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# STANDARD OPERATING PROCEDURES (SOPs) FOR SAMPLING OF MICROBIOME IN DIFFERENT ECOSYSTEMS

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**Keywords:**

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# 1. INTRODUCTION

In 2022, the project SUS-MIRRI.IT "Strengthening the MIRRI Italian Research Infrastructure for Sustainable Bioscience and Bioeconomy" was launched as part of the actions oriented towards Italy's membership of MIRRI-ERIC, the pan-European distributed Research Infrastructure for the preservation, systematic investigation, provision and valorisation of microbial resources and biodiversity. The project SUS-MIRRI.IT, funded by Italy's National Recovery and Resilience Plan – PNRR, is granted by the European Commission's "NextGenerationEU" program with a total budget of about 17M €. The main objective of SUS-MIRRI.IT is to implement the network of microbial biobanks distributed throughout Italy to increase the benefit(s) that society can derive from the knowledge and valorisation of microbial biodiversity, which is a critical asset to face several of the greatest social, economic, and environmental challenges characterizing our time.
The project intends to:

- implement the network of microbial Culture Collection (CCs) by setting up high-standard operative procedures and quality management;
- equipe microbial CCs with cutting-edge laboratory facilities;
- provide more in-depth characterization of microbiomes and individual organisms stored in the CCs as well as identify better long-term preservation methods;
- create the Italian integrated catalogue of microorganisms and their associated metadata;
- set up a single-entry point virtual platform to promote the microbial resources, services, technologies, expertise, training courses, transfer of knowledge activities provided by the Italian CCs;
- strengthen both connections with stakeholders and synergies with other Research Infrastructures at regional, national and international levels;
- develop services and training courses for Academia and Bioindustry.

Microbiomes are defined as a characteristic microbial community (microbiota) occupying a certain habitat (the so-called theatre of activity) and exhibiting distinct and emergent physicochemical properties [1]. Recently, several studies showed that microbiomes represent innovative and frontier tools to address current and future problems in the areas of agriculture, environment, food production, and animal and human health [1, 2, 3, 4, 5].
However, despite this amazing potential of microbiomes, there is a huge gap to be filled in terms of how microbiomes are sampled, characterized, safely stored, and then cultured for further applications. In addition, the lack of standardization in the processes of sampling, extraction of genetic material, execution of -omics technologies, and bioinformatic analyses has resulted in the limited possibility of data comparison [6, 7, 8].
Therefore, in the absence of international standards, the purpose of Work Package 4 of the SUS-MIRRI.IT project is to contribute to the definition and validation of microbiome quality standards to be applied globally in different scientific areas. Specifically, Standard Operating Procedures (SOPs) for sampling different ecosystems (which are: fermented solid and liquid foods, human faeces, animal (canine) faeces, soil/plant environment, insects, water, and sediments) and the subsequent DNA extraction steps have been developed in this document. To properly construct the promised SOPs, the different research units participating in the WP4 gathered relevant information from their own experience/published articles or the available literature to create a solid body of knowledge, based on which to prepare SOPs.
Here, we are thus providing different protocols with a homogeneous and consistent structure organization for the isolation and subsequent analysis of microbiome materials from different matrices. A total of 15 SOPs were created, specifically, 8 for sampling and 7 for DNA extraction.

This new updated version includes the protocols for the analysis of animal (canine) faecal microbiome.

# 2. SOP FOR HUMAN MICROBIOME FAECAL SAMPLING

**Prepared by**: Cristina Costa, Antonio Curtoni, Narcisa Mandras, and Janira Roana on behalf of the SUS-MIRRI.IT consortium.

## 1. **Introduction**

The Microbial Resource Research Infrastructure (MIRRI) is the pan-European distributed Research Infrastructure for the preservation, systematic investigation, provision and valorisation of microbial resources and biodiversity. The project SUS-MIRRI.IT, funded by Italy's National Recovery and Resilience Plan – PNRR, is granted by the European Commission's "NextGenerationEU" program. The project is coordinated by the University of Torino and involves 14 other institutions: CNR, ENEA, National Institute of Oceanography and Applied Geophysics (OGS), the Universities of Cagliari, Genoa, Milano Bicocca, Modena and Reggio Emilia, Naples Federico II, Palermo, Perugia, Parma, Sassari, Verona, and the University of Basilicata. Within the project, specific goals include: i) defining the best strategies to sample and store human microbiome faecal samples for microbiome investigation and ii) optimizing DNA extraction and sequencing techniques to study microbial communities in order to increase the benefit that society can derive from the knowledge and valorisation of microbial biodiversity. This document presents the Standard Operational Procedure (SOP) for human microbiome faecal sample collection, storage, and shipment within the SUS-MIRRI.IT project.

## 2. **Objective**

This SOP is of interest for good practices of DNA extraction from faecal samples in order to characterize the faecal microbiota. The objective of this SOP is to describe the laboratory workflow for preparing human faecal suspension for infusion and optimize data comparisons in the field of the human microbiome by standardizing the protocol for DNA extraction of faecal samples.

## 3. **Principle**

The principle of this SOP (SOP for validated sample collection and storage) is to provide guidance for sample collection and storage based on literature survey in conjunction with a survey of the SUS-MIRRI.IT project partners [9,10]. For each step, tips and recommendations are provided.

## 4. **Persons entitled to use the procedure**

This SOP applies to any person involved in collecting, transportation and processing of faecal samples.

## 5. **Storage sample**

Fresh faecal samples are transferred to sterile specimen containers immediately following defecation. Stool samples should be frozen as soon as possible or at least within 6 hours after sample collection.

## 6. **Materials**

- 50 mL sterile DNA-free plastic containers

- 1 mL microcentrifuge tube
- Pipettes and pipette tips
- BD Columbia Agar with 5% Horse Blood, BD Columbia CNA Agar with 5% Sheep Blood, BD CDC Anaerobe Agar with 5% Sheep Blood
- 0.9% NaCl and glycerol 10%
- Bags with anaerobe pouch
- cryovials

## 7. **Equipment**

- Ultra-freezer (-80°C)
- Laminar flow hood
- Stomacher laboratory homogenizer

## 8. **Step-by-step procedure**

### *8.1. Sampling*

Donors must not have experienced any gastrointestinal infections six months prior to donation, must not have used antibiotics six months prior to donation, must not have suffered from, or be recovering from chronic intestinal diseases such as Crohn's disease, ulcerative colitis, coeliac disease, irritable bowel syndrome, stomach ulcers or colorectal cancer. In addition, donors were required to be free of autoimmune diseases or allergies such as multiple sclerosis, asthma, or psoriasis.

### *8.2. Sample manipulation*

All processing takes place in a laminar flow hood using sterile disposable products [9].
Thirty grams of faeces are recommended from each stool sample. Faeces are diluted in 150 mL of saline solution supplemented with 10% of glycerol (glycerol with NaCl 10% / 0.9% - Monico SPA, Venezia, Italy; 500 mL bottles) and homogenized with stomacher (or vortex) and filtered with disposable sterile cotton gauzes. Usually, 2-6 tubes of 50 mL are filled up with 20 g of faeces and 20 mL of 0.9% NaCl and glycerol 10% solution.
An evaluation of microbial load is made by 1:10 serial dilutions starting from 100 µL of filtered suspension. Each dilution is plated on three different agar plates using a 10-µL calibrated-loop: BD Columbia Agar With 5% Horse Blood (CHAB, Becton Dickinson GmbH, Heidelberg/Germany), BD Columbia CNA Agar with 5% Sheep Blood (CNA, Becton Dickinson GmbH, Heidelberg/Germany) and BD CDC Anaerobe Agar + 5% Sheep Blood, (CDC, Becton Dickinson GmbH, Heidelberg/Germany) to isolate aerobe/anaerobe facultative bacteria, aerobe/anaerobe facultative bacteria nalidixic-acid and colistin resistant and anaerobe obligate bacteria, respectively.
CHAB and CNA are incubated at 37°C for 24-48 h in aerobic conditions while CDC in anaerobic conditions in bags containing an anaerobic pouch. Microbial counts are done at 24-48 hours. The values of colony-forming unit per mL (CFU per mL) is obtained by the following formula:

CFU per mL = (Number of colonies / 0,01 mL) x dilution factor

### *8.3. Defrosting evaluation*

An aliquot of sample is frozen at -80°C in a 1 mL microcentrifuge tube to evaluate the effect of thawing. After defrosting at 37°C or at room temperature (RT), the sample is plated on the agar media

described above: microbial load is evaluated after 48 hours of incubation and compared with microbial load of the fresh culture counted before freezing the sample [9].
The remaining sample is stocked in 100 mL cryovials or in 50 mL tubes, labelled and frozen at -80°C.

## 9. **Metadata requirement**

Metadata is also obtained from the donors including age-range, nationality, ethnicity and diet consumed (i.e. vegan, vegetarian or omnivore). To maintain donor anonymity, no identifying information such as date of birth, name or address was requested.

## 10. **Culture enrichment for subsequent NGS**

Possibility of recovering and maintaining even slow/difficult-growing microorganisms with particular nutritional needs. Possibly useful before analysis of the microbiota using NGS to also identify these microorganisms and possibly OTU (Operational Taxonomic Units) [11,12].

Broad-range medium YCFA (Yeast extract, Casein-hydrolysate, Fatty Acids)

# 3. SOP FOR DNA EXTRACTION FROM HUMAN FAECES

**Prepared by**: Cristina Costa, Antonio Curtoni, Narcisa Mandras, and Janira Roana on behalf of the SUS-MIRRI.IT consortium.

## 1. **Introduction**

The Microbial Resource Research Infrastructure (MIRRI) is the pan-European distributed Research Infrastructure for the preservation, systematic investigation, provision and valorisation of microbial resources and biodiversity. The project SUS-MIRRI.IT, funded by Italy's National Recovery and Resilience Plan – PNRR, is granted by the European Commission's "NextGenerationEU" program. The project is coordinated by the University of Torino and involves 14 other institutions: CNR, ENEA, National Institute of Oceanography and Applied Geophysics (OGS), the Universities of Cagliari, Genoa, Milano Bicocca, Modena and Reggio Emilia, Naples Federico II, Palermo, Perugia, Parma, Sassari, Verona, and the University of Basilicata. Within the project, specific goals include: i) defining the best strategies to sample and store human faecal samples for microbiome investigation and ii) optimizing DNA extraction and sequencing techniques to study microbial communities in order to increase the benefit that society can derive from the knowledge and valorisation of microbial biodiversity. This document presents the Standard Operational Procedure (SOP) for DNA extraction from human faecal samples within the SUS-MIRRI.IT project.

## 2. **Principle**

This SOP aims to optimize data comparisons in the human microbiome by standardizing protocols for DNA extraction of faecal samples. This SOP will be a good practice for DNA extraction from faecal samples to characterize the faecal microbiota.

## 3. **Objective**

The objective of this document is to standardize the faecal samples DNA extraction by giving a step-by-step description of the protocol.

## 4. **Persons entitled to use the protocol**

This SOP applies to any person involved in DNA extraction. This person can be a trainee, fellow, technician or engineer in charge of DNA extraction.

## 5. **DNA Extraction** (Qiagen QIAamp DNA stool kit)

*5.1. Equipment:*

- Microcentrifuge
- 1.5-2 mL DNAse-free microcentrifuge tubes
- Pipettes and pipette DNAse-free tips
- Water bath
- Autoclave

*5.2. Step-by-step procedure*

1. Homogenize the 150 to 200 mg frozen faeces with 1 mL ASL lysis buffer of the kit by vortexing for 2 min in a 2 mL tube containing 0.3 g of sterile zirconia beads Ø 0.1 mm zirconia (BioSpec, Cat. No. 11079101z). (If buffer shows precipitate, heat at 70°C before use);
2. Incubate for 15 min at 95°C;
3. Cells are mechanically lysed by running the Fastprep™ Instrument for 8 min (series of beating 1 min and resting 5 min are preferable);
4. Samples are allowed to cool down on ice for 2 min;
5. Samples are centrifuged at 16000 x g, 4°C, for 5 min;
6. Supernatant is transferred to a new 2mL tube;
7. The pellet is mixed with a 300 µL ASL lysis buffer of the kit, and steps 2-5 are repeated;
8. Supernatants are pooled in the new 2mL tube;
9. Add 260 µL of 10 M ammonium acetate to each lysate tube, mix well, and incubate on ice for 5 min;
10. Centrifuge at 16,000 g, 4°C, for 10 min;
11. Transfer the supernatant to two 1.5mL microcentrifuge tubes, add one volume of isopropanol, mix well, and incubate on ice for 30 min;
12. Centrifuge at 16,000 g, 4°C, 15 min, remove the supernatant using aspiration, wash the nucleic acid pellet with 70% EtOH (0.5 mL) and dry the pellet under vacuum for 3min;
13. Dissolve the nucleic acid pellet in 100 µL of TE (Tris-EDTA) buffer and pool the two aliquots.
14. Add 2 µL of DNase-free RNase (10 mg/mL) and incubate at 37°C, 15 min;
15. Add 15 µL proteinase K and 200µL AL buffer to the supernatant, vortex for 15 sec and incubate at 70°C for 10 min;
16. Add 200 µL of EtOH (96-100%) to the lysate, and mix by vortexing;
17. Transfer to a QIAamp spin column and centrifuge at 16,000 g for 1 min, at RT;
18. Discard flow through, add 500 µL buffer AW1 (Qiagen) and centrifuge at 16,000 g for 1 min, at RT;
19. Discard flow through, add 500 µL buffer AW2 (Qiagen) and centrifuge at 16,000 g for 1 min, at RT;
20. Dry the column by centrifugation at RT for 1 min;
21. Add 200 µL Buffer AE (Qiagen), incubate for 1 min at RT;
22. Centrifuge for 1 min at 16,000 g to elute DNA;
23. Quality control: 1% agarose gel;
24. Sample concentration: use Nanodrop or Qubit.

