

Guidelines for Environmental Microbial Sample Preparation, Storage and Transportation

Content

1 Recommendations on environmental sample	2
2 Recommendations on nucleotides sample	3
3 Sample preparation and storage guidelines	4
3.1 Precautions before sample preparation	4
3.2 Bacteria	4
3.3 Fungi	5
3.3.1 Unicellular fungi	5
3.3.2 Macrofungi	6
3.4 Nucleotides samples	6
3.5 Soil samples	6
3.6 Sludge samples	8
3.7 Water samples	8
3.8 Surface microbial samples	9
3.9 Fecal samples	10
3.10 Intestinal contents samples	10
3.11 Medical microbes samples	10
3.12 Rumen fluid samples	11
4 Packaging, Transportation, and Other Considerations	12
4.1 Sample Naming	12
4.2 Sample Packaging	12
4.3 Sample Labeling	13

1 Recommendations on environmental sample

Recommendations on tissue samples-Microbe

Species	NGS-based Genome	PB	ONT
Bacteria	1 x 10 ⁷ or 0.3 g	≥ 3.5 x 10 ¹⁰ or 0.5 g	≥ 3.5 x 10 ¹⁰ or 0.5 g
Unicellular Fungus	5 x 10 ⁶ -1 x 10 ⁷ or 1 g	≥ 5 x 10 ⁹ or 3 g	≥ 5 x 10 ⁹ or 3 g
Macro Fungus	≥ 2 g	≥ 10 g	≥ 5 g
Other Fungi	≥ 1-2 g	≥ 3-5 g	≥ 3-5 g

Recommendations on environmental sample (For 3 extraction)

Type	Amplicon	NGS-Metagenome	ONT-Metagenome	PB-Metagenome
Soil/Sludge	2 g	3 g	5 g	10 g
Intestinal contents-Animal	0.5-2 g	0.5-2 g	3 g	5 g
Intestinal contents-Insect	0.1-0.25 g	0.5-2 g	3 g	5 g
Feces (Large animals)	0.5-2 g	0.5-2 g	3 g	5 g
Feces (Mouse)	4-6 pieces	4-6 pieces	5-10 pieces	5-10 pieces
Skin/ Genital swab/ Saliva/ Oral soft tissue/Pharyngeal swab/ Rectal swab	5-10 swabs	5-10 swabs		
Surface microorganism	Filter membranes 3 or Swabs 5 or Enriched sediment 1 - 3g	Filter membranes 5 or Swabs 5-10 or Enriched sediment 3 g		
Waterbody/ Air/Liquid/Biofilm	Filter membranes 3 or Enriched sediment 1 - 3g	Filter membranes 5 or Enriched sediment 3g		
Saliva	2 mL	2 mL		
Endophytes	1-2 g	2-3 g		
Dental plaque	0.5-1 g	0.5-1 g		
Rumen fluid	5-10 mL or Sediment 3g	5-10 mL or Sediment 3g		

2 Recommendations on nucleotides sample

Type	Concentration and Purity					
	Conc.(ng/μL)	Amount (μg)	Volume(μL)	OD260/280	OD260/230	Nanodrop /Qubit
NGS-Amplicon	≥ 1	≥ 0.2	≥ 20			
PB-Amplicon	≥ 5	≥ 0.3	≥ 20			
NGS-Metagenome	≥ 1	≥ 0.03	≥ 20			
PB-Metagenome	≥ 50	10 μg/cell	≥ 20	1.8-2.0	≥ 2.0	1.5-2.5
NGS-Microbial Genome	≥ 1	≥ 0.06	≥ 20			
PB-Bacterial Genome	≥ 20	1 μg DNA/1 Gb data Minimum on 1.2 μg	≥ 20	1.7-2.2	≥ 1.0	0.8-2.5
PB-Fungal Genome	≥ 20	1 μg DNA/1 Gb data Minimum on 2.0 μg	≥ 20	1.7-2.2	≥ 1.6	0.8-2.5
ONT-All Microbial Services	≥ 40	≥ 2.0	≥ 20	1.7-2.2	1.0-3.0	0.8-2.5
Type	Conc.(ng/μL)	Amount (μg)	Volume(μL)	OD260/280	OD260/230	RIN
NGS-Metatranscriptome	≥ 50	≥ 1.0	≥ 20	1.7-2.5	0.5-2.5	RIN≥4.5
NGS-Prokaryotic mRNA	≥ 50	≥ 1.0	≥ 20	1.7-2.5	0.5-2.5	RIN≥4.5

Recommended that stored in Nuclease-free Water or DEPC

For agarose gel, all nucleotides should have no or limited contamination, no or with degradation (The main band is clear).

