Foreword

The working group (WG) has been established by the European Commission with the aim to promote the use of Next Generation Sequencing (NGS), and in particular Whole Genome Sequencing (WGS), across the networks of the European Union Reference Laboratories (EURLs) to improve WGS capacity within the European Union (EU) and ensure liaison between the EURLs, the European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC) activities concerning the WGS mandate sent by the Commission. The WG includes all the EURLs operating in the field of the microbiological contamination of food and feed. The present document represents a deliverable of the WG and is meant to be dispatched to the respective networks of the National Reference Laboratories (NRLs).

Guidance document for WGS-laboratory procedures

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Table of contents

1. Introduction……………………………………………………………………………….3

2. Results…………………………………………………………………………………………4

3. Selected methodologies for DNA extraction from bacteria ……………………………….4

4. Selected methodologies for DNA extraction from parasites……………………………..5

5. Selected methodologies for RNA extraction from viruses……………………………….5

6. Selected methodologies for not pathogen-specific DNA extraction……………………….6

7. Library preparation and workflows………………………………………………………..7

8. Appendix 1. List of the SOPs and LOPs…………………………………………………..9

9. Appendix 2. List of the web links for direct downloading of the protocols…………………11

10. Annex. List of the LOPs and SOPs developed at EURLs………………………………..13

1. Introduction

The use of whole genome sequencing (WGS) for diagnostics and surveillance of pathogenic microorganisms is becoming more and more widespread. Common applications include the identification of pathogens (bacteria, viruses, and parasites), investigation of antimicrobial resistance (AMR) determinants, virulence gene detection and investigations of outbreaks.

The availability of high quality DNA is a prerequisite for the successful application of WGS methodologies. In this regard, efficient DNA extraction is strongly influenced by the nature of the pathogen and by the possibility to use in vitro culture systems prior to it. In the case of uncultivable pathogens (e.g., most parasites), enrichment procedures are needed to purify the target organisms from the outnumbering number of unrelated organisms, including the host.

This guidance document provides a list of the available Standard Operating Procedures (SOPs) or Laboratory Operating Procedures (LOPs) for nucleic acid extraction, to validate the performances and suitability for the NRLs network. In addition, information was sought on procedures that can potentially reduce the time from culture to sequencing, using different library preparation and sequencing kits.

2. Results: identification of SOPs and LOPs

Different sources of information were considered. In particular, SOPs and LOPs developed in the context of specific projects (EU COMPARE, ENGAGE and INNUENDO, EFSA projects, and others) were searched by visiting the projects' websites. This resulted in the selection of 16 protocols (Appendix 1 and 2), which address a range of pathogens (bacteria, virus and parasites) and comprise DNA and RNA extraction protocols, combined protocols and complete workflows.

LOPs and SOPs developed at the EURL for *E. coli* (Italy), at the EURL for *Listeria monocytogenes* and at the EURL for coagulase positive Staphylococci (France) were also included and the respective documents are appended at the bottom of this document.

3. Selected methodologies for DNA extraction from bacteria

DNA extraction usually starts from bacterial cultures obtained from a single colony. Two LOPs developed by the Animal and Plant Health Agency (UK) during the COMPARE project have been included in this guidance document. The first describes an automated method based on magnetic-bead technology that extracts high quality DNA from bacterial cells (link 1 in Appendix A). The second LOP describes a rapid and inexpensive method to extract DNA from bacterial cells by boiling. The crude extract generated by this procedure has proven to be suitable for the preparation of libraries for whole genome sequencing (link 2 in Appendix A).

These two LOPs are mentioned in the SOP "DNA extraction and library preparation when using the Illumina sequencing platform" developed by the ENGAGE project (link 3 in Appendix A). This

document also specifies other DNA extraction methods for bacteria, based on commercial kits (Easy-DNA™ Kit, Thermofisher), or on automated procedures (QIAsymphony SP/AS instrument), which have been used at DTU Food (Denmark) and Public Health England (UK), respectively.

Finally, two operating procedures have been developed by the EURL for *Listeria monocytogenes* (France) and by the EURL for coagulase positive Staphylococci (France). These procedures also describe downstream analyses to quantitate and evaluate the purity and integrity of the genomic DNA extracted (see Annexes 1 and 2 appended to this document).

4. Selected methodologies for DNA extraction from parasites

As mentioned in the Introduction, most parasites cannot be grown using *in vitro* systems and it is therefore necessary to purify the stages present in the specific matrix (faeces, host tissues, water and food samples) under analysis. It is also important to consider that the parasite stages present in these matrices are very hard and difficult to lyse. Two LOPs developed by the EURL for Parasites (Italy) during the COMPARE project have been included in this guidance document. These describe all the laboratory procedures that can be used to process faecal samples to purify the oocysts of *Cryptosporidium* (link 4 in Appendix A) or the cysts of *Giardia* (link 5 in Appendix A), and to efficiently extract high quality DNA from these protozoa.

5. Selected methodologies for RNA extraction from viruses

Two LOPs for RNA extraction from viruses, developed by IFREMER (France) and by the Animal and Plant Health Agency (UK) during the COMPARE project, have been included in this guidance document. The first LOP (link 6 in Appendix A) is to be used for liquid samples, and describes the combined use of a lysis buffer, which break viral capsids under chaotropic salt condition, with a magnetic silica bead-based RNA extraction step. The second LOP (link 7 in Appendix A) is to be used for swabs and tissue samples, and describes lysis of virus under highly denaturing conditions using followed by binding of nucleic acids to a silica fibre-containing filter.

A third LOP (link 8 in Appendix A), developed by the Animal and Plant Health Agency (UK) during the COMPARE project, has also been included in this guidance document. The LOP describes the conversion of RNA to double-stranded cDNA, a step necessary prior to next-generation sequencing experiments.

6. Selected methodologies for not pathogen-specific DNA extraction

Four LOPs, developed for nucleic acid extraction from specific matrices and not for a specific group of pathogens, have been included in this guidance document. The extracted DNA/RNA can be used for metagenomics experiments, both targeted (amplicon-based) or untargeted (shotgun). The first LOP, developed at DTU Food (Denmark) during the COMPARE project, describes a procedure to extract DNA from faecal and sewage samples using a commercial kit and a modified protocol (link 9 in Appendix A). The second LOP, developed at University of Bologna (Italy) during the COMPARE project, focuses on rinsates from chicken carcasses, which is a common matrix to detect pathogens from commercially processed broilers. The LOP provides a protocol that uses the whole

rinsate to extract DNA based on a commercial kit (link 10 in Appendix A). The third LOP, also developed at University of Bologna (Italy) during the COMPARE project, describes a protocol to extract DNA from chicken gutsections, including caeca, ilea and crops (link 11 in Appendix A).

The fourth LOP, developed at University of Veterinary Medicine (Hannover, Germany) during the COMPARE project, describes a protocol to extract DNA from formalin-fixed paraffin embedded (FFPE) tissue samples. The protocol combines a de-paraffinization of FFPE tissue sections followed by DNA purification using spin columns (link 12 in Appendix A).

7. Library preparation and workflows

A number of LOPs/SOPs that describe protocols for the library preparation step and/or sequencing on different NGS platforms are also included in this guidance document.

The first LOP, developed by the Animal and Plant Health Agency (UK) during the COMPARE project, focuses on the preparation of sequencing libraries for Illumina instruments using the NexteraXT kit. The starting material includes purified bacterial DNA, double-stranded cDNA, boilates or heat-killed cells (link 13 in Appendix A). A second LOP, also developed by the Animal and Plant Health Agency (UK) during the COMPARE project, describes the steps required to set up a run on the Illumina MiSeq instrument (link 14 in Appendix A).

The third LOP, developed by Ifremer (France) during the COMPARE project, describes a combined protocol for RNA fragmentation, cDNA synthesis and library preparation for NGS applications. The procedure aims at the preferential selection of viral RNA during cDNA synthesis, which is then

fragmented (size range, 200 bp) to produce a library using with a size distribution centred to 300 bp. Sequencing is by the Illumina platform (link 15 in Appendix A).

The fourth LOP, developed by the Erasmus Medical Center (Netherlands) during the COMPARE project, describes the preparation of libraries to be sequenced on the Ion Torrent S5XL platform. The LOP has been tested on different matrices (sewage filtrate, cell culture supernatant and stool), and is based on random priming to allow detection of viral sequences present in the sample without prior knowledge (link 16 in Appendix A).

An additional SOP, developed by the EURL for *Listeria monocytogenes* (France) describes how to prepare libraries using the Nextera XT kit (Illumina) for sequencing on the Illumina MiSeq platform (see Annex 3 appended to this document).

