Laboratory Operating Procedure

Sequencing library preparation for Illumina instruments using the NexteraXT kit

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LOP-Version: 1

Attachments:

Introduction

The purpose of this LOP is to provide a laboratory protocol for the generation of next generation sequence data from purified bacterial DNA, double-stranded cDNA, boilates or heat-killed cells.

Whole Genome Sequencing of bacteria is now possible with the advent of next generation sequencing technologies. This is achieved by determining the DNA sequence of short fragments of genomic DNA and then computationally reassembling them in the correct order. Once RNA has been converted to double stranded cDNA it can also be sequenced in a similar manner.

The principal is that genomic DNA or ds cDNA is randomly broken into small fragments (typically less than 600bp), each of which has Illumina-specific sequencing primers attached to each end.

Sample Material

Heat-killed cell suspension or purified DNA/ds cDNA

Equipment and Reagents

Equipment

- Single & multichannel pipettes suitable for volumes of 0.1 □l – 1000 □l
- Pipette tips (sterile with and without filters) suitable for volumes of 0.1 □l – 1000 □l
- 96-well microtitre plates
- Plate sealing film
- Centrifuge (capable of spinning 96-well plates between 100 x g and 1100 x g, room temperature)
- Thermocyler for 96-well plates
- Vortex mixer
- SPRIPlate Super Magnet Plate
- 1.7 ml microcentrifuge tubes
- Reagent reservoirs

Reagents

- Agencourt Ampure XP
- Molecular Biology Grade Ethanol
- Nextera XT library kit
- Nextera XT index kit
- MiSeq Sequencing Kit
- 10 mM Tris, 1 mM EDTA (TE)
- Molecular Biology Grade Water

General remarks

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Literature

- *(e.g. Kit handbooks, publications)*

Before getting started

- Concentrations of DNA or cDNA in samples should be normalizied to 0.2 ng μl^{-1} by quantifying as per the APHA DNA quantification LOP and diluting accordingly with molecular biology grade water.

- Remove from freezer and thaw the appropriate reagents from a Nextera XT kit: Tagment DNA Buffer, Amplicon Tagment Mix, Nextera PCR Master Mix, Resuspension Buffer and index primers as required.

- Remove the following from fridge and allow to come to room temperature: Neutralize Tagment buffer and Ampure XP.

Procedure

1. - **Library Preparation**

- Pipette 10 µl of Tagment DNA Buffer into the required wells in a new 96-well PCR plate, followed by 5 µl of Amplicon Tagment Mix.

- Add 5 µl of 0.2 ng μl^{-1} sample DNA or cDNA. Cover plate with adhesive film and vortex briefly to mix. Spin down briefly in centrifuge.

- Place the plate in a thermocycler and run the following program (ensuring that the thermocycler lid is heated during the incubation):
    - 55 °C for 5 minutes, hold at 10 °C.

- Once the sample reaches 10°C immediately remove the plate from the thermocycler, spin down briefly, remove the film seal and add 5 µl of NT Buffer to each well. Replace the film and vortex to mix. Incubate at room temperature for 5 minutes.

- Briefly spin down the index primers and arrange the appropriate index primers so that i7 primers can be pipetted into plate columns and i5 primers can be pipetted into rows (as shown in Appendix 1). If there are fewer than 96 samples reduce the numbers of index tubes accordingly.

- Ensure that the combination of adapter primers used for each sample is recorded correctly in the 'Plate Layout and Indexes' tab of the NexteraXT_worksheet.

- Briefly spin plate and remove film. Add 13 µl Nextera XT PCR Master Mix to each well. Using a multichannel pipette add 5 µl of i7 adapter primers to each row of the sample plate. Lids removed from primer tubes should be discarded. Similarly add 5 µl of i5 adapter primers to each column of the sample plate. Cover with adhesive film and vortex to mix. Spin down briefly in centrifuge. Replace lids on primer tubes with new ones: orange for i7 primers and white for i5 primers.

- Perform PCR using the following program on a thermal cycler (ensuring that the thermocycler lid is heated during the incubation):
    - 72°C for 3 minutes
    - 95°C for 30 seconds
    - 12 cycles of:
    — 95°C for 10 seconds
    — 55°C for 30 seconds
    — 72°C for 30 seconds
    - 72°C for 5 minutes
    - Hold at 10°C

- Remove the plate from the thermocycler, spin down briefly in a centrifuge and remove the film. Add 30 µl Ampure XP bead suspension (ensure the suspension is thoroughly mixed before use) to each well. Re-cover and vortex. Incubate at room temperature for 5 minutes.

- Spin down briefly and transfer the plate to the 96-well magnetic stand. When the supernatant has cleared, remove and discard it from all wells using a multichannel pipette.

- Whilst keeping the plate on the magnetic stand wash the beads twice with freshly prepared 80% ethanol as follows: add 200 µl of freshly prepared 80% ethanol to each sample well but do not resuspend the beads. After 30 seconds carefully remove and discard the supernatant.

- After the second wash, ensure that all ethanol has been removed and then allow the beads to air dry for 15 minutes.

- Remove the plate from the magnetic stand and add 23 µl of Resuspension Buffer (from the Nextera XT kit). Cover with film and vortex to mix. After 5 minutes incubation at room temperature briefly spin in a centrifuge to collect the samples at the bottom of the wells.

2. - **Library Normalization and Pooling**

- Library concentrations should be quantifying as per the APHA DNA quantification LOP.

- Libraries can be directly diluted and pooled as required to a final concentration of 2nM in a single-step process

- Perform the dilutions into silanized 1.7 ml microcentrifuge tubes. Pooled libraries and the 96-well plate containing the remaining libraries can be stored in a refrigerator until required.

- Pooled samples are ready for running on the Illumina MiSeq