Note:

1. Due to the uniqueness of some samples, the DNA solution provided may contain colored impurities and viscous insoluble substances, which may affect library construction. The success rate is slightly lower than that of conventional species. Therefore, for these samples, please send at least 1 μg (Qubit) DNA.
2. The nucleic acid concentration of microbial diversity is only used as a reference before amplification; for third-generation microbial diversity, the nucleic acid volume should be increased according to the data volume.

3 Sample preparation and storage guidelines

3.1 Precautions before sample preparation

The quality of the examination is directly affected by whether the specimen collection meets the requirements. Therefore, it is required to be timely, reasonable, effective, and representative:

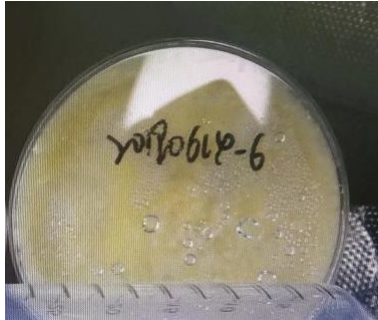
- A. The collected specimen should be placed in a sterile container to ensure that the collected specimen is not contaminated by external sources.
- B. Different methods should be used to collect specimens based on the type of target bacteria (aerobic bacteria, anaerobic bacteria, etc.).
- C. The collected specimen must be representative.
- D. The collection volume should not be too small, otherwise the extracted DNA quantity may be insufficient for subsequent experiments.
- E. Detailed records should be made of the source, collection process, and methods of the samples.

3.2 Bacteria

Note: All bacterial samples must be separated from the bacterial cells or mycelium before submission. Do not send them together with the culture medium, as bacteria with culture medium cannot be extracted.

- 1) Observe the bacterial growth status under a microscope and preferably collect bacteria in the logarithmic growth phase.
- 2) Transfer an appropriate volume of bacterial liquid to a 2 mL sterile screw-capped conical centrifuge tube (free of bacteria and nucleases), and centrifuge at $14,000 \times g$ for 1 minute at room temperature.
- 3) Discard the culture medium and quickly place the bacterial cells precipitate in liquid nitrogen for freezing for at least 1-3 hours (freezing time depends on the tissue amount, ensuring sufficient sample freezing), then transfer to $-80\text{ }^{\circ}\text{C}$ for long-term storage.
- 4) After securely fixing the sample tube on a rack, bury it in the middle section of a dry ice box for transportation of large volumes using dry ice.

Example of **Non**-standardized sample submission:



Example of standardized sample submission:



3.3 Fungi

Fungi exhibit diverse morphologies and are generally classified into unicellular and multicellular fungi. Yeasts are examples of unicellular fungi, while molds and mushrooms (macrofungi) are examples of multicellular fungi.

3.3.1 Unicellular fungi

Yeasts represent the unicellular fungi. The amount of yeast cells required for a single extraction reaction should be $\leq 1 \times 10^7$ cells, with a recommended range of 5×10^6 to 1×10^7 cells. If your project requires a large sample volume, you can divide the sample into separate containers according to the specified quantity requirements.

- 1) Observe the growth status of yeast cells under a microscope and preferably collect yeast cells in the logarithmic growth phase.
- 2) Transfer an appropriate volume of yeast liquid to a 2 mL sterile screw-capped conical centrifuge tube (free of bacteria and nucleases), and centrifuge at $14,000 \times g$ for 1 minute at room temperature.

- 3) Discard the culture medium and quickly place the yeast cell precipitate in liquid nitrogen for freezing for at least 1-3 hours (freezing time depends on the tissue amount, ensuring sufficient sample freezing), then transfer to -80 °C for long-term storage.
- 4) After securely fixing the sample tube on a rack, bury it in the middle section of a dry ice box for transportation of large volumes using dry ice.

