Preparing Onso™ compatible libraries from existing P5/P7 libraries for short-read sequencing

Procedure & checklist

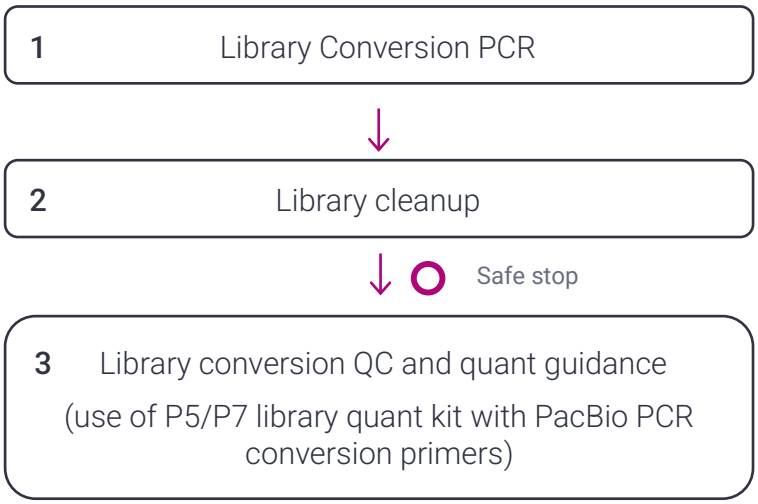
Before you begin

This procedure describes the workflow for converting existing P5/P7-tailed libraries to be compatible for sequencing on PacBio® short-read sequencing systems.

Overview		
Samples per Onso™ library conversion kit		Up to 96 reactions
Workflow time		1 hour (excludes library QC and quant)
Library input		
Quantity	For single libraries	5-100 fmol per library in a volume of up to 20 µL
	For pre-pooled libraries	5-20 fmol per library pool in a volume of up to 20 µL

Workflow

This document applies to researchers and personnel assigned to convert existing P5/P7-tailed libraries to Onso™ compatible libraries.



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Required materials and equipment

Materials	
Onso™ library conversion kit	PacBio® 102-529-500
PCR master mix (2x)	PacBio® 102-552-700
PCR conversion primers	PacBio® 102-554-700
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
Library conversion QC	
Existing P5/P7 library quant kit used to quantify original libraries	Any MLS
qPCR instrumentation	Any MLS

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General best practices

Library input

Though not stated as part of this protocol, starting concentrations should be evaluated with a Qubit fluorometer and/or a qPCR protocol appropriate for the library prep conducted to generate the P5/P7 libraries. DNA inputs can be single libraries or library pools. It is advised that conversion of individual libraries be conducted before pooling to eliminate the possibility of index-crossing from PCR. However, conversion can still be conducted on pre-existing library pools, if necessary.

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.

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

Thaw the PCR conversion primers at room temperature.

Vortex and quick spin all reagents in a microcentrifuge to ensure a homogeneous suspension and 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**
PCR conversion	Light Blue	PCR conversion primers
	Green	PCR master mix (2X)

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

Samples can be stored at 4°C at all safe stopping points listed in the protocol for short-term storage or at -20°C for longer-term storage.

When performing conversion as described here, note that in downstream applications (such as sequencing) there will be reliance on the use of custom sequence primers. Sequences for the conversion primers used here can be found in **Quick reference card – Onso system library preparation adapters**.

Multiplexing

Multiplexed library pools can be converted in the same fashion as a single library. It is recommended that if multiplexed libraries are of interest that similar sized libraries be used for best results.

Thermal cycler program

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

PCR conversion

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	5
	30 sec	65°C	
	30 sec	72°C	
3	5 min	72°C	1
4	Hold	10°C	1

Workflow steps

1. Library conversion PCR

✔	Step	Instructions
	1.1	Bring P5/P7 libraries up to a final volume of **20 µL** each with **nuclease-free water** in 0.2 mL strip tubes.
	1.2	Add the following components in the order and volume listed below to a new microcentrifuge tube. Adjust component volumes for the number of samples 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 (1.3 to 1.5)
	1.3	Vortex to mix **RM1** to ensure a homogeneous suspension.
	1.4	Quick spin **RM1** in a microcentrifuge to collect liquid.
	1.5	Add **30 µL** of the **RM1** to each sample. Total reaction volume should be **50 µL**.
	1.6	Vortex briefly to mix.
	1.7	Quick spin the tube strip in a microcentrifuge to collect liquid.
	1.8	Run the PCR conversion thermal cycler program.
	1.9	Proceed immediately to the next step of the protocol.

Reaction Mix 1 (RM1)

✔	Tube	Component	Volume
	N/A	Nuclease-free water	2.5 µL
	Green	PCR master mix (2X)	25 µL
	Light Blue	PCR conversion primers	2.5 µL
		Total volume	**30 µL**

2. Library cleanup

Converted libraries undergo a cleanup step using either SPRIselect or AMPure XP beads to ensure removal of partial fragments.

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

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

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
	2.1	Add **50 µL** of either SPRIselect or AMPure XP cleanup beads to each sample from the previous step.
	2.2	Pipette mix each sample 10 times and briefly vortex until the solution is homogeneous.
	2.3	Quick spin each sample in a microcentrifuge to collect the liquid.
	2.4	Incubate the samples for **5 minutes** at **room temperature** on the bench to allow DNA to bind to beads.
	2.5	Place tubes in a magnetic separation rack for **5 minutes**, allowing the beads to separate fully from the solution.
	2.6	Slowly pipette off the cleared supernatant without disturbing the beads. **Discard the supernatant.**
	2.7	While each tube remains on the magnet, slowly dispense **200 µL** of **freshly prepared 80% ethanol** into each tube. After **30 seconds**, pipette off and discard the 80% ethanol.
	2.8	Repeat the previous step.
	2.9	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.
	2.10	Remove the tubes containing beads from the magnetic separation rack. **Immediately** add **52 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.
	2.11	Vortex for **1 minute** at **room temperature** to ensure beads are resuspended.
	2.12	Quick spin the samples in a microcentrifuge to collect liquid.
	2.13	Place tubes in a magnetic separation rack for **2 minutes**, allowing beads to collect at the magnet side, and separate fully from the solution.
	2.14	Slowly pipette off **50 µL** of the cleared supernatant without disturbing the beads. Transfer the supernatant to a new tube strip. Discard the old tube strip with beads.
	2.15	Proceed to the next step for library conversion QC and quant guidance.

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

3. Library conversion QC and quant guidance

✔	Step	Instructions
	3.1	Measure library size distribution of stock libraries and converted libraries by way of recommended sizing technology (Agilent 2100 Bioanalyzer, 4150 or 4200 TapeStation, or 5300 or 5400 Fragment Analyzer) following the manufacturer's instructions.
	3.2	Library quantification by qPCR should be performed using P5/P7 quantification kits available from any manufacturer. To accurately assess the amount of Onso compatible library produced, it is recommended that users **substitute P5/P7 quantification primers** with the **PacBio PCR conversion primers** used during conversion. The following considerations should be made when following this recommendation: • PacBio PCR conversion primers will add approximately 42 base pairs to P5/P7 libraries. This size correction for the qPCR should be added to the length of the standards used in qPCR. • PacBio PCR conversion primers should be diluted with **Low TE buffer** to match the primer concentrations used in the P5/P7 quantification kit. • Annealing temperatures for P5/P7 qPCR protocols should be adjusted to 63°C to reflect the recommended annealing temperatures for use with PacBio PCR conversion primers.

PROTOCOL COMPLETE

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