EUCHINASAFE
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Deliverable number: D4.8

Deliverable title: Providing harmonised SOPs describing the application of WGS-based surveillance to support risk management

Work Package No.:	WP 4
Lead beneficiary:	Teagasc
Due date (project month - dd/mm/yyyy):	M42
Actual delivery date (project month - dd/mm/yyyy):	M60 – 19/08/2022

Delivering an Effective, Resilient and Sustainable EU-China Food Safety Partnership

Grant Agreement number:

727864 — EU-China-Safe

Acknowledgements

EU-China-Safe aims at reducing food fraud and improving food safety through focusing on improving food legislation, food inspection and increasing access to information across Europe and China. State-of-the-art technologies including a virtual laboratory will create a unique space to share and demonstrate best practice. The use of innovative technologies will result in improved detection of adulteration of food products as well as increased traceability and transparency of global supply chains.

The project runs from September 2017 to August 2021. It involves 33 partners and is coordinated by QUB (The Queen's University of Belfast, UK).

More information on the project can be found at www.euchinasafe.eu (website in construction)

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Document control page:

Deliverable Title	**Providing detailed SOPs describing the application of WGS-based surveillance to support risk management across the farm-to-fork continuum**
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Version number (VX.Y)	3
Version date (dd/mm/yyyy)	19/08/2022
Last modified by (person and organisation name)	
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Revision history:			
Version	**Date**	**Modified by**	**Comments**

Nature of the deliverable		
ORDP	Open Research Data Pilot	
R	Document, report (excluding the periodic and final reports)	R
DEM	Demonstrator, pilot, prototype, plan designs	
DEC	Websites, patents filing, press & media actions, videos, etc.	
E	Ethics	
OTHER	Software, technical diagram, etc.	

Dissemination Level		
PU	Public, fully open, e.g. web	
CO	Confidential, restricted under conditions set out in Model Grant Agreement	CO
CI	Classified, information as referred to in Commission Decision 2001/844/EC	

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1. SUMMARY

In this deliverable, the details describing the technical steps required to purify bacterial/viral nucleic acid; produce libraries suitable for routine sequencing and undertake short-read high accuracy whole genome sequencing in advance of its subsequent analysis using a harmonised bioinformatics SOP (described in **D4.7**) were developed. The aim of the task was to provide partners in Europe and China with well described, tested and validated SOPs covering bacterial culture, genomic DNA (gDNA) extraction, quantification, library preparation, quality assessments, Illumina MiSeq loading and operation, and WGS pipeline and output analysis.

Furthermore, in light of the covid-19 pandemic, additional protocols were quickly developed and focused on the sequencing of viruses of importance to human health, in this case SARS-CoV-2. These methods are also suitable for use with other viruses of food safety importance.

These methods will enable reproducible and high quality purification of gDNA, library preparation and sequencing of bacteria and enteric viruses in line with approaches being used by competent authority laboratories in Europe and China. In this way, the outputs from this deliverable can support risk assessment and subsequent management of food safety issues, should they arise.

This deliverable is focused only on describing the development of laboratory-based wet microbiological techniques to prepare bacterial DNA for sequencing and the subsequent implementation of these methods was tested in a trial among partners and described in the **D4.7** document.

2. INTRODUCTION

Whole Genome Sequencing (WGS) is a molecular technique whereby purified DNA from a bacterium of interest is fragmented and undergoes a protocol known as library preparation. The prepared DNA is then sequenced on a dedicated platform which generates an output of genomic sequences. The sequencing data generated can be used to identify single nucleotide differences (single nucleotide polymorphisms or SNPs) which describe allelic mutations, allowing the differentiation between genomes of organisms, or simply providing a deeper understanding of their genetic makeup (Land et al., 2015). The application of WGS can provide a range of information, including the presence of mobile genetic elements (MGE e.g., plasmids/bacteriophages), virulence factors such as toxin-encoding genes, or may be used to examine DNA modifications and methylation profiles of foodborne pathogens.

Whole Genome Sequencing may be carried out on a number of platforms including Illumina and Oxford Nanopore Technology. Illumina is the preferred platform for short reads, providing quick turnaround of high-quality results at a relatively low cost (Mitchell et al., 2021), while Oxford Nanopore Technology sequencing, on devices such as MinION, are used for single molecule long read sequencing. While longer sequencing read lengths may help to resolve repetitive DNA repeats and detect epigenetic markers, this technology requires more template DNA and has higher error rates (Quail et al., 2012).

A number of bioinformatic tools allow the analysis of the data output which may provide important insights in terms of outbreak surveillance, forensics, metabolic modelling and metagenome analysis (Land et al., 2015). These tools facilitate genome assembly and subsequent comparative analysis, detection of virulence and AMR genes, SNP calling for genetic comparison between bacterial isolates, and phylogenetic analyses. Following sequencing, steps are taken to decipher the data output to enable the user to draw meaningful conclusions. The general pipeline applied to sequencing output includes quality checks of raw reads (e.g., FastQC), adapter trimming (e.g., Porechop, Trimmomatic), *de novo* genome assembly (e.g., SPAdes), followed by genome annotation (e.g., Prokka) and finally sequence analysis (Mitchell et al., 2021). Then, using databases available over the internet (e.g.,

ResFinder, PlasmidFinder, Virulence Finder Database), AMR-encoding genes, plasmids and virulence genes may be identified within the genome. This information can offer insight into the resistance phenotypes which may be expressed, as well as the level of virulence expected (Bogaerts et al., 2019; Wyres et al., 2014).

The aim of this project was to develop and implement a shared vision of best practice within the EU and China in an effort to enhance food safety, deter food fraud, deliver mutual recognition of data and standards and support the flow of agri-food trade between the two trading blocks in a way that better protects the consumer.

In this work, we set out to harmonise a method for Whole Genome Sequencing of bacterial genomes. This involved:
1. Development of a standard WGS protocols which encompassed all aspects of the process from bacterial culture up to bioinformatic analysis.
2. Selection of twenty-two test isolates, which were subjected to WGS using the SOP in question.
3. Regular communication between the project partners in an effort to ease the process of protocol harmonisation.
4. Comparison of data generated by project partners in the EU and China to validate the process.