3.3.2 Macrofungi

Macrofungi exhibit significant growth form variations due to species differences. When preparing macrofungi samples, you can refer to methods used for plant tissue preparation.

3.4 Nucleotides samples

1) The first option is to directly store the completed DNA solution in a -20 °C freezer. The second option is to precipitate the DNA using sodium acetate-ethanol precipitation method and directly store the sample in a -20 °C freezer. The third option is to add 1 mL of 75 % ethanol to the precipitated DNA pellet and directly store the sample in a -30 °C freezer. The fourth option is to freeze the completed DNA sample into a powder using a low-temperature freeze dryer (can be transported at room temperature).

Note: The first option is recommended.

2) Store the samples in a -20 °C freezer before transportation and transport them using dry ice.

3.5 Soil samples

- 1) Select representative sampling locations and sampling points based on the research objectives.
- 2) Remove the undecomposed litter layer from the soil surface, use a sterilized knife or shovel to collect the top 5 cm of soil, and dig a soil layer from 5 to 20 cm underground. Mix multiple samples from different points uniformly (while preserving appropriate spaces) and label them with sampling location, depth, date, and other information.

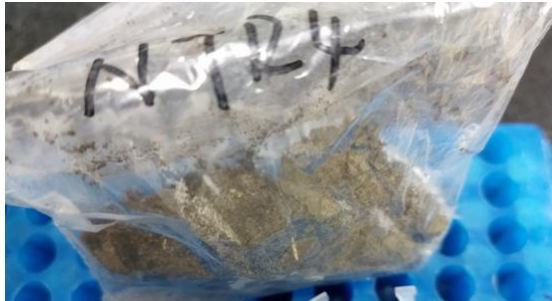
3) After removing visible impurities, screen the soil through a 20-mesh to 60-mesh sieve and transfer it to a 2mL or larger volume EP tube or cryotube. **Each tube should contain approximately 0.25-0.5 g of soil, and the sample quantity should be ensured to be between 1-2 g.** If the soil has a low microbial content, the sample quantity should be increased.

4) Unpackaged samples should not be stored at 4 °C for more than one month. After packaging, store the samples at -80 °C or in liquid nitrogen for long-term storage.

5) Transportation method: Store the samples at 4 °C in an insulated bag for transportation. For samples stored at -80 °C or in liquid nitrogen, transport them using dry ice.

*Note: The laboratory does not have sieving tools, so the teacher needs to sieve the samples before submission. If there are plant residues or other materials in the tissue samples provided, the laboratory will provide feedback and try to avoid but cannot guarantee the complete absence of plant residues during sampling.

Example of **Non**-standardized sample submission:



Example of standardized sample submission:



3.6 Sludge samples

- 1) Select representative sampling locations and sampling points based on the research objectives.
- 2) Collect the sludge samples and place them in sterile centrifuge tubes or other sterile containers.
- 3) Within 4 hours, transfer the samples to 2 mL EP tubes or cryotubes in the laboratory at room temperature. Alternatively, wash the samples with PBS, centrifuge to collect the sediment, and transfer it to 2 mL centrifuge tubes.
- 4) After packaging, store the samples at -80°C or in liquid nitrogen for long-term storage and transport them using dry ice.

3.7 Water samples

Note: Water and air filters should have a diameter of 4 cm, and there should be at least 3 filters (with visible color sediment on the filters).

- 1) Select representative sampling locations and sampling points based on the research objectives.
- 2) Use a multi-point sampling method, avoiding stirring up sediment at the bottom of the water. Avoid contaminating the bottle opening with fingers or other objects.
- 3) Within 4 hours after sampling (during which the samples should be stored at 4°C in the dark), vacuum filter and concentrate the bacteria (parallel replicate samples can be filtered using the same filter). Alternatively, directly centrifuge the water samples at 4°C and 12,000 rpm for 10 minutes, discard the supernatant, freeze the samples in liquid nitrogen, and store them at -80°C for transport using dry ice. Before centrifugation, observe if the water contains abundant organisms. If the biological content in the water is low, centrifuge at least 50 mL of water for enrichment. If the water has a high biological content, centrifuge an appropriate volume of 2-10 mL for enrichment.
- 4) Store the dried and shredded or folded filters with enriched bacteria in 2mL or 5mL sterile EP tubes, and store them at -20°C or -80°C for transport using dry ice.

