Environmental DNA Sampling and Experiment Manual

Version 2.1 (published April 25, 2019)

eDNA Methods Standardization Committee,

The eDNA Society

Table of Contents

1. Introduction

This manual was developed to promote and standardize environmental DNA (eDNA) analysis methods. The information provided here is current as of April 2019. However, it may be updated at any time to keep up with the incessant progress of eDNA analysis technology. It is important, 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/).

Environmental DNA analysis generally follows the workflow starting from water sampling through the collection of eDNA by filtration and extraction of eDNA from the filter to detection of the target species using various molecular techniques. Two methods are mainly used to collect and extract eDNA. One is a method of using a filter cartridge (closed filter), and the other is a disk filter (open filter), both of which are described in this manual. Typical molecular biology techniques for eDNA detection methods include (1) single-species detection using real-time 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 may detect a specific target species accurately and inexpensively, while the method requires time-consuming procedures to design species-specific PCR primers for different target species. The use of eDNA metabarcoding has the advantage of simultaneous detection of multiple species in the biological community using a set of universal PCR primers, although it takes more time and cost than eDNA barcoding. Thus, the two methods may be used in a complementary manner.

General precautions on environmental DNA analysis

Environmental DNA analysis is a technique for detecting and quantifying a very small amount of target DNA in the environment by polymerase chain reaction (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 the analysis results. Highly accurate eDNA analysis may be a battle against contamination in a sense. Therefore, it is necessary to pay particular attention to the following points.

1) Preparation of the experimental environment: It is important to physically separate the rooms for handling dilute DNA (e.g., environmental samples) from the room for handling dense DNA (e.g., PCR products). Also, on the day of the experiment, personnel should strictly adhere to the one-way rule, which restricts movement to unilateral movement from the dilute DNA room to the room handling dense DNA to reduce the risk of contamination as much as possible.

2) Use of DNA-free equipment: The equipment used for experiments should be new and unused or completely decontaminated and DNA-free. Immersion in a sodium hypochlorite solution (for example, a 0.1% concentration) is effective for decontamination. Pipettes and tube racks may be effectively decontaminated under UV irradiation.

3) Wearing gloves: Since samples may be contaminated by any contact during experiments, it is necessary to wear gloves, such as medical rubber gloves, in order to keep the surfaces clean. Gloves should be worn throughout the entire process from the collection of outdoor samples to DNA measurement to prevent contamination by DNA derived from one's own DNA or foods attached to the hands. When samples or reagents are attached to the gloves during an experiment, they should be replaced frequently.

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

5) Use of low DNA adsorption products: Since DNA is predisposed to adsorption to ordinary plastic products, use of low-adsorption microtubes, such as the DNA LoBind Tube (Eppendorf), is recommended, especially for storage.

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 copyright to this manual lies with the eDNA Society, and accountability for the contents lies with the eDNA Society and the eDNA Methods Standardization Committee.

eDNA Standardization Committee

Chairperson

Dr. Toshifumi MINAMOTO, Graduate School of Human Development and Environment, Kobe University (Editor, and author of Chapters 1, 2-2, 3-2, 4-2, and 5-1)

Committee Member (in order of Japanese syllabary)

Dr. Michio KONDOH, Graduate School of Life Sciences, Tohoku University (Reviewer)
Dr. Satoquo SEINO, Graduate School of Engineering, Kyushu University (Author of Chapters 2-1 and 2-3)
Dr. Teruhiko TAKAHARA, Faculty of Life and Environmental Science, Shimane University (Reviewer)
Dr. Hideyuki DOI, Graduate School of Simulation Studies, University of Hyogo (Reviewer)
Dr. Keigo NAKAMURA, Water Environment Research Group, Public Works Research Institute (Reviewer)
Dr. Masaki MIYA, Ecology and Environment Department, Natural History Museum and Institute, Chiba (Author of Chapters 3-1, 4-1, and 5-2)
Dr. Hiroki YAMANAKA, Faculty of Science and Technology, Ryukoku University (Reviewer)

Independent authors (in order of Japanese syllabary)

Dr. Tetsuya SADO, Natural History Museum and Institute, Chiba (Author of Chapters 3-1, 4-1, and 5-2)
Dr. Satoshi YAMAMOTO, Graduate School of Science, Kyoto University (Reviewer)

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

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

2. Selection of sampling sites

General precautions

Industrial wastewater discharged from fish markets, shops, and restaurants is likely to contain DNA from fish and aquatic organisms, which makes it difficult to interpret eDNA analysis results. The same applies to domestic wastewater from houses. Therefore, it is important to select sampling sites that are unlikely to receive inflow from sewage canals, sewage treatment plants, and conduits. In particular, sewage treatment plants, which discharge large volumes of water, may affect survey results. The outlets of treated water (which may be located far away from treatment plants) should be checked, and water should not be sampled in the vicinity of an outlet. Commercial facilities and urban and residential areas may be easily identified from maps, aerial photographs from the Geospatial Information Authority of Japan, and public domain websites in advance of selecting survey sites. These structures and areas may also be visually identified on the site.

On the other hand, some facilities are not visible from maps or during walks around the site. Since outlets are located under embankments and seawalls, they are often not visible from the shore where personnel are located. Personnel should walk along the waterfront before sampling to identify those structures.

Sea surface and the areas located near terrestrial aquaculture facilities and fish preserves should be avoided as well, since wastewater containing DNA from fish reared or preserved in the facilities is often directly released into the water. In addition, bait often includes the mince of fish harvested outside the area. DNA from organisms that do not inhabit the survey area in question, therefore, may be detected.

To avoid any problems with anglers, water should be sampled away from them. In addition, since the bait scattered over the water by anglers may also affect the water, personnel should sample the water from the upstream side of anglers to avoid any effects from their activities, including bait scattering.

In addition, precautionary measures should be implemented to avoid violating any relevant rights, including fishing rights, before conducting surveys. The administrator or owner should be contacted to arrange for the required permissions or licenses.

2-1. Selection of sampling sites in rivers

The distance within which eDNA reflects biological distribution is estimated to be on the order of several hundred meters. Therefore, it is ideal to define sampling points with an interval of several hundred meters on the survey site. However, since sufficient resources are often unavailable, sampling plans should be developed in a manner consistent with budgetary restrictions and objectives.

Water samples collected from rivers upstream of their confluence may help understand the distribution in the rivers.

The following precautions should be taken for determining the survey sites of the rivers. Environmental DNA is diffused under the influence of water flow, and its diffusion is affected by topographical features at various scales. Attention, therefore, should be paid to diffusion on a microhabitat scale. It is also important to take pictures of the environment surrounding the survey site.

1) River topography: River flow velocity and sediment accumulation change, depending on the shape of the river channel. 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. On the other hand, the inner side of the curved area has lower flow velocity. The area is sedimentary, sandbars preferentially form due to sedimentation, and vegetation tends to settle. As a result,

rivers have different micro-topographies and specific ecosystems on the inner and outer banks of curved areas. Different topographies should also be taken into account to select survey points. For example, if a riverside site XX km away from a river mouth is selected for sampling, whether the distance is the direct distance between the two points or the length of the meandering river between the two points should be considered.

2) River flow: A river channel has no uniform flow direction or velocity. Since the centerline of the stream and stagnating and eddying water flow areas have different substance trapping modes, water quality and the organisms inhabiting the stream will be different. Once released from an organism, eDNA has a different diffusion distance depending on flow velocity.

3) Riverbank structure: Natural vegetation forms and grows differently along the left and right banks of a meandering river, depending on the topographical differences as described above. Good habitat is formed under a riverside forest and in riverbank vegetation such as reed beds. On the other hand, artificial structures, such as seawalls and embankments, modify the natural habitat. It is necessary, therefore, to pay attention to the extent of the modification. Indeed, a seawall completely covering a seashore line with concrete may block groundwater leaching from the hinterland and destroy the habitats of interstitial organisms. In contrast, seawalls constructed with stones and concrete blocks allow water to permeate through the gaps, which should be the focus. If the gaps are filled with concrete or other materials, the microhabitats of organisms (e.g. *Leucotomies*) supported by permeating water and discharged groundwater will be destroyed. Embankments have different water permeability levels, depending on whether they are clay structures piled up in the past or concrete structures with the foundation laid underground.

4) Riverbed sediments: Riverbed sediments (materials) have different water permeability levels depending on whether the materials are rock, gravel, sand, or clay. For example, egg-laying sites for salmon have water discharge from beneath the deposited gravel. Closest attention should be paid to water sampling in a riverbed covered largely with fine particles, such as clay. When a water sampler, such as a bucket, comes into contact with the riverbed, the sediment is stirred up, and the water becomes turbid, which makes it difficult to filter the water. In addition, if the riverbed or riverbank is rocky, the water sampler may become caught by rock protrusions and not be recovered. Personnel may fall into the river if they force themselves to recover the samplers. Sampling requires close attention to avoid these risks.

5) Artificial alterations such as river construction: River flow may be disturbed by construction projects undertaken by a river manager (central or local government). If a river channel is excavated to accelerate river flow, the excavation may significantly alter habitat topography, bottom sediment, and vegetation. In particular, if the sandbar is removed, submarine fish will disappear together with their habitat. Pictures of the surrounding environment should be taken during monitoring to detect these disturbances. During construction, muddy water is produced. When river water has higher turbidity, it affects the filtration after sampling.

6) Estuary, brackish, and tide areas: Note that these areas have a mixture of river water and seawater. The same points undergo changing environmental conditions due to tides. The survey time should be decided according to the situation and with reference to the tide table made available by the Japan Meteorological Agency. It is also desirable to document not only the location of the point, but the time, depth, and location (surface, middle, or bottom layers) during sampling in order to determine the influence of the tides. Backstream and mixing patterns of saltwater vary depending on the riverbed gradient and river flow. The influence of strong external forces from the sea toward the estuary includes an increase in the amount of seawater flowing into the river due to rising tide levels and strong waves and the accompanying suppression of river water diffusion into the sea.

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

Although ponds and lakes do not require more precautions for sampling than rivers, which constitute running water areas, care should be used to sample water that well represents the water areas. Small and regular-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 those water areas if it is easily accessible from shore. Although there is a lack of knowledge about the appropriate number of water samples in complex shaped or large ponds and lakes, it is desirable to sample water from as many points as possible. 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.