Finally, the EURL for *E. coli* (Italy) has optimized a protocol for the preparation and sequencing of libraries (400 bp) on the ION Torrent platform (see Annex 4 appended to this document).

Appendix 1. List of the SOPs and LOPs with information on their sources and field of application

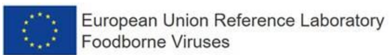
Source	Pathogen(s) targeted	Description	Main application	Method	Link
COMPARE	Bacteria	DNA extraction	WGS	LOP Automated DNA extraction (bacteria)	1
COMPARE	Bacteria	DNA extraction	WGS	LOP Preparation of cell boilates to extract DNA suitable for sequencing library preparation	2
ENGAGE	Bacteria	Workflow/DNA extraction/library preparation	WGS	SOP for DNA extraction and library preparation when using the Illumina sequencing platform	3
COMPARE	Parasites	DNA extraction	WGS	LOP Purification of *Cryptosporidium* oocysts and extraction of DNA for NGS Analyses	4
COMPARE	Parasites	DNA extraction	WGS	LOP Purification of *Giardia* cysts and extraction of DNA for NGS analyses	5
COMPARE	Virus	RNA extraction	WGS	LOP Extraction of total RNA from concentrated viral solution	6
COMPARE	Virus	RNA extraction	Metagenomics	LOP RNA extraction from swabs and tissue samples	7
COMPARE	Non-pathogen specific	cDNA synthesis	All	LOP Conversion of RNA to double-stranded cDNA for NGS	8
COMPARE	Non-pathogen specific	DNA extraction	Metagenomics	SOP Isolation of DNA from fecal and sewage samples using the QIAmp Fast DNA Stool Mini Kit and modified protocol	9
COMPARE	Non-pathogen specific	DNA extraction	Metagenomics	LOP DNA extraction protocol from chicken carcass wash-water	10
COMPARE	Non-pathogen specific	DNA extraction	Metagenomics	LOP DNA extraction protocol from chicken gut	11
COMPARE	Non-pathogen specific	DNA extraction	Metagenomics	LOP Isolation of DNA from formalin-fixed and paraffin-embedded tissue using the QIAamp DNA FFPE Tissue Kit	12

COMPARE	Bacteria	Library preparation	WGS	LOP Sequencing library preparation for Illumina instruments using the NexteraXT Kit	13
COMPARE	Bacteria	Library preparation	WGS	LOP Running prepared sequencing libraries on the Illumina MiSeq platform	14
COMPARE	Virus	Combined protocol	WGS	LOP RNA fragmentation, cDNA synthesis and library preparation for NGS applications	15
COMPARE	Virus	Library preparation	WGS	LOP Agnostic Ion Torrent sequencing	16

Appendix 2. List of the web links for direct downloading of the protocols.

Procedure	Web link
1	www.compare-europe.eu/-/media/Sites/compare-europe/Protocols/LOP_NaE_DNA_BacteriaAutomated_COMPARE_APHA_v1
2	www.compare-europe.eu/-/media/Sites/compare-europe/Protocols/LOP_NaE_DNA_Bacteria_COMPARE_APHA_v1
3	http://www.engage-europe.eu/-/media/Sites/engage-europe/Final-website-documents/ENGAGE_AppC_SOPs-for-DNA-extraction-and-library-preparation_final
4	www.compare-europe.eu/-/media/Sites/compare-europe/Protocols/LOP_NaEWGA_Cryptosporidium_COMPARE_ISS_v1
5	www.compare-europe.eu/-/media/Sites/compare-europe/Protocols/LOP_NaEWGA_Giardia_COMPARE_ISS_v1
6	www.compare-europe.eu/-/media/Sites/compare-europe/Protocols/LOP_NA-extraction_COMPARE_Ifremer_V1
7	www.compare-europe.eu/-/media/Sites/compare-europe/Protocols/LOP_RNA_extraction_COMPARE_APHA_v1
8	www.compare-europe.eu/-/media/Sites/compare-europe/Protocols/LOP_cDNA_synthesis_COMPARE_APHA_v1
9	www.compare-europe.eu/-/media/Sites/compare-europe/Protocols/SOP_DNA_QIAFastDNA_COMPARE_DTU_v3
10	www.compare-europe.eu/-/media/Sites/compare-europe/Protocols/LOP_NaE_DNA_PowerFood_COMPARE_UNIBO_v1
11	www.compare-europe.eu/-/media/Sites/compare-europe/Protocols/LOP_NaE_DNA_chicken-gut_COMPARE_UNIBO_v2

12	www.compare-europe.eu/-/media/Sites/compare-europe/Protocols/LOP_NaE_DNA_QIAamp-DNA-FFPE-Tissue-Kit-_COMPARE_TiHo_v1
13	www.compare-europe.eu/-/media/Sites/compare-europe/Protocols/LOP_Library-Prep_Illumina_COMPARE_APHA_v1
14	www.compare-europe.eu/-/media/Sites/compare-europe/Protocols/LOP_Sequencing_IlluminaMiSeq_COMPARE_APHA_v1
15	www.compare-europe.eu/-/media/Sites/compare-europe/Protocols/LOP_RNA-to-library_COMPARE_Ifremer_V1
16	www.compare-europe.eu/-/media/Sites/compare-europe/Protocols/LOP_Agnostic-IonTorrent-Sequencing_COMPARE_EMC



Annex. List of the LOPs and SOPs developed at EURLs

Source	Pathogen targeted	Description	Main application	Method	
EURL *Lm*	Bacteria	DNA extraction	WGS	LOP Isolating genomic DNA from *Listeria monocytogenes*	Annex 1
EURL *Cps*	Bacteria	DNA extraction	WGS	LOP Isolating genomic DNA from *Staphylococcus aureus*	Annex 2
EURL *Lm*	Bacteria	Library preparation	WGS	SOP Library preparation using the Nextera-XT kit for Illumina MiSeq sequencing	Annex 3
EURL *E.coli*	Bacteria	Library preparation	WGS	LOP Optimization of “NEBNext Fast DNA fragmentation and library preparation set for Ion Torrent” by New England Biolabs for 400 bp libraries	Annex 4

OPERATING PROCEDURE

ISOLATING GENOMIC DNA FROM *LISTERIA MONOCYTOGENES*

Issuer	Author(s)	Validator(s)
Sylvie RUDELLE	**Jean François MARIET** **Benjamin FELIX** **David ALBERT**	**David ALBERT** **Agnes CHAMOIN**

Historic
This is the first version of the operating procedure

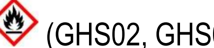
Caution: Trade names or suppliers may be mentioned in the description of the products necessary for applying this method. This information is given as an indication for users of the method and does not constitute an endorsement by the EURL for *Listeria monocytogenes*. Equivalent products may be used if it has been demonstrated that they lead to the same results.

1. MEDIA, REAGENTS AND EQUIPEMENT

CULTURE MEDIA

Non-selective solid media adapted for Lm culture: Tryptone Soy Agar with 0.6% of yeast extract (TSAYE).
Non-selective liquid media adapted for Lm culture: Brain heart medium (BHI).

REAGENTS

- Isopropanol, room temperature  (GHS02, GHS07)
- 70% ethanol, room temperature (H225, H319)
- EDTA (50 mM, pH 8.0)
- Water (molecular biology grade)
- 10mg/ml lysozyme (ref 10 837 059 001, Roche)
- Nuclei Lysis Solution (ref A7941, Promega)
- RNase Solution (ref A797C, Promega)
- Protein Precipitation Solution (ref A795A, Promega)
- Molecular weight 1kb (ref G5711, Roche)
- Loading buffer 6x (ref G190A, Promega)
- Ethidium bromide 10 mg/ml (BET) (ref E-1510, Sigma)
- TBE 10X dilution 1/10 (ref 11 666 703 001, Roche)
- Qubit dsDNA HS Assay Kit (ref Q32854, Invitrogen)
- Seakem GTG Agarose (ref 50070, Lonza)

EQUIPEMENT

- Thermostated centrifuge (6 000-20 000 rcf)
- 1.5ml microcentrifuge tubes (safelock if possible)
- UV-visible spectrophotometer (600 nm) adapted to the measurement of the turbidity of cell density
- Spectrophotometric cuves (4ml PS or PMMA)
- Spectrophotometer cuvette
- Water bath at 37 °C ± 1°C (drybath possible)

- Dry bath 80°C ± 3°C
- Fluorometer (Qubit)
- Micro-volume spectrophotometer (Nanodrop)
- Balance

2. PREPARATION OF SAMPLES

The extraction is performed on confirmed strains of *Listeria monocytogenes* strains according to the method EN ISO 11290-1 and 2.

