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

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Procedure & checklist

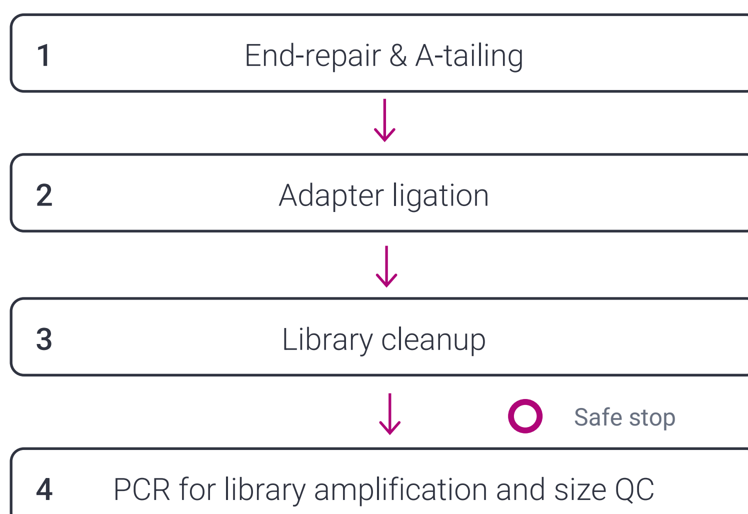
Before you begin

This procedure describes the workflow for constructing Onso libraries from fragmented genomic material 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 DNA library prep kit	Up to 96 reactions	
Workflow time	3 hours (library preparation)	1 hour (library size QC)

DNA input	
Quantity	10-1000 ng per library

Workflow



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Required materials and equipment

Materials	
Onso™ DNA library prep kit	PacBio® 102-431-400 kit
End prep buffer	PacBio® 102-554-300
End prep mix	PacBio® 102-554-200
Ligation mix	PacBio® 102-554-400
Ligation enhancer	PacBio® 102-554-500
EDTA	PacBio® 102-554-600
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 kit	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 Tape Station	Agilent technologies G2992AA or G2991BA
5300 or 5400 Fragment Analyzer	Agilent technologies M5311AA or M5312AA

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General best practices

DNA Input

Samples should be of high quality. Though not stated as part of this protocol, starting concentrations should be evaluated with a Qubit fluorometer and respective reagents. Expected library prep yields are between 50-80% for PCR-free applications.

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 made 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 DNA library prep kit can be aliquoted and stored at room temperature for the duration of library preparation.

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**
End repair and A-tailing	Orange	End prep mix
	Green	End prep buffer
Adapter ligation	N/A	EDTA
	Yellow	Ligation mix
	Red	Ligation enhancer
	N/A	Indexed adapter plate
Library size QC	Green	PCR master mix (2x)
	Light blue	Primer mix

End-repair and A-tailing

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

Vortex the End prep mix 5-8 seconds prior to use for optimal performance and place on ice until use.

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The 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 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 (when 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.

Adapter ligation

Thaw the indexed adapter plate at room temperature. Mix well and store on ice until use.

Prior to use, remove the ligation mix and ligation enhancer from storage and place on ice. Pipette the ligation mix up and down several times to mix.

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.

Thermal cycler program

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

1. End-repair and A-tailing

The 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 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 to 75°C with default ramp rate.

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

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2. 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 the library prep. Refer to the table below for the appropriate number of cycles in step 2:

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)
10	11	6
25	10	5
50	9	4
100	8	3
200	7	2
500	5	*Revert to 1 μL use and guidance
1000	4	*Revert to 1 μL use and guidance

*For generating PCR-amp libraries with input DNA of > 200 ng, the PCR-free library prep yield should likely be sufficient for PCR-amp applications. Users should then revert to the thermal cycler guidance provided for PCR-free library size QC, using only 1 μL of the PCR-free library, but following with a 1X SPRIselect or AMPure XP bead cleanup, as covered in section 4b.

