ODIN

STRENGTHENING ENVIRONMENTAL SURVEILLANCE
TO ADVANCE PUBLIC HEALTH ACTION IN AFRICA

D2.3: ODIN Laboratory Handbook

–

Standard operating procedures for pre-treatment of environmental samples, pathogen analytics and whole genome sequencing

Funded by

the European Union

Deliverable Factsheet

Project Acronym	ODIN
Project Title	STRENGTHENING ENVIRONMENTAL SURVEILLANCE TO ADVANCE PUBLIC HEALTH ACTION
Project Number	101103253
WP Number	2
Lead Beneficiary	THL
Deliverable Title	ODIN Laboratory Handbook – Standard operating procedures for pre-treatment of environmental samples, pathogen analytics and whole genome sequencing
Deliverable Number	D2.3
Deliverable Type	Laboratory handbook
Dissemination Level	Public
Author(s):	Haider Al-Hello (THL, Finland), Outi Nyholm (THL, Finland), Tarja Pitkänen (THL, Finland), Elisa Salmivirta (THL, Finland), Anni Vainio (THL, Finland), Kristiina Valkama (THL, Finland), Vito Baraka (NIMR, Tanzania), Eric Lyimo (NIMR, Tanzania), Steven Mnyawonga (NIMR, Tanzania), Edward Msoma (NIMR, Tanzania), Hillary Sebukoto (NIMR, Tanzania), Modest Chuwa (NPHL, Tanzania), Jackson Claver (NPHL, Tanzania), Ibrahim Mauki (NPHL, Tanzania), Nyambura Moremi (NPHL, Tanzania), Zakaria Garba (IRSS-DRCO, Burkina Faso), Palpouguini Lompo (IRSS-DRCO, Burkina Faso), Yougbaré Sibidou (IRSS-DRCO, Burkina Faso), Marc Tahita (IRSS-DRCO, Burkina Faso), Melissa Kabena Kabengele (UNIKIN, DRC), Vivi Maketa (UNIKIN,

	DRC), Evodie Ngelesi (UNIKIN, DRC), Mays Kisala (INRB Kinshasa, DRC), Brigitte Modadra Madakpa (INRB Goma, DRC), Tavia Bodisa Matamu (INRB Goma, DRC), Marie-Anne Kavira Muhindo (INRB Goma, DRC), Michel Ngimba (INRB Goma, DRC), Pascal Kabuyaya Nzoloka (INRB Goma, DRC), Esperance Tsiwedi Tsilabia (INRB Goma, DRC)
Editor(s):	Outi Nyholm (THL, Finland)
How to cite	Nyholm O, Valkama K, Al-Hello H, Salmivirta E, Vainio A, Lyimo E, Mnyawonga S, Msoma E, Sebukoto H, Chuwa M, Claver J, Mauki I, Moremi N, Garba Z, Lompo P, Sibidou Y, Kabengele M, Ngelesi E, Kisala M, Madakpa B, Matamu T, Muhindo M-A, Ngimba M, Nzoloka P, Tsilabia E, Baraka V, Maketa V, Tahita M, Pitkänen T. ODIN Laboratory Handbook - Standard operating procedures for pre-treatment of environmental samples, pathogen analytics and whole genome sequencing. Nyholm O (editor), The Global Health Network, 2024.

Table of contents

List of abbreviations

AMR	Antimicrobial Resistance
CFU	Colony-forming unit
CP	Carbapenemase-producing
CPE	Carbapenemase-producing Enterobacteriaceae
DRC	the Democratic Republic of the Congo
ESBL	Extended spectrum beta-lactamase
FIB	Fecal Indicator Bacteria
INRB	Institut National de Recherche Biomédicale
IRSS-DRCO	Centre National de la Recherche Technologique et Scientifique
NIMR	National Institute for Medical Research
NPHL	National Public Health Laboratory in Tanzania
PPE	Personal Protective Equipment
SOP	Standard Operating Procedures
THL	Finnish Institute for Health and Welfare
UNIKIN	University of Kinshasa
WGS	Whole genome sequencing

1. Introduction

The ODIN Laboratory Handbook gives guidelines and standard operating procedures for environmental surveillance of pathogens that are posing significant public health burden in Sub-Saharan countries. These priority pathogens are toxigenic *Vibrio cholerae, Salmonella Typhi*, extended spectrum beta-lactamase producing (ESBL) and carbapenemase producing (CP) *Escherichia coli* (*E. coli*) and *Klebsiella pneumoniae* (*K. pneumoniae*). In addition, the numbers of fecal indicator bacteria (FIB) are measured from the water samples to evaluate the magnitude of fecal contamination.

This Handbook includes:

- Guidance for environmental sample collection and sending the samples to laboratory
- Requirements for environmental microbiology laboratory facilities, equipment, and reagents
- Safety guidelines for handling wastewater samples during the sampling and laboratory work
- Sample pretreatment protocols for each sample matrix
- Microbiological and molecular methodology for detection and characterization of priority pathogens from environmental samples
- Protocol for whole genome sequencing (WGS) of bacterial isolates

The Handbook contains the harmonized methods that are applied for analyzing the environmental samples collected during the ODIN consortium research. The following standard methods are needed for conducting the bacteriological water quality investigations and should be available for users of this Handbook:

- ISO 19458:2006 Water quality — Sampling for microbiological analysis
- ISO 8199:2018 Water quality — General requirements and guidance for microbiological examinations by culture
- ISO 11133:2014 Microbiology of food, animal feed and water — Preparation, production, storage and performance testing of culture media
- ISO 7704:2023 Water quality — Requirements for the performance testing of membrane filters used for direct enumeration of microorganisms by culture methods
- ISO/CD 9308-4 Water quality — Enumeration of Escherichia coli and coliform bacteria Part 4: Membrane filtration method for Escherichia coli in water with high levels of background bacteria (draft currently reviewed by the committee)
- ISO 7899-2:2000 Water quality — Detection and enumeration of intestinal enterococci Part 2: Membrane filtration method

The following standard methods are provided as background information and are not necessary for conducting the bacteriological water quality investigations:

- ISO 9308-1:2014 Water quality — Enumeration of Escherichia coli and coliform bacteria. Part 1: Membrane filtration method for waters with low bacterial background flora

- ISO 21872-1:2017 Microbiology of the food chain — Horizontal method for the determination of Vibrio spp. Part 1: Detection of potentially enteropathogenic Vibrio parahaemolyticus, Vibrio cholerae and Vibrio vulnificus
- ISO 19250:2010 Water quality — Detection of Salmonella spp.

The ISO standards can be purchased from the local standards associations, tentatively even with the local languages, or directly from the ISO web shop.

2. Sample collection and sending to the laboratory

2.1 Biosafety precautions during sampling

Working with wastewater may expose laboratory personnel to several potential health hazards. These include exposure to harmful chemicals, pathogens, and biological pollutants. There is also a risk of physical injury from slipping or falling due to the handling of equipment and wet surfaces. In addition, there is the possibility of inhaling harmful gases in the absence of proper ventilation.

Samples are collected following the necessary biosafety precautions by the sampling personnel. Persons performing the sampling from wastewater should use disposable gloves, wear protective clothing and cleanable shoes. Be aware that aerosols may be splashed or otherwise formed in the air from wastewater or contaminated water and thus the personnel performing the sampling should use respirator mask and safety goggles. Respirator masks should preferably be disposable class FFP3 respirator masks. Before starting the sample collection from wastewater, the field team must wear their personal protective equipment.

2.2 Labeling and filling of sample collection form

All the containers that will be used to collect water samples are adequately labeled either prior to or after sampling. The label includes the name of the sampler, the information on the sampling site location (name, GIS), date and time.

The field team will take photographs of the sampling site and fill out sample collection forms.

Information about each sample and sampling event, such as sampling location, sampling date and time, temperature of the water sample at the time of sampling and other observations are recorded to the sample collection form. If some (unusual) events are observed (presence of oil on the water surface, rain, visible fecal debris …), the information must be recorded in the sample collection form.

The sample collection form for recording the sample related metadata and environmental conditions during the sampling event is presented in Annex A.

2.3 Sample collection

Tap water, well water, surface water and wastewater samples are collected according to the predetermined sampling schedule. The sampling program defines whether a grab sample or trap sample such as passive sample is taken. The sampling method is marked to the sample collection form.

In case the samples are taken from tap, where residual chlorine is present, the disinfection chemical needs to be inactivated at the time of sampling by using sodium thiosulphate ($Na_2S_2O_3$ x $5H_2O$). Disinfection chemical inactivation is done by adding 1 ml of sodium thiosulphate with a concentration of 18 mg/ml to one liter of the water sample.

Grab sampling

The water samples can be collected as grab samples into sterile plastic bottles according to ISO 19458 (ISO 19458:2006 Water quality – Sampling for microbiological analysis). Examples of suitable sample volumes for different sample types are presented in Figure 1 and Table 1.

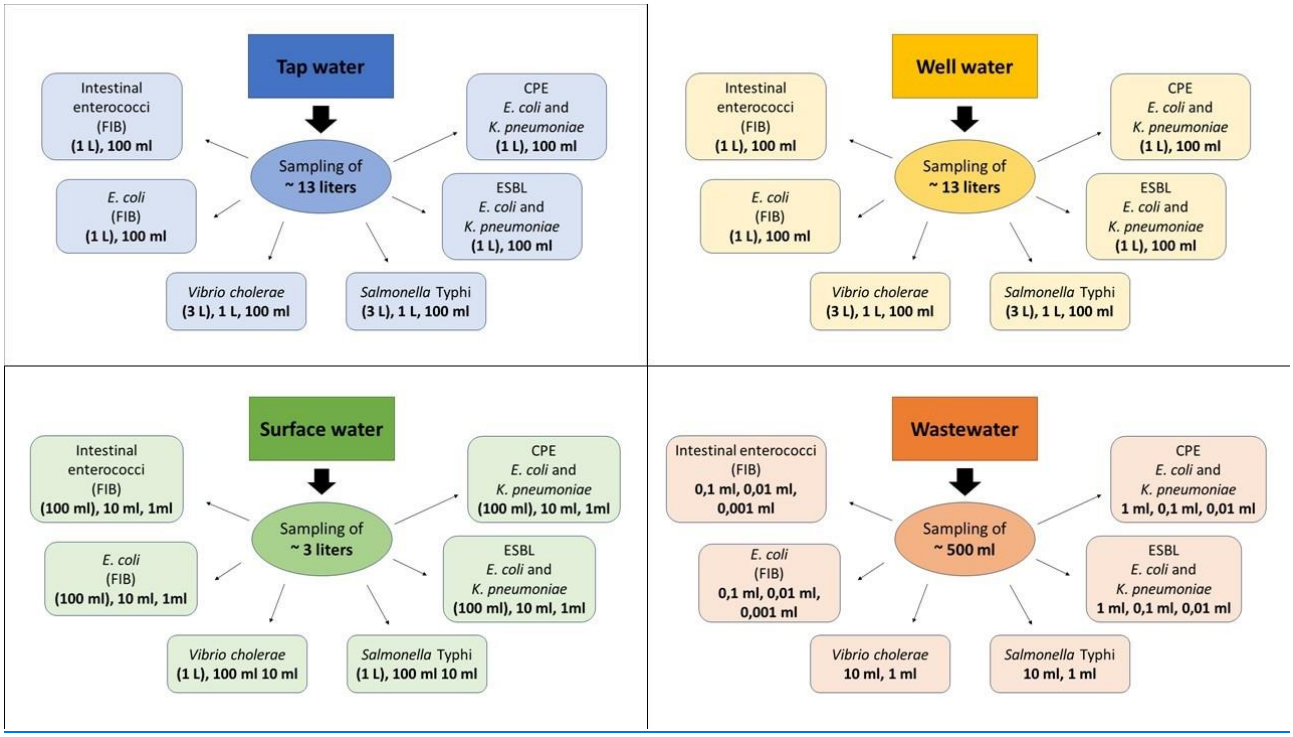


Figure 1. Suitable sampling volumes for tap water, well water, surface water and wastewater. The sample volume in parentheses refers to the sample volume that may be possible for increasing the method sensitivity if the water quality is high.

Table 1. Suitable sampling volumes for tap water, well water, surface water and wastewater (guidance from ISO 19458).

Water matrix	***E. coli* (FIB)**	**Intestinal enterococci (FIB)**	***Vibrio cholerae***	***Salmonella* Typhi**	**ESBL *E. coli* and *K. pneumoniae***	**CPE *E. coli* and *K. pneumoniae***	**In total**
Tap water	(1 l) 100 ml	(1 l) 100 ml	(3 l) 1 l 100 ml	(3 l) 1 l 100 ml	(1 l) 100 ml	(1 l) 100 ml	7 (+ 6) l max. ~ 13 l
Well water	(1 l) 100 ml	(1 l) 100 ml	(3 l) 1 l 100 ml	(3 l) 1 l 100 ml	(1 l) 100 ml	(1 l) 100 ml	7 (+ 6) l max. ~ 13 l
Surface water	(100 ml) 10 ml 1 ml	(100 ml) 10 ml 1 ml	(1 l) 100 ml 10 ml	(1 l) 100 ml 10	(100 ml) 10 ml 1 ml	(100 ml) 10 ml 1 ml	~ 3 l
Wastewater	0,1 ml, 0,01 ml, 0,001 ml	0,1 ml, 0,01 ml, 0,001 ml	10 ml 1 ml	10 ml 1 ml	1 ml, 0,1 ml, 0,01 ml	1 ml, 0,1 ml, 0,01 ml	~ 500 ml

Passive (trap) sampling

Either Moore's swabs (Rafiee et al. 2021, Liu et al. 2022) or 3D[1] printed torpedo samplers (Schang et al. 2021) are used as alternatives for grab sampling of wastewater. Samplers are easy to assemble and can be used to monitor even small catchment areas. In the Project ODIN, passive samplers are used in addition to grab sampling to collect microbes onto absorbent materials, i.e. on cotton swabs, and on electronegative membranes (Figure 2). Absorbed liquid can be used for analyzing the priority pathogens and indicators. Microbes collected onto membrane filters can be removed by bead beating.

Materials

- 3D printed torpedo samplers (3D printer files: https://www.bosl.com.au/wiki/Passive_Sampler), suitable material should withstand wastewater and decontamination processes
- Rope (2-3 mm thick)
- Shade cloth to wrap around the sampler (check that water can pass through)
- Weight to keep sampler submerged
- Adsorbent material to collect biomass

[1]Download link for 3D printer files (https://www.bosl.com.au/wiki/Passive_Sampler)

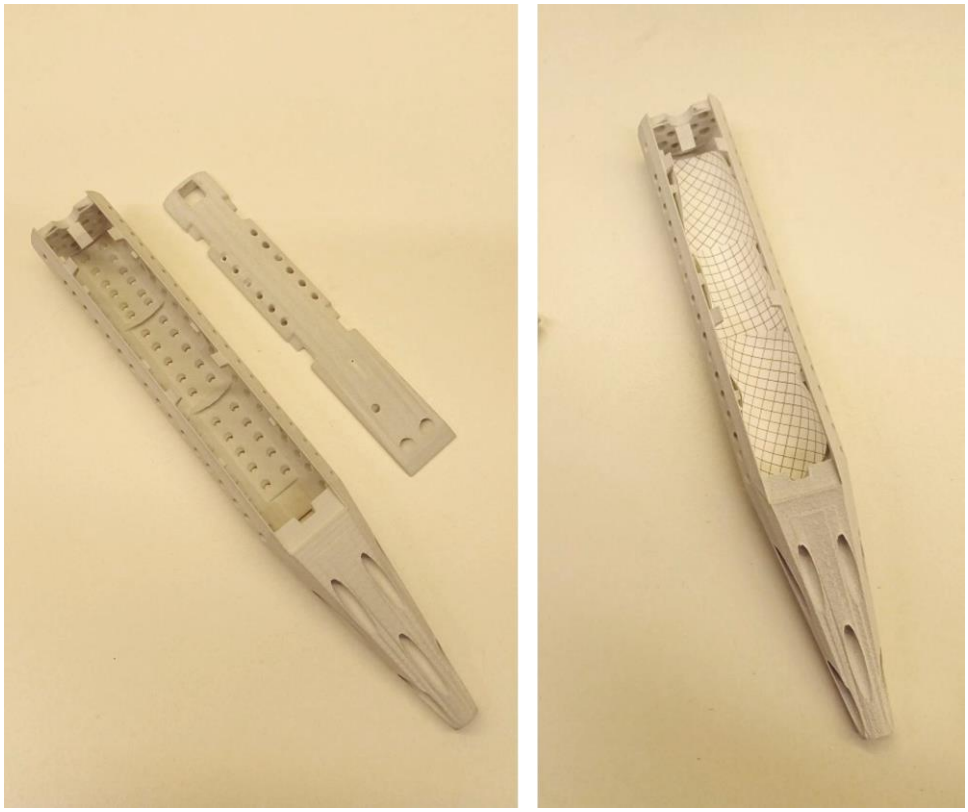


Figure 2. Torpedo-style passive sampler empty (left) and with 0.45 µm membranes inside (right). Photos: THL.

