Environmental DNA Sampling and Experiment Manual

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eDNA Methods Standardization Committee,

The eDNA Society

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1. Introduction

This manual was developed to promote and standardize the methods for environmental DNA (eDNA) analysis. The information provided in this study is current as of August 2024. However, it may be updated at any time to keep up with the incessant progress of eDNA analysis technology. Therefore, to always refer to the latest manual (updated version of this manual). The latest version is available from the website of the eDNA Society of Japan (http://ednasociety.org/).

This is the third revision of the manual, following V2.1 in April 2019 and V2.2 in April 2020. During this intervening period, eDNA has been utilized in various scenarios, and ingenuity and improvements have been made consistent with the actual on-site situations of individual researchers. In this revised version, the content of the manual has been updated based on the methods described in the first edition, and some sections that could not be included in the main text have been introduced in columns. For example, in the section on DNA concentration and extraction method by filtration, we have updated the on-site filtration using syringes and aspirators based on current knowledge. This method has been updated as the filtration method using cartridge filters, as described in the first edition of the manual.

In general, eDNA analysis follows the workflow starting from water sampling through eDNA collection by filtration and eDNA extraction from the filter to the detection of the target species using various molecular techniques. The typical molecular biology techniques for eDNA detection include (1) single-species detection using real-time polymerase chain reaction (PCR, eDNA barcoding) and (2) multiple-species detection for particular taxonomic groups (for example, fish species) using a next-generation sequencing platform (eDNA metabarcoding). The use of eDNA barcoding can detect a specific target species accurately and inexpensively; however, the method requires time-consuming procedures to design specific PCR primers for different target species. The use of eDNA metabarcoding can simultaneously detect multiple species in the biological community using a set of universal PCR primers; however, this technique takes more time and cost than eDNA barcoding. Thus, the two methods may be used in a complementary manner.

This manual provides an overview of the fundamental concepts related to single-species detection, as well as detailed analysis of comprehensive detection methods for fish using MiFish primers. In addition, this revised version has introduced a new section to provide details on eDNA metabarcoding data analysis, which has been requested by many members. Given the constraints of space, items that could not be fully introduced in the main text are presented in the appendix.

Furthermore, although this manual describes standard methods and introduces new technologies, several technologies could not be included in this manual because of limitations in space and the publication schedule. This manual neither negates such technologies nor precludes analysts from making efforts to improve DNA detection sensitivity or minimize labor requirements.

General precautions on environmental DNA analysis

eDNA analysis is a technique for detecting and quantifying a small amount of target DNA in the environment by PCR. Therefore, contamination with high levels of exogenous DNA sequences, including DNA sequences derived from tissue samples or amplification products generated through PCR, often has irreversible effects on analysis results. Thus, performing reliable eDNA analysis is extremely difficult owing to the high chances of contamination. Therefore, the following points must be considered in eDNA analysis:

1) Preparation of the experimental environment: The rooms for handling dilute DNA (e.g.,

environmental samples) must be separated from the rooms for handling dense DNA (e.g., PCR products). In addition, on the day of the experiment, personnel should strictly adhere to the one-way rule, which mandates unilateral movement from the room for handling dilute DNA to the room for handling dense DNA. They should also exercise caution regarding the airflow inside laboratories, including the air conditioning equipment, to reduce the risk of contamination to the maximum possible extent.

2) Use of DNA-free equipment: The equipment to be used in experiments should be new and unused or completely decontaminated and free from residual DNA. For decontamination, immersion in a sodium hypochlorite solution (e.g., 0.1% concentration) is effective. However, the repeated use of this solution can cause metal corrosion and deterioration of plastic components. Various manufacturers provide DNA decontamination reagents that do not result in such deterioration, as well as wet tissue types. Peripheral items, such as tube racks, can also be effectively decontaminated under UV irradiation; however, it should be ensured that there are no shaded areas lacking UV exposure.

3) Wearing gloves: Given that samples may be contaminated by any contact during experiments, wearing disposable gloves, such as medical rubber gloves, is necessary to keep the surfaces clean. When collecting field samples, new gloves must be used at each collection site to prevent sample contamination between sites. Gloves should always be worn throughout the entire process, from the collection of outdoor samples to subsequent DNA measurement, to prevent contamination with one's own DNA or DNA derived from foods on one's hands. In any process, gloves should be replaced frequently when samples or reagents adhere to them during the process.

4) Use of filter tips: The use of filter tips is mandatory to prevent contamination via micropipettes.

5) Use of low DNA adsorption products: As DNA can adsorb to ordinary plastic products, the use of microtubes with low DNA adsorption standard (e.g., DNA LoBind Tubes from Eppendorf) is recommended throughout the process if possible, especially for handling low concentrations of eDNA solutions during extraction.

6) Decontamination of rooms and equipment: Periodic decontamination (preferably on each experimental day) is recommended as contamination may occur through laboratory equipment, including micropipettes and centrifuges. It is also recommended that the equipment be stored in clean, decontaminated, and sealable containers to prevent subsequent contamination. In addition, considering the possibility of contamination through the air, the entire laboratory must be cleaned regularly, air purifiers must be installed, and clean benches appropriate to each process must be used.

Titles to and responsibilities for this manual

This manual was prepared by the members of the eDNA Methods Standardization Committee of the eDNA Society, and experts in eDNA technology. The eDNA Society has copyright to this manual, and the accountability for the contents lies within the eDNA Society and the eDNA Methods Standardization Committee.

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This manual was developed with financial assistance from the eDNA Society. The Committee had independent authority for preparing the text.

For citations to the entire manual or individual chapters, see the “Suggested citations” section at the end of this manual.

2. Selection of sampling sites

General precautions

Biological information in eDNA can be obtained from DNA-containing materials, such as tissue fragments derived from aquatic and marine areas connected to water sampling points. For this reason, it is essential to establish survey sites where samples can be collected in accordance with the research purpose, while paying attention to the upstream environment. Furthermore, eDNA samples may contain various types of DNA apart from those originating from organisms living in the aquatic and marine areas associated with the site. DNA is derived from a diverse array of sources, including fish markets, seafood processing plants, fisheries such as aquaculture farms, sewage water discharged from stores and households, and feed and fertilizer used in agriculture. In addition, DNA may originate from areas distant from rivers and water channels. Although the impact of eDNA is generally assumed to extend to several hundred meters in rivers, it can extend over an even wider area if there is a large mixing volume (concentration × water volume) from the source. It is difficult to completely eliminate the influence of human activities. However, it is recommended to have prior knowledge of the nearby amenities when analyzing the data and, if possible, to set water sampling points that are less affected by such activities. For example, if there is inflow from sewage treatment facilities, such as wastewater treatment facilities, and irrigation/drainage channels, then we may reduce their influence by avoiding areas where the flows converge to the maximum possible extent and by setting up a survey site upstream of the convergence area or on the opposite bank if the channel width is large. Furthermore, when interpreting the results, the water flowing in from such facilities during sampling must be collected and analyzed to identify any potential contaminating species.

Commercial facilities as well as urban and residential areas can be easily identified from maps, aerial photographs from the Geospatial Information Authority of Japan, and public domain websites to select the survey sites. These structures and areas may also be visually identified on the site.

Some facilities are not visible on maps or during walks around the site. Given that outlets are located under embankments and seawalls, they are often not visible from the shore where the personnel are located. The personnel should walk along the waterfront before sampling to identify those structures.

The sea surface and the areas located near terrestrial aquaculture facilities and fish preserves should also be avoided because wastewater containing DNA from fish reared or preserved in facilities is often directly released into the water. These facilities may continuously release high concentrations of DNA, which may have far-reaching effects. In addition, the bait used in aquaculture and fish farming facilities, as well as the sprinkling bait used by anglers, often contain the mince of fish caught outside the area. Thus, there is a risk of detecting DNA from organisms that do not inhabit the water area.

When conducting surveys, consideration must be given to the local residents, as well as to the people and facilities that utilize the water of that area. Water sampling points should be set up to avoid private properties and lands as well as public facilities such as airports and power plants. Depending on the survey site and work, prior permission may be required for some areas. It is also important to avoid collisions with those traveling on the water, such as ships and personal watercraft, and to be considerate of anglers and waterside users. Because permission may be required depending on the site and work, the administrator (or owner in some cases) should be contacted in advance to confirm the required permissions or licenses.

[Column 1] DNA mixed into samples: eDNA samples contain the DNA of the organisms present in the area and the DNA associated with human activities. Apart from the wastewater from sewage treatment facilities, agricultural wastewater, aquaculture and fish farming facilities, and fish markets mentioned in the main text, the DNA present in outdoor campfire remains, trash cans, and feces of dogs, cats, and chickens may have flown into the survey sites during rainy weather. Samples may also be contaminated by the fishing gear and clothing used during the surveys. In addition, contamination

can arise not only from human activities but also from the feces of wild animals. When conducting eDNA analysis that involves the use of minute amounts of DNA, it is difficult to completely prevent the contamination of samples with DNA from organisms other than those present in the area and their effects on the analysis. Therefore, thoroughly assessing and evaluating the suitability of the presence of species in the environment is crucial, instead of solely relying on the list of organisms obtained from the analysis. In this process, data from different periods or nearby areas may be useful. In recent years, there has been progress in the research on environmental RNA, which is believed to have a faster rate of degradation than DNA. We hope that the new analytical techniques will facilitate the assessment of eDNA sources.

[Column 2] Before conducting surveys in downstream river basins and sea areas: In ports, traffic rules have been set out in the Port Regulations Act, which aims to ensure the safety of vessel traffic within ports, and the Maritime Traffic Safety Act, which aims to ensure the safety of vessel traffic in sea areas with heavy vessel traffic, such as Tokyo Bay. Thus, it may be necessary to obtain permission when conducting water or mud sampling. The Port Regulations Act stipulates that anyone who intends to carry out construction or work within a port or near the boundary of a port as specified in Article 2 of the Act must obtain permission from the Japan Coast Guard (such as the Captain of the Port and Chief of the Coast Guard), and measures must be taken to ensure the safety of vessel traffic when obtaining permission. According to the Maritime Traffic Safety Act, permission from the Japan Coast Guard must be obtained when carrying out construction or work on a sea route or in surrounding sea areas. In addition, it is imperative to inform the Japan Coast Guard when conducting construction or work in sea areas other than a sea route or the surrounding sea areas. Therefore, when obtaining permission, measures to ensure the safety of vessel traffic must be taken, as defined in the Port Regulations Act. In areas covered by the law, such construction or work may include not only water sampling using ships but also water sampling from bridges and riverbanks. Thus, when planning a survey, it is necessary to confirm whether the survey site and sampling are covered by such laws and regulations, as well as to submit applications if necessary. In addition, although the work is carried out within an area covered by the Port Regulations Act and the Maritime Traffic Safety Act, permission or notification may not be required depending on the location and work to be undertaken. Therefore, consulting in advance with the Maritime Safety Agency office in the area where the work will be undertaken is recommended.

2-1. Selection of sampling sites in rivers

In rivers, the distance over which eDNA reflects biological distribution is estimated to be several hundred meters, depending on the amount and form of DNA at the source. Therefore, sampling points must be set with an interval of several hundred meters in the survey area, but sufficient resources are often unavailable. Thus, sampling plans should be developed in accordance with budgetary restrictions and objectives while also considering the surrounding and upstream habitat environments.

Water samples collected from rivers upstream of their confluence may provide a comprehensive understanding of the distribution in the rivers.

The following precautions should be taken for determining the survey sites of the rivers. The distribution of eDNA and its diffusion under the influence of water flow are affected by topographical features at various scales. Therefore, attention should be paid at a microhabitat scale. Taking pictures of the environment surrounding the survey site is also important.

1) River channel topography: The flow and sediment deposition conditions, which are important components of a river channel habitat, are influenced by river channel topography (shape). A river has a higher flow velocity on the outer side of curved areas, where the riverbank is eroded and depths are formed with riverside trees overhanging. The inner side of the curved area has a lower flow velocity, which promotes the sedimentation and formation of sandbars that facilitate vegetation colonization. Consequently, microtopographies with different water depths and

riverbed materials are formed on the left and right banks of the curved areas, leading to the formation of specific ecosystems. Depending on the size of the river, the biota of an area may not be accurately captured by sampling from a single bank. In addition, if a survey site is set at XX km from a river mouth, then consideration should be given to whether it is a direct distance between the two points or a distance along the river course, including meandering areas. On the left and right embankments of the river sections managed by the Ministry of Land, Infrastructure, Transport and Tourism, river distance markers are installed on the embankments, with the center of the river as a reference for river management purposes.

2) River flow: Establishing a water sampling point at the site where the flow of the surveyed water area reaches is important. The flow velocity and direction of a river are not uniform even within the same cross-section. The substance-trapping mode differs among the centerline of the stream where the water flow is linear, stagnating aquatic areas, and eddying water flow areas. Thus, the water quality and inhabiting organisms differ accordingly. eDNA released from a certain point has a different diffusion distance depending on the flow velocity, and it may not be sufficiently diffused because of sedimentation in areas with slow flow or other factors. In the vicinity of river convergence areas, surveying each upstream side of the convergence area will aid in identifying the distribution of each tributary.

3) Riverbank structure: As described above, vegetation in a meandering area differs between the left and right banks. For example, riverbank vegetation, such as reed beds, provides a favorable habitat for small swimming fish and juvenile fish. If seawalls are installed at the water edge of riverbanks, attention must be paid to their structure. If seawalls are constructed using stones or environmentally friendly blocks, organisms may inhabit the gaps in the seawalls. Furthermore, if water permeability from behind the block is maintained, allowing groundwater and spring water to flow into the river, it creates a microhabitat for species that prefer low water temperatures.

4) Riverbed sediments: Part of the water in a river that flows on the surface permeates into the river bed, and the ease of permeation varies depending on sediments (riverbed materials, such as rock, gravel, sand, and clay). This flow, which is known as underflow water, has better water quality and less turbidity than river water that flows on the surface because of filtration by gravel. For example, salmon use areas where underflow water is discharged for spawning. Given that substances containing eDNA are filtered and reduced in underflow water, it is necessary to select survey sites where water flowing on the surface can be captured. By contrast, in areas where the riverbed materials contain a large number of fine particles, such as clay, sediments tend to be stirred up when collecting water, which may affect filtration time and analysis results. When water collection is difficult (e.g., at a site where water sampling from a bridge using a water sampler is required or at a site where the water depth is shallow), measures such as slightly shifting the water sampling point must be taken.

5) Artificial changes in the physical field: The physical environment within a river channel may be affected not only by natural disturbances, such as flooding, but also by various artificial factors, such as the maintenance of river channels, bridge construction, construction and renovation of dams, dredging, and gravel extraction. In addition, water flow conditions may change depending on water usage, such as the irrigation and non-irrigation periods downstream of agricultural water intake facilities, as well as night and daytime usage for pumped-storage power generation. It is also important to select a site that is unlikely to be affected by such effects in accordance with the purpose of the survey. In addition, when construction work of river channels is being carried out, the survey work may be affected by restricted access to the site or turbid water. The planned projects can be confirmed through river maintenance plans and other references.

6) Estuary, brackish water, and river tidal areas: In brackish water areas, the state of mixing of river water and seawater, as well as the flow direction and flow speed, fluctuates dynamically because of tidal influences, even at the same site. In addition, the amount and composition of eDNA contained in the water also change. The survey time should be decided based on the purpose of

the survey with reference to the tide table made available by the Japan Meteorological Agency. When sampling water, it is desirable to document not only information on the water sampling location but also the time of water sampling, the surface current direction, and the depth at which the water has been sampled (such as surface, middle, and bottom layers) to determine the influence of tides. The range of backstream and mixing patterns of saltwater varies depending on the riverbed gradient, river flow, the degree of tidal change in the sea area facing the river mouth, and the water temperature of the river. Moreover, in these water bodies, various types of habitats are distributed and changes in flow occur over time because of the tide; these conditions need to be considered while setting the sampling points and time.

2-2. Selection of sampling sites in ponds and lakes

Care should be taken when sampling water that represents the aquatic areas. Small and regularly shaped ponds may not result in significant differences in the detection rate regardless of where the water is sampled. Surface water may be sampled from any point of the water areas if it is easily accessible from shore. Despite the lack of knowledge about the appropriate number of water samples in complex shaped or large ponds and lakes, sampling water from as many points as possible is recommended. The same precautions against any effects of shore structures, sediments, and human activities as described in relation to rivers should be taken for ponds and lakes.

Within a dammed lake, the shallow areas of the lakeshore zone are limited to areas where the original topography is near the water surface of the dam, and steep bedrock often forms below the water surface in the surrounding area. Therefore, the waterside vegetation zone is distributed discontinuously within the dammed lake, and the distribution of the eDNA of organisms that utilize the vegetation zone and have a small range of movement is affected by the distribution of the vegetation zone. Furthermore, lakes have a lower flow velocity and greater water depth than rivers, which hinder the spread of eDNA. Compared with the lakeshore area of the dammed lake, a wider shallow area is formed in the area where the upstream river flows into the lake, and the number of species and individuals of fish caught near the river inflow area is greater than that in the lakeshore area, thereby increasing the likelihood of detecting eDNA from many species. Furthermore, in dammed lakes, fine particles remain suspended even after flooding, which may serve as a PCR inhibitory factor.

The following precautions should be considered when sampling in agricultural canals. Water sources should be identified because canals sometimes receive water pumped from downstream and water supplied through pipelines from other areas. In addition, the volume of water may vary depending on the season. Considerable water is supplied to canals during irrigation, when little water is available during the non-irrigation period. Concrete waterways are slippery, and the water flow may be fast. Therefore, precautions should be taken to avoid accidents. Given that waterways are typically managed by organizations, such as land improvement program entities, consent must be obtained before sampling to avoid problems. In addition, water discharged from paddy fields and other fields may be mixed into the agricultural canals; therefore, the sampled water may contain PCR- inhibiting factors.

2-3. Selection of sampling sites on the coast

Flows in coastal areas are influenced by various factors, including topography, waves, wind direction, and tide level. In addition, given that habitats in coastal areas are also affected by topography at various scales, attention should be paid to flows in coastal areas and the distribution of microhabitats when considering eDNA dispersion. Furthermore, taking pictures of the sampling sites and their surrounding environments is important for confirmation at a later time. The following points should be noted when setting sampling sites on the coast:

1) Coastal topography and bottom sediment: Wave conditions near the shoreline differ depending

on the coastal topography as well as the grain size of the bottom sediment at the shore and the stirring of the sediment caused by the waves. In addition, when water samplers, such as a bucket, come into contact with the bottom sediment, the stirred-up sand and mud can contaminate the water samples, thereby affecting the efficiency of filtration and the quality of the samples. Therefore, it is desirable to confirm the water depth and riverbed materials and select areas where sand or mud is less likely to be stirred up.

A) Rocky or rugged seashores: Water is easily sampled because the sediment is unlikely to be stirred up by waves. These areas have high waves and they are slippery, so safety precautions should be taken to prevent toppling.

B) Sandy beach: The area is relatively accessible. However, the water often contains sand because the bottom sediment is always stirred up near the shoreline (water edge).

C) Tidal flats: The water is often turbid because the water mass always moves over a wide area because of tides and stirs up the sediment. Given the presence of phytoplankton and suspended solids in estuaries and sea areas with high primary production, filtration after sampling may require a long time, and the amount of filtered water will decrease.

2) Coastal flow and water quality: The coastal flow has no uniform flow velocity or direction because the coastal topography is not uniform. The trapping mode of substances in water differs among rocky seashores exposed to waves, the central area of sandy beaches, water stagnation areas around capes and artificial structures, and eddy-flow areas. Thus, these areas have different levels of water quality and biota. Once released from a certain point, eDNA may have a different diffusion distance depending on the flow velocity. Along the coast, water masses move or stagnate in accordance with the tides. However, the water does not flow down and spread in one direction like the freshwater areas of rivers. Considering the effects of freshwater caused by flooding, the persistence of stagnation, and the increase in turbidity is also necessary.

3) Coastal ecosystem structure: Understanding the surrounding ecosystem across the coastal survey site is important. In particular, aquatic organisms and seaweed beds provide a habitat where aquatic organisms live in high density. Their exuberance may be determined by diving surveys and satellite image interpretations.

A) Coastal vegetation varies depending on whether the hinterland is a beach, sand dunes, cliffs, or artificial area (industrial, urban, and residential areas). Thus, interpreting aerial photos provides comprehensive understanding of the wide area surrounding the survey points.

B) Artificial structures, such as seawalls and embankments, may modify the natural habitat; therefore, the extent to which it is modified must be paid attention to. In particular, seawalls completely covering the seashore with concrete block the groundwater leaching from the hinterland and destroy the habitats for interstitial organisms.

C) In the case of seawalls constructed with stones and concrete blocks, the water permeability of the gaps must be considered. If the gaps are filled with concrete or other materials, then the microhabitats of the organisms supported by the permeating water and discharged groundwater are destroyed. Embankments have different levels of water permeability, depending on whether they are clay structures piled up in the past or concrete structures with the foundation laid underground.

4) Artificial changes in the physical field: The physical environment may be artificially altered by dredging, port development projects, creation of reclaimed land, bank protection work, and the installation of wave-dissipating blocks and fish species blocks. It is necessary to understand the spatiotemporal dimensions of these effects, including the scale and continuity of the changes. Recording and photographing the surrounding environment when collecting water are also of immense importance.

A) Landfills eliminate water areas, which may force the monitoring sites to change. In addition, structures protruding into the sea, including breakwaters and jetties, may alter the flow direction and velocity in the surrounding water areas. Therefore, reviewing the maps and personally observing the site in advance e. Construction work for such structures may have begun when the sampling personnel visited the site. The coast, port, or fishing port manager should be contacted to inquire for construction schedules.

B) Submarine drilling, which is performed to develop sea lanes significantly changes the habitat topography, bottom sediment, and vegetation. In particular, when a sandbar is removed, benthic fish as well as their habitats disappears.

C) Turbid water is generated during construction work. The increase in turbidity will affect the filtration work after sampling.

5) Artificial structures (seawalls, embankments, breakwaters, etc.): Many kinds of artificial structures can absorb waves and protect structures. Close attention should be paid to the materials and distances from the shore. Structures constructed away from the shore include foot protection blocks, which are emplaced undersea, tetrapods, which are placed near the water surface; and detached breakwaters, which reduce waves offshore. These structures are constructed with concrete blocks or natural stones. With regard to microtopography, artificial structures have many gaps, and they occasionally provide high permeability, which is contrary to natural coastal reefs. Waves bring the water mass to the structures, which serve as a filter for the water mass. The detached breakwaters are comparable to a small offshore island. Therefore, the structures form a local environment similar to a reef ecosystem.

3. Water sampling and filtration

Water sampling seasons

It has been reported that the detection rate of eDNA decreases in winter. Water sampling, therefore, may have a higher detection rate from spring to autumn. In contrast, the eDNA of some species may be more easily detected during specific seasons or in specific locations because they have unique behavioral characteristics, including seasonal migration. It is important, therefore, to obtain information about the behavior of the target organisms. In addition, externally fertilizing organisms release significant amounts of DNA into the environment during the breeding season. Therefore, species-specific detection of eDNA is expected to have a higher detection rate during the breeding season of the target organism. In contrast, multiple-species detection may have a reduced rate of detecting other species with low amounts of eDNA. For this reason, it is desirable to consider the reproductive season of the target organism according to the characteristics of each method. In addition, because red tides and blue-green algae may cause contamination of sampled water with high levels of PCR inhibitory substances, it is desirable to exclude periods when red tides and blue-green algae break out.

Precautions for safe water sampling

Water sampling for eDNA is performed under many different environmental conditions, depending on the season and location. It is necessary to implement preventive measures against heat stroke and sunburn during the summer, while measures against the cold are required during the winter. Precautionary measures should be implemented to prevent toppling or falling into the water when sampling is conducted along a rocky shore, wet jetty, and the revetments of a reservoir. Water sampling is typically conducted along the water's edge and may cause wetting. It is also important, therefore, to wear water repellent or fast-drying clothing. As a general rule, more than one person should be engaged in the survey and in executing the procedures in the field to prevent contingencies. The use of life jackets is mandatory to ensure safety, especially along the coast and near large rivers. Should personnel drown in Japanese waters, immediately dial the police at 110 if it happens on a river or pond or dial the Coast Guard Hotline at 118 if it happens on the sea.

Equipment, materials, and supplies

The equipment and supplies required for the survey should not be locally procured if possible. There are few DIY or convenience stores in coastal areas, which are often sparsely populated. If any, these stores have a limited variety of products. Ice may be available at supermarkets, fishing gear shops, fresh seafood markets, or convenience stores. These facilities sell seafood, so care should be used to avoid contamination through seafood attached to ice bags. Refrigerants are convenient if they are brought with other supplies in the field. Refrigerants may be stored and cooled in a freezer if sampling personnel stay in an accommodation facility. When conducting field surveys that involve overnight stays, refrigerators and freezers at accommodation facilities may be used to keep "refrigerants" cold or frozen. In such cases, refrigerants should be covered with a plastic bag to protect them against contamination. Dry ice can also be used for freezing.

Filtering methods

Filters used for filtration include glass fiber filters and membrane filters. Some filters are available in sealed containers for ease of on-site use, whereas others are pre-packaged in containers (cartridge filters). This section describes the filtration methods using a filter cartridge on-site after sampling (Section 3-1) and using a glass fiber filter in a laboratory (Section 3-2).

3-1. Water sampling and on-site filtration using a filter cartridge

Precautions for safe water sampling (repeated)

Water sampling for eDNA is performed under many different environmental conditions, depending on the season and location. It is necessary to implement preventive measures against heat stroke and sunburn during the summer, while measures against the cold are required during the winter. Precautionary measures should be implemented to prevent toppling or falling into the water when sampling is conducted along a rocky shore, wet jetty, and the revetments of a reservoir. Water sampling is typically conducted along the water's edge and may cause wetting. It is also important, therefore, to wear water repellent or fast-drying clothing. As a general rule, more than one person should be engaged in the investigation and in executing the procedures in the field to prevent contingencies. The use of life jackets is mandatory to ensure safety and indispensable, especially along the coast and on large rivers. Should personnel drown in Japanese waters, immediately dial the police at 110 if it happens on a river or pond or dial the Coast Guard Hotline at 118 if it happens on the sea.

Tools required to record field data (illustrative)

- Water-resistant field notebooks (e.g., Se-Y11, Kokuyo Co., Ltd.)
- Water-resistant ballpoint pens (e.g., BDWR-40F-B, Pilot Corp.) or pencil
- Handheld GPS (e.g., eTrex20xJ, Garmin International Inc.)
- Data logger conductivity meter (e.g., CD-4307SD, MotherTool Co., Ltd)
- Waterproof digital camera (e.g., Ricoh WG-30, Ricoh Japan Corp.)

