D-JRP15-FED-AMR-WP2.1

Laboratory Operating Procedure (LOP)
Protocol for Whole Genome Sequencing of Bacterial Isolates

Version 1
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A) Description

The current protocol describes the complete procedure for whole genome sequencing of bacterial isolates recovered after cultivation of each sample in the corresponding agar media (described in the annex of the D-JRP15-FED-AMR-WP2.1 and named as “Protocol for Microbiology” and “*C. difficile* culture protocol”). Species to be sequenced include:

- *Escherichia coli* (ideally ESBL-/AmpC-producing *E. coli* and/or positive for *mcr*-variants)
- *Klebsiella pneumoniae* (ideally ESBL-/AmpC-producing *K. pneumoniae* and/or positive for *mcr*-variants)
- *Salmonella* spp
- *Staphylococcus aureus* (ideally methicillin-resistant *S. aureus,* MRSA)
- *Enterococcus faecium/faecalis* (ideally vancomycin-resistant *E. faecium/E. faecalis*, VRE)
- *Clostridium difficile*

B) Equipment

- Sterile 10 µl loop
- 96-well plate shaker
- Heating block
- Microcentrifuge (with rotor for 2 ml tubes)
- 96-well plate centrifuge
- Vortexer
- Pre-cooled 96-well rack
- PCR thermocycler
- MagAttract Magnetic Rack
- 2 ml sample tubes
- 1.5 ml sample tubes
- Pipette tips with aerosol barrier (10, 100, 200, 1000 ul)
- Ethanol (96–100%)
- Lysozyme stock solution (100 mg/ml), frozen.
- Buffer P1 (cat. no.19051, Qiagen)
- Qubit dsDNA BS Assay (Invitrogen)

- Nextera XT DNA Library Preparation Kit (Illumina)
- Nextera XT Index Kit (Illumina)
- Miseq Reagent Kit v3 (600-cycle) (Illumina), containing Reagent Cartridge, HT1, Pr2 Bottle and Miseq Flow Cell.
- PhiX Control v3 (Illumina)
- Microseal ´B´ adhesive film
- 96-well hard shell TCY plate
- TruSeq Index Plate Fixture (Illumina)
- 2.5L ice bucket

C) Procedure

1. Pellet preparation

- Isolates to be sequenced must be fresh. In order to have isolated colonies, streak them in blood agar and incubate the plates at 37°C during 24-48°C
- In sterile 2ml microtubes (as many as isolates) add 800 µl of PBS
- For each isolate, take few colonies from the same plate with a sterile loop and introduce them in a microtube
- Vortex thoroughly
- Short spin at maximum speed
- Eliminate the supernatant without disturbing the pellet
- Using the pellet (up to 2 x 10^9 bacterial cells) proceed to extract the DNA (section C) or otherwise freeze the pellet at -20°C.

2. High Molecular Weight DNA extraction

- For an optimal DNA quality, we recommend to extract the high molecular weight DNA with MagAttract HMW DNA Kit (Qiagen). Extract the HMW according to the manufacturer´s instructions (see MagAttract® HMW DNA Handbook).
- In the last step of this kit, we recommended to substitute buffer AE by 100 µl nuclease free water to perform the elution of the DNA and to maximize the concentration.
- Use BS broad range Qubit assay according to the manufacturer´s instructions to quantify your eluted HMW DNA.
- In new microtubes (1.5 ml) add 2 µl of your HMW DNA and adjust to 0.2 ng/µl with water to achieve a final concentration of 0.2 ng/µl.

- If you do not have Qubit but Nanodrop or Dropsense instead, increase the final concentration to 0.3 ng/µl.

3. *Library preparation*

In order to save costs, the library preparation can be performed at 1/5 of the normal volume for each reagent. If you are an unexperience user, perform the library preparation under the usual conditions (see reference guide from Nextera XT DNA Library Prep Kit). See the components needed and storing conditions before starting.

