Generating hybrid capture libraries for sequencing on the Onso™ system

Procedure & Checklist

Before you begin

This procedure describes how the **Onso DNA Library prep kit** (PacBio® 102-431-400), **Onso Library amp kit** (PacBio 102-410-800) and **Onso Blocker oligos** (PacBio 102-427-200) can be used to support the generation of hybrid capture libraries compatible with sequencing on PacBio short-read sequencing systems.

Note that if the starting material requires fragmentation as part of library preparation, this procedure can be adapted to use the **Onso Fragmentation DNA Library prep kit** (PacBio 102-499-100) rather than the **Onso DNA Library prep kit**. Steps where the **Onso Fragmentation DNA Library prep kit** can be used are noted in their respective sections.

This procedure is intended to be compatible with any commercially available hybrid capture kits and protocols.

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	
Required PacBio® kit(s)	Onso™ DNA library prep kit (PacBio® 102-431-400) Or Onso™ fragmentation DNA library prep kit (PacBio® 102-499-100)
	Onso™ Indexed adapter kit (PacBio® 102-431-700)
	Onso™ Library amp kit (PacBio® 102-410-800)
	Onso™ Blocker oligos (PacBio® 102-427-200)
Required PacBio® Protocols	"Preparing Onso™ libraries from fragmented DNA for short-read sequencing" Or "Preparing Onso™ libraries from genomic DNA for short-read sequencing"
Samples per Onso™ library prep kit	Up to 96 reactions
Workflow time	Varies based upon hybrid capture kit
DNA input	
Quantity	10 – 1000 ng per library for Onso™ DNA library prep kit Or 5 – 500 ng per library for Onso™ fragmentation DNA library prep kit

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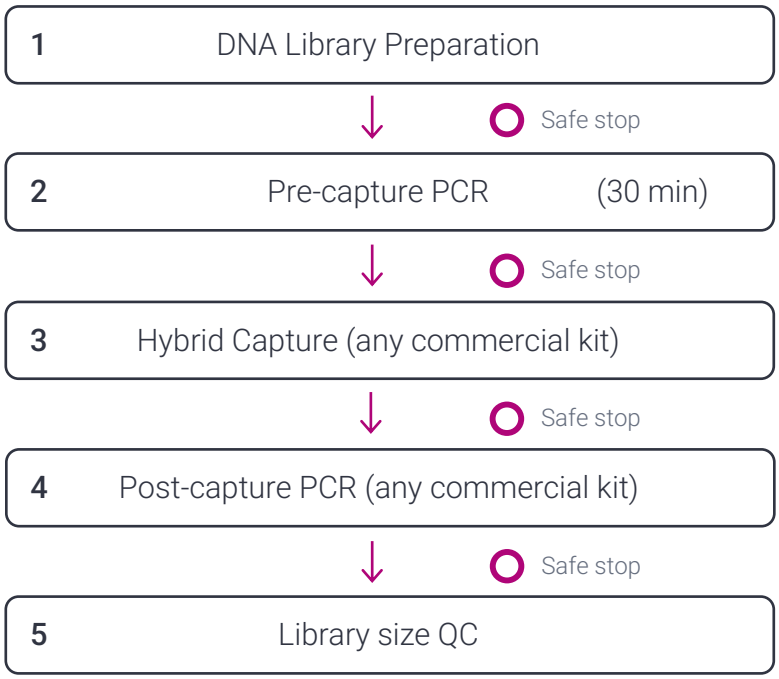
Workflow

This workflow is intended to support commercially available hybrid capture panel products. Although the below workflow and workflow steps are written to support the use of the Onso DNA library prep kit, it can be performed similarly with the Onso fragmentation DNA library prep kit if starting from genomic DNA or DNA that requires fragmentation.