A sampler is equipped with weight to keep it submerged and additional wrapping to reduce collection of solid material (Figure 3)[2]. On day one, the sampler is anchored onto the sewage system (pumping station, manhole, wastewater stream) and collected after 24 hours on day two. Where necessary, a shorter or longer collection times may be used.

Retrieved torpedo samplers are double bagged in plastic and transported as soon as possible in cool boxes with ice packs to the laboratory where samplers are disassembled[3]. Filters are moved to 1.5 ml Eppendorf tubes with sterile forceps and stored in the freezer prior to nucleic acid extraction. Liquid from absorbent pads (e.g. cotton swabs) is squeezed and used for cultivation analysis.

[2] Production of passive sampler https://youtu.be/gG4pA318JJU

[3] Torpedo passive dismantling SOP https://youtu.be/pTXiuIZYgmU

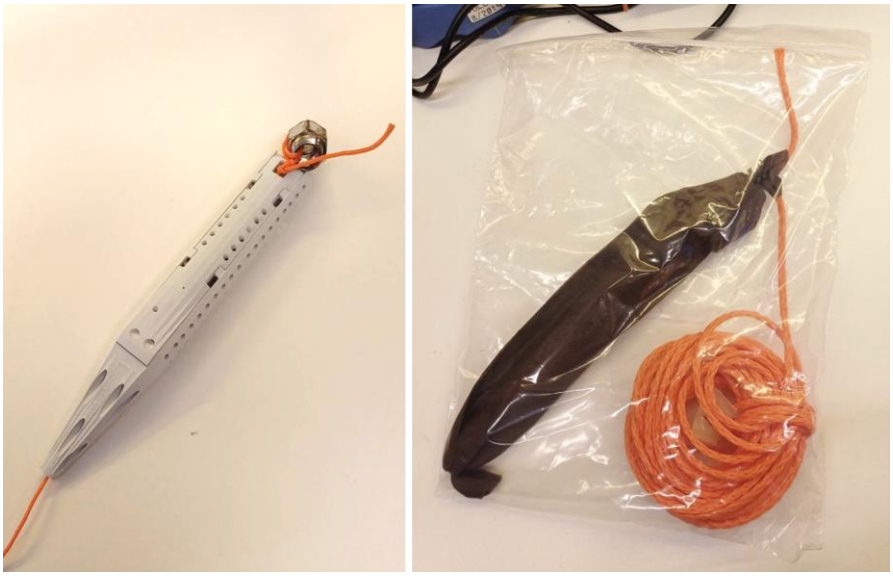


Figure 3. Fully assembled torpedo sampler with weight (left) and additional wrapping with shade cloth (right). Photos: THL.

2.4 Transport and storage of the samples

If the samples are not immediately transported, they must be stored at 5 ± 3 °C according to ISO 19458 standard.

Just before transportation, the samples are packed in cool boxes with frozen cold packs. Enough frozen cold packs should be used in each cool box to upkeep the cold temperature during the transport. More than one sample container or passive sampler can be transported in one cool box. In this case, it must be ensured that there are enough cold packs in the cool box. However, clean water samples (tap water, well water) should be stored and transported separately from wastewater samples. The cold packs are arranged around the sample containers or passive samplers to keep the sample as cold as possible throughout transportation. For example, a bubble wrap can be placed between the sample containers and the cold packs to prevent the samples from freezing during transportation.

Sample containers and passive samplers should be transported to the laboratory as soon as possible, no later than within 24 hours at 5 ± 3 °C (in cool boxes with frozen cold packs). The transportation temperature of the sample can be monitored with a temperature datalogger attached to the cool box. A datalogger can be taped for example to the lid of the cool box to measure the temperature inside the cool box during transportation. The water temperature must be measured during the sampling event and immediately after the transportation when samples arrive to the laboratory. It is important that the temperature is not measured directly from the sample bottle, but from a separate container (from a sample aliquot) as the temperature sensor is not sterile. Temperature measurements are conducted twice - at the time of sampling and when the sample arrives to the laboratory. The steps for temperature measurements are as follows:

1. Mix the water sample in the sample container (bottle).
2. Carefully pour at least 5 ml of the sample into a separate flask or tube.

3. Measure the water temperature immediately from this small sub-fraction of the sample
4. Record the temperature to the sampling sheet.

After the transportation, upon arrival to the laboratory, the laboratories record the arrival time and temperature of the samples to sample collection form (Annex A). The samples must be stored at 5 ± 3 °C for a maximum of 24 h, if the sample analysis is not initiated immediately.

3. Requirements for laboratories and safety at laboratory work

Basic requirements for the laboratory facilities, equipment, and reagents for culture-based microbiological investigations from water samples are described in the international standard ISO 8199 (ISO 8199:2018 Water quality — General requirements and guidance for microbiological examinations by culture). It is essential that the ODIN laboratories follow these basic requirements.

3.1 Working in the biosafety level 2 laboratory with wastewater samples

The aim of the biosafety instructions is to set the necessary requirements for managing the risks associated with the handling, storage and disposal of biological agents and toxins (CDC 2024a). Laboratory technicians must follow both the laboratory's safety instructions and laboratory-specific operating instructions (NIH, 2023). These instructions must be designed according to the local requirements (CDC 2024a, NIH 2023, The University of Chicago 2024).

Working in laboratories with Biosafety Level 2, or BSL-2, provides a set of guidelines and practices aimed at minimizing the risk of exposure to potentially hazardous biological materials. In the context of wastewater, following the biosafety guidelines ensure that laboratory personnel are protected from pathogens and other contaminants that may be present in the samples. The Level 2 of biosafety is crucial to preventing infections and maintaining a safe working environment. (NIH 2023, Caburao 2024, CDC 2024a, CDC 2024b, The University of Chicago 2024)

There are several key safety measures that should be followed. First and foremost, personal protective equipment (PPE), such as gloves, lab coats, and safety goggles, should always be worn. Additionally, proper hand hygiene, including frequent handwashing and the use of hand sanitizers, is essential. It is also important to work in a well-ventilated area and to properly disinfect all equipment and surfaces to prevent cross-contamination. (CDC 2024c, CDC 2024d)

Finally, any personnel working in a BSL-2 laboratory should receive comprehensive training on biosafety protocols, including proper handling and disposal of wastewater samples, understanding the potential hazards and risks associated with the samples, and the correct use of PPE. Regular refresher training sessions should also be conducted to ensure that everyone remains up to date with the latest safety guidelines. (The University of Washington 2019, CDC 2024e)

Risk assessment refers to the identification of hazards occurring at work, determining the magnitude of the risks caused by the hazards, and evaluating the significance of the risks. The introduction of new biological components, the commencement of a new protocol in the laboratory, or substantial changes in the procedure of the protocol, as well as moving to new facilities, are all examples of events that need a new risk assessment or a review of an existing one. (Henderson 2009, Environmental Health and Safety 2024)

After the assessment, the workplace decides on the necessary measures. The risk assessment has to be appropriate to the nature of the risk and preventive, not just reactive to the situation. It should be noted that the risk assessment does not only include matters related to the handling of the biological factor but also the entire operation.

The risk assessment is carried out in accordance with institution's, or workplace's, or laboratory's general practices, but this risk assessment is not necessarily detailed enough in terms of biosecurity. A biorisk assessment can be included, for example, as part of a method or work instructions.

In the first step of the biorisk management process, all hazards related to biorisk are identified and documented. Biological hazards are assessed according to their potential harm to humans, animals, and the environment. All those participating in the work and, if necessary, institution's, or workplace's, or laboratory's safety and risk experts and those responsible for the facility's support services are involved in this process, or they are informed about it. (The University of Washington 2019, NIH 2023, CDC 2024a-e)

4. Culture-based microbiological analyses of the environmental samples

Tap water, well water, surface water and wastewater samples are collected in the ODIN consortium research from the study locations. The culture-based analysis of the samples should be initiated within 24 hours after the sampling events.

The principles of ISO 8199 are followed in water microbiological investigations. The analyzed sample volume varies depending on the estimated fecal contamination level of the samples. When tap water and well water samples are analyzed, 100 ml is used as a standard analysis volume, but for pathogen analysis from clean water, 1000 ml and even higher water sample volumes might be employed.

For surface water samples, 100 ml, 10 ml, and 1 ml volumes are usually suitable for analysis. In case of highly contaminated surface waters, a dilution series might be needed.

Typically, volumes of 10 ml, 1 ml and a 10-fold dilution series in a bacteriological diluent are used for analyzing the wastewater samples. When the wastewater is visibly heavily turbid (high concentration of organic matter), a dilution is needed to have uniform colony growth.

4.1 Enumeration of the Fecal Indicator Bacteria (FIB)

Fecal indicators bacteria are used to evaluate the presence and magnitude of fecal contamination level in the environmental samples. Further, the fecal indicator counts can be used to normalize the priority pathogen results when other normalization information is not available. Normalization with indicators takes account the differences in the fecal contamination levels in the samples between the different sampling times, e.g. due to the differential diluting effects cause by precipitation. Unitless normalized results can be compared over time. Good fecal indicator is stable in a sample and has low geographical and seasonal variability.

Escherichia coli enumeration

E. coli counts are analyzed from the water samples by using the membrane filtration method following the principles of ISO/CD 9308-4 standard method.

> *NOTE: E. coli is the major species (80 – 90 %) of fecal coliforms also called thermotolerant coliforms. The species of fecal coliform group include in addition of E. coli also Klebsiella, Citrobacter and Enterobacter that could also have environmental origins. Herein, we focus on selective colony counting of E. coli, the best-known indicator of fresh fecal contamination.*

The quality control of reagents and materials needs to be conducted according to the standard ISO 11133 (ISO 11133:2014 Microbiology of food, animal feed and water – Preparation, production, storage and performance testing of culture media). ISO 7704 gives quality assurance procedures for membrane filters (ISO 7704:2023 Water quality — Requirements for the performance testing of membrane filters used for direct enumeration of microorganisms by culture methods).

Reagents, materials, and equipment

- Membrane filter
- Membrane filtration equipment
- A solid agar media intended for selective culturing of *E. coli*. The use of tryptone bile X-glucuronide agar medium (TBX) (e.g. Oxoid, Basingstoke, Hampshire, United Kingdom) is recommended.
  > *NOTE: The use of Chromocult Coliform Agar (CCA) according to ISO 9308-1 is an alternative for chlorinated clean water systems where the growth of background microbes is not an issue. CCA is not suitable for surface and wastewaters (ISO 9308-1:2014 Water quality — Enumeration of Escherichia coli and coliform bacteria. Part 1: Membrane filtration method for waters with low bacterial background flora).*
- Diluent according to ISO 8199
- Incubator

Protocol

Using membrane filtration technique, filter undiluted or diluted water sample (depending on the estimated level of fecal contamination) through a membrane filter (Figure 1, Table 1). Dilution of the sample is conducted according to ISO 8199. After filtration, place the membrane filter on the selective agar plate and incubate the plate.

After incubation, count the typical colonies according to the guidance from the culture medium manufacturer.

Calculation and reporting of results

In case that more than one volume of sample has been analyzed, the results are calculated and recorded as weighted mean per volume according to ISO 8199. The final result for tap water, well water and surface water is reported as colony-forming units (CFU)/100 ml and for wastewater as CFU/ml.

Intestinal enterococci enumeration

Intestinal enterococci are analyzed according to the ISO 7899-2 (ISO 7899-2:2000 Water quality – Detection and enumeration of intestinal enterococci – Part 2: Membrane filtration method). Previously, the FIB parameter intestinal enterococci was called as fecal streptococci.

The quality control of reagents and materials needs to be conducted according to the standard ISO 11133 (ISO 11133:2014 Microbiology of food, animal feed and water – Preparation, production, storage and performance testing of culture media). ISO 7704 gives quality assurance procedures for membrane filters (ISO 7704:2023 Water quality — Requirements for the performance testing of membrane filters used for direct enumeration of microorganisms by culture methods).

Reagents, materials, and equipment

- Membrane filter
- Membrane filtration equipment
- Slanetz & Bartley agar medium (S&B) (e.g. Oxoid, Basingstoke, Hampshire, United Kingdom)
- Bile esculin azide agar medium (BEA) (e.g. Merck, Darmstadt, Germany)
- Diluent according to ISO 8199
- Incubator

Protocol

Using membrane filtration technique, filter undiluted or diluted water sample (depending on the estimated level of fecal contamination) through a membrane filter (Figure 1, Table 1). Dilution of the sample is conducted according to ISO 8199. After filtration, place the membrane filter on the S&B plate and incubate plate at (36±2) °C for (44±4) hours.

After incubation, count the typical colonies according to the guidance from the culture medium manufacturer. Examine and count typical colonies as preliminary intestinal enterococci. Transfer the membrane filter with colonies to a BEA plate and incubate plate at (44.0±0.5) °C for 2 hours to confirm the colonies as intestinal enterococci.

Calculation and reporting of results

In case that more than one volume of sample has been analyzed, the results are calculated and recorded as weighted mean per volume according to ISO 8199. The final result for tap water, well water and surface water is reported as colony-forming units (CFU)/100 ml and for wastewater as CFU/ml.

4.2 Detection, enumeration, and isolation of *Vibrio cholerae*

Cultivation of *Vibrio* spp.

Vibrio cholerae is analyzed by selective culture-based membrane filtration method followed by species identification and confirmatory tests to determine the presence of toxigenic strains. The method is based on protocol by Standing Committee of Analysts, UK, – Part 9 – Methods for the isolation of Yersinia, Vibrio and Campylobacter by selective enrichment (Standing Committee of Analysts 2016); except that instead of enrichment step, the membrane filter is placed directly on the solid selective culture medium. The steps of the *Vibrio cholerae* analysis are described in the flowchart (Figure 4.).

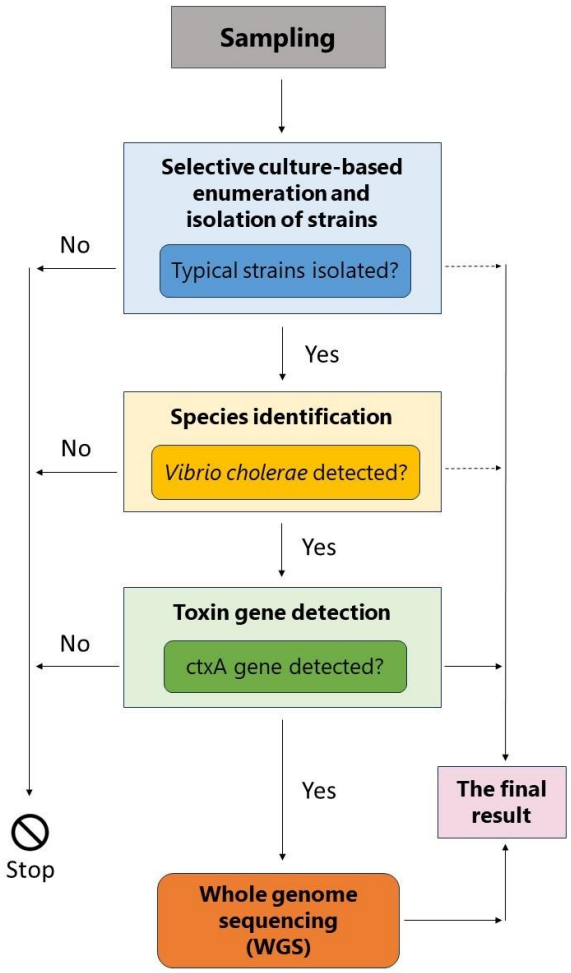


Figure 4. The steps for detection and enumeration of Vibrio cholerae

Vibrio spp. is analyzed without or with enrichment by using the membrane filtration method. Direct analysis with the membrane filtration method without enrichment is recommended, as it enables the enumeration of the *Vibrio* numbers in the samples. For further information about *Vibrio* spp. analyzing with enrichment, see ISO 21872-1 (ISO 21872-1:2017 Microbiology of the food chain — Horizontal method for

the determination of Vibrio spp. Part 1: Detection of potentially enteropathogenic Vibrio parahaemolyticus, Vibrio cholerae and Vibrio vulnificus).