The following precautions should be taken for conduct sampling in agricultural canals. Water sources should be identified because those canals sometime 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. Plenty of water is supplied to canals during irrigation, while little water is available during the non-irrigation period. Concrete waterways are slippery, and the water flow may be fast, so precautions should be taken to avoid accidents. Since the waterways are typically managed by organizations, such as land improvement program entities, it is necessary to obtain consent before any sampling in order to avoid problems. Note also that the water discharged from paddy 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

Precautions for selecting survey points on the coast are discussed below. Since the diffusion of eDNA due to seawater flow is affected by small- and large-scale coast topographies, attention should be paid to the topographical scale and microhabitats. It is important to take photos of the sampling sites and their surrounding environments.

1) Coastal topography and bottom sediment: Wave conditions near the shoreline differ depending on the coastal topography, and the grain size of the bottom sediment at the shore and the stirring of sediment due to waves will differ. Also, when a water sampler, such as a bucket, comes into contact with bottom sediment, sand or mud is stirred up in the water. It is desirable to choose areas where sand or mud is less likely to be stirred up because such sand and mud will affect the quality of the water sample and the efficiency of the filtration.

   A) Rocky or rugged seashores: It is easier to sample water because sediment is unlikely to be stirred up by waves. These areas have high waves and are slippery, so safety precautions should be taken to prevent toppling.

   B) Sandy beach: The area is relatively accessible, but 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 due to tides and stirs up the sediment. Since phytoplankton and suspended solids are present 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. Rocky seashores, which are exposed to waves, the central area of sandy beaches, water stagnation areas around artificial structures, and eddying flow areas trap substances differently in the water, so they have different water quality levels and organisms. Once released from a certain point, eDNA may have a different diffusion distance depending on flow velocity. Along the coast, water masses move or stagnate according to the

tides. Note, however, that the water does not flow down and spread in one direction like a river. It is also necessary to pay attention to the effects of freshwater due to flooding, the persistence of stagnation, and the increase in turbidity.

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

   A) Coastal vegetation varies depending on whether the hinterland is beach, sand dunes, cliffs, or artificial areas (industrial, urban, and residential areas). Aerial photo interpretation helps understand a wide area surrounding the survey points.

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

   C) Seawalls constructed with stones and concrete blocks allow water to permeate through gaps. Close attention should be paid to water permeability in those gaps. If the gaps are filled with concrete or other materials, the microhabitats of organisms supported by 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) Sediments: Seabed sediments (materials) have different water permeability levels depending on whether the materials are rock, gravel, sand, or clay. Close attention should be paid to water sampling from tidal flats, sandy beaches with fine sands, or the seabed covered largely with fine particles, such as clay. When a water sampler, such as a bucket, comes into contact with the seabed, the sediment is stirred up, and the water becomes turbid, which makes it difficult to filter the water. In addition, a water sampler may become caught by rock protrusions along a rocky or rugged seashore and may not be recovered. Operators may fall into the river if they force themselves to recover the samplers. Safety measures should be effectively implemented.

5) Artificial alterations such as coastal construction work: It is necessary to pay attention to disturbances resulting from construction work by a coastal manager (central or local government) and/or a developer. It is necessary to understand the spatiotemporal dimensions of the effects, including the geographical scale of the disturbance and continuity over time. Pictures of the surrounding environment should be taken during monitoring to detect and document these disturbances.

   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 flow direction and velocity in the surrounding water areas. It is, therefore, necessary to review maps and personally observe the site in advance. Construction work for such structures may have begun when sampling personnel visit the site. The coast, port, or fishing port manager should be contacted to inquire about construction schedules.

   B) Submarine drilling to develop sea lanes significantly changes the habitat topography, bottom sediment, and vegetation. In particular, when a sandbar is removed, benthic fishes disappear together with their habitats.

   C) Turbid water is generated during construction work. If turbidity rises, it will affect the filtration work after sampling.

6) Artificial structures (seawalls, embankments, breakwaters, etc.): There are many kinds of

artificial structures intended to absorb waves and protect structures. Close attention should be paid to the materials and distances from 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. The difference in the microtopography between the artificial structures and natural coastal reefs is that the artificial structures have many gaps and occasionally provide high permeability. Waves bring the water mass to the structures, which serve as a filter for the water mass. 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 environmental DNA (eDNA) decreases in winter. Water sampling, therefore, may have a higher detection rate from spring to autumn. On the other hand, 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. Externally fertilizing organisms release significant amounts of DNA into the environment during the breeding season. The breeding season is an important consideration for sampling. If species-specific detection (eDNA barcoding) is intended for some externally fertilizing organisms, their breeding seasons are desirable for sampling. On the other hand, in multiple-species detection (eDNA metabarcoding), dominant species' breeding seasons should be excluded for sampling. Red tides and blooms of blue-green algae may cause contamination of sampled water with high levels of PCR inhibitory substances. It is desirable to exclude the periods when red tides and blue-green algae break out.

Precautions for safe water sampling

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 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. The freezer is often used to stock foodstuffs. Refrigerants should, therefore, be put in a plastic bag before they are placed in a freezer to protect against contamination from foodstuffs. Dry ice can also be used as a freezer.

Filtering methods

Water samples may be filtered on-site or taken back to the laboratory before filtration. There are also two types of filtering equipment: cartridge-type (closed) and glass fiber-type (open or disk) 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)

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 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

- Water-resistant field notebooks (e.g., Se-Y11, Kokuyo Co., Ltd.)
- Water-resistant 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 on-site filtration using syringes

- Filter cartridges (Sterivex, pore size of 0.45 μm, SVHV010RS, Merck KGaA)
- 50 mL lock type syringes (SS-50LZ, Terumo Corp.)
- Parafilm (LMS Co., Ltd.)
- Luer fittings for inlet port (e.g., VRMP6, ISIS Co., Ltd.)
- Luer fittings for outlet port (e.g., VRSP6, ISIS Co., Ltd.)
- 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)
- Rubber gloves (powder-free)
- Paper towels for molecular experiments (e.g., 61440, Nippon Paper Crecia Co., Ltd.)
- RNAlater solution (Ambion)
- 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; Easy Zipper M, S. C. Johnson & Son, Inc.)
- Counter (e.g., tally counter, Plus Corp.)
- 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

• Filter cartridges (Sterivex with a hole diameter of 0.45 μm, SVHV010RS, Merck KGaA)
• Parafilm (LMS Co., Ltd.)
• 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.)
• Sodium hypochlorite solution (Highter for hospitals, Kao Corp.)

3-1-1. Record of field data

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

• Sampling personnel (names of all the sampling team members)
• Date and time (to be written in YYYY-MM-DD format)
• 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 laboratory rubber gloves should be always worn during the following procedures to prevent contamination. Gloves should be replaced every time when personnel move from one

sampling point to another. 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, put two luer fittings or parafilm (approximately six sheets with a size of 1 cm × 5 cm) in Unipack E-4 to plug the inlet and outlet ports of the Sterivex filter units. Put a Unipack E-4 in the kit, which will be used to hold two Sterivex filter units after filtration (Figs. 3-1-2-1, 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: Wear rubber gloves and 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 molecular 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 a case, it is important to record the volume of filtered water.

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 environmental 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) Sealing 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 or parafilm 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.

9) Sealing the inlet port of Sterivex filter unit: Once RNAlater is filled into the Sterivex filter unit, seal the inlet port using a Luer fitting or parafilm (Fig. 3-1-1-14). 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 the filtration is complete, wipe the surface of Sterivex with a 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.

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 rubber 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 DNA will be collected 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.

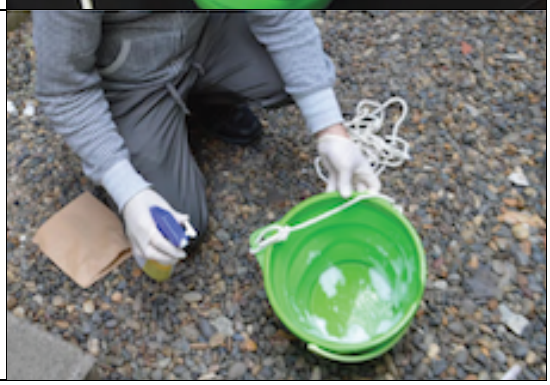
Fig. 3-1-2-1 Contents of on-site filtration kit: 2 × 50 mL syringe, 2 × Sterivex filter unit, 2 × small syringe, 2 × 2 mL tubes with RNAlater, 6 × parafilm, 1 × Unipack (E-4), 1 × Unipack (G-8).	
Fig. 3-1-2-2 The on-site filtration kit is packed in Unipack (G-8). If it is put in one bag like this, it will be easy to use in the field.	
Fig. 3-1-2-3 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.	

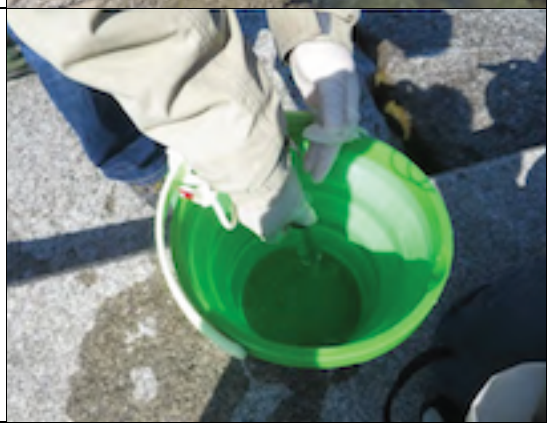
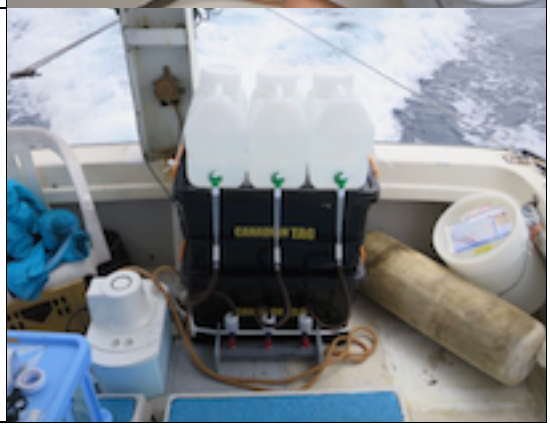
Fig. 3-1-2-5 Wipe thoroughly any residual sodium hypochlorite cleaner with a paper towel. If it is not completely wiped off, bubbles will come out during water sampling and contaminate the environmental water.	
Fig. 3-1-2-6 A bucket is thrown.	
Fig. 3-1-2-7 Haul the rope and recover the bucket with environmental 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 Seal the outlet port of the Sterivex filter unit with a luer fitting or parafilm, 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 Seal the inlet port with luer fitting or parafilm.	
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)

- Surveying field level 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)	More than samples
• Disposable gloves	More than samples
• Water sampling bucket and rope	1 set
• Sodium hypochlorite cleaner spray	1 bottle
• Paper towels	as needed
• Trash bags	as needed
• Boots, chest waders	
• Water quality meter (if necessary)	
• Felt marker, gummed tape, etc.	
• Cooler box	
• Refrigerant	

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	as needed
• Freezer (capable of freezing down to -20˚C or less)	

3-2-1. Record of field data

The elements to be noted in the surveying field level notebook are as follows. They should be written in a water-resistant field notebook with a water-resistant ballpoint pen.