# 4. SOP FOR CANINE MICROBIOME FAECAL SAMPLING

**Prepared by**: Barbara Colitti, Patrizia Nebbia, Andrea Peano, Umra Rasool, Luigi Bertolotti (UNITO) on behalf of the SUS-MIRRI.IT consortium.

## 1. **Introduction**

The Microbial Resource Research Infrastructure (MIRRI) is the pan-European distributed Research Infrastructure for the preservation, systematic investigation, provision and valorisation of microbial resources and biodiversity. The project SUS-MIRRI.IT, funded by Italy's National Recovery and Resilience Plan – PNRR, is granted by the European Commission's Next Generation EU program. The project is coordinated by the University of Torino and involves 14 other institutions: CNR, ENEA, National Institute of Oceanography and Applied Geophysics (OGS), the Universities of Cagliari, Genoa, Milano Bicocca, Modena and Reggio Emilia, Naples Federico II, Palermo, Perugia, Parma, Sassari, Verona and the University of Basilicata. Within the project specific goals include i) defining the best strategies to sample and store dog faecal samples (DFSs) for microbiome investigation and ii) optimizing DNA extraction and sequencing techniques to study microbial communities to reach higher goals such as the implementation of the network of microbial biobanks distributed throughout Italy and to increase the benefit that society can derive from the knowledge and valorisation of microbial biodiversity. This document presents the Standard Operational Procedure (SOP) for canine faecal sample collection, storage, and shipment in the SUS-MIRRI.IT project.

## 2. **Objective**

This SOP aims to establish standardized protocols for dog faeces sampling and collection in order to investigate the canine faecal microbiota. Researchers may assure reliability and consistency in their microbiome analysis by following this SOP, which enables meaningful comparisons between studies.

## 3. **Principle**

The principle of this SOP (SOP for validated sample collection and storage) is to provide guidance for sample collection and storage based on accepted procedures for the collection and processing of fecal samples that have been specially modified for canine microbiome research. In order to effectively characterize the fecal microbiome of dogs, this SOP is based on the principles of assuring sample integrity, limiting contamination, and improving sampling and DNA extraction techniques.

## 4. **Persons entitled to use the procedure**

This SOP applies to any staff member involved in collecting, transporting and processing dog feces samples (DFSs) for microbiome study.

## 5. **Storage sample**

Fresh DFSs must be collected within 15 minutes of defecation. If they are processed immediately after collection, they can be transferred to the laboratory at room temperature. If processed between

4 and 24 hours, they can be kept refrigerated (4°C). Long-term storage of samples should be at -80°C or transported in dry ice and immediately stored at -80°C. In the absence of a -80°C freezer, samples can be stored at -20°C. The literature survey indicated that no storage medium was previously used or is necessary for DFSs storage [13, 14].

## 6. **Materials**

The following materials are required for this SOP:

- Sterile Gloves;
- Sterile DNA-free containers (2 mL Eppendorf tubes only if aliquots are needed);
- Labels;
- Ice Packs;
- Cooler Box;
- Sampling Spatula or Scoop;
- Plastic Bags or Specimen Collection Tubes.



Examples of sterile containers

## 7. **Equipment**

- Refrigerator (+4°C);
- Ultra-freezer (-80°C) or -20°C freezer if an ultra-freezer is not available;
- Laminar flow hood

## 8. **Step by step procedure**

### 8.1. *Sampling*

Collect freshly excreted dog faeces (at least 300 grams per sample).

To reduce contamination, take samples directly from the rectum or immediately after defecation.

To collect and store faecal samples, use sterile gloves and containers.

In order to obtain microbiological diversity, gather numerous samples from various parts of the faeces scoop.

Write specific identification numbers on each sample container, such as the time, date, and dog's ID number.

Aliquot collected faeces (around 300 mg) in sterile tubes or 2 mL Eppendorf tubes using a laminar flow hood if different smaller aliquots are needed.

8.2. *Sample manipulation*

Once taken, all samples must be stored at room temperature and immediately transported to the laboratory.

If processed between 4 and 24 hours, they can be kept refrigerated (4°C) and transported in a cooler box to the laboratory.

Proceed immediately to DNA extraction or store the DFSs at -80°C for more extended storage.

8.3. *Donors requirements for canine microbiome composition studies*

Dogs must not have experienced any gastrointestinal infections six months before donation, must not have used antibiotics six months before donation, and must not have suffered from chronic gastrointestinal diseases (such as IBD, malabsorption, colitis, Parvovirus or Giardia infection, pancreatitis).

## 9. **Metadata requirement**

Metadata to be obtained from the donors include age, gender, breed, body weight, and type of diet consumed (i.e. homemade, barf/raw, commercial [dry or wet], mixed).

## 10. **Culture enrichment for subsequent NGS**

Possibility of recovering and maintaining even slow/difficult-growing microorganisms with particular nutritional needs. Possibly useful before analysis of the microbiota using NGS to also identify these microorganisms and possibly OTU (operational taxonomic units).

Use Broad-range medium YCFA (Yeast extract, Casein-hydrolysate, Fatty Acids) for culture enrichment.

# 5. SOP FOR DNA EXTRACTION FROM CANINE FECAL SAMPLES

**Prepared by**: Barbara Colitti, Patrizia Nebbia, Andrea Peano, Umra Rasool, Luigi Bertolotti (UNITO) on behalf of the SUS-MIRRI.it consortium.

## 1. **Introduction**

The Microbial Resource Research Infrastructure (MIRRI) is the pan-European distributed Research Infrastructure for the preservation, systematic investigation, provision and valorisation of microbial resources and biodiversity. The project SUS-MIRRI.IT, funded by Italy's National Recovery and Resilience Plan – PNRR, is granted by the European Commission's Next Generation EU program. The project is coordinated by the University of Torino and involves 14 other institutions: CNR, ENEA, National Institute of Oceanography and Applied Geophysics (OGS), the Universities of Cagliari, Genoa, Milano Bicocca, Modena and Reggio Emilia, Naples Federico II, Palermo, Perugia, Parma, Sassari, Verona and the University of Basilicata. Within the project specific goals include i) defining the best strategies to sample and store dog faecal samples (DFSs) for microbiome investigation and ii) optimizing DNA extraction and sequencing techniques to study microbial communities to reach higher goals such as the implementation of the network of microbial biobanks distributed throughout Italy and to increase the benefit that society can derive from the knowledge and valorisation of microbial biodiversity. This document presents the Standard Operational Procedure (SOP) for canine faecal sample collection, storage, and shipment in the SUS-MIRRI.IT project.

## 2. **Principle**

This SOP aims to standardize the dog faecal samples (DFSs) DNA extraction by giving a step-by-step description of the protocol.

## 3. **Objective**

This SOP aims to optimize data comparisons in the canine microbiome by standardizing protocols for DNA extraction of faecal samples [13, 14, 15]. This SOP will be a good practice for DNA extraction from faecal samples to characterize the faecal microbiota.

## 4. **Persons entitled to use the protocol**

This SOP applies to any person involved in DNA extraction. This person can be a trainee, fellow, technician or any researcher in charge of DNA extraction.

## 5. **DNA Extraction using Qiagen QIAamp PowerFecal Pro DNA kit**

The method allows to isolate microbial DNA from stool and gut samples. The kit uses QIAGEN's second-generation Inhibitor Removal Technology® (IRT), and is intended for use with samples containing inhibitory substances commonly found in stool, such as polysaccharides, haem compounds and bile salts. Samples are added to a bead-beating tube for rapid and thorough homogenization. Then, a cell lysis occurs by mechanical and chemical methods (Inhibitor Removal Technology to avoid any loss of variability due to inhibitory substances). Total genomic DNA is

captured on a silica membrane in a spin-column format. DNA is then washed and eluted from the membrane and ready for NGS downstream application. In this SOP, the Qiagen PowerFecal Pro kit is used. Alternative validated methods can be evaluated based on relevant scientific literature [16, 17].

5.1. *Material and Equipment*

- Gloves
- DNeasy PowerFecal Pro DNA Kit (QIAGEN)
- Microcentrifuge
- Vortex Adapter for 24 (1.5 or 2 mL) tubes (cat. no. 13,000-V1-24) or TissueLyser II (cat. no. 85300) or similar
- 1.5-2 mL DNAse-free microcentrifuge tubes
- Pipettor (20-1000 µL) and pipette DNAse-free tips

5.2. *Consideration before starting*

- Ensure that the PowerBead Pro Tubes rotate freely in the centrifuge without rubbing.
- Check Solution CD3 for precipitate and heat at 60°C until precipitate dissolves.
- Perform all centrifugation steps at room temperature (15–25°C).

5.3. *Step-by-step procedure (Figure 1)*

0. Thaw faecal samples as collected in SOP_DFS1a on ice or at 4°C.

1. Spin the PowerBead Pro Tube briefly to ensure the beads have settled at the bottom. Add up to 250 mg of stool and 800 µl of Solution CD1. Vortex briefly to mix.

2. Secure the PowerBead Pro Tube horizontally on a Vortex Adapter for 1.5– 2 mL tubes (cat. no. 13,000-V1-24) (or TissueLyser if the microorganism of interest requires stronger homogenization than provided by a vortex). Vortex at maximum speed for 10 min.

Note: If using the Vortex Adapter for more than 12 preps simultaneously, increase the vortexing time by 5–10 min.

3. Centrifuge the PowerBead Pro Tube at 15,000 x g for 1 min.

4. Transfer the supernatant to a clean 2 mL Microcentrifuge Tube.

Note: Expect 500–600 µL. The supernatant may still contain some stool particles.

5. Add 200 µL of Solution CD2 and vortex for 5 s.

6. Centrifuge at 15,000 x g for 1 min. Avoiding the pellet, transfer up to 700 µL of supernatant to a clean 2 ml Microcentrifuge Tube.

Note: Expect 500–600 µL.

7. Add 600 µL of Solution CD3 and vortex for 5 s.

8. Load 650 µL of the lysate onto an MB Spin Column and centrifuge at 15,000 x g for 1 min.

9. Discard the flow-through and repeat step 8 to ensure all the lysate has passed through the MB Spin Column.

10. Carefully place the MB Spin Column into a clean 2 ml Collection Tube.

Note: Avoid splashing any flow-through onto the MB Spin Column.

11. Add 500 µL of Solution EA to the MB Spin Column. Centrifuge at 15,000 x g for 1 min. 12. Discard the flow-through and place the MB Spin Column back into the same 2 ml Collection Tube.

13. Add 500 µL of Solution C5 to the MB Spin Column. Centrifuge at 15,000 x g for 1 min.

14. Discard the flow-through and place the MB Spin Column into a new 2 ml Collection Tube.

15.Centrifuge at up to 16,000 x g for 2 min. Carefully place the MB Spin Column into a new 1.5 mL Elution Tube.

16. Add 50–100 µL of Solution C6 to the centre of the white filter membrane.

17.Centrifuge at 15,000 x g for 1 min. Discard the MB Spin Column. The DNA is now ready for downstream applications.

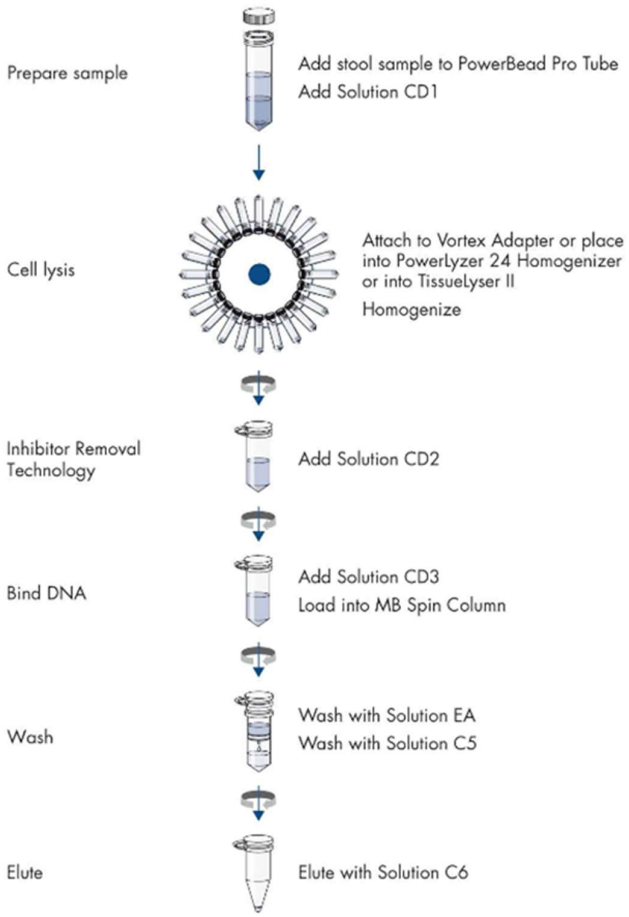


Figure 1. DNA extraction scheme

5.4. *DNA storage*

Store the DNA frozen at -80°C to prevent degradation, as Solution C6 does not contain EDTA.

5.5. *Qualitative and quantitative analysis*

Check DNA for quality using Nanodrop 2000 (Thermofisher).