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Required materials and equipment

Materials	
Onso™ DNA library prep kit	PacBio® 102-431-400
Onso™ Indexed adapter kit	PacBio® 102-431-700
Onso™ Library amp kit	PacBio® 102-410-800
Onso™ Blocker oligos	PacBio® 102-431-600
0.2 mL 8-tube strips or 96-well plate	Any Major Lab Supplier (MLS)
Thermal cycler compatible with 0.2 mL 8-tube strips or 96-well plate	Any MLS
Microcentrifuge	Any MLS
Vortex mixer (or bioshaker)	Any MLS
Single-channel or 8-channel pipettes	Any MLS
Timer	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

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General best practices

DNA input

Starting DNA input concentrations should be evaluated with a Qubit fluorometer and respective reagents.

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.

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.

Note that the reagents in the library prep kit are temperature sensitive and the recommendations in the protocol "Preparing Onso™ libraries from fragmented DNA for short-read sequencing" should be followed carefully.

Temperature-sensitive reagents		
Step used	Tube	Reagent
Pre-capture & post-capture PCR	Green	PCR master mix (2x)
	Light blue	Primer mix
Hybrid Capture	Light blue	Onso Blocker oligos

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Thermal cycler programs

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

Pre-capture PCR

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, the library prep performed, as well as the quality of the DNA samples. The table below provides guidance for the number of PCR cycles for step 2 for libraries prepared from high quality input DNA with the Onso DNA library prep kit.

If using reduced quality DNA samples, or if libraries were prepared with the Onso fragmentation DNA library prep kit, then additional PCR cycles may be necessary.

Input DNA (ng)	Number of cycles for PCR program Step 2
5	9
10	8
25	7
50	6
100	5
200	3
500	2
1000	1

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	Hold

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Workflow steps

1. DNA library preparation

To prepare Onso libraries, refer to "Procedure & Checklist – Preparing Onso libraries from fragmented DNA for short-read sequencing", sections 1-3. Instead of conducting "PCR for library amplification and size QC" in that library prep protocol, proceed with "Pre-capture PCR" steps below.

Conversely, if preparing Onso libraries from DNA that requires fragmentation, refer to "Procedure & Checklist – Preparing Onso libraries from genomic DNA for short-read sequencing", sections 1-3. Instead of conducting "PCR for library amplification and size QC" in that library prep protocol, proceed with "Pre-capture PCR" steps below.

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2. Pre-capture PCR

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

Vortex and quick spin the PCR master mix and the Primer mix prior to use.

<table>
<tr><th>✔</th><th>Step</th><th>Instructions</th></tr>
<tr><td></td><td>1.1</td><td>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.
<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>n/a</td><td>Nuclease-free water</td><td>27 µL</td></tr>
<tr><td></td><td>Green</td><td>PCR master mix (2x)</td><td>50</td></tr>
<tr><td></td><td>Light blue</td><td>Primer mix</td><td>5 µL</td></tr>
<tr><td></td><td></td><td>Total volume</td><td>82 µL</td></tr>
</table></td></tr>
<tr><td></td><td>1.2</td><td>Vortex RM1 briefly to ensure a homogeneous suspension.</td></tr>
<tr><td></td><td>1.3</td><td>Quick spin RM1 in a microcentrifuge to collect liquid.</td></tr>
<tr><td></td><td>1.4</td><td>Add 82 µL of RM1 to a 0.2 mL PCR strip tube for each sample.</td></tr>
<tr><td></td><td>1.5</td><td>Add 18 µL of sample library to the strip tube with RM1. Total reaction volume should be 100 µL.</td></tr>
<tr><td></td><td>1.6</td><td>Vortex briefly to ensure a homogeneous suspension.</td></tr>
<tr><td></td><td>1.7</td><td>Quick spin the strip tube in a microcentrifuge to collect liquid.</td></tr>
<tr><td></td><td>1.8</td><td>Run the Pre-capture PCR thermal cycler program.</td></tr>
<tr><td colspan="3">Library cleanup</td></tr>
<tr><td></td><td>1.9</td><td>Add 100 µL of resuspended SPRIselect or Ampure beads only (not to be confused with EDTA/Bead mix) to each tube to perform a 1X cleanup of each sample.</td></tr>
</table>