- Sampling personnel (names of all the sampling team members)
- Date and time (to be written in YYYY-MM-DD format)
- 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

1) Direct sampling on the waterside: If direct access to the waterside is possible, put a sampling bottle 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. Add 1 mL of 10% benzalkonium chloride solution to the sampled water to inhibit DNA degradation (final concentration 0.01%) and thoroughly mix by inversion (Fig. 3-2-2-3).

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

A) Bucket decontamination: Wear rubber gloves and 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 molecular 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. Add 1 mL of 10% benzalkonium chloride solution to the sampled water to inhibit DNA degradation (final concentration 0.01%) and thoroughly mix by inversion (Fig. 3-2-2-3).

3) Field blank: Open a bottle containing pure water brought from the laboratory to the field, add 1 mL of 10% benzalkonium chloride solution (final concentration 0.01%), and thoroughly mix by inversion.

4) Transport to the laboratory: Transport sampled water to the laboratory away from direct sunlight and high temperatures. Benzalkonium chloride makes it possible to preserve DNA for several days even at room temperature. It is, however, desirable to keep it at as low a temperature as possible (however, currently, it is believed that water samples supplemented with benzalkonium chloride should not be frozen). In addition, ultraviolet rays decompose DNA. Transport samples away from direct sunlight. 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 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: Two glass fiber filters (mean pore size 0.7 μm) are used for filtration of water samples. Filter every 500 mL of the sampled water with each filter (Figs. 3-2-3-2 to -4). One filter may be not sufficient to filter some sampled water up to 500 mL. In such a case, filter smaller amounts of water per filter, and document the amount of filtered water. Even if the amount of filtered water is reduced, the number of filters per sample should be two. 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.

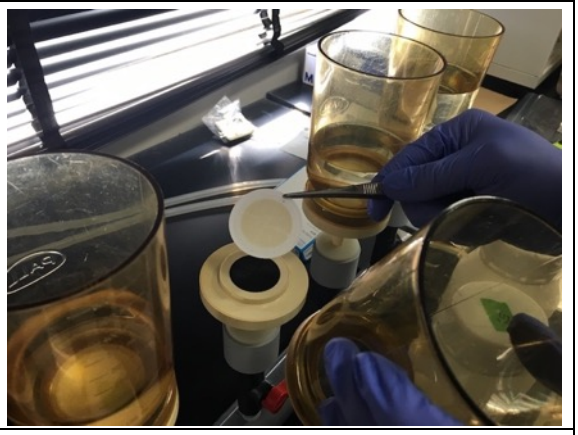
4) Storage of the filter: The filter after filtration is halved with the filtration surface inside; wrap the two filters together in aluminum foil (Fig. 3-2-3-5). Write the sample name on the aluminum foil, put it in a bag such as Unipack, and store it in a freezer (-20˚C or lower) (Fig. 3-2-3-6). In this state, it can be stably stored for several months.

5) Filtration blank: To evaluate contamination during and after filtration, prepare a filtration blank

containing 1 mL of 10% benzalkonium chloride solution and 1 liter of pure water (final concentration 0.01%) once a day and handle it in the same way as the sample. However, a field blank may be substituted for the 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 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

After filtration with a cartridge or glass fiber filter, store samples in a freezer. Pay close attention to the temperature control of the freezer to avoid repeated freezing and thawing.

Common precautions for DNA extraction

DNA extraction from filter cartridges (4-1) and DNA extraction from glass fiber filters (4-2) are described below. To reduce the risk of contamination, it is important to review the DNA extraction procedures scheduled for the day and their sequences in advance. Specifically, DNA extraction procedures, either from filter cartridges or from glass fiber filters, should be physically isolated from the devices and samples used for PCR, and the personnel should not be engaged in DNA extraction after performing post-PCR procedures during the same day.

4-1. DNA extraction from a filter cartridge

Precautions for DNA extraction: Technical tips to reduce contamination risk

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.

In addition, since DNA extraction and subsequent procedures are included in the laboratory process, careful attention should be paid to contamination (with exogenous DNA). In particular, if contamination occurs at this stage, subsequent experiments (real-time PCR for eDNA barcoding and library preparations for eDNA metabarcoding) will fail. To avoid this undesirable situation, a dedicated DNA extraction room should be specified. Also, the DNA extraction room must be sufficiently separated from the PCR room. Care should be used 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

- Fan oven (set to 56˚C)
- Mini rotator (ACR-100, Asone Corp.) and attached 10 mL/15 mL tube holder
- QIAvac Connecting System (QIAvac 24 Plus vacuum manifold and QIAGEN Vacuum Pump, Qiagen KK) [1]
- Tabletop ultracentrifuge (PZ5557-A000, Kubota Corp.)
- High speed refrigerated microcentrifuge (MX-307, Tomy Seiko Co., Ltd.)
- Rotor rack for microcentrifuge (AR015-24, Tomy)
- 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 (VPRM406 Osle Luer lock for 4 mm ID, Isis Co., Ltd.)
- 50 mL conical tubes
- DNeasy Blood & Tissue kit (Qiagen KK)
- Ethanol (for molecular biology)
- 2.0 mL tubes (low DNA binding; Sarstedt K.K.)
- 1.5 mL tubes (low DNA binding; Sarstedt K.K.)
- PBS (–) (phosphate buffered saline without magnesium and calcium, Cell Science Laboratories) [2]
- Parafilm
- Rubber gloves (powder-free)
- Micropipette P-5000, P-1000, P-200, P-100 (Pipetman, Gilson)
- Filter tips (types for the capacities of the micropipettes to be used)
- Scissors (for cutting parafilm)
- One standard tweezers (IPT-12, ASONE Corp.)
- Tube racks for 1.5 mL/2 mL

[1] Instead of QIAvac, RNAlater may be discharged on the tabletop ultracentrifuge using a combination of a 50 mL conical tube and a 2.0 mL tube (see Miya et al. 2016)

[2] This protocol does not suppose the use of the buffer ATL supplied with the DNeasy Blood & Tissue kit.

4-1-1. Preparation for experiment

Be sure to wear rubber 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. (Frozen RNAlater can be relatively quickly thawed at room temperature; Fig. 4-1-1-2.)

4-1-2. Aspiration of RNAlater

1) Remove the cap or parafilm plugged into the Sterivex filter unit outlet port and mount the luer fitting (VPRM406).

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

3) Switch the QIAvac pump on and aspirate 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) Cut the parafilm into 1 cm × 5 cm sizes and make as many as the number of the Sterivex filters units available. (Parafilm is not necessary when using luer fittings [VRSP6].)

5) Remove the Sterivex filter unit from which RNAlater has been aspirated from the manifold and seal the outlet port with parafilm or the luer fitting. (Fig. 4-1-2-2; if parafilm is used, cover the outlet port with several films because the heat of the fan oven expands the inner air, resulting liquid leakage.)

4-1-3. DNA extraction

1) Prepare the premixes using the DNeasy Blood & Tissue kit and PBS (–) (Fig. 4-1-3-1). Mix proteinase-K, AL, and PBS (–) at a ratio of 20 µL, 200 µL, and 220 µL, respectively, per Sterivex filter unit. One more premix should be prepared for the extraction blank for detecting contamination during DNA extraction.

2) Open the inlet port of the Sterivex filter unit and fill the filter unit with the above premix 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.)

3) Cut the parafilm to a size of about 1 cm × 5 cm, and tightly seal the inlet port of the Sterivex filter unit with the parafilm. (Note: Parafilm is not necessary when using the luer fitting [VRMP6]; Fig. 4-1-3 -3.)

4) Insert the Sterivex filter unit into the tube holder of the rotator and attach the tube holder to the rotator body in a manner to make the Sterivex filter unit parallel to the ground.

5) Place the rotator with Sterivex filter units in a fan oven, rotate at 10 rpm, and heat at 56˚C for 20 minutes. (Note: The mini-rotator endurance temperature is 60˚C; Fig. 4-1-3-4.)

6) While warming the Sterivex filter unit to 56˚C, prepare a 2.0 mL tube for DNA recovery (low DNA adsorption) and a 50 mL conical tube (Fig. 4-1-3-5) and put the 2.0 mL tube into the 50

mL conical tube. (Note: Write the necessary information on the cap of the 2.0 mL tube; do not push the tube deeply into the conical tube; Fig. 4-1-3-6.)

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

8) 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). Then, close the cap of the conical tube firmly (Fig. 4-1-3-8).

9) 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 mL tube (Fig. 4-1-3-10).

10) 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.)

11) 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). (Note: Write the necessary information on the column cap.)

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. 4-1-4-2).

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 Buffer AW2 to the column (Fig. 4-1-4-9) and centrifuge at 20,000 g for 3 minutes to dry the DNeasy membrane.

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) Pipet 200 µL Buffer AE (elution buffer) directly onto the DNeasy membrane (Fig. 4-1-4-12). Incubate at room temperature for 1 minute and then centrifuge at 6000 g for 1 minute to elute.

12) After centrifuging, remove the column and tightly cap the tube (Fig. 4-1-4-13). Discard the used column.

13) The purified DNA can be stored stably at –20˚C.

Reference

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

The movie version of this article is available in a low-resolution format at

https://sites.google.com/site/masakimiyalab/publications-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 parafilm.	

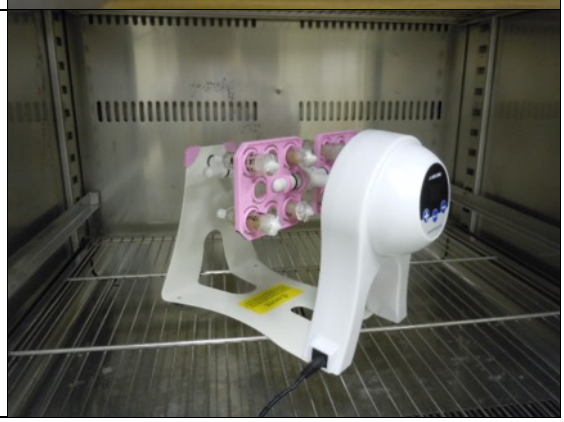
Fig. 4-1-3-1 Make the premixes necessary for DNA extraction available.	
Fig. 4-1-3-2 Open the inlet port of the Sterivex filter unit and fill the above premix using a micropipette (P-1000) and a 1000 μL filter tip.	
Fig. 4-1-3-3 Seal the inlet port of the Sterivex filter unit with parafilm. Tightly seal the units to prevent liquid leakage while heating to 56˚C.	
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.	