The quality control of reagents and materials needs to be conducted according to the standard ISO 11133 (ISO 11133:2014 Microbiology of food, animal feed and water – Preparation, production, storage and performance testing of culture media). ISO 7704 gives quality assurance procedures for membrane filters (ISO 7704:2023 Water quality — Requirements for the performance testing of membrane filters used for direct enumeration of microorganisms by culture methods).

Reagents, materials, and equipment

- Membrane filter
- Membrane filtration equipment
- Thiosulphate citrate bile and sucrose agar medium (TCBS) (e.g. Oxoid, Basingstoke, Hampshire, United Kingdom)
- Diluent according to ISO 8199
- Non-selective solid agar medium for pure culturing e.g. tryptic soy agar medium (TSA) (e.g. Oxoid, Basingstoke, Hampshire, United Kingdom)
- Incubator

Protocol without enrichment

Using membrane filtration technique, filter water sample through a membrane filter (Figure 1, Table 1). After filtration, place membrane filter on the TCBS plate and incubate plate at 37 °C for 18-24 hours.

After the incubation, read the plates according to the guidance given by the medium manufacturer. Typical colonies of *Vibrio cholerae* are usually yellow on TCBS agar medium, with colony size from 2-3 mm (Figure 5.). Also, *Vibrio fluvialis and Vibrio metschnikovii* form yellow colonies on TCBS. Typical colonies of *Vibrio parahaemolyticus* and *Vibrio mimicus* have an appearance from blue to green with colony size 2-5 mm on TCBS, and *Vibrio vulnificus* has a green colony appearance (Harwood et al. 2004). Enterococci and *Proteus* spp. are also able to form colonies on the TCBS agar medium, but these colonies are usually smaller in their size than the *Vibrio* spp. colonies.

Cultivate pure cultures from typical colonies on non-selective plates, e.g. TSA, and incubate plates at (36±2) °C for approximately 24 hours for further confirmation.

Figure 5. Yellow colonies of Vibrio cholerae on the TCBS plate with a membrane filter. Photo: THL.

Methods to species identification of *Vibrio spp.* strains

Maldi-Tof

Bacterial strains are identified using a Maldi-ToF MS (engl. matrix-assisted laser desorption ionization time of flight mass spectrometry) method according to guide provided by the manufacturer. Maldi-ToF method is an efficient and reliable method for speciation of *Vibri*o spp. strains.

Follow the manufacturer's instructions while reading the results. It is recommended to identify two parallel spots from the same isolate. Identification is considered reliable if the score value is 2.0 – 3.0.

The isolates identified as being *Vibrio cholerae*, are further characterized to determine their toxigenicity.

Other methods

In case when Maldi-ToF is not available, biochemical tests, such as API 20E (BioMérieux, Marcy l'Etoile, France) or VITEK 2 (BioMérieux, Marcy l'Etoile, France) can be used for speciation of the *Vibrio spp.* strains.

The isolates identified as being *Vibrio cholerae*, are further characterized to determine their toxigenicity.

Methods to verify toxigenic *Vibrio cholerae* isolates

PCR-confirmation of the presence of cholera toxin gene is the primary method for use. Alternatively, serogrouping with antisera agglutination can be done with *Vibrio cholerae* O1 and O139 antisera.

Conventional PCR to detect Vibrio cholerae ctxA gene

Purpose of the test: To detect the cholera toxin-determining *ctxA* gene of *Vibrio cholerae*.

Principle: Cholera toxin belongs to enterotoxins, which consist of two polypeptides. PCR is used to selectively amplify a part (554 bp) of the 777 basepair (bp) *ctxA* gene located in *Vibrio* pathogenicity island 1 using specific primers. Primers have been previously published by Nandi et al. (2000) and the method has been set up in THL's bacteriology laboratory.

Sample: Fresh pure culture on a plate.

Controls: Positive control: *ctxA*-positive *Vibrio cholera*e isolate

Negative control: a non-toxigenic strain of *V. cholerae*

Negative controls for DNA extraction and PCR

DNA from the sample strains and control strains is extracted by heating. The extracted DNAs are stored in a -20 °C freezer and batches are used until they run out, the products start to fade, or the result is wrong.

Reagents:

PCR reagents:

- Pyrogen-free sterile water (e.g. HyPureTM Molecular Biology Grade Water, manufacturer HyClone), storage in a refrigerator (+2…+8 °C) divided into e.g. 1.5 ml Eppendorf tubes in 1 ml portions in the mastermix room, stored divided in the freezer (below -18 °C).
- A multiplex PCR kit (e.g. 5× FIREPol® Master Mix ready use (250 rxn), cat. No: 04-11-00125, Solis BioDyne, or QIAGEN® Multiplex PCR Kit manufactured by QIAGEN; catalog number: 206143 (100 pcs of 50 µl reaction), 206145 (1000 pcs of 50 µl reaction), contains:
    - 2x QIAGEN Multiplex PCR Master Mix (HotStarTaq® DNA polymerase, Multiplex PCR Buffer, dNTP Mix)
    - Q-Solution (5x)
    - sterile water
    - Storage below -18 °C in the freezer
- Primers (Nandi et al. 2000)
    - ctxAF (5' CTCAGACGGGGATTGTTAGGCACG 3')
    - ctxAR (5' TCTATCTCTGTAGCCCCTATTACG 3')
    - The primers are ordered dissolved in sterile water (100.0 pmol/µl), from which working dilutions of 1:10 are made (10 µl primer + 90 µl sterile H2O).
    - Both stock solutions and working dilutions are stored below -18 °C in a freezer.

Agarose gel electrophoresis reagents:

- Agarose, e.g. SeaKem-LE agarose (Lonza), VWR: 50004 (500g)
    - Storage RT
- 5×TBE buffer (Tris-borate-EDTA)
    - A working solution of 0.5×TBE is prepared by diluting it 1:10 in purified water
    - Storage RT
- 6×Loading Dye – sample buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll 400)
    - Storage in the refrigerator (+2...+8 °C)

  NOTE: Master mix 5× FIREPol® is already colored and no need for a loading dye in case this product is used for PCR.
- Size marker, e.g. GeneRuler 100 bp Plus DNA Ladder (100-3000 bp), ThermoFisher # SM0322 (5×50 µg)
    - Store below -18 °C in a freezer.
    - The operating tube is stored in the refrigerator (+2...+8 °C).
- SYBR Safe 10.000X (S33102, Invitrogen)
    - Storage RT shield from the light
    - Expiry 6 months after opening the reagent tube
    - 1X working solution (diluted in TBE Buffer) storage in the refrigerator (+2...+8 °C) – working solution stays good for one week

Equipment, instruments and supplies:

- sterile 1.5 ml and 0.5 ml plastic Eppendorf tubes
- pipettes
- filter pipette tips
- nitrile protective gloves

- crushed ice
- 250 ml Erlenmeyer flask
- microwave oven
- PCR machine
- agarose gel running equipment (gel casting tray, well comb, running device and power supply)
- UV gel imaging device and camera
- Eppendorf centrifuge
- Refrigerator (+2…+8 °C)
- below -18 °C freezer
- test tube mixer (Vortex)

Performance of the work:

1. Sample (template) pretreatment
    - Take 1–2 colonies from the plate
    - Suspend the sample in 150 µl of 0.9% NaCl
    - Centrifuge for 5 min at 3,000 rpm
    - Boil the sample or hold in a heating block at 100 °C for 10 min
    - Centrifuge the sample for 10 sec at 3,000 rpm
    - Keep the tubes in a cool/ice bath during the preparation of the Master-Mix
    - Also make one DNA extraction control tube to which no bacteria are added
2. PCR reaction
    - Calculate the required amount of master-mix: samples, controls, DNA isolation control, master-mix water control and 1 pipetting spare, use a run form in e.g. Excel.
    - Mastermix for one sample:

Reagent	Volume per reaction (µl)
Pyrogen-free sterile H2O	8.0
Q-solution	2.5
Primer ctxAF 126 (working concentration 10 pM)	1.0
Primer ctxAR 127 (working concentration 10 pM)	1.0
2xQiagen MM	12.5
Total	25

    - Prepare the Master-mix and distribute a' 25 µl into the tubes.
    - Add template 1 µl / tube, final volume in PCR tube 26 µl.
    - Start the PCR program.

PCR program:

Step	Temp	Time
1.	95 °C	15 min
2.	94 °C	30 sec
3.	60 °C	90 sec
4.	72 °C	92 sec
5	-	Return to step 2. 30 times
6.	72 °C	10 min
7.	4 °C	∞
8.	-	The program ends

3. Agarose gel electrophoresis:
    - Run the PCR products in a 1.5%-2% agarose gel.
    - Molecular weight marker GeneRuler 100 bp, pipette 3 µl of the prepared mixture.
    - If the master mix is not colored already, add 3 µl of 6 x Loading Dye buffer to the PCR product. Pipette 10 µl of the mixture onto the gel.
    - Gel running conditions: 50–60 min, 120-140V.
    - Stain with Sybr Safe and image with AlphaImager.
        - To make the staining solution, dilute the 10,000x Sybr Safe reagent to a 1x concentration using the 0.5x TBE buffer (working solution). Prepare enough of the 1x staining solution to fully submerge the entire gel. For example 10 µl 10.000x Sybr Safe to 100 ml 0.5x TBE buffer.
        - Staining protocol: submerge gel onto staining solution and incubate for 30 min. If possible, agitate gel on an orbital shaker at 50 rpm or gently shake gel staining tray a few times during the intubation period. After incubation, gel is ready for imaging.
        - Gels stained with Sybr Safe can be disposed as normal waste

Interpretation of the results and acceptance criteria:

The results of the samples are compared with control strains and molecular markers. If the strain has the *Vibrio cholerae ctxA* gene, a 301 bp product is amplified in the PCR. If the sample does not form a product of the correct size, the strain does not have the *ctxA* gene. The positive control must be positive, and the negative controls must be negative for the result to be accepted. Otherwise, the test will be repeated.

PCR reactions use several reagents and have many work steps, so the method is prone to errors. There is also a high risk of contamination in PCR methods, which is tried to be minimized by using separate workspaces for PCR work, by ensuring the cleanliness of the spaces and reagents, and by using a negative control in PCR reactions. If a PCR product is amplified in the negative control, the DNA is isolated again, and the reaction is repeated using new lots of reagents.

Serogrouping with antisera

Purpose of the test: To define the O-serogroup of a *Vibrio cholerae* isolate.

Principle: There are more than 100 different O-serogroups of *V. cholerae*. Two of them, O1 and O139, can cause epidemic cholera. *V. cholerae* strains that are not serogroup O1 or O139 (common name non-O1, non-O139) are common in marine and brackish waters and shellfish. They, like some other species of the *Vibrio* genus, are usually harmless, but can cause e.g. diarrhea, ear infections and wound infections. All *Vibrio* isolates that are suspected to be *V. cholerae* are subjected to serogroup determination (O1, O139) by antigen-antibody agglutination on glass slides using commercial antisera.

Sample: Fresh pure culture on a non-selective media such as blood agar.

Reagents:

- Non-selective plate e.g. TSA or blood agar
- 0.9% and 4% NaCl
    - For determining rough shapes
    - Storage in the refrigerator in plastic bottles, 1.5 ml/bottle
    - Remain usable for several months
- Antisera (e.g. Denka Seiken Co, LTD, Tokyo, Japan)
    - O1 polyvalent group antiserum
    - Optional: O1 Ogawa antiserum
    - Optional: O1 Inaba antiserum
    - O139 (Bengal) antiserum
    - Pre-diluted for use
    - Expiry date marked on the bottles
    - Can also be used after the expiry date if the control strains are working
    - Storage in the refrigerator

Controls: Positive control strains *V. cholerae* O1 and *V. cholerae* O139

Optional: V. cholerae O1 Inaba and *V. cholerae* O1 Ogawa

Negative control *V. cholerae* non-O1, non-O139

Performance of the work:

1. Cultivate a colony from a TCBS plate onto a non-selective plate e.g. TSA or blood agar.

2. Incubate at +35… +37 °C overnight.

3. Drop a drop of O1 group serum onto the glass slide.

4. Using toothpicks, suspend the bacterial mass from the plate into a drop on the glass slide.

5. Turn the glass slide carefully to mix; agglutination is visible to the naked eye within 30 s to 1 min.

6. Record the result of the reaction (+++ for clear precipitation; – for no precipitation; + or ++ can be used if there is an unclear result and the test needs to be repeated).

7. If the strain does not agglutinate with O1 group serum, test the strain with O139 antiserum.

8. If the strain agglutinates with all antisera, confirm with 0.9% and 4% NaCl that it is not the rough (R) form, which has no O antigen at all.

7. Dispose of waste in accordance with waste management guidelines.

Interpretation of the results:

A rapid, strong reaction is a positive result and indicates the serogroup of the strain (O1 or O139). If no reaction is observed, the result is negative (non-O1/non-O139).

Calculation and reporting of results

In case that more than one volume of sample has been analyzed, the results are calculated and recorded as weighted mean per volume according to ISO 8199. The final result for tap water, well water and surface water is reported as colony-forming units (CFU)/100 ml and for wastewater as CFU/ml.

The isolates identified as being toxigenic *Vibrio cholerae* (by positive *ctxA* PCR test) or *Vibrio cholerae* O1 and O139 (by serogrouping test) are further characterized by WGS.

4.3 Detection and selective isolation of *Salmonella enterica* subsp. *enterica* serotype Typhi, i.e. *Salmonella* Typhi

Description for *Salmonella* spp. selective isolation method

Salmonella spp. is analyzed according to the ISO 19250 (ISO 19250:2010 Water quality – Detection of *Salmonella* spp.). However, local and/or technical modifications are necessary. E.g. in Finland, semi-solid MSRV is used instead of RVS-broth enrichment. The steps of the *Salmonella* analysis are described in the flowchart (Figure 6.).

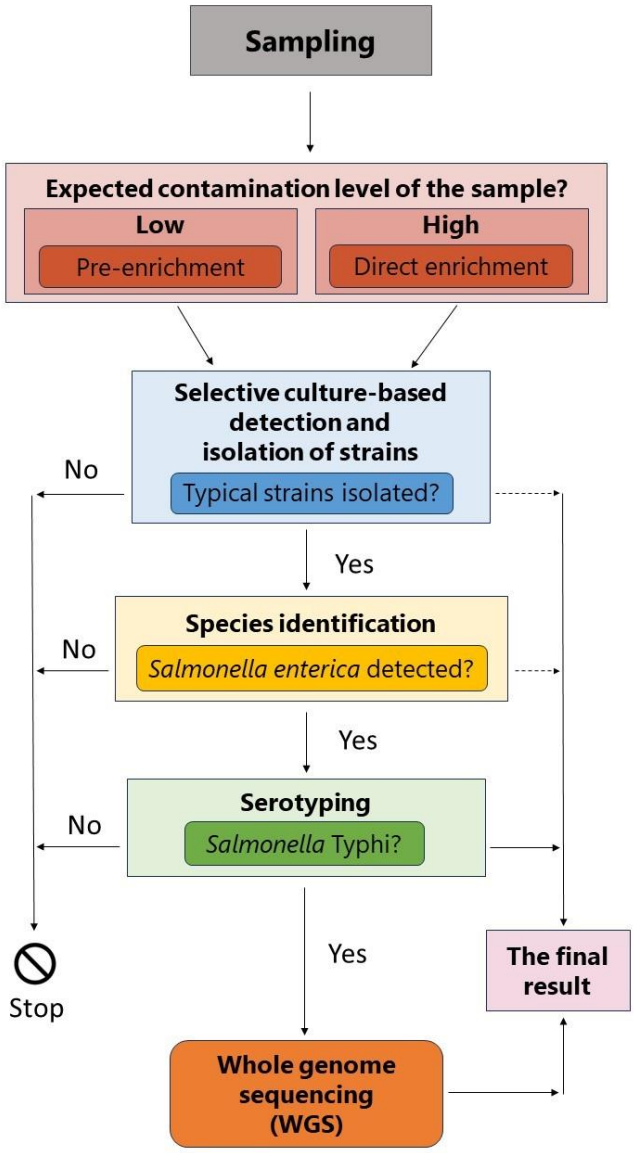


Figure 6. The steps for detection and selective isolation of Salmonella enterica subsp. enterica serotype Typhi

The quality control of reagents and materials needs to be conducted according to the standard ISO 11133 (ISO 11133:2014 Microbiology of food, animal feed and water – Preparation, production, storage and performance testing of culture media). ISO 7704 gives quality assurance procedures for membrane filters (ISO 7704:2023 Water quality — Requirements for the performance testing of membrane filters used for direct enumeration of microorganisms by culture methods).