Check DNA quantity using a fluorometric method such as Qubit 3.0 fluorometer (Invitrogen, Life Technology) and Qubit dsDNA HS Assay Kit.

# 6. SOP FOR INSECT GUT SAMPLING

**Prepared by**: Elena Gonella, Aya Elsayed, and Irene Stefanini on behalf of the SUS-MIRRI.IT consortium.

## 1. **Introduction**

The Microbial Resource Research Infrastructure (MIRRI) is the pan-European distributed Research Infrastructure for the preservation, systematic investigation, provision and valorisation of microbial resources and biodiversity. The project SUS-MIRRI.IT, funded by Italy's National Recovery and Resilience Plan – PNRR, is granted by the European Commission's "NextGenerationEU" program. The project is coordinated by the University of Torino and involves 14 other institutions: CNR, ENEA, National Institute of Oceanography and Applied Geophysics (OGS), the Universities of Cagliari, Genoa, Milano Bicocca, Modena and Reggio Emilia, Naples Federico II, Palermo, Perugia, Parma, Sassari, Verona, and the University of Basilicata. Within the project, specific goals include: i) defining the best strategies to sample and store insect gut samples for microbiome investigation and ii) optimizing DNA extraction and sequencing techniques to study microbial communities in order to increase the benefit that society can derive from the knowledge and valorisation of microbial biodiversity. This document presents the Standard Operational Procedure (SOP) for insect gut sample collection, storage, and shipment within the SUS-MIRRI.IT project.

## 2. **Objective**

This SOP aims to standardize insect gut sampling for microbiome analysis (insect group: fruit flies; model species *Bactrocera oleae*), by defining the rearing strategy and the procedures for gut dissection and collection.

## 3. **Principle**

The principle of this SOP (SOP for validated sample collection and storage) is to provide guidance for insect sample collection and storage based on literature surveys. In drafting this SOP, rearing and gut dissection of dipteran insects are considered to obtain the most representative sample collection method in terms of microbial community composition. For each step, tips and recommendations are provided.

## 4. **Persons entitled to use the procedure**

Procedures listed in this technical report can be conducted by any person involved in insect rearing studies and guts microbiomes.

## 5. **Preliminary steps**

### *5.1. Sampling material*

- Plastic net
- Paper Towels
- Cotton
- Plexi-glass
- Parafilm

- Forceps
- Bleach
- Absolute EtOH

*5.2. Fly cages design*

For the flies to oviposit, laboratory cages measuring 40 × 30 × 30 cm are fitted with wax cones. Three sides of the cages are netted to facilitate airflow, while the top and bottom are made of plexiglass. The top portion has three 8 cm-diameter holes for placing wax cones in the cages. For oviposition, three funnel-shaped wax cones (8 cm in diameter by 30 cm in height) are placed within the cages. A piece of sponge is adhered to the plexiglass lid of each cone to retain humidity.

*5.3. Larval diet components*

For 1 kilogramme of larval diet, the following components are used: tap water (550 mL), extra virgin olive oil (20 mL), Tween® 80 emulsifier (7.5 mL), potassium sorbate (0.5 g), Nipagin® (2 g), sugar (20 g), brewer's yeast (75 g), soy hydrolysate (30 g), hydrochloric acid 2N (30 mL), and cellulose powder (275 g) as the bulking agent [18, 20].

*5.4. Adults rearing*

Adults are kept in colony wooden cages (30 x 30 x 30 cm) under laboratory conditions (24 ± 1.5°C, RH 40 ± 5%, L:D 14:10), fed a diet of sugar, yeast hydrolysate, and water (4:1:5) (no antibiotic added). Egg yolk powder is administered ad libitum as an additional source of protein. A moistened cotton stick is protruded from a small water container to deliver water [19,21].

6. **Step-by-step procedures**

1. *Bactrocera oleae* adults are obtained from the larvae in infested olive fruits;
2. Adult flies are provided with the diet and oviposition wax cones to obtain new generation;
3. Larvae are reared on the larval diet;
4. Pupae are collected every 3 days and kept in another cage until they emerge;
5. Reared larvae and adult flies are sampled for gut dissection. Surface sterilization is applied using 70% alcohol by wiping the insect cuticle before processing dissecting the insect gut;
6. Guts are dissected by removing the entire gut, from crop to anus, with sterile forceps under a stereoscopic microscope [22];
7. Guts are used immediately for DNA extraction or stored at -20°C.

# 7. SOP FOR INSECT GUT DNA EXTRACTION

**Prepared by**: Elena Gonella, Aya Elsayed, and Irene Stefanini on behalf of the SUS-MIRRI.IT consortium.

## 1. **Introduction**

The Microbial Resource Research Infrastructure (MIRRI) is the pan-European distributed Research Infrastructure for the preservation, systematic investigation, provision and valorisation of microbial resources and biodiversity. The project SUS-MIRRI.IT, funded by Italy's National Recovery and Resilience Plan – PNRR, is granted by the European Commission's "NextGenerationEU" program. The project is coordinated by the University of Torino and involves 14 other institutions: CNR, ENEA, National Institute of Oceanography and Applied Geophysics (OGS), the Universities of Cagliari, Genoa, Milano Bicocca, Modena and Reggio Emilia, Naples Federico II, Palermo, Perugia, Parma, Sassari, Verona, and the University of Basilicata. Within the project, specific goals include: i) defining the best strategies to sample and store insect gut samples for microbiome investigation and ii) optimizing DNA extraction and sequencing techniques to study microbial communities in order to increase the benefit that society can derive from the knowledge and valorisation of microbial biodiversity. This document presents the Standard Operational Procedure (SOP) for DNA extraction insect gut samples within the SUS-MIRRI.IT project.

## 2. **Objective**

This SOP aims to standardize DNA extraction procedures from insect gut samples to optimize data comparisons in the insect microbiome field.

## 3. **Principle**

This SOP provides a description of the necessary equipment and a step-by-step description of the procedures.

## 4. **Persons entitled to use the protocol**

This SOP applies to any person involved in SFFs and LFFs DNA extraction. This person can be a trainee, fellow, technician or engineer in charge of insect gut DNA extraction.

## 5. **Insect gut DNA extraction**

### *5.1. Materials and Equipment*

- Gloves
- Microcentrifuge (10,000 x g)
- Pipettors (50 µL - 500 µL)
- 1.5 mL microcentrifuge tubes
- Vortex and adaptors
- 1X TE buffer
- Ethidium bromide Stain
- DNA ladder 1 Kb
- Transilluminator, UV

- Microvolume Spectrophotometer
- Fluorimeter

*5.2. Step-by-step Procedure*

*5.2.1 DNA Extraction*

1. Pestle insect guts with sterile pestles in 300 µL of TE buffer;
2. Without any delay, 200 µL of chloroform and 150 µL of 6 M NaCl should be added, and the tubes will then invert ten times;
3. Centrifuge for 5 minutes at a speed of 10,000 rpm;
4. Transfer the supernatant to a new 1.5 mL microcentrifuge tube;
5. Add one volume of absolute EtOH to the tube in a controlled manner, along the tube's inner wall;
6. Centrifuge the mixture at 10,000 rpm for 5 min afterwards, discard the supernatant;
7. Transfer via pipette the precipitate DNA to a fresh microcentrifuge tube and apply two rounds of washing with 70% EtOH through centrifugation at 5,000 rpm for a duration of 5 minutes;
8. The pellet is purified by an air-drying process for a duration of 10 minutes at RT;
9. Dissolve the pellet in 50 µL of TE buffer (pH 8) [23].

*5.2.2. Gel Electrophoresis*

**TRIS EDTA 10X BUFFER PREPARATION**

1. Prepare 800 mL of distilled water in a suitable container.
2. Add 15.759 g of Tris-Cl (0.1 M) to the solution.
3. Add 2.92 g of EDTA (0.01 M pH 8) to the solution.
4. Add double-distilled water until the volume is 1 L and mix with a magnetic anchor.

**GEL PREPARATION**

1. Dilute tris EDTA 10X solution to obtain 1X with double-distilled water.
2. Dissolve 1 g of agarose in 100 mL of TE buffer 1X and thoroughly mix with a magnetic anchor.
3. Heat the solution until boiling (approx. 5 mins).
4. Add 1 µL of ethidium bromide stain and mix the solution.
5. Rapidly pour the solution obtained into the housing, place the comb and let it cool.
6. Add 500 mL of TE 1X buffer to the electrophoresis tray.

**ELECTROPHORESIS**

1. Add 2 µL DNA ladder (1Kb) to any well selected to house the marker.
2. Add 2 µL of samples to the wells annotating their spatial position.
3. Run the gel for 30 mins under 60 V.
4. Examine results using a transilluminator.

## 6. **Qualitative and quantitative analysis**

*6.1. Nucleic Acid/Protein Ratio*

1. Turn on the spectrophotometer and select Nucleic Acid application.
2. -Select the type of sample to be measured from the drop-down list.

3. Pipette 1-2 µL of the appropriate blanking solution onto the bottom pedestal, lower the arm and click the Blank button. Wait for measurement and wipe clean.
4. Pipette 1-2 µL of samples and click Measure button. Wipe clean after every measurement.
5. Visualize results on software [24].

### *6.2. DNA Concentration*

The assessment of DNA concentrations of samples prior to genomic library construction can be conducted through the utilization of a Fluorometer. Samples possessing a deoxyribonucleic acid concentration that falls below 1 nanogram per litre shall be declined [25].

# 8. SOP FOR GEOTHERMAL SPRING SEDIMENTS SAMPLING

**Prepared by**: Antonino Pollio, Donato Giovannelli, Feliciana Oliva, Nunzia Nappi, and Marzia Licata on behalf of the SUS-MIRRI.IT consortium.

## 1. **Introduction**

The Microbial Resource Research Infrastructure (MIRRI) is the pan-European distributed Research Infrastructure for the preservation, systematic investigation, provision and valorisation of microbial resources and biodiversity. The project SUS-MIRRI.IT, funded by Italy's National Recovery and Resilience Plan – PNRR, is granted by the European Commission's "NextGenerationEU" program. The project is coordinated by the University of Torino and involves 14 other institutions: CNR, ENEA, National Institute of Oceanography and Applied Geophysics (OGS), the Universities of Cagliari, Genoa, Milano Bicocca, Modena and Reggio Emilia, Naples Federico II, Palermo, Perugia, Parma, Sassari, Verona, and the University of Basilicata. Within the project, specific goals include: i) defining the best strategies to sample and store geothermal spring sediments for microbiome investigation and ii) optimizing DNA extraction and sequencing techniques to study microbial communities in order to increase the benefit that society can derive from the knowledge and valorisation of microbial biodiversity. This document presents the Standard Operational Procedure (SOP) for geothermal spring sediments sample collection, storage, and shipment within the SUS-MIRRI.IT project.

## 2. **Objective**

This SOP aims to standardize procedures for geothermal spring sediments and background sampling.

## 3. **Principle**

The principle of this SOP is to provide guidance for sample collection and storage. Sediment is collected in the immediate vicinity of geothermal spring venting orifice. The background is taken near the source basin to evaluate the contribution of the soil community to the subsurface community (non-hydrothermally altered if feasible).

## 4. **Persons entitled to use the procedure**

This SOP applies to any person involved in collecting, transportation and processing of geothermal spring samples. This person can be any sampling personnel.

## 5. **Storage temperature**

Immediately after collection, sediment samples are stored on dry ice or frozen in liquid nitrogen. In the absence of dry ice storage at 4°C up to a maximum of 6 hours. Long-term storage of samples should be at -20°C. Samples for the growth and isolation of microorganisms are stored immediately and long-term at 4°C.

## 6. **Materials**

- 2 x 50 mL Falcon tubes

- 1 x 120 mL culture vial
- Spatula stainless steel
- Spoon stainless steel
- Gloves.

7. **Equipment**:

- Multiprobe
- Cooler
- Crimper
- Field thermometer

8. **Step-by-step procedure**

*8.1. Sampling*

Geographical coordinates are recorded for each sampling site. Subsequently, a combination of field observations is performed using a field thermometer for detecting the temperature and a multi-sensor probe that detects environmental parameters such as pH, temperature, conductivity, and redox potential.
After recording the parameters, sediment and background soil samples are collected. Sediment and background soil samples for DNA extraction should be collected using a sterile spatula in sterile centrifuge tubes. The top 0.5 cm of sediment or background soil should be collected. The tubes should be filled up to 40 mL, avoiding excess liquid if possible, and dead organic matter (twigs, leaves, moss, and biofilm). The sediments must be collected as close as possible to the spring, while the background soil must be collected nearby the spring. After collection, samples are stored at -20°C upon return to the laboratory, being careful to homogenize the sample in the tube before freezing.
For growth and isolation of microorganisms, sediments, fluids, and biofilm are collected from the spring, added to 120 mL vials, and stored at +4°C [26, 27].

*8.2. Sampling manipulation*

After collection, all samples are stored in a portable cooler filled with ice packs for transport to the laboratory. Whenever possible, it would be good practice to store samples in liquid nitrogen.

9. **Metadata requirement**

Sheets showing the most significant characteristics of the sampling site, synthetic and standardized description, used for data recording such as: expedition code, the country where the sample is located, full site name, date of sampling, sample types collected, latitude, longitude, altitude from sea level, temperature, pH, dissolved oxygen, conductivity, salinity, $H_2S$, and alkalinity.

# 9. SOP FOR GEOTHERMAL SPRING SEDIMENTS AND BIOFILM DNA EXTRACTION

**Prepared by**: Antonino Pollio, Donato Giovannelli, Feliciana Oliva, Nunzia Nappi, and Marzia Licata on behalf of the SUS-MIRRI.IT consortium.