3.1 Tagment genomic DNA

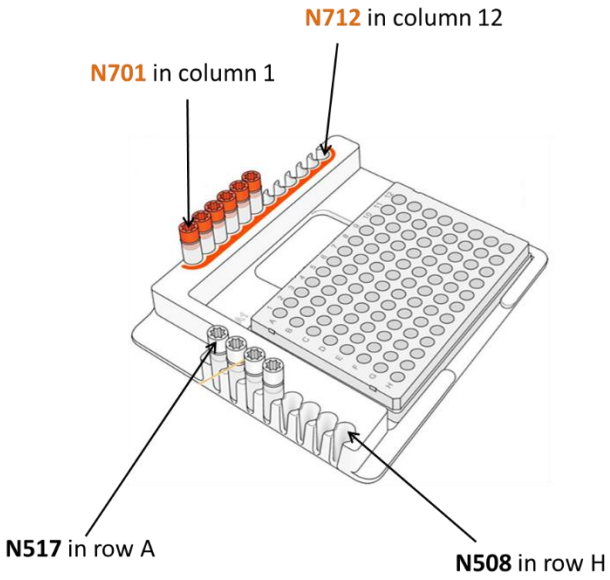
- Thaw the index kit, Amplicon Tagment Mix (ATM) and the Tagment DNA Buffer in the fridge (previously frozen)
- Bring the NPM (Nextera PCR Master Mix) to room temperature
- Vortex the Neutralize Tagment Buffer (NT) if a precipitate is visible (stored at RT).
- Invert all tubes 3-5 times
- Shortly spin down all tubes
- Label a 96 well plate as TAGMENT plate
- Pipette 2 µl of TD Buffer in each well (according to your number of samples)
- Add 1 µl gDNA (0,2ng/µl Qubit,) in each well
- Add 1 µl ATM (Amplicon Tagment Mix) in each well
- Pipet up and down to mix
- Seal the TAGMENT plate with sealing film
- Spin down for 1 min in a microcentrifuge
- Place the TAGMENT plate on a PCR thermocycler and run the tagmentation program with the following programme:
  - 5 min at 55°C
  - NTA55 hold at 10°C
- As soon as cycler reaches 10°C continue immediately with the NEUTRALISATION of the tagment plate because the transposome is still active. DO NOT INTERRUPT WORKFLOW!

3.2. Neutralization

- Shortly spin down tagment plate in a plate centrifuge
- carefully remove sealing film
- pipette 1 µl NT (Neutralize Tagment Buffer) in each well of the tagment plate
- mix the sample and the NT Buffer by pipetting up and down
- seal tagment plate
- spin down for 1 min in a microcentrifuge
- incubate tagment plate for 5 min at RT.

3.3. *Amplification*

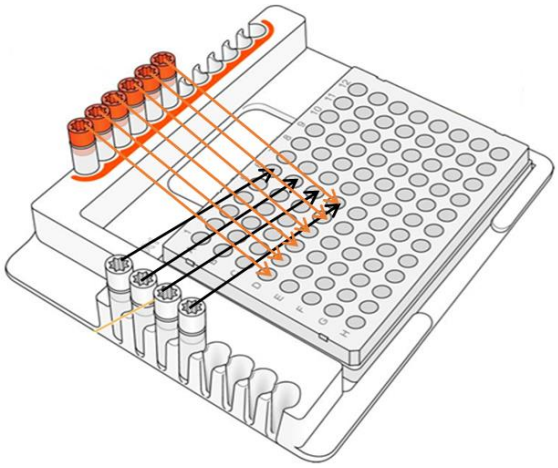
- Invert all tubes and shortly spin down
- For 96 samples arrange the index primers in the Truseq Index Plate Fixture as follows:
    - arrange Index 1 (i7) adapters (orange) in columns 1-12 of the TruSeq Index Plate
    - arrange Index 2 (i5) adapters (white) in rows A-H of the Truseq Index Plate



    - record position of Index tubes in your protocol in a chart like the following one:

	N701	N702	N703	N704	N705	N706	N707	N708
S517								
S502								
S503								
S504								
S505								
S506								
S507								
S508								

- Place TAGMENT plate in the TruSeq Index Plate Fixture
- Pipet 3 µl NPM (Nextera PCR Master Mix) into each well
- Using a multichannel pipette add 1 µl index 1 adapters (orange) down each column.
- Mix sample and index adapters by pipetting up and down
- Using a multichannel pipette add 1 µl index 2 adapters (white) across each row.
- Mix sample and index adapters by pipetting up and down



- replace the old caps of the white and orange index adapters by new ones
- seal TAGMENT plate
- spin down TAGMENT plate for 1 min in a plate centrifuge
- place TAGMENT plate in the PCR thermocycler using the following conditions:

72°C, 3 min
95°C, 30 sec
12 cycles of:
    95°C 10 sec
    55°C 30 sec
    72°C 30 sec
72°C, 5 min
Hold at 10°C

- This a **SAFE STOPPING POINT!** You can now store the tagment plate at 2°C to 8°C for up to 2 days