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

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Workflow steps

1. End-repair and A-tailing

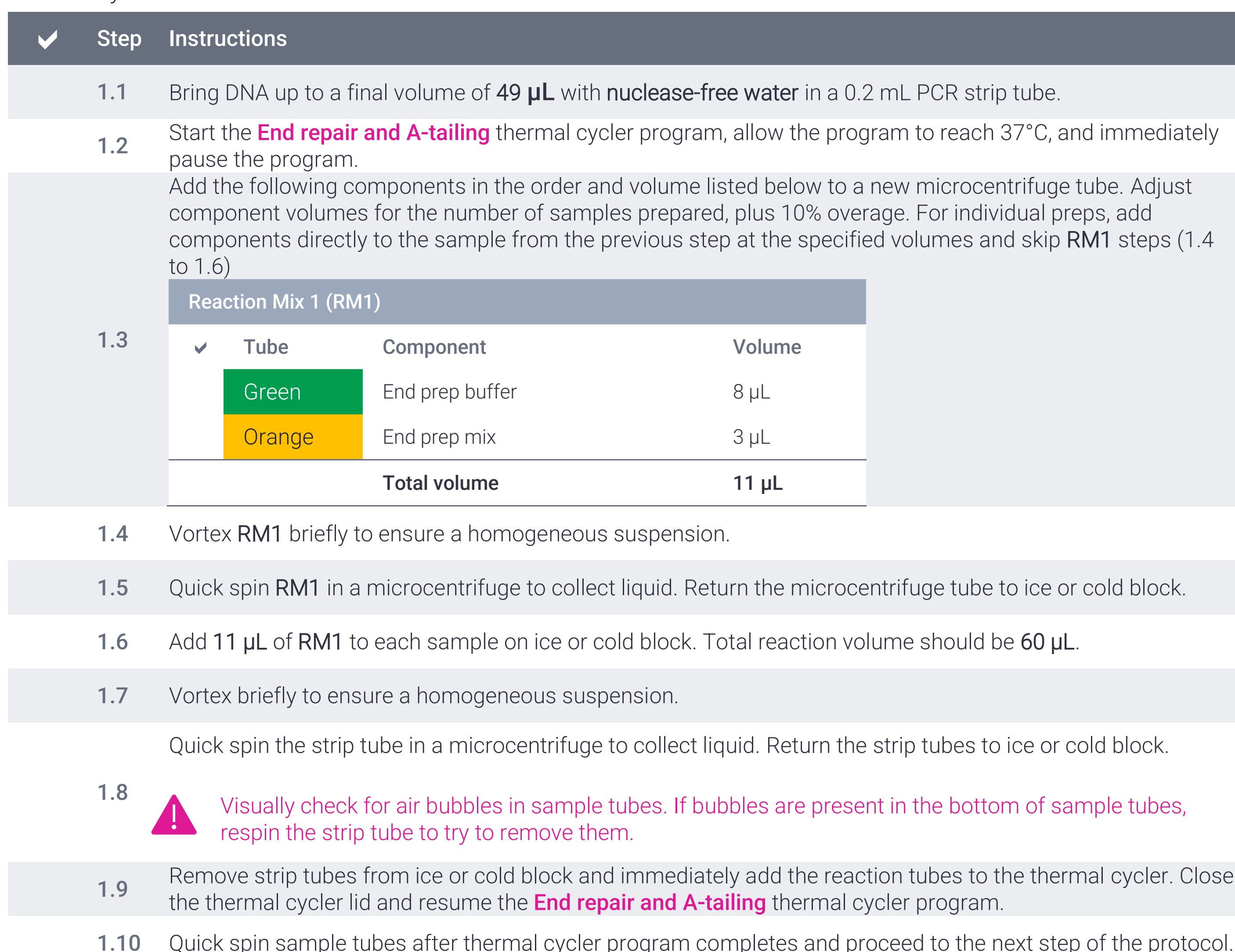
DNA input should be within 10-1000 ng per library, in a volume less than or equal to 49 µL.

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

Vortex the End prep mix 5-8 seconds prior to use for optimal performance and place on ice until use.