Notes:

- A. For filtering large volumes of low microbial content clear water samples, use $0.22\ \mu\text{m}$ polyethersulfone filters. Each sample should have at least 1L of water.

- B. For turbid water samples that filter slowly or are prone to clogging with 0.22 μm filters, it is recommended to use 0.45 μm mixed cellulose ester filters (cellulose acetate, nitrocellulose). Avoid using such filters if the water contains insecticides and herbicides. Each sample should have 0.5 L-1 L of water, and if there is clogging, store multiple filters from the same sample in one tube.
- C. If there are many insoluble particles in the water sample, use 2-5 μm pore size filters to remove the insoluble particle impurities, and then use 0.22 μm or 0.45 μm filters to concentrate the bacteria. Each sample should have 0.5 L-1 L of water, and if there is clogging, store multiple filters from the same sample in one tube.
- D. If the study focuses on viruses, first filter out bacteria and other large cells from the water using 0.22 μm filters, and then use positively charged filters (such as Virozorb's 1 MDS or NanoCeram's Virus Sampler cartridges) to concentrate the viruses. Each sample should have 20 L of water.

3.8 Surface microbial samples

Note: Visible changes should be present on the swab surface.

- 1) Select representative sampling locations and sampling points based on the research objectives.
- 2) Collect samples: For surface microbial samples from fruits, plant roots, etc., rinse them repeatedly with sterilized water and centrifuge for enrichment (be careful not to damage the surface of the fruits and avoid collecting juice in the sample). For skin samples, use a cotton swab to scrape the surface approximately 20-30 times. Place the samples in sterile centrifuge tubes or other sterile containers and freeze them at $-80\text{ }^{\circ}\text{C}$.
- 3) For other surface microbial samples, bring them back to the laboratory at room temperature within 4 hours after collection. Wash them with PBS, centrifuge to collect the sediment, and transfer it to 2 mL centrifuge tubes.
- 4) After packaging, store the samples at $-80\text{ }^{\circ}\text{C}$ or in liquid nitrogen for long-term storage and transport them using dry ice.

3.9 Fecal samples

Note: It is not recommended to send fecal samples with preservative solution. Directly sending fecal samples yields better extraction results.

- 1) Wear gloves to collect fresh fecal samples.
- 2) Use sterile cotton swabs or fecal sampling tools to collect the inner portion of the sample (avoiding surface cells from the intestinal mucosa). The external surface is prone to contamination, and bacterial DNA may degrade upon exposure to air.
- 3) Transfer the collected fecal samples into 2 mL sterile EP tubes or sterile cryovials. Each tube should contain 0.5-2 g of fecal material. Prepare 2-3 backup tubes for each sample.
- 4) After packaging, promptly place the samples in liquid nitrogen for rapid freezing. Subsequently, store them at -80 °C or in liquid nitrogen for long-term storage. Transport the samples using dry ice.

3.10 Intestinal contents samples

- 1) After the experimental subject has died, under sterile conditions, remove the entire intestine and use a sterile dissecting knife to extract the contents of the desired intestinal segment.
- 2) Use a sterile surgical blade to scoop the contents and immediately place them on ice for packaging and labeling.
- 3) Transfer the collected samples into 2 mL sterile EP tubes or sterile cryovials. Each tube should contain 0.5-2 g of tissue. Prepare 2-3 backup tubes for each sample.
- 4) After packaging, promptly place the samples in liquid nitrogen for rapid freezing. Subsequently, store them at -80 °C or in liquid nitrogen for long-term storage. Transport the samples using dry ice.

3.11 Medical microbes samples

- 1) Saliva: After selecting individuals that represent the research population, rinse the mouth with sterile water at least 30 minutes in advance. Cleanse the oral cavity of food debris. After 30 minutes, collect the saliva using an appropriate container (preferably a screw-top centrifuge tube). Freeze the sample rapidly in liquid nitrogen and store at low temperature. If rapid freezing in liquid nitrogen is not possible within a short time, specialized collection tubes for sputum/saliva (containing a

preservative) can be used. Use one tube per sample and store at room temperature for 7-15 days.