3.4. Clean up libraries

- Bring Ampure Beads to RT
- According to the number of samples, freshly prepare 80% ethanol (20 ml H2O + 80 ml 100% EtOH)
- Thaw RSB (Resuspension Buffer)
- Spin down TAGMENT plate for 1 min in the plate centrifuge

- Label a MIDI plate (deep well plate) as CAA plate (Clean Amplified Plate)
- Transfer 10 µl PCR product from each well of the TAGMENT plate to the CAA plate
- Vortex AMPure XP Beads for 30 sec
- Add 6 µl AMPure XP Beads to each well of the CAA plate
- Seal the CAA plate
- Shake at 1800 rpm for 2 min
- Incubate for 5 min at RT (without shaking)
- Place on magnetic stand and wait until liquid is clear (~ 2 min)
- Remove and discard all supernatant from each well while the plate is still placed on the magnetic stand. If you take beads up by removing the supernatant, leave the plate on the magnetic stand for another 2 min and repeat this step.
- Wash 2 times as follows:
  - Add 40 µl freshly prepared 80% EtOH to each well while plate is placed on magnetic stand. Do not resuspend beads!!!
  - Incubate for 30 sec on magnetic stand. Do not disturb beads!!!
  - Remove and discard all supernatant from each well
- Using a 10µl pipette carefully remove residual EtOH from each well
- Air-dry on magnetic stand for 15 min
- Remove CAA plate from magnetic stand
- Add 10,5 µl RSB (Resuspension Buffer) to each well
- Seal the plate
- Shake at 1800 rpm for 2 min
- If the sample is not homogenously spread in RSB, pipette up and down
- Incubate at RT for 2 min (without shaking)

- Place on magnetic stand and wait until liquid is clear (~ 2 min)
- Label new 96 well plate as CAN plate (Clean Amplified NTA Plate)
- Transfer 10 µl of the supernatant from the CAA plate (still placed on magnetic stand) to CAN plate.
- This a **SAFE STOPPING POINT!** You can now store the CAN plate at -20°C for up to 1 week

3.5. Checking libraries with Bioanalyzer (optional)

Run 1 µl of library on a Bioanalyzer using a high-sensitivity DNA chip to check library size. As long as you have a distribution of sample from approximately 200bp to 1.5kb, you can proceed withsequencin

3.6. Normalization of the libraries

This set of reagents in the normalization of the library contains formamide, an aliphatic amide that is a probable reproductive toxin. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Wear protective equipment, including eye protection, gloves, and laboratory coat. Work under a fume hood.

- Thaw LNA1 (Library Normalization Additives 1)
- Vortex LNA1 - watch out for precipitate
- Bring LNB1 (Library Normalization Beads 1) to RT
- Bring LNW1 (Library Normalization Wash 1) to RT
- Vortex LNB1 vigorously until no beads are present at the bottom of the tube
- Label a new MIDI (deep well) plate as LNP (Library Normalization Plate)
- Transfer 4 µl from CAN to LNP
- For each sample pipette 7,8 µl LNA1 in a 1,5 ml microtube using a 1000 µl pipette set to 1000 µl pipette LNB1 15-20 x up and down until pellet vanishes
- Immediately add LNB1 to LNA1 in the 1,5 ml tube (1,4 µl LNB/sample)
- Invert tube 15-20 x
- Add 9 µl LNA1/LNB1 mix to each well of the LNP plate
- Seal LNP plate
- Shake the plate at 1800 rpm for 30 min
- Place LNP plate on magnetic stand and wait until liquid is clear (~ 2 min)
- Using a 50 µl pipette set to 20 µl remove and discard supernatant from each well while the plate is still placed on magnetic stand
- Remove LNP from magnetic stand

- Work now under the fume hood and wash two times as follows:
  - add 9 µl LNW1 (Library Normalization Wash 1) to each well
  - seal LNP
  - shake LNP for 5 min at 1800 rpm
  - place LNP plate on magnetic stand and wait until liquid is clear (~ 2 min)
  - using a 10 µl pipette set to 10 µl remove and discard supernatant from each well while LNP is still placed on magnetic stand
  - remove LNP from magnetic stand
- Add 6 µl 0,1 N NaOH solution to each well
- Discard the rest (do not put 0,1N NaOH back in freezer)
- Seal LNP
- Shake LNP for 5 min at 1800 rpm
- Label a new 96 well plate as SGP (Storage Plate)
- Pipette 6 µl LNS1 Buffer in each well of SGP
- Make sure that all samples in the LNP are resuspended, if not, pipette to mix and put back on shaker for another 5 min at 1800 rpm
- Place LNP plate on magnetic stand and wait until liquid is clear (~ 2 min)
- Transfer 6 µl of the supernatant from LNP to SGP
- Seal SGP
- Spin down SGP for 1 min at 1,000 g
- This a **SAFE STOPPING POINT!** You can now store the SGP plate at -20°C for up to 1 week or continue with the pooling of the libraries.