## 1. **Introduction**

The Microbial Resource Research Infrastructure (MIRRI) is the pan-European distributed Research Infrastructure for the preservation, systematic investigation, provision and valorisation of microbial resources and biodiversity. The project SUS-MIRRI.IT, funded by Italy's National Recovery and Resilience Plan – PNRR, is granted by the European Commission's "NextGenerationEU" program. The project is coordinated by the University of Torino and involves 14 other institutions: CNR, ENEA, National Institute of Oceanography and Applied Geophysics (OGS), the Universities of Cagliari, Genoa, Milano Bicocca, Modena and Reggio Emilia, Naples Federico II, Palermo, Perugia, Parma, Sassari, Verona, and the University of Basilicata. Within the project, specific goals include: i) defining the best strategies to sample and store geothermal spring sediments and biofilm samples for microbiome investigation and ii) optimizing DNA extraction and sequencing techniques to study microbial communities in order to increase the benefit that society can derive from the knowledge and valorisation of microbial biodiversity. This document presents the Standard Operational Procedure (SOP) for DNA extraction from geothermal spring sediments and biofilm samples within the SUS-MIRRI.IT project.

## 2. **Objective**

This SOP aims to standardize the protocol for geothermal spring sediments and biofilm DNA extraction.

## 3. **Principle**

This SOP aims to standardize the DNA extraction of geothermal spring sediments/biofilm by providing a detailed description of the protocol.

## 4. **Persons entitled to use the procedure**

This SOP applies to any person involved in geothermal spring sediment/biofilm DNA extraction.

## 5. **Protocol**

Geothermal spring sediments/biofilm DNA extraction with protocol according to [27] and [28].

### *5.1. Materials*

- 15 mL centrifuge tubes
- 2 mL Eppendorf tubes Lowbind
- Tips P25000, P10000, P5000, P1000 and P200

*5.2. Equipment*

- Centrifuge up to 10,000 x g
- Vortex
- Dry incubator at 37°C
- Water bath warmed at 65°C
- Chemical Hood
- Pipette P5000, P1000 and P200
- Qubit (Thermo Fisher Scientific, Qubit Flex Fluorometer)
- NanoDrop (Thermo Scientific ND 1000)

*5.3. Solutions*

*DNA Extraction Buffer 1X (100 mM Sodium Phosphate Buffer pH 8.0 + 100 mM EDTA pH 8.0 + 100 mM TrisHCl pH 8.0 + 0.5 M NaCl + 1% CTAB)

*For samples at low pH (< 4.0), DNA extraction buffer 2X is used (200 mM Sodium Phosphate Buffer pH 8.0 + 100 mM EDTA pH 8.0 + 200 mM TrisHCl pH 8.0 + 1.5 M NaCl + 1% CTAB).

SDS 20%
Lysozyme 100 mg/mL
Proteinase K 20 mg/mL
Phenol:Chloroform:Isoamyl alcohol (25:24:1)
Chloroform:Isoamyl alcohol (24:1)
Isopropanol 100%
Na-acetate (3 M)
Tris-HCl buffer pH 8.0 50 mM

*5.4. Stock solutions*

Sodium Phosphate buffer 1 M, pH 8.0 (9.32 mL $Na_2HPO_4$ 1 M + 0.68 mL $NaH_2PO_4$ 1 M)
$Na_2HPO_4$ 1 M
$NaH_2PO_4$ 1 M
Tris-HCl 2 M, pH 8.0 (adjust pH with HCl 37% or 12 M)
EDTA 0.5 M, pH 8.0 (adjust pH with ~ 750 µL NaOH 5 M)

6. **Step-by-step procedure**

1. Resuspend 3-5 g of sediment in 5 mL of DNA Extraction Buffer 1X* and shake the tubes several times to mix sediment and Ext. Buffer;
2. Incubate shaking at 37°C for 1 h to equilibrate the buffer (vortex briefly/ invert every 10 min);
3. Add 200 µL of Lysozyme and incubate shaking at 37°C for 30 min. Vortex briefly to the maximum speed each 3 min;
4. Add 50 µL of Proteinase K and incubate shaking at 37°C for 30 min. Vortex briefly to the maximum speed each 3 min;
5. Add 500 µL of SDS (20 %) to the sample;
6. Perform 5 steps of freeze-thawing in dry ice/65°C;
7. Incubate the sample in a water bath at 65°C for 1 hour. Invert to mix each 15 min;
8. Centrifuge the samples at 4,000 rpm for 10 min at RT;
9. Recover the supernatant in a sterile centrifuge 15 mL tube;
10. Add 3 mL of DNA Extraction Buffer 1X* and shake the tubes several times to mix;

**11.** Incubate water bath warmed the sample at 65°C for 30 min. Invert to mix each 10 min;
**12.** Centrifuge the samples at 4,000 rpm for 10 min at RT;
**13.** Recover this second supernatant and add it in the same sterile centrifuge 15 mL tube where the supernatant from step 9 was recovered combining the two supernatants;
**14.** Add to the supernatants, the same volume of a solution of Phenol:Chloroform:Isoamyl alcohol (25:24:1). Invert until completely mixed;
**15.** Centrifuge at 7,000 rpm for 10 min at RT;
**16.** Recover The supernatant is recovered in a sterile centrifuge 15 mL tube;
**17.** Add to the supernatants, the same volume of a solution of Chloroform:Isoamylic alcohol (24:1). Invert until completely mixed;
**18.** Centrifuge the sample is centrifuged at 7,000 rpm for 10 min at RT. Repeat from step 17 if the aqueous phase is not clean;
**19.** Recover the aqueous phase in a sterile centrifuge 15 mL tube;
**20.** Add 0.6 x volume of Isopropanol + 0.1 vol of Na-acetate (3 M) to the sample and incubate overnight at RT;
**21.** Then, centrifuge the sample at 9,400 rpm for 30 min at +4°C;
**22.** Remove the supernatant and centrifuge at 9,400 rpm for 2 min at +4 °C, remove the remaining supernatant;
**23.** Dry the pellet at 37 °C and resuspended in 50 µL of Tris-HCl pH 8.0 50 mM;
**24.** Assess DNA integrity and quantification with NanoDrop and Qubit.

# 10. SOP FOR SOIL/PLANT SAMPLING IN MICROBIAL GENOMIC STUDIES

**Prepared by**: Annamaria Bevivino, Manuela Costanzo, Luciana Di Gregorio, Alessia Fiore, Valeria Poscente, Federico Sbarra, Filippo Sevi, Silvia Tabacchioni, Marco Garello, Davide Spadaro, Cristina Varese, Benedetta Turchetti, Ciro Sannino, Gianmarco Mugnai, Andrea Franzetti, Valeria Tatangelo, Silvia Lampis, Giovanna Felis, Elisa Salvetti, and Marco Andreolli, on behalf of the SUS-MIRRI.IT consortium.

## 1. **Introduction**

The Microbial Resource Research Infrastructure (MIRRI) is the pan-European distributed Research Infrastructure for the preservation, systematic investigation, provision and valorisation of microbial resources and biodiversity. The project SUS-MIRRI.IT, funded by Italy's National Recovery and Resilience Plan – PNRR, is granted by the European Commission's "NextGenerationEU" program. The project is coordinated by the University of Torino and involves 14 other institutions: CNR, ENEA, National Institute of Oceanography and Applied Geophysics (OGS), the Universities of Cagliari, Genoa, Milano Bicocca, Modena and Reggio Emilia, Naples Federico II, Palermo, Perugia, Parma, Sassari, Verona, and the University of Basilicata. Within the project, specific goals include: i) defining the best strategies to sample and store soil/plant samples for microbiome investigation and ii) optimising DNA extraction and sequencing techniques to study microbial communities in order to increase the benefit that society can derive from the knowledge and valorisation of microbial biodiversity. This document presents the Standard Operational Procedure (SOP) for soil/plant sample collection, storage, and shipment within the SUS-MIRRI.IT project.

## 2. **Objective**

The purpose of this SOP is to standardise the procedures for the collection of soil/plant representative samples (bulk soil, rhizosphere, roots, phyllosphere) that will be carried out during the SUS-MIRRI.IT project. This SOP will be followed and validated by the WP4 partners for the analysis of the microbiome in soil/plant ecosystems and include the description of the necessary equipment and best practices for shipping and storage of samples.

## 3. **Principle**

The objective of this document is to define the protocol for soil/plants sampling to assure the highest homogeneity of samples and representativeness of field. It will list all facilities needed to execute in-field sampling and all step-by-step procedures aimed to obtain the most representative samples in terms of microbial community composition. Plants strictly interact with many microorganisms that can regulate their physiology, determining the holobiont health [29]. The endophytic and epiphytic bacterial and fungal communities, associated with the different below- and above- ground compartments, are influenced by ecological dynamics and biotic/abiotic factors. Considering the holobiont definition, we can assume that plant health is closely linked to the interactions between the plant and its microbiota, as it is in the animal kingdom. Conventionally, the soil-plant system is divided in different compartments: bulk soil, rhizosphere, root, phyllosphere and endosphere. Bulk-soil refers to non-adhering peripheral soil in the immediate area outside plant roots. The term "rhizosphere" was first coined in 1904 by the German agronomist and plant physiologist Lorenz Hiltner to describe the plant-root interface [30]. The rhizosphere represents the area around a plant root colonised by a unique population of microorganisms strictly regulated by plants through specific

root depositions (e.g. exudates) [31]. It is not possible to define a unique rhizosphere size or shape, being plant root systems highly biodiverse and complex. It is correct to assess that the rhizosphere consists of a gradient in chemical, biological and physical properties changing radially and longitudinally along the root. The phyllosphere is defined as the aerial plant habitat *sensus lato* or the leaf surface in relation to the external environment, the above-ground aerial region of the plant inhabited by microbes [32, 33]. Plants with their root system represent unique ecological niches for soil microbiome which colonises the rhizosphere, roots and to a certain extent above ground parts [34, 35].

## 4. **Person entitled to use the protocol**

Procedures listed in this technical report can be conducted by any person involved in soil and plant-associated microbial communities' studies.

## 5. **Preliminary steps**

### *5.1. Site establishment*

Agricultural fields with well-established cropping areas across contrasting environmental conditions (e.g. soil types) will be identified. Location (i.e. latitude and longitude) and other characteristics of each field should be recorded as reported in Table 1. In the case of croplands, the crop type (e.g. wheat) and main variety for each species (i.e. *Triticum aestivum*) should be recorded.

**Table 1.** Template for site characterisation

| | |
|---|---|
| **Field name:** | e.g. Country_sitename_fieldname |
| **Latitude:** | xx° xx' xx" N |
| **Longitude:** | xx° xx' xx" E |
| **Crop variety:** | e.g. *Triticum aestivum* |
| **Visual plant cover [%]:** | xx % |
| **Dominant plant species:** | e.g. *Triticum aestivum* |
| **Plot name:** | e.g. Country_sitename_fieldname_plotname |

Additional information can be carried out such as:

- Crop details (phenological state; crop rotation; etc.)
- Irrigation
- Pruning
- Fertilization
- Tillage
- Phenological state
- Pesticide application
- Crop rotation
- Weather data

*5.2 Sampling and shipment equipment*

- Gloves
- 70% EtOH
- Shovel
- 2 x Pot spades
- 2 x Scalpels
- Scissors or shears
- Soil coring equipment
- Zipped plastic bags
- Permanent markers
- 50-mL and 15-mL centrifuge tubes
- Thermo-stable shipping box
- Buckets
- Dry ice or ice packs

Wear clean gloves always sterilized with EtOH and replace the gloves between each sample to prevent contamination. Sterilize all equipment with 70% EtOH and wipe between samples.

*5.3. Sampling design*

*5.3.1. Herbaceous crops*

Within the defined sampling area, a 30 m x 30 m plot should be established (or equivalent area depending on cropping system), as shown in Figure 1. Minimum six to maximum 12 biological replicates will be composed by randomly selected individual plants equally distributed. Plants must be at a similar phenological state and soil should be recently irrigated to ease the process of sampling. Steps that require sample processing can be held in one same working area paying attention to equipment sterility between each replicate processing. In the case of rhizosphere/root/phyllosphere samples, individual plants will be harvested for each of the 6-12 replicates. For bulk-soil, each replicate will be formed by mixing 5 soil cores extracted in an x-shaped pattern (see red X in Figure 1) around the 6-12 selected plants. For the phyllosphere, leaves will be collected in tubes using flamed scissors and forceps. In order to ensure sufficient plant material for subsequent processing, tubes will be filled with several leaves of each individual plant.