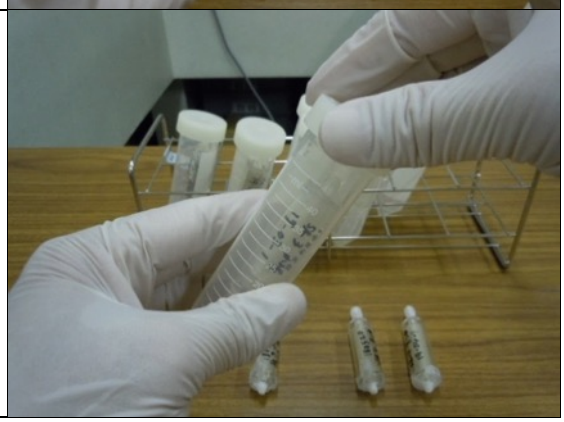
Fig. 4-1-3-5 While heating Sterivex filter unit units, prepare a new 2.0 mL tube (low DNA adsorption) and a 50 mL conical tube available for DNA collection.	
Fig. 4-1-3-6 A 2.0 mL tube is inserted into a 50 mL conical tube.	
Fig. 4-1-3-7 Insert the inlet port of the Sterivex filter unit into the 2.0 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.	

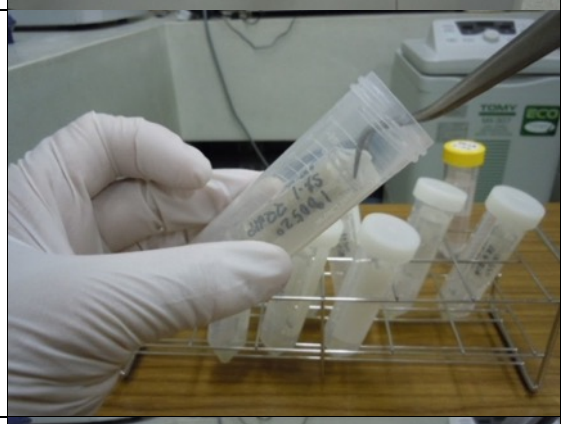
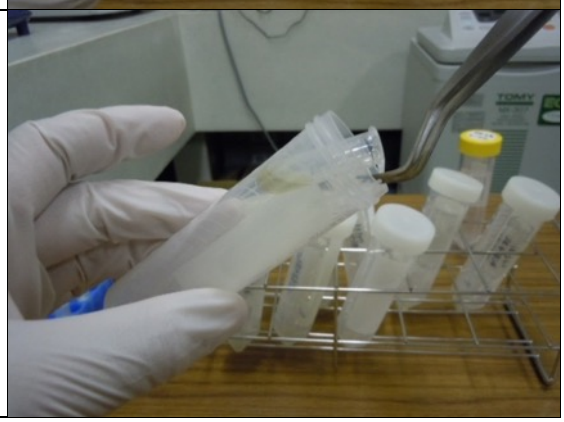
Fig. 4-1-3-9 Centrifuge the conical tube containing the Sterivex filter unit at 6000 g for 1 minute.	
Fig. 4-1-3-10 Extracted DNA is collected in a 2.0 mL tube within a conical tube.	
Fig. 4-1-3-11 Remove the Sterivex filter unit carefully from the conical tube using tweezers.	
Fig. 4-1-3-12 Next, remove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

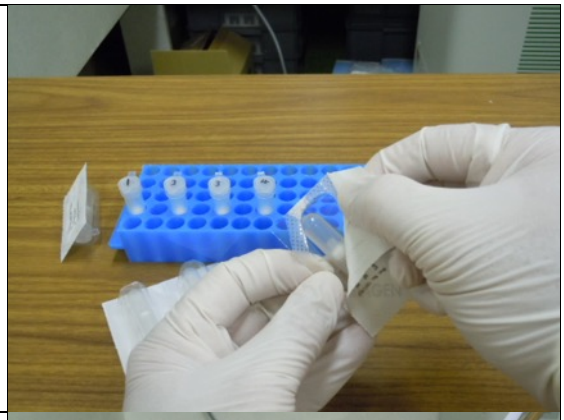
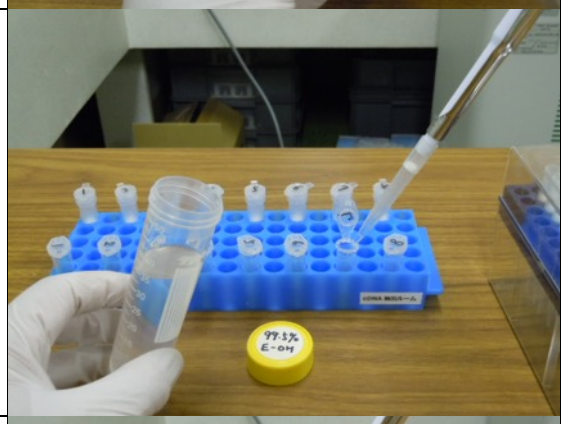
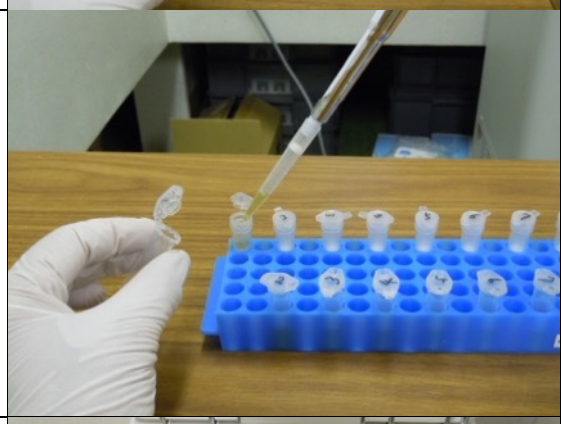
Fig. 4-1-4-1 Open the package of the column (with collection tube) supplied with the DNeasy Blood & Tissue kit and place it in a tube rack.	
Fig. 4-1-4-2 Add 200 µL ethanol (96% to 100%) to the 2.0 mL tube containing the extracted DNA and mix thoroughly with a pipette.	
Fig. 4-1-4-3 Place the extracted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

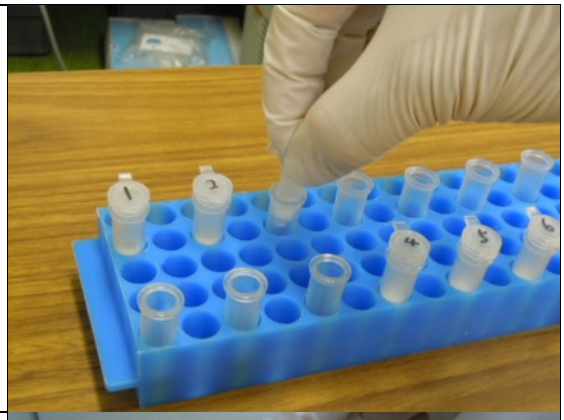
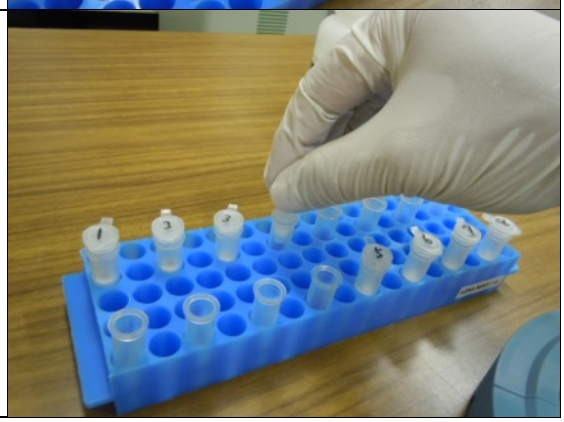
Fig. 4-1-4-5 After centrifuging, remove the collection tubes from the column and place them to a new 2 mL collection tube.	
Fig. 4-1-4-6 Discard used collection tubes.	
Fig. 4-1-4-7 Add 500 µL Buffer AW1 to the columns.	
Fig. 4-1-4-8 After centrifuging, place the columns to new 2 mL collection tubes.	

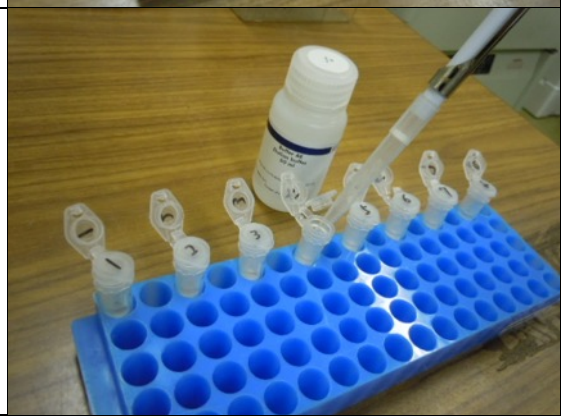
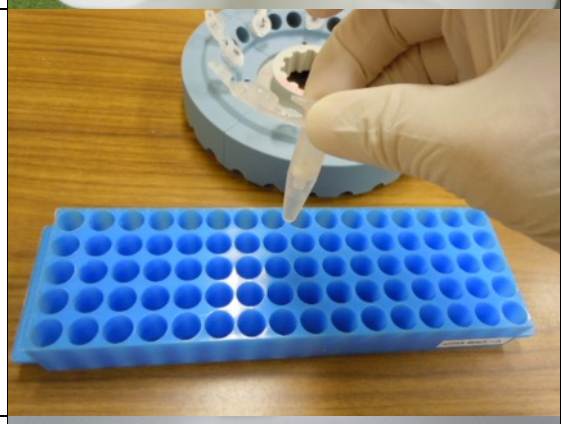
Fig. 4-1-4-9 Add 500 µL Buffer AW2 to the columns.	
Fig. 4-1-4-10 Prepare 1.5 mL low DNA bind tubes available and write the necessary information on the caps.	
Fig. 4-1-4-11 After centrifuging, move the columns to the 1.5 mL tubes made available in Fig. 4-1-4-10.	
Fig. 4-1-4-12 Pipet 200 µL Buffer AE (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. 4-1-4-13 After centrifuging, remove the column and close the tube cap tightly.	
Fig. 4-1-4-14 The purified eDNA can be stably stored at –20˚C.	