3. STANDARD OPERATING PROCEDURES (SOPS)

3.1. BACTERIAL CULTURE AND EXTRACTION

3.1.1. Culture

A loopful of frozen stock cultures were streaked onto Brain Heart Infusion (BHI) agar and incubated at 37 °C overnight. Three to five colonies from the overnight plate were inoculated directly into approximately 6 ml of Brain Heart Infusion (BHI) Broth, and incubated overnight at 37 °C.

3.1.2. DNA Extraction

DNA extraction was carried out from bacterial overnight culture using the QIAgen DNeasy Ultraclean Microbial Kit. 1.8 ml of overnight culture was transferred into a 2 ml centrifuge tube and centrifuged at 10,000 x g for 3 min. The supernatant was decanted, and any remaining liquid pipetted off, ensuring the pellet was not disturbed. Note that for certain microbial cultures, less than 3 min centrifugation may be sufficient. The pellet was resuspended in 300 µl of PowerBead solution and vortexed gently. The resuspended cell solution was transferred to the PowerBead tube. Solution SL was added (50 µL) before securing the PowerBead tubes to a vortex adapter tube holder and vortexing at maximum speed for 10 min. Tubes were centrifuged at a maximum of 10,000 x g for 30 sec at room temperature. The supernatant was then transferred to a clean 2 ml tube, and 100 µl of Solution IRS added. The tube was vortexed for 5 sec and incubated at 4 °C for 5 min, with subsequent centrifugation at 10,000 x g for 1 min at room temperature. The entire volume of supernatant was transferred to a clean 2 ml collection tube, avoiding the pellet, and 900 µl of Solution SB was added and the mixture vortexed briefly. Approximately, 700 µl of mixture was loaded into a MB Spin Column and centrifuged at 10,000 x g for 30 sec at room temperature. After discarding the flow-through, the remaining supernatant was added to the MB Spin Column and centrifuged again at 10,000 x g for 30 sec. All flow-through liquid was discarded and 300 µl of Solution CB was added on top of the spin column, prior to centrifugation at 10,000 x g for 30 sec at room temperature. The flow-through was once again discarded and centrifugation repeated at 10,000 x g for 1 min at room temperature. The MB Spin Column was subsequently placed in a new 2 ml collection tube, and Solution EB (50 µl) added to the centre of the filter membrane. The tube was centrifuged at 10,000 x g for 30 sec at room temperature. The MB Spin Column was discarded and the DNA remaining in the tube was

successfully prepared. DNA was frozen until needed for downstream applications (-20 to – 80 °C).

3.1.3. Quantification of bacterial genomic DNA

Quantification of bacterial genomic DNA may be carried out using the Qubit™ 2.0 Fluorometer in combination with the Qubit™ dsDNA HS (High Sensitivity) Assay Kit, or alternatively using the Nanodrop™ 1000 Fluorospectrometer.

The Qubit™ 2.0 Fluorometer is a touch screen operated, self-contained unit. In order to quantify DNA, the Qubit™ dsDNA HS Assay Kit was used. This kit includes the Qubit™ dsDNA HS Working Solution and two standard solutions. Clear, optical Real-Time PCR tubes (0.5 ml) were prepared for each sample with 198 µl of working solution and 2 µl of sample. Standards (1 & 2) were prepared with 190 µl of working solution and 10 µl of standard. Samples were mixed and incubated for 2 min before reading. Light was excluded from samples as much as possible to prevent degradation of the fluorophore. Using the touch screen DNA and the dsDNA HS Assay kit were selected. Standards are read individually before each sample in turn. More information is available in the Qubit User manual.

The Nanodrop™ 1000 Fluorospectrometer utilizes patented sample retention technology which uses surface tension to hold the sample in place on the sample pedestal, removing the need for cuvettes or sample tubes. Furthermore, Nanodrop™ has the ability to measure highly concentrated samples and does not require the addition of buffers/working solutions. Onscreen instructions on the companion PC software are followed, selecting nucleic acid and then DNA. The system requires a "blank" measurement prior to reading samples and this is carried out by placing either 2 µl of water or 2 µl of buffer used during extraction (in this case Solution EB). Prior to each sample measurement, both pedestals are wiped with a lint-free tissue. 2 µl of sample is loaded to the lower pedestal and the arm is loaded to connect the upper and lower measurement pedestal. As

light array passes through the sample the output is relayed to a PC and can be interpreted. The purity of the nucleic acid is assessed by the ratio of sample absorbance at 260 and 280 nm. A ratio of ~1.8 is generally accepted as "pure" for DNA. The ratio of sample absorbance at 260 and 230 nm is a secondary measure of nucleic acid purity. This value is often higher than the respective 260/280 values and are commonly within the range of 1.8-2.2. If this ratio is substantially lower, it may be indicative of the presence of co-purified contaminants.

A target of 19-25 ng/µl of purified DNA is needed for library preparation. If purified DNA exceeds this target, it is diluted as appropriate in Solution EB. Concentration and purity should be rechecked after dilution. Typically, 50 – 60 µl of diluted purified DNA is prepared in order to ensure enough starting material for the library prep step plus further quantification.

3.2 LIBRARY PREPARATION

3.2.6 Fragmentation/End Prep

DNA fragmentation was carried out using the NEBNext® Ultra™ II FS DNA Library Prep Kit for Illumina®. The protocol for bacterial genome sequencing library prep falls under the category of "Protocol for Large Fragment Sizes (>550bp) and is available in full in Chapter 3 of the "NEBNext® Ultra™ II FS DNA Library Prep Kit for Illumina® Instruction Manual" available on the New England BioLabs website.

All regents in the NEBNext® Ultra™ II FS DNA Library Prep Kit for Illumina® were fully defrosted, vortexed and held on ice until use. Input material of 26 µl of DNA was used for the fragmentation step. 7 µl of NEBNext® Ultra™ II FS Reaction Buffer and 2 µl of NEBNext® Ultra™ II FS Enzyme Mix was used for each reaction. All components were added to 0.2 µl PCR tubes or 96-well plates for larger library preps. Reactions were vortexed for 5 sec and spun briefly in the microcentrifuge. In a thermocycler, with the heated lid set to 75 °C, the following program was run: 5 min at 37 °C; 30 min at 65 °C; hold at 4 °C. At this point, samples may be stored at -20 °C, however, a slight loss in yield

may occur. For this reason, it is recommended to continue to adaptor ligation.

