Preparing multiplexed WGS/MetaG SMRTbell libraries with the Express TPK2.0 for the PacBio Sequel2

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Protocol status: Working
We use this protocol and it's working

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ABSTRACT

The preparation of (meta)genomic libraries using the PacBio Express Template Kit 2.0 (TPK2.0) with the Barcoded Adapter Plate 3.0 at the IMR.

Based upon PacBio protocol 101-696-100 (Dec.2019).

MATERIALS

The following materials list contains those consumables+quantities used specifically at the IMR to complete the present protocol for 48 bacterial-sized genomes (or shallow metagenomes) in 1 SMRTcell.

Raw Sample Quantification

Quant-iT 1X HS dsDNA Kit (1000 samples)
Optical plate
PCR microplates 96-well Bio-Rad
PCR microplates sealing film Bio-Rad
Tips ClipTip 20
Tips ClipTip 200
Tips ClipTip 300
Reservoir
UltraPure water

DNA Verification/Size-Selection

Coastal Genomics Size-Selection gels
PCR microplates 96-well Bio-Rad
Hamilton Tips 300
Tips ClipTip 20
Tips ClipTip 300

Covaris g-TUBE Shearing (optional)

Covaris g-TUBEs

PCR microplates 96-well Bio-Rad
Manual p100 tips

SMRTbell Standard Library Preparation
SMRTbell Express Template Prep Kit 2.0 (18-96 samples)
SMRTbell Barcoded Adapter Plate 3.0
Ampure PB Beads
PCR microplates 96-well Bio-Rad
PCR microplates sealing film Bio-Rad
1.5 mL Eppendorf tubes
PCR tube strips (Axygen, 8 x 0.2 mL)
Ethanol
UltraPure water
Reservoir
Tips ClipTip 20
Tips ClipTip 200
Manual p10 tips
Manual p100 tips
Manual p1000 tips

Final Quantification and Pooling
Quant-iT 1X HS dsDNA Kit (1000 samples)
Optical plate
PCR tube strips (Axygen, 8 x 0.2 mL)
1.5 mL Eppendorf tubes
Tips ClipTip 20
Tips ClipTip 200
Tips ClipTip 300

ABC
Sequel 2 Binding 2.0 and Internal Ctrl Kit 1.0 (24 samples)
Quant-iT 1X HS dsDNA Kit (1000 samples)
Qubit Tubes
PCR tube strips (Axygen, 8 x 0.2 mL)
1.5 mL Eppendorf tubes
1.5 mL LoBind tubes
Ampure PB Beads
Manual p10 tips
Manual p100 tips
Manual p1000 tips

Sequel Sequencing
Sequel 2 Sequencing Kit 2.0 (4 rxn)
SMRT Cell 8M Tray (4 cells)

Sequel Pipet Tips v2
PCR microplates 96-well Bio-Rad
ABgene heat seals
Sequel Mixing Plate (fr.Eppendorf)
Tube Septa
Sequel SMRT Cell Oil
N2 tank

gDNA Shearing with Covaris g-TUBEs

2m

1 Dilute 1 µg gDNA with water into 100 µL to get a final concentration of [M] 10 ng/µL.

2 Transfer gDNA to the g-TUBE and centrifuge (cap up) at 860 x g, 00:05:00 (MBI centrifuge D3024 fixed-angle rotor) to achieve a target mode size of **9-10 kb**. Repeat the spin until the entire gDNA sample has passed through the orifice (this may take 2-3 spins). 5m

Note

The force and time you will need to spin in order to achieve the desired fragment range will vary depending on your centrifuge+rotor combination. Use the guide provided with the g-TUBEs and then conduct tests with your equipment the first time attempting shearing (gel or BioAnalyzer verification will be required).

Note

If after the first spin the sample has not completely passed from the upper chamber to the lower chamber, carefully open the g-TUBE cap to release any pressure. Tighten the g-TUBE cap and perform a second spin (cap-up) at 860 x g, 00:05:00 . If after two spins, there is still a small volume of DNA that has not passed to the lower chamber, perform a third spin at 5000 x g to recover the full volume of sample in the lower chamber.