Reagents, materials, and equipment

- Membrane filter
- Membrane filtration equipment
- Pre-enrichment:
    - Buffered peptone water (BPW) (e.g. Oxoid, Basingstoke, Hampshire, United Kingdom)

- Selective enrichment (alternatives given and have varying performance)
    - Modified semi-solid rappaport-vassiliadis (MSRV) (e.g. Oxoid, Basingstoke, Hampshire, United Kingdom)
    - Mueller-kaufman tetrathionate-novobiocin broth (MKTTNn) (e.g. Oxoid, Basingstoke, Hampshire, United Kingdom)
    - Rappaport-Vassiliadis broth with soya (RVS) (e.g. Oxoid, Basingstoke, Hampshire, United Kingdom)
- Selective isolation
    - E.g. xylose lysine deoxycholate agar medium (XLD) (e.g. Oxoid, Basingstoke, Hampshire, United Kingdom)
- Non-selective solid agar medium for pure culturing e.g. tryptic soy agar medium (TSA) (e.g. Oxoid, Basingstoke, Hampshire, United Kingdom)
- Diluent according to ISO 8199
- Incubator

Protocol with pre-enrichment (for water intended for human consumption and for surface water)

Using membrane filtration technique, filter water sample through a membrane filter (Figure 1, Table 1). After filtration, transfer membrane filter into buffered peptone water (BPW). Incubate BPW with membrane filter at (36±2) °C for (18±2) hours.

After pre-enrichment, transfer 100 ul of BPW broth as three drops to a semi-solid MSRV plate. Incubate MSRV plate in (41,5) °C (24±3) hours.

Alternatively, instead of MSRV medium, RVS broth enrichment can be done. After the BPW pre-enrichment, add each BPW broth to RVS broth and incubate at (41,5±1) °C for (24 ±3) hours. Enrichment can be done also with MKTTn broth. After the BPW pre-enrichment, add each BPW broth to MKTTn broth and incubate at (36±2) °C for (24 ±3) hours.

After incubation, inspect the MSRV plate for mobility of the bacteria. Transfer material from typical mobile growth to solid XLD agar plate. In case RVS or MKTTn broth have been used, transfer a loopful of broth to selective solid plate (e.g. XLD).

Incubate the selective solid agar plate at (36±2) °C for (24 ±3) hours and inspect the plates for typical growth according to medium manufacturer instructions.

Cultivate pure cultures from typical *Salmonella* spp. colonies on non-selective plates, e.g. TSA, and incubate plates at (36±2) °C for approximately 24 hours for further confirmation.

Protocol without pre-enrichment (for wastewater)

Using membrane filtration technique, filter wastewater sample and transfer membrane filter into the RVS broth or MKTTn broth. Incubate RVS broth at (41,5 ±1) °C for (24 ±3) hours and MKTTn broth at (36±2) °C for (24 ±3) hours.

After enrichment, transfer RVS broth or MKTTn broth to XLD plate. Incubate XLD plate (36 ±2) °C (24 ±3) hours and inspect the plates for typical growth according to medium manufacturer instructions.

Cultivate pure cultures from typical *Salmonella* spp. colonies on non-selective plates, e.g. TSA, and incubate plates at (36±2) °C for approximately 24 hours for further confirmation.

Methods to species identification of *Salmonella* spp. strains

Salmonella spp. colonies can be identified by using Maldi-ToF method. Maldi-ToF method is an efficient and reliable method for speciation of the *Salmonella spp.* strains. In case when Maldi-ToF is not available, biochemical tests, such as API 20E (BioMérieux, Marcy l'Etoile, France) or VITEK 2 (BioMérieux, Marcy l'Etoile, France) can be used for speciation of the *Salmonella spp.* strains.

After the species is verified as *Salmonella enterica,* further characterization of the isolate needs to be performed to identify serotype *Salmonella* Typhi.

Serotyping to identify serotype *Salmonella* Typhi

Purpose of the test: To test if the isolated *Salmonella enterica* is serotype *Salmonella* Typhi.

Principle: Antigenic structure of *Salmonella* is conducted by slide agglutination with Rabbit antisera. The O and H serotypes of the *Salmonella* strain are determined. The somatic O-antigen is a part of the lipopolysaccharide (LPS) on the surface of the bacterial cell, which consists of sugar chains. If the sugar chains are complete, the strain is the normal smooth (S) form. If the sugar chains are partially or completely missing, the strain is rough (R) form. *Salmonella* are able to move actively with the help of their cilia or flagella. Flagellar H-antigens are located on the surface of the bacterial cilia. Most *Salmonella* are able to form two types of cilia (H1 and H2 phases). The presence of O and H antigens is confirmed by antigen-antibody agglutination on glass slides, using commercial or home-made group, polyvalent and absorbed antisera.

The antigenic structure of serotype *Salmonella* Typhi is 9, Vi:d:-. Other *Salmonella s*erotypes have unique antigen structures.

Safety procedures: Live cultures of *S.* Typhi should be handled in a biosafety cabinet if possible. Otherwise, PPE should be used.

Sample: Fresh pure culture on a plate.

Controls: Positive control: *S.* Typhi

Negative control(s): Some other serotype(s) of *Salmonella enterica* subsp. *enterica* that do not agglutinate with the same antisera as *S.* Typhi.

Reagents:

Specific antisera for detection of antigenic structure of *S.* Typhi:

- OMA: O group pool (1,2,3,4,5,9,10,12,15,19,21,46) Rabbit antiserum (Staten Serum Institute, SSI)
- O:9: O group Rabbit antiserum (SSI)
- Vi: O group Rabbit antiserum (SSI)
- H:d: H phase Rabbit antiserum (SSI)
- Negative control: a couple of other O and H antisera

Swarm agar plate

Drigalski agar plate

Glass slides

Toothpicks

(Table-top) lamp inside a biosafety cabinet or on a laboratory table

Performance of the work:

1. Cultivate one smooth-looking colony on a Swarm plate (H serotyping) by gently drawing a short line on the center of the plate with a culture stick and use the same stick to make a dispersal culture on a Drigalski plate (O serotyping). *S.* Typhi does not move and the growth on the Swarm plate should not spread.

2. Incubate plates at +35...+37 ºC overnight.

3. O-serotyping: Drop a drop of O group pool serum onto the glass slide. Using a toothpick, suspend the bacterial mass from the Drigalski plate into a drop on the glass slide. Turn the glass slide carefully to mix for up to 1 min. You can hold the slide in front of a lamp to see the reaction clearly. A positive reaction can appear even in seconds. Record the result of the reaction (+++ for clear precipitation; – for no precipitation). Repeat with O:9 and Vi sera and negative control sera (e.g. O46). If desired, up to three drops of different antisera are able to fit on the same glass slide e.g. O:9, O:46, and Vi.

4. H-serotyping: Drop the H:d phase serum on the glass plate. Using a toothpick, suspend the bacterial mass from the growth on the Swarm plate. Turn the glass slide carefully to mix for up to 1 min. A positive reaction can appear even in seconds. Record the result of the reaction (+++ for clear precipitation; – for no precipitation). Repeat with negative control sera.

Calculation and reporting of results

The results is reported as presence/absence in the analyzed sample volume. In case that more than one volume of sample has been analyzed, it is possible to provide a semi-quantitative count estimate.

The isolates identified as being serotype *Salmonella* Typhi, are further characterized by WGS.

4.4 Detection, enumeration, and isolation of extended spectrum beta-lactamase (ESBL) and carbapenemase-producing Enterobacteriaceae (CPE) *Escherichia coli* and *Klebsiella pneumoniae*

Culture-based methods with chromogenic agars are used to detect ESBL and CPE *E. coli* and *K. pneumoniae* (Heljanko et al. 2024, Tiwari et al. 2024).

The quality control of reagents and materials needs to be conducted according to the standard ISO 11133 (ISO 11133:2014 Microbiology of food, animal feed and water – Preparation, production, storage and performance testing of culture media). ISO 7704 gives quality assurance procedures for membrane filters

(ISO 7704:2023 Water quality — Requirements for the performance testing of membrane filters used for direct enumeration of microorganisms by culture methods).

Reagents, materials and equipment

- Membrane filter
- Membrane filtration equipment
- ESBL: CHROMagar Orientation + CHROMagar ESBL -supplement (e.g. CHROMagar, Paris, France)
- CPE: CHROMagar mSuperCARBA (e.g. CHROMagar, Paris, France)
- Non-selective solid agar medium for pure culturing e.g. tryptic soy agar medium (TSA) e.g. Oxoid, Basingstoke, Hampshire, United Kingdom)
- Diluent e.g. phosphate buffer
- Incubator

Protocol

Using membrane filtration technique, filter water sample through a membrane filter (Figure 1, Table 1). After filtration, place membrane filter on the solid agar medium plates (CHROMagar Orientation + CHROMagar ESBL -supplement and CHROMagar mSuperCARBA) and incubate plates at (36±2) °C for 18–24 hours.

For wastewater prepare dilution series using e.g. phosphate buffer as the diluent. Pipette wastewater sample and/or its 10-fold dilutions to solid agar medium plates (CHROMagar Orientation + CHROMagar ESBL -supplement and CHROMagar mSuperCARBA) and spread the inoculant. Dilution of the sample and inoculation on culture medium are conducted according to ISO 8199. Incubate plates at (36±2)°C for 18–24 hours.

After incubation, count the typical colonies on each chromogenic plate according to the guidance from the culture medium manufacturer.

Cultivate pure cultures from typical colonies on non-selective plates, e.g. TSA, and incubate plates at (36±2) °C for approximately 24 hours for further confirmation.

Species identification of ESBL/CPE Escherichia coli and Klebsiella pneumoniae

Species identification of ESBL and CPE *E. coli* and *K. pneumoniae* can be done by using the Maldi-ToF method. Maldi-ToF method is an efficient and reliable method for speciation of the *Enterobacterales* strains. In case when Maldi-ToF is not available, biochemical tests such as API 20E (BioMérieux, Marcy l'Etoile, France) or VITEK 2 (BioMérieux, Marcy l'Etoile, France) can be used for species identification.

Calculation and reporting of results

In case that more than one volume of sample has been analyzed, the results are calculated and recorded as weighted mean per volume according to ISO 8199. The final result for tap water, well water and surface water is reported as colony-forming units (CFU)/100 ml and for wastewater as CFU/ml

The isolates identified as *E. coli* and *K. pneumoniae* are further characterized by WGS.

5. Whole genome sequencing of bacterial isolates

Laboratory equipment and reagents for bacterial whole genome sequencing have been listed in the ODIN Handbook Annex B Laboratory reagents and equipment for bacterial WGS.

5.1 DNA extraction from bacterial isolates

There are several options to perform DNA extraction. Here two kits, MagAttract and QIAmp DNA mini kit, are described.

Protocol with the MagAttract kit

DNA extraction with the commercial MagAttract (Qiagen) extraction kit is based on a simple protocol using magnetic beads. The method can be used to purify DNA between 100 and 200 kilobases. The isolation method is highly selective and minimizes shearing stress to the DNA, thus producing long-chain DNA. Isolated DNA is very pure and can be used directly for next-generation sequencing reactions or genotyping.

The isolation method consists of four steps: lysis, DNA binding to magnetic beads, washing, and elution. In different bacterial species, the initial stage of extraction, i.e. killing the bacterium and breaking down the cells, can be carried out in different ways. Optimized buffers and enzymes gently lyse samples while ensuring minimized fragmentation of genomic DNA. Next, DNA binds to the surface of the magnetic beads and the sample is washed to get rid of contaminants and PCR inhibitors. Finally, pure DNA is eluted in a buffer where it can be stored frozen.

Sample

The bacterial strain used is a fresh pure culture on a suitable non-selective plate.

Reagents for the extraction

MagAttract HMW DNA Kit (manufacturer Qiagen, Germany, product number 67563), keep at room temperature for one year after opening.

Contents of the kit:

ATL buffer
AL buffer
MB buffer
MW1 buffer
PE buffer
AE buffer
Proteinase K
RNAase A (100mg/ml)
Nuclease-free water

Ethanol, 95-99%

Lysostaphin (1mg/ml), SIGMA, Lysostaphin from Staphylococcus staphylolyticus, Ref Nr. L7386-15MG, storage -20°C.

Lysozyme (100 mg/ml), storage -20°C

Mutanolysin (1mg/ml), SIGMA, Mutanolysin from Streptomyces globisporus ATCC 21553, Ref Nr. M9901-10KU, storage -20°C

P1 buffer (50 mM Tris, 10 mM EDTA, pH 8.0), storage at room temperature

TE buffer (1M Tris-HCl, 0.5M EDTA pH8.0), storage at room temperature

EDTA, 0.1 mM+Tris-HCl, 10 mM (Low-TE buffer), pH 8.0 Sterile, storage at room temperature

Qubit Measurement Reagents

Qubit dsDNA BR Assay Kit Reagents (Invitrogen): ThermoFisher SKU Q32850

- Qubit dsDNA BR Reagent, storage at room temperature protected from light

- Qubit dsDNA BR buffer, storage at room temperature

- Qubit dsDNA BR standard 1#, storage in the refrigerator (+2 - +8 °C)

- Qubit dsDNA BR standard 2#, storage in the refrigerator (+2 - +8 °C)

Agarose gel electrophoresis reagents

E.g. SeaKem LE Agarose (Lonza Rockland, ME USA, art. no 50005) can be used, storage at room temperature

5×TBE buffer (Tris-Borate-EDTA)
- prepare a working solution of 0.5×TBE by diluting 1:10 in purified water
- storage at room temperature

Purified milliQ water, non-sterile, storage at room temperature

Molecular weight marker: 1kb DNA Extension ladder (e.g. Invitrogen, product number 10511–012; marker strands as base pairs (bp): 517/506, 1018, 1636, 2036, 3054, 4072,
5090/5000, 6108, 8144, 10,000 and 40,000). Storage in freezer (below -18°C)

6x DNA Loading Dye (e.g. Thermo Scientific Part No. R0611 (6 x DNA Loadin Dye comes with GeneRuler 100bp DNA Ladder), store in a freezer (below -18°C)

SYBR Safe 10.000X (S33102, Invitrogen)
- Storage RT shield from the light
- Expiry 6 months after opening the reagent tube
- 1X working solution (diluted in TBE Buffer) storage in the refrigerator (+2...+8 °C) – working solution stays good for one week

Equipment

- Vortex

- Heating block/ heating bath +100 °C
- Thermal shaker (Thermomixer)
- Scale
- Microwave
- Centrifuge
- Agarose gel run system (gel box and power supply)
- UV Gel Imaging System
- Refrigerator +2...+8 °C
- Freezer below -18 °C
- Laminar flow cabinet
- Qubit 2.0 fluorometer

Consumables

- Sterile 2 ml plastic Eppendorf SafeSeal tubes
- Sterile 1.5 ml plastic Eppendorf tubes with screw caps Sterile 1.5 ml plastic Eppendorf tubes
- Sterile 0.5 ml plastic Eppendorf tubes: Qubit assay tubes (set of 500, Cat.no. Q32856, Life technologies) or Axygen PCR-05-C tubes (VWR, part no.10011– 830)
- 2 ml plastic tubes containing Lysing Matrix D - ceramic beads. (MP Biomedicals, CAT no: 6913050)
- Tube racks
- Pipettes
- Disposable gloves (nitrile gloves for handling EtBr)
- Filtered pipette tips
- Magnetic rack
- Erlenmeyer bottle, e.g. 250 ml

Work facilities

The work is done in the DNA extraction room.