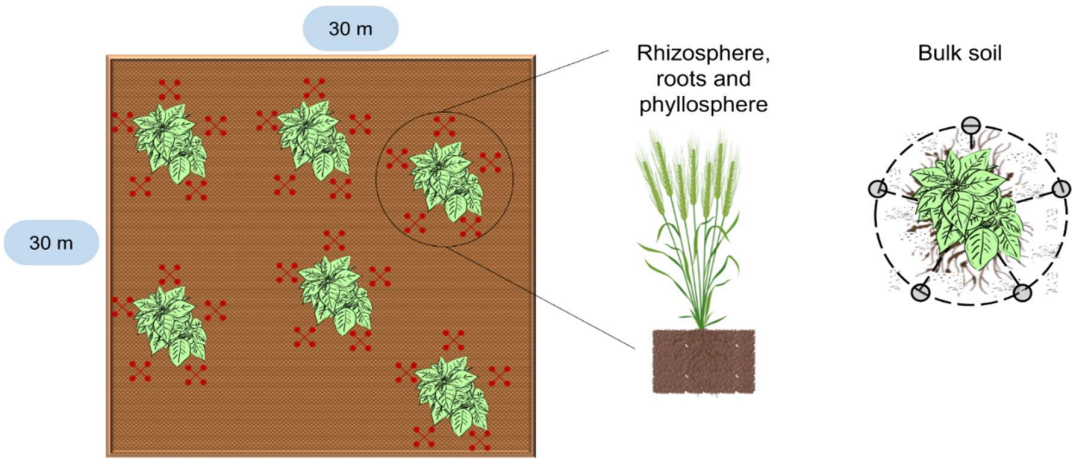


**Figure 1.** *Schematic representation of sampling within the plot.*

*5.3.1. Non-herbaceous plants*

Minimum six to maximum12 biological replicates will be composed by randomly selected individual plants equally distributed. Steps that require sample processing can be held in one same working area paying attention to equipment sterility between each replicate processing. For rhizosphere/roots, 5 points in a x-shaped pattern around the plants' trunk will be selected and sampled by soil coring, based on root system size at least three equidistant points from plants' trunk will be selected and sampled by soil coring, based on root system size, figure 2. The latter (i.e. the material associated to the 5 points) will be pooled into a composite sample. The operator will dig with a shovel to expose portions of the root system. Exposed portions of roots should be representative of the entire system. Younger, smaller, less lignified roots should be preferred. For bulk-soil 6-12 composite soil samples derived from 5 cores extracted in an x-shaped pattern (red X in figure 1) will be collected in the immediate areas outside selected plant roots. For the phyllosphere, different leaf replicates will be collected at an average height. The operator will cut leaves from branches using metal scissors and tweezers, both sterilised each time with ethanol, and immediately put in sterile plastic bags to prevent any exogenous DNA.

## 6. **Step-by-step procedures for sampling and shipment**

*6.1. Bulk-Soil*

1. Sterilise soil core sampler with 70% EtOH between each biological replicate and wipe clean it.
2. Use a soil core sampler with adequate diameter and extension based on plant dimension.
3. Collect 5 soil cores in a radial pattern for each biological replicate as shown in Figure 2.
4. Pour the collected soil in a bucket and thoroughly mix to produce composite soil for each biological replicate.
5. Remove vegetation residues, grass and litter, if present, from the surface before sampling and from the composite sample.
6. Sieve the bulk-soil at 2-mm and transfer to a labelled zipped plastic bag stored in ice during field work.
7. Ship samples to the laboratory in ice (0-4°C, e.g. thermo-stable shipping box).
8. Once received, transfer collected soil in a 50- or 15-mL centrifuge tube (20-40 g of soil) and store it at -80°C until DNA extraction. Repeat this step for each biological replicate.

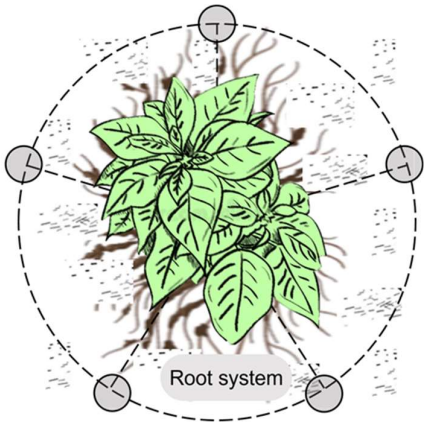


**Figure 2.** *Sampling design for bulk-soil.*

*6.2. Rhizosphere soil*

**1.** Sterilise 2 scalpels with 70% EtOH between each biological replicate and wipe clean it;
**2.1.** For **herbaceous plants**, dig with the shovel to take out the entire root within 10-cm radius around the plant and down to 20-cm soil depth. Manually remove excessive non-adhering soil by vigorous shaking and using a scalpel or pot spade;
**2.2.** For **non-herbaceous plants,** select 5 equidistant points from the plant stem, inside the root system radius, in an x-shaped pattern similar to the one used for bulk-soil (Figure 2). Dig with the shovel and collect isovolumic soil detaching from roots for each point with a pot spade;
**3.** Collect approximately 10-30 g of soil detaching from different areas of the plant root system and transfer in labelled zipped plastic bags stored in ice during field work, as shown in Figure 3. A different scalpel or pot spade should be used for each step;
**4.** Repeat steps 1-3 for each replicate;
**5.** Ship samples collected to the laboratory are preserved in ice (0-4°C);
**6.** Once received in the laboratory, transfer the soil collected in a labelled 50-mL or 15-mL centrifuge tube and store it at -80°C until DNA extraction. Repeat this step for each replicate.


**Figure 3.** *Step procedures of rhizosphere sampling.*

*6.3. Root*

**1.** Sterilise 2 scalpels with 70% EtOH between each replicate and wipe clean it. In addition, for non-herbaceous plants, a pair of scissors and/or shears should also be sterilised with 70% EtOH. Wear clean gloves always sterilised with EtOH and replace the gloves between each sample to prevent contaminations;
**2.a** For herbaceous plants, dig with the shovel to take out the entire root system within a 10-cm radius around the plant and down to 20-cm soil depth;
**2.b** For non-herbaceous plants, dig with the shovel to expose portions of the root system. Exposed portions of the root system should be representative of the entire system, and for this reason at least 5 points will be selected, based on root system size, in an x-shaped pattern similar to the one used for bulk-soil (Figure 2). Digging depth depends on root system depth. Once the root system has been exposed, cut enough roots to fill a 50-mL Falcon tube, picking equally from all points. Younger,

smaller, less lignified roots should be preferred. Also, if endophytic organisms are the focus, breaking roots should be minimised, as cutting the roots could lead to endophytic organisms being washed out;
**3.** Manually remove excessive non-adhering soil by vigorous shaking and using a scalpel;
**4.** Repeat steps 1-3 for each replicate. Store the root system (or portion of root system) in ice (0-4°C) during field work;
**5.** Ship samples collected to the laboratory are preserved in ice (0-4°C);
**6.** Once received in the laboratory, if roots are not immediately used, store samples at -80°C until further processing;
**7.** To wash the roots, add approximately 20 mL of chilled (4°C) sterile water to the root fraction. Wash the root by shaking vigorously (by hand or mixer, for 15 - 30 s), and then drain the water. Repeat this step at least twice, until no soil remains on the root surface. If clumps of soil are still trapped between the roots, use sterile tweezers to remove them. If necessary, use sterile gloves to keep the root steady during the operation. Gloves should be changed between replicates;
**8.** Store the roots in sterile 50 mL Falcon tubes, flash freeze the roots in liquid $N_2$, and store the samples at -80 °C. Otherwise, proceed to step 10;
**9.** Chill a sterilised mortar and pestle using liquid $N_2$. Measure out 600 to 700 mg of root tissue and place the tissue into the mortar. Carefully, add enough liquid $N_2$ to cover the roots.
**10.** Grind the roots into small pieces. Continue the process of adding liquid $N_2$ and grinding (at least two times, be consistent between samples), until the roots are a fine powder. Ensure that the root tissue does not thaw during this step;
**11.** Quickly, before the root powder begins to thaw, use a sterile spatula to transfer the root powder into pre-weighed 1.5 mL microcentrifuge tubes on ice. Record the weight of the tube and powder. Typically, 300 - 400 mg of powder is transferred;
**12.** If follow-up DNA extraction cannot be performed immediately, store the ground powder at -80°C.

### *6.4. Phyllosphere sampling*

**Leaf, bud, branch and trunk sampling for both epiphyte and endophyte analysis**

Wearing sterilised gloves cut at least 50 grams of plant materials with sterilised scissors to fill in a sterile 50-mL conical tube or plastic bag.

**Branch and trunk sampling for epiphyte analysis**

Collect epiphytic communities on branches and trunk using a sterile swab on an area of at least 50 cm2 and put in a sterile plastic bag.

**Sample storage and shipment**

1. Ship samples collected to the laboratory preserved in ice (0-4°C);
2. Store swab at -80°C until DNA extraction;
3. Pre-process the samples for epiphyte and endophyte analysis within 12 h.

#### *6.4.1. Sample pretreatment for epiphyte and endophyte analysis*

**Procedure to detach epiphytes form leaf and bud samples:**

1. Add 5 mL sterile leaf wash solution [36] (1.2% Triton, 1 M TRIS, 50 mM EDTA) to the conical tube or bag containing leaves, buds or branches and sonicate them for 10 min;
2. Vortex for 5 min at maximum speed the conical tubes or shake for 5 min at 200 rpm on an orbital shaker the bags;
3. Manually remove leaves, buds or branches and filter on 0.22 µm pore-size nitrocellulose membranes;
4. Store the samples at -80°C until DNA extraction.

**Sample pretreatment for endophyte analysis:**

1. Sterilise by consecutive immersion for 1 min in 75% EtOH, 3 min in 1% sodium hypochlorite, and 30 s in 75% EtOH, followed by three rinses with sterile water;
2. Freeze-dry and homogenise the sterilised plant material as previously described for roots (§ 5.3, step 10 and 11);
3. Store the samples at -80°C until DNA extraction.

*6.5. Debris/soil*

1. Sterilise 1 scalpel with 70% EtOH between each replicate and wipe clean it.
2. Debris/soil are collected at a depth of about 10-20 cm depending on the type of debris/soil. Soil samples are collected below the rhizosphere removing the entire root within 10-cm radius around the plant and down to 20-cm soil depth.
3. Sterilise the sieve with 70% EtOH and sieve the debris/soil to 4.0 and 4.8 mm size to remove bigger soil particles and stones.
4. Using a scalpel to collect approximately 20 g of debris/soil for each replicate within the designed plots, and transfer in labelled zipped sterile plastic bags stored in ice during field work.
5. Repeat steps 1-4 for each replicate.
6. Preserve ship samples collected to the laboratory in ice (0-4°C).
7. Transfer the soil collected in a labelled 50-mL centrifuge tube and store it at -80°C until DNA extraction. Repeat this step for each replicate.

## 7. **Metadata requirement**

Mandatory for field (site description and sampling) field and plot name; geographic location (Country + Site); geographic coordinates (latitude and longitude); visual plant coverage [%]; dominant plant species; plant species of interest (i.e. crop species); current crop details (cultivar; phenological stage, crop age); history (organic/integrated/conventional regimen; previous crops); fertilisation (type, amount, n° of applications; last/next application); irrigation (system, annual rate/timing, last/next date); tillage (n° of operations, last/next repeat, depth); horizon (slope); sample collection time; composite bulk soil sample name; composite soil field; storage conditions.

Recommended: average temperature (last three months); cumulative precipitations (last three months); soil core number per composite; soil core sample depth; soil core diameter.

Mandatory for bulk soil and rhizosphere soil (soil chemical and physical properties): pH; soil organic matter (SOM); particle size distribution (PSD) (sand, silt, and clay content); bulk density; available water capacity; exchangeable acidity; cation exchange capacity (CEC); total organic C, total N content, C:N ratio; P content; extractable K content; calcium carbonates ($CaCO_3$); electrical conductivity; sample weight for DNA extraction.

Recommended: micronutrients; Mg; Na; Fe; Cu; Mn; Zn; B.

Mandatory for plant (root and phyllosphere): root sample name; phyllosphere sample name; photosynthetic activity; fresh weight roots; fresh weight shoots; dry weight roots; dry weight shoots; plant height; stem diameter; visual coverage of leaves (%).

Recommended: normal difference vegetation index.

## 8. **Recommendations**

It is recommended to store samples in liquid $N_2$, when available, during field work or transportation. When this is not possible, samples can be stored in dry ice but minimising the time delay between sampling and shipment. Long-term storage should be at -80°C to prevent changes in soil physicochemical properties and avoid DNA degradation.

# 11. SOP FOR DNA EXTRACTION FROM SOIL/PLANT SAMPLES

**Prepared by**: Annamaria Bevivino, Manuela Costanzo, Luciana Di Gregorio, Alessia Fiore, Valeria Poscente, Federico Sbarra, Filippo Sevi, Silvia Tabacchioni, Marco Garello, Davide Spadaro, Cristina Varese, Benedetta Turchetti, Ciro Sannino, Gianmarco Mugnai, Andrea Franzetti, Valeria Tatangelo, Silvia Lampis, Giovanna Felis, Elisa Salvetti, Marco Andreolli, Paola Quatrini, and Giuseppe Gallo on behalf of the SUS-MIRRI.IT consortium.

## 1. **Introduction**

The Microbial Resource Research Infrastructure (MIRRI) is the pan-European distributed Research Infrastructure for the preservation, systematic investigation, provision and valorisation of microbial resources and biodiversity. The project SUS-MIRRI.IT, funded by Italy's National Recovery and Resilience Plan – PNRR, is granted by the European Commission's "NextGenerationEU" program. The project is coordinated by the University of Torino and involves 14 other institutions: CNR, ENEA, National Institute of Oceanography and Applied Geophysics (OGS), the Universities of Cagliari, Genoa, Milano Bicocca, Modena and Reggio Emilia, Naples Federico II, Palermo, Perugia, Parma, Sassari, Verona, and the University of Basilicata. Within the project, specific goals include: i) defining the best strategies to sample and store soil/plant samples for microbiome investigation and ii) optimizing DNA extraction and sequencing techniques to study microbial communities in order to increase the benefit that society can derive from the knowledge and valorisation of microbial biodiversity. This document presents the Standard Operational Procedure (SOP) for DNA extraction from rhizosphere/soil samples within the SUS-MIRRI.IT project.