3 Invert the g-TUBE and repeat the above spin/procedure.

4 Collect the sheared gDNA from the 48 individual g-TUBEs and transfer them to the first 6 columns of a new 96-well PCR plate.

AMPure PB Clean-up

10m

5 Add 0.4X volume of AMPure PB beads to each sheared gDNA sample in the 96-well plate, then seal the plate and pulse vortex it to mix. Finally, quickly spin down the plate (for 2-3 seconds) to collect the bead mixture at the bottom of the plate.

6 Incubate the beads on the benchtop for 00:10:00 at Room temperature . 10m

7 Place the plate on a magnetic bead rack to collect the beads to the side/bottom of the wells and, once cleared, slowly pipette off and discard the cleared supernatant without disturbing the bead pellet.

> Note
>
> Alternatively, the supernatant can be saved in another plate until recovery of DNA is confirmed.

8 Wash the beads with freshly prepared 80% ethanol: 30s

- With the plate on the magnetic rack, slowly fill the plate with sufficient volume of 80% ethanol without waste or cross-contamination.
- Do not disturb the bead pellet.
- After 00:00:30 , slowly pipette off the 80% ethanol and discard.

Note

Using freshly prepared 80% ethanol is ideal in order to achieve optimal results, but stored 80% ethanol in a tightly capped polypropylene tube can be used up to 3 days with minimal adverse performance.

9 Repeat **Step 8** above for the 2nd ethanol wash.

10 Remove any residual 80% ethanol:

- Remove the plate from the magnetic rack and quickly spin to collect the beads.
- Place the plate back on the magnetic rack.
- Pipette off and discard any remaining 80% ethanol.

11 Remove the plate from the magnetic rack and allow the beads to air-dry for 00:02:00. 2m

12 Add 20 µL of Elution Buffer to the beads to elute the DNA: 2m

- Seal the plate and pulse vortex it, followed by a quick spin down.
- Elute the DNA by letting the mixture incubate for 00:02:00 at Room temperature.
- Place the plate back on the magnetic rack and wait for the supernatant to clear completely, then, without disturbing the bead pellet, transfer the supernatant to a new 96-well plate and label as "Clean sheared gDNA".

Sheared gDNA Quantification and Sizing

13 Quantify the **Clean sheared gDNA** using the Invitrogen Quant-iT dsDNA HS assay kit on the fluorescent 96-well plate reader. Use 1 µL of 1:10 diluted gDNA of each sample (1 µL of gDNA + 9 µL of water) to quantify. Use the remaining 9 µL of the 1:10 dilution to evaluate the size distribution of the sheared gDNA on an analytical platform: the IMR uses 0.5% precast gels with 3+20 kb DNA markers on the Coastal Genomics Nimbus Select robot.

SMRTbell Library Construction

15m

14 Remove Single-Strand Overhangs

15m

- Aliquot at least 480 ng of each sheared gDNA and add Elution Buffer to make a total volume of 14.6 µL for each into a new 96-well plate. PacBio low input for the Sequel II System (1 sample) requires a minimum of > 400 ng. PacBio low input for multiplexing with the Sequel II System (2 samples up to 600 Mbp per genome) requires a minimum of > 300 ng per sample.

- Dilute the DNA Prep Additive 1:5 with Enzyme Dilution Buffer as follows:

Component	Volume
Enzyme Dilution Buffer	16 µL
DNA Prep Additive	4 µL
Total Volume	20 µL

- Prepare DNA Prep Master Mix as follows:

Component	1 rxn	50-plex (48+extra)
DNA Prep Buffer	2.33 µL	116.5 µL
NAD	0.33 µL	16.5 µL
Diluted DNA Prep Additive (from above)	0.33 µL	16.5 µL
DNA Prep Enzyme	0.33 µL	16.5 µL
Total Volume	3.32 µL	166.0 µL

- For each sample, add 3.3 µL of the DNA Prep Master Mix to the above 14.6 µL of sheared gDNA.

