program as “Library Amplification”. 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*
	30 sec	65°C	
	30 sec	72°C	
3	5 min	72°C	1
4	Hold	10°C	1

*The number of cycles depends 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.

Use 1 µL of the library and follow the recommended number of cycles below to QC the library.

If you plan to PCR-amplify the library for clustering, use 15 µL of the library and follow the recommended number of cycles below. The resulting amplified library should be sufficient for size-QC and clustering.



Input DNA (ng)	Recommended number of cycles	
	For size-QC only Use 1 µL of PCR-free library QC	For amplification use 15 µL of library
5	NA	9
10	NA	8
25	NA	7
50	NA	6
100	NA	5
200	9	4 (if necessary)
500	7	2 (if necessary)

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.



✓	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).																			
1.2		<table border="1"> <thead> <tr> <th colspan="3">Reaction Mix 1 (RM1)</th> </tr> <tr> <th>✓</th> <th>Tube</th> <th>Component</th> <th>Volume</th> </tr> </thead> <tbody> <tr> <td></td> <td>Blue</td> <td>Fragmentation buffer</td> <td>8 µL</td> </tr> <tr> <td></td> <td>Purple</td> <td>Fragmentation enzyme</td> <td>3 µL</td> </tr> <tr> <td colspan="2">Total volume</td> <td></td> <td>11 µL</td> </tr> </tbody> </table>	Reaction Mix 1 (RM1)			✓	Tube	Component	Volume		Blue	Fragmentation buffer	8 µL		Purple	Fragmentation enzyme	3 µL	Total volume			11 µL
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	<p>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.</p> <p>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).</p> <p> 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.</p>																			
	2.2	<table border="1"> <thead> <tr> <th colspan="3">Reaction Mix 2 (RM2)</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</td> <td>30 μL</td> </tr> <tr> <td></td> <td>Red</td> <td>Ligation enhancer</td> <td>1.0 μL</td> </tr> <tr> <td colspan="3">Total volume</td> <td>31 μL</td> </tr> </tbody> </table>	Reaction Mix 2 (RM2)			✓	Tube	Component	Volume		Yellow	Ligation mix	30 μ L		Red	Ligation enhancer	1.0 μ L	Total volume			31 μ L
Reaction Mix 2 (RM2)																					
✓	Tube	Component	Volume																		
	Yellow	Ligation mix	30 μ L																		
	Red	Ligation enhancer	1.0 μ L																		
Total volume			31 μ L																		
	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	<p>Quick spin the strip tube in a microcentrifuge to collect liquid.</p> <p> 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.</p>																			
	2.8	Run the Adapter ligation thermal cycler program.																			
	2.9	Quick spin sample tubes after thermal cycler program completes and immediately proceed to the next step of the protocol.																			

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.

Step	Instructions
------	--------------

EDTA/Bead Mix preparation

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.

EDTA/Bead Mix

3.1	✓ Tube	Component	Volume
	n/a	Beads	125 µL
	n/a	EDTA	25 µL
Total volume			150 µL

3.2 Mix the **EDTA/Bead Mix** by first pipetting up and down 10 times, followed by a pulse vortex, to achieve a homogeneous solution.

3.3 Quick spin **EDTA/Bead Mix** in a microcentrifuge to collect liquid.

Bead binding for double-sided size selection

Select the 1st and 2nd bead addition ratios for the library size desired.

Double-sided bead-to-sample volume ratio selection for library sizes

Desired insert size distribution	150-250 bp	300-350 bp
Final library size (insert + adapter)	300-400 bp	450-500 bp
1 st Bead Addition	0.50X	0.35X
2 nd Bead Addition	0.25X	0.25X

3.4 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) = 24.0 µL resuspended EDTA/Bead Mix.

3.5 Pipette up and down 10 times to mix, then vortex briefly to resuspend the beads. Check that the contents are well mixed.

3.6 Quick spin the samples in a microcentrifuge to collect liquid.

3.7 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.

3.8 Place tubes in a magnetic separation rack for **5 minutes**, allowing the beads to separate fully from the solution.

3.9 Transfer each **sample's supernatant** to a **new strip tube** without disturbing beads and **discard beads**.

3.10 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) = 17.0 µL resuspended EDTA/Bead Mix.

3.11 Pipette up and down 10 times to mix., then vortex briefly to resuspend the beads. Check that the contents are well mixed.

