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Preparing multiplexed 16S/18S/ITS amplicon SMRTbell libraries with the Express TPK2.0 for the PacBio Sequel2 V.1

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Protocol status: Working

We use this protocol and it's working

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Abstract

The preparation of amplicon libraries using the PacBio Express Template Kit 2.0 (TPK2.0) with fusion primers at the IMR.

Based upon PacBio protocol 101-599-700 (Jun.2019).

Materials

The following materials list contains those consumables+quantities used specifically at the IMR to complete the present protocol for 190 amplicons (2 plates) in 1 SMRTcell.

Barcoded PCRs and Gel Verification

As per the IMR standard MiSeq PCR protocol.

Sample Pooling and AMPure Clean-up

Ampure PB Beads

PCR tube strips (Axygen, 8 × 0.2 mL)

1.5 mL Eppendorf tubes

15 mL Falcon tubes

Ethanol

Tips ClipTip 20

Manual p100 tips

Manual p1000 tips

UltraPure water

Quantification and Combining of Cleaned Pools

Quant-iT 1X HS dsDNA Kit (1000 samples)

Qubit Tubes

1.5 mL Eppendorf tubes

various tips

SMRTbell Standard Library Preparation

SMRTbell Express Template Prep Kit 2.0 (18-96 samples)

Ampure PB Beads

1.5 mL Eppendorf tubes

1.5 mL LoBind tubes

PCR tube strips (Axygen, 8 × 0.2 mL)

Ethanol

UltraPure water

Manual p10 tips

Manual p100 tips

Manual p1000 tips

ABC

Sequel 2 Binding 2.1 and Internal Ctrl Kit 1.0 (24 samples)

Quant-iT 1X HS dsDNA Kit (1000 samples)

Qubit Tubes

PCR tube strips (Axygen, 8 × 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



Barcoded PCRs

- 1 Prepare PCRs and verify by Coastal Genomics gels as in the IMR protocol **Preparing multiplexed 16S/18S/ITS amplicons for the Illumina MiSeq**, except only using Plates 1+2 (F1R1 + F1R2) of the PacBio working primers from the IMR protocol **Preparing Combined Indexed Primer Plates (IDT Standard) for the PacBio Sequel2 - Sequel Dual Indices**.
- 2 To pool the 96 samples from the remaining  30 μL of each well in the Aggregated PCR Plate 1, transfer  10 μL of each column into one column of a new 96-well plate named the Amplicon Pool Plate (remaining columns to be used in subsequent poolings). Once complete, pipette  100 μL of each of the 8 wells into one 1.5 mL Eppendorf tube and label it Plate 1 PCR Pool. Repeat this step for the Aggregated PCR Plate 2 and label that tube as the Plate 2 PCR Pool.

AMPure PB Clean-up

10m

- 3 Mix the below volume of AMPure PB beads with  75 μL of the Plate 1 PCR Pool in a new 1.5 mL Eppendorf tube:

Amplicon Target	AMPure Volume
16S amplicon (~1500 bp)	0.6X
18S amplicon (~1850 bp)	0.6X
ITS amplicon (~750 bp)	1.2X

Note

Use  100-150 μL of PCR Pool if many of the samples were poorly amplified on that plate to get enough DNA after beads purification.

- 4 Gently finger mix (tap the bottom of the tube with your finger) the DNA/bead mixture and then incubate the on the benchtop for  00:10:00 at  Room temperature . For improved recovery, you can additionally finger mix the tube 2-3 times during the incubation. After incubation, quickly spin down the tube to collect all the liquid into the bottom of the tube.

10m



- 5 Place the tube on a magnetic bead rack to collect the beads to the side/bottom of the tube 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.

- 6 Wash the beads with freshly prepared 80% ethanol:

30s

- With the tube on the magnetic rack, slowly fill the tube 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.

- 7 Remove any residual 80% ethanol:

- Remove the tube 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.

- 8 Remove the tube from the magnetic rack and allow the beads to air-dry for

2m

 00:02:00 .