4-2. DNA extraction from glass fiber filters

Before DNA extraction: measures for reducing the risk of contamination

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).

In addition, since DNA extraction and subsequent procedures are included in a laboratory process, careful attention should be paid to contamination (with foreign DNA). In particular, if contamination occurs at this stage, subsequent experiments (library adjustments) will fail. To avoid this, a laboratory dedicated to DNA extraction (DNA extraction chamber) should be used. Also, 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

• Centrifuge (capable of rotating Salivette tubes)	
• Centrifuge (capable of rotating 1.5 mL tubes)	
• Heat block or incubator (set to 56 ˚C)	
• Salivette tubes	as many as samples
• Tweezersas many as samples	
• DNeasy Blood & Tissue Kits	as many as samples
• Buffer AL and Proteinase K	
• 1.5 mL Eppendorf tubes (low adsorption)	as many as samples
• Ethanol (for molecular biology)	as needed
• TE buffer (pH 8.0: for molecular biology)	as needed
• Rubber gloves (powder-free)	
• Various micropipettes	
• Various filter chips	
• 1.5 mL/2 mL tube racks	

[1] This protocol does not suppose the use of buffer ATL supplied with the DNeasy kits.

4-2-1. Preparation for experiment

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

1) Set the temperature of the thermostatic heat block to 56˚C. (Increase the temperature of the heat block in advance because it takes time to warm up.)

2) Put the filter used for filtering water samples into Salivette tubes (Figs. 4-2-1-1, -2). If two filters are used for one sample, put 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).

4-2-2. Proteinase digestion

1) Add 400 µL of Buffer AL and 40 µL of ProK per sample (Fig. 4-2-2-1). If the sample size is n, it

is recommended to prepare n + one reagent units before dispensing them.

2) Incubate for 30 minutes at 56˚C in a thermostatic heat block (Fig. 4-2-2-2). When heated in a heat block, caps of the Salivette tubes may be blown away. Loosen the portion between the basket and the lower tube part a little, and put the samples upright in the heat block.

3) Then, centrifuge at 3000 g for 3 minutes (Fig. 4-2-2-3). At this point, 800 to 1,000 µL of filtrate is found at the bottom of the Salivette tube (Fig. 4-2-2-4).

4) To further collect DNA remaining on the filter within the Salivette tube, add 220 µL of TE (Fig. 4-2-2-5) and allow TE to stand for 1 minute to collect it further. Then, centrifuge at 3000 g for 3 minutes. Always wear rubber gloves during the experiment.

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 ethanol to the lower DNA solution (Fig. 4-2-3-1) (Fig. 4-2-3-2).

2) Mix by pipetting (Fig. 4-2-3-3), then transfer approximately 650 µL (about half) to DNeasy column (Fig. 4-2-3-4) (Fig. 4-2-3-5), 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, -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 tubes (Fig. 4-2-3-12), add 500 µL Buffer AW2 (Fig. 4-2-3-13), and then centrifuge for 2 minutes at the maximum speed for the centrifuge in use.

6) When removing from the centrifuge and transferring the columns, be careful not to shake and to ensure that the lower liquid is not attached to the tip of the upper column.

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

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

9) Add 100–200 µL of Buffer AE (Fig. 4-2-3-17) and allow 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.

Reference

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

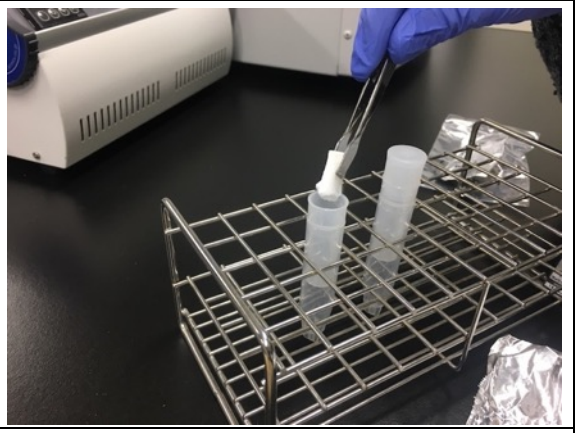
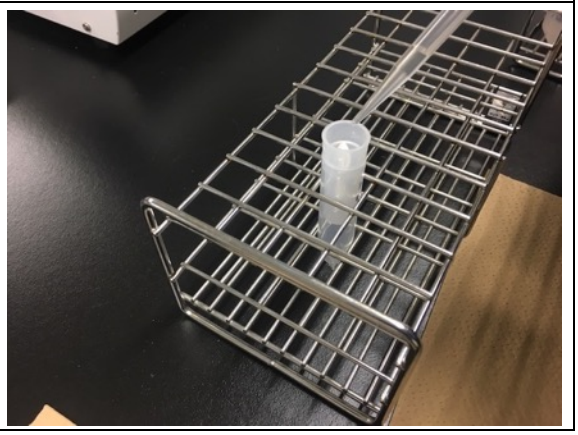
Fig. 4-2-1-1 Remove the filters from the freezer and transfer them to Salivette tubes.	
Fig. 4-2-1-2 A filter in a Salivette tube.	
Fig. 4-2-1-3 Write the sample numbers on the top and lower part of the Salivette tube.	
Fig. 4-2-2-1 Add a mixture of Buffer AL and Proteinase K.	

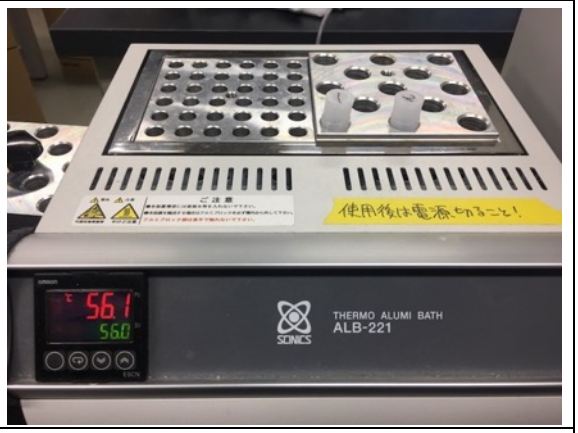
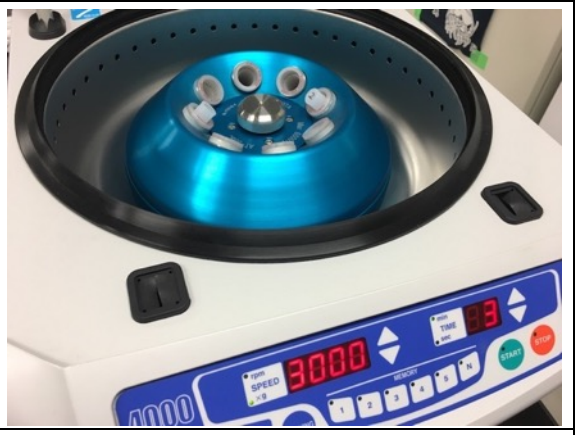
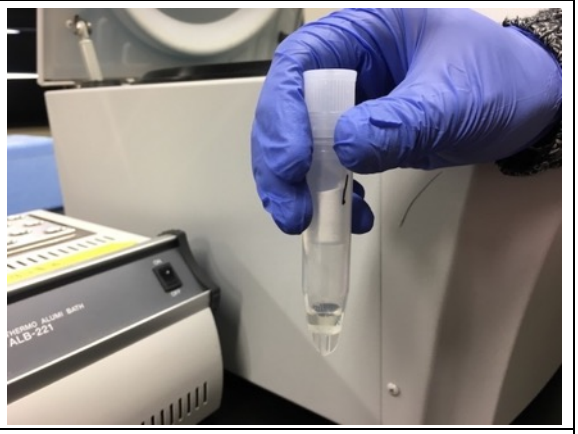
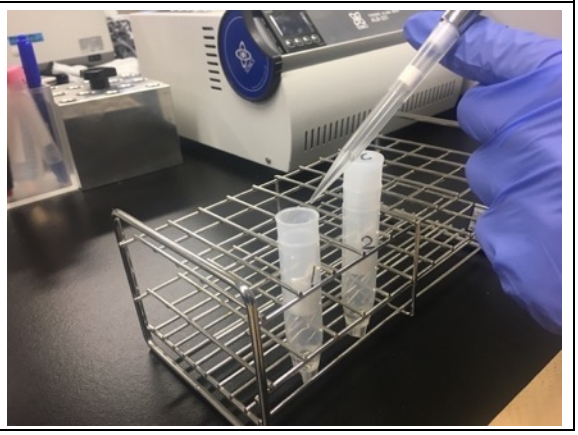
Fig. 4-2-2-2 Incubate at 56˚C for 30 minutes.	
Fig. 4-2-2-3 Centrifuge and move the liquid on the filter toward the bottom of the Salivette tube.	
Fig. 4-2-2-4 Salivette tube after centrifuging. Approximately 800 to 1000 µL of liquid is collected at the bottom of the tube.	
Fig. 4-2-2-5 Add Buffer TE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

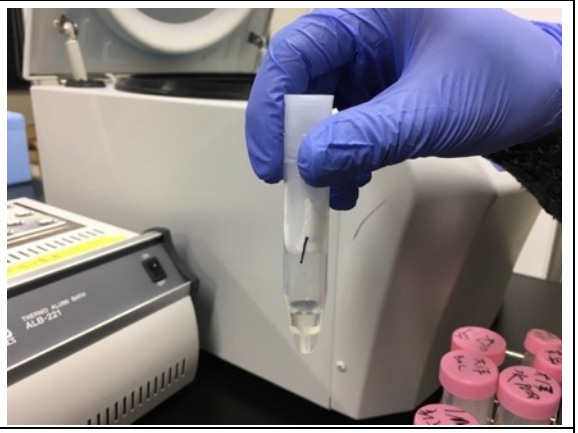
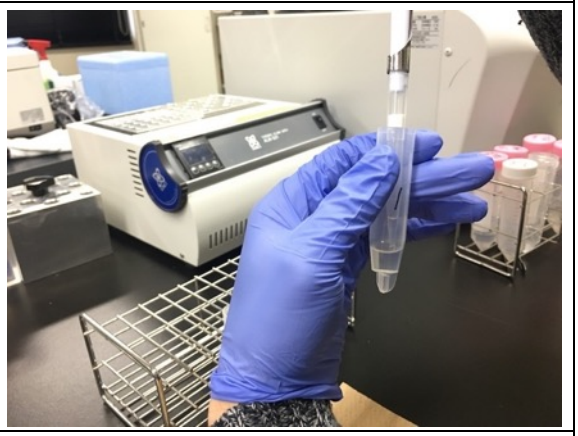
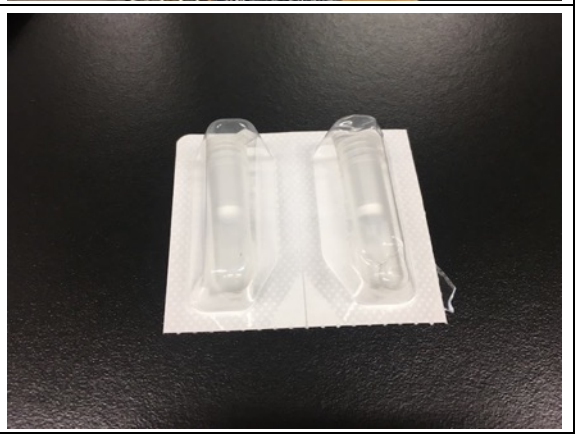
Fig. 4-2-3-1 The Salivette tube after the second centrifuge.	
Fig. 4-2-3-2 Remove the upper part of the Salivette tube and add ethanol to the lower part.	
Fig. 4-2-3-3 After adding ethanol, mix well by pipetting.	
Fig. 4-2-3-4 DNeasy column.	