1. Each strain to be analysed is isolated on TSAYE agar and incubated at 37°C±1°C for 18-24 hours.

2. One colony is inoculated in BHI liquid medium, at 37°C±1°C for 16-18h.

3. OPERATING PROCEDURE

1. Transfer 2 ml of the BHI culture into a spectrophotometer cuvette and measure OD at 600 nm (expected value: 1.0 to 1.8). If the measurement is too weak extend the incubation of the BHI, if the measurement is too high, dilute the culture with non-inoculated BHI

2. Add 1.4 ml from cuve to a 1.5 ml microcentrifuge tube.

3. Centrifuge at 6 000 rcf and room temperature for 5 minutes to pellet the cells.

4. Remove the supernatant by rapid inversion and dry the sides of the tube with a clean absorbent paper. This step can also be done with a micropipette.

5. Resuspend the cells thoroughly in 480 µl of 50 mM EDTA.

6. Add 120 µl lysozyme to the resuspended cell pellet and gently pipet to mix. The purpose of this pretreatment is to weaken the cell wall of Lm so that efficient cell lysis can take place.

7. Incubate the sample at 37 °C for 60 minutes in a waterbath.

8. Centrifuge for 2 minutes at 16 000 *rcf*

9. Remove the supernatant by rapid inversion and dry the sides of the tube with a clean absorbent paper. This step can also be done by pipetting.

10. Add 600 µl of Nuclei Lysis Solution. Gently pipet until full resuspension of cells, if after 20 pipetting pellet is not fully relaxed proceed to step 11.

11. Incubate at 80 °C (dry bath) for at least 10 minutes to lyse the cells; if necessary gently invert the tube to allow complete dispersion of the pellet; repeat inversions each five minutes, in the limit of 45 minutes; then cool to room temperature.

12. Add 3µl of RNase Solution to the cell lysate. Invert the tube 6 times to mix.

13. Incubate at 37 °C for 60 minutes. Cool the sample to room temperature.

14. Add 200 µl of Protein Precipitation Solution to cell lysate. Mix by vortexing vigorously at high speed for 20 seconds to mix the Protein Precipitation Solution with the cell lysate. Treat each sample sequentially.

15. Incubate the sample on ice for at least 30 minutes and with a maximum of 1 hour.

16. Centrifuge at 20 000 rcf for 3 minutes. (16 000 rcf is possible).

17. Transfer the supernatant containing the DNA (≈ 700 µl) to a clean 1.5ml microcentrifuge tube containing 600 µl of room temperature isopropanol.

Note: Be careful not to transfer the white protein precipitate into the new tube. Leave some residual liquid in the tube to avoid contamination of the DNA solution with the precipitated protein.

18. Gently mix by inversion until the thread-like strands of DNA form a visible mass.

19. Centrifuge at 20 000 rcf for 2 minutes.

20. Remove the supernatant by rapid inversion and dry the sides of the tube with a clean absorbent paper. This step can also be done by pipetting.

21. Add 600 µl of room temperature 70% ethanol and gently invert the tube several times to wash the DNA pellet. If necessary, flip the bottom of the tube to allow release of the DNA pellet.

22. Centrifuge at 20 000 rcf for 2 minutes.

23. Carefully aspirate the ethanol with a micropipette or pour-off by fast inversion. Drain the tube on clean absorbent paper and allow the pellet to air-dry for 15 minutes until complete ethanol evaporation.

24. Add 100 µl of water (molecular biology grade) to the tube and rehydrate the DNA by incubating the solution overnight at 5°C ± 3°C.

25. Store at 5°C ± 3°C if the DNA is used within 48h or at a temperature ≤ -18 °C for long-term storage.

4. DNA QUANTITATION AND QC

QUALITY CONTROL OF DNA PURITY USING UV VISIBLE SPECTROPHOTOMETER (NANODROP)

1. DNA quantitation (Absorbance measured at 260nm)
2. Purity 260nm/280nm ratio (protein contamination) (Acceptable value around 2) and 260nm/230nm ratio (organic compounds contamination) (Acceptable value around 2)
3. Spectral curve (check the shape of the curve)

Use 2 µl of sample for measurement.

FLUOROMETRIC QUANTITATION (QUBIT 3.0 + QUBIT™ DSDNA HS ASSAY KIT)

1. Use a 10 fold dilution of extracted DNA.
2. Prepare High sensitivity mix buffer according to manufacturer (1µl Qubit™ dsDNA HS Reagent + 199µl Qubit™ dsDNA HS Buffer).
3. Mix 10 µl 1/10-dilution DNA with 190 µl Mix buffer.
4. Incubate 3 min and measure according to manufacturer instruction

GENOME INTEGRITY

1. Prepare a Seakem GTG Agarose gel at 0.8% (any basic agarose is acceptable if allowing resolution of DNA > 1kb)
2. Fill each well with 200 ng of DNA (Qubit measured)
3. Migration 3 - 4h with an electrical field at 120V/105mA in TBE 1X

DNA extraction & quality controls flowchart

Option B: to be used if option A does not lead to satisfactory results.

Cell preparation

Option A

- Lm Plating on TSAYE Agar 18-24h 37 ± 1°C
- Pick 1 isolated colony in 10 ml of BHi 16-18h 37 ± 1°C
- 2 ml of BHi in 4 ml PS spectrophotometric cuve OD_{600} target: 1.0 to 1.8
- Transfer 1.4 ml in a 1.5 ml safelock microtube
  Centrifuge 5 min at 6000 rcf at room temperature
  Remove supernatant by inversion or pipetting
  Resuspend cells in 480 µl EDTA 50 mM pH 8.0 (freshly prepared)

Option B

- Plating on TSAYE Agar 18-24h 37 ± 1°C
- Scratch culture from plate and resuspend it in 2 ml TE buffer OD_{600} target: 1.0 to 1.8
- Transfer 480 µl in a 1.5 ml safelock microtube
  Resuspend pellet by 10 s vortexing

Both options:

- Add 120 µl Lysozyme solution 10 mg/ml
  Resuspend pellet by 10 s vortexing
  Incubation 60 min in 37 °C ± 1°C (waterbath or drybath)

Cell lysis

- Centrifuge 2 min at 16000 rcf at room temperature
  Remove supernatant by inversion and wipe tubes
  Add 600 µl Nuclei Lysis
- Pellet resuspension by 10 gentle pipetting
  Incubation 10 min at 80 °C
  Gently shake by inversion 4 times
  If cells remain clogged incubate 5 min more, then shake 8 times by inversion
  Repeat until full dispersion (less than 45 min)
- Cool down tube to room temperature, add 3 µl RNase A solution
  Invert tubes six times to mix
  Incubation 60 min in 37 °C (waterbath or drybath)

Protein precipitation

- Cool to room temperature
  Add 200 µl Protein precipitation solution. Vortex 20 s.
  Incubate on ice 30 min (1 hour max)
  Centrifuge 3 min 20000 rcf

DNA precipitation

- Transfer supernatant (≈ 700 µl) to 600 µl room temperature Isopropanol in 1.5 ml safelock tube
  Shake by gentle inversion and centrifuge 2 min 20000 rcf
  Remove supernatant by inversion or pipetting
- Add 600 µl 70% ethanol and shake by inversion and/or flipping to free DNA pellet
  Centrifuge 2 min 20000 rcf then remove supernatant by inversion or pipetting
  Air dry 10-15 min at room temperature

Quality controls

DNA Purity
UV Absorbance (Nanodrop)
260 nm / 280 nm ratio
260 nm / 230 nm ratio
DNA quantitation (relative absorbance 260 nm)

DNA quantitation
Fluorometric quantitation (Qubit)
10 fold dilution of sample in wtaer
Based on result prepare 200 ng aliquots for electrophoresis

→ **DNA integrity and RNA contamination**
Agarose Gel electrophoresis
0,8 % agarose in 1x TBE buffer
3h migration 120V
Staining in Ethidium bromide

Only the electronic version of this document is authentic

Adrien ASSERE 07/04/2019 Applicable immédiatement

MODE OPERATOIRE

GENOMIC DNA EXTRACTION OF S.AUREUS USING THE PROMEGA WIZARD KIT

EMETTEUR	REDACTEUR(S)	SIGNATAIRE(S)
Isabelle MUTEL	D. MERDA; N. VINGADASSALON	Yacine NIA Agnes CHAMOIN

OBJET DES MODIFICATIONS
Création de document

Les modifications sont repérées dans le texte par un trait dans la marge.