1.10	Vortex briefly. Check to ensure contents are well mixed.
1.11	Quick spin the strip tube in a microcentrifuge to collect liquid.
1.12	Incubate samples for **5 minutes** at **room temperature.**
1.13	Place tubes on a magnetic separation rack and incubate for **5 minutes**, allowing the beads to separate fully from the solution.
	Bead washing and sample elution
1.14	Slowly pipette off the cleared supernatant without disturbing the beads. **Discard the supernatant.**
1.15	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.
1.16	Repeat the previous step.
1.17	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.
1.18	Remove the tubes containing beads from the magnetic separation rack. **Immediately** add **17 µL** of **nuclease-free water** to each tube and resuspend the beads by pipetting 10 times to ensure beads are in solution.
1.19	Quick spin the samples in a microcentrifuge to collect liquid.
1.20	Resuspend the beads by vortexing briefly.
1.21	Quick spin the samples in a microcentrifuge to collect liquid.
1.22	Incubate the samples for **1 minute** at **room temperature** to elute DNA.
1.23	Place the tube(s) in a magnetic separation rack for **2 minutes**, allowing the beads to collect at the magnet and separate fully from the solution.
1.24	Slowly pipette off **15 µL** of the cleared supernatant without disturbing the beads. Transfer supernatant to a **new tube strip**. Discard the old tube strip with beads.
1.25	For each sample, measure the library size distribution using the PCR-amplified library product with the recommended sizing technology (Agilent 2100 Bioanalyzer, 4150 or 4200 TapeStation, or 5300 or 5400 Fragment Analyzer) following the manufacturer's instructions.
1.26	From the sizing technology output, obtain the calculated average size (bp) from the peak table, and determine the desired amount (ng) to input into hybrid capture. Transfer this necessary amount into clean strip tubes.
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Use the TapeStation or Bioanalyzer sizing technology to approximate the library size distribution, checking for average fragment size, region molarity, and total molarity. Use the Qubit system for quantification of the libraries, as it provides a more accurate quantification. Refer to the desired hybrid capture commercial kit to be used for guidance on appropriate input DNA amounts per sample needed to proceed into hybrid capture.

3. Hybrid capture

During the probe hybridization, use the PacBio Onso blocker oligos to prevent nonspecific capture of off-target sequences. Note that other commercially available kit blockers will not be effective on Onso adapters. When preparing a block mix, if the adapter-specific blocker oligos supplied by another manufacturer are a separate component, switch them out with the Onso blocker oligos. If they are provided premixed with nonspecific blockers, then add the Onso blockers to the supplied block mix reaction and adjust the reaction volume to account for the extra volume of Onso blocker oligos being used (as described below):

- For appropriate use of Onso blocker oligos, add 0.6 µL of Onso blocker oligos per 1 µg of library.
- If the commercially available hybrid capture block reaction instructions state to target a final volume, account for the 0.6 µL blocker oligo volume from the library volume.
- In the instance where the commercially available kit block reaction instructions require concentrating down after preparation of the block reaction, and then reconstituting to a final designated volume, then no adjustment to the library concentration needs to be made to account for the addition of the Onso blocker oligos.

No other changes need to be made from the original hybrid capture protocol for compatibility with other commercially available hybrid capture kits.

SAFE STOPPING POINT - Store according to commercial hybrid capture kit recommendations

4. Post-capture PCR

Post-capture PCR and any necessary library cleanup steps can be completed by using the hybrid capture manufacturer kit reagents and guidelines, making note to switch out the manufacturer primer mix with the Onso amp kit primer mix, and adjusting the PCR annealing temperature to 65°C.

Note that the Onso amp kit primer mix is supplied as a 20X stock intended to target a 1X final concentration within PCR reactions.

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

5. Library size QC

✔	Step	Instructions
	5.1	For each sample, measure the library size distribution using the PCR-amplified library product from section 4 “Post-capture PCR” with the recommended sizing technology (Agilent 2100 Bioanalyzer, 4150 or 4200 TapeStation, or 5300 or 5400 Fragment Analyzer) following the manufacturer’s instructions.
	5.2	For each sample, take **2 µL** of the PCR-amplified library product from section 4 “Post-capture PCR” 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 5.2 can be conducted simultaneously with step 5.1.
	5.3	Once complete, use the product of section 4 above for subsequent clustering and sequencing efforts.

PROTOCOL COMPLETE

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