The 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 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.



<table>
<tr><th>✔</th><th>Step</th><th>Instructions</th></tr>
<tr><td></td><td>1.1</td><td>Bring DNA up to a final volume of 49 µL with nuclease-free water in a 0.2 mL PCR strip tube.</td></tr>
<tr><td></td><td>1.2</td><td>Start the End repair and A-tailing thermal cycler program, allow the program to reach 37°C, and immediately pause the program.</td></tr>
<tr><td></td><td>1.3</td><td>Add the following components in the order and volume listed below to a new microcentrifuge tube. 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)
<table>
<tr><th colspan="4">Reaction Mix 1 (RM1)</th></tr>
<tr><th>✔</th><th>Tube</th><th>Component</th><th>Volume</th></tr>
<tr><td></td><td>Green</td><td>End prep buffer</td><td>8 µL</td></tr>
<tr><td></td><td>Orange</td><td>End prep mix</td><td>3 µL</td></tr>
<tr><td></td><td></td><td>Total volume</td><td>11 µL</td></tr>
</table></td></tr>
<tr><td></td><td>1.4</td><td>Vortex RM1 briefly to ensure a homogeneous suspension.</td></tr>
<tr><td></td><td>1.5</td><td>Quick spin RM1 in a microcentrifuge to collect liquid. Return the microcentrifuge tube to ice or cold block.</td></tr>
<tr><td></td><td>1.6</td><td>Add 11 µL of RM1 to each sample on ice or cold block. Total reaction volume should be 60 µL.</td></tr>
<tr><td></td><td>1.7</td><td>Vortex briefly to ensure a homogeneous suspension.</td></tr>
<tr><td></td><td>1.8</td><td>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, respin the strip tube to try to remove them.</td></tr>
<tr><td></td><td>1.9</td><td>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 End repair and A-tailing thermal cycler program.</td></tr>
<tr><td></td><td>1.10</td><td>Quick spin sample tubes after thermal cycler program completes and proceed to the next step of the protocol.</td></tr>
</table>

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2. 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.

Indexed adapter plate wells are intended for single use.

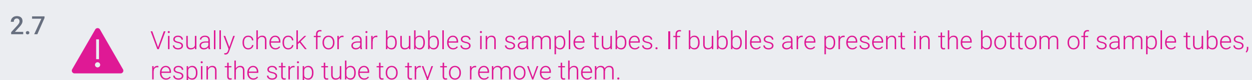
Remove the Ligation mix and Ligation enhancer from storage just prior to use and place on ice or cold block.

✔	Step	Instructions
	2.1	Manually pierce the foil seal of each indexed adapter plate well to be used with a fresh pipette tip. 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 1.10.
	2.2	Add the following components in the order and volume listed below to a new microcentrifuge tube. Adjust component volumes for the number of samples being prepared, plus 10% overage. For individual preps, add components directly to each sample from step 1.10, in the order and volume listed below, and 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.

Reaction Mix 2 (RM2)			
✔	Tube	Component	Volume
	Yellow	Ligation mix	30 µL
	Red	Ligation enhancer	1 µL
		Total volume	**31 µL**

✔	Step	Instructions
	2.3	Vortex **RM2** briefly to ensure a homogeneous suspension.
	2.4	Quick spin **RM2** in a microcentrifuge to collect the liquid.
	2.5	Add **31 µL** of **RM2** to each sample from step 1.10. Total reaction volume should be **93.5 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, respin the strip tube to try to remove them.

✔	Step	Instructions
	2.8	**Immediately** add the reaction tubes to the thermal cycler and close the thermal cycler lid. Run the Adapter ligation thermal cycler program.
	2.9	Quick spin sample tubes after thermal cycler program completes.
	2.10	**Immediately** proceed to the next step of the protocol.

