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Quality control and assurance for S. Typhi amplicon sequencing

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Abstract

The following quality control points should be followed alongside the amplicon sequencing protocol for genotyping *Salmonella* Typhi and help to ensure the protocol has been performed correctly and the results are valid.

1. Lab setup

(i) A unidirectional workflow should be used to reduce the potential for contamination i.e. Sample preparation room (DNA extraction) > Reagent preparation room (PCR master mix) > Post-PCR room (sequencing). If you cannot use separate rooms for these steps, using separate benches and ensuring you keep the areas clean should be sufficient.

(ii) Gloves should be changed before leaving and entering each laboratory section and each time contaminating DNA is potentially encountered.

(iii) The equipment should not be moved between the sample and reagent preparation rooms.

2. Equipment

(i) The laboratory should have a schedule for maintaining equipment including the setup, calibration, repair, record-keeping, and normal operation of all equipment. This is to verify that the equipment is functioning properly.

(ii) Temperatures of equipment (incubators, water baths, heat blocks, refrigerators, laboratory freezers) should be monitored and recorded at least once a day for each workday in use. Alternatively, temperatures may be monitored continuously using a computer-based alarm system.

(iii) The block temperature of a thermocycler should be tested at least twice a year by the laboratories or under the maintenance contract.

(iv) Use a PCR machine with a heated lid or layer/ use paraffin oil over the master mix to ensure the sample does not evaporate.

(v) The centrifuge should be balanced before use to increase bearing life and minimise vibrations that can unsettle concentrates. Separate refrigerators and freezers for samples, reagents, and final amplification products should be maintained in the designated laboratory room.

3. Methodology

(i) General

- All work surfaces and pipettes should be cleaned with a disinfectant and/or 70% ethanol before and after each use.
- Tubes containing stored samples and reagents should be centrifuged briefly (~ 30 seconds) before opening to ensure that all liquids are at the bottom of the tubes.

- Do not vortex any enzymes. Use Insulated bench-top coolers or ice to keep the enzyme cold while setting up the experiment.
- Molecular-grade water or its equivalent from commercial sources should be used for all assays.
- The use of barrier tips and aerosol-resistant tips, both of which minimize cross-contamination of samples during pipetting is recommended.
- Amplified products should always be kept in a separate freezer from reagents (such as master mix), samples, and sample concentrates.

(ii) PCR

- Every lot of new oligos should be checked for contamination with a PCR positive and negative control.

  o The positive control should produce expected bands during electrophoresis. If no amplification bands are observed in the positive control, verify the primer sequence or contact the primer supplier (assuming all other reagents are functioning properly). Use a reference strain (e.g., *S.* Typhi H58 or *S.* Typhi CT18) as a positive control for the genotyping panel. For the MDR panel, use g block amplicons for each plasmid target.

  o No positive results should be detected in the negative control. If there are amplification bands present in the negative control, it indicates contamination in the primers, and the contaminated primer should be discarded, (assuming other reagents are not contaminated and working properly).

- Use appropriate controls with every PCR run to ensure the accuracy of the PCR results.
  - o Positive control: Use the tviD amplicon (g blocks) as the positive control.
  - o Negative control: Include a no-template control (nuclease-free water) with every PCR run.
- Store LongAmp ® Taq 2X Master Mix at proper temperature to ensure the polymerase activity is not lost. The master mix is suitable for 15 freeze-thaw cycles at -20°C and stable for three months at 4°C for frequent use.
- Use thin-walled PCR tubes because they provide the best heat transfer ensuring that the reaction volume reaches its specified temperature in the shortest amount of time, thereby improving specificity and reproducibility.

(iii) Gel electrophoresis/tape station

- This step is optional but is recommended as a quality control step before proceeding with sequencing.

- Run the PCR products on a 1% agarose gel for gel electrophoresis or on Tapestation using D5000 screentape and reagents to check the amplification of PCR products.

- The positive control should always show amplification bands. If there are no bands, it indicates that the samples testing negative by PCR may not be truly negative and all samples should be retested.

o Before repeating check the cycling conditions on the thermocycler are correct and that none of the reagents have expired or been stored incorrectly. If there is amplification in the negative control, it indicates contamination of one or more reagents or contamination during pipetting. In this case, the samples testing positive by PCR may not be truly positive and should be repeated.

o If repeating the negative control still shows a band in the gel/Tapestation, do a thorough decontamination of all pipettes, equipment and the work space, then try using fresh reagents. When repeating the testing after deep cleaning the lab, test the negative control first to confirm as negative and then repeat the samples.

(iv) Sequencing

a. Bead cleanup (this applies to all the bead clean-up steps throughout the protocol)

- Before using the Ampure beads make sure they are vortexed well to ensure all beads are completely suspended in the solution. If the beads are not vortexed well, they will remain settled at the bottom and there will not be enough beads to capture all the DNA for clean up resulting in product loss.

- During the washing steps, avoid touching the magnetic bead pellet on the side of the well to minimise product loss.