Tools required for on-site filtration using syringes (illustrative)

- Filter cartridges (Sterivex, pore size of 0.45 μm, SVHV010RS, Merck KGaA)
- 50 mL lock type syringes (SS-50LZ, Terumo Corp.)
- Luer fittings for inlet port (e.g., VRMP6, ISIS Co., Ltd.)
- Luer fittings for outlet port (e.g., VRSP6, ISIS Co., Ltd.)
- Life jackets
- Buckets (e.g., folding soft bucket 8-type I-484, Iseto Inc.)
- Ropes (Cremona solid cord braided rope with a diameter of 6 mm, Yutaka Make)
- Disposable gloves (powder-free)
- Paper towels for experiments (e.g., Kim Towel, Nippon Paper Crecia Co., Ltd.)
- RNA stabilization solution for tissues (RNAlater, Thermo Fisher Scientific K.K.)
- Disposable pipettes (E-243, Nihon Medical Science, Inc.)
- 2.0 mL tubes (low DNA binding; Sarstedt AG & CO. KG)
- Sodium hypochlorite solution (Highter 1000 400 mL, foam cleaner for medical facilities, Kao Corp.)
- Purified water (e.g., Purified Water P One-touch Cap 500 mL, Kenei Pharmaceutical Co., Ltd.)
- Plastic bags with zippers (140 mm × 200 mm; Unipack G-8, Production Japan)
- Plastic bags with zippers (100 mm × 140 mm; Unipack E-4, Production Japan)
- Plastic bags with zippers (17.7 cm × 20.3 cm; Ziploc Easy Zipper M, S. C. Johnson & Son, Inc./ Asahi Kasei Home Products)
- Counter (e.g., tally counter)
- Writing instruments (felt pen) (e.g., MO-150-MCBK3, Zebra Co., Ltd.)
- Cooler (e.g., Hyper sub-zero temperatures cooler M, Logos Corp.)
- Refrigerant (e.g., Double-speed below freezing Pack M, Logos Corp.)

• Cooling bags, S size (e.g., Cooling flat bag S, Morofuji Corp.)

Tools required for on-site filtration using an aspirator (illustrative)

- Filter cartridges (Sterivex with a hole diameter of 0.45 μm, SVHV010RS, Merck KGaA)
- Luer fittings for inlet port (VRMP6, Isis Co., Ltd.)
- Luer fittings for outlet port (VRSP6, Isis Co., Ltd.)
- Plastic tank 10 L (1-2169-01, ASONE Corp.) with handheld flat bottle
- epTIPS standard 1-10 mL (30000765, Eppendorf AG)
- Luer fittings, male Luer lock 4.0 mm (VPRM406, Isis Co., Ltd.)
- Luer fittings, Mestaper 5.0 mm (VRF506, Isis Co., Ltd.)
- Rubber tube for exhaust (6-590-01, ASONE)
- Tube I type joints (6-663-02, ASONE Corp.)
- Silicon plugs with holes (1-7650-07, ASONE Corp.)
- Aspirator (Gas-1, ASONE Corp.)
- Filter holder manifold (2-258-01, ASONE Corp.)
- Disposable gloves (powder-free)
- Sodium hypochlorite solution (Highter for hospitals, Kao Corp.)*1

*1 Confirm that there are no problems with the expiration date (such as changes in chlorine concentration) before use.

3-1-1. Record of field data

These may be adjusted depending on the purpose of the survey. When recording data in a water-resistant field notebook, it is recommended to use a water-resistant ballpoint pen, pencil, or mechanical pencil.

- Sampling personnel (names of all the sampling team members)
- Date (to be written in YYYY-MM-DD format, preferably with time if needed)
- Survey point number and name of water sampling point (e.g., abbreviation of the project name + survey number + survey point number)
- Latitude and longitude (decimal notations such as 35.101252 N, 139.293012 E are convenient)
- Classification of Riverbank/Lakeshore/Coast/Sediment: Sandy beach, gravel beach, reef, coral reef, seawall (concrete, tetrapods, abandoned stone, etc.)
- Weather and sea conditions (including wind direction, wind force and wave height)
- Water temperature (°C): Measured using a portable water quality meter
- Tide (spring, middle, neap, transitional and long tides) and flux and reflux (high tide, low tide, flood tide, falling tide) (in the sea and river tidal area)
- Salinity (‰): Measured using a portable water quality meter (in the sea to brackish water area)
- Transparency (transparent, slightly turbid, turbid)
- When water is sampled in rivers, record discharges from a dams and power plant if known.
- Filtered water volume (mL): Be sure to record it.
- Visually identified fish and other organisms: Since extracted eDNA contains DNA from organisms other than fish, records of jellyfish and other visually identified organism species may be useful later.
- Photographs (photos were taken or not)
- Other: Any events or conditions likely to have an impact on environmental water (presence of anglers; any discharge or inflows; water management in the surrounding paddy fields)

3-1-2. Water sampling and on-site filtration using syringes

This section describes on-site filtration, which involves loading a Sterivex filter unit in a syringe.

Disposable gloves should be always worn and changed at each water sampling point to prevent cross-contamination. The descriptions below relate the sampling method of using a bucket. Water may be also sampled simply using bottles or syringes.

1) Preparation of filtration kits: For example, when filtration is performed using two Sterivex kits at one sampling point (maximally 1 L × 2 tubes = 2 L), a kit is prepared from two Sterivex kits, which are defined as one set. Put two Sterivex filter units and two syringes in a Unipack G-8. In addition, put two 2.0 mL tubes containing RNAlater and two disposable pipettes together in a small Unipack (E-4). Then, in each Unipack E-4, place two luer fittings for plugging the inlet and outlet ports of the Sterivex filter units. Additionally, place one Unipack E-4 in the kit, which will be used to hold two Sterivex filter units after filtration (Figs. 3-1-2-1 and -2).

2) Preparation of water sampling tools: Since water is typically sampled from a location higher than the water surface, including a jetty, breakwater, and seawall, an approximately 15 m long rope should be firmly tied to a bucket with a cutout. If a soft bucket 8-type I-484 is used, a rope should be tied to two points: the hole on its body edge and its handle because the handle may come off (Fig. 3-1-2-3).

3) Bucket decontamination: Spray the foam cleaner of sodium hypochlorite on the inside of the bucket and the tip of the rope tied to the bucket (Fig. 3-2-4). After allowing the bucket to stand for a few minutes, wipe the cleaner foam with a paper towel for experiments (Fig. 3-1-2-5). Then, wash the bucket and rope tip together twice with environmental water. Any residual cleaner may degrade DNA in the sample.

4) Water sampling with a bucket: Tie the end of the rope to a rock or bridge railing to prevent the bucket from being lost. Throw the bucket (Fig. 3-1-2-6) and pull the rope around to recover the bucket with environmental water (Fig. 3-1-2-7). In order to prevent data bias, sample the water 10 times using a bucket for one sampling and filter 100 mL of environmental water from each water sample using the same syringe. This procedure amounts to filtering 1 L of water per syringe. However, turbid environmental water may clog the filter after several hundred mL of water is filtered, which may make filtration impossible. In such cases, it is important to record the volume of water filtered up to that point.

5) On-site filtration using Sterivex filter units: Aspirate 50 mL of environmental water sampled in a bucket into a syringe (Fig. 3-1-2-8). Take more than 50 mL of environmental water into the syringe and push out any air and excess water from the syringe with the syringe facing up. After adjusting the amount of environmental water to 50 mL, attach a Sterivex filter unit to the syringe and perform pressure filtration (Fig. 3-1-2-9). Be careful not to overtighten the luer lock because the Sterivex filter unit has to be repeatedly attached to and removed from the syringe. Filter the same environmental water sampled with a bucket stroke twice (50 mL × 2 = 100 mL) and then discard the remaining bucket water. Collect the water with a bucket and repeat this procedure 10 times until the total amount of filtered water reaches 1 L (a total of 20 filtration with syringes). Because of the monotonous work, use a counter (tally counter) to avoid mistakes in counting.

6) Removal of water from the Sterivex filter unit: Once all the above filtration procedures are complete, remove the Sterivex and fill the syringe with air. Attach the Sterivex to the syringe again and push the water out of the cartridge (Fig. 3-1-2-10). Repeat this procedure several times to remove as much water as possible.

7) Closing the outlet port of the Sterivex filter unit: When the water inside the Sterivex filter unit is almost removed, plug the outlet port with a luer fitting while the Sterivex filter unit is still attached to the syringe (Fig. 3-1-2-11).

8) Injection of RNAlater: Remove Sterivex from the syringe and use a disposable pipette (Fig. 3-1-2-12) to inject 1–2 mL of RNAlater from the inlet (Fig. 3-1-2-13). Since there is a ledge at the

junction between the inside of the inlet port and the cartridge, RNAlater does not enter well if the tip of a disposable pipette is caught in the ledge. RNAlater can be injected smoothly by inserting the tip of a disposable pipette deeply. In addition, a study has reported that the use of Buffer ATL increases the yield (Wu & Minamoto 2023), and such a method may also be used. In that case, approximately 1–2 mL of Buffer ATL can be used.

9) Closing the inlet port of Sterivex filter unit: Once RNAlater is filled into the Sterivex filter unit, seal the inlet port using a luer fitting (Fig. 3-1-2-14). Be careful not to overtighten the luer fittings. In order to prevent degradation of the DNA collected on the filter in the Sterivex filter unit, it is desirable to complete this procedure at the sampling site.

10) Labeling Sterivex: After injecting RNAlater, thoroughly wipe the moisture on the surface of Sterivex with a Kimtowel paper towel. Write the necessary information, such as date and survey point number, on the Sterivex with a felt pen (Fig. 3-1-2-15).

11) Storage of the Sterivex filter unit: After labeling the Sterivex, place the cartridge in Unipack E-4. Put the Unipack containing the Sterivex filter unit in a zipper lock bag, put the zipper lock bag in a cooler box containing refrigerant and store the box under cool and dark conditions, and take it to the lab (Fig. 3-1-2-16). Store the Sterivex at −20°C or lower temperature.

12) Preparation of blanks: A field blank is prepared by carrying out the same procedure as above using purified water (pure water). The necessity and frequency of field blanks should be carefully determined by each project entity according to the purpose of the survey.

3-1-3. On-site filtration using an aspirator

This section describes an effective method for filtering a large amount of water in a laboratory or on a ship with a 100 V AC power supply. Use a 10 L plastic tank with a stopcock for filtration. Attach a 10 mL pipette tip to the stopcock and screw the inlet port of the Sterivex filter unit into the tip. Furthermore, connect the aspirator via the luer fitting to the outlet port of the Sterivex filter unit and filter a large amount of water by suction filtration. Always wear disposable gloves for experiments to prevent contamination in the following procedures.

1) Decontamination of a polyethylene container: Put a commercially available sodium hypochlorite solution in a polyethylene container and add on-site seawater or tap water to adjust the effective sodium hypochlorite concentration to 0.1% or more (Fig. 3-1-3-1). Shake the container well and then wash three times with on-site seawater to be filtered to remove the bleach.

2) Assembling the filtration unit: Connect a 10 mL pipette tip to the stopcock of the polyethylene container (Fig. 3-1-3-2). Attach a luer fitting (male luer lock) to the outlet port of the Sterivex filter unit (Fig. 3-1-3-3). Connect the rubber tube to the tube I-type joint and attach the silicon stopper with a hole to the tube (Fig. 3-1-3-4). Connect the outlet port of the filter holder manifold to the aspirator with a rubber tube (Figs. 3-1-3-5/6). Attach the above rubber tube to the suction hole of the filter holder manifold (Fig. 3-1-3-7). Finally, firmly push the end of the tip connected to the polyethylene container into the inlet port of the Sterivex filter unit (Fig. 3-1-3-8) to complete the mass filtration system (Figs. 3-1-3-9, -10).

3) Filtration: Add water to the aspirator tank. Use a decontaminated bucket to collect environmental water and place it in a polyethylene container. Switch the aspirator on, water will flow from the polyethylene container toward the aspirator, and eDNA will be trapped on the filter of the Sterivex. Lines marked on the container indicating a volume of 1 L may be useful to check the amount of water aspirated and filtered.

4) Removal of residual water: When filtration is completed, remove the Sterivex filter unit from the pipette tip, continue aspiration and filtration, and the water inside the Sterivex filter unit will

be removed.

5) Treatment of the Sterivex filter unit after filtration: Continue the procedures mentioned in 7) and later in 3-1-2 before storing the Sterivex filter.

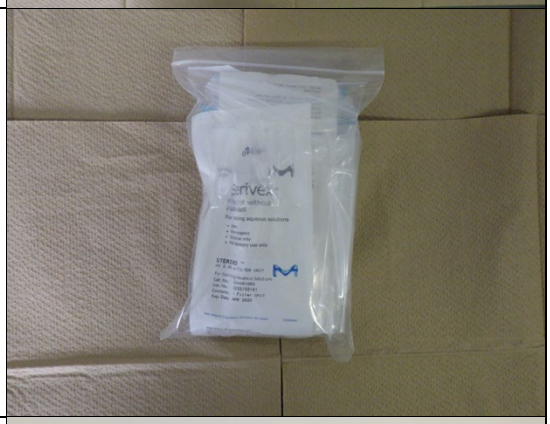
Fig. 3-1-2-1 Contents of on-site filtration kit: 2 × 50 mL syringe, 2 × Sterivex filter unit, 2 × small syringe, 2 × 2 mL tubes with RNAlater, 1 × Unipack (E-4), 1 × Unipack (G-8).	
Fig. 3-1-2-2 The on-site filtration kit is packed in Unipack (G-8). If it is put in one bag like this, it will be easy to use in the field.	
Fig. 3-1-2-3 A life jacket and a bucket. An approximately 15 m long Cremona solid cord braided rope is cut out and firmly attached to a folding bucket (Soft Bucket 8 type I-484). Because the handle is easily detached, make sure that the rope end is tied to the small hole at the bucket edge.	 

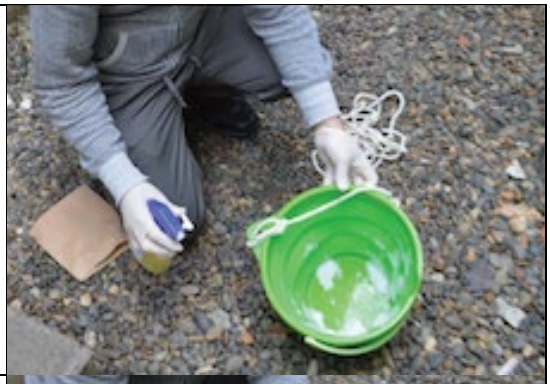
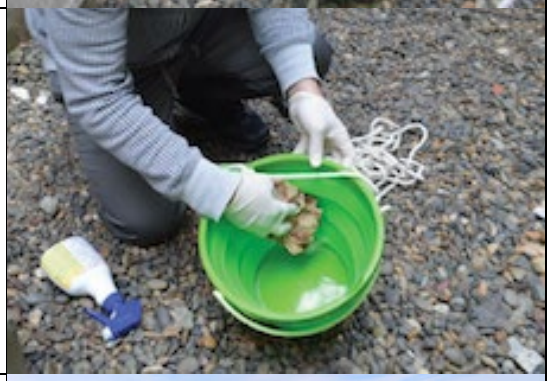
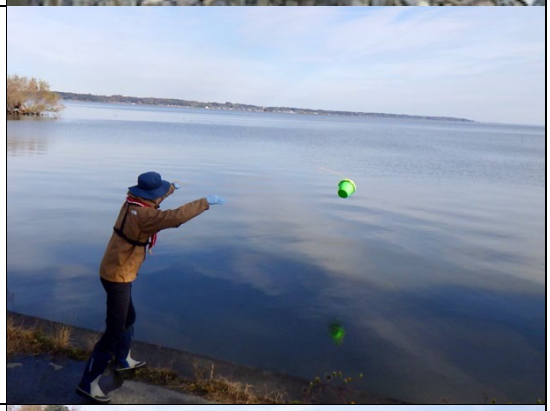
Fig. 3-1-2-4 Decontaminate the inside of the bucket and the tip of the rope attached to the bucket by spraying the foam cleaner of sodium hypochlorite.	
Fig. 3-1-2-5 Wipe thoroughly any residual sodium hypochlorite cleaner with a paper towel. If it is not completely wiped off, bubbles will come out during water sampling and contaminate the environmental water.	
Fig. 3-1-2-6 A bucket is thrown.	
Fig. 3-1-2-7 Haul the rope and recover the bucket with environmental water.	

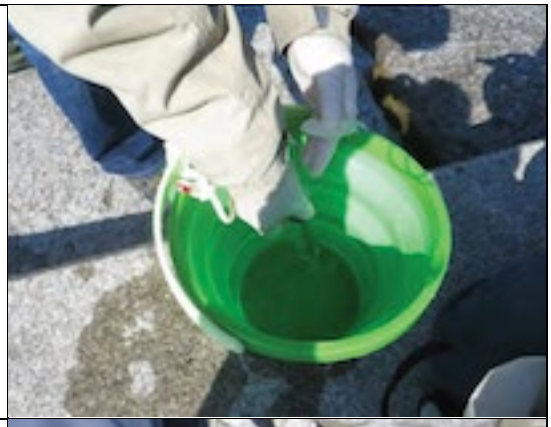
Fig. 3-1-2-8 Aspirate the environmental water from the bucket using a 50 mL syringe.	
Fig. 3-1-2-9 Attach a Sterivex filter unit and perform pressure filtration.	
Fig. 3-1-2-10 When the filtration is finished, remove the Sterivex filter unit and fill the syringe with air. Reattach the Sterivex filter unit to the syringe, and push out the residual moisture from the cartridge. Repeat this procedure several times until no water comes out of the cartridge.	
Fig. 3-1-2-11 Close the outlet port of the Sterivex filter unit with a luer fitting, while the Sterivex filter unit remains attached to the syringe	

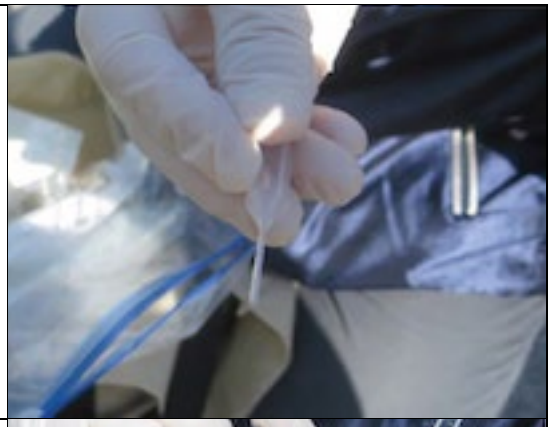
Fig. 3-1-2-12 Aspirate RNAlater from a 2.0 mL tube using a disposable pipette.	
Fig. 3-1-2-13 Inject the aspirated RNAlater from the inlet port using a disposable pipette.	
Fig. 3-1-2-14 Close the inlet port with a luer fitting.	
Fig. 3-1-2-15 Wipe the surface of the Sterivex filter unit with a paper towel, and write the necessary information, including sampling date and survey point number, on the Sterivex with a felt pen.	

Fig. 3-1-2-16 Put two Sterivex filter units from the same site in a Unipack, and put the Unipack in a zipper lock bag. Store the zipper lock bag in a cooler containing refrigerant.	
Fig. 3-1-3-1 Put commercially available bleach solution in a polyethylene container and add on-site seawater or tap water to achieve effective chlorine concentration of 0.1% or more.	
Fig. 3-1-3-2 Push a 10 mL pipette tip into the tip of the stopcock of the plastic tank.	
Fig. 3-1-3-3 Attach a luer fitting (male luer lock) to the outlet port of the Sterivex filter unit.	

Fig. 3-1-3-4 Connect the rubber tube and the silicon with holes through the tube I joint (white joint).	
Fig. 3-1-3-5 Connect the outlet port of the filter holder manifold with a rubber tube.	
Fig. 3-1-3-6 Connect the rubber tube with the aspirator.	
Fig. 3-1-3-7 Attach the above rubber tube to the water absorption hole of the filter holder manifold.	

Fig. 3-1-3-8 Insert the end of a 10 mL pipette tip attached to the stopcock of the polyethylene container into the Sterivex filter unit insertion slot.	
Fig. 3-1-3-9 Mass filtration system using the Sterivex filter units is complete.	
Fig. 3-1-3-10 If there is a power supply of 100 V AC, mass filtration can be easily performed on a fishing boat as shown in the photo.	

3-2. Water sampling and filtration using glass fiber filters in the laboratory

Precautions for safe water sampling (repeated)

Environmental DNA sampling (water sampling) is performed under many different environmental conditions, depending on the season and location. It is necessary to implement preventive measures against heat stroke and sunburn during the summer, while measures against the cold are required during the winter. Precautionary measures should be implemented to prevent toppling or falling into the water when sampling is conducted along a rocky shore, wet jetty, and the revetments of a reservoir. Water sampling is typically conducted along the water's edge and may cause wetting. It is also important, therefore, to wear water repellent or fast-drying clothing. As a general rule, more than one person should be engaged in the investigation and in executing the procedures in the field to prevent contingencies. The use of life jackets is mandatory to ensure safety and indispensable, especially along the coast and on large rivers. Should personnel drown, immediately dial the police at 110 if it happens on a river or pond or dial the Coast Guard Hotline at 118 if it happens on the sea.

Tools required to record field data (illustrative)

- Water-resistant field notebooks (e.g., Se-Y11, Kokuyo Co., Ltd.)
- Water-resistant pressurized ballpoint pens (e.g., BDWR-40F-B, Pilot Corp.)
- Handheld GPS (e.g., eTrex20xJ, Garmin International Inc.)
- Data logger conductivity meter (e.g., CD-4307SD, MotherTool Co., Ltd)
- Waterproof digital camera (e.g., Ricoh WG-30, Ricoh Japan Corp.)

Tools required for water sampling and transportation to a laboratory (illustrative)

• Water sampling bottles (with a volume of 1 L or more, pre-bleached)	More than samples
• Water sampling bottles (containing 1 L of pure water)	1 bottle per day
• 10% benzalkonium chloride solution (divided in 1 mL)[*1]	More than samples
• Disposable gloves (powder-free)	More than samples
• Life jackets	
• Water sampling bucket and rope	1 set
• Sodium hypochlorite cleaner spray	1 bottle
• Pure water[*2]	as needed
• Paper towels	as needed
• Trash bags	as needed
• Boots, chest waders	
• Water quality meter (if necessary)	
• Felt marker, gummed tape, etc.	
• Cooler box	
• Refrigerant	

*1 The use of benzalkonium chloride solution has been patented. Thus, royalty fees may be required for its use, and each user must confirm this in advance. Methods to suppress DNA degradation in water samples include the use of benzalkonium chloride solution and the preservation of water samples by refrigeration or freezing.

*2 This refers to water of a quality that exhibits no trace of DNA residue in PCR and other tests, such

as commercially available purified water and distilled water.

Tools required for filtration using glass fiber filters in laboratory (illustrative)

• Filter holders (pre-bleached [Fig. 3-2-2-1])	as needed
• Aspirator or vacuum pump	as needed
• Glass fiber filters (with mean pore size of 0.7 μm)	Twice as many as filtrations
• Tweezers (pre-bleached)	as needed
• Aluminum foil	as needed
• Chuck bags	as needed
• Bleached buckets	as needed
• Sodium hypochlorite solution	as needed
• Pure water	as needed
• Disposable gloves (powder-free)	as needed
• Freezer (capable of freezing down to −20°C or less)	

3-2-1. Record of field data

The elements to be noted are as follows. They should be written in a water-resistant field notebook with a water-resistant ballpoint pen, pencil, or mechanical pencil.

- Sampling personnel (names of all the sampling team members)
- Date (to be written in YYYY-MM-DD format, preferably with time if needed)
- Survey site number and name of water sampling site (abbreviation of the project name + survey number + survey site number)
- Latitude and longitude (Decimal notations such as 35.101252 N, 139.293012 E are convenient)
- Classification of riverbank/lakeshore/coast/sediment: Sandy beach, gravel beach, reef, coral reef, seawall (concrete, tetrapods, abandoned stone, etc.)
- Weather and sea conditions (including wind direction, wind force, and wave height)
- Water temperature (°C): Measured using a portable water quality meter
- Tide (spring, middle, neap, transitional, and long tides) and flux and reflux (high tide, low tide, flood tide, falling tide) (in the sea)
- Salinity (‰): Measured using a portable water quality meter (in the sea)
- Transparency (transparent, slightly turbid, turbid)
- Filtered water volume (mL): Be sure to record it if the volume is less than 1000 mL.
- Visually identified fish and other organisms: Since extracted eDNA contains DNA from organisms other than fish, records of jellyfish and other visually identified organism species may be useful later.
- Photographs (photos were taken or not)
- Other: Any events or conditions likely to have an impact on environmental water (presence of anglers, any discharge or inflows, and water management in surrounding paddy fields)

3-2-2. Water sampling and transportation to a laboratory

Always wear disposable gloves and change them at each water sampling point to prevent cross-contamination.

1) Direct sampling on the waterside: If direct access to the waterside is possible, sample water by placing a sampling bottle (e.g., a bottle that has been disinfected with chlorine in advance; Figure 3-2-2-1) directly into the water. After washing the bottle twice with on-site environmental water, sample a little more than 1 liter of environmental water. To prevent contamination, ensure that the environmental water discarded after washing is not mixed with environmental water to be sampled. If water is sampled in a river, discard the wastewater used for washing downstream (Fig. 3-2-2-2). Be careful not to stir up mud during the sampling.

2) Use of a bucket: If the waterside is not accessible, use a bucket to sample river water.

   A) Bucket decontamination: Spray the foam cleaner of sodium hypochlorite on the inside of the bucket and the tip of the rope attached to the bucket (Fig. 3-2-2-4). After allowing them to stand for a few minutes, wipe any residual cleaner with a paper towel for experiments (Fig. 3-2-2-5). Then, wash the bucket and rope tip twice with environmental water. Any residual cleaner may degrade the DNA in the sample.

   B) Bucket sampling: Throw the bucket and haul the rope to recover the bucket containing the environmental water (Fig. 3-2-2-6). Wash the bucket twice with the collected water. Then wash the bottles twice with the collected water and sample slightly more than 1 liter of environmental water. To prevent contamination, ensure that the environmental water discarded after washing is not mixed with the environmental water to be sampled. If water is sampled from a river, discard the wastewater used for washing downstream.

3) Field blank: In the field, open the bottle containing pure water brought from the laboratory and treat it in the same manner as the samples.

4) Transport to the laboratory: Transport sampled water to the laboratory away from direct sunlight and high temperatures. There are several ways to preserve the collected water: (1) To suppress DNA degradation, add 1 mL of 10% benzalkonium chloride solution to 1 L of the sample (final concentration: 0.01%), seal it, mix it thoroughly by inverting, and transport the sample to the laboratory (Figure 3-2-2-3). (2) Place the collected water in a cooler on the spot, refrigerate it, and take it to the laboratory. Benzalkonium chloride, if added, enables the preservation of DNA for several days, even at room temperature. It is, however, desirable to keep the water at as low a temperature as possible. In addition, transport samples away from direct sunlight, as ultraviolet rays damage DNA. Immediately after transportation, the following filtrations should be promptly performed.

3-2-3. Filtration using glass fiber filters

Filter samples taken back to the laboratory as soon as possible (within 48 hours after sampling). The personnel who perform filtration should wear disposable gloves throughout.

1) Preparation for bleaching: Put tap water in a bucket and add a commercially available sodium hypochlorite solution to a working chlorine concentration of 0.1% or more.