- 9 Add  20 μL of Elution Buffer to the beads to elute the DNA:

2m



- Gently finger mix the tube.
- Elute the DNA by letting the mixture incubate for  00:02:00 at  Room temperature .
- Quickly spin the tube to collect the bead mixture, then place it back on the magnetic rack.
- Wait for the supernatant to clear completely, then, without disturbing the bead pellet, transfer the supernatant to a new 1.5 mL Eppendorf tube and label as Plate 1 Cleaned PCR Pool.

10 Repeat Steps 3-9 for the Plate 2 PCR Pool and label the final 1.5 mL Eppendorf tube as Plate 2 Cleaned PCR Pool.

Quantification and Combining of Cleaned Pools

- 11 Quantify both P1+P2 Cleaned PCR Pools using the Invitrogen Qubit dsDNA HS assay as in the IMR standard MiSeq PCR protocol.
- 12 Combine equal molar quantities of both P1+P2 Cleaned PCR Pools in a total mass of at least  350 ng in a new 1.5 mL Eppendorf tube. Adjust the final Combined Pool volume to  23.5 μ L to prepare the SMRTbell library.

Note

In general,  175 ng of each pool is combined together to make total mass of  350 ng . However, the total mass should be adjusted when combining 16S/18S pools (close in size) with ITS pools (which are only half the size or less). For example, a 16S Pool 1 and an ITS Pool 2, which are both at the same concentration (such as), would require  175 ng of Pool 1 and only  87.5 ng of Pool 2 = approx.  263 ng final amount in order to maintain equal molar ratios between the two pools.

SMRTbell Library Construction

15m

13 DNA Damage Repair

30m



- Prepare DNA Damage Repair reaction ("Reaction Mix 1") as follows in one 0.2 mL PCR tube:

Component	Volume
DNA Prep Buffer	3.5 µL
DNA Damage Repair Mix v2	1.0 µL
NAD	0.5 µL
Combined PCR Pool	23.5 µL
Total Volume	28.5 µL

- Gently finger mix the contents of the tube, 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.

14 End-Repair/A-Tailing

1h

- Add 1.5 µL of End Prep Mix to the above 28.5 µL tube of Reaction Mix 1 to make a total volume of 30 µL of "Reaction Mix 2".
- Gently finger mix the contents of the tube, followed by a quick spin down.
- Incubate at 20 °C for 00:30:00 , followed by 65 °C for 00:30:00 , then return the reaction to 4 °C . Proceed to the next step.

15 Adapter Ligation

1h 10m

- Prepare the Adapter Ligation Reaction by adding the following components, in order, to the above Reaction Mix 2 tube:

Component	Volume
Reaction Mix 2	30.0 µL
Overhang Adapter v3	2.5 µL
Ligation Mix	15.0 µL
Ligation Additive	0.5 µL



Component	Volume
Ligation Enhancer	0.5 μL
Total Volume	48.5 μL

- Gently finger mix the contents of the tube, followed by a quick spin down.
- Incubate at $20\text{ }^{\circ}\text{C}$ for 01:00:00 , followed by $65\text{ }^{\circ}\text{C}$ for 00:10:00 to heat kill the ligation reaction, then return the reaction to $4\text{ }^{\circ}\text{C}$. Proceed to the next step.

Note

If not proceeding to the next step on the same day, the ligation reaction can be stored at $4\text{ }^{\circ}\text{C}$.

Purification of SMRTbell Templates

- 16
- Follow **Steps 3-9** above with the addition of $58.2\text{ }\mu\text{L}$ (1.2X) of AMPure PB beads to the above $48.5\text{ }\mu\text{L}$ of final SMRTbell Template and using $20\text{ }\mu\text{L}$ of Elution Buffer to elute the DNA from the beads.
 - Quantify the eluted Purified SMRTbell Template as above in **Step 11**.
 - Purified SMRTbell Template is stored at $-20\text{ }^{\circ}\text{C}$.

Note

Use 1.2X volume of AMPure PB beads for combined 16S/18S with ITS amplicons, or full runs of ITS, to fully recover the smaller ITS SMRTbell templates.

Anneal, Bind and Clean Final SMRTbell Templates

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- For primer annealing and polymerase binding, follow the instructions in the SMRT Link Sample Setup print-out. Use the **Sequel II Binding Kit 2.1** and **Sequencing Primer v4** for



all libraries.