3.2.7 Adaptor Ligation

The FS reaction mixture from the previous step (3.2.1) was carried on to adaptor ligation (35 µl). NEBNext® Ultra™ II Ligation Master Mix (30 µl) and NEBNext® Ligation Enhancer (1 µl) was added directly on top of the FS reaction mixture for each reaction. NEBNext® Adaptor for Illumina® (2.5 µl), from the NEBNext® Multiplex Oligos for Illumina® kit, was added. It is important to add the adaptor last. Ensure to keep all regents and tubes/plates on ice. Pipette mix each reaction mixture and centrifuge briefly. In a thermocycler, with the heated lid off, the reaction was incubated at 20 °C for 15 min. Following incubation, 3 µl of USER enzyme (NEBNext® Multiplex Oligos for Illumina® kit) was added. After mixing, the mixture was incubated at 37 °C for 15 min with the heated lid set to ≥ 47 °C. At this point samples may be stored overnight at -20 °C without affecting yield.

3.2.8 Size Selection of Adaptor-ligated DNA fragment Sizes >550 bp

Size selection is carried out using sample purification beads. We recommend AMPure XP Beads or alternatively SPRIselect beads. If using AMPure XP beads, allow beads to warm to room temperature for at least 30 min before use. It is important to note that the proportion of beads used at each step throughout the library prep is directly related to the amount of sample present and is specifically optimised to isolate the fragments of interest.

Reaction volume from the previous step (step 3.2.2; 71.5 µl) was taken forward to the size selection step. The volume was brought up to 100 µl by adding 28.5 µl 0.1 X TE (1X TE diluted 1:10 with water). Size selection beads were thoroughly vortexed, and pipette mixed in order to resuspend. 40 µl (0.4 X) resuspended beads were added to the adaptor ligation reaction and mixed well by pipetting and vortexing (3-5 sec). Samples were centrifuged briefly, ensuring that the beads did not settle out. Samples were incubated for at least 5 min at room temperature, and subsequently placed on a magnetic stand for a further 5 min. After 5 min or when the solution had cleared, the supernatant was carefully removed and discarded (taking care not to disturb the beads). 200 µl of freshly prepared 80% ethanol was added to the tube/plate while in the magnetic stand and incubated for 30 seconds, before removing carefully in such a way as to not disturb the beads. The ethanol wash was repeated for a total of two washes. Any residual ethanol was removed with a p20 pipette tip. Beads were air dried for up to 5 min while the tube/plate remained on the magnet with the lid open. It is important during this step to ensure that the beads do not over-dry, which may result in a lower recovery of DNA. Over-dried beads appear light brown and may begin to crack – beads should be eluted when dark brown and gloss and all visible liquid has evaporated.

When the size selection beads had dried, the tube/plate was removed from the magnetic stand and the DNA target was eluted from the beads by adding 102 µl 0.1X TE buffer. Samples were mixed well by pipette mixing and spun briefly before incubation for at least 2 min at room temperature. The tube/plate was placed back on the magnetic stand for 5 minutes, before 100 µl was transferred to a new PCR plate.

A volume of 50 µl (0.5X) of resuspended sample purification bead were added to the sample and mixed thoroughly by pipette mixing, ensuring to expel all liquid from the tip. Centrifugation after mixing was extremely brief to prevent the beads form settling out. Samples were incubated at room temperature for at least 5 min and then placed on the magnetic stand to separate the beads from the supernatant for a further 5 min. The supernatant was carefully removed and discarded, and the protocol continued with the beads. 200 µl of freshly prepared 80% ethanol was added to the tube/plate while in the magnetic stand and incubated for 30 seconds, before removing carefully in such a way as to not disturb the beads. The ethanol wash was repeated for a total of two washes. Any residual ethanol was removed with a p20 pipette tip. Beads were air

dried for up to 5 min while the tube/plate remained on the magnet with the lid open, ensuring not to over-dry the beads. When the size selection beads had dried, the tube/plate was removed from the magnetic stand and the DNA target was eluted from the beads by adding 17 µl 0.1X TE buffer. Samples were mixed well by pipette mixing and spun briefly before incubation for at least 2 min at room temperature. The tube/plate was placed back on the magnetic stand for 5 minutes, before 15 µl was transferred to a new PCR plate.

These cleaned-up adaptor-ligated fragments were carried into the next step, PCR Enrichment. At this point, the sample can be stored at -20 °C, without negatively affecting yield.

3.2.9 PCR Enrichment of Adaptor-Ligated DNA

PCR enrichment of Adaptor-Ligated DNA is carried out to add "indexes" to the samples being tested. This step means that samples may be individually identified when added to the sequencing run in a pool with the other samples. In this way, we attach two sequences a Universal i5 Primer and an Index i7 primer. There are eight i5 primers, and twelve i7 primers in the NEBNext Multiplex Oligos for Illumina, allowing up to 96 samples to be indexed with a different combination of primers for easy identification. To the resulting adaptor ligated DNA fragments from the previous step (15 µl), 25 µl pf NEBNext Ultra II Q5 Master Mix, 5 µl of index primer i7 and 5 µl of universal primer i5 were added, for a total volume of 50 µl. The reaction mixture was mixed thoroughly by either pipette mixing or vortexing (1 min at 1800 rpm) and briefly centrifuged (centrifuge stopped when reached 200 rpm). Using a thermocycler, the following cycling conditions were used to carry out PCR amplification: initial denaturation at 98 °C for 30 sec, three to eight cycles of 98 °C for 10 seconds (denaturation) and 65 °C for 75 seconds, followed by final extension at 65 °C for 5 minutes and a 4 °C hold. It is important when carrying out bacterial sequencing that the PCR amplification step be optimised, as the number of cycles necessary can vary from one organism to another. The number

of cycles should be high enough to provide sufficient library fragments for a successful sequencing run, but low enough to avoid PCR artefacts and over-cycling. Further information can be found in the NEBNext® Ultra™ II FS DNA Library Prep Kit for Illumina® Instruction Manual.

3.2.10 Clean-up of PCR Reaction

SPRIselect or AMPure XP beads are used in this step. It is important to note that when using AMPure XP beads, these must equilibrate to room temperature (~30 min) prior to use. It is also essential (regardless of type used) that beads are thoroughly resuspended. The volumes indicated are specifically designed in a ratio with the product volumes of this step to isolate fragments of a particular size. It is important to only use the ratios indicated at the step intended, as to alter these can have adverse effects. More information on the use of size selection beads can be found in the user manual for these products.