## 2. **Principle**

This SOP aims to standardize DNA extraction procedures from soil/plant samples performed during the SUS-MIRRI.IT project by providing a description of the necessary equipment, a step-by-step description of the procedures and best practices for storage and shipping of samples.

## 3. **Objective**

The objective of this document is to define the protocols for the extraction of DNA from soil/plant samples, storage, and shipment. The PowerSoil DNA Pro Kit from QIAGENtm is recommended to obtain high yield and quality of microbial DNA from samples [37]. All procedures for microbial lysis and DNA extraction are referred to the user manual provided with the kit. Protocols to measure sample integrity and evaluate the quantity and quality of DNAs are also included in this document. Moreover, the best preservation strategies for metagenomic samples will be elucidated.

## 4. **Person entitled to use the protocol**

Procedures listed in this technical report can be conducted by any person involved in soil and plant-associated microbial communities' studies.

## 5. **Preliminary steps**

### *5.1. Equipment needed*

- Gloves

- DNaesy PowerSoil Pro Kit (QIAGENtm)
- Microcentrifuge (10,000 x g)
- Pipettors (50 µL - 500 µL)
- 2 mL Bead Tubes
- Vortex and adaptors
- 1X TAE buffer (Sigma Aldrich)
- GelRed Nucleic Acid Stain (Sigma Aldrich) 10,000X dissolved in water
- Lambda DNA/HindIII Marker
- Bio Red Molecular Imager ChemiDoc XRS+
- NanoDrop 2000 (Thermo Fisher Scientific)
- Qubit 4.0 fluorimeter (Invitrogen, Life technology

*5.2. Experimental procedure*

For sample collecting procedures follow Standard Operating Protocols for soil/plant sampling in microbial genomic studies (SOP_07).

Soil, rhizosphere, plant samples, swabs and nitrocellulose membranes stored at -80°C will be transferred in dry ice (0-4°C) prior to the DNA extraction. For each sample replicate, 3 sub-samples will be aliquoted and will undergo the DNA extraction procedure. DNAs obtained by the extraction method will run in gel electrophoresis to evaluate their integrity and then analysed for their quantity and quality, as shown in Figure 1. Extracted DNA will be stored at -80°C as indicated in the kit user manual until further experiments will be conducted (e.g. sequencing).


**Figure 1.** *DNA extraction and analysis.*

6. **Step-by-step procedure**

*6.1. DNA extraction*

All reagents and equipment are provided with the kit. Solution CD2 should be stored at 2-8°C upon arrival.

1. The DNA must be extracted in three replicates for each sample;
2. Place samples in dry ice (0-4°C);
3. Spin the PowerBead Pro Tubes briefly to ensure that beads have settled at the bottom.
4. Add 0.25 g of sample (in case of nitrocellulose membranes and swabs, add one third of the sample) to the PowerBeads Pro Tube and 800 µL of Solution CD1. Vortex to mix;
5. Secure the PowerBead Pro Tube horizontally on a Vortex Adapter for 1.5–2 mL tubes (cat. no. 13,000-V1-24). Vortex at maximum speed for 10 min;

**Note**: If using the Vortex Adapter for more than 12 preps simultaneously, increase the vortexing time by 5–10 min.

**Note**: For more information about other bead beating methods, see the "Protocol: Detailed" section of DNeasy® PowerSoil® Pro Kit Handbook [38].

6. Centrifuge the PowerBead Pro Tube at 15,000 x g for 1 min;
7. Transfer the supernatant to a clean 2 mL microcentrifuge tube;

**Note**: Expect 500–600 µL. The supernatant may still contain some soil particles.

8. Add 200 µL of Solution CD2 and vortex for 5 s;
9. Centrifuge at 15,000 x g for 1 min at RT. Avoiding the pellet, transfer up to 700 µL of supernatant to a clean 2 mL microcentrifuge tube;

**Note**: Expect 500–600 µL.

10. Add 600 µL of Solution CD3 and vortex for 5 s;
11. Load 650 µL of the lysate onto an MB Spin Column and centrifuge at 15,000 x g for 1 min;
12. Discard the flow-through and repeat step 8 to ensure that all the lysate has passed through the MB Spin Column;
13. Carefully place the MB Spin Column into a clean 2 mL collection tube. Avoid splashing any flow-through onto the MB Spin Column;
14. Add 500 µL of Solution EA to the MB Spin Column. Centrifuge at 15,000 x g for 1 min;
15. Discard the flow-through and place the MB Spin Column back into the same 2 mL collection tube;
16. Add 500 µL of Solution C5 to the MB Spin Column. Centrifuge at 15,000 x g for 1 min;
17. Discard the flow-through and place the MB Spin Column into a new 2 mL collection tube;
18. Centrifuge at up to 16,000 x g for 2 min. Carefully place the MB Spin Column into a new 1.5 mL Elution tube;
19. Add 50–100 µL of Solution C6 to the centre of the white filter membrane;
20. Centrifuge at 15,000 x g for 1 min. Discard the MB Spin Column. The DNA is now ready for downstream applications.

It is recommended to store the DNA frozen (–30 to –15°C or –90 to –65°C) as Solution C6 does not contain EDTA. To concentrate DNA, please refer to the Troubleshooting Guide.

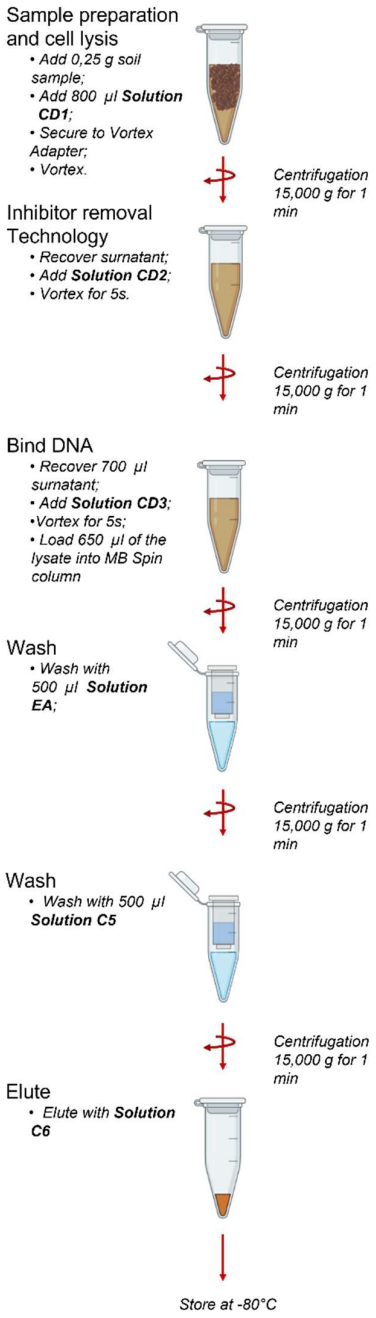


**Figure 2**. *DNA extraction scheme.*

*6. 2. Gel electrophoresis*

**Tris acetate EDTA 50X buffer preparation**

1. Dissolve 242 g of tris base in 800 mL double-distilled water.
2. Add 100 mL 0.5 M EDTA solution (pH 8.0) and 27.1 mL of glacial acetic acid to the solution.
3. Adjust volume to 1 L with double-distilled water and mix with a magnetic anchor.
4. Autoclave at 121°C for 20 min.

**Gel preparation**

1. Dilute tris acetate EDTA 50X solution to obtain 1X with double-distilled water.
2. Dissolve 1.5 g of agarose in 100 mL of TAE buffer 1X and thoroughly mix with a magnetic anchor.
3. Heat the solution until boiling (approx. 5 min).
4. Add 10 µL of GelRed Nucleic Acid Stain and mix the solution.
5. Place the comb into the electrophoretic tray, rapidly pour the obtained solution into the housing and let it cool.
6. Add TAE 1X buffer to the electrophoresis tray until it reaches the limit line.

**Electrophoresis**

1. Add 2 µL Lambda DNA/HindIII to any well selected to house the marker.
2. Add 2 µL of samples to the wells annotating their spatial position.
3. Run the gel for 45 min under 90 V.
4. Examine results using Bio Red Molecular Imager ChemiDoc and produce images of every run.

*6.3. Qualitative and quantitative analysis*

**Nucleic acid/Protein Ratio**

1. Turn on NanoDrop 2000 (Thermofisher) and select Nucleic Acid application.
2. Select the type of sample to be measured from the drop-down list.
3. Select Add to report to save sample data to a workbook.
4. Pipette 1.5-2 µL of the appropriate blanking solution onto the bottom pedestal, lower the arm and click the Blank button. Wait for measurement and wipe clean.
5. Pipette 1.5-2 µL of samples and click Measure button. Wipe clean after every measurement.
6. Visualize the results on software.

**DNA concentration**

All reagents and equipment are provided with the kit.

1. Set up 2 assay tubes for the standards and 1 assay tube for each sample.
2. Prepare the Qubit working solution by diluting the Qubit reagent 1:200 in Qubit buffer. Prepare 200 µL of working solution for each standard and sample.
3. Prepare the assay tubes according to the table below.

**Table 1**. Assay tubes preparation (schematic).

| | **Standard assay tubes** | **User sample assay tubes** |
|---|---|---|
| Working solutions (from step 2) | 190 µL | 180-199 µL |
| Standard (from kit) | 10 µL | - |
| User sample | - | 1-20 µL |
| Total Volume in each assay tube | 200 µL | 200 µL |

4. Vortex all tubes for 2-3 seconds and incubate for 2 min at RT.

5. Calibrate the instrument running standards prepared for the desired assay (dsDNA). Select assay type and press read standards.
6. Run samples selecting sample volume added to the solution and measuring units. Insert the sample tube and press read. The reading takes approximately 3 seconds for the quantitation assays and approximately 5 seconds for the quality assays.
7. Visualize the results on screen.

### *6.4. DNA pool, storage, and sample shipment*

Collection tubes containing the extracted DNA from sub-samples should be now pooled into a single clean labelled tube. Keep tubes in ice during this step. Extracted DNA should be stored at -80°C to prevent degradation. DNA may be eluted with sterile DNA-free PCR grade water. Samples need to be shipped in dry ice (0-4°C) and for further information (e.g. DNA concentration and quality) contact service providers for DNA sequencing.

## 7. **Recommendations**

Transfer the samples from -80°C to dry ice prior to the extraction and keep them during the entire experiment. Every sample will be extracted in triplicate. DNAs extracted can be stored at -80°C.

# 12. SOP FOR WATER SAMPLING

**Prepared by**: Paola Quatrini, Giuseppe Gallo, Raimondo Gaglio, Rosa Alduina, Benedetta Turchetti, Ciro Sannino, Gianmarco Mugnai, Andrea Franzetti, Valeria Tatangelo, Silvia Lampis, and Marco Andreolli on behalf of the SUS-MIRRI.IT consortium.

## 1. **Introduction**

The Microbial Resource Research Infrastructure (MIRRI) is the pan-European distributed Research Infrastructure for the preservation, systematic investigation, provision and valorisation of microbial resources and biodiversity. The project SUS-MIRRI.IT, funded by Italy's National Recovery and Resilience Plan – PNRR, is granted by the European Commission's "NextGenerationEU" program. The project is coordinated by the University of Torino and involves 14 other institutions: CNR, ENEA, National Institute of Oceanography and Applied Geophysics (OGS), the Universities of Cagliari, Genoa, Milano Bicocca, Modena and Reggio Emilia, Naples Federico II, Palermo, Perugia, Parma, Sassari, Verona, and the University of Basilicata. Within the project, specific goals include: i) defining the best strategies to sample and store water samples for microbiome investigation and ii) optimizing DNA extraction and sequencing techniques to study microbial communities in order to increase the benefit that society can derive from the knowledge and valorisation of microbial biodiversity. This document presents the Standard Operational Procedure (SOP) for water sample collection, storage, and shipment within the SUS-MIRRI.IT project.

## 2. **Objective**

This SOP aims to optimize data comparisons in the aquatic microbiome field by the standardization of the procedure for water sampling.

## 3. **Principle**

The principle of this SOP (SOP for validated sample collection and storage) is to provide guidance for sample collection and storage based on literature survey in conjunction with a survey of the SUS-MIRRI.it partners and using already existing SOPs. In drafting this SOP, several kinds of waters were taken into account (wastewater, groundwater, marine and lake water). For each step, tips and recommendations are provided.

## 4. **Persons entitled to use the procedure**

This SOP applies to any person involved in collecting, transportation and processing of water samples. This person can be a trainee, fellow, technician or engineer, or any agent in charge of sampling.

## 5. **Storage temperature of water**

During the transport to the laboratory, keep water samples in refrigerated conditions (4°C). Store samples at -80°C before processing. If microbial biomass is necessary, filter the water and store the filters at -80°C.

6. **Materials**

- Gloves
- Sterile 500-1000 mL glass (like Pirex or equivalent) bottles (preferably black)
- 50 mL sterile DNA-free crew cap plastic tubes
- Pipettes and pipette tips
- Sterile plastic bags
- Dry ice
- Glass 5 L beakers (n = 4)
- Glass funnels

7. **Equipment**

- Centrifuge
- Ultra-freezer (-80°C) or -20°C freezer if an ultra-freezer is not available
- Ice core extraction tool or ice axe
- Peristaltic pump and sterile tubing

For groundwater sampling:

- Sampling pump - Submersible or bladder pumps with adjustable rate controls
- Inert tubing
- Flow-measurement equipment
- Multi-parameter probe with flow-through cell
- Glass dark bottles (1 L) or pressure sterile filter unit with cartridges (e.g. Sterivex)
- Vacuum filtration set
- Cellulose nitrate (CN) membrane, 0.22 µm pore size

8. **Step-by-step Procedure**

*8.1. Sampling*

Collect the requested amount of water in suitable sterile containers such as 50 mL sterile DNA-free crew cap plastic tubes or sterile glass bottle by direct and complete immersion (if wastewater is contained in a tank provided with a tap, it can be used to collect sample). The amount of water depends on the putative bacterial load (50 mL-1 L) [39].