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3. Library cleanup

Addition of EDTA to beads in both steps help 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 mixture preparation
	3.1	Prepare the EDTA/Bead mixture at volumes as listed below, to a new microcentrifuge tube. Adjust component volumes for the number of samples being prepared. Note that volumes listed for the beads and EDTA mix are for one library prep.
	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 the liquid.
		Bead binding
	3.4	Add **84.2 µL** of resuspended **EDTA/Bead Mix** to each tube from step 2.10 to perform a 0.9X cleanup of the ligation reaction volume.
	3.5	Pipette up and down 10 times to mix, only. Check that the contents are well mixed. Note: Due to the high volume retained in the tube, do not vortex to mix at this step.
	3.6	Quick spin each sample in a microcentrifuge to collect the liquid.
	3.7	Incubate samples for **5 minutes** at **room temperature** on the bench.
	3.8	Place tubes in a magnetic separation rack for **5 minutes**, allowing the beads to separate fully from the solution.
	3.9	Slowly pipette off the cleared supernatant without disturbing the beads. **Discard the supernatant.**
	3.10	Remove the tubes containing beads from the magnetic separation rack. Add **50 µL** of **Low TE** to each tube.
	3.11	Vortex briefly to resuspend the beads. Check to see that the contents are well mixed.
	3.12	Quick spin each sample in a microcentrifuge to collect the liquid.
	3.13	Add **60 µL** of resuspended **EDTA/Bead Mix** to each tube to perform a 1.2X cleanup of the sample volume.
	3.14	Pipette up and down 10 times to mix, then vortex briefly to resuspend the beads. Check that the contents are well mixed.
	3.15	Incubate sample for **5 minutes** at **room temperature.**
	3.16	Quick spin the samples in a microcentrifuge to collect liquid.
	3.17	Incubate samples on a magnetic separation rack for **5 minutes**, allowing the beads to separate fully from the solution.

EDTA/Bead Mix (step 3.1)

✔	Tube	Component	Volume
	n/a	Beads	125 µL
	n/a	EDTA	25 µL
		Total volume	**150 µL**

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Bead washing and sample elution

3.18	Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant.
3.19	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.
3.20	Repeat the previous step.
3.21	Remove residual 80% ethanol: • Remove tube strip from the magnetic separation rack. • Quick spin tube strip in a microcentrifuge. • Place tube strip back on a magnetic separation rack until beads separate fully from the solution. • Using a P20 pipette, aspirate off residual 80% ethanol and discard.
3.22	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.
3.23	Vortex briefly to ensure beads are resuspended.
3.24	Quick spin the strip tubes in a microcentrifuge to collect liquid.
3.25	Place strip tubes on a magnetic separation rack for 2 minutes, allowing beads to collect at the magnet side, and separate fully from the solution.
3.26	Slowly pipette off 18 µL of the cleared supernatant without disturbing the beads. Transfer supernatant to a new tube strip. Discard the old tube strip with beads.
3.27	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.

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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.
	4a.2	Vortex **RM3** briefly to ensure a homogeneous suspension.
	4a.3	Quick spin **RM3** in a microcentrifuge to collect the 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** 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.
	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 3.27 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 3.27 for subsequent clustering and sequencing efforts.

Reaction Mix 3 (RM3) (step 4a.1)

✔	Tube	Component	Volume per sample
	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**

PROTOCOL COMPLETE

OR (see next page)

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✔	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. (See Reaction Mix 3 (RM3) table below.)
	4b.2	Vortex **RM3** briefly to ensure a homogeneous suspension.
	4b.3	Quick spin **RM3** in a microcentrifuge to collect the 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.
	4b.9	Add **50 µL** of resuspended SPRIselect or AMPure XP beads only (not to be confused with EDTA/Bead mix) to each tube to perform a 1X cleanup of each sample.
	4b.10	Repeat steps 3.14 - 3.21 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 on 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 **48 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 4b.16.
	4b.18	Once complete, use the PCR-amplified library products of step 4b.15 for subsequent clustering and sequencing efforts.
		PROTOCOL COMPLETE

Reaction Mix 3 (RM3)

✔	Tube	Component	Volume per sample
	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**

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

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