0.6X (30 µl) resuspended beads was added to the PCR reaction, mixed well by vertexing at 1800 rpm for 1 min (or pipette mixing 10 times), and centrifuged briefly ensuring the beads did not come out of solution. Samples were incubated at room temperature for 5 minutes, and subsequently placed on a magnetic stand for a further 5 minutes. When the solution was clean, the supernatant was carefully removed and discarded, taking care not to disturb the beads containing genetic targets.

A volume of 200 µl of freshly prepared 80% ethanol was added to the plate/tube while in the magnetic stand and incubated at room temperature for 30 secs, then carefully removed and discarded without disturbing the beads. The ethanol wash was repeated once more for a total of 2 washes. Following the second wash, all visible liquid was removed by spinning briefly and removing the remaining ethanol with a P10 pipette tip. Beads were air dried for up to 5 minutes, ensuring not to over-dry, which can result in a lower recovery of DNA. The plate was removed from the magnetic stand and the beads eluted by adding 33 µl of 0.1X TE buffer. Samples were mixed thoroughly, spun briefly and

incubated for a minimum of 2 mins. The tube/plate was plated on the magnetic stand, and after 5 min, 30 µl was transferred to a new PCR plate/tube. The resultant sample can be stored at -20 °C until needed.

3.2.11 Normalisation

In order to normalise each of the samples within the library, two measurements for each sample are needed - fragment size and DNA concentration. Quantification of bacterial genomic DNA may be carried out using the Qubit™ 2.0 Fluorometer in combination with the Qubit™ dsDNA HS (High Sensitivity) Assay Kit, or alternatively using the Nanodrop™ 1000 Fluorospectrometer. Details of how to use these instruments was discussed in section 3.1.3 above.

Read length refers to the number of base pairs (bp) sequenced from a DNA fragment. Following sequencing, the regions of overlap between reads allow assembly and alignment of reads to reconstruct the full DNA sequence. Read lengths correspond directly to the reagents/chemistry used on the Illumina platform i.e., more chemistry cycles allow generation of longer reads. Illumina is a short-read sequencing platform, and the largest kit available on the MiSeq Platform allows for 2 x 300 bp. This means that the machine will read 300bp (including adapters and indexes) from each end of the fragment. In this way, longer fragments which are more than 600bp, will not be fully covered and may lead to a lack of completion of the genome. It is for this reason that optimisation of the fragmentation step, as mentioned above (3.2.1), is of the utmost importance to ensure an output of quality data. Standard Illumina sequencing libraries tend to have a fragment size of approximately 200 to 500 bp, excluding adapters. While larger fragments of up to 1000bp will cluster, these larger sizes will result in a lower efficiency and lower yield.

Fragment size is assessed using the Agilent 4200 Tapestation System, an automated electrophoresis tool used for DNA and RNA sample quality control. The Agilent High Sensitivity D5000 ScreenTape and reagents are used to assess fragment size for Illumina Sequencing. 2 µl of

D5000 sample buffer and 2 µl of sample were combined in a skirted PCR plate or a PCR tube and centrifuged. In a PCR tube, a ladder solution was prepared with 2 µl DNA ladder and 2 µl of D5000 sample buffer per screentape device. Screentape devices contain 16 lanes sufficient for 1 ladder and 15 samples per device. Using the PC software installed, on-screen instructions will navigate the user through the setup of the machine, and sample output is displayed on the screen after the process has finished. Images on the NEB website will indicate how the graph should look, and any necessary troubleshooting required. Older Agilent models, such as the Agilent 2100 Bioanalyzer System are also appropriate for fragment size estimation and information on operation of this device can be found on the Agilent Website.

The molar concentration of each sample to be diluted was calculated using the average fragment size (Tapestation) and the concentration (Qubit/Nanodrop), using the following equation:

$$\frac{concentration\ ng\ per\ \mu L}{660\ g\ per\ mole \times avg\ fragment\ size} \times 10^6$$

µL/L = concentration (nM).

Each sample was normalised to 4 nM. 5 µL of each normalized sample was pooled together in one 1.5 ml LoBind tube. In order to confirm the library concentration (4 nM), a qPCR can be carried out using the NEBNext® Library Quant Kit for Illumina® and the concentration adjusted accordingly (see NEB website for more details).

3.2.12 Library Denaturation

0.2 N NaOH (200 µL 1M NaOH and 800 µL nuclease-free water) was prepared. 5 µl of normalised library pool and 5 µL of 0.2 N NaOH were added to a 1.5 ml LoBind tube and vortexed briefly, before centrifugation at 280 x g for 1 min. After incubation for 5 min, 990 µL of pre-chilled HT1 was added to the tube and mixed. The result was a 1 ml 20 pM denatured library. The library was diluted to 18 pM by adding 540 µl of the 20 pM library to 60 µl of pre-chilled HT1. 6 µl of the library was removed from the tube, and 6 µl of the PhiX reference library was added. The

denatured library was placed on ice until Miseq loading.

3.2.13 Miseq Loading

Selecting the Miseq Reagent chemistry is dependent on the fragments to be sequenced, as well as the outputs expected. Information on what chemistry is right for each project can be found at https://emea.illumina.com/systems/sequencing-platforms/miseq/specifications.html.

In the case of common bacterial pathogens (e.g. *Listeria* spp., *Salmonella* spp., *E. coli,* etc.), larger fragment sizes and a bigger output are most common due to the size of the genome. For this reason, the Miseq Reagent Kit v3 (2 x 300 bp - 600 cycles) kit is used. The kit comprises a reagent cartridge, incorporation buffer and a flow cell. The cartridge is stored at - 20 ℃, while the buffer and flow cell are stored at refrigeration temperatures. It is important to allow the cartridge to fully defrost prior to use. This can be done so by placing it in the fridge overnight, or alternatively, by placing it in a container of water (up to the indicated water-fill line) ~2 hours before use. Ensure to invert both the cartridge and the incorporation buffer to ensure all reagents are well mixed before use.