3.12 Quick spin the samples in a microcentrifuge to collect liquid.

3.13 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.

3.14 Place tubes in a magnetic separation rack for **5 minutes**, allowing the beads to separate fully from the solution.

Bead washing and sample elution for double-sided size selection

3.15 Slowly pipette off the cleared supernatant without disturbing the beads. **Discard the supernatant.**

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.

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.

3.18

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.

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.

3.31

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



The resulting library is a PCR-free library. Proceed to section 4 "Size-QC and quantification".

4. Size-QC and quantification

To determine the size of the library, PCR amplification **must** be performed. Refer to section “**Thermal Cycler Program**”, under “Library amplification for PCR conditions. To quantify the library, it is highly recommended to use qPCR to accurately measure the concentration of the library for clustering.

The product from section 3 is used for PCR-free applications. Refer to **section 4a**. To perform library size QC and quantification, 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 subsequent clustering.

Refer to section 4b for PCR-amplified libraries.

✓ Step	4a. Library size QC																
	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)																
4a.1	<table border="1"> <thead> <tr> <th>Tube</th> <th>Component</th> <th>Volume</th> </tr> </thead> <tbody> <tr> <td>n/a</td> <td>Nuclease-free water</td> <td>21.5 µL</td> </tr> <tr> <td>Green</td> <td>PCR master mix (2x)</td> <td>25</td> </tr> <tr> <td>Light blue</td> <td>Primer mix</td> <td>2.5 µL</td> </tr> <tr> <td></td> <td>Total volume</td> <td>49 µL</td> </tr> </tbody> </table>	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	
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 Library Amplification program, based on the amount of DNA input used in the library prep.. Using 1.0 ul of the library, use the number of cycles as recommended below:																
	<table border="1"> <thead> <tr> <th>Input DNA (ng)</th> <th>Recommended number of cycles</th> </tr> </thead> <tbody> <tr> <td>200</td> <td>9</td> </tr> <tr> <td>500</td> <td>7</td> </tr> </tbody> </table>		Input DNA (ng)	Recommended number of cycles	200	9	500	7									
Input DNA (ng)	Recommended number of cycles																
200	9																
500	7																
4a.9	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	To quantify, Take 2 µL of the library from step 3.37 and use the 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. Optional: Qubit may be used in this step but qPCR for quantification is preferred because of its accuracy.																
	Note: Step 4a.10 can be conducted simultaneously with step 4a.9.																
4a.11	Once QC is complete, use the libraries from step 3.37 for subsequent clustering and sequencing efforts.																

For **PCR-amplified** library applications, amplified product is generated in the section below (**4b**), using 15 μL of the library from section 3, which should be sufficient for QC (sizing and qPCR quantification) and amplification. The resulting amplified library should be used for subsequent clustering.

✓ Step	4b. Library amplification and QC for PCR-amplified library applications		
	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)		
4b.1	✓ 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.		
	Run the Library Amplification program, based on the amount of DNA input used in the library prep. Using 15.0 μL of the library, follow the number of cycles as recommended below. The amplified library is sufficient for size-QC and clustering.		
	Input DNA (ng)	Recommended number of cycles	
4b.8	5	9	
	10	8	
	25	7	
	50	6	
	100	5	
	200	4 (if necessary)	
	500	2 (if necessary)	
4b.9	Clean-up the amplified library by adding 50 μL of SPRIselect or AMPure beads 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 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 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. **Optional:** Qubit may be used in this step but qPCR for quantification is preferred because of its accuracy.
- 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
Added thermal cycler recommendations of pages 4 + 5. Other QC and quantification updates throughout.	02	MAR 2025

Research use only. Not for use in diagnostic procedures. © 2025 Pacific Biosciences of California, Inc. ("PacBio"). All rights reserved. Information in this document is subject to change without notice. PacBio assumes no responsibility for any errors or omissions in this document. Certain notices, terms, conditions and/or use restrictions may pertain to your use of PacBio products and/or third-party products. Refer to the applicable PacBio terms and conditions of sale and to the applicable license terms at pacb.com/license. Pacific Biosciences, the PacBio logo, PacBio, Circulomics, Omniome, SMRT, SMRTbell, Iso-Seq, Sequel, Nanobind, SBB, Revio, Onso, Apton, Kinnex, PureTarget, SPRQ, and Vega are trademarks of PacBio.