3.7. Pooling of libraries

- Set thermomixer to 98°C
- Thaw MiSeq reagent cartridge in cold water bath
- Prepare ice bucket: 3 parts ice cubes 1 part water
- Thaw SGP at RT
- Spin down SGP 1 min 1,000 g
- If SGP was stored at -20°C pipet each sample that will be pooled up and down to mix
- Label a 1,5 ml microtube as PAL (Pooled Amplicon Library)
- Pool a volume (in µl) of each sample from SGP to the PAL tube according the table below:

Genus	Species	Genome Size (Mb)	Pooling factor	Volume

Staphylococcus	*aureus*	2.8	1,5	7,5 µl
Enterococcus	*faecium*	2.9	1	6 µl
Enterococcus	*faecalis*	2.9	1	6 µl
Salmonella	*enterica*	4.9	2	10 µl
Escherichia	*coli*	5.5	2	10 µl
Klebsiella	*pneumoniae*	5.5	2	10 µl
Peptoclostridium	*difficile*	4.3	2	10 µl

- Mix PAL thoroughly by pipetting up and down

- Label new microtube as DAL (Diluted Amplicon Library)
- Pipet 570 µl HT1 (Hybridization buffer) into DAL
- Transfer 24 µl from PAL to DAL (note: if you take more PAL, you have to take less HT1 accordingly in order that the total volume remains 600 µl)
- Store PAL for up to one week at -20°C!
- Mix DAL thoroughly by pipetting up and down
- Vortex DAL at max. speed
- Incubate DAL 2 min at 98°C in thermomixer (OR optional: incubate DAL 4 min shaking at 850 rpm)
- Invert DAL 1-2 x
- Place DAL immediately on ice and leave there for 5 min
- Thaw phiX control (20 pM solution not older than 3 weeks)
- In the meantime remove cartridge from water bath, invert 10x, tap on workbench 3-4x and
- Pierce the "Load samples reservoir" using a pipette tip
- Add 6 µl 20 pM phiX control to the DAL tube
- Pipet 600 µl (total volume) DAL into the "Load Samples reservoir" of the cartridge.

4. Sequencing

- Proceed directly to the run setup steps using the MiSeq Control Software (MCS) interface (accordingly with the Miseq System Guide from Illumina) to perform paired-end sequencing (2 × 300 bp).

5. Quality check, de novo assembly and genomic analysis

- Your analysis software will check for adapters or determine the base quality within the reads, among other parameters. Analyze your raw reads with a quality control check software such as FastQC. If this step is already included in your pipeline, the quality check will be performed automatically and those reads with a Phred quality score below 20 will not pass the quality filter.

- Afterwards, trim your reads in order to have sufficient read quality for subsequent analysis and to eliminate adaptor sequences. Command-based tools like Trimmomatic or FastX-toolkit can be used at this point to improve the quality of your reads.
- Check again the quality of your trimmed sequences with FastQC.
- *de novo* assemble the resultant raw reads into draft genomes (FASTA format) using SPAdes.
- Establish now a filter in your genotyping software (e.g: Seqpshere) to select only for genotyping those assemblies with a minimum coverage of 5-fold and minimum length of 200 bp.
- Extract *in silico* the 7 loci composing the MLST targets for each species with specific software (e.g., Ridom Seqsphere +, Bionumerics, CLC Genomics Workbench), command-line tools (e.g. MLST Github) or alternatively retrieve them by inserting the sequences in FASTA format in https://pubmlst.org/. When available, perform cgMLST for your isolates and assess their genetic relatedness by generating a Minimum Spanning Tree (MST). Use the default threshold for each species.
- Run each FASTA file in CARD database (Analyze>RGI) with default parameters to retrieve the ARGs present in each isolate.

D) References

Illumina MiSeq System Guide. Document#15027617v05Material#20000262. August 2019. Available at: https://support.illumina.com/content/dam/illumina-support/documents/documentation/system_documentation/miseq/miseq-system-guide-for-local-run-manager-15027617-05.pdf

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Andrews, S. (2010). FastQC: A Quality Control Tool for High Throughput Sequence Data [Online]. Available online at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc/.

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