Protocol

DNA extraction is always done from a fresh bacterial pure culture. Ensure that the bacterial culture has been grown in optimal conditions (temperature and atmosphere) on a non-selective medium suitable for the species. Always make sure that the bacteria to be extracted for the DNA grows clean and there is no contamination on the plate.

- Culture ESBL/CPE *E. coli* and *K. pneumoniae* on MH II plate and incubate at +35-37°C overnigth
- Culture *Vibrio cholerae* and *Salmonella* Typhi on R1 plate and incubate at +35- 37°C; if necessary, can also be grown on blood agar plate.

DNA Extraction

Initial stage of DNA extraction: ESBL/CPE *E. coli* and *K. pneumoniae* and *Salmonella* Typhi (gram-negative)

Preheat the heat block or water bath in advance.

Pipet 180 µl ATL buffer into a 2 ml SafeSeal microtubes, collect bacterial mass from the plate with an inoculation rod and gently dissolve it in the buffer by pulse vortexing. NOTE! Work must be done in a biosafety cabinet.

Use a cap lock on the Eppendorf tubes.

Heat tubes at +100°C on a heat block or in a water bath for 10 minutes.

Cool before the next step. Centrifuge briefly.

Continue to isolate the DNA.

Initial stage of DNA extraction: *Vibrio cholerae* (gram-negative)

1. Pipet 300 µl TE buffer into a 2 ml SafeSeal microtube and collect bacterial mass from the plate with an inoculation rod or a small loop. Centrifuge for 2 minutes at 13000 x g and remove the supernatant.

2. Dissolve the bacterial pellet (resuspend) in 180 µl ATL buffer

3. Continue to DNA extraction.

DNA Extraction

Check that the MW1 and PE buffer from the DNA extraction kit have been dissolved in ethanol. If you open a new kit, document the necessary information in the Reagent Tracking folder.

1. Heat the thermal shaker to + 56°C.
2. Add 20 µl of proteinase K and mix by tapping the tube.
3. Incubate at +56 °C for 30 minutes (900 rpm). Centrifuge briefly.
4. Add 4 µl RNAase A (100 mg/ml), mix by pulsed vortexing (or tap the tube several times) and incubate for 2-15 min at room temperature (15 - 25 °C). Please note that cooling the heat shaker from 56°C to > 23°C takes about 15 minutes.
5. Add 15 µl MagAttract suspension G (vortex before use) and 280 µl MB buffer to the sample. Mix with pulsed vortexing. Place tubes with sample into the tube rack.
6. Place the tubes and tube rack onto the thermal mixer and incubate at room temperature (15 - 25 °C) for 3 minutes at 1400 rpm.
7. Place the tube holder on the magnetic rack, wait (~ 1 min) until separation of the beads is complete and remove the supernatant (remove about 500–800 µl). Try to avoid touching the magnetic beads when removing the supernatant.
8. Remove the tubes from the magnetic rack and add 700 µl of MW buffer to the tubes and place the tubes in the thermal shaker. Incubate at room temperature (15 – 25°C) for 1 min 1400 rpm.
9. Repeat steps 7-8, perform step 7 again.
10. Remove the tubes from the magnetic rack and add 700 µl of PE buffer to the tubes and place the tubes in the thermal shaker. Incubate at room temperature (15 – 25°C) for 1 min 1400 rpm.
11. Repeat steps 7 and 10, perform step 7 again, and carefully remove all traces of PE buffer with a small pipette.
12. Do not remove the tubes from the magnetic rack. Rinse the particles with 700 µl of nuclease-free water while the beads are attached to the wall of the sample tube. Incubate for 1 min at room temperature (15 - 25 °C) on a magnetic rack and remove the supernatant. IMPORTANT: Do not pipet water directly into the bead pellet, but pipet it against the wall opposite the bead pellet of the sample tube.
13. Repeat step 12.

14. Remove the tubes off the magnetic rack and add an appropriate volume of AE buffer, 100 - 200 µl, usually 150 µl. Place the sample tubes in the thermal shaker and incubate at room temperature (15 - 25°C) for 3 min 1400 rpm.
15. Transfer the tubes to the magnetic rack, wait (~ 1 min) until the magnetic beads have separated. Transfer the supernatant containing the DNA into a new clean sample tube. Mark the date and the person who performed the extraction on the tubes. Store extracted DNA at -20 °C.

Determination of DNA concentration with Qubit

All reagents must be at room temperature (15–25°C) before use. Do not hold reagents or tubes in your hands for too long to prevent them from getting too warm (an increase in temperature can affect the result). When enabling new reagents, document the required information to Reagent Monitoring.

1. Count the required number of needed Qubit tubes (0.5 ml)
2. Dilute the required amount of Qubit Working Solution:

   Qubit dsDNA BR reagent is diluted 1:200 in Qubit dsDNA BR buffer. Calculate 199µl buffer and 1µl reagent per sample. The buffer foams easily, so it may be necessary to calculate a pipetting allowance for one extra sample.
   Standards are measured every time.

3. Pipette working solution into tubes:

   Standard: 190µl working solution + 10µl standard
   Sample: 195µl working solution + 5µl extracted DNA
   (also possible to use 180-199µl working solution + 1-20µl isolated DNA)
4. Vortex the tubes for 2-3 seconds and incubate at room temperature for 2 minutes.
5. Measuring a sample with a Qubit reader:

   Touch the screen to open the Qubit instrument

   Select DNA-> select dsDNA Broad range

   Select whether to read new calibrations, i.e. standard samples. If you do, select Yes and insert the first standard tube into the machine and select Read. Remove standard 1# and read standard 2#. If you do not want to read the standards, select No.

   Select Read/Read Next Sample to read the samples. The value that appears on the display indicates the DNA concentration of the tube. Press Calculate Stock Conc. to find out the DNA content of the sample. Select the amount of DNA pipetted (usually 5 µl) and the unit ng/l to see the DNA content of the sample.

6. Record the results e.g. to (electronic) laboratory notebook or Excel spreadsheet.
7. After saving the result, select Read Next Sample and read the next sample.

   A good DNA concentration is considered to be above 1 ng/µl. If the concentration is below this, the DNA extraction will be renewed. Concentrations are taken into account for the dilution of DNA for the making of the library.

Verification of DNA quality by agarose gel electrophoresis

In case of problems and when extracting DNA from a new bacterial species, a sample of the extracted DNA is taken and run on 1.0% agarose gel (1.0 g SeaKem LE/ 100 ml 0.5 x TBE buffer). The molecular weight standard used is a 1kb DNA Extension ladder (fragments 500 - 40000 bp), which is used for approx. 0.1 g/1 mm gel well width. (High molecular weight DNA is greater than 50 kb in size and shows minimal smearing.) The sample for gel run is made as follows:

Extracted DNA	5 µl
6 x DNA Loading Dye	1 µl

The molecular weight standard is made as follows:

1kb DNA Extension Ladder	2 µl
PCR water	3 µl
6 x DNA Loading Dye	1 µl

Pipet the entire (6 µl) sample into the gel well. Pipet 3 µl of the molecular weight standard into both ends of the gel.

Run the samples in the gel at 80 V, 120 min.

Dye the gel with Sybr Safe or RefSafeTM Nucleic Acid Staining Solution. Image with the AlphaImager.

Example protocol: Stain with Sybr Safe staining

- To make the staining solution, dilute the 10,000x Sybr Safe reagent to a 1x concentration by using the 0.5x TBE buffer (staining solution). Prepare enough of the 1x staining solution to fully submerge the entire gel. For example 10 µl 10.000x Sybr Safe to 100 ml 0.5x TBE buffer.
- Staining protocol: submerge gel onto staining solution and incubate for 30 min. If possible, agitate gel on an orbital shaker at 50 rpm or gently shake gel staining tray a few times during the intubation period. After incubation, gel is ready for imaging.
- Gels stained with Sybr Safe can be disposed as normal waste

The size and quality of the sample are visually assessed from the gel image. If the fragment is very poorly visible or not visible at all, or if RNA (extra bands) or fragmented DNA (veil) can be seen in the image, the DNA is extracted again. Figure 7 shows an example of low-quality and good-quality DNA on the gel. Figure 7 a) shows additional zones and a lot of smearing. In Figure 7 b), the DNA zones are clear and about 40kb in size.

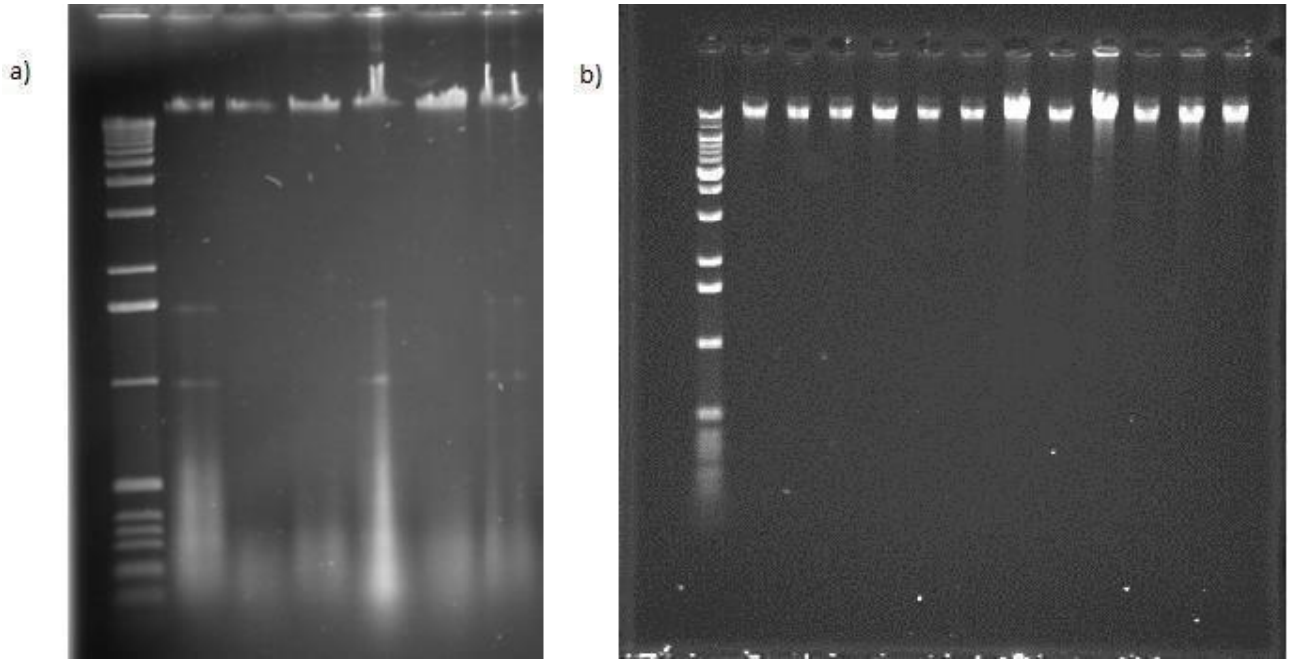


Figure 7. Poor quality (a) and good quality (b) DNA samples run onto a gel.

Protocol with the QIAamp DNA Mini Kit

Notes before starting

- If a precipitate has formed in Buffer ATL or Buffer AL, dissolve by incubating at 56°C.
- Add ethanol to Buffer AW1 and Buffer AW2 concentrates, as indicated on the bottle. Equilibrate samples to room temperature (15–25°C).

1. Culture the bacteria and perform the initial steps of DNA extraction as described in the chapter *Protocol with the MagAttact kit*.
2. Add 180µL of buffer ATL into Eppendorf tube (1.5 ml sterile DNA free tube).
3. Pick (2-5) colonies of bacteria from a fresh culture (overnight) and mix in tube containing buffer ATL.
4. Vortex for a few seconds.
5. Add proteinase K (20µL), mix by vortexing and incubate at 56^{o}C for 2hrs.
6. Add 200µL buffer AL to the sample, mix by vortexing for 15s and incubate at 70^{o}C for 10 mins. After mixing briefly centrifuge the 1.5ml microcentrifuge tube to remove drops from inside the lid.
7. Add 200µL ethanol (96-100%) to the sample and mix by vortexing for 15s. After mixing, briefly centrifuge the 1.5 ml tube to remove drops from inside the lid.
8. Carefully apply the mixture from step 7 (including the precipitate) to the QIAamp mini spin column (in a clean 2ml collection tube) without wetting the rim. Close the cap and centrifuge 6000 x g

(8000rpm) for 1 min. Place the QIAamp mini spin column in a clean 2ml collection tube and discard the tube containing the filtrate.

9. Carefully open the QIAamp mini spin column and add 500µL buffer AW1 without wetting the rim. Close the cap and centrifuge at 6000 x g (8000rpm) for 1 min. Place the QIAamp mini spin column in a clean 2 ml collection tube(provided) and discard the collection tube containing the filtrate.
10. Carefully open the QIAamp Mini spin column and add 500µL buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x g;14,000rpm) for 3min.
11. Recommended: Place the QIAamp Mini spin column in a new 2ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed (14,000rpm) for 1 minute.
12. Place the QIAamp Mini spin column in a 1.5ml microcentrifuge tube (not provided) and discard the old collection tube with the filtrate. Carefully open the QIAamp Mini spin column and add 50µL buffer AE OR distilled water. Incubate at room temperature for 1 min, and then centrifuge at 6000 x g (8000 rpm) for 1 min.
13. Add 50µL buffer AE or distilled water again to the mini spin column. Incubate at room temperature for 1 min, and then centrifuge at 6000 x g (8000rpm) for 1 min.
14. Determine the DNA concentration and check the quality as described in the chapter *Protocol with the* MagAttact kit.

5.2 WGS library preparation with Illumina Nextera XT kit and sequencing with MiSeq instrument

The Illumina Nextera XT kit can be used to create a library for whole-genome sequencing. The sample DNA is labeled with specific index primers and amplified using PCR. With the help of index primers, the different samples in the library are separated from each other after sequencing. The generated PCR product is purified, and the amount of DNA is normalized by measuring the size of the library with a BioAnalyzer instrument and determining the concentration with a Qubit instrument or a method based on magnetic particles. The magnetic particles bind a certain amount of DNA, which is finally eluted from the magnetic particles. Finally, all samples in the library are pooled and the pooled library is denatured before sequencing. The sequencing reaction is performed with MiSeq instrument, and the results are processed with different analysis programs depending on the bacterial species.

Sample

DNA, extracted with the MagAttract kit or QIAmp DNA mini kit, and the concentration of which has been measured with the Qubit device.

Reagents

The reagents used in the method are presented in Annex B Laboratory reagents and equipment for bacterial WGS.

Equipment

Vortex
PCR machine
Plate centrifuge (and adapter + counterweight)
Centrifuge (and adapters for index primer tubes)

Plate mixer (heating)
Pipettes
MiSeq instrument
(Electronic) pipettes
Heat block +100˚C
Magnetic holder

Consumables

24-well plates
foil seals/adhesive seals
Factory clean 1.5 ml Eppendorf tubes
Pipette tips with filters
Protective gloves
50 ml tubes
100 ml measuring cylinder

Work facilities

The work is done in the DNA extraction and PCR laboratory facilities. Some of the work steps are performed in pre-PCR facilities and some in post-PCR facilities.