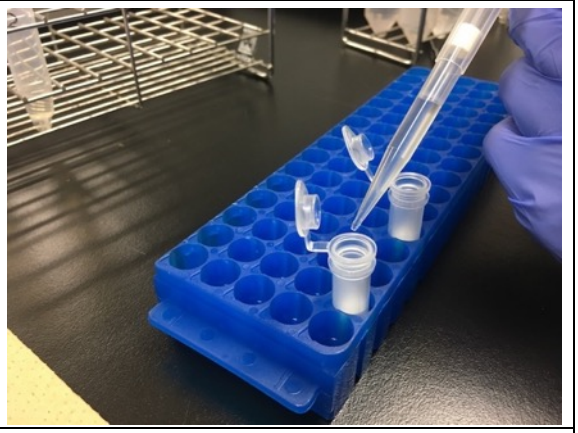
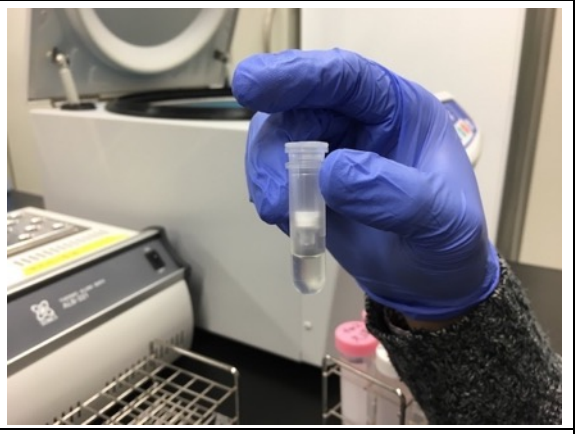
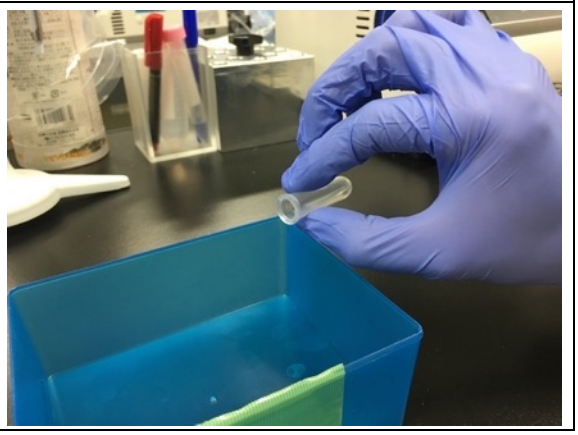
Fig. 4-2-3-5 Transfer the solution in the lower part of the Salivette tube to the DNeasy column.	
Fig. 4-2-3-6 Pass the solution through the column by centrifuging.	
Fig. 4-2-3-7 Since DNA is trapped in the column part, the lower liquid is liquid waste.	
Fig. 4-2-3-8 Discard the liquid waste.	

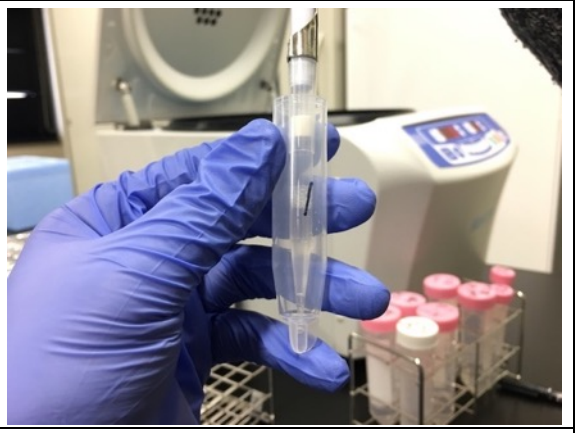
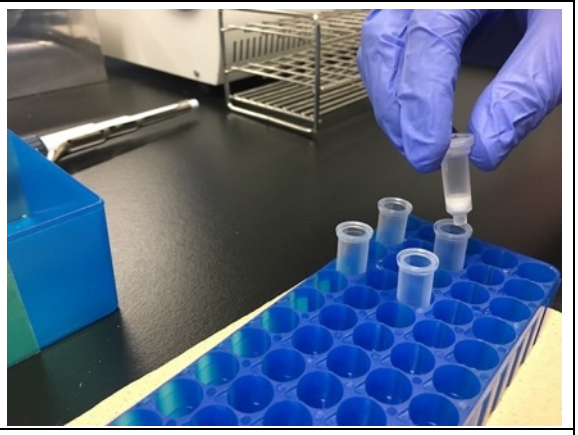
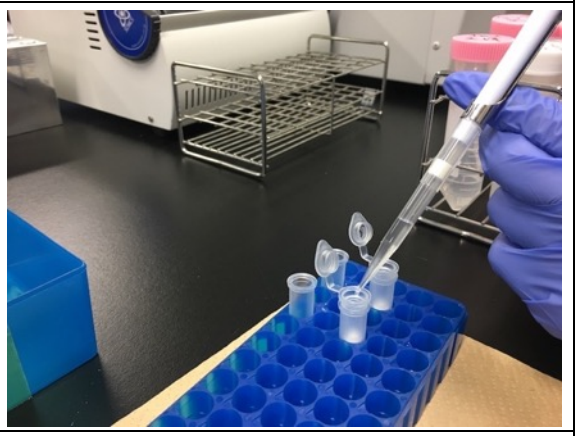
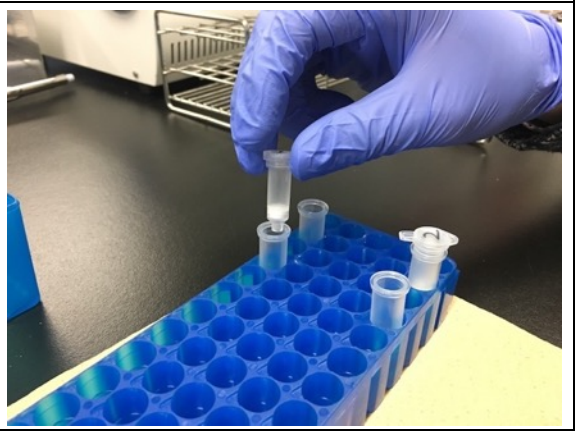
Fig. 4-2-3-9 Once more, transfer the DNA solution from the Salivette tube to the column and centrifuge.	
Fig. 4-2-3-10 After centrifuging, transfer the column to a new 2 mL tube.	
Fig. 4-2-3-11 Pour buffer AW1.	
Fig. 4-2-3-12 After centrifuging, transfer the column to a new 2 mL tube.	

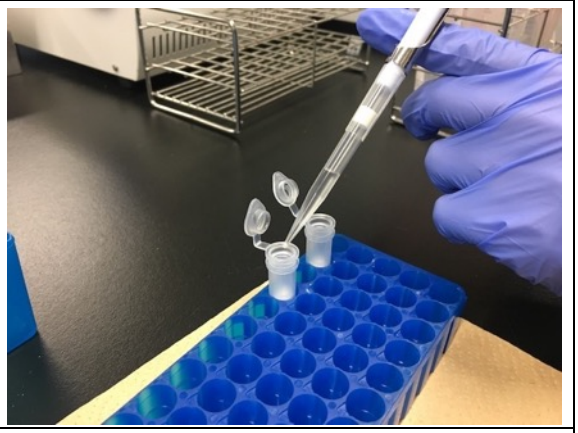
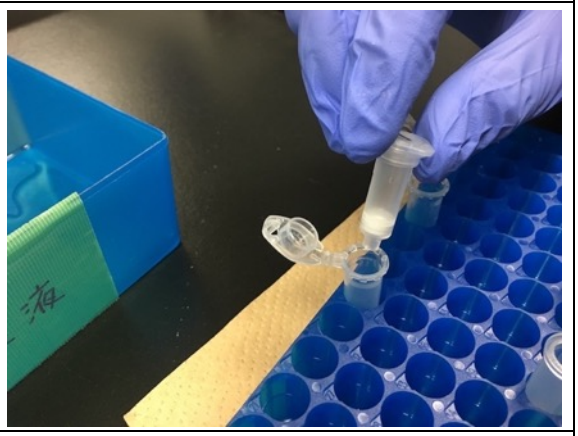
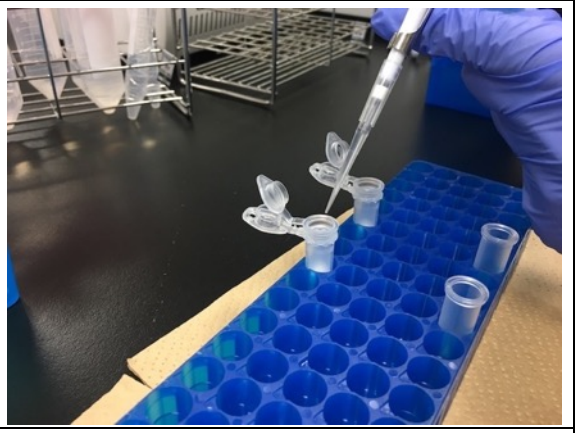
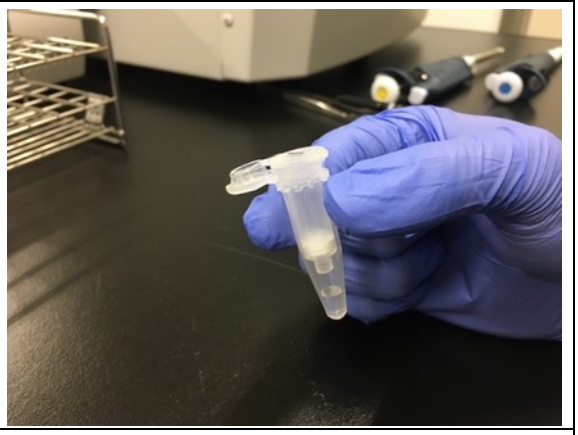
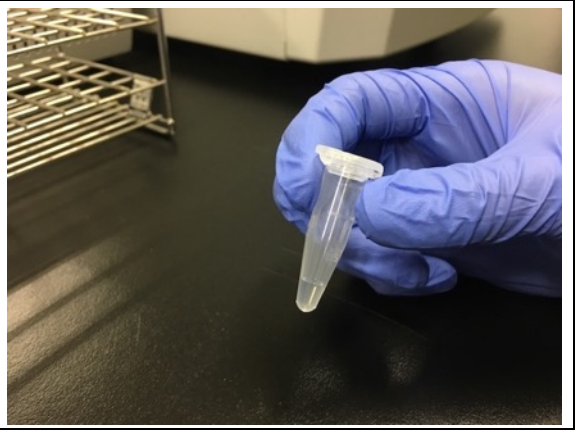
Fig. 4-2-3-13 Pour buffer AW2 and centrifuge.	
Fig. 4-2-3-14 Make a 1.5 mL low binding tube available and write the sample number.	
Fig. 4-2-3-15 Transfer the centrifuged column to the 1.5 mL tube.	
Fig. 4-2-3-16 The columns are put in 1.5 mL tubes.	