2) Bleaching of tools: Immerse filter holders and tweezers in a bleaching bucket for more than 5 minutes before use (Fig. 3-2-3-1). Rinse them with tap water and then rinse with distilled water before use. This bleaching must be done every time a new sample is handled. Since it is necessary to decontaminate the entire bottle, including the bottle surface, after use, immerse the entire bottle in a bleaching bucket for 5 or more minutes for decontamination before use in the next survey.

3) Filtration: One or two glass fiber filters (mean pore size: 0.7 μm) are used for the filtration of a 1 L water sample (Figs. 3-2-3-2 to -4). In addition, it may be difficult to filter 1 L depending on the sample water. In such a case, be sure to document the amount of water filtered. Even if the amount of filtered water is reduced, it is recommended to use no more than two filters per sample. Care

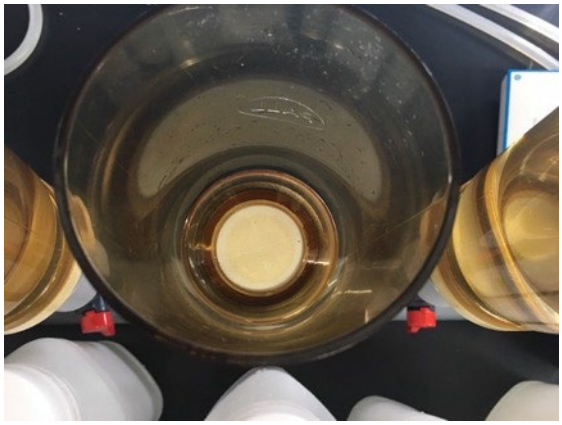
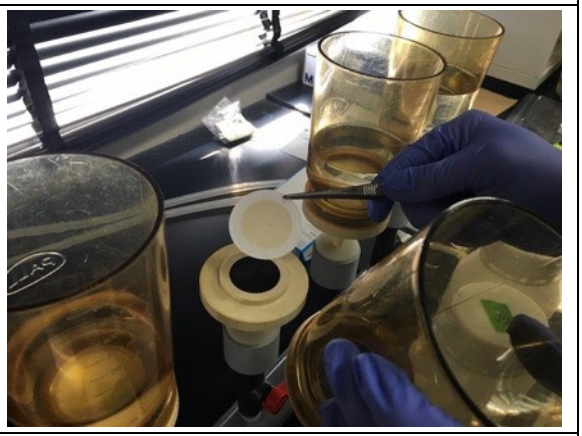
must be taken to keep filters higher than the sample water and not to leave the filter container open, in order to prevent contamination due to sample water being applied to the unused filter.

4) Storage of the filter: The filter after filtration is halved with the filtration surface inside; wrap the two filters together in aluminum foil (Fig. 3-2-3-5). Write the sample name on the aluminum foil, put it in a bag such as Unipack, and store it in a freezer (−20°C or lower) (Fig. 3-2-3-6). In addition, it is recommended to promptly proceed with the next process (DNA extraction).

5) Filtration blank: To evaluate the presence or absence of contamination during and after filtration, prepare a “filtration blank” of 1 L pure water once per day of work and treat it in the same manner as the samples. However, a “field blank” may be used as a substitute for the filtration blank. If benzalkonium chloride is added at the time of transportation, similarly add 1 mL of 10% benzalkonium chloride solution to 1 L of pure water (final concentration: 0.01%), seal it, mix it thoroughly by inverting, and treat it as a “filtration blank”.

Fig. 3-2-2-1 Decontaminate sampling bottles with a sodium hypochlorite solution before use.	
Fig. 3-2-2-2 Discard the environmental water used for washing bottles at a location, including a downstream area, where the discarded water will not affect the sample.	
Fig. 3-2-2-3 (Optional) After sampling water, add 1 mL of 10% benzalkonium chloride solution.	
Fig. 3-2-2-4 Decontaminate the inside of the bucket and the tip of the rope attached to the bucket by spraying the foam cleaner of sodium hypochlorite.	

Fig. 3-2-2-5 Wipe thoroughly any residual sodium hypochlorite solution with a paper towel for molecular experiments. If it is not completely wiped off, bubbles will come out during water sampling and contaminate the environmental water.	
Fig. 3-2-2-6 Haul the rope and recover the bucket with environmental water.	
Fig. 3-2-3-1 Decontaminate equipment used for filtration with a sodium hypochlorite solution before use.	
Fig. 3-2-3-2 Samples are filtered.	

Fig. 3-2-3-3 Filter after filtration.	
Fig. 3-2-3-4 Filter after filtration.	
Fig. 3-2-3-5 Shade the filtered filter with aluminum foil.	
Fig. 3-2-3-6 Write sample information on aluminum foil and store it in a bag, such as a Unipack	

4. Extraction of DNA

Precautions for sample storage

Filters that have been used to filter environmental water need to be stored in a freezer. Pay close attention to the temperature control of the freezer to avoid repeated freezing and thawing.

Common precautions for DNA extraction

To reduce the risk of contamination, it is important to review the procedures scheduled for the day and their sequences in advance. Specifically, the work area used for DNA extraction should be physically isolated from the equipment and samples used after PCR, and it should be ensured that personnel are not engaged in DNA extraction or prior procedures after performing post-PCR procedures on the same day. In addition, it is recommended to decontaminate the work area, tools, and equipment before and after work.

DNA extraction from filter cartridges (4-1) and DNA extraction from glass fiber filters (4-2) are described below.

4-1. DNA extraction from a filter cartridge

Introduction

This section describes a method for extracting DNA from a Sterivex filter cartridge. This method is a slightly modified version of the method published in the *Journal of Visualized Science* (Miya et al., 2016), a video journal, and a series of techniques are visually illustrated in the video. This protocol does not use Buffer ATL supplied with the DNeasy kit, but a study has reported that the use of Buffer ATL increases the yield (Wu & Minamoto, 2023; Fukuzawa et al., 2023). Furthermore, a study has shown that the yield can be increased by increasing the amount of reagents used in DNA extraction (Wong et al., 2020), suggesting that these approaches may be equally effective.

From this point on, careful attention must be paid to contamination with exogenous DNA, as the procedures take place in the laboratory. In particular, contamination at this stage results in the failure of subsequent experiments (preparation of real-time PCR samples and next-generation sequencing libraries). To avoid this undesirable situation, a room dedicated to DNA extraction should be set up, which must be sufficiently separated from the rooms used for PCR-related procedures. In addition, great care must be taken not to enter the DNA extraction room after handling DNA extracted from tissues or PCR products on the same day.

Laboratory tools, reagents, and consumables required for DNA extraction (illustrative)

- Incubator (capable of operating at 56°C, with a mini-rotator installed inside)
- Mini rotator (ACR-100, Asone Corp.) and attached 10 mL/15 mL tube holder
- QIAvac 24 Plus System (connection adapter set including QIAvac 24 Plus Vacuum Manifold (QIAvac 24 Plus), vacuum trap and connecting tube, installation tray set (QIAvac Connecting System), and vacuum pump; all from Qiagen KK) ※1
- Centrifuge (capable of centrifuging 50 mL conical tubes)
- High speed refrigerated microcentrifuge (capable of centrifuging 2.0 mL tubes and DNeasy columns)
- Desktop small centrifuge (Micro Six MS-1, Asone Crop)
- Vortex mixer (Vortex-Genie 2 Mixer, MS Equipment Co., Ltd.) and 3-inch platform
- Luer fitting (for connecting to a suction device, VPRM406, Isis Co., Ltd.)
- Luer fitting (for the inlet port, VRMP6, Isis Co., Ltd.)
- Luer fitting (for the outlet port, VRSP6, Isis Co., Ltd.)
- 50 mL conical tubes (Nippon Genetics)
- DNeasy Blood & Tissue kit (Qiagen KK)
- 96%–99.5% Ethanol (for molecular biology, Fujifilm Wako Pure Chemical Industries, Ltd.)
- Low DNA binding 2.0 mL tubes (Sarstedt K.K.)
- Low DNA binding 1.5 mL tubes (Sarstedt K.K.)
- PBS (–) (phosphate buffered saline without magnesium and calcium, Cell Science Laboratories) [2]
- Disposable gloves (powder-free)
- Micropipette P-1000, P-200, P-100 (Pipetman, Gilson)
- Low DNA binding filter tips (Various types according to the capacity of the micropipette used. They need to be compatible with the micropipette used.)
- One standard tweezers (IPT-12, ASONE Corp.)
- Tube racks for 1.5 mL/2 mL

※1 Instead of using the QIAvac apparatus, RNAlater may be removed using a centrifuge after combining a 50 mL conical tube and a 2.0 mL tube (see Miya et al. 2016).

※2 This protocol does not use buffer ATL supplied with the DNeasy kit. However, it has been reported that the use of Buffer ATL is effective (Wu & Minamoto, 2023; Fukuzawa et al., 2023), and such a method may also be used.

4-1-1. Preparation for experiment

Be sure to wear disposable gloves during the experiment. (If the gloves are contaminated during the work, replace them immediately.)

1) Set the temperature of the fan oven to 56°C. (Increase the temperature of the oven well in advance because it takes time to warm up; Fig. 4-1-1-1.)

2) Prepare filtered Sterivex filter cartridges filled with RNAlater. If it has been stored in a frozen state, thaw it thoroughly at room temperature before proceeding (Fig. 4-1-1-2).

4-1-2. Aspiration of RNAlater

1) Prepare the extraction solution (premix) using the DNeasy Blood and Tissue kit and PBS (-) (Figure 4-1-3-1). Prepare a premix with 20 µL of Proteinase K (600 mAU/ml, provided in the kit), 200 µL of Buffer AL, and 220 µL of PBS (-) per Sterivex. This also includes one “extraction blank” to detect contamination during DNA extraction.

2) Connect the QIAvac 24 Plus vacuum manifold with the Sterivex filter unit via the luer fitting (Fig. 4-1-2-1).

3) Turn on the QIAvac pump, and aspirate and remove the RNAlater from the inlet port toward the outlet port. (A slight amount of RNAlater remains within the Sterivex cartridge because of its structure; the residual RNAlater will cause no problem during DNA extraction.)

4) Prepare the same number of luer fittings (VRSP6) as the Sterivex filters.

5) Disconnect the Sterivex filter unit, from which RNAlater has been removed, from the manifold and seal the outlet port with a luer fitting (Fig. 4-1-2-2).

4-1-3. DNA extraction

1) Open the inlet port of the Sterivex filter unit and fill the filter unit with the above premix (Fig. 4-1-3-1) using a micropipette (P-1000) and a 1000 µL filter tip. (Caution: there is a ledge at the junction between inside the inlet port and the cartridge; the liquid may overflow if the tip is not properly inserted; Fig. 4-1-3-2.)

2) Seal the inlet port of the Sterivex filter unit with a luer fitting (VRMP6) (Fig. 4-1-3-3).

3) Insert the Sterivex filter unit into the 10 mL/15 mL tube holder of the mini-rotator and attach the tube holder to the rotator body so that the Sterivex filter unit is horizontal.

4) Place the rotator with Sterivex filter units attached in an incubator, rotate it at 10 rpm, and heat it at 56°C for 20 minutes (Note: The endurance temperature of the mini-rotator is 60°C; Fig. 4-1-3-4).

5) While heating the Sterivex filter unit, prepare a 2.0 mL tube for DNA recovery (low DNA adsorption) and a 50 mL conical tube (Fig. 4-1-3-5), bend the cap of the 2.0 mL tube, and place it in the 50 mL conical tube (Note: Write the necessary information on the cap of the 2.0 mL tube, such as the number, in advance. Do not push the tube all the way into the conical tube; Fig.

4-1-3-6).

6) After completion of warming, carefully remove the luer fitting on the inlet port of the Sterivex filter unit, while preventing liquid inside from leaking.

7) Insert the inlet port of the Sterivex filter unit into the 2.0 mL tube contained in the conical tube and lightly push it down to the bottom of the 50 mL conical tube (Fig. 4-1-3-7). The 2.0 mL tubes could incur damage during centrifugation if they are not properly fully inserted. Then, close the cap of the conical tube firmly (Fig. 4-1-3-8).

8) Centrifuge the conical tube containing the Sterivex filter unit at 6,000 g for 1 minute (Fig. 4-1-3-9) and collect the extracted DNA in a 2.0 mL tube (Fig. 4-1-3-10).

9) Remove the 50 mL conical tube from the centrifuge and remove the Sterivex filter unit (Fig. 4-1-3-11) and 2.0 mL tube in order using tweezers. (Fig. 4-1-3-12) (Note: The 2.0 mL tube is uncapped; handle it carefully.)

10) Discard the used Sterivex filter unit and firmly cap the 2.0 mL tube.

4-1-4. DNA purification using a commercial kit

1) Make as many columns attached to the DNeasy Blood & Tissue kits (DNeasy) as the Sterivex filter unit filter units plus one extraction blank available (Fig. 4-1-4-1). Write the necessary information on the column cap.

2) Add 200 µL ethanol (96% to 99.5%) to the 2.0 mL tube containing the extracted DNA and mix thoroughly with a pipette (Fig. 4-1-4-2).

3) Set the suction volume of the pipette (P-1000) at 700 µL and pipet the extracted DNA into the column. (Note: The solution may reach a larger volume than 640 µL because of a small amount of residual RNAlater; Fig. 4-1-4-3). The extraction blank is obtained by adding 200 µL ethanol (96% to 100%) to 440 µL of the mixture prepared in 4-1-3 (see above) and mixing the mixture with a pipette.

4) Centrifuge the column containing the solution at 6000 g for 1 minute (Fig. 4-1-4-4).

5) After centrifuging, remove the column collection tube and place the column on a new 2 mL collection tube (Fig. 4-1-4-5). Discard the used collection tube (Fig. 4-1-4-6).

6) Add 500 µL Buffer AW1 to the column (Fig. 4-1-4-7) and centrifuge at 6000 g for 1 minute.

7) After centrifuging, place the column to a new 2 mL collection tube (Fig. 4-1-4-8). Discard used collection tubes.

8) Add 500 µL of Buffer AW2 to the column (Fig. 4-1-4-9) and centrifuge for 3 minutes at the maximum speed of the centrifuge used.

9) Prepare a new 1.5 mL tube with low DNA adsorption and write the necessary information on the cap (Fig. 4-1-4-10).

10) After centrifuging, place the column in the new 1.5 mL tube (Fig. 4-1-4-11). Discard used collection tubes.

11) Add 100–200 µL of Buffer AE (elution buffer) onto the column membrane (Fig. 4-1-4-12), incubate at room temperature for 1 minute, and then centrifuge at 6,000 g for 1 minute (Fig. 4-1-4-13). If the concentration of recovered DNA is expected to be low, the amount of elution

buffer can be reduced to approximately 50 μL. Record the amount of buffer used for elution.

12) After centrifuging, remove the column and tightly cap the tube (Fig. 4-1-4-14). Discard the used column.

13) The purified DNA can be stored stably at –20°C (Fig. 4-1-4-15).

Reference

Fukuzawa, T., Shirakura, H., Nishizawa, N., Nagata, H., Kameda, Y. & Doi, H. 2023, "Environmental DNA extraction method from water for a high and consistent DNA yield." *Environmental DNA* 5 (4): 627–633. doi: 10.1002/edn3.406

Miya, M., Minamoto, T., Yamanaka, H., Oka, S., Sato, K., Yamamoto, S., Sado, T. & Doi, H. 2016. "Use of a filter cartridge for filtration of water samples and extraction of environmental DNA." *Journal of Visualized Experiments,* (117): e54741. doi: 10.3791/54741

Wong, M.K.S, Nakao, M. & Hyodo, S. 2020, "Field application of an improved protocol for environmental DNA extraction, purification, and measurement using Sterivex filter." *Scientific Reports* 10: 21531. doi: 10.1038/s41598-020-77304-7

Wu, Q. & Minamoto, T. 2023. "Improvement of recovery yield of macro-organismal environmental DNA from seawater samples." *Analytical Sciences* 39, 713-720. doi: 10.1007/s44211-023-00280-1

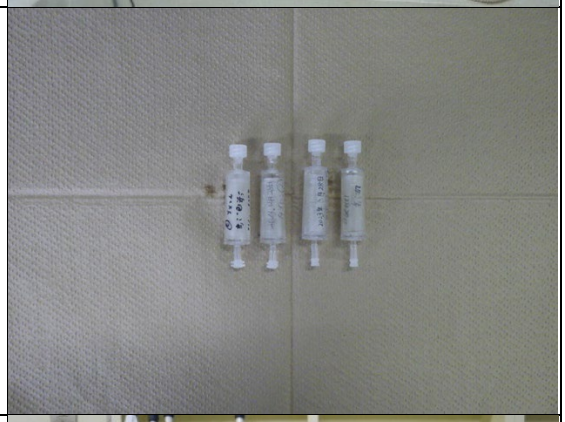
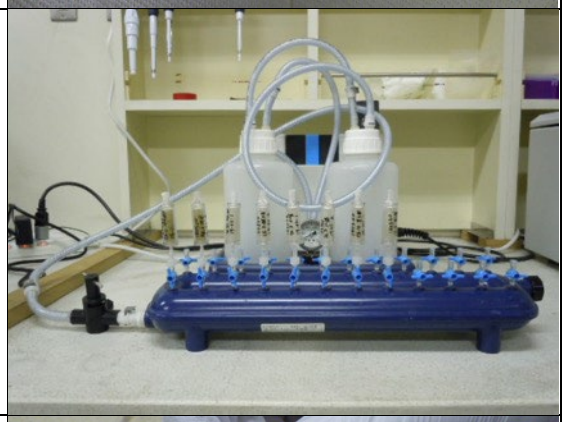
Fig. 4-1-1-1 Set the fan oven at 56°C. Since it takes time to reach the specified temperature, start heating the oven sufficiently before the DNA extraction.	
Fig. 4-1-1-2 Thaw the Sterivex filter units frozen at –20°C. Sterivex filter units stored in a refrigerator do not have to be thawed (Fig. 4-1-1-2).	
Fig. 4-1-2-1 Open the outlet ports of the Sterivex filter units, connect them with QIAvac via luer fittings, and extract RNAlater filled in Sterivex filter units.	
Fig. 4-1-2-2 Again, close the outlet port with the luer fitting.	

Fig. 4-1-3-1 Make the premixes necessary for DNA extraction available.	
Fig. 4-1-3-2 Open the inlet port of the Sterivex filter unit and fill the above premix using a micropipette (P-1000) and a 1000 μL filter tip.	
Fig. 4-1-3-3 Close the inlet port of the Sterivex filter unit with the luer fitting.	
Fig. 4-1-3-4 Place the rotator equipped with Sterivex filter units within a fan oven, rotate at 10 rpm, and maintain the temperature at 56°C for 20 minutes.	

Fig. 4-1-3-5 While heating Sterivex filter unit units, prepare a new 2.0 mL tube (low DNA adsorption) and a 50 mL conical tube available for DNA collection.	
Fig. 4-1-3-6 A 2.0 mL tube is inserted into a 50 mL conical tube.	
Fig. 4-1-3-7 Insert the inlet port of the Sterivex filter unit into the 2.0 mL tube and push it down to the bottom of the 50 mL conical tube.	
Fig. 4-1-3-8 Push the Sterivex filter unit and the 2.0 mL tube into the bottom of the conical tube and then close the cap of the conical tube firmly.	

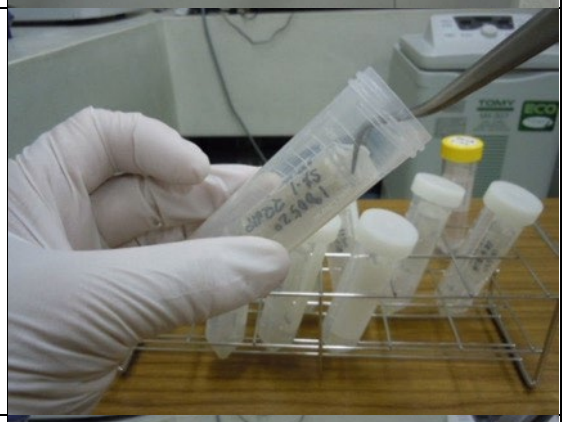
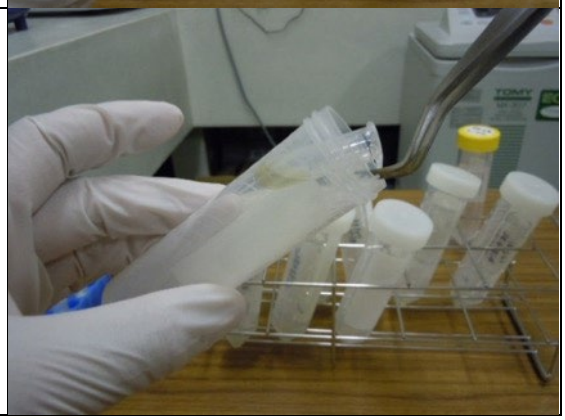
Fig. 4-1-3-9 Centrifuge the conical tube containing the Sterivex filter unit at 6000 g for 1 minute.	
Fig. 4-1-3-10 Extracted DNA is collected in a 2.0 mL tube within a conical tube.	
Fig. 4-1-3-11 Remove the Sterivex filter unit carefully from the conical tube using tweezers.	
Fig. 4-1-3-12 Next, remove the 2.0 mL tube from the conical tube using tweezers. Remove the tube carefully because the tube remains open.	

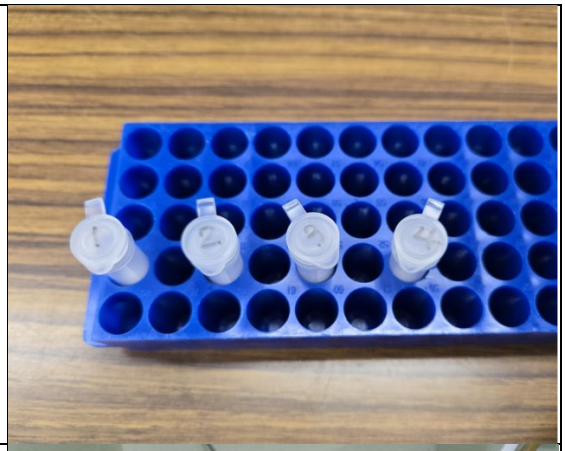
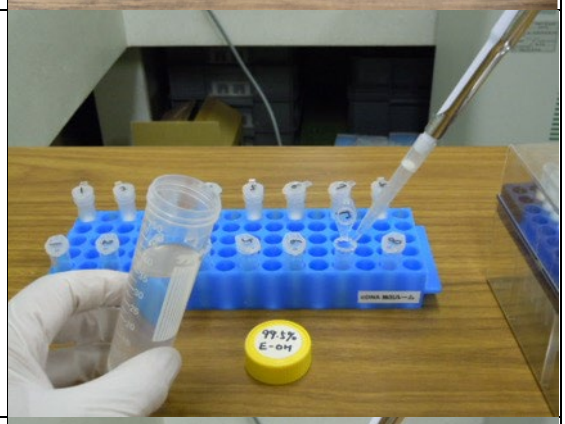
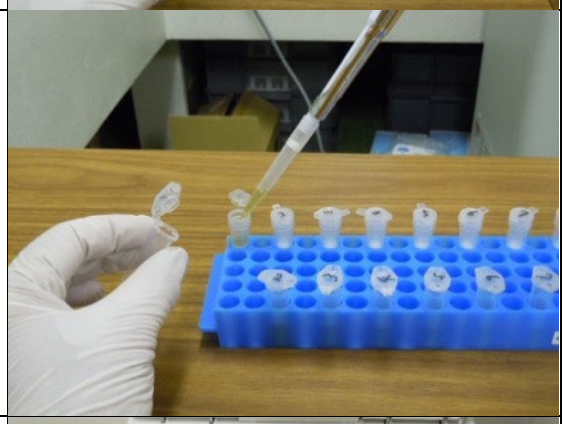
Fig. 4-1-4-1 Place the column (with collection tube) supplied with the DNeasy Blood & Tissue kit in a tube rack.	
Fig. 4-1-4-2 Add 200 µL ethanol (96% to 100%) to the 2.0 mL tube containing the extracted DNA and mix thoroughly with a pipette.	
Fig. 4-1-4-3 Place the extracted DNA in the column.	
Fig. 4-1-4-4 Centrifuge the column containing the extracted DNA at 6000 g for 1 minute.	

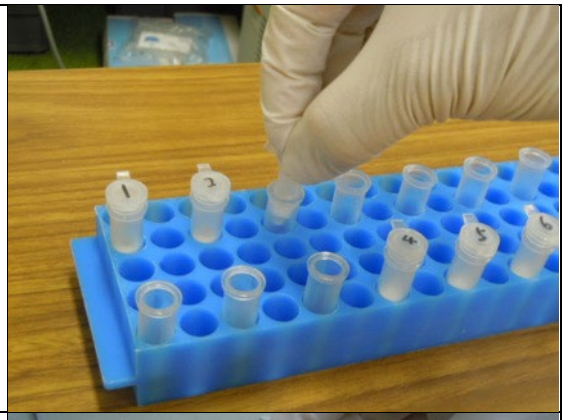
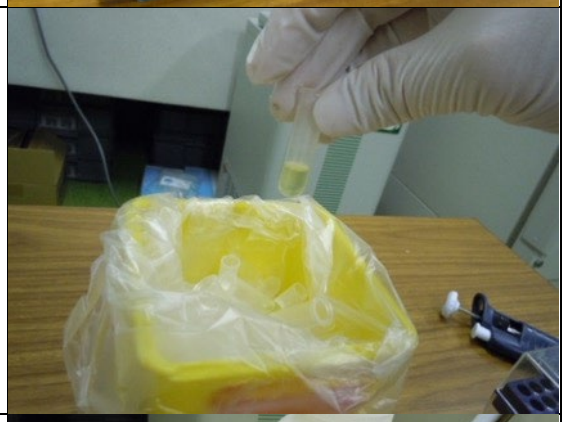
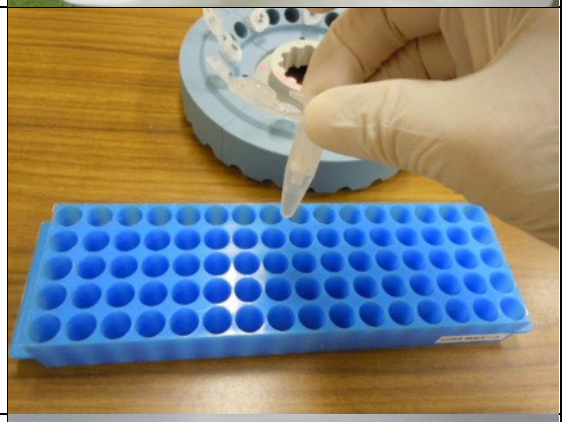
Fig. 4-1-4-5 After centrifuging, remove the collection tubes from the column and place them to a new 2 mL collection tube.	
Fig. 4-1-4-6 Discard used collection tubes.	
Fig. 4-1-4-7 Add 500 µL Buffer AW1 to the columns.	
Fig. 4-1-4-8 After centrifuging, place the columns to new 2 mL collection tubes.	