Seuls la version informatique et les exemplaires en diffusion contrôlée font foi.

Caution: **Trade names or suppliers may be mentioned in the description of the products necessary for applying this method. This information is given as an indication for users of the method and does not constitute an endorsement by the EURL for Coagulase Positive Staphylococci (CPS). Equivalent products may be used if it has been demonstrated that they lead to the same results.**

1. MEDIA, REAGENTS AND EQUIPMENT

CULTURE MEDIA

- Non-selective solid media adapted for CPS culture: Milk Plate Count Agar (milk-PCA).
- Non-selective liquid media adapted for CPS culture: Brain heart medium (BHI).

REAGENTS

- Milk-PCA (ref AEB 620717, Biomérieux).
- EDTA (50 mM, pH 8.0)
- 10mg/ml lysozyme (L6876-5MG, Sigma)
- 10mg/ml lysostaphine (L7386-5MG, Sigma)
- Nuclei Lysis Solution (ref A7941, Promega)
- RNase Solution (ref A797C, Promega)
- Protein Precipitation Solution (ref A795A, Promega)
- Isopropanol, room temperature (GHS02, GHS07)
- 70% ethanol, room temperature (H225, H319)
- Water (molecular biology grade MBG) (ref W3513, Sigma)
- Molecular weight 1kb (ref G5711, Roche)
- Loading buffer 6x (ref G190A, Promega)
- TBE 10X dilution 1/10 (ref 11 666 703 001, Roche)
- Ethidium bromide 10 mg/ml (BET) (ref E-1510, Sigma)
- Qubit dsDNA HS Assay Kit (ref Q32854, Invitrogen)
- Seakem GTG Agarose (ref 50070, Lonza)
- Nusieve Agarose GTG (ref 50080, Lonza)

EQUIPEMENT

- UV-visible spectrophotometer (600 nm) adapted to the measurement of the turbidity of cell density
- Thermostated centrifuge (6 000 - 20 000 rcf)
- 1.5 ml microcentrifuge tubes (safe lock if possible and max recovery)

- Spectrophotometer cuvette
- Dry bath at 37 °C ± 1°C
- Dry bath with shaking 80°C ± 3°C (thermomixer)
- Ice machine
- Micro-volume spectrophotometer (Nanodrop)
- Fluorometer (Qubit)
- Balance
- Microwave
- Water bath (50°C ± 2°C,)
- Electrophoresis tank
- Staining bath
- UV gel instrument

2. PREPARATION OF SAMPLES

The extraction is performed on confirmed strains of *Staphylococcus aureus* strains.

Pick one isolated colony from Milk-PCA and suspend in 10ml of BHI. Incubate it 16 – 18 h at 37°C.

3. OPERATING PROCEDURE

1. Transfer 2 ml of the BHI culture into a spectrophotometer cuvette and measure OD at 600 nm (expected value: 1.0 to 2.5). If the measurement is too weak extend the incubation of the BHI, if the measurement is too high, dilute the culture with non-inoculated BHI

2. Add 1.5 ml of the BHI culture into a 1.5 ml safe lock microcentrifuge tube.

3. Centrifuge at 16 000 rcf and room temperature for 2 min to pellet the cells.

4. Remove the supernatant by rapid inversion and the rest with a micropipette.

5. Resuspend the cells thoroughly in 480 µl of 50 mM EDTA.

6. Add 105 µl lysozyme and 15 µl lysostaphine to the resuspended cell pellet and gently pipet to mix. The purpose of this pretreatment is to weaken the cell wall of CPS so that efficient cell lysis can take place.

7. Incubate the sample at 37 °C for 60 min in a dry bath.

8. Centrifuge for 8 min at 16 000 *rcf* and 10°C

9. Remove the supernatant by rapid inversion and the rest by pipetting.

10. Add 600 µl of Nuclei Lysis Solution. Gently pipet until full resuspension of cells, if after 20 pipetting pellet is not fully relaxed proceed to step 11.

11. Incubate at 80 °C (thermomixer) for 30 min with shaking (3000 rpm) to lyse the cells then let it cool down 5 min at 4°C; if necessary gently invert the tube to allow complete dispersion of the pellet; repeat inversions each five min, in the limit of 45 min; then cool to room temperature.

12. Add 3 µl of RNase Solution to the cell lysate. Invert the tube 6 times to mix.

13. Incubate at 37 °C for 60 min in a dry bath. Cool the sample at room temperature.

14. Add 200 µl of Protein Precipitation Solution to cell lysate. Mix by vortexing vigorously at high speed for 20 seconds to mix the Protein Precipitation Solution with the cell lysate. Treat each sample sequentially.

15. Incubate the sample on ice for at least 20 min and maximum 1 hour.

16. Centrifuge at 16 000 rcf for 3 min and 10 °C.

17. Simultaneously, prepare 1.5 ml max recovery (low bindind) tubes with 600 µl isopropanol of room temperature.

18. Transfer the supernatant containing the DNA (≈ 700 µl) to this isopropanol tubes.

Note: Be careful not to transfer the white protein precipitate into the new tube. Leave some residual liquid in the tube to avoid contamination of the DNA solution with the precipitated protein.

19. Gently mix by inversion until the thread-like strands of DNA form a visible mass.

20. Centrifuge at 16 000 rcf for 2 min.

21. Remove the supernatant by rapid inversion and the rest by pipetting.

22. Add 600 µl of room temperature 70% ethanol and gently invert the tube several times to wash the DNA pellet. If necessary, flip the bottom of the tube to allow release of the DNA pellet.

23. Centrifuge at 16 000 rcf for 2 min and 10°C.

24. Carefully aspirate the ethanol with a micropipette or pour-off by fast inversion. Drain the tube on clean absorbent paper and allow the pellet to air-dry for 15 min until complete ethanol evaporation.

25. Add 100 µl of water (molecular biology grade) to the tube and rehydrate the DNA by incubating the solution overnight at 4°C.

26. Store at 4°C the DNA before use for the sequencing.

Seuls la version informatique et les exemplaires en diffusion contrôlée font foi.

QUALITY CONTROL OF DNA PURITY USING UV VISIBLE SPECTROPHOTOMETER (NANODROP)

1. DNA quantitation (Absorbance measured at 260 nm)

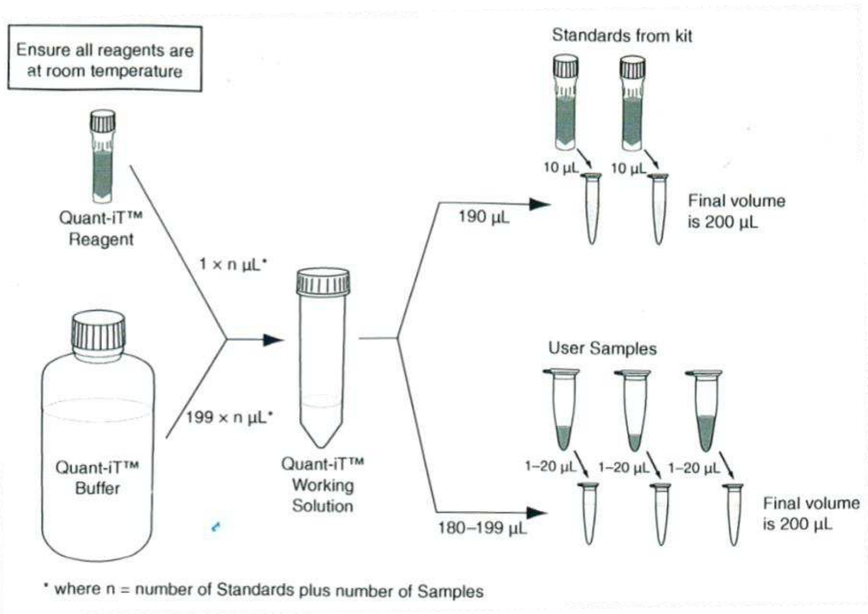
2. Purity 260 nm / 280 nm ratio (protein contamination) (Acceptable value around 2 and between 1.80 and 2.20) and 260 nm / 230 nm ratio (organic compounds contamination) (Acceptable value around 2 and between 1.80 and 2.20)

3. Make a blank with water (molecular biology grade). Use 2 µl of DNA sample for measurement. Spectral curve (check the shape of the curve).