Protocol

Occupational safety

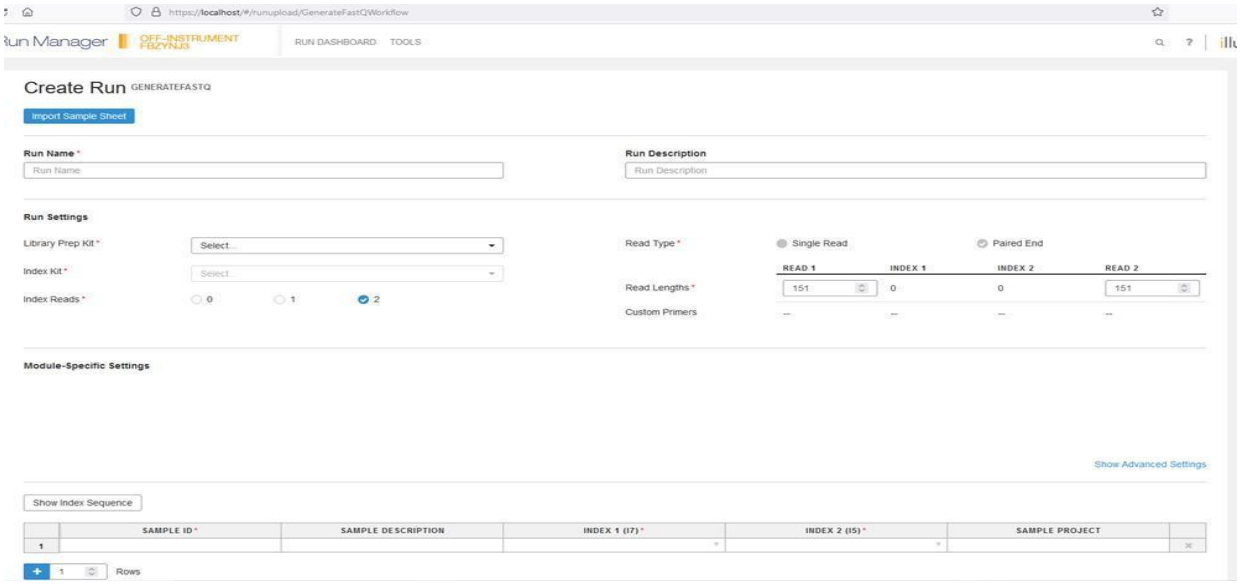
Some of the reagents used in the work contain Formamide (LNA1 and LNW1 reagents and the reagent cartridge used in the MiSeq runs). Formamide is absorbed into the body by inhalation, through the skin and when swallowed. Formamide can irritate eyes and skin. Formamide is classified as a reproductive hazard and can damage a fetus.

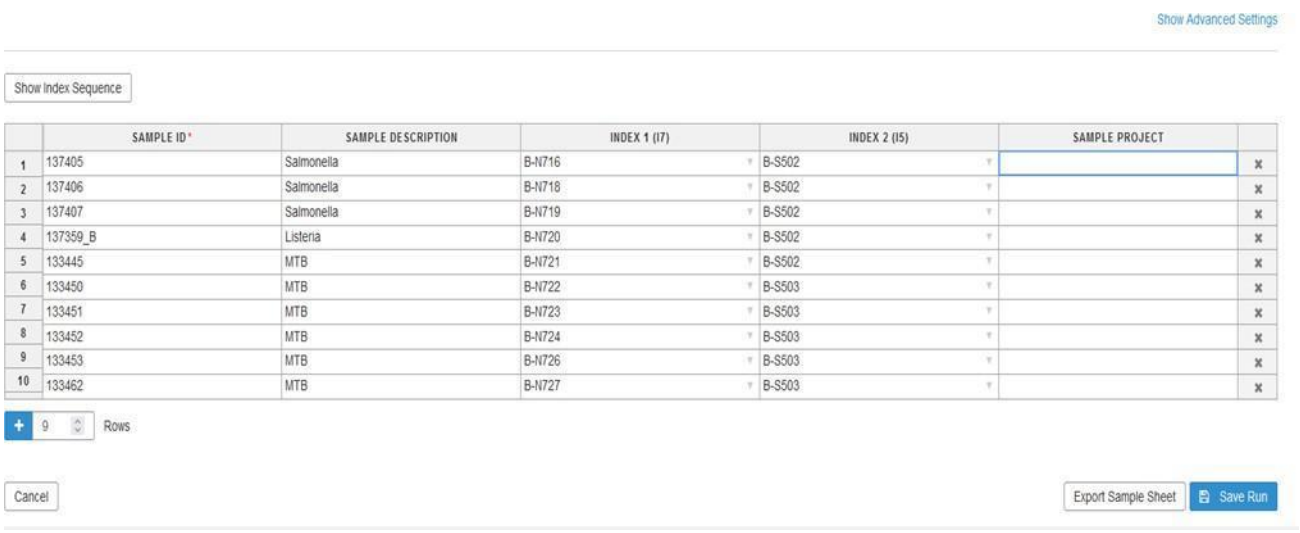
Always wear a protective coat and nitrile gloves when working. Some of the work steps are carried out in a fume hood.

The workflow

Before starting, design the Index primer pairs with the Local Run Manager program (the LRM program must be installed on your machine or use the program on the MiSeq device).

Local Run Manager -> **Create Run** -> select **GenerateFastQ** -> **Fill in the information required for the run (mandatory marked with red *)** -> **Run Name** date of the run -> **Library Prep Kit** from the pull-down menu Nextera XT -> **Index Kit** from the pull-down menu the index kit to be used (A or B) -> other parameters as in the picture below -> In the **Rows** section lines are added from the + - sign or by writing a number as many as there are samples in the library -> **Sample ID** in the strain number -> **Sample Description** in the bacteria name (note! do not use spaces or prohibited characters such as: ? ( ) [ ] / \ = + < > : ; " ' , * ^ | & . in any column), the sample project section can be left blank -> finally you select **Export sample sheet** -> Save the csv file to the lab network in a suitable folder (or alternatively to a memory stick)





You should use the indexes evenly and use the consumption monitoring file. No two identical index pairs may be in the same run. The program will present an error if this happens and ask to change the index pairs. You should take a printscreen of the index table and print it for yourself as a guide for pipetting.

Note! Index kit V2 A and B indexes are used alternately in the runs to prevent the "carryover" effect of indexes.

Controls and template

The success of the library preparation is checked in every run. A successful analysis of the strain indicates that the sequencing of the strain has been successful. In addition, the success of the library preparation is controlled by sequencing the genomic DNA of a well characterized known isolate, e.g. an ATCC strain of a

suitable species, for instance *E. coli*. A library is made from the control DNA, and it is run together with other strains once a month and analyzed like other strains of that species. This control is valid for all strains, not only *E. coli* strains.

In addition to the positive control, a "zero sample" control is sequenced at least 4 times a year, i.e. a sample that does not contain bacterial material. The zero sample is made starting from DNA isolation (no bacteria, only TE buffer) just as a "normal strain" would be made. The purpose of the control is to examine the amount of background (e.g. with the Kraken2 program).

NOTE! Halved reagent amounts are mainly used to make the library, in which case the pipetting amounts indicated in the brackets of the instructions are followed. If there are problems with preparing the library (e.g. too little DNA content before normalization), alternatively, when renewing the library, you can use full amounts.

Dilute the DNA samples in the DNA extraction room to a concentration of 0.2 ng/µl (**0.4 ng/µl**) in low TE buffer (or nuclease-free water).

Tagmentation (fragmentation and addition of adapters)

Go to the Template Room or work on the dedicated bench for template adding. Work in a biosafety cabinet if possible.

- Take the TD buffer and ATM to thaw from the freezer.
- Invert TD buffer and ATM 3-5 times to mix. Do not vortex.
- Take a 24-well plate and label it with the letter combination NTA (=Nextera Tagment Amplicon Plate)
- Cut a piece of protective foil the size of the plate
- Add to each well used (**halved**)

  10 µl **(5 µl)** TD buffer

  5 µl **(2,5 µl)** DNA sample

  5 µl **(2,5 µl)** ATM

- Close the protective foil carefully, mix with a vortex (about 5 seconds) and centrifuge for about a minute in a plate centrifuge. Make sure the centrifuge has a counterweight and use a 96-well plate as an adapter.
- Put the disk in the PCR instrument and run the following program:

  55 °C for 5 min

  10 °C for ∞

- Remove the well plate from the PCR instrument as soon as the sample temperature has reached 10 °C.
- Take index primers and NPM out to thaw from the freezer.
- Replace protective foil and add 5 µl (**2.5 µl**) of NT buffer (storage at RT) to each well

- Seal carefully with foil, mix with a vortex (about 5 seconds) and centrifuge for a minute with a plate centrifuge.
- Leave at room temperature for 5 minutes.

Index-PCR

- Thaw the NPM and index primers well in advance of this step
- Invert each tube (NPM and primers) 3–5 times and centrifuge the solutions to the bottom. Use adapters (empty Eppendorf tubes) for centrifugation.
- Add to each sample well
  - 15 µl **(7,5 µl)** NPM
  - 5 µl (**2.5 µl**) index 2 primer (white caps) into each sample well (open only one cap at a time). Replace it with a new cap each time.
  - 5 µl **(2,5 µl)** index 1 primer (orange caps) into each sample well (open only one cap at a time). Replace it with a new cap each time.
- Take a new protective foil and close it carefully, mix with a vortex (about 5 seconds) and centrifuge for about a minute in a plate centrifuge.
- Take the plate to the PCR machine and run the following program:
  - 72 °C for 3 min
  - 95 °C for 30 s
  - 12 cycles:

    95 °C – 10 s

    55 °C – 30 s

    72 °C – 30 s

    72 °C for 5 min

    10 °C ∞

- If you have to stop working at this point, you can store the plate in the refrigerator for 1-2 days.

PCR product purification

- Continue the work in the biosafety cabinet of the post-PCR room.
- Take the AmPure XP bead suspension out to room temperature from the refrigerator ahead of time (about 15-30 minutes before starting work).
- Take the RSB (resuspension buffer) out to thaw.
- Prepare 80% ethanol from absolute alcohol. Dilute ethanol in nuclease-free water. 360 µl (**400 µl**) of ethanol is required per sample.
  - E.g. for 12 strains 4 ml abs. EtOH + 1 ml aqua = 5 ml 80% EtOH
- Centrifuge the plate with a plate centrifuge, about 1 min.
- Vortex the Ampure XP bead suspension for 30 seconds.
- Add 30 µl (**15 µl**) of Ampure XP bead suspension to each well.
- Mix in a shaker for 2 minutes at 1800 rpm.
- Incubate at room temperature for 5 minutes.
- Place the well plate in a magnetic rack and wait for about 2 minutes or until the supernatant has become clear.

- Hold the plate in the magnetic rack and carefully remove the supernatant into the waste tube (90 µl in a pipette). Be careful not to touch the magnetic beads.
- Keep the plate still in the magnetic rack and wash the particles with 80% ethanol row by row (Tip: hold the plate when pipetting, do not touch the magnetic beads, but pipet from the other side of the tube):
    - add 180 µL (**200 µL**) of 80% ethanol to each well. Do not resuspend the beads!!
    - Incubate for 30 seconds.
    - Remove the supernatant (220 µl in a pipette).
- Repeat washing as above.
- Carefully remove the remaining ethanol (50 µl in a pipette) and let the pellet dry for 5 **(3)** minutes at room temperature without protective foil.
- Remove the plate from the magnetic rack and add 53 µl **(26.4 µl)** of RSB to each well (select the pipet/mix setting of the electronic pipette or gently pipet back and forth ten times to mix the beads properly). You can briefly centrifuge the plate with a plate centrifuge or make sure by pipetting that the liquid is at the bottom of the tube. You can use EB buffer instead of RSB if needed.
- Incubate at room temperature for 2 minutes.
- Place the plate in the magnetic rack and wait for about 2 minutes or until the supernatant has become clear.
- Prepare two new 24-well plates:
- CAN (=clean amplified NTA plate (backup), keeps for 1 week -15 °C-25 °C)
- LNP (=Library Normalization plate)
- Transfer 20 µl (**about 15 µl for LNP plate and about 8 µl for CAN plate**) of supernatant from each well to both new plates.

5.3 Library normalization

Qubit+Bioanalyzer method

Normalization of libraries is mainly done with Qubit+BioAnalyzer assays. Normalization can alternatively be done using the magnetic bead method, the instructions of which can be found under title *Library normalization using the magnetic bead method (Bead-based).*

- Get ice and take the CAN plate out to thaw.

<u>Measuring the concentration with Qubit</u>

- Measure the concentration from the CAN plate with the Qubit instrument and the Qubit dsDNA HS kit.
- Count the required number of Qubit tubes (0.5 ml).
- Dilute the required amount of Qubit working solution:
- Qubit dsDNA HS reagent is diluted 1:200 in Qubit dsDNA HS buffer. Calculate 199 µl of buffer and 1 µl of reagent per sample. The buffer foams easily, so it may be necessary to calculate a 1-fold pipetting allowance.
- Standards are measured every time.

- Pipette the working solution into the tubes:
  - Standard: 190 µl working solution + 10 µl standard
  - Sample: 198 µl working solution + 2 µl extracted DNA
- Vortex the tubes for 2-3 seconds and incubate for 2 minutes at room temperature.
- Measuring the sample with the Qubit instrument (NOTE! Do not hold the tubes in your hand for a long time, the increase in temperature affects the result)
  - Touch the screen to turn on the Qubit instrument.
  - Select dsDNA-> select dsDNA High Sensitivity
  - Choose to read new calibrations, i.e. standard samples. Press Yes and put the first standard tube in the machine and press Read. Remove standard 1# and read standard 2#.
  - Read the samples by selecting Read/Read Next Sample. The value that appears on the screen indicates the DNA concentration in the tube. Press Calculate Stock Conc. to find out the DNA concentration of the sample. Select the amount of pipetted DNA (usually 2µl) and the unit ng/µl, to display the DNA concentration of the sample. Record the result. Alternatively, you can save the results by pressing Save and transfer the results to a computer with a memory stick.
- When you have recorded the result, press Read Next Sample and read the next sample.
- Qubit values should be between 4–6 ng/µl for the Bioanalyzer run. If the values are more than 10 ng/µl, dilute e.g. 1:2 in RSB buffer or EB buffer (2 µl DNA + 4 µl RSB) for the run, or if the qubit value is approx. 20 ng/µl dilute 1:4 (2 µl DNA + 8 µl RSB).
- Acceptance criterion: if the concentrations of all strains in the library are below 1 ng/µl, the library preparation is repeated. A concentration of about 1 ng/µl can be accepted for individual strains, and in this case the strain is pooled undiluted.

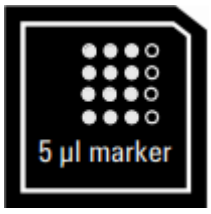
<u>Bioanalyzer assay</u>

- One BioAnalyzer run can accommodate 11 strains. If there are more than 11 strains in the library, the calculated average size of the strain (either the size of the same species in the same run or the average size of 1000 bp) is added to the normalization table.
- NOTE! The kit contains dimethyl sulfoxide (DMSO), which binds to nucleic acid and is therefore a potential mutagen. Use nitrile gloves! Waste is collected as hazardous waste in its own bag in a fume hood.
- Take the reagents out to room temperature at least 30 min before starting and protect the reagents from light.
- Turn on the instrument.
- Always wash the electrodes of the Bioanalyzer instrument before and after a run. Also wash between runs.
  - Take the wash plate and slowly load it with 400 µl of milliQ water through one well. The water spreads over the entire plate.
  - Place the wash plate in the instrument and close the lid for approx. 10 seconds.
  - Remove the wash plate and leave the lid open for approx. 10 seconds to let the water evaporate from the electrodes before closing the lid.
  - Store the washplate in an empty Eppendorf storage box or minigrip bag.
  - Always use a new wash plate when using a new kit.

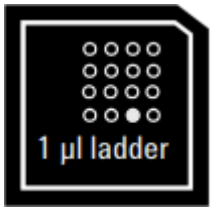
- Prepare the gel (1 gel makes 4 full runs):
- Add 15 µl High Sensitivity DNA dye (blue cap) into the High Sensitivity DNA gel matrix tube (red cap), vortex, and spin the liquid down to the bottom of the tube.
- Transfer the solution into the filter tube and centrifuge at 6000 rpm (2240 g+-20%) for 10 min. Label the tube with the preparation date, protect the solution from light, and store at +4 – +8 °C in a refrigerator. The gel will keep for 6 weeks after preparation.
- Load the gel onto the plate:
    - If you are using a previously made gel, let it get warm for 30 min at RT before use.
    - Take a new High Sensitivity DNA plate and put it in the gel loading device.
    - Pipette 9 µl of the gel into the well marked with G (white inside the black circle).

- Check that the syringe plunger is at 1 ml and close the gel loading device.
- Push the piston down until the clip holds the piston down. Wait 60 s and release the piston.
- Wait 5 s for the piston to rise by itself.
- Carefully open the gel loading device (hold the plate with one hand).
- pipette 9 µl of gel into the other wells marked with G (3 pcs.).