Fig. 4-1-4-9 Add 500 µL Buffer AW2 to the columns.	
Fig. 4-1-4-10 Prepare 1.5 mL low DNA bind tubes available and write the necessary information on the caps.	
Fig. 4-1-4-11 After centrifuging, move the columns to the 1.5 mL tubes made available in Fig. 4-1-4-10.	
Fig. 4-1-4-12 Pipet 200 µL Buffer AE (elution buffer) onto the column membranes.	

Fig. 4-1-4-13 Incubate the columns at room temperature for 1 minute and then centrifuge at 6000 g for 1 minute.	
Fig. 4-1-4-14 After centrifuging, remove the column and close the tube cap tightly.	
Fig. 4-1-4-15 The purified eDNA can be stably stored at −20°C.	

4-2. DNA extraction from glass fiber filters

Introduction

This section describes how to extract DNA from glass fiber filters. This method is a slightly modified version of the method published by Uchii et al. (2016).

This protocol does not use Buffer ATL supplied with the DNeasy kit, but it has been reported that the use of Buffer ATL increases the yield (Wu & Minamoto, 2023; Fukuzawa et al., 2023). In addition, a study has shown that the yield can be increased by increasing the amount of reagents used in DNA extraction (Wong et al. 2020.), and these methods may also be used.

In addition, careful attention must be paid to avoid contamination with exogenous DNA in the procedures from this point on, which takes place in the laboratory. In particular, contamination at this stage results in the failure of subsequent experiments (preparation of real-time PCR samples and next-generation sequencing libraries). To avoid this, a laboratory dedicated to DNA extraction (DNA extraction chamber) should be used. In addition, the DNA extraction room must be sufficiently separated spatially from the PCR room. Care should be used to not to enter the DNA extraction room after handling DNA extracted from tissues or PCR products during the same day.

Laboratory tools, reagents, and consumables required for DNA extraction (illustrative)

• Centrifuge (capable of rotating Salivette tubes)	
• Micro high-speed refrigerated centrifuge (capable of centrifuging 2.0 mL tubes and DNeasy columns)	
• Incubator (set to 56°C. Heat block may also be used.)	
• Salivette tubes	as many as samples
• Tweezers	as many as samples
• DNeasy Blood & Tissue Kits	as many as samples
• Buffer AL and Proteinase K	
• 1.5 mL Eppendorf tubes (low DNA adsorption)	as many as samples
• 96% to 99.5% Ethanol (for molecular biology)	as needed
• TE buffer (pH 8.0: for molecular biology)	as needed
• Disposable gloves (powder-free)	
• Micropipette P-1000, P-200, P-100 (e.g., Pipetman, Gilson Company Inc.)	
• Various filter chips	
• 1.5 mL/2 mL tube racks	

4-2-1. Preparation for experiment

Be sure to wear disposable gloves during the experiment. (If the gloves are contaminated during the work, replace them immediately.)

1) Prepare an incubator set at 56°C (prepare this in advance as it takes time to warm up).

2) Place the filter[※1], which has been used for filtering water samples and folded, into a Salivette tube, with the folded side facing down (Figs. 4-2-1-1 and -2). If two filters are used for one

sample, place them together in one Salivette tube.

3) Write sample numbers on the top and lower part of the Salivette tubes (Fig. 4-2-1-3).

※1 It has been noted that if the moisture remaining in the filter contains benzalkonium chloride, it may result in a decrease or variation in DNA yield. Thus, a step to remove moisture (e.g., centrifugation at 5,000 × *g* for 1 minute) may be added prior to this treatment, if necessary.

4-2-2. Proteinase digestion

1) Add 400 µL of Buffer AL and 40 µL of Proteinase-K (600 mAU/ml) per sample (Fig. 4-2-2-1). If there are n samples, it is recommended to prepare n+1 units of each reagent in a batch and then dispense them.

2) Incubate in an incubator at 56°C for 30 minutes (Fig. 4-2-2-2). When heated, the caps of the Salivette tubes may pop out. Thus, slightly loosen the portion between the basket and the lower tube part and place them upright in the incubator.

3) Then, centrifuge at 3000–5,000 × *g* for 3 minutes (Fig. 4-2-2-3). The Salivette tube may be placed inside a 50 mL conical tube and centrifuged. At this point, 800–1,000 µL of filtrate is found at the bottom of the Salivette tube (Fig. 4-2-2-4).

4) To collect the DNA remaining on the filter in the Salivette tube, add 220 µL of TE (Fig. 4-2-2-5) and allow it to stand for 1 minute. Then, centrifuge at 3000–5,000 × *g* for 3 minutes.

4-2-3. Purification of DNA using a commercial kit

1) Remove and discard the upper part of the Salivette tube containing the filter and add 400 µL of 96% to 99.5% ethanol to the lower DNA solution (Fig. 4-2-3-1 and -2).

2) Mix by pipetting (Fig. 4-2-3-3), then transfer approximately 650 µL (about half) to DNeasy column (Fig. 4-2-3-4 and -5), and centrifuge for 1 minute at 6000 g (Fig. 4-2-3-6).

3) Discard the filtrate remaining in the bottom 2 mL collection tube (Fig. 4-2-3-7 and -8), transfer the DNA solution remaining at the bottom of the Salivette tube to the column again (Fig. 4-2-3-9), and centrifuge for 1 minute at 6000 g. Repeat this process until the DNA solution is exhausted.

4) Transfer the column in a new 2 mL collection tube (Fig. 4-2-3-10), add 500 µL of Buffer AW1 (Fig. 4-2-3-11), and centrifuge at 6000 g for 1 minute.

5) Transfer the columns to new 2 mL collection tubes (Fig. 4-2-3-12), add 500 µL of Buffer AW2 (Fig. 4-2-3-13), and centrifuge for 2 minutes at the maximum speed of the centrifuge used.

6) When removing the tubes from the centrifuge and transferring, be careful not to let the lower liquid adhere to the tip of the upper column due to shaking.

7) Make a low DNA binding 1.5 mL tube available and write the sample name (Fig. 4-2-3-14).

8) Transfer the columns to a low DNA binding 1.5 mL tube (Fig. 4-2-3-15 and -16).

9) Add 100–200 µL of Buffer AE (elution buffer) (Fig. 4-2-3-17) and allow it to stand for 1 minute. After centrifuging at 6000 × *g* for 1 minute (Fig. 4-2-3-18), remove the columns in a manner to ensure that the outlet part of the column is not attached to the extraction solution and tighten the lid of the 1.5 mL tube (Fig. 4-2-3-19). It can be stored stably at –20°C in this condition. Furthermore, if the concentration of recovered DNA is expected to be low, the amount of elution

buffer can be reduced to approximately 50 μL. Record the amount of buffer used for elution.

Supplementary information

If no appropriate-sized centrifuge is available for centrifuging Salivette tubes, an extraction method using small spin columns may be used instead of Salivette tubes. For details, please refer to Yamanaka et al. (2016).

Reference

Fukuzawa, T., Shirakura, H., Nishizawa, N., Nagata, H., Kameda, Y. & Doi, H. 2023, "Environmental DNA extraction method from water for a high and consistent DNA yield." *Environmental DNA* 5 (4): 627–633. doi: 10.1002/edn3.406

Uchii, K., Doi, H. & Minamoto, T. 2016, "A novel environmental DNA approach to quantify the cryptic invasion of non-native genotypes." *Molecular Ecology Resources* 16 (2): 415-422. Doi: 10.1111/1755-0998.12460

Wong, M. K. S, Nakao, M. & Hyodo, S. 2020, "Field application of an improved protocol for environmental DNA extraction, purification, and measurement using Sterivex filter." *Scientific Reports* 10: 21531. doi: 10.1038/s41598-020-77304-7

Wu, Q. & Minamoto,T. 2023. "Improvement of recovery yield of macro-organismal environmental DNA from seawater samples." *Analytical Sciences* 39, 713-720. doi: 10.1007/s44211-023-00280-

Yamanaka, H., Motozawa, H., Tsuji, S., Miyazawa, R. C., Takahara, T. & Minamoto, T. 2016. "On-site filtration of water samples for environmental DNA analysis to avoid DNA degradation during transportation." *Ecological Research* 31 (6): 963-967. doi: 10.1007/s11284-016-1400-9

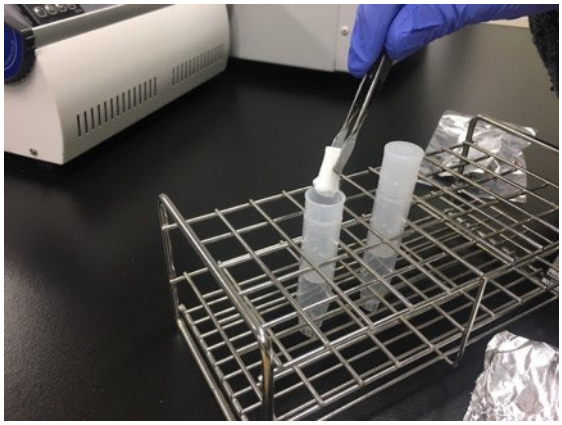
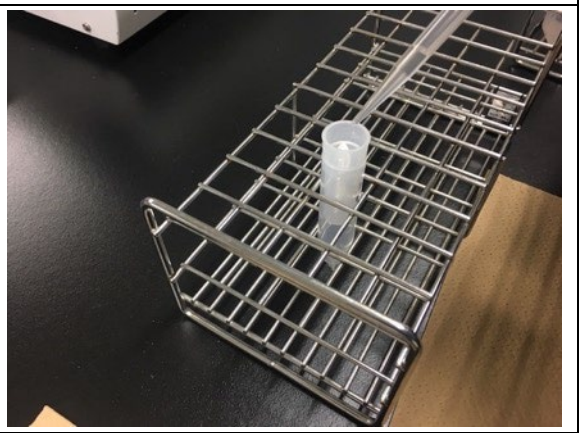
Fig. 4-2-1-1 Remove the filters from the freezer and transfer them to Salivette tubes.	
Fig. 4-2-1-2 A filter in a Salivette tube.	
Fig. 4-2-1-3 Write the sample numbers on the top and lower part of the Salivette tube.	
Fig. 4-2-2-1 Add a mixture of Buffer AL and Proteinase K.	

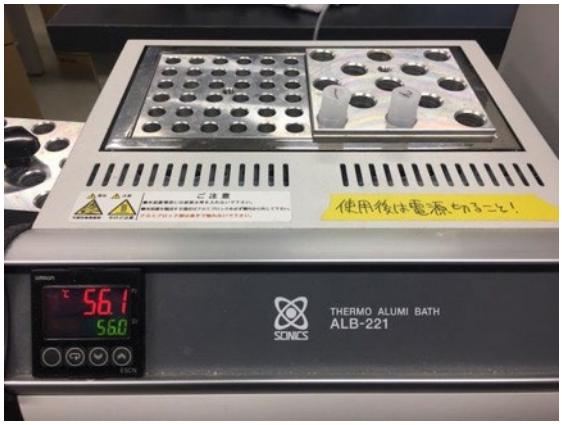
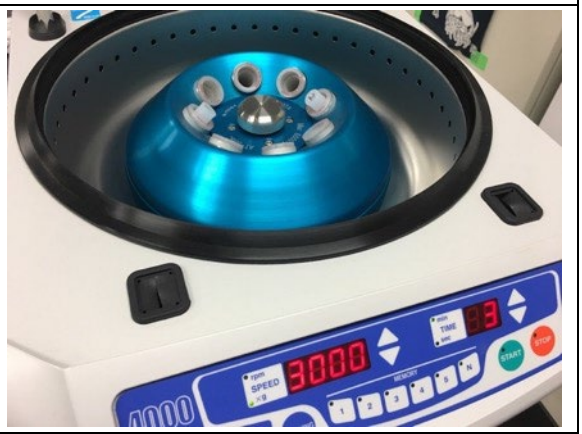
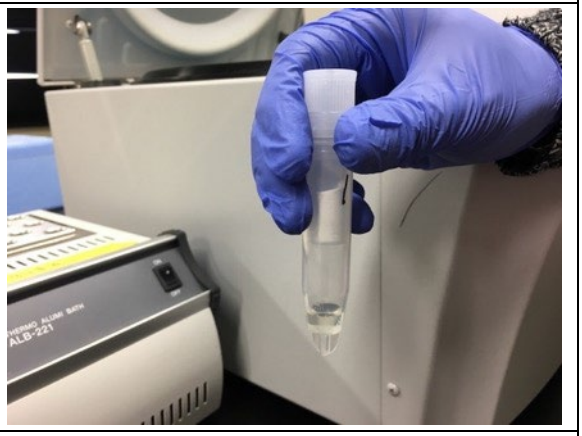
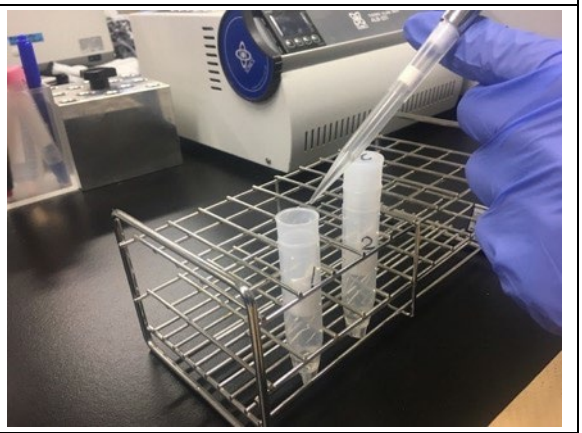
Fig. 4-2-2-2 Incubate at 56°C for 30 minutes.	
Fig. 4-2-2-3 Centrifuge and move the liquid on the filter toward the bottom of the Salivette tube.	
Fig. 4-2-2-4 Salivette tube after centrifuging. Approximately 800 to 1000 µL of liquid is collected at the bottom of the tube.	
Fig. 4-2-2-5 Add Buffer TE to the filter.	

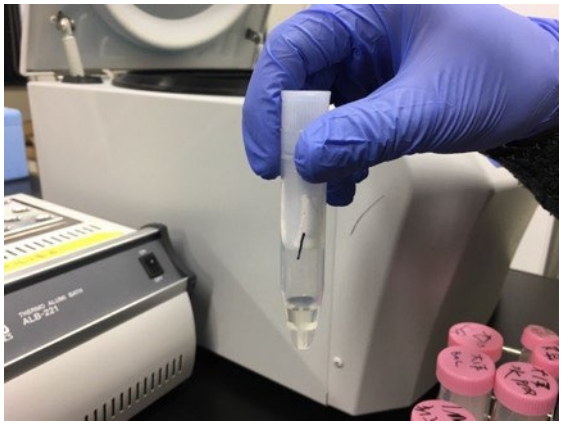
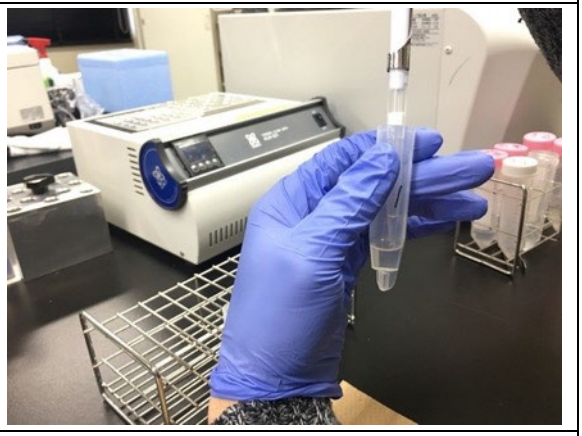
Fig. 4-2-3-1 The Salivette tube after the second centrifuge.	
Fig. 4-2-3-2 Remove the upper part of the Salivette tube and add ethanol to the lower part.	
Fig. 4-2-3-3 After adding ethanol, mix well by pipetting.	
Fig. 4-2-3-4 DNeasy column.	

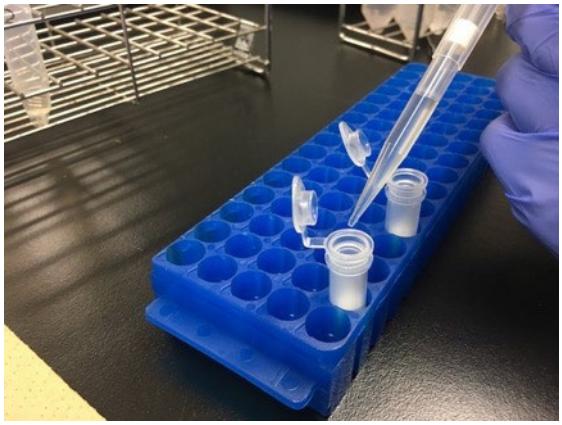
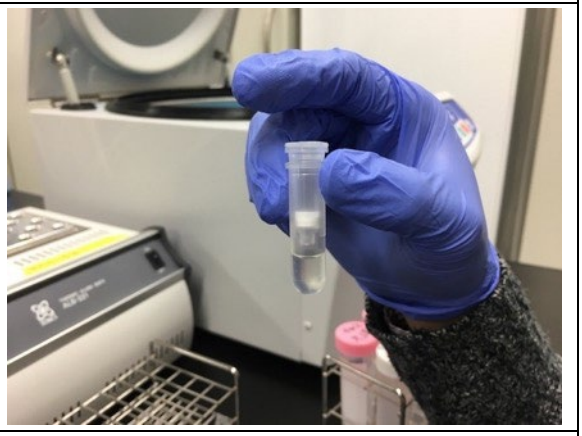
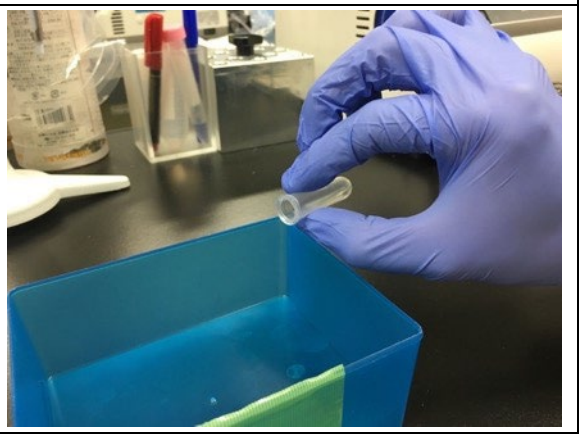
Fig. 4-2-3-5 Transfer the solution in the lower part of the Salivette tube to the DNeasy column.	
Fig. 4-2-3-6 Pass the solution through the column by centrifuging.	
Fig. 4-2-3-7 Since DNA is trapped in the column part, the lower liquid is liquid waste.	
Fig. 4-2-3-8 Discard the liquid waste.	

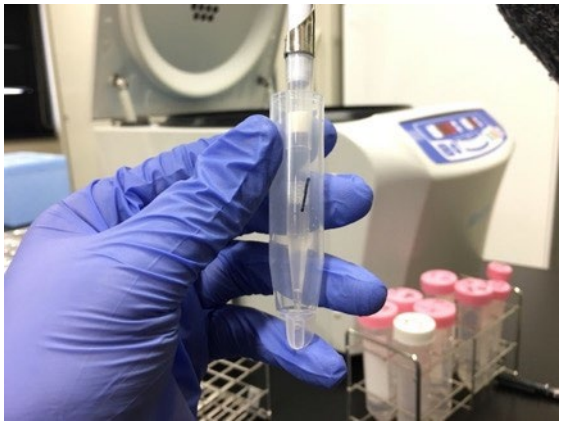
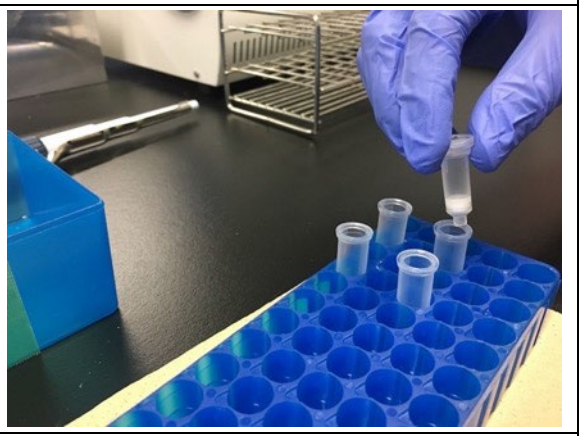
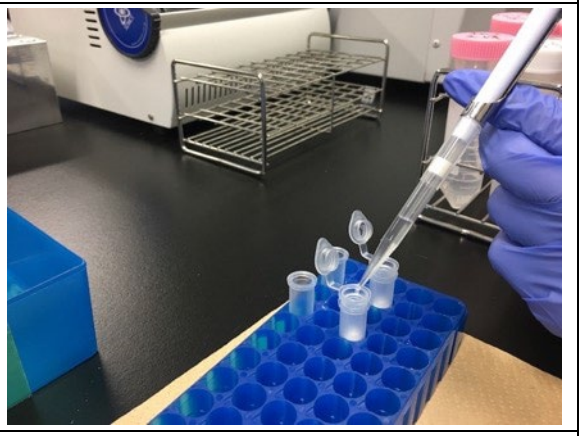
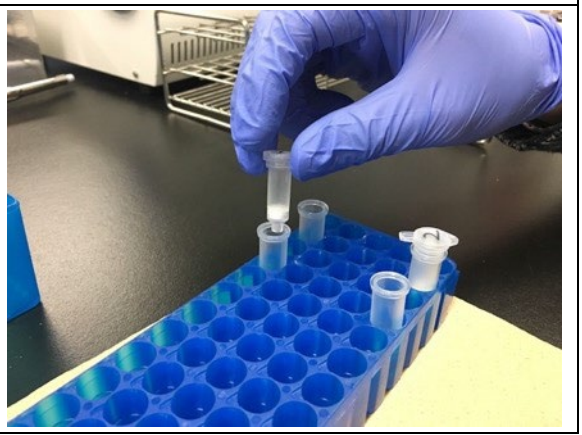
Fig. 4-2-3-9 Once more, transfer the DNA solution from the Salivette tube to the column and centrifuge.	
Fig. 4-2-3-10 After centrifuging, transfer the column to a new 2 mL tube.	
Fig. 4-2-3-11 Pour buffer AW1.	
Fig. 4-2-3-12 After centrifuging, transfer the column to a new 2 mL tube.	

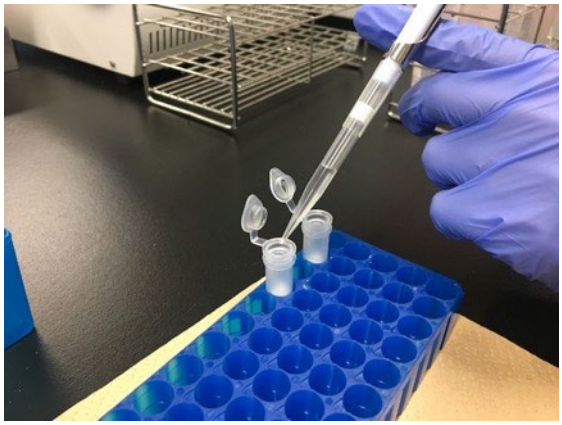
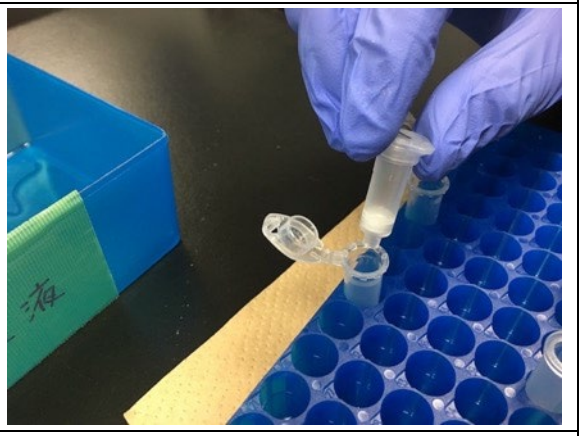
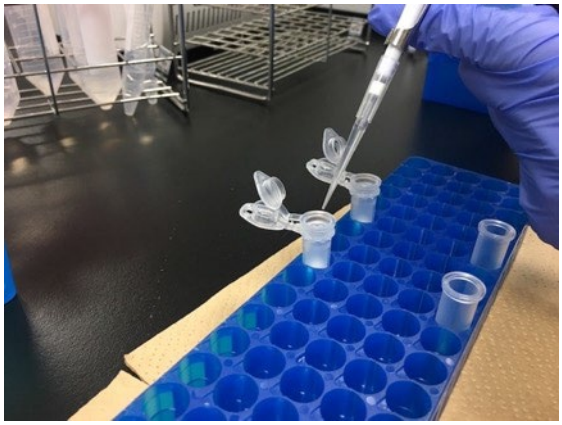
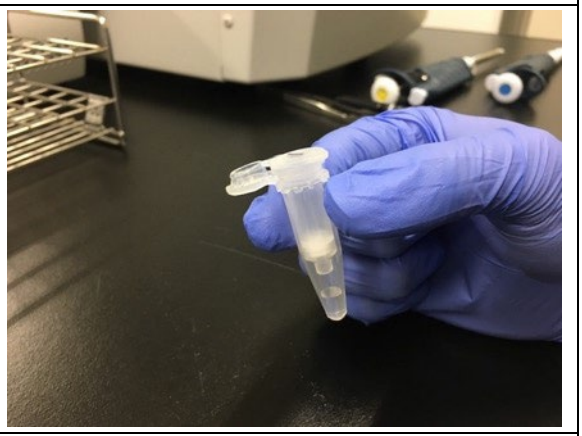
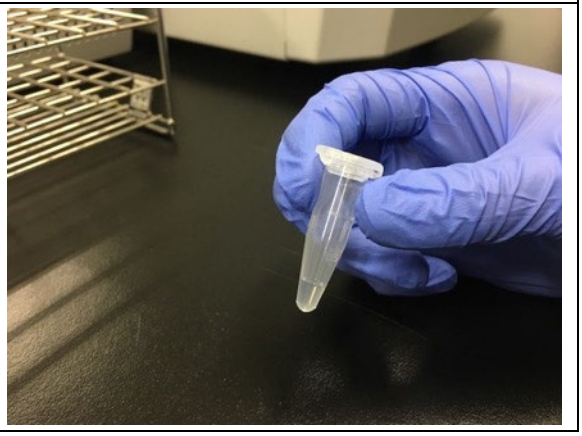
Fig. 4-2-3-13 Pour buffer AW2 and centrifuge.	
Fig. 4-2-3-14 Make a 1.5 mL low binding tube available and write the sample number.	
Fig. 4-2-3-15 Transfer the centrifuged column to the 1.5 mL tube.	
Fig. 4-2-3-16 The columns are put in 1.5 mL tubes.	

Fig. 4-2-3-17 Pour buffer AE, allow the tube to stand for 1 minute, and centrifuge.	
Fig. 4-2-3-18 The tube after centrifuging. At this point, the solution that is in the bottom is the DNA sample. Do not discard it.	
Fig. 4-2-3-19 Remove the column, put the lid on the tube, and store frozen.	

5. DNA analysis

5-1. Single species detection and quantification of eDNA

Introduction

This section describes single-species detection and quantification of eDNA using real-time PCR. Since single-species detection requires the design of different detection assays for specific target species, only an example will be described below. Experimental conditions, therefore, should be individually developed for different detection assays.