4. Export the results table.

FLUOROMETRIC QUANTITATION (QUBIT 3.0 + QUBIT™ DS DNA HS ASSAY KIT)

1. Use a 100 fold dilution of extracted DNA. (10 µl DNA sample in 90 µl of water (molecular biology grade) for 1/10 dilution then 10 µl of this 1/10 sample in 90 µl water (molecular biology grade) for 1/100 sample).
2. Prepare High sensitivity mix buffer according to manufacturer (1 µl Qubit dsDNA HS Reagent + 199µl QubitTM dsDNA HS Buffer).



3. Mix 10 µl 1/100-dilution DNA with 190 µl Mix buffer. Vortexing 10 s

4. Incubate 3 min and measure according to manufacturer instruction

5. Read the standard sample then the DNA sample.

6. Export the results table.

Seuls la version informatique et les exemplaires en diffusion contrôlée font foi.

Genome Integrity

1. Prepare a Seakem GTG Agarose gel at 0.8%. Mixture of both agarose Seakeam GTG and Nusieve agarose.

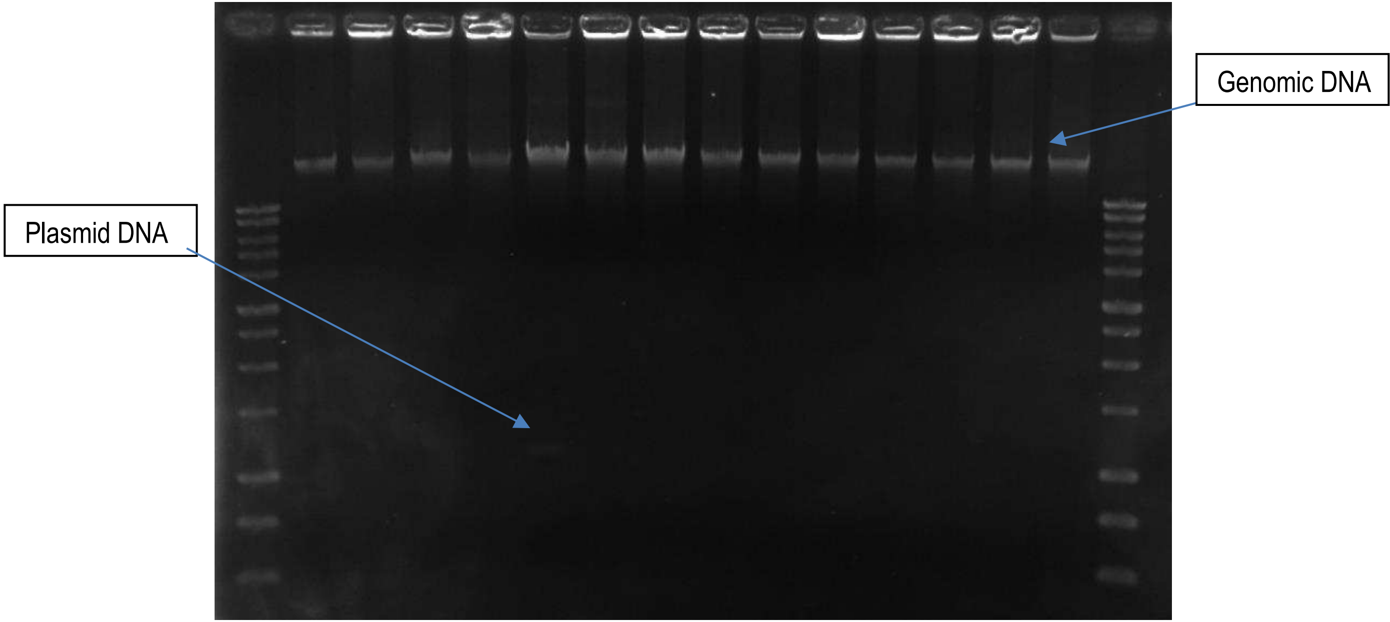
Gel size	Seakem GTG agarose (g)	Nusieve agarose (g)	1X TBE buffer (ml)
Small (12 samples)	0.34	0.34	80
Regular (20 - 40 samples)	0.50	0.50	125

2. Fill each well with 200 ng of DNA ((depending on the Qubit measurement)

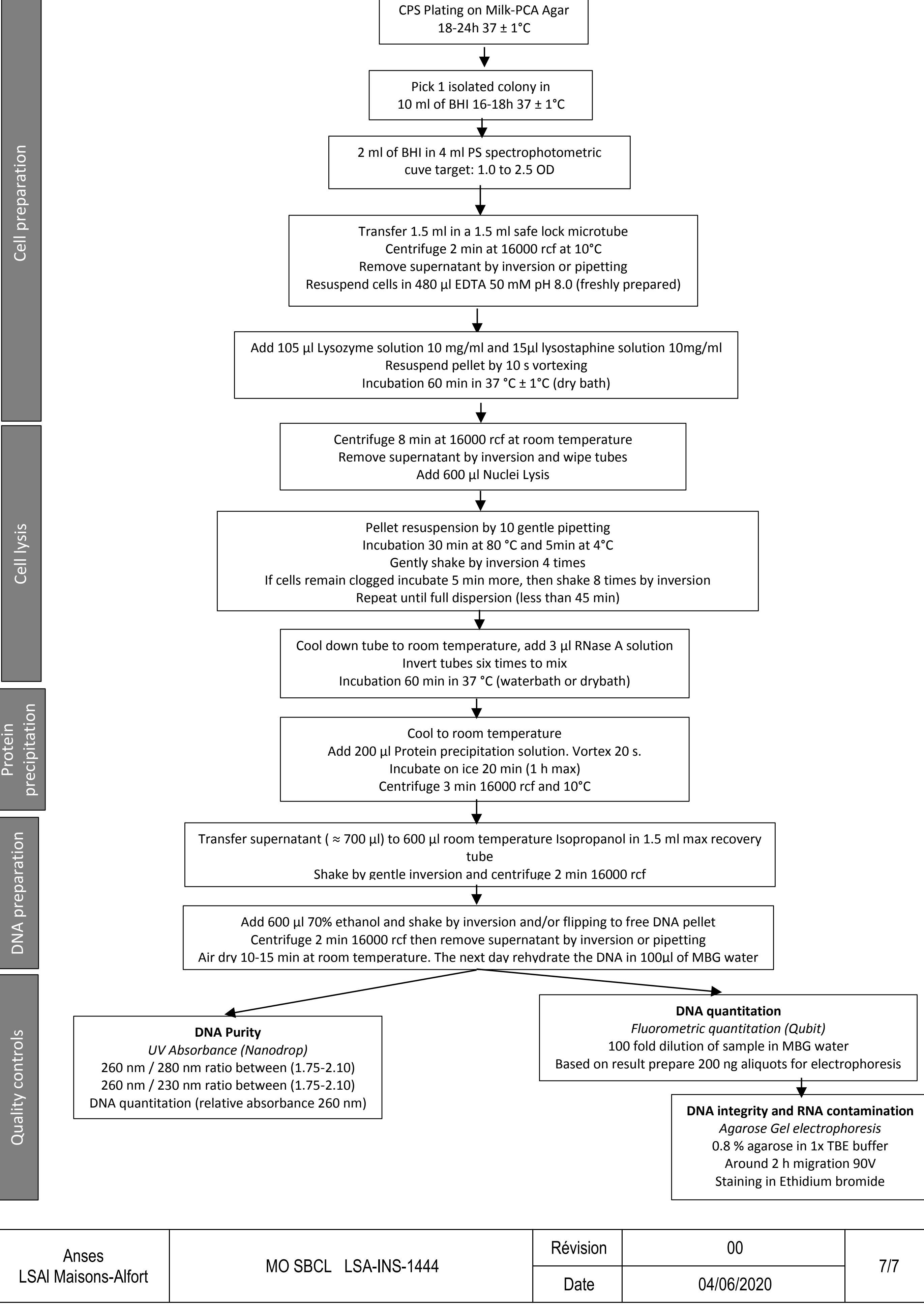
- Volume for 200 ng (µl) = Original sample concentration (ng/µl) / 200
- Water volume to dilute (µl) = 10 - Volume for 200 ng

3. Migration around 2 h with an electrical field at 90V in TBE 1X.

Overview of expected results on the agarose gel:



DNA extraction & quality controls flowchart



STANDARD OPERATING PROCEDURE

LIBRARY PREPARATION USING THE NEXTERA XT KIT FOR ILLUMINA MISEQ SEQUENCING

ISSUER	AUTHOR(S)	ENDORSER(S)
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LIST OF MODIFICATIONS
Title and frontpage update

Modifications are identified in the text by a line in the margin.

1. GENERALITIES

1.1. OBJECTIVES

The objective of this procedure is to detail how to prepare libraries using the Nextera XT kit (Illumina) for sequencing on the MiSeq (Illumina).