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 (eDNA barcoding) of eDNA and quantification of the DNA 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 specific bases in the target species compared with closely related species and design primers (Fig. 5-1-1-1). There should be a base specific to the target species near the 3' end of the primer. 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 living 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. 5-1-1-2).

5) Sequencing of eDNA derived amplicons: If DNA amplification derived from environmental samples is confirmed, sequence the PCR amplification products (amplicons) and verify that the DNA of the target species is 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

- 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
• Rubber gloves (powder-free)
• A variety of micropipettes
• A variety of filter tips*

*1 It is necessary to design primers and probes individually for different targets.

Reagent composition per PCR reaction

• Environmental Master Mix 2.0	10.0 μL
• AmpErase Uracil N-Glycosylase	0.1 μL
• Assay Mix	1.0 μL
• DNA	2.0–5.0 μL
• Ultra-Pure Water	appropriate amount
• Total	20.0 μL

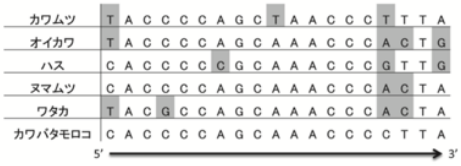
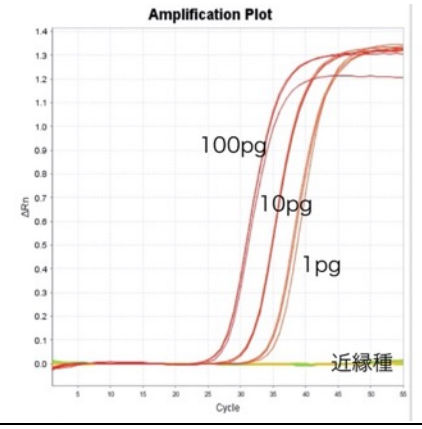
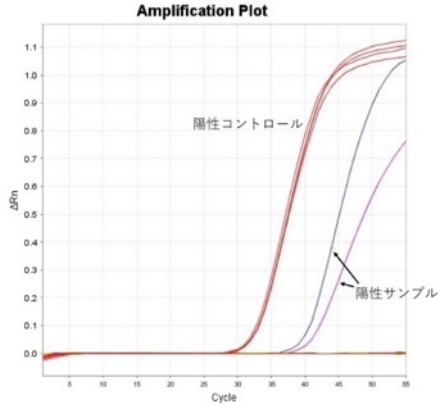
Perform all the PCR reactions (including positive controls [quantification standard], eDNA samples, field blanks, filtration blanks, and PCR blanks) with three or more replicates. Use an artificially synthesized gene as the quantification standard and a four or more fold dilution series to quantify the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

Since the conditions of the PCR reaction 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.

If three replicates of positive controls (or quantitative standard) and PCR blanks are put on each PCR plate, the number of samples (including field blanks and filtration blanks) that can be analyzed at one time is limited to a maximum of 27 (for quantification) or 30 samples (for presence/absence detection).

Any eDNA sample is determined positive if at least one of three replications proves positive (Fig. 5-1-2). Quantify the DNA using the quantification standard data. Sequence the PCR amplification product from at least a portion of the sample found to be positive to verify that the amplified DNA is the DNA of interest. 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.

Fig. 5-1-1-1 Primer design. An example of primer design for *Hemigrammocypris rasborella* (Source: Fukuoka et al. 2016)	
Fig. 5-1-1-2 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.	

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)

5-2. Multiple species detection using MiFish primers

5-2-1. Library preparation—1: First-round PCR (1st PCR)

Before experiment: Reducing contamination risks

MiFish metabarcoding uses the polymerase chain reaction (PCR) to amplify the target eDNA to the extent that it can be analyzed with current molecular techniques, while it appends various adapters to both ends of the PCR products to analyze with a next-generation sequencing platform at the same time (library preparation). PCR, on the other hand, synthesizes exceptionally large amounts of DNA fragments and is likely to contaminate the experiments. Therefore, the laboratory for PCR preparation (pre-PCR room) and the laboratory for performing PCR and handling PCR products (post-PCR room) should be spatially separated. 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 MiFish metabarcoding employs two-step PCR, it is necessary to dilute the first-round PCR (1st PCR) product as a template for the second-round PCR (2nd PCR). Therefore, it is necessary to install a clean bench or an equivalent (KOACH T 500, Koken Co., Ltd.) that creates a clean (open) space in the latter laboratory (post-PCR room) in order to prevent contamination during this procedure. Micropipettes, tips, tubes, tube racks, and Milli-Q water should be decontaminated in advance using a UV sterilizer light bulb (e.g., NB-5, Nichiban Co., Ltd.). Experimental tables should also be decontaminated using the foaming bleach.

Laboratory instruments, reagents, and consumables required for 1st PCR

- Thermal cyclers (e.g., GeneAmp PCR System 9700, Applied Biosystems)
- UV sterilizer light bulb (e.g., NB-5, Nippon Steel Industry Co., Ltd.)
- KAPA HiFi HS ReadyMix (KK2602, KAPA Biosystems Inc.) [1]
- MiFish primer stock solution (stock solution diluted to 100 μM with TE buffer is convenient, if it is available)

Primers for elasmobranchs (primers optimized for sharks and rays)

MiFish-E-F-v2 (5′–3′; 61 mer):
ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNNRGTTGGTAAATCTCGTGCCAGC
MiFish-E-R-v2 (5′–3′; 68 mer):
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNGCATAGTGGGGTATCTAATCCTAGTTTG

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

MiFish-U-F (5′–3′; 60 mer):
ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNNGTCGGTAAAACTCGTGCCAGC
MiFish-U-R (5′–3′; 67 mer):
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNCATAGTGGGGTATCTAATCCCAGTTTG

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

MiFish-U2-F (5′–3′; 60 mer):
ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNNGCCGGTAAAACTCGTGCC

AGC
MiFish-U2-R (5′–3′; 67 mer):
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNCATAGGAGGGTGTCTAA
TCCCCGTTTG

• Milli-Q water (or high-grade sterilized water for molecular biology experiments) [2]
• TE buffer (high grade for molecular biology experiments)
• 8-strip tubes
• 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-strip tubes
• Rubber gloves (powder-free)

[1] DNA extracted from environmental water (e.g., terrestrial waters) 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 obtained using KOD FX Neo (Toyobo Inc.). Amplification is performed according to the protocol provided by the vendor. If PCR is not successful because of the interference of PCR inhibitors, 10-fold dilution of the template DNA may often lead to successful PCR amplification. In addition, it is also recommended to perform more PCR cycles during the 1st PCR. However, you should not perform more than 40 cycles (see 5-2-1-1) to avoid undesirable amplification of exogenous DNA.

[2] If Milli-Q water is shared across different laboratory rooms, it can easily cause contamination. Milli-Q water should be decontaminated using a UV lamp before each experiment. Otherwise, you should purchase sterile water for molecular biology experiments.

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

• High-speed cooling centrifuge (e.g., MX-307, Tomy Seiko Co., Ltd.)
• Vortex mixer (Vortex-Genie 2 Mixer, MS Equipment Co., Ltd.)
• Desktop centrifuge for 8-strip tubes (Micro PCR spinner, MS-PCR, ASONE Corp.)
• Desktop centrifuge for 1.5 mL/2.0 mL (Micro Six MS-1, ASONE Corp.)
• GeneRead Size Selection Kit (180514, Qiagen K.K.) [1]
• 2.0 mL collection tubes (19201, Qiagen K.K.; purchased separately because the kit does not include as many tubes as needed.)
• 1.5 mL tubes (low DNA bind)
• 80% ethanol (appropriate amount)
• Micropipette P-1000, P-200, P-100, P-20, P-2 (E.g., Pipetman, Gilson Company Inc.)
• Filter tips (various types for different micropipette capacities)
• Rubber gloves (powder-free)

[1] Good results have also been obtained by purifying with DNA purification reagent kits using beads, such as Agencourt AMPure XP and SPRIselect beads (both Beckman Coulter Inc.). The volume of Agencourt AMPure XP or SPRIselect reagent equivalent to that of the PCR product is used, and the protocol supplied by the vendor is followed.

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

- TapeStation 2200 (Agilent Technology Inc.) [1]
- High Sensitivity D1000 Screen Tape (5067-5584, Agilent Technologies Inc.)
- High Sensitivity D1000 Reagents (5067-5585, Agilent Technologies Inc.)
- Pipette tips for TapeStation (5067-5153, Agilent Technologies Inc.)
- Table stirrer (Vortex MS3 Basic, IKA Japan K.K)
- Tabletop centrifuge for 8-strip tubes (e.g., Micro PCR spinner MS-PCR, ASONE Corp.)
- Desktop 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-2 (e.g., Pipetman, Gilson Company Inc.)
- Filter chip 10 μL
- 8-strip tube
- 8-strip tube rack
- Rubber gloves (powder-free)
- Milli-Q® Direct Ultra-Pure Water

[1] The BioAnalyzer from the same company has also yielded good results. The protocol supplied by the vendor is followed as is.