Prior to running the Miseq instrument, a maintenance wash must be carried out. This comprises 3 washes (1 0.5% Tween 20; and 2 water) and is essential in ensuring quality runs and removing reads from previous samples. After washing, it is important to raise the sippers before starting a sequencing run, as to skip this step can damage the machine. This can be done by pressing a button on the wash menu. The Miseq is an extremely user-friendly platform and will prompt the user when and where to insert all relevant reagents in sequence. In order to ensure success in each run, it is important to clean both the flow cell and the reagent cartridge before use. Using some tissue paper, ensure that each well of the reagent cartridge is free of droplets of water that may enter the sequencing run. The flow cell is extremely fragile and should be handled with care. On inspection, two lanes can be seen which the samples will flow through in order to be read by the machine. It is important that the area surrounding these lanes is clean and clear of any artefacts. Water and lint free tissue may be used to gently clean the flow cell. In cases of stubborn artefacts, ethanol may be used, but every care should be taken in removing as much residual ethanol as possible.

When the items are clean, the sample may be loaded into the Miseq Cartridge. On the cartridge, position 17 is highlighted with "Load Sample" in bright orange. Using a clean pipette tip and some light force, pierce a hole in the foil at the loading position. Following this, pipette 600 µl of prepared library from above (3.2.7) into position 17, ensuring not to lose any sample in the process. The cartridge can now be inserted into the sequencer, and the run may be commenced. A previously prepared sample sheet must be uploaded to the machine, detailing the run details and identifying the indexes for each sample. A sample of these sheets is attached in the appendices of this document.

After the sample sheet has been uploaded, the machine will perform a number of checks prior to running. Success of the sequencing run can be measured by a number of parameters measured by the Miseq, including Q30, cluster density, etc. More details of these, what they mean, and how to troubleshoot any errors, is available online on the Illumina website.

PubMLST), Resistome analysis (ABRicate + Resfinder), Virulome analysis (ABRicate + VFDB) and Variant identification (Snippy). Other Antimicrobial Resistance Gene Databases such as ARDB, BacMet, CARD, etc were then searched, followed by Plasmid sequence detection with Plasmid Finder, etc. Finally, the GO annotation was prepared.

A schematic representation is shown in **Figure-2.**

Schematic representation of a WGS
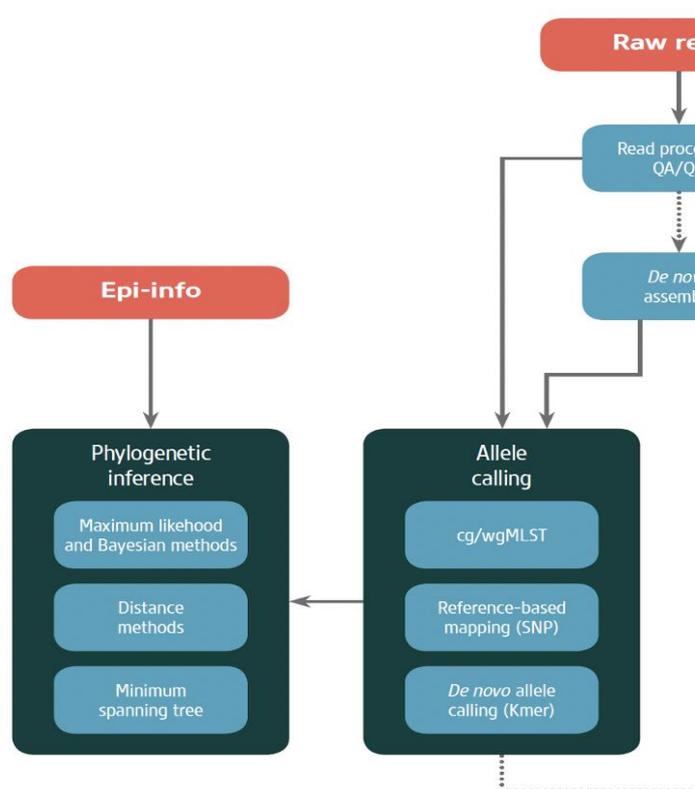
Figure-2. A schematic representation showing the individual steps used as part of a typical bioinformatics workflow

Figure 1. Illumina Miseq Instrument. (Courtesy of Illumina, Inc.)

4. BIOINFORMATICS WORKFLOW

The Bioinformatics Pipeline used is detailed in full in the appendices of this document. However, the general scheme involved initial quality control (FastQC, MultiQC), followed by filtering and trimming of reads. Quality Control was then repeated and subsequently reads filtering and trimming were repeated as necessary. K-mer analysis against known genome databases was performed for species identification. De novo assembly using SPADES was carried out. File ordering was carried out to allow for collection and renaming of scaffolds. Contigs were examined and counted, and small contigs removed. Following this, the quality of results were assessed using QUAST genome assembly quality assessment tool, and basic data statistics were performed. Genome Size estimation was carried out using K-mer analysis. Annotation using Prokka was carried out, followed by MLST (mlst +

5. WHOLE VIRAL GENOME SEQUENCING OF SARS-COV-2

5.1.1. Viral particles extraction from soft fruits, frozen fruits, ready-to-eat salads, and fresh/frozen vegetables.

For the extraction of virus particles from soft fruits, frozen fruits, ready-to-eat salads, and fresh/frozen vegetables, 25 g of sample was transferred to the sample compartment of a mesh filter stomacher bag. Large soft fruits were coarsely chopped into pieces of approximately 2.5 cm × 2.5 cm × 2.5 cm. A volume of 40 ml TGBE buffer was added along with pectinase (30 units of pectinase from *Aspergillus niger*, or 1,140 units of pectinase from Aspergillus aculeatus) and 10 l of MeV (process control virus material). After incubation at room temperature with constant rocking at approximately 60 rpm for 20 min (pH adjusted to 9.5), the eluate was decanted from the filtered compartment and centrifuged at 10,000 x g for 30 minutes (min) at 4 °C. The supernatant obtained was adjusted to pH 7.0 and 0.25 volumes of 5 × PEG/NaCl solution were then added. The mixture was homogenised by shaking for 1 min and then incubated with constant rocking at around 60 rpm at 5 °C for 60 min and centrifuged at 10,000 x g for 30 min at 4 °C. The pellet was concentrated by centrifugation at 10,000 x g for 5 min at 5 °C and resuspended in 500 l of PBS for an equal volume purification with chloroform-butanol (vortexed and incubated at room temperature for 5 min). A final step of centrifugation (10,000 x g for 15 min at 5 °C) allowed to obtain an aqueous phase that was transferred to a fresh tube and retained for RNA extraction and purification.

