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

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FEB 09, 2023

OPEN ACCESS

DOI:
dx.doi.org/10.17504/protocols.io.j8nlkk89wl5r/v1

Protocol Citation: André M Comeau, Gina V Filloramo 2023. Preparing multiplexed 16S/18S/ITS amplicon SMRTbell libraries with the Express TPK2.0 for the PacBio Sequel2. **protocols.io** https://dx.doi.org/10.17504/protocols.io.j8nlkk89wl5r/v1

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

Created: Mar 16, 2022

Last Modified: Feb 09, 2023

PROTOCOL integer ID: 59525

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 x 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 x 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 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

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 00:02:00. 2m

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 µL 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
	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 °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

16
- Follow **Steps 3-9** above with the addition of 58.2 µL (1.2X) of AMPure PB beads to the above 48.5 µL of final SMRTbell Template and using 20 µL 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 °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

17 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.