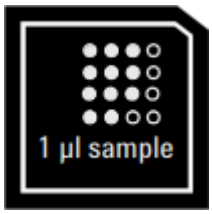
- Load the marker onto the plate:
    - Pipette 5 µl of marker (green cap) onto the plate into all sample wells and into the marker well. Do not leave any wells empty.



- Load the ladder and samples onto the plate
    - Pipette 1 µl of the ladder (yellow cap) into the well marked with a ladder.



- Pipette 1 µl of each sample into the 11 sample wells. Pipette 1 µl of marker into wells where no sample will be placed.



- Put the plate in the vortex intended for it and vortex for 1 min at 2400 rpm (you can set the rpm, but the time is automatically 1 min).
- Start the Bioanalyzer run within 5 min of plate completion.

Run start with Agilent 2100 Bioanalyzer

- Make sure you have washed the electrodes before the run.
- Open the 2100 Expert program and select instrument DE13804121 BioAnalyzer 1 or DEDAE02199 BioAnalyzer 2 -> assays -> Electrophoresis -> dsDNA -> High sensitivity DNA.
- Enter the names of the samples (sample name) and choose how many samples you will run (Run sample).
- Place the plate in the device and close the lid.
- When the device has recognized the plate and everything in the start run check list is green, start the run by pressing Start (a run with 11 samples takes about 40 min).
- Remove the plate immediately after the run and do not leave it, e.g. overnight or over the weekend.
- Wash the electrodes as above.

Analyzing the results

- Under the Contexts bar, select Data-> all samples.
- Change the values so that Global: slope threshold 20.
- Region Table (Illumina recommends) -> Inspect the range 400-3000 bp. Adjust the value to the right: click on one of the samples and right-click on the image. Select "modify region" and change the values to 400 and 3000. You can also change the value for all samples at once: All samples menu on the right, global advanced sample setpoints smear analysis regions set the desired value in the window 400-3000 bp and press ok.

- If there have been problems finding the size standards, you can set them manually (there should be 15 spikes). First, select the lowest and highest ladder spike. That might be enough in some cases.
- If you don't have 15 Ladder spikes and you can't add them, you have to repeat the run.
    - Ladder -> Peak table. If any peak has a value of 0 (a peak with no size), right-click -> manual integration-> add Peak for those without a value.
    - If the analysis is still not successful and error messages are visible, repeat the Bioanalyzer run.
    - If it is a "late migration" problem, open the peak table of that sample and go to the upper marker, right-click "Manually Set Upper marker". This should fix the problem.
- The marker spikes must be visible, and the library spike must remain between the marker spikes. The spike of the library should be relatively narrow and sharp, but the shape is not critical for sequencing success.
- If the migration of the library fails (average size of the strain over 1200 bp), the calculated average size of the strain (1000 bp) is added to the normalization table.
- Record the average size of the samples and enter the sizes.
- Save the run in PDF format on a suitable disk drive and folder with the Print function
- Turn off the instrument
- Freeze the CAN plate at -15 °C-25 °C and proceed to normalization with the LNP plate.

Library normalization using the magnetic bead method (Bead-based)

- Normalization using the magnetic bead method is only done for libraries that have been prepared with full reagent amounts.
    - Prepare **fresh 0.1 M NaOH each time**, á 30 µl/sample. E.g. 100 µl 1 M NaOH + 900 µl water.
    - Note! 1M NaOH only lasts for 2 weeks, after which a new stock must be dissolved.
- Steps that use LNA1 and LNW1 reagents are done **in the fume hood** of the post-PCR room because they contain formamide. Put all waste containing formamide (liquids, pipette tips) in the formamide waste container! Use nitrile gloves and a protective coat.
- Thaw LNA1 (=Libary Normalization Additives). Once LNA1 is at room temperature, vortex thoroughly to ensure all precipitates are dissolved.
- Take LNB1 (=Library Normalization **Beads**) and LNW1 (=Libary Normalization **Wash**) out from the refrigerator to room temperature.
- Make sure that LNS1 (=Libary Normalization Storage buffer) is at room temperature before starting (storage at RT).
- Vortex the LNB1 for at least 1 minute or until no precipitate is visible at the bottom.
- Pipette **44 µL** of LNA1 **per sample** into a clean 1.5 mL Eppendorf tube. NO NEED TO COUNT THE PIPETTING ALLOWANCE!
- Pipette the LNB1 back and forth 10-15 times to mix the beads properly. Transfer **8 µL** of freshly mixed LNB1 **per sample** to the Eppendorf tube containing LNA1.
- Mix by pipetting back and forth and dispense 45 µL into each LNP sample well of the plate.
- Cover the well plate with protective foil and shake in a plate shaker at 1800 rpm for exactly 30 minutes.
- Quickly centrifuge in a plate centrifuge and place the plate in a magnetic rack for 2 minutes or until the supernatant is clear.

- Carefully remove the supernatant (70 µl in a pipette).
- Move the plate off the magnetic rack and wash the beads with LNW1 as follows (do the pipetting in the fume hood):
    - Add 45 µL of LNW1 to each sample well and cover the wells with a protective foil. Shake in a plate shaker at 1800 rpm for 5 minutes. Spin quickly with a plate centrifuge if you notice bubbles in the tubes or the liquid is not all at the bottom. Place the plate in the magnetic rack and wait 2 minutes or until the supernatant is clear. Carefully remove the supernatant (70 µl in a pipette).
    - Repeat washing as above. Make sure you remove all of the LNW1 after the second wash.
- You can do the following steps on the benchtop.
- Move the plate off the magnetic rack and add 30 µL of 0.1 M NaOH to each sample well. Resuspend the pellet by pipetting back and forth.
- Cover the wells with protective foil and shake in a plate shaker at 1800 rpm for 5 minutes.
- Prepare a new 24-well plate by marking it with SGP (=storage plate) and cutting a suitably sized protective foil.
- If necessary, quickly centrifuge with a plate centrifuge and place the plate in a magnetic rack for 2 minutes or until the supernatant is clear.
- During this, add 30ul LNS1 to each well of the SGP plate.
- Transfer 30 µl of supernatant from LNP plate to SGP plate.
- Cover the SGP plate with protective foil, vortex for about 5 seconds and quickly centrifuge in a plate centrifuge.
- Store the plate in the freezer (-15 °C-25 °C) until pooling (1 - 3 weeks).

5.4 Library dilution and pooling

Preparation and denaturation of PhiX 20 pM stock (running control):

- Add to an Eppendorf tube:
    - 2 µl of 10 nM PhiX library
    - 3 µl 10mM Tris-Cl, pH 8.5, 0,1% Tween 20
    - 5 µl of fresh 0.2 M NaOH
- Vortex and centrifuge for about 1 min and incubate the tube at room temperature for 5 min.
- Dilute the solution to a volume of 20 pM by adding 990 µl cold HT1 buffer to the tube.
- The solution stays for 3 weeks.

Library pooling (after library normalization with the Qubit+Bioanalyzer method)

- In general, the desired concentration for pooling is 4 nM (for a halved and full library, or 2 nM for a long-run library) and the desired volume is 20 µl.
- After entering the Qubit and Bioanalyzer results into the dilution table, you can make the dilutions from the LNP plate to EB or RSB buffer in a new 24-well plate. Vortex and spin briefly.
- Pre-label five 1.5 ml Eppendorf tubes (Pool 1, Pool 2, Pool 3, NaOH 0.2 M and PhiX 12.5 pM).
- Prepare fresh 0.2 M NaOH in an Eppendorf tube (200 µl 1 M NaOH+800 µl HyClo water).
- **Pool 1**: Pipette 5 µl of each diluted sample into a clean Eppendorf tube, vortex, and spin.

- If you get negative values from the dilution table, you can pool the undiluted sample 5 µl (mark it in the table).
- **Pool 2:** take pooled library (pool 1) 5 µl and fresh 0.2 M NaOH 5 µl (1 M NaOH 200 µl + HyClo water 800 µl) and incubate at room temperature for 5 min.
- Add 990 µl HT1 buffer, making the library concentration 20 pM.
- **Pool 3 (12pM)**: Dilute library further (usually long run library): take 360 µl 20 pM library + 240 µl HT1 buffer. Now the library concentration is **12 pM OR**
- **Pool 3 (15pM)**: Dilute the library further (halved or unhalved library): take 450 µl 20 pM library + 150 µl HT1 buffer. Now the library concentration is **15 pM.**
- **Pool 3 (10pM)**: Dilute the library further: take 300µl 20 pM library + 300µl HT1 buffer. Now the concentration of the library is **10pM.**

- Dilute 20 pM PhiX control in HT1 buffer (37.5 µl 20 pM PhiX+22.5 µl HT1 buffer).
- Add 6 µL of 12.5 pM PhiX control to the pooled library (pool 3).
- Pipette 600 µl into the chamber of the run cartridge and start the run according to 7.2.6.

Library pooling (after library normalization by the magnetic bead method)

- Get ice and heat the heat block to 96 °C for denaturation. Take the library and PhiX-stock out to thaw on the ice.
- After that, combine the libraries (pool) as follows:
    - Label a clean Eppendorf tube with the letters PAL (=pooled Amplified library) and transfer 5 µl of each sample from the SGP plate to the PAL tube, vortex, and centrifuge briefly.
    - Label the **screw cap** Eppendorf tube with the letters DAL (=diluted Amplified library) and pipette 600 µl of HT1 buffer into the DAL tube.
    - Transfer 23 µL from the PAL tube to the DAL tube. Pipette back and forth 3-5 times.
    - Vortex the DAL tube and rapidly centrifuge.
    - Incubate in the heat block at 96 degrees for exactly 2 minutes.
    - Put the tube on ice immediately afterwards. Keep the tube on ice for at least 5 minutes or until you load it into the reagent cartridge.
    - Use of PhiX control: 20pM stock is diluted to 12.5pM -> 37.5µl 20pM PhiX + 22.5µl HT1.
    - Add 6 µl of 12.5 pM PhiX control to the DAL tube, making the concentration about 1%.
    - Time from denaturation to loading the cartridge should be as short as possible.

5.5 Preparing the reagent cartridge, using the MiSeq instrument and starting the run

- Take the reagent cartridge and HT1 (Hybridization Buffer) out of the freezer to thaw at +4 °C the day before. The cartridge can also be thawed at room temperature or in lukewarm water (1 hour) if, for example, you want to make sure that the library has been successful. Always make sure before use that the reagents in the cartridge are completely melted. When the HT1 buffer has melted, store it in the refrigerator until use. Also keep the thawed reagent cartridge in the refrigerator until the sample is loaded.

- Power cycling must be done before the run, and it is good to do a post-run wash (20 min) so that the liquid flow of the instrument works properly. (Instructions: Annex C).
- Save a sample sheet onto the instrument: Open the LRM program with the Miseq device → Click on Create Run in the upper right corner and GenerateFASTQ → select Import Sample Sheet in the upper left corner and search for your run's sample sheet on a folder → finally select Save Run in the lower right corner.
- Open Illumina's program, Click Sequence → Click Open Local Run Manager → Check Use BaseSpaceTM Sequence Hub for this run and Run analysis, collaboration, and storage → Next → log in. Select "next".
    - MiSeq asks you to install the flow cell that came with the reagent kit.
    - Take the flow cell out of its case and carefully rinse off the salt water with water. DO NOT TOUCH THE BLACK DOT (flow cell port gasket). Dry gently with Kimwipe paper.
    - You can also wipe the glass with 80% EtOH on lint-free paper. Scratches, bubbles, fingerprints, or paper lint must not be visible on the glass. When the glass is completely clear, remove the old glass and insert the new glass into the instrument. Make sure MiSeq can read the code on the flow cell. After that, select "next".
    - Next, MiSeq asks you to empty the waste container and install the PR2 reagent bottle that came with the flow cell in its place. Gently invert the PR2 reagent a few times and place it in the instrument. Wait for the MiSeq to read the code from the bottom of the bottle. Select "next".
    - MiSeq asks you to load a reagent cartridge. Check that the reagents are melted and mixed properly. Precipitates must not be visible in the reagents. Invert it gently about ten times to mix the reagents. DO NOT SHAKE! Tap the reagent cartridge against the table to break up air bubbles.
    - Use a 1000 µl pipette tip and break the protective film over the top of the sample chamber marked orange “LOAD SAMPLES”.
    - Pipette 600 µl of sample from the Pool 3/DAL tube into the chamber of the sample cartridge and immediately transfer the cartridge into the MiSeq.
    - Select "Change sample sheet", then import your own file from the desktop (if named with the cartridge code, Miseq can find it automatically).
    - Select "Save and continue" and then "next".

Wait until MiSeq has checked that everything is OK and the green approvals appear on screen. If everything is OK, press "Start Run".

You can follow the progress of the run in Illumina's BaceSpace in real time from your own computer.

(https://euc1.sh.basespace.illumina.com/home/index).

End of run

When the run is finished, the Template Line-wash is performed (Annex C). When washing, the waste container is emptied from the instrument into the formamide waste container located in the fume hood (remember nitrile gloves and protective coat). Well number 8 of the reagent cartridge is emptied into the formamide waste container with a disposable Pasteur pipette, after which the cartridge can be disposed of among other waste.

5.6 Quality assurance of the sequence reads and the sequence data download

The quality and success of the run is evaluated in Illumina's BaceSpace using various parameters. The most important of these are Cluster density (density of clusters on the flow cell, optimum 800-1200 K/mm2), Cluster PF % (access of clusters through the filter, usually >85%) and Q30 total % [measures the possibility of errors in determining specific bases (=Base Call) the higher the better, usually >90%]. These run parameters are recorded. A sufficient number of reads passing the cluster (reads passing filter, Reads PF) indicates successful sequencing (24–30 million Paired-End Reads).

Each species of bacteria is analyzed according to separate instructions. Suboptimal run parameters do not necessarily prevent the successful analysis of the genome. The success of the sequencing is determined definitively in the analysis processes and according to the criteria defined in them. If the analysis of the strain is not successful according to the criteria and limits set for it, the library and sequencing will be redone for the strain.

Using the Phix control:

PhiX enables internal quality control, which monitors the success of sequencing (we test that a certain known sequence, phiX, is obtained correctly and there are no errors, base call). Usually, 1% PhiX control is loaded into the run and the number of errors in its sequencing must not exceed 6% (Illumina's recommendation).

Recommended parameters for different kits:
http://www.illumina.com/systems/miseq/performance_specifications.html

The sequences are downloaded from BaseSpace server if the server is used. Alternatively, the sequence data is stored locally in MiSeq and the sequence files can be downloaded and stored elsewhere.

6. Storing the sequence data

Sequence data requires an adequate amount of storage space. For instance, a gzipped fastq read pair of *Vibrio cholerae* whole genome sequenced using MiSeq and 300 cycle kit aiming at 100 x average coverage of the assembled genome is approximately 400-500 megabytes. A storage system must be able to manage and handle growing amounts of data. The system must be well documented so that a single sequence file can be traced and retrieved from the system when needed.

7. References

Caburao EA. 2024. Understanding Best Practices in Hazardous Materials Management. Safety culture. Available at: https://safetyculture.com/topics/hazardous-substances/hazardous-materials-management/ Accessed: 18th Dec 2024

CDC. 2024a. Biosafety trainings. Available at: https://www.cdc.gov/safe-labs/php/biosafety-training/index.html Accessed: 18th Dec 2024

CDC. 2024b. Biorisk management. Available at: https://www.cdc.gov/safe-labs/php/biorisk-management/index.html Accessed: 18th Dec 2024

CDC. 2024c. A Stepwise Process to Improve Biorisk Management Systems. Available at: https://www.cdc.gov/safe-labs/php/echo-biosafety/stepwise-process-improve-biorisk-management-systems.html Accessed: 18th Dec 2024

CDC. 2024d. Biological Risk Assessment Process. Available at: https://www.cdc.gov/safe-labs/php/biological-risk-assessment/process.html Accessed: 18th Dec 2024

CDC. 2024e. Biological Risk Assessment. Available at: https://www.cdc.gov/safe-labs/php/biological-risk-assessment/index.html Accessed: 18th Dec 2024

Environmental Health and Safety. 2024. Personal Protective Equipment Requirements for Laboratories. Available at: https://ehs.ncsu.edu/laboratory-safety/personal-protective-equipment-requirements-for-laboratories/ Accessed: 18th Dec 2024

Harwood V, Gandhi J, Wright A. 2004. Methods for isolation and confirmation of *Vibrio vulnificus* from oysters and environmental sources: a review. Journal of Microbiological Methods 59 (2004) 301–316. doi:10.1016/j.mimet.2004.08.001.