5.1.2. Virus Extraction from meats and crustacean

Virus particles from meat product, meat preparations and crustacean were extracted from 5 g of well-mixed matrices. The extraction included the addition of 7 ml Trizol and 5 ml PBS to the sample in a stomacher bag spiked with 50 l of internal process control (MeV). The liquid fraction was transferred into a 50 ml sterile tube and centrifuged (10,000 x g for 20 min at 4°C). The supernatant was treated with 200 l of chloroform per 1 ml of supernatant recovered (~1.4 ml), mixed for 15 seconds and incubated at room temperature for 10 min. After a centrifugation step (10,000 x g for 15 min at 4°C), the aqueous

layer was used as a template for the RNA extraction and purification.

5.1.3. Virus Extraction from Bivalve Molluscan Shellfish (BMS)

Virus extraction specimens were sampled live and intact. Examples of suitable bivalve molluscan shellfish (BMS) and crustacean for this protocol included oysters, mussels, clams, etc. The digestive glands from BMS were used as a template for the extraction of virus particles and a minimum combined gland mass of 2 g was required. The sample material was finely chopped with a sterile scalpel and transferred into a centrifuge tube and spiked with 10 l of the process control virus material (MV). A volume of 2 ml proteinase K solution was added, and the mixture was incubated at 37 °C with shaking (320 rpm) for 60 min. Secondary incubation was carried out by placing the tube in a water bath at 60 °C for 15 min. The supernatant obtained by centrifugation (3,000 x g for 5 min) was retained for RNA extraction and purification.

5.1.4. Swabs from high-touch surfaces and food skin and packaging

Swab samples from high touch surfaces and food surface skin and associated packaging materials were taken using individually wrapped swabs immersed in viral transport media (VTM). Excess VTM was removed, and the swab used to sample surface areas of interest (25 cm3 is commonly used). The swab was taken using moderate pressure, moving in at least two directions, and rotating the swab in order to use the entire surface area of the cotton swab. It is recommended to avoid the swab drying completely. Immediately after sampling, the swab was placed into the transport container containing VTM, the stick cut at the breakpoint by bending gently before the cap was closed tightly. The outside of the transport container was wiped with an alcohol wipe and labelled clearly with unique sample identifiers. A field blank was submitted on each sampling day. In this instance, the control swab was handled in the same way as the environmental samples, i.e., in the work area, the swab was opened and

removed from packaging and placed into the transport container without swabbing any surface.

In the case where samples were taken outside of the laboratory environment (e.g. from a food processing environment), samples were packaged as follows for transport. Following sampling, all samples were placed into a clean bag and sealed. The outside of the bag was wiped with an alcohol wipe and placed inside another clean bag and sealed (the samples are now double bagged). A final wipe down of the exterior bag was performed with an alcohol wipe. The SARSfood sample form was filled out and placed inside a separate bag. Samples were packed to avoid damage during transport and were wrapped in absorbent material and shipped with an ice pack. The outside of the shipping container was labelled in a clear, legible manner, e.g., TEST CODE CFS2020WHO - PCR LAB ONLY. Samples were shipped on the same day where possible. In instances where this was not feasible, samples were refrigerated for a maximum of 72 hours.

5.1.5. RNA Extraction and Purification (SARS-CoV-2)

The RNA was extracted from the different samples using a purification protocol for viral RNA tested both in a manual setting and automated purification using the QIAamp Viral RNA Mini Kit (Qiagen, UK) on QIAcube Connect (Qiagen, UK) based on the principles that viral RNA binds specifically to the silica membrane, and pure viral RNA is eluted in either water or a buffer provided with the kit. The RNA was used immediately as the template for the RT-qPCR or stored at -80°C for the later analysis.

5.1.6. RT-qPCR (SARS-CoV-2)

In this study, the RT-qPCR was used as a detection assay adopting primer sets targeting the nucleocapsid protein region 1 (N1), nucleocapsid region 2 (N2) and Human RNase P (RP) based on the "2019-nCoV CDC EUA authorised RT-qPCR Probe Assay primer/probe mix" (IDT, UK). A process control virus (Mengo Virus - MV) was included in the panel for the evaluation of the performances of the protocol, along with negative

controls and positive controls consisting of purified RNA previously tested positive for the presence of RNA of SARS-CoV-2 and SARS-CoV-2 control plasmids, contain the complete nucleocapsid gene from 2019-nCoV, SARS, and MERS (IDT, UK). Luna Universal Probe qPCR Master Mix (NEB, UK) was used for preparing mastermix reactions for each target and loaded in 96-well (0.1 ml) on a QuantStudio 5 (Applied Biosystems, UK).

5.1.7. Characterisation of RT-qPCR positive samples by Whole Virus Genome Sequencing (WvGS)

A whole virus genome sequencing (WvGS) amplicon strategy has been developed and used to obtain high-quality consensus sequences of SARS-CoV-2. The application of this approach has allowed for higher resolution analysis, enhancing the understanding of transmission patterns, whilst guiding policies designed to prevent disease spread. A high-throughput Illumina MiSeq-based platform for WvGS characterisation of virus taken from positive RT-qPCR tests was already in place and providing comparative genomic analysis and it was applied in a previous study focused on uncovering transmission routes within MPPs and elsewhere, and in particular the transmission of the virus from healthcare workers and patients. The Illumina MiSeq-based assay was deployed to support hospital outbreak investigations. RNA from positive RT-qPCR samples (from food processing plant sewage outlets and food matrices, in WP-1) were reverse-transcribed into cDNA and characterised by WvGS, according to the protocol provided by the ARTIC Network initiative. Bowtie 2 will then be used for aligning the sequencing reads to the reference genome for SARS-CoV-2 (GenBank number, MN908947.3) and SAMtools for manipulating the alignments. SNPs defined clusters and a median-joining network were used to examine relationships with an additional 1,000 strains collected from GISAID. Clade annotation was included using Pangolin, GISAID and NextStrain platforms.

Samples were prepared for sequencing starting from cDNA synthesis (reverse transcription) using

LunaScript RT SuperMix (New England Biolabs, Ipswich, UK). The sequencing library were obtained following ARTIC tiling-amplicon approach, prepared using the NEBNext Ultra II kit (New England Biolabs, Ipswich, UK) and sequenced on an Illumina MiSeq using 500-cycle v2 reagent kits (Illumina, Cambridge, UK).