- Seal the plate and pulse vortex it, followed by a quick spin down.

- Incubate at 37 °C for 00:15:00, then return the reactions to 4 °C. Proceed to the next step.

15 DNA Damage Repair

30m

- Prepare DNA Damage Master Mix as follows:

	Component	1 rxn	50-plex (48+extra)
	DNA Damage Repair Mix v2	0.67 µL	33.5 µL
	Enzyme Dilution Buffer	0.33 µL	16.5 µL
	Total Volume	1.00 µL	50.0 µL

- For each sample, add 1 µL of the DNA Damage Master Mix to the above 17.9 µL of digested gDNA.
- Seal the plate and pulse vortex it, followed by a quick spin down.
- Incubate at 37 °C for 00:30:00, then return the reactions to 4 °C. Proceed to the next step.

16 End-Repair/A-Tailing

40m

- For each sample, add 1 µL of End Prep Mix to the above 18.9 µL of damage-repaired gDNA.
- Seal the plate and pulse vortex it, followed by a quick spin down.
- Incubate at 20 °C for 00:10:00, followed by 65 °C for 00:30:00, then return the reaction to 4 °C. Proceed to the next step.

17 Ligate Barcoded Overhang Adapters

1h 10m

- Add 2 µL of Barcoded Overhang Adapters from the SMRTbell Barcoded Adapter Plate 3.0 to the above 19.9 µL of A-tailed gDNA.
- Seal the plate and pulse vortex it, followed by a quick spin down.
- Prepare Ligation Master Mix as follows:

	Component	1 rxn	50-plex (48+extra)
	Ligation Mix	10.00 µL	500.0 µL
	Ligation Additive	0.33 µL	16.5 µL
	Ligation Enhancer	0.33 µL	16.5 µL

	Component	1 rxn	50-plex (48+extra)
	Total Volume	10.66 µL	533.0 µL

- For each sample, add 10.7 µL of Ligation Master Mix to the above 21.9 µL of barcoded gDNA.
- Seal the plate and pulse vortex it, followed by a quick spin down.
- Incubate at 20 °C for 01:00:00, followed by 65 °C for 00:10:00 to heat kill the ligation reaction, then return the reaction to 4 °C. Proceed to the next step.

Note

If not proceeding to the next step on the same day, the ligation reaction can be stored at 4 °C.

Purification of SMRTbell Templates

18

- Follow **Steps 5-12** above with the addition of 14.7 µL (0.45X) of AMPure PB beads to the above 32.6 µL of final SMRTbell Templates and using 20 µL of Elution Buffer to elute the DNA from the beads.
- Quantify the eluted Purified SMRTbell Templates as above in **Step 13**.
- Purified SMRTbell Templates are stored at -20 °C.

Pooling Calculation and Final Quantification

19

- Pool purified SMRTbell Template libraries in equimolar amounts, if so desired, or use the PB Multiplex Calculator for more complex cases.
- Quantify the final library pool in preparation for ABC below.
- (Optional): Proceed with a gel size-selection (via 0.5% precast gel on the Coastal Genomics Nimbus Select robot, Pippin-Prep, or similar) of the final library pool to ensure absolute removal of any potential inhibitors (see note below).

Note

We have observed low P1 occupancy (and failed runs) due to carryover of impurities during AMPure bead clean-up steps. We have increased P1 productivity by using gel size-selected SMRTbell Template libraries as input for ABC. In our hands, and with the libraries attempted so far, we have not found that the addition of RNase, as recommended by PacBio for resolving low P1, has lead to much success. However, what has improved or completely rescued certain WGS libraries is the use of PB's SMRTbell Enzyme Clean-Up Kit 2.0.

Anneal, Bind and Clean Final SMRTbell Templates (ABC)

20 For primer annealing and polymerase binding, follow the instructions in the SMRT Link Sample Setup print-out. Use the **Sequel II Binding Kit 2.0** and **Sequencing Primer v4** for all libraries.