5-1-1. Design of species-specific primers (and probes)

The species-specific primers should be capable of efficiently amplifying the DNA of the target species without non-specific amplification of the DNA of closely related species that share the same habitat. The primer designing procedure is as follows.

1) Acquisition of sequence information: Download nucleotide sequence information of the target species and closely related sympatric species. Databases such as NCBI (https://www.ncbi.nlm.nih.gov/genbank/) and BOLD (http://www.barcodinglife.org) are used as appropriate. However, since the databases may contain erroneous information, it is desirable to refer to more than one sequence information source.

2) Primer design: Search for regions with differences in base sequences between the target species and closely related species, and design primers (Fig. 5-1-1-2). There should be a base specific to the target species near the 3' end of the primer. When designing primers, it is recommended to refer to general precautions for primer design, such as whether the Tm value is within an appropriate range. Create TaqMan probes also as needed.

3) In silico check: Identify primer specificity using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Verify that other sympatrically inhabiting species are not amplified.

4) In vitro check: Perform PCR using DNA samples extracted from the tissues of the target species and closely related species, and check whether the DNA is amplified to confirm its specificity (Fig. 5-1-1-4).

5) Sequencing of eDNA derived amplicons: If DNA amplification derived from environmental samples is confirmed, perform direct sequencing of the PCR amplicons and verify that the DNA of the target species has been correctly amplified.

5-1-2. Real-time PCR experiments

The following is an example. It is necessary to adjust the protocol according to the equipment, reagents, and target species.

Laboratory instruments, reagents, and consumables (illustrative)

- Real-time PCR device (96 wells)
- PCR reagent (2 × Environmental Master Mix 2.0, Thermo Fisher Scientific K.K.)

• UNG enzyme (AmpErase Uracil N-Glycosylase: Thermo Fisher Scientific K.K.)
• Assay Mix (with a 18 μM primer and a 2.5 μM TaqMan probe [*1]
• 96-well PCR plates and seals
• Disposable gloves (powder-free)
• Micropipette P-1000, P-200, P-100 (e.g., Pipetman, Gilson Company Inc.)
• A variety of filter tips

※1 It is necessary to design primers and probes individually for different targets. In addition, when preparing a TaqMan probe, setting the Tm value (the temperature at which 50% of double-stranded DNA dissociates into single-stranded DNA) of the probe higher than that of the primer is expected to improve specificity.

Reagent composition per PCR (illustrative)

• Environmental Master Mix 2.0	10.0 μL
• AmpErase Uracil N-Glycosylase (UNG enzyme)	0.1 μL
• Assay Mix	1.0 μL
• eDNA sample	2.0–5.0 μL
• DNase/RNase-Free Water	appropriate amount
• Total	20.0 μL

Perform all the PCR (including positive controls [quantification standard], eDNA samples, field blanks, filtration blanks, and PCR blanks) with three or more replicates. Use an artificially synthesized gene as the quantification standard and a four or more fold dilution series to quantify the DNA.

Since the conditions of the PCR vary widely, as an example, two-step PCR as performed under the conditions that include the above-mentioned reagent composition and Tm value of around 60°C for the primer is described below.

> After an initial step consisting of amplifications for 2 minutes at 50°C and 10 minutes at 95°C, a cycle consisting of 15 seconds at 95°C and 1 minute at 60°C is repeated for 50–55 times.

A positive control (or quantitative standard) and a PCR blank are placed on each PCR plate. Therefore, if all PCR are performed in triplicate, the number of samples (including field blanks and filtration blanks) that can be analyzed simultaneously is 27 samples (for quantification) or 30 samples (for presence/absence detection).

If eDNA samples are run in triplicate, any sample that is determined positive in any one of the triplicates is considered positive (Fig. 5-1-3). Quantify the DNA using the quantification standard data[※2]. Perform direct sequencing using PCR amplicons for at least a portion of eDNA samples found to be positive and confirm that it is indeed the target DNA. It is desirable to confirm the sequence, especially for a sample for which a large Cq value (Ct value) is obtained during real-time PCR.

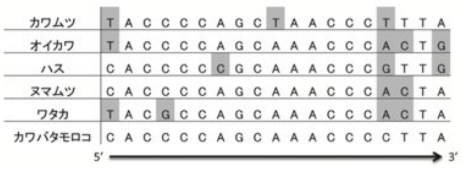
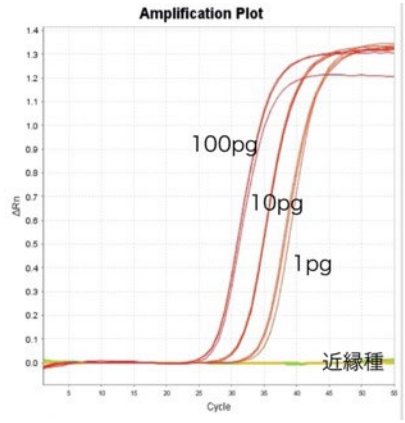
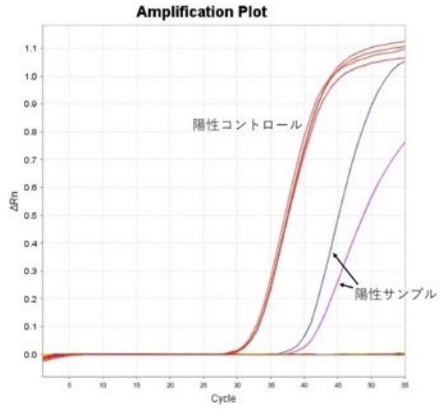
※2 Methods for synthesizing quantitative standards include those using plasmids, linear double-stranded DNA (e.g., gBlooks® Gene Fragments from IDT can be purchased), and PCR products. In addition, the yields of purchased artificially synthesized genes are approximate, and they are likely affected by impurities as they have not been purified. Therefore, it is desirable to purify them using a purification kit or other techniques. The concentration is then measured using Qubit, digital PCR, or other methods, and they are diluted to the desired concentration for use.

Response to negativity due to PCR inhibition

eDNA samples may contain PCR inhibitors, such as humic acid. Although the PCR system introduced here is considered to be relatively unaffected by PCR inhibition, false negative results may sometimes be obtained due to PCR inhibition. If a false negative due to PCR inhibition is suspected, the presence or absence of an inhibitory effect can be examined by methods such as spiking in DNA that has been shown to amplify as an internal positive control (internal standard). If inhibition is observed, diluting the DNA solution prior to PCR may improve the results. In addition, several kits are available to remove inhibitors contained in DNA.

References

Fukuoka, A., Takahara, T., Matsumoto, M. Biology Club of Hyogo Prefectural Agricultural High School, Ushimaru, A. & Minamoto, T. 2016. "Establishment of detection system for native rare species, *Hemigrammocypris rasborella*, using environmental DNA" *Journal of the Ecological Society of Japan* 66 (3): 613-620. (in Japanese)

Fig. 5-1-1-2 Primer design. An example of primer design for *Hemigrammocypris rasborella* (Source: Fukuoka et al. 2016) ※3	
Fig. 5-1-1-4 In vitro amplification check. Verify that the DNA of the species in question is amplified (red line) while the DNA of closely related species is not amplified (other colors).	
Fig. 5-1-2 An example of the result from real-time PCR.	

※3 Depending on the target species, intraspecific polymorphisms may be found in the target DNA sequence. Therefore, it is necessary to be aware that there may be local populations of the target species that are difficult to detect using the created (published) primers/probes (false negatives). It is known that the primers/probes described here do not match perfectly in some local populations.

5-2. eDNA metabarcoding

5-2-1. Library preparation – 1: 1st PCR

Introduction

eDNA metabarcoding uses the polymerase chain reaction (PCR) to amplify the DNA of the target taxonomic group to the extent that it can be analyzed, while it appends various adapters to both ends of the PCR products, processing them into molecules that can be analyzed with a next-generation sequencing platform (library preparation).

PCR produces exceptionally large amounts of DNA fragments and is likely to contaminate the experiments. Therefore, the laboratory for performing PCR preparation, such as mixing PCR reagents, (pre-PCR room) should be spatially separated from the laboratory for performing PCR and handling PCR products (post-PCR room). It is also necessary to implement measures to reduce contamination risks. Specifically, the personnel should not be engaged in DNA extraction or other experiments after handling PCR products during the same day. Furthermore, since it employs two-step PCR, it is necessary to dilute the first-round PCR (1st PCR) product and use it as a template for the second-round PCR (2nd PCR). Accordingly, it is necessary to install a clean bench to prevent contamination that occurs during the procedure or the Table Coach (KOACH T 500, Koken) to create a clean, open space. In addition, micropipettes, tube racks, and experimental tables should be decontaminated in advance using foaming bleach or other products. Furthermore, decontaminated consumables, such as tips, tubes, and water to be used for PCR assays should be purchased from a trusted manufacturer whenever possible.

Below, we describe an example of metabarcoding sample preparation using MiFish primers.

Laboratory instruments, reagents, and consumables required for 1st PCR (illustrative)

- Thermal cyclers (preferably those that can accommodate a 96-well plate)
- KAPA HiFi HS ReadyMix (KK2602, KAPA Biosystems Inc.) [1]
- MiFish primer ※2, ※3, ※4

Primers for elasmobranchs (primers optimized for sharks and rays)

MiFish-E-F-v2 (61 mer):
5’-ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT NNN NNN RGT TGG TAA ATC TCG TGC CAG C-3’
MiFish-E-R-v2 (68 mer):
5’-GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATC TNN NNN NGC ATA GTG GGG TAT CTA ATC CTA GTT TG-3’

Primers for actinopterygians (i) (universal primer for ray-finned fishes)

MiFish-U-F (60 mer):
5’-ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT NNN NNN GTC GGT AAA ACT CGT GCC AGC-3’
MiFish-U-R (67 mer):
5’-GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATC TNN NNN NCA TAG TGG GGT

ATC TAA TCC CAG TTT G-3’

Primers for actinopterygians (ii) (primers optimized for perch sculpin, which is a common species in the temperate coastal waters of Japan)

MiFish-U2-F (60mer):
5’-ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT NNN NNN GCC GGT AAA ACT CGT GCC AGC-3’
MiFish-U2-R (67 mer):
5’-GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATC TNN NNN NCA TAG GAG GGT GTC TAA TCC CCG TTT G-3’

- DNase/RNase-free water (for molecular biology experiments)
- TE buffer (for molecular biology experiments)
- 8-tube strip with integrated caps
- 1.5 mL tubes (low DNA bind tubes)
- Micropipette P-1000, P-200, P-100, P-20, P-2 (e.g., Pipetman, Gilson Company Inc.)
- Filter tips (various according to different micropipette capacities)
- Electric micropipettes 0.5–10 μL, 5–100 μL (e.g., Xplorer Plus, Eppendorf AG)
- Tube racks for 1.5 mL/2.0 mL tubes
- Tube racks for 8-tube strip
- Disposable gloves (powder-free)

※1 DNA extracted from terrestrial environmental water and mud samples often contain PCR inhibitors (e.g., humic acid). The 1st PCR sometimes fails for those DNA with KAPA HiFi HS. In such a case, good amplification may be achieved by using KOD FX Neo, KOD One (Toyobo Inc.), Platinum SuperFi II DNA Polymerase (Thermo Fisher Scientific K.K.), or similar products. However, because enzymes have different characteristics, such as the likelihood of non-specific amplification and the amplification efficiency for each sequence, it is necessary to understand the differences in their characteristics when using them. In addition, when conducting comparative analysis, it is best practice to avoid combining results from different enzymes. Regarding PCR protocol, the one provided by the manufacturer can be basically used without substantial modifications. Additionally, if amplification is not successful because of the influence of PCR inhibitors, successful amplification may be achieved by diluting the sample. There are also several kits available that remove inhibitors contained in DNA.

※2 It is convenient to purchase the one that has been diluted to 100 μM in TE buffer. There may be deviations in the mixed bases depending on the synthesis manufacturer.

※3 Some fish species, such as sweetfish, smelt, and lamprey, have mutations in the primer regions, which reduce amplification efficiency. When used in an environment inhabited by these fish species, detection is possible by mixing in small amounts of primers with modified sequences.

※4 Here, we show an example of primers for analyzing fish species for which prior research has been conducted. A primer set should be appropriately selected based on the required taxonomic resolution and the read length that can be handled by the sequencer used. In addition, the subsequent analysis steps describe an example using MiFish primers.

Laboratory instruments, reagents, and consumables required for purification and concentration of first PCR product (illustrative)

- Desktop compact centrifuge (Force Mini SBC-140, LabNet)

- Plate centrifuge (PC-2R, AS ONE Corp.)
- 1.5 mL tubes (low DNA bind)
- 96-well plates（low DNA bind）
- SPRIselect※1 (Beckman Coulter, Inc.)
- Buffer EB (Qiagen KK)
- 85% ethanol
- Magnetic plate for 96-well plates ※2 (DynaMag-96 Side, Thermo Fisher Scientific K.K.)
- Centrifuge for 96-well plates (for spin-down)
- Micropipette P-1000, P-200, P-100 (e.g., Pipetman, Gilson Company Inc.)
- 8-channel micropipettes P-200, P-10
- Filter tips (various types according to the capacity of the micropipette used. They need to be compatible with the micropipette used.)
- Disposable gloves (powder-free)

※1 Purification and concentration can also be performed using spin columns for PCR product purification (with no strict size cutoff). However, a low content of the target fragment in the extracted eDNA solution leads to a high content of primer-derived products, which typically manifest around 100–150 bp as a result of PCR. Thus, purification conditions may vary depending on the characteristics of the column used. Furthermore, spin columns capable of strict size fractionation are available and may be used if the user is particular about the column method. However, the performance of columns and beads, such as fragment recovery amount and sequence composition, is not necessarily the same across manufacturers. Therefore, to obtain consistent data, one specific product should always be used for each primer set used.

※2 A variety of magnetic plates for 96-well plates are available from various companies, but the ones with a sufficiently strong magnetic force should be selected. In addition, for the procedures explained here, a type that collects beads on the side of the well is more convenient than a type that collects them at the bottom of the well.

Laboratory instruments, reagents, and consumables required for quantification of purified and concentrated 1st PCR products (illustrative)

- TapeStation 4150 (Agilent Technology Inc.)
- High Sensitivity D1000 Screen Tape (5067-5584, Agilent Technologies Inc.)
- High Sensitivity D1000 Reagents (5067-5585, Agilent Technologies Inc.)
- 4150/4200 TapeStation Loading Tips (5067-5598, Agilent Technologies Inc.)
- Table stirrer (Vortex MS3 Basic, IKA Japan K.K)
- Tabletop centrifuge for 8-tube strip (e.g., Micro PCR spinner MS-PCR, ASONE Corp.)
- Tabletop centrifuge for 1.5 mL/2.0 mL (e.g., Micro Six MS-1, ASONE Corp.)
- Electric micropipette 0.5–10 μL (Xplorer Plus, Eppendorf Inc.)
- Micropipette P-10 or P-2 (e.g., Pipetman, Gilson Company Inc.)
- Filter chip 10 μL (those compatible with Pipetman)
- 8-tube strip
- 8-tube strip rack
- Disposable gloves (powder-free)
- DNase/RNase-free Water

5-2-1-1. 1st PCR

When PCR is performed for eDNA, the amount of fish-derived DNA in the extracted DNA is not known in advance. Therefore, it is important to define the optimum experimental conditions, such as the number of PCR cycles, by conducting preliminary experiments before the experiment.

Be sure to wear rubber gloves during the experiment. If the gloves are apparently contaminated during the work, replace them immediately.

1) Turn on the thermal cycler before starting the experiment.

2) Primer dilution: Dilute the stock primer solution (100 µM) 20 times with a commercially available TE buffer. Mix the diluted primers in the following ratio to make a primer mix; MiFish-E-F/R-v2: MiFish-U-F/R: MiFish-U2-F/R = 1: 2: 1. (If DNA includes only freshwater fish, only MiFish-U-F/R is required.)

3) Reagent composition: When PCR is performed in a total volume of 12 µL (including 2 µL of DNA), the composition of the premix per tube is as follows. (Caution: The amount of reagent should be 1.1 to 1.2 times the required amount. Otherwise, it may be insufficient when dispensing with an electric pipette.) The final concentration of each of the forward primer mix and reverse primer mix is approximately 583 nM.

KAPA HiFi HS ReadyMix	6.0 µL
Primer mix	2.8 µL
DNase/RNase-free Water	1.2 µL

4) PCR replicates: To minimize PCR dropouts, it is recommended to perform PCR multiple times on the same sample (eDNA). For example, PCR is performed eight times using one 8-tube strip. Furthermore, to monitor the contamination that occurs during the 1st PCR, add a new blank (1st PCR blank) to each 1st PCR set, in addition to the filtration blank and extraction blank. For the 1st PCR blank, DNase/RNase-free water is used as the template instead of extracted DNA (Fig. 5-2-1-1-1).

5) Prepare a premix by mixing the above reagents about 1.1 times the required amount and dispense 10 µL each into 8-tube strip with an electric pipette. Then, dispense 2 µL each of the extracted eDNA into the tubes with an electric pipette. If PCR is performed eight times, 16 µL of extracted DNA (2 µL × 8) is required.

6) The thermal cycler settings are as follows:

DNA denaturation at 95°C for 3 minutes (initial denaturation and activation of HotStart enzyme)
Denaturation at 98°C for 20 seconds
Annealing at 65°C for 15 seconds
Extension at 72°C for 15 seconds
} 35 cycles
Extension reaction at 72°C for 5 minutes (final extension)
4°C

7) After the PCR is finished, remove the tubes immediately and freeze them at -20°C, and proceed to the purification step as soon as possible.

Note 1: The annealing temperature is set considerably higher due to the characteristics of KAPA HiFi ReadyMix (at least 60°C). If the temperature is higher than 65°C, some species may not be detected because of mismatches between the primer and template sequences. Conversely, if the

temperature is lower than 60°C, non-specific products (products presumed to be derived from 16S rRNA of microorganisms) may increase. Considering the trade-off between the two, this annealing temperature seems appropriate, but it should be optimized according to the purpose of each.

Note 2: Regarding the number of cycles, sufficient amplification may not be observed with 35 cycles in seasons with low fish activity, such as winter, or due to the influence of PCR inhibitors. In addition, if the number of cycles is greatly increased due to the absence of PCR products, it may result in non-specific amplification, hindering analysis. The optimal number of cycles should be considered according to one's own situation.

5-2-1-2. Purification and concentration of 1st PCR products

Here, we describe an example of the purification and concentration of 1st PCR products using magnetic beads.

Always wear disposable gloves. Change gloves immediately if you feel that they have been contaminated during procedures.

1) The 1st PCR products from technical replicates are combined into one 1.5 mL tube (Fig. 5-2-1-2-1). Because 12.0 µL of 1st PCR product is obtained per well in each tube, 8 repeats result in a total of 96.0 µL, for example.

2) Stir the SPRIselect bottle well to thoroughly disperse the precipitated beads (Fig. 5-2-1-2-2). Prepare 300 µL of 85% ethanol per sample.

3) Add an equal amount of SPRIselect to the PCR product solution, mix thoroughly by pipetting 10 times, and then transfer the entire amount to a 96-well plate (Fig. 5-2-1-2-3). Allow it to stand at room temperature for 1 minute.

4) Place the 96-well plate on a magnetic plate for 96-well plates and allow it to stand until the solution becomes clear (Fig. 5-2-1-2-4).

5) Carefully remove the supernatant without aspirating the beads (Fig. 5-2-1-2-5).

6) While the plate is still set on the magnetic plate, add 150 µL of 85% ethanol (Fig. 5-2-1-2-6) and allow it to stand at room temperature for 30 seconds. Carefully remove the supernatant using a P-200 8-channel pipette without aspirating the beads.

7) While the plate is still set on the magnetic plate, add 150 µL of 85% ethanol again and allow it to stand at room temperature for 30 seconds. Carefully remove the supernatant using a P-200 8-channel pipette without aspirating the beads (Fig. 5-2-1-2-7).

8) Spin down the plate, place it on the magnetic plate again, and completely remove residual ethanol using a P-10 8-channel pipette and tips (Fig. 5-2-1-2-7).

9) Remove the plate, add 25 µL of Buffer EB, and mix thoroughly by pipetting 10 times. Allow it to stand at room temperature for 1 minute (Fig. 5-2-1-2-7).

10) Place the plate on the magnetic plate and allow it to stand until the solution becomes clear (Fig. 5-2-1-2-8).

11) While being careful not to aspirate the beads, collect all the clear supernatant and transfer each to a new 1.5 mL tube (low DNA bind) (Fig. 5-2-1-2-9). In this state, the samples can be stably stored at –20°C.

5-2-1-3. Quantification of the purified and concentrated 1st PCR product

This procedure verifies that primer and adapter dimers have been removed and simultaneously quantifies the amount of the target DNA. This protocol includes the use of the TapeStation 4150 (Agilent Technologies Inc.) and a High Sensitivity D1000 ScreenTape System kit. The ScreenTape is pre-filled with gel in 16 lanes, and up to 16 PCR products can be quantified simultaneously. When a DNA ladder is used, 15 products can be quantified. Similarly, the analysis can be performed using equivalent equipment from other companies in addition to TapeStation.

Always wear disposable gloves.

1) Allow the reagent to equilibrate at room temperature for 30 minutes (Fig. 5-2-1-3-1).

2) Launch the TapeStation computer. After the startup, turn on the TapeStation 4150 main unit (Fig. 5-2-1-3-2).

3) After confirming the connection between the TapeStation and the computer, launch the TapeStation 4150 controller software and set the High Sensitivity D1000 ScreenTape and the required number of 4150/4200 TapeStation Loading Tips (Fig. 5-2-1-3-3).

4) Prepare 8-tube strip and dispense 2 µL of High Sensitivity D1000 Sample Buffer for the total number of one High Sensitivity D1000 Ladder and samples (Fig. 5-2-1-3-4).

5) Add 2 µL of the ladder or purified and concentrated 1st PCR product to the 2 µL of buffer as dispensed (Figs. 5-2-1-3-5 and -6) and close the caps. After brief centrifuging, stir for 1 minute at 2000 rpm using a vortex (Fig. 5-2-1-3-7). Centrifuge briefly again after stirring and bring the solution to the tube bottom.

6) Carefully open the cap in a manner not to scatter the solution and set it in the TapeStation 4150 cassette (Fig. 5-2-1-3-8).

7) Once electrophoresis is started following the instructions of the TapeStation 4150 controller (Figs. 5-2-1-3-9 and -10), the target band (approximately 310 bp) will be displayed along with the pherogram approximately 20 minutes later (Fig. 5-2-1-3-11). There are rare cases where the marker peak cannot be read, but in that case, correct it manually as appropriate.

8) Read the concentration of the target band and dilute it in DNase/RNase-free water. The measurement range of High Sensitivity D1000 is 10–1000 pg. Thus, if the band concentration exceeds this range, dilute the sample to an appropriate dilution percentage and measure again to determine the dilution percentage within the appropriate range. In principle, no signal can be obtained for blanks. Therefore, dilute it using the mean dilution percentage for positive samples for convenience. However, for example, if non-specific amplification is significant, the concentration should be adjusted after the 2nd PCR. In that case, purify and measure DNA concentration separately, without combining the samples into one tube in step (11) of 5-2-2-1.

Note: When diluting the purified and concentrated 1st PCR product, it is recommended that DNase/RNase-free water be pre-dispensed in 1.5 mL tubes at a volume necessary for diluting the sample for each tube in the PCR preparation room and then brought into the PCR room. Note that the purified and concentrated 1st PCR products brought into the PCR preparation room may cause significant cross-contamination. If the concentration does not reach the specified level, dilute to 0.05 ng/µL to increase the number of PCR cycles.

9) Use the diluted 1st PCR on the same day it is prepared and do not store it in a refrigerator or freezer. The undiluted 1st PCR product can be stored at –20°C; however, measures should be taken in subsequent procedures to minimize freezing and thawing to the maximum possible extent.

5-2-2. Library preparation – 2: 2nd PCR

Before starting the experiment

This section describes how to perform second-round PCR (2nd PCR) using a quantified 1st PCR product as a template. The main purpose of the 2nd PCR is to append an index (tag) sequence and a flow cell binding sequence to the template (quantified 1st PCR product) with sequence primers added. Since the purpose is not to amplify the template, the cycles are usually limited to 10 times. If a sufficient amount of the 1st PCR product is not obtained (only less than 0.1 ng/μL of PCR product is prepared), it may be necessary to increase the number of 2nd PCR cycles. In addition, in order to minimize the effect of carryover from one run to another (the library of the last run that may remain in the channel inside MiSeq), a combination of index sequences used in the previous few runs (two to three runs) should not be used.

Laboratory instruments, reagents, and consumables for the 2nd PCR (illustrative)

- Thermal cycler (preferably those that can accommodate a 96-well plate)
- Quantified 1st PCR product (0.1 ng/μL)
- KAPA HiFi HS ReadyMix (KK2602, KAPA Biosystems Inc.)
- Primers with indices (information related with the index sequence is provided at the end of this section) ※1, ※2

A forward primer (blue indicates an eight-base index sequence)

5'-AAT GAT ACG GCG ACC ACC GAG ATC TAC ACx xxx xxx xAC ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC T-3'

A reverse primer (red indicates an eight-base index sequence)

5'-CAA GCA GAA GAC GGC ATA CGA GAT xxx xxx xxG TGA CTG GAG TTC AGA CGT GTG CTC TTC CGA TCT-3'

- DNase/RNase-free water (or high-grade sterilized water with molecular biology grade)
- TE buffer (molecular biology grade)
- 8-tube strip
- 1.5 mL tubes (low DNA bind)
- Micropipette P-1000, P-200, P-100, P-20, P-2 (e.g., Pipetman, Gilson Company Inc.)
- Filter tips (various according to different micropipette capacities)
- Electric micropipettes 0.5–10 μL, 5–100 μL (e.g., Xplorer Plus, Eppendorf AG)
- Tube racks for 1.5 mL/2.0 mL tubes
- Tube racks for 8-strip tubes
- Disposable gloves (powder free)

※1 It is convenient to purchase those that have been pre-diluted to 100 μM in TE buffer.
※2 The sequence must be changed according to the primers used in the 1st PCR. Here, we show an example of using the MiFish primers, shown in this manual, for the 1st PCR.