5.1.8. Computational Analysis

The raw data produced by sequencing were used as input for the generation of consensus sequences. Different methods, pipelines and tools were evaluated for the computational analysis, including commercial tools such as Geneious and CLC Genomics Workbench. In this study, free, open source tools were preferred and the pipeline included the following steps:

1. Trim reads for quality (Q25+) and for adapters on both ends. Then trim primer sequences (a hard 30 bases on each end), keeping only sequences that are at least 75 bases. The step was performed using cutadapt.
2. Map reads to reference sequence using bwa and sorting with SAMtools
3. Trimming of adaptors ARTIC scheme using iVar and sorting with SAMtools
4. Call variants and generate consensus sequence

Comparative sequence analysis was conducted by phylogenetic inference maximum likelihood (ML) method with RAxML using the multiple sequences aligned by MAFFT. Clades and variants were assigned respectively using Nextstrain and Pangolin COVID-19 lineage assigner (http://pangolin.cog- uk.io/).

6. CONCLUDING REMARKS

Detailed technical methods required for bacterial culture, purification of bacterial gDNA or viral DNA/RNA, library preparation and subsequent sequencing were described. These protocols have been shared among the partners and tested then validated, to provide for a harmonised approach to whole genome

sequencing of bacteria of importance to human health. In addition, a parallel protocol to similarly sequence SARS-CoV-2 was developed, distributed and also tested by partners in both EU and China.

These laboratory-based methods described in this deliverable along with the adoption and implementation of a harmonised bioinformatic pipeline (described in **D4.7**), now provide a platform for the detailed harmonised production and analysis of genome data, independent of the biological hazard under investigation. Furthermore, the methods described in this task (along with the steps for their bioinformatic-based analyses described in *D4.7*) are in line with similar technical strategies used by regulatory laboratories, thereby enabling a parallel approach to risk assessment and subsequent management, for the protection of brand reputation and public health alike.

Outputs from this deliverable **D4.8** provide for a harmonised laboratory-based approach to the management of bacterial and viral hazards for the preparation of sequencing libraries. In addition, **D4.7**, described the application of a harmonised bioinformatics pipeline, to be used in conjunction with the outputs from this task, to support the risk assessment of these biological hazards, for the protection of human health

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8. APPENDIX

10.1 Miseq Sample Sheets

samplesheet1
(1).csv

samplesheet2
(1).csv

	A	B	C	D	E	F
1	[Header]					
2	IEMFileVe	5				
3	Date	########				
4	Workflow	GenerateFASTQ				
5	Applicatic	FASTQ Only				
6	Instrumer	MiSeq				
7	Assay	NEBNext Ultra II FS DNA Library Prep Kit for Illumina				
8	Index Ada	NEBNext Multiplex Oligos for Illumina (Dual Index Pr				
9	Description	MS6906919-600V3				
10	Chemistry	Amplicon				
11						
12	[Reads]					
13	301					
14	301					
15						
16	[Settings]					
17	ReverseCo	0				
18	Adapter	AGATCGGAAGAGCACACGTCTGAACTCCAGTCA				
19	AdapterRe	AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT				
20						
21	[Data]					
22	Sample_IC	Sample_N	Sample_P	Sample_W	I7_Index_	index
23	1				i701	ATTACTCG i
24	2				i702	TCCGGAG, i
25	3				i703	CGCTCATT i
26	4				i704	GAGATTC(i
27	5				i705	ATTCAGA/ i
28	6				i706	GAATTCG1 i
29	7				i707	CTGAAGC i
30	8				i708	TAATGCG(i
31	9				i709	CGGCTAT(i
32	10				i710	TCCGCGA/ i
33	11				i711	TCTCGCG(i
34	12				i712	AGCGATA i
35	13				i701	ATTACTCG i
36	14				i702	TCCGGAG, i
37	15				i703	CGCTCATT i
38	16				i704	GAGATTC(i

10.2 Bioinformatics Pipeline

```
#########################################
################
######UCD-CFS WGS pipeline
#############################
##curated by Guerrino Macori, Séamus
Fanning###################
```

```
#########################################
################
```

#set number of THREADS= $THREADS and name of files = ${INPUT}

1. Quality Control
FastQC
(http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) Remarks: FastQC
usage: http://www.plob.org/2013/07/16/5987.html
MultiQC https://multiqc.info/

$fastqc -t $THREADS ${SAMPLE} -o outputFolder/
$multiqc .

2. Reads filtering and trimming
fastP (https://github.com/OpenGene/fastp)

$fastp -i "$1"_L001_R1_001.fastq.gz -I
"$1"_L001_R2_001.fastq.gz -f {your value} -F
{your value} -t {your value} -T {your value} -o
/outputFolder/"$1"_R1.fastp.fq.gz -O
"$1"_R2.fastp.fq.gz -h "$1".html -j "$1".json

2.1 Repeat quality control and Reads filtering and trimming accordingly

3. Species identification
k-mer analysis against known genome database
(Kraken, Kraken2, Centrifuge)

4. De novo assembly
SPAdes (https://github.com/ablab/spades)
$./spades.py -1 "$1"_R1.fastp.fq.gz -2
"$1"_R2.fastp.fq.gz --careful --only-assembler -t
$THREADS --cov-cutoff auto -o
/outputFolder/"$1"

5. File ordering, useful curated scripts:
5.1 #to collect and rename the scaffold output of SPAdes from different folders:
#In your spades output folder:

$find . -type f -name "scaffolds.fasta" -printf
"/%P\n" | while read FILE ; do DIR=$(dirname
"$FILE");mv ."$FILE"

/outputFolder/"$DIR".fasta;done

5.2 #count the contigs
grep -c ">" scaffolds.fasta

5.3 #remove small contigs
#write the following program using nano and
called removesmalls.pl
#copy and paste the command below in the file

```perl
#!/usr/bin/perl
use strict;
use warnings;

my $minlen = shift or die "Error: `minlen`
parameter not provided\n";
{
   local $/=">";
   while(<>) {
     chomp;
     next unless /\w/;
     s/>$//gs;
     my @chunk = split /\n/;
     my $header = shift @chunk;
     my $seqlen = length join "", @chunk;
     print ">$_" if($seqlen >= $minlen);
   }
   local $/="\n";
}
```