For groundwater sampling, purge groundwater from the well preferably with low-flow (0.1 – 0.5 L/min) for at least 3 well volumes and until stabilization of water-quality-indicator parameters of pH, oxidation-reduction potential (ORP), dissolved oxygen (DO); collect 1 L of groundwater in the sample bottle or pass it through a sterilizing cartridge; filter 1 L of water sample on CN membrane. Use more than one membrane in case of clogging, store the CN membrane at -80°C until DNA extraction.

For ice sampling, ice cores/blocks are collected after removing and discarding about 5–10 cm of ice surface from the glacier, using a specific ice core extraction tool or ice axe. The ice cores/blocks are placed into sterile bags and maintained on boxes containing dry ice (to prevent melting). The decontamination of the outer part of ice is mandatory to remove the possible contamination made during the sampling. The decontamination procedure was optimized following the protocol described by Rogers and colleagues [40]. Using a laminar flow cabinet, the ice is dipped into 2 L of NaClO solution at 5.25% for 20 seconds, then dipped into 2 L of sterile cold (4°C) distilled water for 20

seconds for three times using three different beakers with sterile water. The ice core is then transferred in sterile funnels for melting and collection of the water. Considering the low concentration of microorganisms, the amount of water to be collected ranges from 200 to 500 mL depending on the microbial load and turbidity of the sample.

For hypersaline brines sampling the peristaltic pump and sterile tubing are necessary. The brines are collected in sterile Pyrex bottles.

Once taken, all samples must be stored in a portable cooler filled with ice packs for transport to the laboratory.

## 9. **Metadata requirement**

The basic parameters to be analysed are pH, temperature, redox potential, dissolved oxygen (better with the use of a multiparametric probe). Other parameters are conductivity, inorganic nitrogen and phosphate contents, total Chemical Oxygen Demands (tCOD) and soluble Chemical Oxygen Demands (sCOD). Some specific kinds of analyses may be required depending on the water samples: for wastewater, Total Suspended Solids (TSS), Volatile Suspended Solids (VSS), Volatile Fatty Acids (VFAs), Total Organic Carbon, protein, and carbohydrate contents; for groundwater, sea and lake water, depth distance from the coastline, organic contaminants, and heavy metal content.

# 13. SOP FOR DNA EXTRACTION FROM WATER SAMPLES

**Prepared by**: Paola Quatrini, Giuseppe Gallo, Raimondo Gaglio, Rosa Alduina, Benedetta Turchetti, Ciro Sannino, Gianmarco Mugnai, Andrea Franzetti, Valeria Tatangelo, Silvia Lampis, and Marco Andreolli on behalf of the SUS-MIRRI.IT consortium.

## 1. **Introduction**

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## 2. **Objective**

This SOP aims to optimize data comparisons of microbiomes characterizing water and aqueous samples by the standardization of the protocols for operating procedures for DNA extraction.

## 3. **Principle**

This SOP aims to standardize the procedures for DNA extraction from microorganisms residing in water and aqueous samples by giving a step-by-step description of the protocol.

## 4. **Persons entitled to use the protocol**

This SOP applies to any person involved in DNA extraction. This person can be a trainee, fellow, technician or engineer in charge of DNA extraction.

## 5. **DNA extraction from microbial cells residing in water**

The commercial DNeasy PowerWater Kit is recommended for the DNA extraction from microbial cells residing in water; the amount of water to use can be chosen on the basis of the bacterial load (from 100 to 550 mL) [39].

### *5.1 Equipment*

- Centrifuge for 15 mL tubes
- Disposable/reusable filter funnels
- Filter membranes

- Microcentrifuge
- Tilt shaker
- Thermomixer
- Vortex
- Vacuum Filtration System
- 2L Beaker (n = 3)
- 50 mL screw cap DNase-free tubes
- 1.5-2 mL microcentrifuge DNase-free tubes
- Pipettes and pipette DNase-free tips
- Autoclave

*5.2 Step-by-step procedure*

1. filter 100-500 mL of water using a reusable or disposable filter funnel attached to a vacuum source;
2. using two sets of sterile forceps, pick up the white filter membrane at opposite edges and roll the filter into a cylinder with the top side facing inward;
3. insert the filter into the 5 mL PowerWater® Bead Tube;
4. add 1 mL of Solution PW1 to the PowerWater® Bead Tube;
5. heat the PowerWater® Bead Tube at 65°C for 10 min;
6. secure the tube horizontally and vortex at maximum speed for 5 min;
7. centrifuge the tubes ≤ 4,000 x g for 1 min at RT;
8. transfer all the supernatant to a clean 2 mL collection tube. Draw up the supernatant using a 1 mL pipette tip by placing it down into the beads;
9. centrifuge at 13,000 x g for 1 min. Avoiding the pellet, transfer the supernatant to a clean 2 mL collection tube;
10. add 200 µL of Solution IRS and vortex briefly to mix. Incubate at 2–8°C for 5 min;
11. centrifuge the tubes at 13,000 x g for 1 min;
12. avoiding the pellet, transfer the supernatant to a clean 2 mL collection tube;
13. add 650 µL of Solution PW3 and vortex briefly to mix;
14. load 650 µL of supernatant onto a MB Spin Column. Centrifuge at 13,000 x g for 1 min. Discard the flow-through. Repeat until all the supernatant has been processed;
15. place the MB Spin Column Filter into a clean 2 mL collection tube;
16. add 650 µL of Solution PW4 (shake before use). Centrifuge at 13,000 x g for 1 min;
17. discard the flow-through and add 650 µL of EtOH (provided) and centrifuge at 13,000 x g for 1 min;
18. discard the flow-through and centrifuge again at 13,000 x g for 2 min;
19. place the MB Spin Column into a clean 2 mL collection tube;
20. add 100 µL of Solution EB or nuclease free water to the centre of the white filter membrane;
21. centrifuge at 13,000 x g for 1 min;
22. discard the MB Spin Column. The DNA is now ready for downstream applications.

# 14. SOP FOR SOLID FERMENTED FOODS (SFFs) SAMPLING

**Prepared by**: Luca Cocolin, Sahar Maghrebi, Ilario Ferrocino, Vittorio Capozzi, Massimo Ferrara, Giacomo Zara, Angela Bianco, Teresa Zotta, Anna Reale, Filomena Nazzaro, and Maria Gullo on behalf of the SUS-MIRRI.IT consortium.

## 1. **Introduction**

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## 2. **Objective**

This SOP aims to optimize data comparisons in the food microbiome field by the standardization of the procedure for SFFs sampling.

## 3. **Principle**

The principle of this SOP is to provide guidance for sample collection and storage based on literature surveys in conjunction with a survey of the SUS-MIRRI.it partners and using already existing SOPs. In drafting this SOP, several SFFs were taken into account (Table 1). For each step, tips and recommendations are provided.

**Table 1**: The various solid fermented foods considered in this SOP.

| | **Solid Fermented Foods** |
|---|---|
| 1 | Fermented meats |
| 2 | Fermented legumes |
| 3 | Sourdoughs |
| 4 | Table olives |
| 5 | Cheeses |

4. **Persons entitled to use the procedure**

This SOP applies to any person involved in collecting, transportation and processing of solid fermented food samples. This person can be a trainee, fellow, technician or engineer, or any agent in charge of sampling.

5. **Storage temperature of SFFs**

If SFFs are processed immediately after collection, they must be kept in refrigerated conditions (4°C) within 3h. Long-term storage of samples should be at -80°C or transported in dry ice and immediately stored at -80°C. In case of absence of a -80°C freezer, samples can be stored at -20°C. The literature survey indicated that no storage medium was previously used or is necessary for SFF samples storage.

6. **Materials**

The following materials are required for this SOP:

- 50 mL and 200 mL sterile DNA-free plastic containers
- Sterile plastic stomacher bags, with plastic ties for samples collection
- Ringer solution (concentration as per manufacturer's instructions)
- 2 mL microcentrifuge tubes
- Pipettes and pipette tips
- Lab spoon/spatula
- Balance
- Cylinder
- Beaker
- Laminar flow hood

7. **Equipment**

- Stomacher blender
- Centrifuge
- Ultra-freezer (-80°C) or -20°C freezer if an ultra-freezer is not available

8. **Step-by-step procedure**

*8.1 Sampling*

Put the product (unit/portion), at least 100 g in a sterile plastic stomacher bag closed by using plastic ties.

*8.2 Samples manipulation*

Once taken, all samples must be stored in a portable cooler filled with ice packs and immediately transported to the laboratory within max 4h.

**For fermented meats and fermented legumes** [41, 42]

1. Based on the size or shape of the products, collect 10 g of sample (prior casing removal for fermented meats) with a sterile lab spoon/spatula;
2. Put the sample in the stomacher blender bag;

3. Measure 90 mL of Ringer solution using a graduated cylinder;
4. Add the Ringer solution to the sample into the stomacher blender bag;
5. Put the stomacher blender bag into the stomacher blender for 3 min at RT;
6. Add 1.8 mL of sample to a 2 mL collection tube;
7. Centrifuge at 13,000 x g for 10 min at RT;
8. Discard the supernatant;
9. Spin the tubes at 13,000 x g for 1 min at 4°C;
10. Remove the remaining supernatant completely with a pipette tip;
11. Pass immediately to DNA extraction immediately or store the pellet at -80°C.

**Recommendation**: Pooling multiple aliquots to increase cell concentration.

**For sourdoughs**

1. Aseptically collect 5 g of fresh sample with a sterile spatula and put in a sterile stomacher bag.
2. Add 45 mL of Ringer solution and homogenize for 3-min into the stomacher blender Centrifuge 1.25 mL of the homogenized sample in a 2 mL collection tube at 13,000 x g for 1 min at RT. Discard the supernatant.
3. Repeat step 2 by adding the homogenized sample directly in the same centrifuge tube to the already obtained cell pellet. Discard the supernatant.

**Note**: this step concentrates and pellets the microbial cells from a total of 2.5 mL of the homogenized sample, corresponding to 25% of the collected material (i.e. 1.25 g)

4. Remove the remaining supernatant completely with a pipette tip.
5. Pass immediately to DNA extraction immediately or store the pellet at -80°C.

**For fermented table olives**

1. Based on the size or shape of the products, collect 25 g of sample (representative of the complex microbial composition of the global sample) with a sterile lab spoon/spatula;
2. Put the sample in the stomacher blender bag;
3. Measure 90 mL of Ringer solution using a graduated cylinder;
4. Add the Ringer solution to the sample into the stomacher blender bag;
5. Put the stomacher blender bag into the stomacher blender for 3 min at RT;
6. Add 1.8 mL of sample to a 2 mL collection tube;
7. Centrifuge at 800 x g for 5 min at RT;
8. Collect the supernatant in a new sterile 2 mL tube without disturbing the pellet;
9. Centrifuge the supernatant at 13,000 x g for 5 min at 4°C;
10. Discard the supernatant;
11. Spin the tubes at 13,000 x g for 1 min at 4°C;
12. Remove the remaining supernatant completely with a pipette tip;
13. Proceed immediately to DNA extraction or store the pellet at -80°C.

**For cheese and dairy products**

1. Handle the cheese sample under a laminar flow hood, to ensure the sterile conditions;
2. Weigh the cheese sample (10 g) under a laminar flow hood (use a sterile tray or, directly, the sterile filter bag for homogenization step);

**Note 1**: if necessary, freeze the weighted cheese samples at -80°C (directly in a sterile bag) until the genomic DNA extraction step (and start from the homogenization step).

**Note 2**: if the sample has been frozen, proceed with slow thawing (overnight, 4°C) before the homogenization step.

3. Measure 90 mL of Sodium citrate (2% w/v) solution using a graduated cylinder;
4. Add the Sodium citrate (2% w/v) solution to the sample into the sterile filter bag containing the cheese sample;
5. Put the sterile filter bag into the stomacher blender 2 min at RT;
6. Centrifuge (10,000 x g, 5 min, 4°C) 18 mL of strained homogenates in 50 mL sterile DNA-free plastic containers and discard the supernatant;
7. Resuspend the pellet in 1.8 mL of STE buffer (100 mmol/L NaCl, 10 mmol/L Tris, 1 mmol/L EDTA, pH 8) to obtain the initial microbial concentration;
8. Wash the pellet 3 times with STE buffer (12,000 x g, 5 min, 4°C);
9. Discard the supernatant;
10. Remove the remaining supernatant completely with a pipette tip;
11. Pass to DNA extraction immediately or store the pellet at -80°C.

## 9. **Metadata requirement**

Mandatory for fermented meats: recipe, pH and aw determinations. Recommended: fermentation and ripening conditions (temperatures and RH%), standard Microbiological analysis (total viable counts, Lactobacillaceae, Staphylococcaceae and Enterobacteriaceae)

Mandatory for fermented legumes: recipe, pH, TTA and aw determinations. Recommended: standard Microbiological analysis (total viable counts, Lactobacillaceae, Staphylococcaceae, Enterobacteriaceae, yeasts and moulds, Bacillaceae).