Laboratory instruments, reagents, and consumables for the size selection (illustrative)

- E-Gel Power Snap Plus Electrophoresis Systems (G9110, Thermo Fisher Scientific K.K.)
- E-gel SizeSelect II 2% (G661012, Thermo Fisher Scientific K.K.)
- Molecular size markers (those that can distinguish molecular weight around the target size)
- 2nd PCR products (individually or several tubes per library)
- DNase/RNase-free water
- 1.5 mL tubes (low DNA bind)
- Micropipette P-100 (Pipetman, Gilson Company Inc.)
- Filter tips 100 µL
- Tube racks for 1.5 mL/2.0 mL tubes
- Disposable gloves (powder-free)

Laboratory instruments, reagents, and consumables for the quantification (illustrative)

- Qubit 4.0 Fluorometer (Thermo Fisher Scientific K.K.)
- Qubit dsDNA HS Assay (Q32851 [for 100 samples], Thermo Fisher Scientific K.K.) (Q32854 [for 500 samples], Thermo Fisher Scientific K.K.)
- Qubit assay 500 µL tube (Q32856, Thermo Fisher Scientific K.K.)
- Tube racks for 500 µL tubes
- Desktop small centrifuge (e.g., Micro Six MS-1, AS ONE Crop)
- Micropipette P-1000, P-200, P-100, P-20, P-2 (e.g., Pipetman, Gilson Company Inc.)
- Filter tips (various types compatible with Pipetman, according to the capacity of the micropipettes used)
- 2nd PCR products recovered with E-Gel
- Disposable gloves (powder-free)

5-2-2-1. 2nd PCR

Be sure to wear disposable gloves during the experiment (disposable gloves should be quickly replaced if contaminated during the procedure). The method described below is developed so that numbers of reads for each library obtained by the 2nd PCR are as constant as possible while reducing the amount of reagents used.

Note: Be sure to prepare PCR in the PCR preparation room (pre-PCR room). PCR templates contain PCR products, although the products are diluted. Therefore, templates should not be brought into the PCR preparation room. Templates should be dispensed on a clean bench (or equivalent space) in the PCR room (post-PCR room). When dispensing templates in the PCR room, switch off the fans in the room to prevent dust in the laboratory from rising.

1) Turn on the thermal cycler before starting the experiment.

2) Primer dilution: Dilute the primer stock solution to 5 µM using a commercially available TE buffer.

3) Reagent composition: When PCR is performed in a total volume of 15 µL (including 1.86 µL of

DNA), the composition per tube is as follows. (Caution: More than 1.2 times of the required amount of reagent should be prepared. Otherwise, the reagent may not be dispensed to all the tubes with an electric pipette.) The final concentration of each of the forward and reverse primers is approximately 293 nM.

KAPA HiFi HS ReadyMix	7.5 µL
Primer	0.88 µL each
DNase/RNase-free water	3.88 µL
Quantified first PCR product	1.86 µL

Note: In the 2nd PCR, the combination of index sequences in the forward and reverse primers changes for each sample. This allows different samples to be identified after sequencing. Although there are various methods to make different combinations of the index sequences, we here adopt a method of changing the index sequences for "rows" and "columns" when arranging 8-tube strips in a tube rack (Combinatorial Dual Index method; CDI). Since 8-tube strips are placed in the rack, the row is always composed of 8 tubes. Columns, however, can be increased or decreased in number by adjusting the number of 8-tube strips. As an example, a combination that enables parallel sequences consisting of 32 libraries (= samples) by using eight types of forward primer (index sequences: D501–D508) and four types of reverse primer (index sequences: A701–A704) is described. Furthermore, as discussed at the beginning, to minimize the effect of carryover between runs (the library of the previous runs remaining in the flow paths inside MiSeq), the use of a combination of index sequences that has been once used should be avoided in several subsequent runs (2-3 runs).

D501/A701	D501/A702	D501/A703	D501/A704
D502/A701	D502/A702	D502/A703	D502/A704
D503/A701	D503/A702	D503/A703	D503/A704
D504/A701	D504/A702	D504/A703	D504/A704
D505/A701	D505/A702	D505/A703	D505/A704
D506/A701	D506/A702	D506/A703	D506/A704
D507/A701	D507/A702	D507/A703	D507/A704
D508/A701	D508/A702	D508/A703	D508/A704

To create the combinations listed above, create eight different premixes consisting of four tubes containing the forward primers (D501–508) in the direction of the row (horizontal direction) and four different premixes consisting of eight tubes containing the reverse primers (A701–704) in the direction of the column (vertical direction) (Fig. 5-2-2-1-1). By creating premixes containing primers in such a manner (rather than dispensing only one primer solution at a time), reaction solutions can be accurately prepared even if the total volume of the PCR is small (15 µL in this case).

4) After dispensing, lightly close the caps of the 8-tube strips and centrifuge it in a small tabletop centrifuge to spin down the solution to the bottom of the tube.

5) Take the 8-tube strips together with the tube rack to the clean bench in the PCR room (or equivalent space).

6) Carefully open the caps of the 8-tube strips to prevent the premix from scattering, and add 1.86 µL of each diluted 1st PCR product to the designated tubes.

7) Tightly close the caps of the 8-tube strips and centrifuge it in a small tabletop centrifuge to spin down the solution to the bottom of the tube.

8) In the 2nd PCR, perform shuttle PCR that combines annealing and extension reactions. Set up the thermal cycler as follows:

DNA denaturation at 95°C for 3 minutes (initial denaturation and activation of HotStart enzyme)
Denaturation at 98°C for 20 seconds
Annealing plus extension at 72°C for 15 seconds } 10 cycles
Extension reaction at 72°C for 5 minutes (final extension)
4°C

9) Set the 8-tube strips on the thermal cycler and start the 2nd PCR.

10) After the reaction is complete, immediately remove it from the thermal cycler and store it frozen at –20°C until the next step.

11) By the completion of the 2nd PCR, a unique index has been added to each sample. This allows facilitates combining samples into one tube after enzyme inactivation or index primer removal. Combine samples to be analyzed together (e.g., 10 samples taken in one survey) into one tube (Fig. 5-2-2-1-2). If dilution is performed after the 1st PCR, no particular concentration adjustment is required. However, if the samples contain PCR inhibitors, the amplification rate may remain affected even after dilution, reducing the number of obtained reads.

※1 When using Illumina sequencers, to minimize the effect of index hopping (swapping of tags added for sample identification) that occurs during sequencing, the forward and reverse primers used in the 2nd PCR may not be shared between columns or rows, and a completely different combination of primers may be used for each sample (Unique Dual Index method; UDI). In particular, the incidence of index hopping is high in sequencers that use patterned flow cells, such as iSeq. Therefore, use of UDI is considered essential when using such models for metabarcoding analysis.

5-2-2-2. Purification of 2nd PCR product

The 2nd PCR product consists of a 1st PCR product region (about 300 bp) with a flow cell binding sequence (total 53 bp) and an index sequence (total 16 bp) at its ends, which are necessary for massively parallel sequencing using MiSeq. A 1st PCR product often includes a non-specific fragment (presumed to be a product derived from 16S rRNA of a microorganism) as large as 70 bp larger than a target fish-derived product. In this section, we describe the method of excising only the 2nd PCR product (approximately 370 bp) derived from fish by gel electrophoresis using E-Gel (practically, it is the pipette aspiration method).

Always wear disposable gloves for experiments when working.

1) Turn on the E-Gel Power Snap Plus Electrophoresis Systems (hereinafter referred to as Power Snap).

2) Open the package containing E-gel SizeSelect II 2% (gel enclosed in a plastic case, hereafter called “gel”), carefully remove the two combs inserted into the gel wells (Fig. 5-2-2-2-1), and insert it into the Power Snap (Figure 5-2-2-2-2).

3) Prepare molecular size markers appropriately to achieve a total volume of 25 µL. In addition, mix 22.5 µL of the 2nd PCR product with 2.5 µL of the included Loading Buffer to achieve a total volume of 25 µL.

4) After adding 50 µL of water to each well, load a molecular size marker and the 2nd PCR product into each well in the upper row. Load the molecular size marker into a well other than the sample

wells.

5) Close the filter cover of the Power Snap (Figure 5-2-2-2-3). Operate the “Category” and “Type” on the screen, select “E-Gel EX 2%,” set the electrophoresis time to 14 minutes initially, and start the electrophoresis.

6) After approximately 10 minutes from the start, use the “View Gel” function to check the status of the product. While adjusting the electrophoresis time, continue the electrophoresis until the target band of approximately 370 bp approaches the collection well of the gel.

7) Stop the electrophoresis when the target band is sufficiently close to the collection well (Figure 5-2-2-2-4). Even after stopping, electrophoresis will continue slightly due to inertia. Thus, attention must be paid to the timing of stopping.

8) Refill the collection well with 25 µL of DNase/RNase-free water.

9) Carefully restart the electrophoresis. Stop the electrophoresis once the entire band has entered the collection well.

10) Recover the target band from the collection well using a pipette and collect the band into one 1.5 mL tube per library. Avoid piercing the agarose during collection.

Note: In many cases, enough of the 2nd PCR product can be recovered from the collection well, but the concentration may be insufficiently high to adjust the product to 4 nM after quantification. In this case, you may repeat a sequence of the following procedures: fill the collection wells with DNase/RNase-free water and restart the electrophoresis. Then, bring the band on the downstream side of the collection well, set the electrophoresis mode to Reverse E-Gel, return the band to the collection well, and recover the target band again. Finally, a sufficiently high concentration of the 2nd PCR product may be obtained by concentrating the product using a spin column or other methods.

5-2-2-3. Quantification of the excised 2nd PCR product

This section describes a simple method for measuring the concentration of a library excised from E-Gel using Qubit. To determine the concentration more accurately, Illumina recommends measurement using qPCR, but it is empirically expected that Qubit measurement is sufficient. Here, we explain an example of using Qubit 4.0.

Always wear disposable gloves for experiments when working.

1) Remove the Qubit dsDNA HS Assay Kit from the refrigerator and let it equilibrate to room temperature over 30 or more minutes (Fig. 5-2-2-3-1).

2) The number of tubes required to measure the concentration using Qubit should be counted including the calibration Standards #1 and #2 in addition to the libraries..

3) Once the temperature of the kit has returned to room temperature, prepare the required number of 200 µL premixes, which contain 1 µL of Qubit dsDNA HS Reagent and 199 µL of Qubit dsDNA HS Buffer, per measurement (Figs. 5-2-2-3-2 and -3). If only one library requires concentration measurement, a total of three libraries, including two calibration standards, are needed. Thus, prepare the premix by adding 597 µL of Qubit Buffer to 3 µL of Qubit Reagent.

4) Prepare two 0.5 mL tubes for the Qubit measurement and dispense 190 µL each of premix to calibration Standards #1 and #2. Then, add 10 µL of Standard #1 and #2 supplied with the kit to the tubes (Fig. 5-2-2-3-4), respectively. Close the lids, vortex the tubes for 2–3 seconds, and

centrifuge them briefly.

5) Prepare the required number of 0.5 mL tubes for libraries, dispense 198 µL each of premix into the tubes, and then add 2 µL of the library to each (Fig. 5-2-2-3-5). Close the caps, vortex the tubes for 2–3 seconds, and centrifuge them gently. Allow all tubes to incubate at room temperature for 2 minutes (Fig. 5-2-2-3-6).

Note: Incubate at room temperature for at least 2 minutes or measurements will not stabilize.

6) Turn on and start up the Qubit Fluorometer (Fig. 5-2-2-3-7). Select "dsDNA" on the screen and then select the kit to be used ("dsDNA High Sensitivity" in this case). Select calibration using calibration standards and measure Standards #1 and #2 prepared in (4) in order according to the Qubit instructions (Fig. 5-2-2-3-8).

7) Next, measure the concentration of the libraries. Set the tubes, touch "Calculate Stock Conc," set the sample volume at 2 µL, and measure the concentration (Figs. 5-2-2-3-9 and -10). It is advisable to repeat the concentration measurement of the same sample until the measured value becomes stable.

8) Qubit 4.0 displays the sample concentration (ng/µL) and the measured value (ng/mL) of the measurement solution prepared by diluting the sample 100-fold (Figs. 5-2-2-3-10 and -11). If the sample concentration is less than 1.0 ng/µL, some ingenuity will be required, such as recovering the same library from multiple wells and concentrating it using a column.

5-2-2-4. Index information

The index sequences can be selected from those listed by Hamady et al. (2008). However, when using multiple i5 sequences or multiple i7 sequences, attention must be paid to the color balance of the index sequences used simultaneously, and those that differ by at least three bases must be selected. When filling out the sample sheet for the MiSeq run, the i7 sequence needs to be converted to the reverse complement sequence. However, it should be noted that the sheet is filled out differently depending on the model, such as iSeq100 and NextSeq550. Cross-contamination between runs (contamination that originates from the library from the previous run remaining in the flow path inside MiSeq) can be avoided by changing the index for each run. Therefore, it is recommended to have a large number of index sequences readily available.

Referemce

Hamady, M., Walker, J. J., Harris, J. K., Gold, N. J. & Knight, R. (2008) "Error-correcting barcoded primers for pyrosequencing hundreds of samples in multiplex." *Nature Methods* 5: 235–237.

5-2-3. Massively parallel sequencing using MiSeq

Before starting sequencing

This section describes the massively parallel sequencing method using Illumina MiSeq. The most important point for successful sequencing using this method is to prepare a high-quality library that consists exclusively of MiFish amplicons without adapter dimers or non-specific products. In order to obtain such high-quality libraries and, at the same time, obtain almost as many reads across the libraries, this protocol provides that the 1st PCR products are purified and quantified, and the 2nd PCR was performed using the 1st PCR product as a template adjusted to a fixed concentration (0.1 ng/μL). In addition, since maintenance of MiSeq is very important in order to successfully perform runs as well, information related to maintenance is listed first. Furthermore, an analysis using MiFish primers has shown that comparable taxonomic detection results were obtained using Illumina's iSeq100 (which uses a patterned flow cell) and MiSeq (which uses a random flow cell) (Nakao et al. 2021).

Reagents and consumables required for MiSeq maintenance (illustrative)

- Tween 20 (P7949, Sigma Aldrich Japan GK)
- DNase/RNase-free water
- 50 mL freestanding centrifuge tubes
- 500 mL washing bottles
- 0.01% sodium hypochlorite solution (NaOCl)
- MiSeq Disposable Wash Tubes (MS-102-999, Illumina Inc.)
- Disposable gloves (powder-free)

Laboratory instruments, reagents, and consumables for sequencing (illustrative)

- MiSeq (Illumina Inc.)
- MiSeq Reagent Kit v2 (300 cycles) (MS-102-2002, Illumina Inc.): Use this product when a total of 15 million reads is required. If fewer reads are sufficient, use MiSeq Reagent Micro Kit v2 (MS-103-1002; a total of approximately 4 million reads) or MiSeq Reagent Nano Kit v2 (MS-103-1001; a total of approximately 1 million reads) depending on the number of reads required.
- Libraries with known concentrations
- 0.2 N NaOH (prepared for each experiment from a stock with a high concentration, such as 2 N)
- PhiX Control v3 (FC-110-3001, Illumina Inc.)
- 99.5% ethanol with molecular biology grade (Fujifilm Wako Pure Chemical Industries, Ltd.)
- DNase/RNase-free water
- Deionized water
- 1.5 mL tubes (low DNA bind)
- Vortex mixer (VORTEX-GENIE 2 Mixer, M&S Instruments Inc.) and 3-inch platform
- Micropipette P-1000, P-200, P-100, P-20, P-2 (e.g., Pipetman, Gilson Company Inc.)
- Filter tips (types for the volumes of the micropipettes to be used)
- 1000 μL pipette tips
- Lens cleaning tissue (2105-841, Whatman Inc.)
- Desktop small centrifuge (e.g., Micro Six MS-1, ASONE Corp.)
- Bat for thawing reagent kit (Approximately 373 W × 273 D × 63 H) (e.g., PB-2, ASONE Corp.)
- Disposable gloves (powder-free)

The MiSeq Reagent Kit includes 1) an HyB Buffer tube and 2) a reagent cartridge (Fig. 5-2-3-A) as frozen components as well as 3) a PR2 bottle and 4) a flow cell (Fig. 5-2-3-B) as refrigerated components. Be careful not to confuse refrigerated and frozen storage. In addition, once frozen products have been thawed, they must not be re-frozen.

5-2-3-1. Maintenance of MiSeq

The following three types of cleaning are applied to MiSeq. 1) Maintenance wash: The wash has to be performed once a month, consisting of the 3 × 20-minute-long washes with two replacements of the wash solution (the MiSeq kit requests a maintenance wash if it is left without washing for over one month after the last run). 2) Standby wash: 3 × 50-minute-long washes with one replacement of the wash solution (the MiSeq kit requests a wash if it is left in the idle condition for more than 1 week). 3) Post-run wash: 1 × 20-minute-long wash without replacement of the wash fluid after the run. Note that a protocol known as the sodium hypochlorite wash, which uses an adapter provided by Illumina Inc., should be applied to remove residual library carried over from the last run from the microtube inside the MiSeq. After a run is completed, the post-run wash is performed, and the switch is turned off. Then, a series of washes as provided below are performed before the next run. Note that the date of the last wash is displayed on the top screen of the wash.

(i) Switch on MiSeq main unit (MiSeq control software starts up)
↓
(ii) Maintenance wash (If the system is not used for more than one month after the last run)
↓
(iii) Post-run wash using a sodium hypochlorite solution (for removing the library carried over from the last run)
↓
(iv) Start MiSeq run
↓
(v) Normal post-run wash (do not use a sodium hypochlorite solution)
↓
(vi) Switch off the MiSeq main unit

Procedure common to all wash processes

Always wear disposable gloves for experiments when working.

1) Preparation of 10% Tween 20 wash solution: Dilute the Tween 20 stock solution (Fig. 5-2-3-1-1) to make a 10% Tween 20 solution (stock available), and further dilute it to prepare a 0.5% Tween 20 wash solution, which will actually be used for the washes. Tween 20 prepared to 10% may be purchased and used. Carefully pour 5 mL of the Tween 20 into 50 mL centrifuge tubes (be careful not to pour too much of the Tween 20 because it is very viscous; Fig. 5-2-3-1-2). Add DNase/RNase-free water to the centrifuge tubes to make 50 mL. Shake the centrifuge tubes well to mix the solution. Precisely 25 mL of the 10% Tween 20 solution is used in the first wash, and this is the wash solution for two washes.

2) Preparation of 0.5% Tween 20 wash solution: Put 25 mL of the above 10% Tween 20 solution into a 500 mL wash bottle (Fig. 5-2-3-1-3) and add DNase/RNase-free water until the solution reaches 500 mL on the scale of the wash bottle. Mix by inverting about 10 times.

3) Filling of the wash solution: Inject wash solution into the wash cartridge using the washing bottle containing the solution (Fig. 5-2-3-1-4). When wash solution is poured up to the height of approximately 1 cm from the top (hole) of the wash cartridge, the cartridge will contain

approximately 6 mL. Once the solution has been completely poured, transfer the remaining wash solution to a wash bottle (about 350 mL) (Fig. 5-2-3-1-5).

4) Prepare the wash: Press the “Perform Wash” button in the lower left of the top screen of the MiSeq control software (MCS) (Fig. 5-2-3-1-6) to display three types of washes (Fig. 5-2-3-1-7). Select the wash you want to perform. Check that the used flow cell (the flow cell used in the last run) is set in the flow cell compartment (Fig. 5-2-3-1-8). When you press “Next” in the lower right of the screen, a screen opens indicating the procedure for taking out the wash cartridges, wash bottles, and waste liquid bottles.

5) Setting a wash cartridge: After a while, an image of taking out cartridges and bottles is displayed on the screen (Fig. 5-2-3-1-9). Open the reagent compartment door of the MiSeq and then open the reagent chiller door. Pull out the cartridge used for the last run from the reagent chiller or the wash cartridge used for the last wash, insert the wash cartridge to the end (Fig. 5-2-3-1-10), and close the reagent chiller door.

6) Setting a wash bottle and a waste liquid bottle: Pull up the sipper handle of the reagent compartment. Set the prepared wash bottle (Fig. 5-2-3-1-11), empty the waste liquid bottle and set it in place, and then pull down the shipper handle (Fig. 5-2-3-1-12).

7) Start wash: When the wash cartridge, wash bottle, and waste liquid bottle are set, close the reagent compartment door of MiSeq and press “Next” in the bottom right of the screen to start the wash (Fig. 5-2-3-1-13).

8) End of post-run wash: When the wash is finished, "Done" is displayed in the lower right corner. When you press it, you will return to the top screen of the MiSeq control software, indicating that the entire wash process is completed. For maintenance and standby washes, the wash solution needs to be replaced as described below. Therefore, thoroughly wash the wash bottle used earlier with tap water, rinse it with Milli-Q, and prepare a 0.5% Tween 20 wash solution again. It is recommended to prepare the amount to be used at once.

9) Replacement of the wash solution: When the first round of maintenance or standby wash is completed, a message prompting replacement of the wash solution is displayed. Remove the wash cartridge, wash bottle from the reagent compartment of MiSeq, and discard the solution remaining in them. Rinse the cartridge port and bottle interior thoroughly with tap water and then rinse thoroughly with DNase/RNase-free water. Fill the ports and bottles with the newly prepared 0.5% Tween 20 wash solution as described above and set it in the reagent compartment of the MiSeq again. Since the waste solution does not accumulate so much, the waste liquid bottle may be used as it is.

10) End of wash: When all the steps of maintenance or standby wash are completed, "Done" is displayed in the lower right corner (Fig. 5-2-3-1-14). Press “Done” to return to the top screen of the MiSeq control software, indicating that the entire wash process is completed.

Wash using a sodium hypochlorite solution

1) Prepare a wash cartridge and insert MiSeq Disposable Wash Tubes, which contain 1 mL of sodium hypochlorite solution adjusted to 0.01%, in port 17 (Figs. 5-2-3-1-15 and -16). Pour the 0.5% Tween 20 wash solution into the ports, excluding the 17th port, in the same manner as in the usual post-run wash, and pour the rest in the wash bottle.

2) When the preparation is completed, press the “Perform Wash” button in the lower left of the top screen. Three types of wash are displayed. Select "Post-Run wash." Another screen opens slightly below the center containing a checkbox asking whether the sodium hypochlorite solution is used. Check the box.

3) Press “Next” in the lower right. The subsequent steps are the same as the above-described wash. When the wash is completed, "Done" is displayed in the lower right corner. When you press the button, you will return to the top screen of the MiSeq control software.

Post-run wash after the run

When the run is completed, "Start Wash" will be displayed in the lower right. The subsequent steps are the same as the steps of the wash described above.

Boot up of MiSeq main unit

Turn on the switch on the right back side of the MiSeq main unit. Windows, the OS supplied with the main unit, boots up. MiSeq control software (MCS) is set to start automatically when the startup is completed (Fig. 5-2-3-1-17). It takes approximately 5 minutes to complete the start-up. The MiSeq main unit should be started up in a manner to coincide with the start of the run. In addition, to avoid issues such as an interrupted run, it is recommended that the MiSeq main unit be restarted once all pre-run washes have been completed.

5-2-3-2. Preparing for MiSeq sequencing

This section describes the procedures that should be completed at least one hour before actually starting any sequencing. Always wear disposable gloves for experiments when working.

1) Thaw the frozen components: Put the frozen reagent cartridge and HyB buffer tube in a stainless tray and gently pour deionized water up to the maximum water volume line marked on the side of the cartridge (Fig. 5-2-3-2-1). Leave them immersed in the deionized water for approximately 1 hour to fully thaw the reagents inside the kit. They may be thawed at 4°C overnight the day before. The reagents can be stored stably at 4°C for up to 1 week.

2) Prepare 0.2 N NaOH solution: The 0.2 N NaOH solution is used to denature the DNA of the library into single-stranded DNA. Prepare a fresh 0.2 N NaOH solution by diluting from 2 N NaOH solution (can be stocked) with DNase/RNase-free water for each run. Since the amount to be used is very small, 1 mL can be prepared in a 1.5 mL tube, which results in minimal errors during dilution while achieving a sufficient amount. Store the prepared 0.2 N NaOH solution on ice or in the refrigerator until use; it should be used within 12 hours. It can be used for other runs as long as it is within 12 hours. Dispose of the remaining NaOH solution according to the waste disposal standards of each facility.

3) Generate a sample sheet: Start the software Illumina Experiment Manager (IEM) installed in the MiSeq main unit and generate a sample sheet according to the instructions on the screen. Record the reagent cartridge number (example: MS-XXXXXXX-300V2.csv; Fig. 5-2-3-2-2) on the side of the cartridge. The number will be required to create the sample sheet later. In addition, if it is saved in CSV format, the sample sheet can be edited as a spreadsheet in Excel or other software. Therefore, it is more efficient to generate a base file with IEM and re-edit it as a spreadsheet. Store the generated sample sheets in the Illumina/Illumina Control Software folder of the MiSeq main unit.

5-2-3-3. Final adjustment of library concentration

This section describes an experimental procedure for forming clusters at an appropriate density on a flow cell in which sequencing reactions are performed and for more effectively separating one cluster from another. The number of reads expected to be obtained by performing the following procedures is the total number of reads listed on the kit minus the proportion of spiked-in PhiX.

1) Adjustment of the library to 4 nM: Adjust the library to 4 nM based on the concentration measured by Qubit. The number of moles is determined based on library length (mean 372 bp) and molecular weight per base (660 g/mol). Since the concentrations must be calculated across multiple units (micro/nano/pico), it is better to include the mole number conversion formula in the spreadsheet beforehand. In addition, when multiple libraries with different numbers of samples are sequenced simultaneously, the mixing ratios are calculated based on the number of samples (excluding blanks) and the allocation of the desired number of reads for each sample, and the mixing amount of each is determined. Libraries selected according to the size of the 2nd PCR products have a mean library length of 372 bp. When the weight concentration of the solution is α ng/μL, the molar concentration can be obtained by the calculation formula $\alpha*10^6/372*660$.

2) Denaturation of DNA: Pipet 5 μL of the library adjusted to 4 nM and 5 μL of 0.2 N NaOH into a new 1.5 mL tube. Then, vortex for a few seconds, briefly centrifuge to collect the solution at the tube bottom, and let stand at room temperature for 5 minutes to denature the DNA in the library. At this point, denatured DNA has a concentration of 2 nM.

3) Adjust to 20 pM: Add 990 μL of HyB Buffer to the denatured DNA and adjust it to 20 pM (100-fold dilution).

4) Adjust the final concentration to 12 pM: When the final concentration of the MiFish amplicon is adjusted to 12 pM, the amplicon will have a cluster density of 800–1000 K/mm^2 and almost as many reads as recommended by the vendor. To achieve a final concentration of 12 pM, add 240 μL of HyB buffer to 360 μL of the library solution. To control the cluster density more precisely, evaluate the concentration of only the fragments that can form clusters using a qPCR kit for Illumina library quantification (e.g., GenNext NGS Library Quantification Kit from Toyobo Inc.) and adjust the final concentration.

5) PhiX spike-in: To improve signal separation between clusters formed on the flow cell, add 10-25% PhiX control v3 (Illumina Inc.) as a spike-in. Denature it with 0.2 N NaOH in the same manner as to denature the sample DNA and adjust it to 20 pM with the HyB buffer. When using 10% PhiX, for example, extract 60 μL from the 600 μL library above and add 36 μL of PhiX adjusted to 20 pM and 24 μL of HyB Buffer. The appropriate amount of PhiX to be added must be determined depending on the library.

The above procedure has resulted in the preparation of 600 μL of library adjusted to 12 pM. Inject it into the reagent cartridge and load it into MiSeq.

5-2-3-4. Procedures before and after start of sequencing

This section describes the procedures required before and immediately after the start of sequencing. The library has already been adjusted to 12 pM and is ready for sequencing once the instruments are set up correctly.

Always wear disposable gloves for experiments when working.

