Samples Preparation for Foodborne Pathogen Detection and Tracking project

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Foodborne Pathogens Detection and Tracking

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ABSTRACT

Our foodborne Pathogen detection and tracking project main goal is to create openly available FAIR workflows capable of detecting and tracking all kinds of pathogens in any metagenomics sample, specifically in food serving public health purposes. To test our workflows, our first step was to create our test datasets. That are mainly food samples, which are spiked with particular strain of a pathogen and the workflows should detect it and tack its trace among all samples.

The following steps will show you how our samples, as test datasets, are prepared

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We use this protocol and it's working

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Materials and Methods

1 5d

Bacteria stock cultures Salmonella enterica subsp. Houtenae DSM 9221, Salmonella enterica subsp. Enterica DSM 554, Salmonella enterica subsp. Salamae DSM 9220, Campylobacter jejuni subsp. Jejuni DSM 4688, Campylobacter lari subsp. Lari DSM 11375, Listeria monocytogenes DSM 20600, Listeria monocytogenes DSM 19094 and Listeria ivanovii subsp. Ivanovii DSM 20750 have been used for sequencing the pure strains.
Salmonella have been grown on a XLD plate and enriched in buffered pepton water at 37 °C for 24:00:00. Listeria have been grown on Brilliance ™ Listeria agar plate and enriched in half fraser broth at 30 °C for 48:00:00. Campylobacter has been cultured on Brilliance CampyCount Agar, Campylobacter Selective Blood Free Agar (CCDA) plates and enriched in Preston selectiv bouillon at 42 °C for 24:00:00.
Salmonella have been used in spiked samples with chicken meat with and without enrichment. For the sequencing setups without enrichment bacteria from fluidic cultures have been used, 1 ml has been centrifuged and the pellet together with the meat was used for DNA isolation. In the enrichment step the spiked Salmonella samples were incubated at 37 °C for 24:00:00 before DNA isolation.

DNA isolation

2 1h

Genomic DNA of the samples was extracted by using the STAR BEADS Pathogen DNA/RNA Extraction kit (CYANAGEN SRL, Bologna, Italy) a magnetic bead based nucleic acid extraction method. Samples have been incubated at 56 °C for 01:00:00 with lysis buffer and Proteinase K 20 ng /µl followed by extraction according to the manufacturer‘s instructions.

DNA quality and quantity

3 DNA concentrations were measured with the Qubit® 4.0 Fluorometer (Thermo Fisher Scientific) using the double-stranded DNA (dsDNA) High-Sensitivity (HS) assay kit (Thermo Fisher Scientific), following the manufacturer's protocol. The quality was evaluated with a Nanodrop® 1000 (Themo Fisher Scientific), assessing the 260/280 nm and 260/230 nm ratios. 260/280 and 260/230 ratios were close to the expected ranges 1.8–2.0 and 2.0–2.2, respectively.

Nanopore sequencing

1d 0h 53m

4 Nanopore sequencing was performed by Oxford Nanopore sequencing technology for genomic DNA by using the Native barcoding genomic DNA (with EXP-NBD104, EXP-NBD114, and SQK-LSK109) protocol (Oxford Nanopore). In the first step, DNA repair and end-preparation for the adapter ligation were performed. The NEBNext FFPE DNA Repair Mix (NEB) and NEBNext Ultra II End repair / dA-tailing Module (NEB) reagents were prepared according to the manufacturer's instructions. DNA tested samples were diluted with nuclease-free water according to the protocol.

5 5m For R9.4.1 flow cells, transfer 1 µg (or 100-200 fmol) genomic DNA into a 1.5 mL Eppendorf DNA LoBind tube and adjusted to the volume of 48 µL with nuclease-free water in 1.5 mL Eppendorf DNA LoBind tubes, mixed thoroughly by flicking the tube (to avoid DNA fragmentation), and spun down. 3.5 µL of NEBNext FFPE DNA Repair Buffer, 2 µL of NEBNext FFPE DNA Repair Mix, 3.5 µL of Ultra II End-prep reaction buffer and 3 µL of Ultra II End-prep enzyme mix were combined. After gentle mixing by flicking the tube, it was spun down and incubated for 5 min at 20 °C following with incubation for 00:05:00 at 65 °C followed by the sample purification by using Agencourt AMPure XP beads (Beckman Coulter) was performed.