Mandatory for sourdoughs: pH and aw determinations. It is advised to fill out a special questionnaire to get information on the method of sourdough preparation: i) the type of flour; ii) the type of "starter" (fruit, honey, flowers, yogurt, etc.); iii) the method of storage (fridge, freezer, RT); iv) the type of bread produced.

Mandatory for fermented table olives: origin of the sample, variety, ripening stage at harvest, fermentation period, temperature of fermentation, sample location (e.g. latitude, longitude), pH. Standard Microbiological analysis (total viable counts, Lactobacillaceae, Staphylococcaceae and Enterobacteriaceae; yeasts and filamentous fungi)

Mandatory for cheese: type of milk (i.e. cow, goat, sheep, other), type of thermal treatments (i.e. none, T°C/time of the treatment), use of starter culture and/or adjunct culture (i.e. indicate the type of starter/adjuncts), ripening conditions (T°C, RH%, time), type of ripening (internal, surface), pH, aw and NaCl% at sampling time; standard microbiological analysis (total viable counts, Enterobacteriaceae, coliforms, lactobacilli and streptococci by using dedicated differential medium)

# 15. SOP FOR LIQUID FERMENTED FOODS (LFFs) SAMPLING

**Prepared by**: Luca Cocolin, Sahar Maghrebi, Ilario Ferrocino, Vittorio Capozzi, Massimo Ferrara, Giacomo Zara, Angela Bianco, Teresa Zotta, Anna Reale, Filomena Nazzaro, and Maria Gullo on behalf of the SUS-MIRRI.IT consortium.

## 1. **Introduction**

The Microbial Resource Research Infrastructure (MIRRI) is the pan-European distributed Research Infrastructure for the preservation, systematic investigation, provision and valorisation of microbial resources and biodiversity. The project SUS-MIRRI.IT, funded by Italy's National Recovery and Resilience Plan – PNRR, is granted by the European Commission's "NextGenerationEU" program. The project is coordinated by the University of Torino and involves 14 other institutions: CNR, ENEA, National Institute of Oceanography and Applied Geophysics (OGS), the Universities of Cagliari, Genoa, Milano Bicocca, Modena and Reggio Emilia, Naples Federico II, Palermo, Perugia, Parma, Sassari, Verona, and the University of Basilicata. Within the project, specific goals include: i) defining the best strategies to sample and store liquid fermented foods (LFFs) for microbiome investigation and ii) optimizing DNA extraction and sequencing techniques to study microbial communities in order to increase the benefit that society can derive from the knowledge and valorisation of microbial biodiversity. This document presents the Standard Operational Procedure (SOP) for LFFs sample collection, storage, and shipment within the SUS-MIRRI.IT project.

## 2. **Objective**

This SOP aims to optimize data comparisons in the food microbiome field by the standardization of the procedure for LFFs sampling.

## 3. **Principle**

The principle of this SOP (SOP for validated sample collection and storage) is to provide guidance for sample collection and storage based on literature survey in conjunction with a survey of the SUS-MIRRI.it partners and using already existing SOPs. In drafting this SOP, several LFFs were taken into account (Table 1). For each step, tips and recommendations are provided.

**Table 1**: The various liquid fermented foods considered in this SOP.

| | **Liquid Fermented Foods** |
|---|---|
| 1 | Fermented beverages |
| 2 | Vinegars |
| 3 | Table olive brines |
| 4 | Milk and fermented milk |

4. **Persons entitled to use the procedure**

This SOP applies to any person involved in collecting, transportation and processing of solid fermented foods samples. This person can be a trainee, fellow, technician or engineer, or any agent in charge of sampling.

5. **Storage temperature of LFFs**

If LFFs are processed immediately after collection, they must be kept in refrigerated conditions (4°C) within 3h. Long-term storage of samples should be at -80°C or transported in dry ice and immediately stored at -80°C. In case of absence of a -80°C freezer, samples can be stored at -20°C. The literature survey indicated that no storage medium was previously used or is necessary for LFF samples storage.

6. **Materials**

- 50 mL sterile DNA-free plastic containers
- Ringer solution (concentration as per manufacturer's instructions)
- 2 mL microcentrifuge tubes
- Pipettes and pipette tips

7. **Equipment**

- Centrifuge
- Ultra-freezer (-80°C) or -20°C freezer if an ultra-freezer is not available

8. **Step-by-step procedure**

*8.1. Sampling*

Put the product (unit/portion) in sterile DNA-free plastic containers.

*8.2. Sample manipulation*

Once taken, all samples are stored in a portable cooler filled with ice packs for transport to the laboratory at 4°C.

**For fermented beverages**

1. Add 1.8 mL of sample to a 2 mL collection tube;
2. Centrifuge at 2,500 x g for 20 min at 4°C;
3. Centrifuge at 13,000 x g for 3 min at RT;
4. Discard the supernatant;
5. Pass immediately to DNA extraction immediately or store the pellet at -20°C.

**For milk and fermented milk** [43]

1. Handle the milk sample under a laminar flow hood, to ensure the sterile conditions;
2. Weigh the sample (10-20 mL) under a laminar flow hood (use a sterile pipet or, directly, the sterile filter bag for homogenization step);

**Note 1**: if necessary, freeze the weighed samples at -80°C (directly in a sterile bag) until the genomic DNA extraction step (and start from the homogenization step).

**Note 2**: if the sample has been frozen, proceed with slow thawing (overnight, 4°C) before the homogenization step.

3. Measure (20-40 mL) of Sodium citrate (2% w/v) solution using a graduated cylinder;
4. Add the Sodium citrate (2% w/v) solution to the sample into the sterile filter bag containing the sample;
5. Put the sterile filter bag into the stomacher blender for 2 min at RT;
6. Centrifuge (10,000 x g, 5 min, 4°C) 18 mL of strained homogenates in 50 mL sterile DNA-free plastic containers and discard the supernatant;
7. Resuspend the pellet in 1.8 mL of STE buffer (100 mmol/L NaCl, 10 mmol/L Tris, 1 mmol/L EDTA, pH 8) to obtain the initial microbial concentration;
8. Wash the pellet 3 times with STE buffer (12,000 x g, 5 min, 4°C);
9. Discard the supernatant;
10. Remove the remaining supernatant completely with a pipette tip;
11. Pass to DNA extraction immediately or store the pellet at -80°C.

**For fermented table olive brine**:

1. Collect 50 mL of samples (representative of the complex microbial composition of the global sample) with a sterile pipette;
2. Centrifuge at 800 x g for 5 min at RT;
3. Collect the supernatant in a new 50 mL sterile tube without disturbing the pellet;
4. Centrifuge the supernatant at 11,000 x g for 15 min at 4°C;
5. Discard the supernatant;
6. Spin the tubes at 11,000 x g for 3 min at 4°C;
7. Remove the remaining supernatant completely with a pipette tip;
8. Proceed immediately to DNA extraction or store the pellet at -80°C.

## 9. **Metadata requirement**

Mandatory for fermented beverages and vinegar: procedure, acetification parameters. Recommended: fermentation system and refilling procedure, standard microbiological analysis (total viable counts).

Mandatory for fermented table olives brine: origin of the sample, variety, ripening stage at harvest, fermentation period, temperature of fermentation, sample location (e.g. latitude, longitude), pH. Standard microbiological analyses (total viable counts, Lactobacillaceae, Staphylococcaceae and Enterobacteriaceae; yeasts and filamentous fungi)

Mandatory for milk: type of milk (i.e. cow, goat, sheep, other), type of thermal treatments (i.e. none, T°C/time of the treatment), use of starter culture and/or adjunct culture (i.e. indicate the type of starter/adjuncts), storage/fermentation conditions (T°C, RH%, time), pH; standard microbiological analyses (total viable counts, Enterobacteriaceae, coliforms, lactobacilli and streptococci by using dedicated differential medium).

# 16. SOP FOR FERMENTED FOODS DNA EXTRACTION

**Prepared by**: Luca Cocolin, Sahar Maghrebi, Ilario Ferrocino, Vittorio Capozzi, Massimo Ferrara, Giacomo Zara, Angela Bianco, Teresa Zotta, Anna Reale, Filomena Nazzaro, and Maria Gullo on behalf of the SUS-MIRRI.IT consortium.

## 1. **Introduction**

The Microbial Resource Research Infrastructure (MIRRI) is the pan-European distributed Research Infrastructure for the preservation, systematic investigation, provision and valorisation of microbial resources and biodiversity. The project SUS-MIRRI.IT, funded by Italy's National Recovery and Resilience Plan – PNRR, is granted by the European Commission's Next Generation EU program. The project is coordinated by the University of Torino and involves 14 other institutions: CNR, ENEA, National Institute of Oceanography and Applied Geophysics (OGS), the Universities of Cagliari, Genoa, Milano Bicocca, Modena and Reggio Emilia, Naples Federico II, Palermo, Perugia, Parma, Sassari, Verona, and the University of Basilicata. Within the project, specific goals include: i) defining the best strategies to sample and store solid and liquid fermented foods (SFFs and LFFs) for microbiome investigation and ii) optimizing DNA extraction and sequencing techniques to study microbial communities in order to increase the benefit that society can derive from the knowledge and valorisation of microbial biodiversity. This document presents the standard operational procedure (SOP) for DNA extraction from solid and liquid fermented food samples within the SUS-MIRRI.IT project.

## 2. **Principle**

This SOP aims to optimize data comparisons in the food microbiome field by the standardization of the protocol for SFFs and LFFs DNA extraction.

## 3. **Objective**

The objective of this document is to standardize the SFFs and LFFs DNA extraction by giving a step-by-step description of the protocol.

## 4. **Persons entitled to use the procedure**

This SOP applies to any person involved in SFFs and LFFs DNA extraction. This person can be a trainee, fellow, technician or engineer in charge of SFFs and LFFs DNA extraction.

## 5. **DNA extraction with the use of DNeasy® powerfood® microbial kit**

### *5.1 Principle*

Microbial food culture is pelleted by centrifugation and resuspended in lysis buffer. The lysed cells are transferred to a bead beating tube containing beads designed for small-cell (microbial) lysis and vortexed using a Vortex Adapter. After protein and inhibitor-removal steps, the DNA released from the lysed cells is bound to a silica spin filter. The spin filter is washed, and the DNA is recovered in Solution EB. Commercial kit such as DNeasy PowerFood Kit from QIAGEN is suggested to be used to isolate high yield and quality of microbial DNA. All procedures for microbial lysis and DNA extraction are referred to the user manual provided with the kit.

*5.2 Materials*

- Gloves
- DNeasy PowerFood Kit (QIAGEN)
- Microcentrifuge (13,000 x g)
- Pipettors (50 µl – 1,000 µl)
- Vortex
- Vortex Adapter for 24 (1.5–2.0 mL) tubes (cat. no. 13,000-V1-24)

*5.3 Conditions and usage constraints to follow*

- All components and reagents of the DNeasy PowerFood Microbial Kit should be stored at RT.
- Solution MBL must be warmed at 55°C for 5–10 min to dissolve precipitates prior to use and used while still warm.
- If Solution MR precipitates, warm at 55°C for 5–10 min. Solution MR can be used while still warm.
- Shake to mix Solution PW before use.
- Ensure that the PowerBead Pro tubes rotate freely in the centrifuge without rubbing.
- Perform all centrifugation steps at RT.

*5.4 Step-by-step procedure*

1. Prepare and homogenize the food sample as already explained in the SFFs and LFFs sampling procedure;
2. Resuspend the cell pellet in 450 µL of Solution MBL;
3. Transfer the resuspended cells to a PowerBead tube;

**Note**: To increase yields or for difficult cells, please refer to the Alternative Lysis Methods section in the Troubleshooting Guide.

4. Secure PowerBead tubes horizontally to a Vortex Adapter (cat. no. 13000-V1-24);
5. Vortex at maximum speed for 10 min;

**Note**: To reduce DNA shearing, please refer to the Alternative Lysis Methods section in the Troubleshooting Guide.

6. Centrifuge the tubes at a maximum of 13,000 x g for 1 min at RT;
7. Transfer the supernatant to a clean 2 mL collection tube (provided);

**Note**: Expect approximately 400 µL of supernatant.

8. Add 100 µL of Solution IRS and vortex briefly to mix. Incubate at 2–8°C for 5 min;
9. Centrifuge the tubes at 13,000 x g for 1 min at RT;
10. Avoiding the pellet, transfer the entire volume of supernatant to a clean 2 mL collection tube (provided);

**Note**: Expect approximately 450 µL of supernatant.

11. Add 900 µL of Solution MR and vortex to mix;
12. Load 650 µL of supernatant onto an MB Spin Column and centrifuge at 13,000 x g for 1 min. Discard the flow-through and repeat until all the supernatant has been loaded onto the MB Spin Column;

**Note**: A total of two loads are required for each sample processed.

**13.** Place the MB Spin Column into a clean 2 mL collection tube (provided);
**14.** Add 650 µL of Solution PW. Centrifuge at 13,000 x g for 1 min at RT;
**15.** Discard the flow-through and add 650 µL of EtOH (provided) and centrifuge at 13,000 x g for 1 min at RT;
**16.** Discard the flow-through and centrifuge at 13,000 x g for 2 min;
**17.** Place the MB Spin Column into a clean 2 mL collection tube (provided);
**18.** Add 60 µL of Solution EB to the centre of the white filter membrane and centrifuge at 13,000 x g for 5 min;
**19.** Discard the MB Spin Column. The DNA is now ready for downstream applications;

**Note**: We recommend storing DNA frozen (–20°C to –80°C) as Solution EB does not contain EDTA.

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