1) Check that the reagent in the reagent cartridge is completely thawed and wipe off any water droplets on the outside of the cartridge with a paper towel. Gently invert the reagent cartridge approximately 10 times to mix the reagent in the cartridge. To remove air bubbles from the reagent, tap the cartridge several times on the tabletop covered with the paper towel (Fig. 5-2-3-4-1).

2) Use a 1000 μL pipette tip to piece the foil at the library injection port (marked by the orange color) (Fig. 5-2-3-4-2). Discard the used chip.

3) Use a new 1200 µL or 1000 µL pipette tips to place 600 µL of the library adjusted to 12 pM into the injection port of the reagent cartridge (Fig. 5-2-3-4-3). Be careful on pipetting to prevent bubbles from entering the library. Cover the injection port with parafilm, protect it from light, and store it at 4℃ until run.

4) Boot up the MiSeq Control Software (MCS) and press the "SEQUENCE" button to proceed to run setup.

5) If MiSeq is connected to the Internet, a confirmation button appears asking whether you want to use BaseSpace (a cloud environment provided by Illumina Inc.) after pressing the SEQUENCE button. (Fig. 5-2-3-4-4). User registration with MyIllumina is required before using BaseSpace.

6) To use BaseSpace, check the "Use BaseSpace" box for storage and analysis and enter the information used when registering your MyIllumina account in the display field. If you are not using BaseSpace, leave this box blank. After selecting, press the "Next" button to go to the flow cell setting screen.

7) Remove the chilled flow cell from the container using polyethylene tweezers. Because the flow cell is stored immersed in the buffer inside a container, rinse the glass and plastic parts of the flow cell thoroughly with DNase/RNase-free water before setting it on the MiSeq. Since buffer is often left in the polyethylene case of the flow cell, rinse the area thoroughly and gently and carefully shake the flow cell to discharge any water from the polyethylene case. At this time, be careful to keep the water flow from intensely contacting the gasket (opening of the flow path to the reagent or sample).

8) Apply a small amount of 99.5% ethanol to the lens cleaning tissue and gently wipe any water off the glass part of the flow cell. Check the glass for dirt and stains and lightly polish with a lens cleaning tissue until it is clean. Be careful not to touch the two-hole gasket port on the plastic part of the flow cell.

9) Open the MiSeq flow cell compartment door and then open the flow cell latch and remove the used flow cell. When you open the latch, you should press the silver button for opening the latch while keeping a hand on the latch in order to prevent the flow cell from suddenly jumping up. If there is dust or precipitated buffer on the pedestal of the flow cell latch, wipe them off with the lens cleaning tissue used for polishing the flow cell.

10) Place the flow cell in place on the pedestal and gently push down on the latch until it is fixed.

11) Check the lower left of the MiSeq control software (MCS) screen to make sure that the ID (RFID) written on the flow cell has been read successfully. If RFID is being read, close the flow cell compartment door and press the "Next" button.

12) Take out the refrigerated PR2 bottle (sequence buffer), mix gently, and remove the screw cap.

13) Open the MiSeq reagent compartment door and pull up the sipper handle. Place the refrigerated PR2 bottle on the right side and empty the waste liquid bottle. When the setting is completed, be sure to pull down the sipper handle.

Note: The run will start whether or not you pull down the sipper handle. If you do not pull it down, you will not be able to aspirate the buffer, which will (probably) stop the run.

14) Check the lower left of the MCS screen to make sure that the ID (RFID) written on the PR2 bottle has been read.

15) Open the door of the reagent chiller and wait until the sipper inserted in the wash cartridge is completely pulled up. Note that if you try to pull out the wash cartridge before the sipper is

pulled up, the sipper may break.

16) Firmly insert the reagent cartridge filled with the library all the way into the reagent chiller.

17) Close the reagent chiller door. Check the lower left of the MCS screen to make sure that the ID (RFID) on the reagent cartridge has been read successfully.

18) The experiment name and the analysis workflow will be displayed on the screen. Check the hierarchy of sample sheet folders in the bottom left of the screen and press “Next.”

19) Move to the pre-run check screen. Checkboxes are displayed on the screen. When all the boxes are checked, the Start Run button becomes active. Press the button to start the run.

20) Sequencing often experiences initial problems for a while after pressing the “Start Run” button. You may, however, restart the run until the reagent flows into the flow cell. It is desirable to watch the MiSeq runs in front of the instrument for about 5 minutes.

Reference

Nakao, R., Inui, R., Akamatsu, Y., Goto, M., Doi, H. & Matsuoka, S. 2021. Illumina iSeq 100 and MiSeq exhibit similar performance in freshwater fish environmental DNA metabarcoding. *Scientific Reports* 11: 15763. Doi: 10.1038/s41598-021-95360-5

Miya, M., Sato, Y., Fukunaga, T., Sado, T., Poulsen, J. Y., Sato, K., Minamoto, T., Yamamoto, S., Yamanaka, H., Araki, H., Kondoh, M. & Iwasaki, W. 2015. “MiFish, a set of universal PCR primers for metabarcoding environmental DNA from fishes: detection of more than 230 subtropical marine species.” *Royal Society Open Science,* 2 (7): 150088. doi: 10.1098/rsos.150088

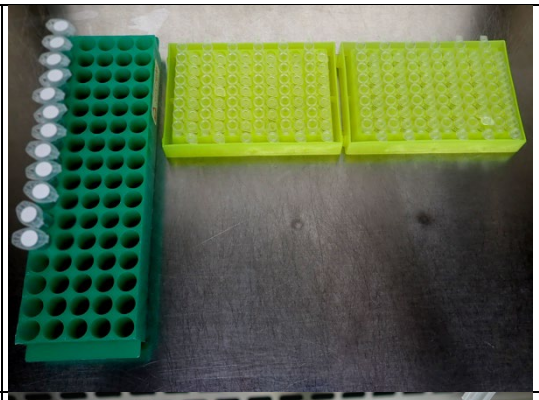
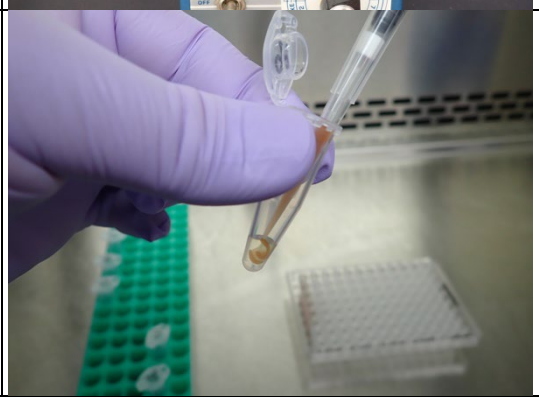
Fig. 5-2-1-1-1 Eight repeats of 1st PCR were set up for the same sample using one 8-tube strip.	
Fig. 5-2-1-2-1 Replicate reactions are pooled together for each sample.	
Fig. 5-2-1-2-2 Stir the SPRIselect bottle thoroughly to disperse the magnetic beads.	
Fig. 5-2-1-2-3 An equal amount of SPRIselect is added to the pooled PCR products from all repeats, mixed by pipetting, and transferred to a 96-well plate (low DNA binding).	

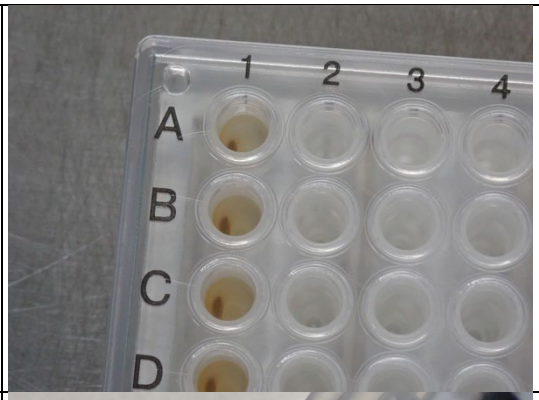
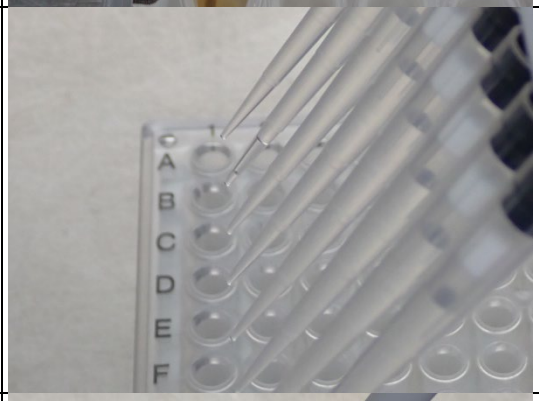
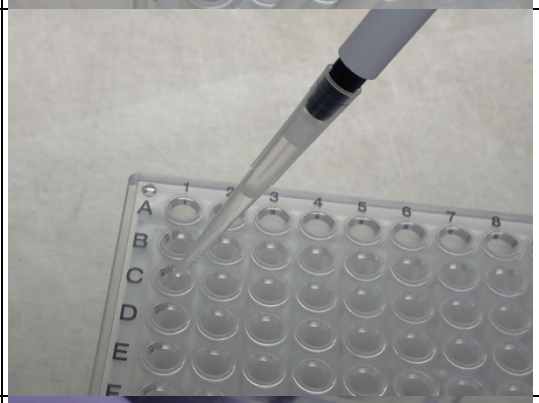
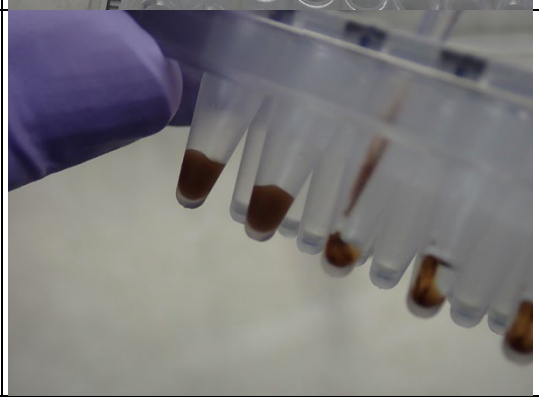
Fig. 5-2-1-2-4 Place on a magnetic plate and leave until the liquid becomes clear.	
Fig. 5-2-1-2-5 Carefully remove the supernatant.	
Fig. 5-2-1-2-6 Add 85% ethanol while still on the magnetic plate, leave for 30 seconds, and carefully remove the supernatant. Repeat this process twice.	
Fig. 5-2-1-2-7 The supernatant remaining on the wall is collected by centrifugation, and placed on the magnetic plate again to completely remove residual ethanol from the magnetic beads. Then, the plate is removed from the magnet and 25 μL of Buffer EB is added to thoroughly disperse the beads.	

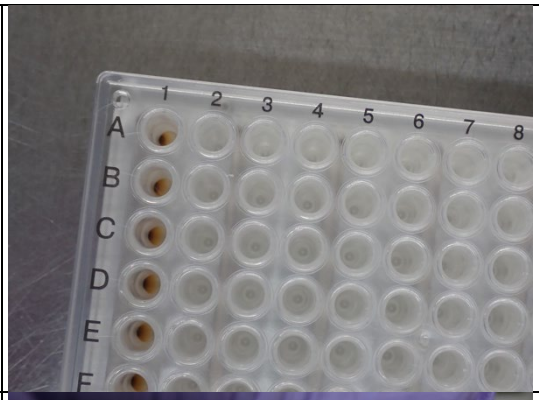
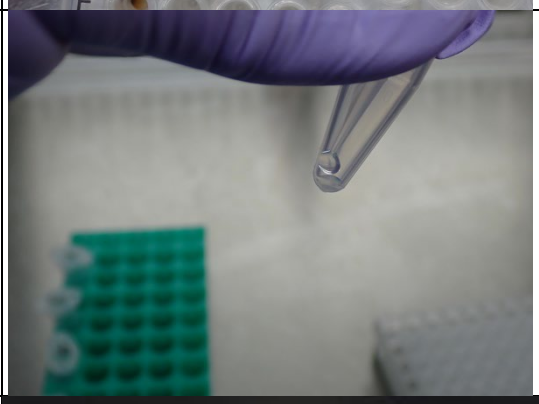
Fig. 5-2-1-2-8 Place it back on the magnetic plate and leave it until the liquid becomes clear.	
Fig. 5-2-1-2-9 Collect all the supernatant, being careful not to suck up the magnetic beads.	
Fig. 5-2-1-3-1 Kit for TapeStation 4150. High Sensitivity D1000 Screen Tape (left) and reagent (right).	
Fig. 5-2-1-3-2 Boot up the computer supplied with the TapeStation 4150.	

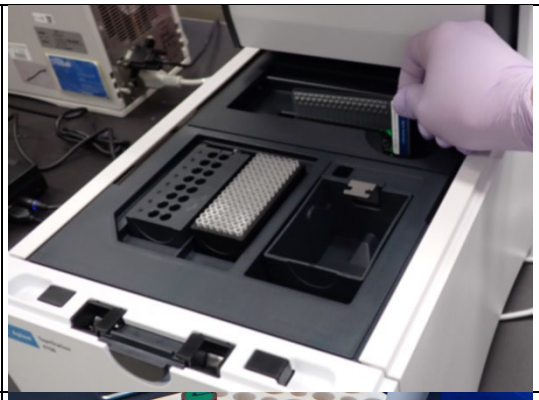
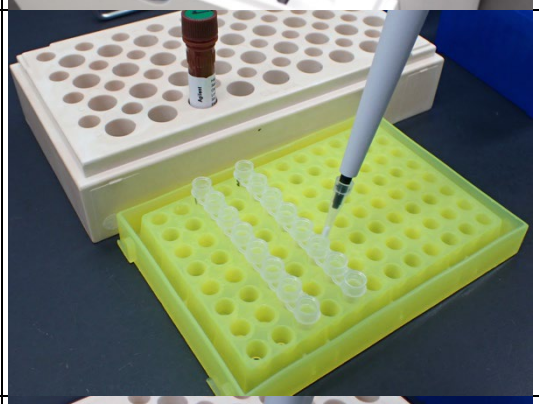
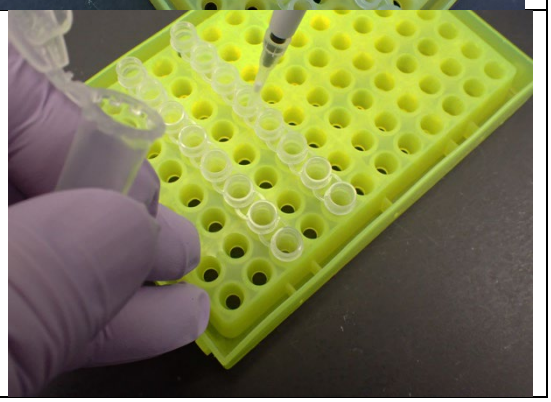
Fig. 5-2-1-3-3 Load the High Sensitivity D1000 Screen TapeScreenTape and the required number of 4150/4200 TapeStation Loading Tips pipette tips.	
Fig. 5-2-1-3-4 Dispense 2µL of High Sensitivity D1000 Sample Buffer into each well.	
Fig. 5-2-1-3-5 Dispense 2µL of High Sensitivity D1000 Ladder into the first tube.	
Fig. 5-2-1-3-6 Add 2 µL of the purified and concentrated 1st PCR product to the remaining tubes.	

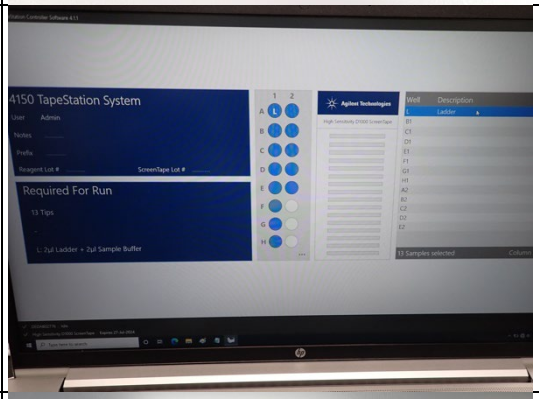
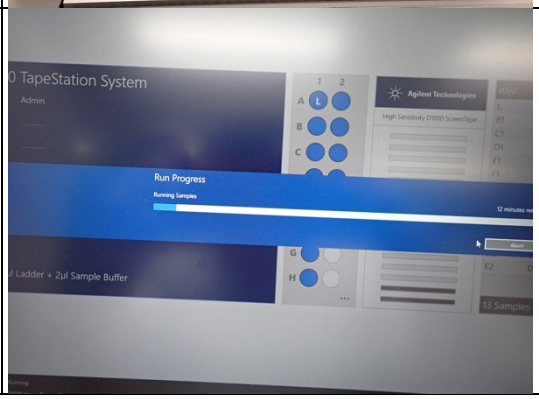
Fig. 5-2-1-3-7 After stirring at 2,000 rpm for 1 minute, the liquid is collected at the bottom of the tube using a tabletop mini centrifuge.	
Fig. 5-2-1-3-8 Carefully open the caps of the 8-tube strips so as not to spill the liquid inside, and place them in the cassette of the TapeStation 4150.	
Fig. 5-2-1-3-9 In the 8-tube strip image on the left, specify the ladder and the arrangement of the PCR products to be measured on the tube.	
Fig. 5-2-1-3-10 When analysis is started, it will automatically perform the analysis after self-diagnosis. The remaining time will be displayed during the electrophoresis.	

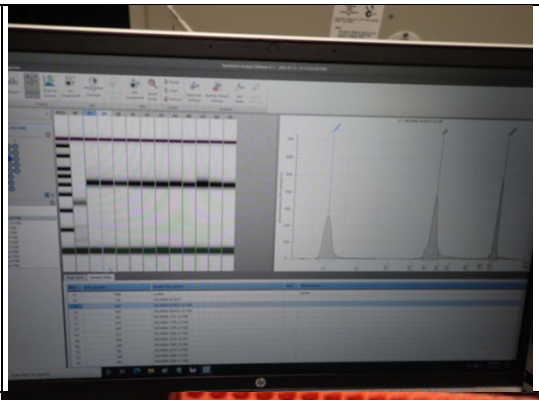
Fig. 5-2-1-3-11 When the electrophoresis is complete, the target band (approximately 310 bp) is displayed along with a pherogram.	
Fig. 5-2-2-1-1 When the number of samples is 40.	
Fig. 5-2-2-1-2 Collect the reaction solution in a single tube.	
Fig. 5-2-2-2-1 Carefully remove the comb.	

Fig. 5-2-2-2-2 Insert the cartridge from the right side of the gel into the iBase.	
Fig. 5-2-2-2-3 Close the rid of the Power Snap.	
Fig. 5-2-2-2-4 The target product (approximately 370 bp) arrived just above the baseline collection well. The ladder (size marker) is in the center. The target product (370 bp) is slightly above the collection well. The bands that are occasionally seen with a smear above the target band are non-specific products, so be careful not to put them into the collection well.	

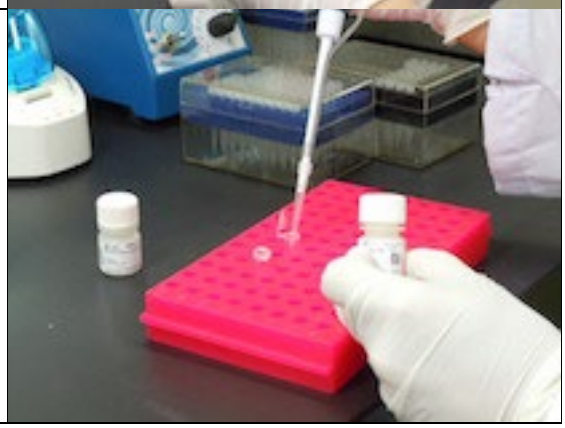
Fig. 5-2-2-3-1 Remove the Qubit dsDNA HS Assay Kit from the refrigerator and return its temperature to room temperature for 30 or more minutes.	
Fig. 5-2-2-3-2 Place the Qubit dsDNA HS Buffer in a new tube.	
Fig. 5-2-2-3-3 Add the Qubit dsDNA HS Reagent.	
Fig. 5-2-2-3-4 Add 10 µL each of standard #1 and #2 to tubes separately.	

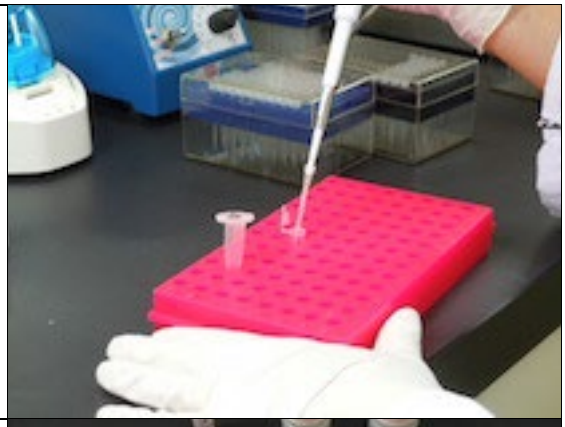
Fig. 5-2-2-3-5 Add 2 μL of the library to the tubes.	
Fig. 5-2-2-3-6 Let stand at room temperature for 2 minutes. If this procedure is omitted, no stable measurements will be obtained.	
Fig. 5-2-2-3-7 Power on and start the Qubit 4 Fluorometer.	

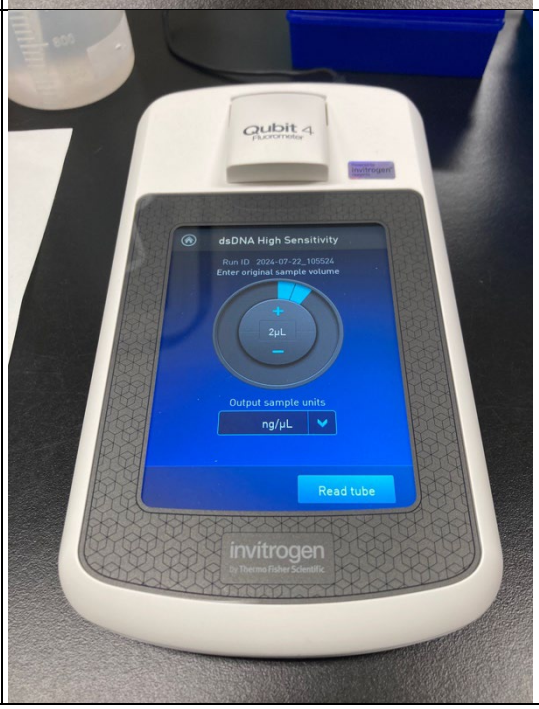
Fig. 5-2-2-3-8 Measure Standards #1 and #2 in that order according to the instructions from Qubit, and create a calibration curve.	
Fig. 5-2-2-3-9 Enter the volume of sample added to the Qubit tube, the units in which the results are displayed, etc.	

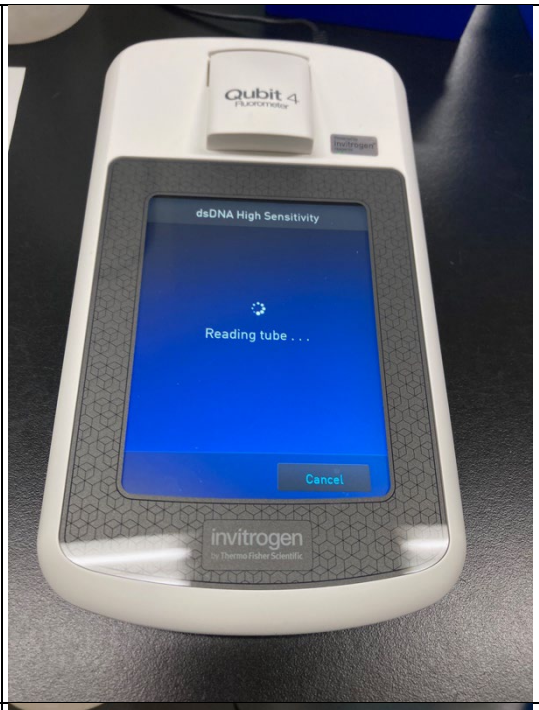
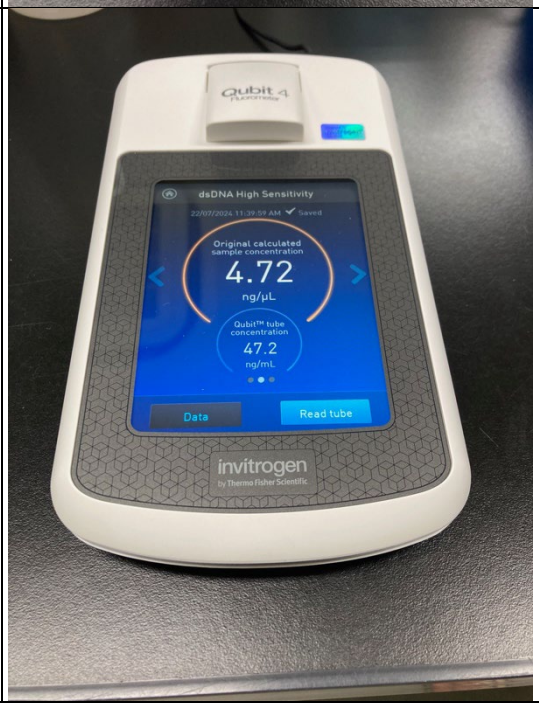
Fig. 5-2-2-3-10 Measuring the concentration of the sample.	
Fig. 5-2-2-3-11 When the sample is subsequently measured, the concentration of the sample DNA is displayed. The top line shows the concentration of the sample, and the bottom line shows the concentration of DNA in the measurement tube.	

Fig. 5-2-3-A Frozen components; HT1 tube (right front) and reagent cartridge (left front).	
Fig. 5-2-3-B Refrigerated components; PR2 bottle (center) and flow cell (right).	
Fig. 5-2-3-1-1 Tween 20 stock solution.	
Fig. 5-2-3-1-2 Carefully pour 5 mL of Tween 20 into a 50 mL centrifuge tube. The Tween stock solution is highly viscous and should be handled with care.	

Fig. 5-2-3-1-3 Place 25 mL of 10% Tween 20 solution into a 500 mL wash bottle.	
Fig. 5-2-3-1-4 Inject the wash solution into the wash cartridge using the wash bottle.	
Fig. 5-2-3-1-5 After the wash solution is injected into the cartridge, transfer the remaining wash solution to a wash bottle (about 350 mL).	
Fig. 5-2-3-1-6 Top screen of the MiSeq Control Software (MCS).	