1.2. EQUIPMENTS

- Nanodrop
- Qubit
- Microcentrifuge
- PeqStar thermocycler
- IKA microplate shaker (for Option B : Bead-based normalization)
- BioAnalyzer (or TapeStation) (for Option A : standard normalization)
- MiSeq (check reservation planning on the shared network drive)

1.3. MATERIALS AND REAGENTS

- Qubit dsDNA high sensitivity kit (reagents + standards) (ThermoFisher Q32854)
- Qubit assay tubes (ThermoFisher Q32856)
- Eppendorf 0.5-ml LoBind tubes
- Aluminium foil
- 15-ml conical tubes
- 1.5-ml microcentrifuge tubes
- Illumina Nextera XT kit (Illumina FC-131-1024 or FC-131-1096)
- Illumina Index and replacement caps (Illumina FC-131-1001, FC-131-2001)
- AMPure XP beads (or equivalent size-selection beads) (Beckman Coulter A63880 or A63881; Amplitech AC-60005 or AC-60050; Macherey Nagel 744970.5 or 744970.50)
- 96-well plate or PCR strips (depending on how many samples are processed at once)
- 96-well plate film (Biorad microseal B and Roche LC480 film)
- Multichannel pipette and corresponding tips
- Various pipettes and corresponding tips
- Absolute ethanol
- Magnetic stand
- Molecular biology grade water
- 1M NaOH (freshly prepared or from frozen stock)
- Illumina MiSeq reagent cartridge

1.4. PREREQUISITE BEFORE STARTING

Important:

Some reagents in this procedure contain formamide or di-methyl formamide which are known teratogens. Consequently, Option B of this procedure is strictly forbidden for pregnant women.

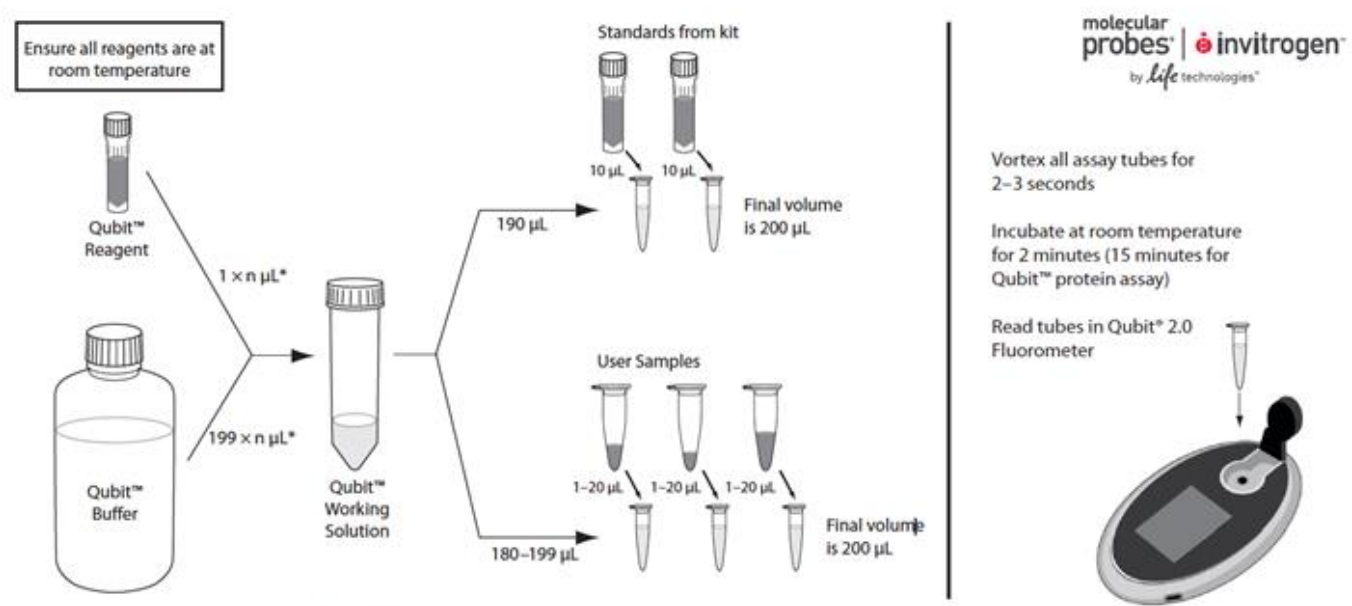
Before starting check that:
- The devices are available
- Reagents / consumables needed are present and in sufficient quantity

Remarks :

- Thoroughly centrifuge the plates before pipetting
- The different steps take place in different rooms. Respect the different areas (especially for post-PCR manipulations).
- Many steps require precise timing. Be sure to have a timer.
- Some steps require ice
- Please decontaminate all areas with bleach once you're finished!

2. QUBIT QUANTIFICATION USING GDNA EXTRACTIONS

Samples with a concentration lower than 100ng/uL can be quantified on the Qubit.



Pre quantify your samples after genomic extractions using the Nanodrop to make sure you are below 100ng/uL.

1. Plug in the Qubit. Choose “DNA”, then “dsDNA high sensitivity”,
2. Prepare a mix for your samples (x) + standards (2): n=x+2,
3. n*199uL of Qubit buffer + n*1uL of light sensitive Qubit reagent,
4. Cover the rack with aluminum foil,
5. Dispend 190uL of the Qubit working solution into 2 small “Probe Molecular” tubes labeled S1 and S2. Protect from light,
6. Add 10ul of the Standard 1 and Standard 2 on tubes (S1 and S2), respectively,
7. Protecting the tubes with a glove, vortex the tubes and incubate at room temperature protected from light for 3 minutes,
8. Briefly spin down the tubes and insert into the Qubit,
9. Select “Read Standard 1”,
10. Insert the second standard and select “Read Standard 2”. Then the machine is ready to quantify your samples,
11. Prepare x tubes with 199uL of Qubit working solution,
12. By batches of few tubes, add 1uL of gDNA sample in each tube,
13. Protecting the tubes with a glove, vortex the tubes and incubate at room temperature protected from light for 3 minutes,
14. Briefly spin down the tubes and insert into the Qubit,
15. Select “Read Sample”,
16. When the result appears select “Stock Concentration”,
17. Crawl down to 1uL (sample size),
18. Select “ng/uL” as unit for the concentration and record the concentration in the following table on the “Qc” column,
19. Proceed the same for each sample,
20. Using the table, calculate the Final volume (Vf=Qc*10) and the volume of water (VH2O=Vf-2),
21. Based on these calculations make dilutions of your samples to reach the theoretical concentration of 0.2ng/uL. Mix 2uL of your sample with VH2O of molecular grade water.

Sample name		Read Concentration (ng/uL)	Final volume (uL)	V of water (uL)
		Qc	*= Qc*10*	*= Vf-2*
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				
13				
14				
15				
16				
17				
18				
19				
20				
21				
22				
23				
24				

NB: If the dilution factor is important, check the new concentration with the Qubit dsDNA high sensitivity kit

3. LIBRARY PREPARATION USING PRE-QUANTIFIED GDNA EXTRACTION

Do not vortex any buffer of the Nextera XT kit, Invert few times to homogenate before utilization.

1. First you need to design your entire WGS array. Fill the lab tracking sheet to assign indexes to each of your strains, so they will all have their specific code (Illumina Experiment Manager).
2. Remove reagents from the freezer and let them thaw for 30 minutes in ice bucket:
   ATM (Amplicon Tagment Mix)
   TD (Tagment DNA Buffer)
   indexes
   NPM (Nextera PCR Master Mix)
   RSB (Resuspension Buffer)
   NOTE: Turn on thermocycler to allow it to begin warming.