Sputum or saliva can also be collected using cotton swabs. The pre-processing steps are the same as mentioned before. After moistening the cotton swab with sputum or saliva, store the swab and freeze it rapidly in liquid nitrogen for low-temperature storage (this method is recommended).

2) For sample collection such as lung fluid or ascites during surgery, directly transfer the collected liquid into a suitable collection tube (15 mL), with a minimum volume of 2 mL. If the sample volume is large, centrifuge it at 4 °C, 12000 rpm to separate the lower liquid layer and precipitate. After thorough mixing, freeze the sample rapidly in liquid nitrogen for low-temperature storage.

3.12 Rumen fluid samples

1) In Vivo Sampling (Oral Intubation Method)

Applicable Scenarios: Dynamic monitoring in live animals without the need for slaughter.

Procedure: Immobilize the animal's head. Gently insert a sterilized stomach tube through the mouth into the rumen (insertion depth for adult cattle is approximately 1.5–2 meters). Aspirate rumen fluid using negative pressure or a syringe. Discard the initial portion of the fluid, which may be contaminated with saliva. Collect the middle portion of the sample into suitable collection tubes (15 mL–50 mL). Subsequently, aliquot the sample into 2 mL sterile centrifuge tubes.

Precautions: The procedure must be performed gently to avoid injury to the esophagus or rumen wall. Allow sufficient intervals (at least 2 hours) between multiple samplings to avoid disturbing the rumen environment.

2) Post-Slaughter Sampling

Applicable Scenarios: Obtaining large volumes of samples or full-thick tissue in a single session.

Procedure: Immediately after slaughter, open the abdominal cavity to expose the rumen. Use a sterile scalpel or scissors to incise the rumen wall. Collect the contents directly, taking care to avoid contact with the rumen wall surface to prevent contamination. Place the collected sample into suitable collection tubes (15 mL–50 mL), then aliquot into 2 mL sterile centrifuge tubes. To improve representativeness, mix samples from different rumen sites (e.g., dorsal and ventral sacs).

Precautions: The time from slaughter to sampling should be minimized (ideally <30 minutes) to prevent shifts in the microbial community.

3) Post-Sampling Processing and Storage

Immediately after collection, place the samples on ice or snap-freeze in liquid nitrogen to inhibit microbial metabolism and nucleic acid degradation.

If isolation of microbial cells is required, centrifuge the sample (e.g., $10,000 \times g$ for 10 minutes) to remove particulate material, retaining either the pellet or the supernatant as needed.

Storage Conditions:

Short-term storage: At $4\text{ }^{\circ}\text{C}$ for no more than 24 hours.

Long-term storage: In a $-80\text{ }^{\circ}\text{C}$ ultra-low temperature freezer or liquid nitrogen tank.

4 Packaging, Transportation, and Other Considerations

4.1 Sample Naming

Please use letters and numbers to represent the samples, keeping the length within 8 characters. For backup samples of the same sample, please clearly indicate it in the information form.

4.2 Sample Packaging

When removing tissue samples from liquid nitrogen or $-80\text{ }^{\circ}\text{C}$ freezer (it is recommended to store tissue samples in cryovials; if using snap-cap centrifuge tubes, make sure to seal the tube with Parafilm), place them in an insulated foam box (recommended thickness of at least 2 cm) for sample storage. Prepare approximately 8-10 kg of block dry ice (the specific amount can be consulted with the sample center) to provide a low-temperature environment for sample preservation. Different types of extracted samples must be packaged separately. For example, if extracting root, stem, and leaf tissues from a plant, make sure to package these three types of tissues separately before submitting the samples. For example, when extracting pulp and seeds, make sure to package the seeds and pulp separately before rapid freezing in liquid nitrogen.

For projects with a large number of samples, to expedite the handover and storage of samples, please package every 5-10 samples into a small package according to the sample order in the name or information form. Finally, consolidate the small packages into a large package.

4.3 Sample Labeling

Please clearly and concisely label the sample name on each sample tube (use a high-quality oil-based pen for marking and avoid contact with organic solvents such as ethanol). Do not directly label or attach easily detachable labels on the aluminum foil package to avoid label detachment or unclear numbering during low-temperature storage, which may result in the inability to extract the samples. Seal the sample tubes with Parafilm. The sample name on the tube should match exactly with the sample name provided in the information form.