#You would then invoke the script like so:

perl removesmalls.pl 500 <file.fasta> >
<finalname.fasta>

6. Quality of the results
QUAST
(http://bioinf.spbau.ru/quast or http://quast.sourceforge.net/quast): Genome assembly quality
assessment tool QUAST instruction
document: http://quast.bioinf.spbau.ru/manual.html
$./quast.py test_data/contigs_1.fasta \
     test_data/contigs_2.fasta \
   -r test_data/reference.fasta.gz \
   -g test_data/genes.txt \

```
    -1 test_data/reads1.fastq.gz -2
test_data/reads2.fastq.gz \
    -o quast_test_output
```

6.1 Basic data statistics after assembly
assembly-stats (https://github.com/sanger-pathogens/assembly-stats)
assembly-stats (https://github.com/rjchallis/assembly-stats)
assemblyStatics (https://github.com/WenchaoLin/assemblyStatics)
velvet-stats (https://github.com/ajmazurie/velvet-stats)
gstawk (https://github.com/mspopgen/gstawk)
seqStats(https://github.com/peteashton/seqStats): Two figures are produced: one contains the length distribution histogram and a cumulative length plot, the other plots GC vs sequence length.
TBtools(https://github.com/CJ-Chen/TBtools)

6.2 Kmer analysis gene size evaluation
GCE (ftp://ftp.genomics.org.cn/pub/gce/): BGI's software for genome evaluation
GCE literature: https://www.researchgate.net/publication/255722390_Estimation_of_genomic_characteristics_by_analyzing_k-mer_frequency_in_de_novo_genome_projects In structions for use
blog: https://www.plob.org/article/9388.html
KmerGenie (http://kmergenie.bx.psu.edu/)
Jellyfish (http://www.genome.umd.edu/jellyfish.html)
How to use Jellyfish: http://www.chenlianfu.com/?p=806
KmerFreq

7. Annotation
Add features to assembly using Prokka
prokka (http://www.vicbioinformatics.com/software.prokka.shtml): Annotation for pronuclear

7.1 MLST
From assembly w/ automatic scheme detection (mlst + PubMLST)

7.2 Resistome
From assembly (abricate + Resfinder)

7.3 Virulome
From assembly (abricate + VFDB)

7.4 Variants
From reads aligned to reference (snippy)

7.5 Other Antimicrobial Resistance Gene Database:
ARDB: http://ardb.cbcb.umd.edu/index.html
BacMet (http://bacmet.biomedicine.gu.se/): Antibacterial biocide and metal resistance genes database # BacMet has a supporting search and annotation tool, and its execution is as follows: perl /sdg/database/BacMet_v1.1/BacMet-Scan_v1.1 .pl -i ./final.scaffold.fa -o E6.3 -d /sdg/database/BacMet_v1.1/BacMet_EXP.704 -blast -e 0.00001 -cpu 10 -columns all -p 20 -table -report -counts -v
CARD: https://card.mcmaster.ca/
Resfams: http://www.dantaslab.org/resfams
NCBI Bacterial Antimicrobial Resistance Reference Gene Database: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA313047
ARG-ANNOT: http://en.mediterranee-infection.com/article.php?laref=283%26titre=arg-annot
EcOH: https://github.com/katholt/srst2/tree/master/data

7.6 Plasmid sequence detection
PlasmidFinder: https://cge.cbs.dtu.dk/services/PlasmidFinder/ PlasmidFinder identifies plasmids in total or partial sequenced isolates of bacteria. PlasmidFinder, which searches for matches in a replicon database, had the highest precision (1.0) but was restricted by the contents of its database and the contig length obtained from de novo assembly (recall = 0.33). PlasmidFinder database download link: https://cge.cbs.dtu.dk//services/data.php
cBAR (http://csbl.bmb.uga.edu/~ffzhou/cBar/) recall and precision of 0.77 and 0.63.
Recycler (https://github.com/Shamir-

Lab/Recycler) It correctly predicted small plasmids but failed with long plasmids (recall = 0.12, precision = 0.28).

PlasmidSPAdes (http://spades.bioinf.spbau.ru/plasmidSPAdes/)

PLACNET (https://sourceforge.net/projects/placnet/)

PLACNET2FASTA (https://github.com/tomdeman-bio/PLACNET2FASTA): Converts PLACNET output to a FASTA file containing plasmid contigs

7.7 GO annotation
blast2go (https://www.blast2go.com/)
GO_Annotation_Plot (https://github.com/ZhihaoXie/GO_Annotation_Plot.git)

10.3 Whole Viral Genome Sequencing of SARS-CoV-2

PDF

sars-cov-2-whole-g
enome-sequencing-

protocols.io

SARS-CoV-2 Whole Genome Sequencing on Illumina V. (bwpvpdn6)

Guerrino Macori[1], Lauren Russell[1], Seamus Fanning[1]

[1]University College Dublin

bwpvpdn6

1 Works for me
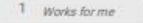
Guerrino Macori
University College Dublin

DISCLAIMER

In development
We are still developing and optimizing this protocol. Comments and feedback appreciated.

ABSTRACT

This SOP describes the procedure for generating cDNA from SARS-CoV-2 viral nucleic acid extracts and subsequently obtaining, through the amplicons tiling, the whole viral genome using V3 nCov-2019 primers (ARTIC). This is followed by library construction and pooling of samples and quantitation, prior to sequencing on the Illumina MiSeq.
The SOP is adapted from the nCoV-2019 sequencing protocol: https://www.protocols.io/view/ncov-2019-sequencing-protocol-bbmuik6w, and it was used in this study:
Lucey M, Macori G, Mullane N, Sutton-Fitzpatrick U, Gonzalez G, Coughlan S, Purcell A, Fenelon L, Fanning S, Schaffer K. Whole-genome Sequencing to Track Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) Transmission in Nosocomial Outbreaks. Clinical Infectious Diseases. 2020.
https://academic.oup.com/cid/advance-article/doi/10.1093/cid/ciaa1433/5909421

PROTOCOL INFO

Guerrino Macori, Lauren Russell, Seamus Fanning . SARS-CoV-2 Whole Genome Sequencing on Illumina .
protocols.io
https://protocols.io/view/sars-cov-2-whole-genome-sequencing-on-illumina-bwpvpdn6

MANUSCRIPT CITATION please remember to cite the following publication along with this protocol

https://academic.oup.com/cid/advance-article/doi/10.1093/cid/ciaa1433/5909421

KEYWORDS

Tiling PCR, WGS, SARS-CoV-2, nCoV-2019, nCoV19, WvGS

CREATED

Jul 19, 2021

LAST MODIFIED

Oct 04, 2021

PROTOCOL INTEGER ID

51669