6 7m 30s Agencourt AMPure XP beads were resuspended by vortexing, and the DNA sample was transferred into a 1.5 mL Eppendorf DNA LoBind tube. A total of 60 µL of resuspended beads were added, the sample was mixing by flicking the tube, and incubated on a Hula mixer for 00:05:00 at **room temperature**. For the washing step, 70% ethanol solution was freshly prepared by using nuclease free water. The sample was spun down and placed on a magnet unit until a pellet was formed and with the eluate clear and colorless. Although the tube is on a magnet unit, the supernatant was pipetted off, and the pellet was washed twice with 200 µL of freshly prepared 70% ethanol. After a brief spin down, the sample was placed on a magnetic unit, residual ethanol was pipetted off, and the pellet was dried for about 00:00:30. Then the pellet was resuspended in 25 µL of nuclease-free water for 00:02:00 at **room temperature**, placed on a magnetic unit, and clear and colorless DNA sample was separated from the pellet. The DNA sample (1 µL) was quantified by using a Qubit 4 fluorometer.

7 The repaired and end-prepped DNA was taken forward into the native barcode ligation step. 500 35m 30s ng of the end-prepped DNA was combined with Native Barcode 2.5 µL and NEB Blunt/TA Ligase Master Mix 25 µL and incubated for 00:10:00 at **room temperature**.
Then, the sample purification by using Agencourt AMPure XP beads (Beckman Coulter) was performed as described before. The pellet was resuspended in 26 µL of nuclease-free water. The DNA sample (1 µL) was quantified by using a Qubit 4 fluorometer. Equimolar amounts of each barcoded sample was pooled into a 1.5 mL Eppendorf DNA LoBind tube, ensuring that sufficient sample is combined to produce a pooled sample of 700 ng total for the R9.4.1 flow cell. The pooled and barcoded DNA was quantified by the Qubit fluorometer and continued with the adapter ligation and clean-up step.
AMII adapter mix (OxfordNanopore) and Quick T4 Ligase (NEB) were spun down and placed on ice. The ligation buffer (LNB, Oxford Nanopore), elution buffer (EB, Oxford-Nanopore), and long fragment buffer (LFB, Oxford Nanopore) was thawed at **room temperature**, spun down, and mixed by pipetting. 700 ng pooled barcoded sample (65 µL) was combined with Adapter Mix II (AMII) 5 µL , NEBNext Quick Ligation Reaction Buffer (5X) 20 µL and Quick T4 DNA Ligase 10 µL , gently mixed by pipetting, spun down, and incubated for 00:10:00 at **room temperature**. The mixture was purified by using AMPure XP beads; 50 µL of resuspended beads were added to the DNA sample, mixed by pipetting and incubated for 00:05:00 on a Hula mixer at **room temperature**, then spun down and placed on a magnet unit. After pellet formation, the supernatant was pipetted off. The pellet was washed with 250 µL of LFB, then the beads were resuspended by pipetting and placed on a magnet for a pellet formation. A clear and colorless supernatant was removed and the washing procedure was repeated. After the second washing with LFB, the sample was dried for 00:00:30 , resuspended in 15 µL of EB and incubated for 00:10:00 at **room temperature**. Then the sample was placed on a magnetic unit to form a pellet until the supernatant is clear and colorless. A total of 15 µL of the sample were retained in a 1.5 mL DNA LoBind tube and 1 µL was quantified by using a Qubit 4 fluorometer. The prepared DNA library was then stored on ice until it was loaded into a flow cell.

8 For the flow cell loading procedure, the sequencing buffer (SQB), loading beads (LB), flush tether 5m (FLT), and flush buffer (FB) all from OxfordNanopore, were thawed at **room temperature** and placed on ice. SQB, FB, and FLT were mixed by vortexing and spun down.
To prepare the flow cell priming mix, 30 µL of thawed and mixed Flush Tether (FLT) was mixed directly to the tube of thawed and mixed Flush Buffer (FB).
The priming port was opened and check for a small air bubble under the cover by drawing back a small volume to remove any bubbles. 800 µl of the priming mix was added into the flow cell via the priming port, avoiding the introduction of air bubbles and incubated for 00:05:00 .
During this time, prepare the library for loading by following the steps below.
For the preparation of the library for loading 37.5 µL Sequencing Buffer II (SBII) was mixed

with 25.5 µL Loading Solution (LS) and 12 µL DNA library in a total volume of 75 µL. For completing the flow cell priming the SpotON sample port was opened as well and 200 µL of the priming mix was loaded into the flow cell via the priming port, avoiding the introduction of air bubbles.
The DNA library was loaded directly after this step on a R9.4.1 MinION Mk flow cell (OxfordNanopore). SpotON sample port cover and priming port were closed and sequencing was started. The sequencing device control, data acquisition and real-time basecalling were carried out by the MinKNOW software the MinION Mk1C device.

Several independent sequencing runs and data collection procedures were performed in this project.