Foreword

The WG has been established by the European Commission with the aim to promote the use of NGS across the EURLs' networks, build NGS capacity within the EU and ensure liaison with the work of the EURLs and the work of EFSA and ECDC on the NGS mandate sent by the Commission. The WG includes all the EURLs operating in the field of the microbiological contamination of food and feed and this document represents a deliverable of the WG and is meant to be diffused to all the respective networks of NRLs.

Supporting document for preparing high quality DNA for Whole Genome Sequencing

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1. Introduction

In the framework of the activities of the Inter EURLs Working Group on Next Generation Sequencing, the need for guidelines for checking the quality of extracted DNA to be used for sequencing was highlighted. This document is meant to provide guidance in this crucial preliminary step of the laboratory work to produce NGS data.
It was agreed to start considering the three pathogens *Listeria monocytogenes*, *Salmonella* and Shiga toxin-producing *E. coli* (STEC), on which the Joint EFSA-ECDC molecular typing data collection is currently focused, with the aim of expanding the topic to other pathogens in a second step.

DNA extraction

A document dedicated to wet-lab procedures performed as preliminary steps for Whole Genome Sequencing (WGS), including DNA extraction protocols, was prepared in the framework of the activities of this working group and is available at the following link:
https://www.iss.it/documents/5430402/0/Guidance+document+for+WGS-laboratory+procedures.pdf/2d0f3edb-7a46-754e-a0b3-2c4612b27de8?t=1637739547899
DNA extraction protocols based on different principles have been shown to extract suitable DNA in terms of purity and concentration to be used for downstream protocols needed for WGS. Nevertheless, some common practices such as preparing boiling lysates to be used as DNA preparation or DNA extraction protocols based on salt and ethanol precipitation are generally not recommended to be used for WGS (1). In detail, protocols based on magnetic-beads extraction and spin-columns kits, either used through automatic workstations or by single-sample treatment, were proved to produce DNA of adequate quality for WGS. Nevertheless, performing a quality check of the extracted DNA before using it for WGS application is advisable to avoid submitting low quality samples to sequencing, which could provide issues in the library preparation and in the sequencing protocol. Despite requiring some additional time, this is meant to avoid eventual repetition of shipment of the DNA samples to the sequencing centre and double treatment of samples for sequencing, finally resulting in saving time, which is crucial especially in situation of emergency such as, for example, during the investigation for a potential outbreak.

For this reason, here we summarize the main steps considered crucial for preparing pure and high quality DNA for WGS of pathogenic bacteria.

Selection of an isolated bacterial colony for DNA extraction
When testing bacteria, selecting a single isolated colony for DNA extraction is the first preliminary step. A minimal contamination of the starting material, either with other bacterial species or with other isolates of the same species potentially present in the sample, would impair the genomic analyses. For this reason, it is important to start from a plate containing a pure culture of the strain intended for genomic sequencing, from where selecting a single colony for the chosen DNA extraction protocol.

Control of the main genetic features characterizing the isolate
In order to avoid unintended sequencing of non-target strains present in the initial culture or sequencing of strains which could have eventually lost virulence genes (e.g. loss of Shiga-toxin coding genes from STEC

isolates) checking the presence of the main characteristics of the strains by PCR in the single colony selected for sequencing, eventually directly in the DNA extracted for sequencing, is advisable. This is especially important for pathogens for which loss of virulence genes is occasionally reported (e.g. STEC).

Plasmids

It has been recently shown that some DNA extraction protocols are impaired in terms of plasmid extraction. Virulence genes of pathogenic bacteria and antimicrobial resistance genes are often carried on large plasmids. Impaired plasmid extraction would still allow strain-by-strain comparison through cgMLST, as the genes representing the target of such analysis are harboured on the chromosome, but would hinder a proper characterization of the isolate. For this reason, it is advisable to avoid salting-out extraction methods based on alcoholic precipitation, in order to obtain a complete picture of bacterial genome through WGS (2).

DNA manipulation

It's important to avoid fragmentation of DNA, for example minimizing the steps of freeze/thawing. Fragmentation would impact the result of sequencing, especially when long-reads technologies are applied (3).

Elution buffers

Library preparation kits used for DNA sequencing generally make use of enzymes whose activity could be impaired by the presence of EDTA in the DNA solution. On the other hand, many DNA extraction kits involve a final elution/rehydratation step in EDTA-containing buffers. It was demonstrated that 10mM Tris-HCl (pH 8.5) or nuclease-free water can be used as valid alternatives not causing problems in downstream WGS protocols (2).

DNA quality check

Despite no specific quality criteria are generally required for input DNA by library preparation kits, assessing the quality of the DNA sample is advisable after extraction, to ensure its pureness and inspect fragmentation and to avoid problems in downstream protocols needed for WGS.
As a quality check, agarose gel or capillary electrophoresis can be performed, to inspect if fragmentation appears with smeared bands at low molecular weight and to visualize absence of evident rRNA bands. Spectrophotometric analysis of the DNA can also be performed to assess the ratio between the absorbance at 260 nm and 280 nm. The value of the 260/280 absorbance ratio should within the range 1,75-2,05 (3).

DNA concentration

The concentration of the DNA samples must be measured to fulfil minimum criteria of the library preparation kits used for WGS. The DNA extraction kits available on the market are generally able to extract adequate quantity of DNA of the target organisms for the majority of the library preparation kits. In detail, some WGS protocols require only 1 ng of DNA in 5 µl (1), but other may require 100 ng of DNA in 8 µl (https://www.iss.it/documents/5430402/0/Guidance+document+for+WGS-

laboratory+procedures.pdf/2d0f3edb-7a46-754e-a0b3-2c4612b27de8?t=1637739547899). When no specific information is provided by the sequencing service, it is advisable to prepare an amount of DNA which could serve for more than one assay with any sequencing protocol, which currently corresponds to 200 ng of pure DNA in 16 µl of nuclease-free water or 10mM Tris-HCl (pH 8.5).
The concentration should be measured for each DNA sample using a fluorometric system (e.g. the Qubit fluorometer) and the corresponding reagents kit, avoiding spectrophotometric methods measuring absorbance, which provide useful information in the quality check step but are less accurate for DNA quantification.

If the abovementioned quality and quantity parameters are not fulfilled, the DNA isolation step should be repeated and troubleshooted.

Bibliography

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2- Nouws S. et al "Impact of DNA extraction on whole genome sequencing analysis for characterization and relatedness of Shiga toxin-producing Escherichia coli isolates" Scientific Reports (2020) 10:14649

3- ISO 23418:2022 "Microbiology of the food chain — Whole genome sequencing for typing and genomic characterization of bacteria — General requirements and guidance"; Technical Committee: ISO/TC 34/SC 9 Microbiology; Publication date: 2022-06