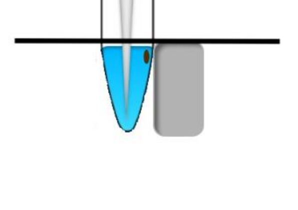
3. Remove reagents from cooler and let them warm up at room temperature:
   AMPure XP beads
   NT (Neutralize Tagment Buffer)

4. Following 30 minutes on ice, remove indexes from ice and allow them to begin warming to room temperature.
5. Using PCR strip tubes (or 96 well-plate), add 10uL of TD buffer to each well to be used for this assay.
   NOTE: Change tips each time to ensure the delivered volume is correct.
6. Add 1ng of input **DNA** to each well (5µl of dilution at 0.2 ng/µl).
7. Add **5uL of ATM buffer** to each well. Be careful, as this buffer is viscous.
   NOTE: Change tips each time to ensure the delivered volume is correct.
   **Also, this is a time sensitive step; too much time will result in fragments that are too small.*
8. **Mix well but gently and slowly by pipetting as pictured on the right**.
   NOTE: use a 100uL pipette set to 15uL.
9. Close the tubes (or seal plate with film B).
10. Centrifuge at 300xg at room temperature for one minute.
11. Incubate in thermocycler and run the following program: (NEXTERA, NTA program, V=20uL).
    a. 55C for 5 min
    b. Hold at 10C for 1 min.
    NOTE: Because this step is time and temperature sensitive, do not leave the thermal cycler.
12. Add **5uL of NT buffer** in each well. Mix well by pipetting as described in step 8.
    NOTE: this is also a time sensitive step, use a multichannel pipette whenever possible
13. Close the tubes (or seal plate with film LC480) and centrifuge at 300xg at room temperature for one minute.
14. Incubate the samples at room temperature for 5 minutes.
    NOTE: This time can be used to mix and centrifuge the indexes.
15. Add **5uL of the appropriate white-capped indexes** in each well. Be really careful to avoid cross-contamination. Discard previous cap and replace with a new one .
16. Add **5uL of the appropriate orange-capped indexes** in each well. Be really careful to avoid cross-contamination. Discard previous cap and replace with a new one.
17. Mix well by pipetting as described in step 8.
18. Add **15uL of NPM buffer** in each well.
    NOTE: Change tips each time to ensure the delivered volume is correct.
19. Close the tubes (or seal plate) and centrifuge at 300xg at room temperature for one minute.
    NOTE: This is a critical step. The plate needs to be tightly sealed with a microseal to avoid sample evaporation.
20. Perform the following PCR program: (NEXTERA, PCR program, V=50uL)
    a. 72C for 3 minutes
    b. 95C for 30 sec
    c. 12 cycles of: 95C for 10 sec, 55C for 30 sec, 72C for 30 sec
    d. 72C for 5 minutes
    e. Hold at 10C
    NOTE: Vortex the AMPure XP beads for 30 sec. This will allow time for the bubbles to settle out of the solution before use in step 24.
21. Centrifuge samples at 300xg at room temperature for one minute.

NOTE: The next steps are to be performed in the post-PCR room

22. Transfer PCR products into a PCR strip or 96-well plate (v=50uL)
23. Vortex strongly the AMPure XP beads for 5 additional sec.
24. For 2x150 runs on the Miseq, add **30uL of AMPure XP beads** in each well.
    For 2x250 runs on the MiSeq, add **25 µL of AMPure XP beads** in each well.
    NOTE: Change tips each time to ensure the delivered volume is correct.
    NOTE: change volume of beads according to the desired size of insert
    NOTE: Avoid bead carryover by confirming no liquid droplets are on your pipette tip. This will affect the ratio of beads to PCR reaction and affects the insert size.

25. Mix well by pipetting as described in step 8. Inspect the mixture; it is important that the brown beads are suspended well throughout the entire solution.
26. Incubate at room temperature for 5 to 10 minutes.
    NOTE: Use this time to prepare 400uL of 80% ethanol per sample (320uL of absolute ethanol + 80uL of e-pure water).



27. Place the plate or tubes on a magnetic stand for 2 minutes.
    a. It is important for the beads to collect into a pellet just below the solution's surface.
    b. This can best be achieved by not forcing the tube to the bottom of the magnetic stand or by using a spare pipette tip to wedge the tube at an appropriate angle.
28. Discard supernatant. Avoid disturbing the pellet.
    NOTE: It may be easier to avoid the pellet with a small pipette (200uL) for steps 29-34.
29. Add **200uL of 80% Ethanol solution** without disrupting the beads.
30. Incubate 30 sec.
31. Discard supernatant. *(Avoid disturbing the pellet.)*
32. Add **200uL of 80% Ethanol solution** without disrupting the beads.
33. Incubate 30 sec.
34. Discard all the supernatant. *(Avoid disturbing the pellet.)*
35. Let air-dry for a minimum of **10 minutes** on the magnetic stand.
    NOTE: Exceeding the maximum air-dry period can make resuspension difficult and is not recommended. Over-drying is indicated by cracks in the bead pellets.

    NOTE: Make sure that RSB buffer is well thawed and mix well by vortexing.
36. "Rehydrate" the beads with **52.5uL of RSB buffer** and mix by pipetting as described in step 8**.**
37. Incubate at room temperature for 2 minutes.
38. Place the plate on the magnetic stand for 2 minutes.
39. **Carefully** transfer 30uL of supernatant into a new strip of PCR tube. *(Avoid disturbing the pellet.)*
40. Safe stopping point. Store the samples at +4°C or -20°C.

4. OPTION A: STANDARD LIBRARY NORMALIZATION

NOTE: For small number of libraries or tagmented libraries concentration < 15 nM

1. Quantify the samples with Qubit dsDNA high sensitivity kit (cf p2).
2. Determine the average size of the libraries using the Bioanalyzer (cf Procedure for qualifying libraries using the bioanalyzer 2100 High Sensitivity DNA Assay) or TapeStation.
3. Calculate the molarity (in nM) of each library according to the table below.
4. Transfer 20 µl of each library to a new tube (or plate) and dilute in to 2 nM with RSB according to the table below.

Sample name		Concentration (ng/uL)	Average size (bp)	Molarity (nM)	Final volume (uL)	V of RSB (uL)
		Qc	*Size*	*Mc=* *(Qc / (Size * 660))*1.10⁶*	*Vf=Mc*10*	*=Vf-20*
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						
11						

5. OPTION A: LIBRARY POOLING AND MISEQ SAMPLE LOADING

1- Remove a **MiSeq reagent cartridge** from the freezer and thaw at room temperature in a water bath (Need approximately 1 hour).
2- Remove **HT1** (Hybridization buffer) from freezer and thaw at room temperature.
3- In an ice bucket prepare an ice-water bath with 3 parts ice and 1 part water.
4- Prepare fresh 0.2N NaOH from a 1N frozen stock solution (40 µl H2O + 10 µl 1N NaOH)
5- Transfer 10 µl of each diluted sample in a single 1.5 ml LoBind tube.
6- Denature the 2 nM library pool by combining 5µl of the 2 nM library pool and 5 µl 0.2N NaOH.
7- Vortex **briefly** and spin down
8- Incubate for 5 minutes at room temperature
9- Add 990 µl pre-chilled HT1 to the tube containing the denatured library. The result is 1 ml of 10 pM denatured library.
NOTE: Keep the denatured library in ice-water until ready to load.

NOTE: During that time invert the MiSeq reagent cartridge several times to mix all reagents.
10- Load Diluted libraries into the Load Samples reservoir of the MiSeq reagent cartridge.

6. OPTION B: BEAD-BASED LIBRARY NORMALIZATION

Note: the following steps are to be performed under a chemical hood.

1. Remove reagents from freezer and let them thaw to room temperature
   LNA1 (Library Normalization additives 1)
   NOTE: Before use, vortex vigorously to make sure there is no precipitate or crystal.
   NOTE: contains formamide, handle with gloves.
2. Remove reagents from fridge and let them thaw to room temperature
   LNB1 (Library Normalization Beads 1)
   LNW1 (Library Normalization Wash 1)
   NOTE: Vigorously vortex LNB1 for at least 1 minute
3. Transfer 20 µl of the library to a 96-well plate (Bio-Rad TCY plate).
   NOTE: Change tips each time to avoid cross-contamination
   NOTE: Perform the next steps under a fume hood.
4. For 96 samples, add 4.4 ml of LNA1 to a fresh tube.
   NOTE: Scale down according to actual number of samples +1
5. Resuspend LNB1 by pipetting up and down 15-20 times with a P1000 pipette set at 800 µl.
6. For 96 samples, immediately transfer 800 µl of LNB1 to the tube containing LNA1. Mix well by inverting the tube 15-20 times.
   NOTE: Scale down according to actual number of samples +1
7. Add 45 µl of the combined LNA1/LNB1 to each well of the plate containing libraries.
8. Seal the plate with microseal B.
9. Shake the plate on the IKA microplate shaker at exactly 1800 rpm for 30 minutes.
   NOTE: this is a time sensitive step. Incubation of greater or less than 30 minutes may affect library representation and cluster density.
   NOTE: This time can be used to prepare fresh 0.1 N NaOH
10. Place the plate on a magnetic stand for 2 minutes.
11. With the plate on the magnetic stand, with a pipette set to 80 µl, carefully remove and discard the supernatant.
12. Remove the plate from the magnetic stand and wash with LNW1 as follows:
    a. Add 45 µl of LNW1 to each well.
    b. Seal the plate with microseal B
    c. Shake on the IKA shaker at 1800 rpm for 5 minutes
    d. Place the plate on the magnetic stand for 2 minutes
    e. Carefully remove the supernatant
13. Remove the plate from the magnetic stand and repeat the wash with LNW1:
    a. Add 45 µl of LNW1 to each well.
    b. Seal the plate with microseal B
    c. Shake on the IKA shaker at 1800 rpm for 5 minutes
    d. Place the plate on the magnetic stand for 2 minutes
    e. Carefully remove the supernatant
14. Remove the plate from the magnetic stand and add 30 µl of fresh 0.1N NaOH.
15. Seal the plate with microseal B.
16. Shake the plate on the IKA shaker at 1800 rpm for 5 minutes.
    NOTE: Make sure that all samples are completely resuspended.
17. Place the plate on the magnetic stand for 2 minutes.
18. Add 30 µl LNS1 (Library Normalization Storage buffer, stored at RT) to each well of a new fresh plate.
19. Transfer the supernatant from the library to the fresh plate containing LNS1
    NOTE: Change tips each time to avoid cross-contamination
    NOTE: Make sure NOT to aspirate any bead.
20. Seal the plate with microseal B and centrifuge at 1000g for 1 minute.
21. Proceed to library pooling or store the plate at -20°C for up to a week.