Heljanko V, Tyni O, Johansson V, Virtanen J-P, Räisänen K, Lehto K-M, Lipponen A, Oikarinen S, Pitkänen T, WastPan Study Group and Heikinheimo A. 2024. Clinically relevant sequence types of carbapenemase-producing *Escherichia coli* and *Klebsiella pneumoniae* detected in Finnish wastewater in 2021–2022. Antimicrobial Resistance & Infection Control (2024) 13:14.https://doi.org/10.1186/s13756-024-01370-z.

Henderson T. 2009. Personal Protective Equipment in the Lab. Labmanager. Available at: https://www.labmanager.com/personal-protective-equipment-in-the-lab-20269 Accessed: 18th Dec 2024

International Organization for Standardization (ISO). 2000. ISO 7899-2:2000. Water quality — Detection and enumeration of intestinal enterococci. Part 2: Membrane filtration method.

International Organization for Standardization (ISO). 2006. ISO 19458:2006 Water quality — Sampling for microbiological analysis.

International Organization for Standardization (ISO). 2010. ISO 19250:2010 Water quality — Detection of Salmonella spp.

International Organization for Standardization (ISO). 2014. ISO 11133:2014 Microbiology of food, animal feed and water — Preparation, production, storage and performance testing of culture media.

International Organization for Standardization (ISO). 2014. ISO 9308-1:2014 Water quality — Enumeration of Escherichia coli and coliform bacteria. Part 1: Membrane filtration method for waters with low bacterial background flora.

International Organization for Standardization (ISO). 2017. ISO 21872-1 (ISO 21872-1:2017 Microbiology of the food chain — Horizontal method for the determination of Vibrio spp. Part 1: Detection of potentially enteropathogenic Vibrio parahaemolyticus, Vibrio cholerae and Vibrio vulnificus).

International Organization for Standardization (ISO). 2018. ISO 8199:2018 Water quality — General requirements and guidance for microbiological examinations by culture.

International Organization for Standardization (ISO). 2023. ISO 7704:2023 Water quality — Requirements for the performance testing of membrane filters used for direct enumeration of microorganisms by culture methods.

Liu P, Ibaraki M, VanTassell J, Geith K, Cavallo M, Kann R, Guo L, Moe CL. 2022. A Sensitive, Simple, and Low-Cost Method for COVID-19 Wastewater Surveillance at an Institutional Level. Science of The Total Environment 807:151047. https://doi.org/10.1016/j.scitotenv.2021.151047.

Nandi B, Nandy RK, Mukhopadhyay S, Nair GB, Shimada T, Ghose AC. 2000. Rapid Method for Species Specific Identification of Vibrio cholera Using Primers Targeted to the Gene of Outer Membrane Protein OmpW. J. Clin. Microbiol. 38:4145-4151.

National Institutes of Health (NIH). 2023. Laboratory Biosafety Manual BSL-2 and BSL 2/3. Available at: https://ors.od.nih.gov/sr/dohs/Documents/bsl-2-lab-safety-manual.pdf Accessed: 18th Dec 2024

Rafiee M, Isazadeh S, Mohseni-Bandpei A, Mohebbi SR, Jahangiri-Rad M, Eslami A, Dabiri H, Roostaei K, Tanhaei M, Amereh F. 2021. Moore Swab Performs Equal to Composite and Outperforms Grab Sampling for SARS-CoV-2 Monitoring in Wastewater. Science of The Total Environment 790:148205. https://doi.org/10.1016/j.scitotenv.2021.148205.

Schang C, Crosbie ND, Nolan M, Poon R, Wang M, Jex A, John N, et al. 2021. Passive Sampling of SARS-CoV-2 for Wastewater Surveillance. Environmental Science & Technology 55 (15): 10432–41. https://doi.org/10.1021/acs.est.1c01530.

Standing Committee of Analysts. 2016. The Microbiology of Recreational and Environmental Waters (2016) – Part 9 – Methods for the isolation of *Yersinia*, *Vibrio* and *Campylobacter* by selective enrichment. Methods for the Examination of Waters and Associated Materials. https://standingcommitteeofanalysts.co.uk/microbiology-working-group/ Accessed: 18th Dec 2024

The University of Chicago. Biological Safety Training. Available at: https://researchsafety.uchicago.edu/training/biological-safety-training/ Accessed: 18th Dec 2024

The University of Washington. 2019. Laboratory Risk Assessment Tool. Available at: https://www.ehs.washington.edu/resource/laboratory-risk-assessment-tool-lab-rat-843 Accessed: 18th Dec 2024

Tiwari A, Lehto KM, Paspaliari D, Al-Mustapha A, Sarekoski A, Hokajärvi A-M, Länsivaara A, Hyder R, Luomala O, Lipponen A, Oikarinen S, Heikinheimo A, Pitkänen T and WastPan Study Group. 2024. Developing wastewater-based surveillance schemes for multiple pathogens: The WastPan project in Finland. Science of The Total Environment 926 (2024) 171401. https://doi.org/10.1016/j.scitotenv.2024.171401

ANNEXES

ANNEX A. Sample collection form

O D I N

STRENGTHENING ENVIRONMENTAL SURVEILLANCE TO ADVANCE PUBLIC HEALTH ACTION IN AFRICA

SAMPLE COLLECTION FORM

In the field:

1. Name of person(s) conducting the sampling: _____
2. Sampling location (city, municipality, sampling location name and precise sampling point):

   _____

   _____
3. Sample code: _____
4. Sampling date (day/month/year) and time (hour/minute): _____
5. Air temperature at the time of sample collection: _____˚C
6. Temperature of the water sample at time of sample collection (Note! to be measured from a fraction of the sample poured away from the sample container; thermometer is not sterile): _____˚C
7. Is the sampling container sterile? Yes ☐ No ☐
8. Sample matrix:

   Tap water ☐ Well water ☐ Surface water ☐ Wastewater ☐

9. Specific observations related to the sampling event (e.g. changes in the nearby environment, precipitation events, etc.):

_____

_____

FILL OUT FOR TAP WATER SAMPLING (Questions 10-17)

10. Technique used for sampling: Grap ☐ Passive ☐
11. Is the tap disinfected before sampling? Yes ☐ No ☐

    If yes, describe how: _____
12. Are attached devices and inserts removed before sampling?

    Yes ☐ No ☐
13. Is water from the tap flushed before sampling? Yes ☐ No ☐
14. Total volume of the water sample collected? _____ Liters; Number of sample containers? _____
15. Does sample contain residual chlorine? Yes ☐ No ☐
16. Has residual chlorine been inactivated (usually by using sodium thiosulphate)?

    Yes ☐ No ☐
17. Any other observations? Yes ☐ No ☐

    If yes, describe:

_____

FILL OUT FOR WELL WATER SAMPLING (Questions 18-20)

18. Technique used for sampling: Grap ☐ Passive ☐
19. Describe sampling process and used equipment:

_____

20. Any other observations (Rainy season, dry season etc.)? Yes ☐ No ☐

If yes, describe:

_____

FILL OUT FOR SURFACE WATER SAMPLING (Questions 21-23)

21. Technique used for sampling: Grap ☐ Passive ☐
22. Are there any facilities possibly discharging chemical effluents upstream of the sample collection point? Yes ☐ No ☐

    If yes, describe what kind of facility?

    _____
23. Any other observations (Rainy season, dry season etc.)? Yes ☐ No ☐

    If yes, describe:

_____

FILL OUT FOR WASTEWATER SAMPLING (Questions 24-27)

24. Type of wastewater treatment plant/sewage system? Open ☐ Closed ☐
25. Technique used for sampling: Grap ☐ Passive ☐
26. Strength of flow at the time of collection?

    Strong ☐ Weak ☐ No flow ☐
27. Any other observations (Rainy season, dry season etc.)? Yes ☐ No ☐

    If yes, describe:

_____

In the laboratory

1. Name of person receiving sample at laboratory: _____
2. Date (day/month/year) and time (hour/minute) of sample received at the laboratory: _____

3. Temperature of the water sample at time of sample arrived at laboratory (Note! must be measured from a fraction poured into laboratory tube after mixing the sample container): _____˚C
4. Is there anything notable or unusual about the sample upon arrival?

   Yes ☐ No ☐

   If yes, describe:

   _____

   5. Date (day/month/year) and time of analysis initiation: _____

ANNEX B. Laboratory reagents and equipment for bacterial WGS

Reagents

DNA extraction and Measurement of DNA concentration

Reagent	Temperature	Work phase	infective laboratory
DNA-extraction kit for bacteria	Depens on the kit contents	DNA extraction	x
Qubit dsDNA BR-Kit	Room Temperature	Measurement of DNA concentration	x

Library preparation

Reagent	Spesific name	Included in Illumina Nextera XT kit	Temperature	Work phase	Pre-PCR	Post-PCR
TD-buffer	Tagment DNA buffer	x	Freezer -20°C	Tagmentation	x	
ATM	Amplicon Tagment Mix, 96 RXN	x	Freezer -20°C	Tagmentation	x	
NT-buffer	Neutralize Tagment Buffer	x	Room temperature	Tagmentation	x	
NPM	Nextera PCR Master Mix	x	Freezer -20°C	Index-PCR	x	
Indexes (primers), Illumina			Freezer -20°C	Index-PCR	x	
AmPure XP - partikkelit			Refrigerator 2-8°C	PCR product purification		x
80% EtOH (stock 96-99%)			Dilution from 96%-99% EtOH, Room temperature	PCR product purification, Cleaning flow cell		x
RSB	Resuspension buffer	x	Freezer -20°C	PCR-product purification		x
0,1 M NaOH (stock 1 M)			1M NaOH stock (max 2 weeks old) dilute 0,1M just before use, Room Temperature	Library Normalization		x
LNA1	Library Normalization Additives 1	x	Freezer -20°C	Library Normalization		x
LNB1	Library Normalization Beads 1	x	Refrigerator 2-8°C	Library Normalization		x

LNW1	Library Normalization Wash 1	x	Refrigerator 2-8°C	Library Normalization		x
LNS1	Library Normalization Storage Buffer 1	x	Room temperature	Library Normalization		x
EB-buffer (Qiagen)	10 mM Tris-Cl, pH 8.5		Room temperature	Dilution of the library		x
EDTA, 0.1 mM+Tris-HCl, 10 mM (Low-TE-Buffer)	0.1 mM+Tris-HCl, 10 mM		Room temperature	Dilution of DNA	x	
Nuclease-free water	HyClone™ HyPure, Molecular Biology Grade		Refrigerator 2-8°C	EtOH dilution		x

Miseq Sequencing

Reagent	**Spesific name**	**Included in Illumina Miseq Reagent kit**	**Temperature**	**Work phase**	**Pre-PCR**	**Post-PCR**
MiSeq Reagent Kit v2 (300-cycles)	MiSeq® v2 RGT Kit 300 cyc PE-Bx 1 of 2	x	Freezer -20°C	Sequencing		x
	MiSeq® v2 Reagent Kit Box 2 of2	x	Refrigerator 2-8°C	Sequencing		x
Tween 20			use dilution 0,5%, Room temperature	Washing Miseq		x
Sodium hypochlorite	5% NaOCl		Refrigerator 2-8°C	Miseq Template Line Wash		x
PhiX, Illumina			Freezer -20°C	Sequencing control		x

Option: Normalization for library

Reagent	**Temperature**	**Work phase**	**Pre-PCR**	**Post-PCR**
Qubit dsDNA HS-Kit	Room temperature	Measurement of library DNA concentration		x
Agilent High Sensitivity DNA Kit	Room temperature, Refrigerator 2-8°C	Measurement of library size		x

Equipment

Equipment: DNA extraction (depends on the method!)	Spesifications	Number	pre-PCR	post-PCR	Infective lab
Biosafety Cabinet class II		1			x
Centrifuge	for 1,5 ml tubes	1		x	x
Measurement of DNA concentration (eg. Qubit)		1			x
Thermal mixer for Magattrack method					
Equipment and plastics: Library preparation	Spesifications	Number	pre-PCR	post-PCR	Infective lab
Miseq		1		x	
Thermal cycler	for 96-well plates	2	x	x	
Vortex		2	x	x	
plate centrifuge/spinner	spinner, for 96-well plates	2	x	x	
tube centrifuge	spinner, for 1,5 ml tubes	2	x	x	
plate shaker	for 96-well plates	1		x	
pipets	different sizes: 1-10ul, 10-100ul, 20-200ul, 100-1000ul		x	x	
filter tips for pipets			x	x	
1,5 ml eppendorf-tubes			x	x	x
96- or 24-well plates	Suitable for PCR-instrument		x	x	
50 ml Falcon tubes				x	
Nitrile gloves			x	x	
Magnetic stand	for 96-well plates	1		x	
100 ml measuring glass				x	
96-Well Plate Cover Foil/Adhesive PCR Plate Foils			x	x	
Heating block +100C	for 1,5 ml tubes	1		x	
Qubit spesific tubes	0,5 ml		x		
fume chamber		1		x	
PCR cabinet or equivalent		1	x		
Freezer -20°C		1	x	x	
Refrigerator 2-8°C		1	x	x	
Measuring pipet and pipet boy	10ml, 25 ml	1		x	

Option: for size and consentration based normalization	Spesifications	Number	pre-PCR	post-PCR	Infective lab
BioAnalyzer		1		x	
Qubit		1		x	
Centrifuge	for 1,5 ml tubes	1		x	

ANNEX C. MiSeq device maintenance and power cycling

POWER CYCLING

- You should complete it before starting the run
- Manage -> Shut down -> When the screen is dark, power off the back of the device for more than 60 seconds and then turn it on again.

WASHING

GENERAL

0.5% Tween 20 as washing liquid in all washes.

- First prepare 50 ml of 10% Tween 20 (5 ml of Tween 20 + 45 ml of H2O). The solution is enough for two washes.
- Make 500 ml of this washing solution (25 ml 10% Tween 20 + 475 ml H2O) in a washing bottle and mix by turning.
- Pipette about 6 ml of washing solution from the bottle into each well of the washing cartridge, so that at least 350 ml of solution should remain in the washing bottle (black line on the side of the bottle).
- Place the washing cartridge and washing bottle in the device and, if necessary, empty the waste bottle properly.

After washing, leave the used bottles and cartridge in the machine and record the washing you have done in the usage diary.

POST-RUN WASH

- Carry out washing before each run, which ensures the functionality of the instrument's fluid flow.
- Duration of washing approx. 20 min.

TEMPLATE LINE WASH (Post-run wash with Sodium Hypochlorite)

- Wash after every run, no later than the day after the run.
- Washing can also be done before starting a run.
- Duration of washing approx. 20 min.
- First prepare 0.5% Tween 20 washing liquid in the normal way.

- Prepare fresh NaOCl (in the refrigerator in a small brown bottle):

  add 36 µl 5% NaOCl + 864 µl H2O (1:25 solution).Add 50 µl 1:25 NaOCl solution + 950 µl H2O to a separate Miseq tube.

- Fill the other wells of the washing cartridge normally, put the tube filled with NaOCl in hole number 17.
- Select Post-Run Wash as usual, but put a tick in Perform optional template line Wash.

MAINTENANCE WASH

- Wash approximately every 30 days (schedule attached to the usage log).
- Duration of washing approx. 60 min (washing stages 3*20 min). This requires 3*500 ml of washing solution. Always make fresh washing solutions at each step.
- During the maintenance wash, clear the computer's back up files from the folders D:\Illumina\MiSeq analysis and D:\Illumina\MiSeq Output so that (at least) the files of the current month remain in the folder. The file folder size of one run is about 15 GB. Also empty the computer's trashbin.

STANDBY WASH

- If the device will not be used for ≥ 7 days, a standby wash will be performed.
- Duration of washing approx. 120 min (2*60 min). This requires 2*500 ml washing solution.

  When the device is used after the standby phase, a maintenance wash must be performed before a run.