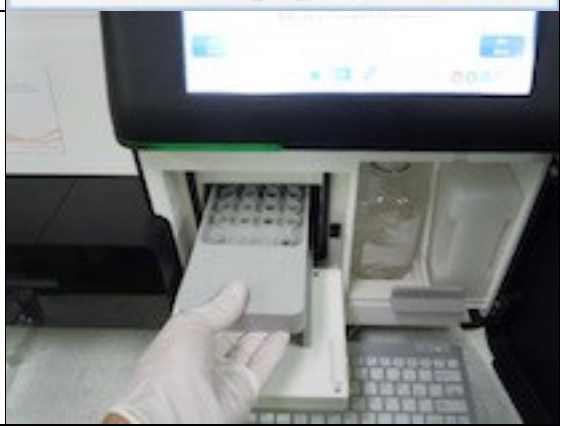
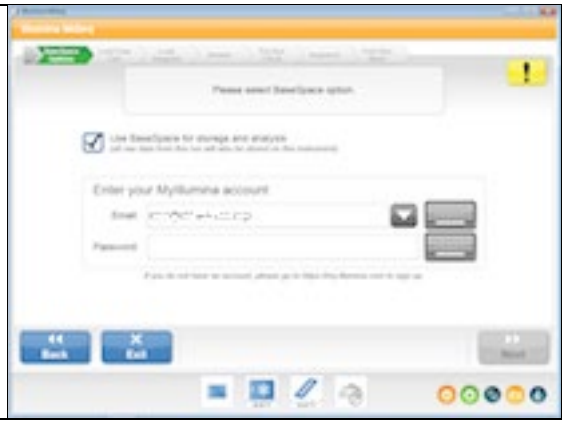
Fig. 5-2-3-1-7 Press the Perform Wash button. Three different wash types are displayed.	
Fig. 5-2-3-1-8 Check that the used flow cell is set in the flow cell compartment.	
Fig. 5-2-3-1-9 The screen will show the reagent cartridges and bottles being removed.	
Fig. 5-2-3-1-10 Place the wash cartridge in the reagent chiller.	

Fig. 5-2-3-1-11 Set the wash bottle.	
Fig. 5-2-3-1-12 After the bottle is set, pull down the sipper handle. Note that even if you forget to pull down the handle, the run will automatically start.	
Fig. 5-2-3-1-13 Press Next in the lower right of the screen to start the wash.	
Fig. 5-2-3-1-14 After all processes are completed, “Done” is displayed in the lower right.	

Fig. 5-2-3-1-15 A special tube for sodium hypochlorite solution.	
Fig. 5-2-3-1-16 Insert a special tube containing sodium hypochlorite solution into port 17.	
Fig. 5-2-3-1-17 The MiSeq control software boots up and starts preparing for the sequence.	
Fig. 5-2-3-2-1 Remove the HyB Buffer tube and gently pour deionized water up to the maximum water volume line marked on the side of the cartridge. Let stand for 1 hour to thaw the reagent completely.	

Fig. 5-2-3-2-2 The reagent cartridge number is written immediately under the barcode. Be careful to correctly transcribe the number because it is small and difficult to read.	
Fig. 5-2-3-4-1 Gently tap the cartridge several times against the test bench covered with the Kim Towel to bring the reagents down to the tube bottom.	
Fig. 5-2-3-4-2 Make a large hole in the injection ports using a 1000 µL pipette tip (orange port 17). Then, make a small hole in the center of the ports to prevent the tubes from getting broken when the sipper is inserted. (Be careful to keep the pipette tip from touching the reagent inside the tube.)	
Fig. 5-2-3-4-3 Place 600 µL of the prepared library into the reagent cartridge injection port (orange) using a 1200 µL pipette tip.	

Fig. 5-2-3-4-4 When you press SEQUENCE button, a confirmation button for BaseSpace will be appeared.	

5-2-4. eDNA metabarcoding sequence data analysis

Introduction

Large amounts of data obtained through environmental DNA (eDNA) metabarcoding are analyzed using a combination of several software programs, referred to as a "pipeline". Different types of pipelines, each with unique operability and algorithms, are available, and the choice of pipeline depends on the user's needs. Here, we present an example of the procedure for using the "MiFish pipeline" and provide an overview of "Claident,".

5-2-4-1. MiFish pipeline

Preparation of FASTQ files

Upon completion of sequencing as described in the workflow in Section 5-2-3, FASTQ files will be generated in the MiSeqOutput/BaseCalls folder (if MiSeq is used). Each FASTQ file corresponds to a single sample, and the filename includes the sample name (Section 5-2-3-2), such as samplename_S1_L001_R1_001.fastq.gz. For detailed information on the filename, structure of FASTQ files, and the workflow for generating FASTQ files using MiSeq, please refer to the following link:

https://support.illumina.com/content/dam/illumina-support/documents/documentation/software_documentation/miseqreporter/miseq-reporter-generate-fastq-workflow-guide-15042322-01.pdf.

Procedures for analyzing a single sample

For the analysis of FASTQ files, we introduce the use of the online MiFish pipeline. In addition, the pipeline is available for downloaded from GitHub (a software development platform) and can be installed on a local computer.

1) Access the MiFish pipeline web platform (https://mitofish.aori.u-tokyo.ac.jp/mifish/) using a web browser.
2) For a single sample, upload the two FASTQ files (read1 and read2) to the web platform (Fig. 5-2-4-1-1).
3) Verify that the uploaded files have been correctly recognized (Fig. 5-2-4-1-2).
4) Upload the FASTQ files to the MiFish server (Fig. 5-2-4-1-3).
5) View and adjust parameters. The default settings are appropriate for most scenarios (Fig. 5-2-4-1-4).
    a) MiFish Ref. DB: Select a reference database. By default, the latest version, which includes the largest number of fish species, is selected. Older versions can be selected for the purpose of reproducing the results of previous analyses.
    b) Filter reads by length: The default values are appropriate for MiFish primers.

c) BLASTN identity: By default, denoised haploid sequences are assigned to the target organism if the identify is greater than 97%. Increasing this value would lead to a more accurate, but less comprehensive results.

6) Click the "Run" button. A separate tab (result page) will be opened in the web browser, which can be saved as a bookmark for future reference.

Note: The result page will be stored on the MiFish server for 1 month. It is recommended to save all tables and figures.

How to read the results of single-sample-analysis

The result page provides statistical information of the raw sequencing reads, the full list of identified fish species, and the results of downstream analysis.

1) Quality control results (Fig. 5-2-4-1-5). This figure can be exported by clicking the ≡ button on the top right. Data files can be exported by clicking the ⬇ button on the top right.
   a) Raw reads
   b) High-quality reads
   c) Merged reads
   d) Non-ambiguous reads without "N" bases
   e) Length-filtered reads (Step 5b in Section 5-2-4-2)
   f) Unique reads
   g) Denoised haploid sequences
   h) Identified fish species

Note: If the number of length-filtered reads is significantly fewer than the number of non-ambiguous reads without "N" bases (Fig. 5-2-4-1-6), there may be errors in the sequencing data, such as significant off-target (non-fish species) amplification or mistakes in library construction.

2) The full list of identified fish species (Fig. 5-2-4-1-7), which includes the following six columns:
   a) Photos from FishBase (https://www.fishbase.se/search.php). Species without available photos are displayed with a default icon.
   b) Scientific names, common names, and some important ecological features. The names are sourced from NCBI Taxonomy (https://www.ncbi.nlm.nih.gov/taxonomy), and data on ecological features are obtained from FishBase.
   c) Labels (high/moderate/low) indicating the confidence level of the species identification. A low confidence level indicates that the identified fish species is ambiguous due to the presence of other fish species with identical or similar barcode sequences.
   d) Number of reads that originate from that species.
   e) Number of haploid sequences. By clicking the corresponding 👁 button, a new box will pop up, displaying the list of haploids (Fig. 5-2-4-1-8).
   f) Representative barcode sequences. By clicking the corresponding 🔍 button, a new tab will open, allowing the user to search for similar sequences in the MitoFish database.

The table is paginated at every 10 rows. The entire table can be exported in Excel format by clicking the ⬇ button on the top right.

Note: The table may include non-fish species due to off-target amplification or contamination.

3) Phylogenetic tree of all haploids contained in this sample (Fig. 5-2-4-1-9). The number of eDNA reads (read abundance) is indicated by circles of different colors and sizes. The file can be exported in SVG and NEWICK formats by clicking the button on the top right.

4) Stacked bar graph showing relative abundance at different taxonomic levels (Fig. 5-2-4-1-10). Different taxonomic levels (class, order, family, and genus) can be switched by clicking the tabs above the figure. This figure can be exported by clicking the button on the top right.

Procedure for analyzing multiple sample groups

The MiFish pipeline can also analyze multiple datasets, including samples obtained from different locations or time points, as well as samples obtained from biological replicates under the same condition (Fig. 5-2-4-1-11). The procedure is almost the same as that described in Section 5-2-4-2, but samples can be divided into groups in Step 3 (Fig. 5-2-4-1-12). Typically, samples with different sampling conditions are placed in different groups, and samples with the same conditions (replicates) are placed in the same group.

How to read the results of multiple-sample-analysis

The structure of the result page is similar to that described in "Single Sample Data" above. The analysis results for different samples can be switched by clicking the navigation tab of "Taxonomic Classification" (Fig. 5-2-4-1-13). In addition, when analyzing multiple groups, the results of α and β diversity between groups are displayed in scatter plots and heatmaps, respectively (Figs. 5-2-4-1-14 and -15). The method for outputting to an external file is the same as described in Section 5-2-4-2.

Note 1: When it is difficult to identify species based on the analysis results, species may be estimated by considering the habitat at the sampling site. However, in such cases, it is desirable to take appropriate measures, such as adding notes to the results.

Note 2: The results of species identification may vary due to the status of database maintenance. Therefore, it is necessary to clearly state the version information of the MiFish pipeline and the gene database used for analysis.

Q & A

Q: What is a reference database?

A: A reference database for metabarcoding is a comprehensive set of DNA sequences of target primer (e.g., MiFish primer) regions of potential target species (e.g., various fish species). Reference databases are crucial for metabarcoding analysis, as DNA sequences and species are linked by similarity searches with reference data. The reference database built into the MiFish pipeline is regularly updated monthly to maintain its completeness and is curated by experts to ensure its accuracy.

Q: What happens after the "Run" button is pressed?

A: Once the "Run" button is pressed, the uploaded FASTQ files undergo a series of pre-processing

steps and are compared against a reference database to identify the species contained in the data. These steps include quality and length filtering, cutting into amplicon regions, denoising, similarity search, phylogenetic analysis, and biodiversity analysis. For a detailed explanation of the workflow, please refer to the following link: https://mitofish.aori.u-tokyo.ac.jp/mifish/help/

Q: What are filtering reads? (Fig. 5-2-4-1-5)

A: Raw Illumina sequencing reads contain noisy data, which must be removed before their comparison with the reference database. Examples of noisy data include the following:

a) Sequencing errors shown as low-quality regions of base-calling
b) Excessive primers or adapters linked to amplicons
c) Off-target amplicons with abnormal lengths

The MiFish pipeline integrates tools that facilitate the automatic removal of such noisy reads.

Q: What is denoising (noise removal)? (Fig. 5-2-4-1-5)

A: In eDNA metabarcoding, low-abundance DNA sequences may be observed because of minor species (i.e., true positives) or artificial sequencing errors (i.e., false positives). Denoising is a technique used to distinguish between true and false positives based on the fact that if such a low-abundance DNA sequence is false positive, it will be accompanied by a nearly identical sequence with high abundance. The MiFish pipeline obtains true positive sequences through denoising.

Q: What is alpha diversity? (Fig. 5-2-4-1-14)

A: Alpha diversity is a value that indicates the diversity in the composition of a biological community present in a particular site or a sample, in terms of species and individuals. It can be assessed using various parameters, including those based on the number of species richness (e.g. chao index) and those based on the relative abundance distribution of species (e.g. Shannon and Simpson indices).

Q: What is beta diversity? (Fig. 5-2-4-1-15)

A: Beta diversity refers to the variation in biological communities observed across different sites or samples. A higher value of beta diversity indicates a greater difference in species composition between samples.

References

Iwasaki, W., Fukunaga, T., Isagozawa, R., Yamada, K., Maeda, Y., Satoh, TP., Sado, T., Mabuchi, K., Takeshima, H., Miya, M. & Nishida, M. 2013. “MitoFish and MitoAnnotator: a mitochondrial genome database of fish with an accurate and automatic annotation pipeline”. *Molecular Biology and Evolution* 30:2531-2540.

Sato, Y., Miya, M., Fukunaga, T., Sado, T. & Iwasaki, W. 2018. “MitoFish and MiFish Pipeline: A Mitochondrial Genome Database of Fish with an Analysis Pipeline for Environmental DNA Metabarcoding”. *Molecular Biology and Evolution* 35:1553-1555.

Zhu, T., Sato, Y., Sado, T., Miya, M. & Iwasaki, W. 2023. “MitoFish, MitoAnnotator, and MiFish Pipeline: Updates in ten years”. *Molecular Biology and Evolution* 40: msad035.

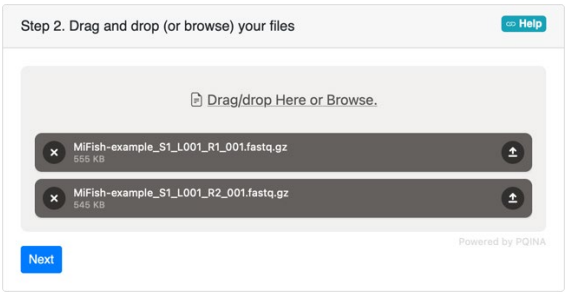
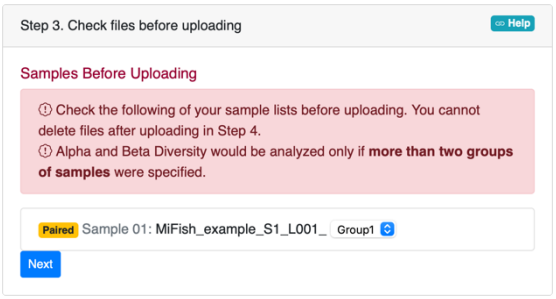
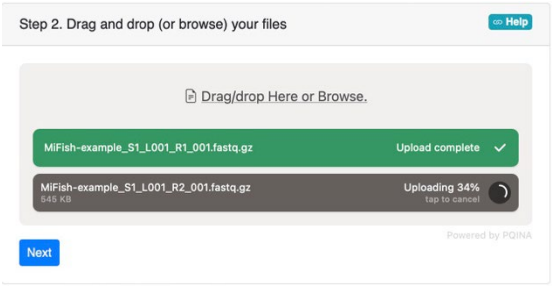
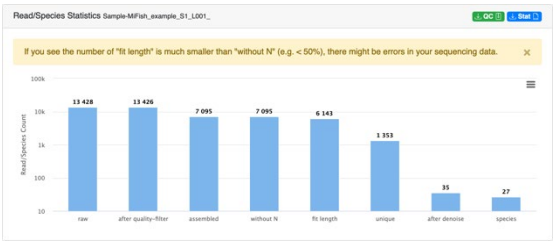
Fig. 5-2-4-1-1 Upload the FASTQ files of your sample onto the web platform. Only compressed files (with extension .gz, .bz2, or .xz) are supported. To remove any uploaded file, simply click on the × button located on the left side.	
Fig. 5-2-4-1-2 Confirmation of whether the uploaded files have been correctly recognized.	
Fig. 5-2-4-1-3 Upload the FASTQ files onto the MiFish server by clicking the ↑ button on the right side. Files that have been successfully uploaded will be highlighted in green and cannot be deleted.	
Fig. 5-2-4-1-4 View and/or adjust parameters as needed. The default settings are suitable for most cases, and it is usually not necessary to change them.	
Fig. 5-2-4-1-5 Bar graph showing the quality control results of raw sequencing reads and the number of denoised haploids and identified species.	
Fig. 5-2-4-1-6 An example of sequencing data that may contain errors. The number of reads decreases abruptly after a certain sequence length.	

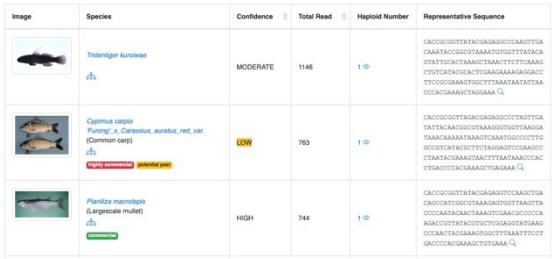
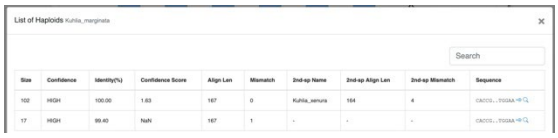
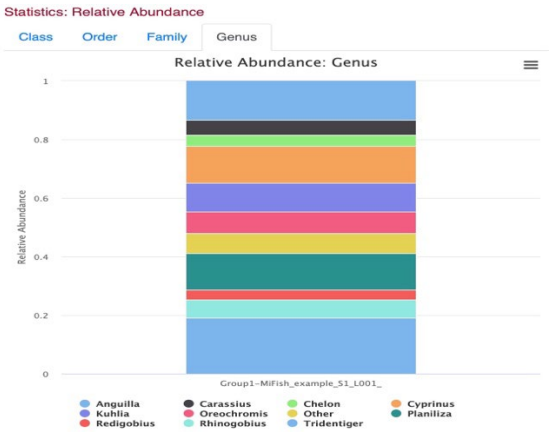
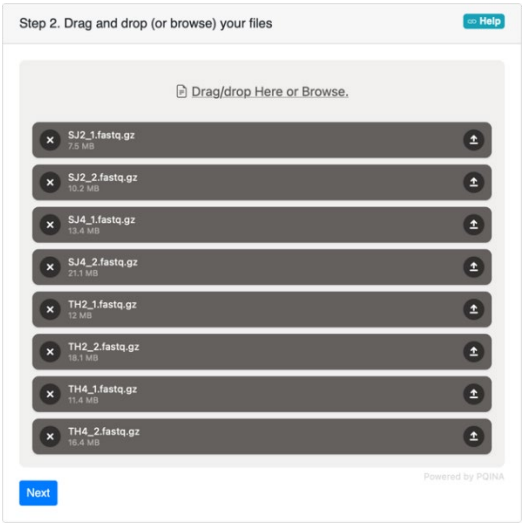
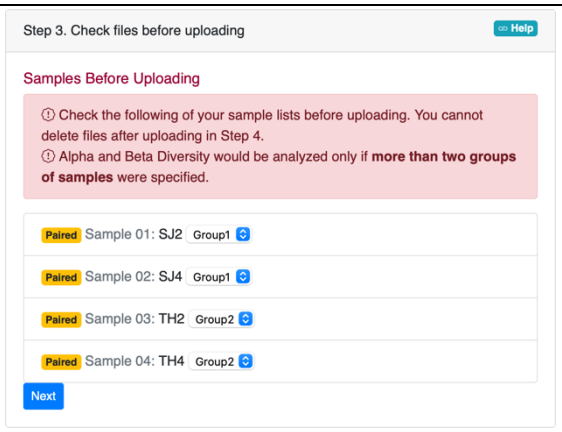
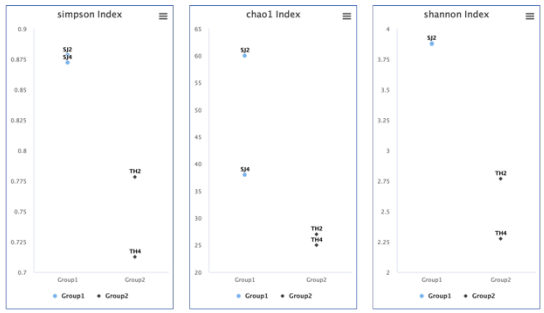
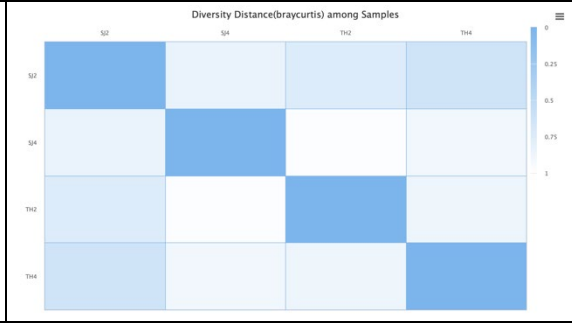
Fig. 5-2-4-1-7 List of all identified fish species.	
Fig. 5-2-4-1-8 List of distinct haploids (denoised distinct reads) belonging to the same fish species.	
Fig. 5-2-4-1-9 Phylogenetic tree of all the distinct haploids within the same sample.	
Fig. 5-2-4-1-10 Relative abundance statistics at different taxonomic levels.	
Fig. 5-2-4-1-11 Analysis of multiple samples using the MiFish pipeline.	

Fig. 5-2-4-1-12 Multiple samples can be assigned to groups by selecting from the attached menus. Up to three groups are supported. This figure shows two sample groups, each containing two biological replicates.	
Fig. 5-2-4-1-13 Switching of results between different samples. Results can be switched by clicking the navigation tabs.	
Fig. 5-2-4-1-14 Alpha diversity shown in scatter plots. This figure shows two sample groups (Groups 1 and 2), each with two biological replicates (SJ2 and SJ4 in Group 1 and TH2 and TH4 in Group 2).	
Fig. 5-2-4-1-15 Beta diversity shown in heatmap. Darker color indicates a shorter distance between two samples (i.e. higher similarity).	

5-2-4-2. Claident

5-2-4-2-1. Introduction

Claident is a collection of programs designed for base sequence data analysis in metabarcoding and DNA barcoding. Its key differences from the MiFish pipeline are as follows:

- Claident supports not only fish metabarcoding data using MiFish primers but also data from all genetic loci of all organisms and viruses.
- Claident supports both non-quantitative and quantitative metabarcoding.
- Claident offers more flexible and detailed analysis options.
- Analysis is performed locally on the user's own computer without the need for an online service.
- Claident requires certain prerequisite knowledge and necessary tools for use.

Here, we explain the installation of Claident and a method for quantitative metabarcoding using internal standard DNA. For further assistance, please refer to the support page for Claident at the URL below as necessary.

- https://github.com/astanabe/eDNAmanual

Additionally, please refer to the URL below for sample data, sample files, and manuscript files related to this chapter.

- https://www.claident.org/

In addition, the full version of Section 5-2-4-2 is available as an attached document at the URL below.
https://github.com/astanabe/eDNAmanual/blob/main/metabarcodinganalysisswithClaident.pdf

5-2-4-2-2. Operating environment

Claident is designed to operate in the following environments:

- Debian 11 or later (including WSL environments on Windows)
- Ubuntu 20.04 or later (including WSL environments on Windows)
- Linux Mint 20 or later
- RedHat Enterprise Linux 8 or later
- AlmaLinux 8 or later (including WSL environments on Windows)
- Rocky Linux 8 or later
- macOS with Homebrew installed
- macOS with MacPorts installed

For detailed instructions on installation of Claindent, please refer to the URL below (full version attached).
https://github.com/astanabe/eDNAmanual/blob/main/metabarcodinganalysisswithClaident.pdf

5-2-4-2-3. Overall flow of data analysis and prerequisites

Data analysis using Claident is performed as follows:

1. Demultiplexing
2. Concatenation of paired-end sequences
3. Removal of low-quality sequences
4. Denoising
5. Chimera removal without using a reference sequence database
6. Internal standard sequence clustering
7. Chimera removal using a reference sequence database
8. Index-hopping removal
9. Decontamination using negative controls
10. Molecular identification
11. Creation and processing of OTU composition table
12. Coverage-based rarefaction
13. Estimation of DNA concentration using the number of internal standard DNA reads

The final OTU composition table is processed in R or other statistical analysis environments to generate plots, summarize data, and test hypotheses. Claident itself does not include statistical analysis functionality. Claident can be used to analyze most metabarcoding data, but we here proceed with the explanation assuming the data below. Data that do not meet the following criteria can still be analyzed, but they are not covered in this chapter.

- eDNA samples extracted from filtration filters that have been used for filtering environmental water, along with a field blank as a negative control, are included.
- The library is prepared using the following method:
  - Add multiple internal standard DNAs with known concentrations and use MiFish primers for tailed PCR (1st PCR) (5-2-1. Library preparation – 1: 1st PCR, p.60)
  - Use index primers with 1st PCR product as template, for tailed PCR (2nd PCR) (5-2-2. Library preparation – 2: 2nd PCR, p.66)
  - The resulting library is a dual-indexed library with indices at both ends.

- The 2nd PCR products of each sample are mixed and sequenced on an Illumina sequencer in a single run or in a single lane.
  - Overlapping (i.e., concatenable) paired-end sequences
  - Run data containing .bcl or undemultiplexed FASTQ including index sequences are available on hand.

Therefore, the following information must be known for each sample and blank:

- Whether it is a sample or blank
- Amount of filtered water
- Amount of extracted DNA solution (the amount of solution used in the final elution, not the amount of solution collected)
- Internal standard DNA base sequence
- Internal standard DNA concentration
- Of the primer sequences during 1st PCR, the partial sequence where the sequencer starts reading

- Of the primer sequences during 2nd PCR, the partial sequence that is read as an index

For a detailed explanation of operations such as base sequence data processing, please see below (full version attached).
https://github.com/astanabe/eDNAmanual/blob/main/metabarcodinganalysiswithClaident.pdf

Appendix

Akifumi Tanabe 2024. Quantitative metabarcoding analysis using Claident.
https://github.com/astanabe/eDNAmanual/blob/main/metabarcodinganalysiswithClaident.pdf (full version)

Suggested citations

Whole manual

The eDNA Society (2024) Environmental DNA Sampling and Experiment Manual Version 3.0. Otsu, Japan.

Chapter 2

Seino, S. and Minamoto, T. (2024) Selection of sampling sites. Pages 6–10 *in* Environmental DNA sampling and experimental manual version 3.0. Ed. by eDNA Methods Standardization Committee, The eDNA Society, Otsu, Japan.

Chapter 3

Miya, M. and Sado, T. (2024) Water sampling and on-site filtration using a filter cartridge. Pages 12–22 *in* Environmental DNA sampling and experimental manual version 3.0. Ed. by eDNA Methods Standardization Committee, The eDNA Society, Otsu, Japan.

Minamoto, T. (2024) Water sampling and filtration using glass fiber filters in the laboratory. Pages 23-29 *in* Environmental DNA sampling and experimental manual version 3.0. Ed. by eDNA Methods Standardization Committee, The eDNA Society, Otsu, Japan.

Chapter 4

Miya, M. and Sado, T. (2024) DNA extraction from a filter cartridge. Pages 31–42 in Environmental DNA sampling and experimental manual version 3.0. Ed. by eDNA Methods Standardization Committee, The eDNA Society, Otsu, Japan.

Minamoto, T. (2024) DNA extraction from glass fiber filters. Pages 43–51 in Environmental DNA sampling and experimental manual version 3.0. Ed. by eDNA Methods Standardization Committee, The eDNA Society, Otsu, Japan.

Chapter 5

Minamoto, T. (2024) Single species detection and quantification of eDNA. Pages 52–54 in Environmental DNA sampling and experimental manual version 3.0. Ed. by eDNA Methods Standardization Committee, The eDNA Society, Otsu, Japan.

Miya, M. and Sado, T. (2024) Multiple species detection using MiFish primers. Pages 55–92 in Environmental DNA sampling and experimental manual version 3.0. Ed. by eDNA Methods Standardization Committee, The eDNA Society, Otsu, Japan.

Zhu, T and Iwasaki, W (2024) eDNA metabarcoding sequence data analysis: MiFishpipeline. Pages xx-xx in Environmental DNA sampling and experimental manual version 3.0. Ed. by eDNA Methods Standardization Committee, The eDNA Society, Otsu, Japan.

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