- After the final wash with ethanol, air dry the samples to remove any traces of ethanol. Any residual ethanol carryover will interfere with sequencing. Do not over-dry the samples to the point where the pellet appears cracked as it would reduce the recovery of DNA. This is because DNA fragments bind strongly to the beads and may be difficult to elute off after drying onto the beads completely.

b. Qubit quantification

- Use Qubit dsDNA broad range kit for this protocol. Store the buffer and reagents from the kit at room temperature and the standards at 4°C. Reagent will freeze if stored below room temperature and temperature fluctuations can affect the

accuracy of the assay. The kit is stable for 6 months when stored at directed conditions.

- Use only thin-wall, clear, 0.5-mL PCR tubes from the brands recommended by the manufacturer which include Qubit® assay tubes (Cat. no. Q32856) or Axygen® PCR-05-C tubes (part no. 10011-830).
- Do not label the side of the tube as this could interfere with the sample reading. Label any information on the tube cap.
- Do not hold the assay tubes in your hand before reading as it will warm the solution and result in a low reading.
- For each assay perform calibration with the standards and insert the tubes in the correct order i.e. Standard 1 followed by Standard 2. The value of Standard 2 should be at least 10- to 50-fold higher than the value of Standard 1.
- If the values are incorrect or the 'standards incorrect' message is displayed then check that you used the correct standards with the broad range kit, used 10 µL of the standard and 190 µL of the Qubit® dye working solution in the appropriate tubes and tubes were inserted in the correct order.
- The Qubit fluorometer will usually read the sample concentration as out of range (too low) for negative controls or samples that do not have amplification in PCR. If this is unexpected then check that products have amplified in PCR or if the amplicons were lost during the bead cleaning process.

c. 200fmol calculation

- Use the template provided with the protocol for calculation. Confirm the final concentration of DNA required using the appropriate calculator. Round off the calculations to remove decimal values, which may lead to pipetting errors.
- Save any leftover PCR products until the results have been analysed.

d. End Prep

- It is important that the NEBNext Ultra II End Prep Reaction Buffer is thoroughly mixed by vortexing to dissolve any precipitates.
- Do not vortex the NEBNext Ultra II End Prep Enzyme.
- If the End prep DNA will be left in the thermocycler for an extended period make sure the program is set to lower the temperature to cool the products after the incubation cycles are completed.
- The protocol can be paused at this point but store the end-prepped DNA in the low-bind tubes.

e. Barcoding and Ligation

- Always check that the barcoding kit is compatible with the flow cell. For this protocol, we use the native barcoding kit SQK-NBD114 which is only compatible with R10.4.1 flow cells.
- If using the 96 barcode plate, spin down the plate briefly (~30 seconds) to make sure that no liquid is stuck to the film sealing the barcode plate.
- Note down the barcodes that you are using for your samples. Be careful while dispensing the barcodes into the tube to avoid splashing or creating aerosols to prevent contamination.
- Thoroughly mix the EDTA to stop the barcoding reaction. Use the correct volume of EDTA based on the concentration provided with the barcoding kit.

f. Flow cell prep and loading

- Store the flowcell at 4°C in a flat position. Do not freeze the flow cell.
- Perform the flowcell check before every run. The quality of the flow cell will be shown as one of the three outcomes on the Sequencing Overview page:

  o Yellow exclamation mark: The number of sequencing pores is below warranty. If this is the first time using the flow cell and it is within the warranty period (within 3 months of receipt), ONT can replace it for free, please contact your supplier for more details.

  o Green tick: The number of sequencing pores is above warranty and ready for sequencing.

  o Question mark: A flow cell check has not been run on the flow cell during this MinKNOW session.
- Use only bovine serum albumin and not any other albumin types (e.g. recombinant human serum albumin).
- The Native Adapter (NA) used in this kit and protocol is not interchangeable with other sequencing adapters.
- Take care when drawing back the buffer from the flow cell. Do not remove more than 20-30 µl, and make sure that the array of pores is covered by buffer at all times. Introducing air bubbles into the array can irreversibly damage pores.
- The Library Beads (LIB) tube contains a suspension of beads. These beads settle very quickly. Ensure they are mixed immediately before use.

- Install the light shield on your flow cell as soon as the library has been loaded for optimal sequencing output.
- It is recommended to leave the light shield on the flow cell when the library is loaded, including during any washing and reloading steps. The shield can be removed from the flow cell when the library has been removed.

g. Software

- Ensure the Laptop is connected to Wifi so that the software is connected to the Nanopore cloud system through telemetry. This is needed for pore scans that take place during the sequencing run.
- Ensure the laptop has enough battery supply for the sequencing run. It is safest to connect it to the charger for the duration of any sequencing run.
- Check the system messages in MinKNOW to see if the laptop has enough space for the sequencing run to complete. If not, move the data from the previous sequencing runs to make space on the system.
- When a sequencing run is complete, check the MinKNOW run report to confirm that you have enough data for analysing your results. If data is insufficient, you may start the sequencing run again for the same library already loaded on the flowcell.
- To restart the sequencing library from the previous run, select "join existing" on MinKNOW and choose the same folder to save your sequencing data, as in the previous run.

h. Washing flowcell

- While washing the flowcell after the sequencing run, slowly load the flowcell wash mix to ensure that all library contents are pushed out and the pores are well covered with the wash mix.
- Remove any waste from the waste port to ensure no liquid is left behind and the waste ports are not blocked before storage.