7. OPTION B: LIBRARY POOLING AND MISEQ SAMPLE LOADING

1. Remove a **MiSeq reagent cartridge** from the freezer and thaw at room temperature in a water bath (Need approx 1 hour).
2. Remove **HT1** (Hybridization buffer) from freezer and thaw at room temperature.
3. In an ice bucket prepare an ice-water bath with 3 parts ice and 1 part water.
4. Set a heat-block to 96°C.
5. Transfer 5 µl of each library into a single 1.5 ml tube
6. Transfer 576 µl of HT1 into a second 1.5 ml tube.
7. Transfer 24 µl of the pooled libraries to the tube containing HT1. Using the same tip, pipette up and down 3-5 times.
   NOTE: The recommended volumes for diluting the pooled libraries with HT1 represent a 25-fold dilution. If less than 5 libraries are pooled increase the volume used to reach 24 µl. However, if cluster density is found to be too high or too low, the dilution ratio can be changed.
   NOTE: if genomes of different sizes are to be sequenced together, don't mix equal amounts of each. Longer genomes will require MORE of the library (because it requires more reads for the same coverage)
8. Mix by vortexing at top speed.
9. Incubate on heat block at 96°C for 2 minutes.
10. Invert 1-2 times to mix and immediately place in ice-water bath.
11. Incubate in ice-water bath for 5 minutes.
    NOTE: During that time invert the MiSeq reagent cartridge several times to mix all reagents.
12. Load Diluted libraries into the Load Samples reservoir of the MiSeq reagent cartridge.

Optimized NebNext Fast DNA Fragmentation & Library Protocol from New England Biolabs for 400 bp libraries for Ion Torrent

Manuela Marra(1), Valeria Michelacci(2)

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Bring the following reagents at room temperature before starting:

1) TE 0.1x
2) BARCODE Adapters (on ice)
3) Et-OH 80%

Starting material

100 - 1000 ng of DNA (quantification Qubit Fluorometer is required)

Fragmentation and End Repair

1) Mix the following components in a sterile microfuge tube on ice:

Component	Volume
H_2O	**variable**
DNA	**0.5–7.75 µl**
• (green) NEBNext DNA Fragm. **Reaction Buffer**	**1 µl**
• (green) NEBNext DNA Fragm. **Master Mix** *	**0.75 µl**
final volume	**10 µl**

* Vortex the vial of **NEBNext DNA Fragmentation Master Mix** for 3 seconds

Note: For DNA with an AT content ≥ 70%, add 0.75 µl of • (green) NEBNext Fragmentation Master Mix and 0.75 µl sterile H2O.

3) Incubate in a **thermal cycler** for **15 minutes at 25°C**, followed by **10 minutes at 70°C**, **hold at 10°C.**

4) Pulse spin the microfuge tube and **place on ice.**

! A precipitate can form upon thawing of the NEBNext Q5 Hot Start HiFi PCR Master Mix. To ensure optimal performance, place the master mix at room temperature while performing size selection/cleanup of adaptor-ligated DNA. Once thawed, gently mix by inverting the tube several times.

Preparation of Adaptor Ligated DNA

1) Mix the following components:

Component	Volume
• (red) **T4 DNA Ligase Buffer** for Ion Torrent	**2.0 µl**
• (red) **Bst 2.0 WarmStart DNA Polymerase**	**0.5 µl**
• (red) **T4 DNA Ligase**	**2.0 µl**
Sterile **H2O**	**3.5 µl**
P1 adaptor	**1.0 µl**
final volume	**9 µl**

Add 9 µl of Mix to each Fragmentated DNA sample

2) add 1 µl of different "Ion Xpress Barcode Adapter" (ThermoFisher) to each sample

The **total volume in the microfuge tube should be 20 µl**. Mix the contents by pipetting up and down several times.

3) Incubate in a **thermal cycler** for **15 minutes at 25°C**, followed by **5 minutes at 65°C, hold at 10°C.**

4. Add **2.5 µl** ○ (white) **Stop Buffer**, vortex and pulse-spin.

Cleanup of Adaptor Ligated DNA

1) **Add 81 µl** (1.8X volume) of **AMPure XP Beads*** to the sample and mix by pipetting up and down. (* room temperature)

2) Incubate **5 min at room temp**.

3) Pulse-spin and transfer in a new 1.5 ml DNA LoBind tube. Place in a **magnetic rack for 2-3 min**.

4) Remove and discard the supernatant

5) Keep the tube on the magnet and **add 500 µl of 80% ethanol**. Incubate at room temp. **For 1-2 min**

6) Discard the supernatant.

7) **Repeat steps 5-6**

8) Keeping the tube in the magnetic rack, with the cap open, **air dry** the beads **for 5 minutes at room temp**.
Caution: Do not overdry the beads. This may result in lower recovery of DNA target.

9) Remove the tube from the magnet and **Resuspend** the beads in **22 µl of 0.1X TE.**
(NB volume may be adjusted for specific size selection protocol).

Incubate 5 min at room temp.

10) Pulse spin. Place in the magnetic rack until the beads have collected to the side of the tube and the solution is clear.

11) **Transfer approximately 22µl of the supernatant to a clean tube (LoBind).** Be careful not to transfer any beads

Proceed to **E-Gel size selection**:
select adaptor ligated DNA in the 450-480 bp range for 400 bp libraries

PCR Amplification of Adaptor Ligated DNA

1) Mix the following components in a sterile microfuge tube:

For 100 ng – 1 µg

• (blue) Primers	**2 µl**
Sterile **H_2O**	**1 µl**
• (blue) NEBNext Q5 Hot Start HiFi PCR Master Mix	**25 µl**

2) Add 28 µl of Mix **to 22 µl of Adaptor ligated DNA** in a 0.2 ml PCR tube

3) Incubate in a **thermal cycler** at the following thermal conditions:

98° C, 30 seconds
[98° C, 10 seconds; 58° C, 30 seconds, 65° C, 30 seconds for **4-12 cycles***]
65°C, 5 minutes, hold at 4°

* 8 cycles for 100 ng of DNA (10-12 cycles for 10 ng; 4-6 cycles for 1 µg

Cleanup of Amplified Library

1) **Add 45 µl** (0.9X volume) of **AMPure XP Beads*** to each tube and mix by pipetting up and down e riunire in una provetta LoBind (* mai congelare e usare a t.amb.)

2) Incubate **5 min at room temp**.

3) Pulse-spin. Place in a **magnetic rack for 2-3 min**.

4) Remove and discard the supernatant

5) Keep the tube on the magnet and **add 500 µl of 80% ethanol**. Incubate to room temp. For 1-2 min provetta). Discard the supernatant.

6) **Repeat step 5.**

7) Keeping the tube in the magnetic rack, with the cap open, air dry the beads for 5 min
Caution: Do not overdry the beads. This may result in lower recovery of DNA target.

8) Remove the tube from the magnet. **Resuspend** the beads in **25 µl of 0.1X TE.**
Mix well by pipetting up and down, and **incubate 5 min. at room temp**.

9) Put the tube on the magnetic rack until the solution is clear. **Transfer** approx. **20 µl** to a clean tube.

10) Assess library quality **(1 µl)** on the **Bioanalyzer.**