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.)

KAPA HiFi HS ReadyMix	6.0 μL
Primary mix	2.8 μL
Milli-Q® Direct Ultra-Pure Water	1.2 μL

4) PCR runs: To minimize PCR dropouts, perform PCR eight times on the same sample (eDNA) using one 8-strip tube (8 technical replicates). In order to monitor the contamination that occurs during the 1st PCR, be sure to include at least one blank in the 1st PCR. (Perform one run and put Milli-Q water as the template instead of extracted DNA.) Even when the PCR run is performed on a filtration blank and an extraction blank at the same time, be sure to include the

PCR blank at the 1st PCR stage (Fig. 5-2-1-1).

5) Prepare a premix by mixing the above reagents about 1.1 times the required amount and dispense 10 µL each into 8-strip tubes 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:

Insert initial denaturation at 95˚C for 3 minutes
Denaturation at 98˚C for 20 seconds
Annealing at 65˚C for 15 seconds
Extension at 72˚C for 15 seconds } 35 cycles
Insert final extension at 72˚C for 5 minutes

Note: 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 concerns, the suggested annealing temperature level seems to be appropriate, but further study is necessary in the future.

7) The number of 1st PCR cycles: When the activity of fish is low, such as during wintertime, 35 cycles may not achieve sufficient PCR amplification. The cycles, therefore, should be increased. For example, you should increase the number of cycles up to 40 cycles or less to recover a sufficient amount of DNA. For example, a sufficient amount of 1st PCR product (filtered 1 L of seawater) was obtained after 35 cycles for the coastal water samples from the Boso Peninsula during warm periods (from spring to autumn months), while 38 cycles were required to obtain the same amount of 1st PCR products during the winter months. More than 40 cycles should not be performed, however, because excessive cycles may cause false positive detections, including generation of distinct PCR products in a blank. PCR cycles, therefore, should be carefully set to avoid the undesirable amplification.

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

This section describes the purification and concentration method for the 1st PCR products using a spin column (GeneRead Size Selection Kit; hereafter called “column”: Fig. 5-2-1-2-1). During the 1st PCR, a small amount of DNA with an unknown concentration is amplified to a sufficient amount for a series of subsequent experiments. Thus, the 1st PCR is likely to generate primer and adapter dimers that inhibit subsequent experiments. It is critically important to remove those dimers to prepare a good library; therefore, the column purification is repeated twice in the following protocol using the same column. Although a single purification may be sufficient, the two purification procedures can remove dimers to a level that they cannot be detected on the gel.

1) Collect the 1st PCR products from the eight PCR replicates into a single 1.5 mL tube (Fig. 5-2-1-2-2). Since the PCR is performed in a volume of 12.0 µL per tube, the total volume amounts to 96.0 µL. The blank has a volume of 12.0 µL because it is amplified once.

2) Add four times the volume of Buffer SB1 (384 µL; the volume of blank SB1 solution is 48 µL) to each 1.5 mL tube (Fig. 5-2-1-2-3), mix well, and centrifuge briefly. Let the solution stand at room temperature for 1 minute.

3) Prepare as many columns (with collection tubes) supplied with the GeneRead Size Selection kit as needed and write the necessary information on the cap (Fig. 5-2-1-2-4).

4) To bind the DNA, apply the mixture (PCR product mixed with SB1) to the column (Fig. 5-2-1-2-5) and centrifuge at 20,000 g for 1 minute (Fig. 5-2-1-2-6).

5) After centrifuging, remove the collection tube and place the column to a new collection tube (Fig. 5-2-1-2-7). Discard the used collection tube (Fig. 5-2-1-2-8).

6) To wash the DNA, add 700 µL of 80% ethanol to the column (Fig. 5-2-1-2-9) and centrifuge at 20,000 g for 1 min (Fig. 5-2-1-2-10). After centrifuging, transfer the column to a new collection tube. Discard used collection tubes. Perform this procedure (purification with 80% ethanol) twice.

7) Prepare 1.5 mL tubes provided by the GeneRead Size Selection kit and write the necessary information on the cap. Place the column to the 1.5 mL tube (Fig. 5-2-1-2-11).

8) Add 90 µL Buffer TE (provided) to the column (Fig. 5-2-1-2-12) and let the column stand at room temperature for 1 minute.

9) Centrifuge at 20,000 g for 1 minute.

10) Perform a second purification on 90 µL of the product collected in a 1.5 mL tube. Add four times the amount of Buffer SB1 (360 µL) extracted DNA (90 µL) eluted with TE buffer to a 1.5 mL tube (Fig. 5-2-1-2-13), mix well, and then centrifuge briefly. Let the tube stand at room temperature for 1 minute.

11) Place the column in a new collection tube and add the above purified product to the column (Fig. 5-2-1-2-14). Centrifuge for 1 minute at 20,000 g.

12) After centrifuging, remove the collection tube and transfer the column to a new collection tube. Discard used collection tubes.

13) Add 700 µL of 80% ethanol to the column and centrifuge at 20,000 g for 1 minute. After completion, transfer the column to a new collection tube. Discard used collection tubes. This procedure (purification with 80% ethanol) is performed twice.

14) Prepare a 1.5 mL low DNA binding tube and write the necessary information on the cap. Place the column on the tube with it uncapped.

15) For elution, carefully add 17 µL Buffer EB (provided) to the center of the column membrane (Fig. 5-2-1-2-15) and let the column stand at room temperature for 1 minute.

16) Centrifuge the column at 20,000 g for 1 minute. After centrifuging, remove the column from the tube and close the cap tightly (Fig. 5-2-1-2-16). Discard the column. The capped tube may be stored stably at –20˚C (Fig. 5-2-1-2-17).

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 2200 (Agilent Technologies Inc.). Note that the protocol may be applied using other systems comparable to the TapeStation, such as the BioAnalyzer. In the case of the TapeStation 2200, a High Sensitivity D1000 ScreenTape System kit will be used. The ScreenTape is pre-filled with gel in 16 lanes, and up to 16 PCR products can be quantified simultaneously.

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 2200 main unit (Fig.

5-2-1-3-2).

3) After verifying the connection between the TapeStation and the computer, launch the TapeStation 2200 controller software and set the ScreenTape and pipette tips (Fig. 5-2-1-3-3).

4) Prepare the 8-strip tubes and dispense 2 µL of High Sensitivity D1000 Sample Buffer to as many tubes as the DNA ladder plus 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. 5-2-1-3-5/6) 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 2200 cassette (Fig. 5-2-1-3-8).

7) Follow the instructions for the TapeStation 2200 controller (Figs. 5-2-1-3-9/10) to start electrophoresis, and the target band (about 310 bp) will be displayed along with the pherogram about 20 minutes later (Fig. 5-2-1-3-11).

8) Read the concentration of the target band and dilute it to 0.1 ng/µL with Milli-Q water. Dilute the blank to the mean dilution percentage for positive samples for convenience.

Note: When diluting the purified and concentrated 1st PCR product, it is recommended that Milli-Q 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. In addition, the sample is more accurately diluted by calculating the amount of Milli-Q water required for dilution (NOT for the total amount of purified and concentrated 1st PCR product), but for a portion (e.g., 10.0 µL) of the product. The calculation makes the amount (reads) of the product almost constant. If the concentration does not reach the specified level, dilute to 0.05 ng/µL to increase the number of PCR cycles.

9) Store the diluted 1st PCR product and the remaining undiluted 1st PCR product at –20˚C.

5-2-2. Library preparation—2: Second-round PCR (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 sequence and a flow cell binding sequence to a template (a quantified 1st PCR product) using primer sequences added during the 1st PCR. 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

- Thermal cyclers (e.g., GeneAmp PCR System 9700, Applied Biosystems, Thermo Fisher Scientific K.K.)
- UV sterilizer light bulb (e.g., NB-5, Nichiban Co., Ltd.)
- Quantified 1st PCR product (0.1 ng/μL)
- KAPA HiFi HS ReadyMix (KK2602, KAPA Biosystems Inc.)
- Primer stock solutions with indices (primer stock solutions diluted to 100 μM with TE buffer are convenient, which are available to order. The information related with the index sequence is given at the end of this section)

Example of a forward primer (blue indicates an eight-base index sequence)

F D501 (5′–3′; 70 mer)
AATGATACGGCGACCACCGAGATCTACACTATAGCCTACACTCTTTCCCTACACGACG
CTCTTCCGATCT

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

R D701 (5′–3′; 66 mer)
CAAGCAGAAGACGGCATACGAGATCGAGTAATGTGACTGGAGTTCAGACGTGTGCTC
TTCCGATCT

- Milli-Q water (or high-grade sterilized water with molecular biology grade)
- TE buffer (molecular biology grade)
- 8-strip tubes
- 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
- Rubber gloves for experiments (powder free)

Laboratory instruments, reagents, and consumables for the size selection

- E-Gel iBase Power System (G6400, Invitrogen, Thermo Fisher Scientific K.K.)
- E-Gel Safe Imager E-Gel Real-Time Transilluminator (G6500, Invitrogen, Thermo Fisher Scientific K.K.)
- E-gel SizeSelect II 2% (G661012, Invitrogen, Thermo Fisher Scientific K.K.)
- Molecular size markers: 50 bp DNA Ladder (10416014, Invitrogen, Thermo Fisher Scientific K.K.)
- 2nd PCR products (individually or several tubes per library)
- Milli-Q 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
- Rubber gloves (powder-free)

Laboratory instruments, reagents, and consumables for the quantification

- Qubit 2.0 Fluorometer (Life Technologies Japan Ltd)
- Qubit dsDNA HS Assay (Q32851 [for 100 samples], Life Technologies Japan Ltd.) (Q32854 [for 500 samples], Life Technologies Japan Ltd.)
- Qubit assay 500 µL tube (Q32856, Life Technologies Japan Ltd.)
- 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 (types for the volumes of the micropipettes to be used)
- 1st PCR products recovered with E-Gel
- Rubber gloves (powder-free)

5-2-2-1. 2nd PCR

Be sure to wear rubber gloves during the experiment (rubber gloves should be quickly replaced if contaminated during the procedure). Before the experiments, pipettes and tubes should be decontaminated for approximately 20 minutes using a UV sterilizer light bulb (Fig. 5-2-1-1-1). 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: If the amount of reagent is not 1.2 times

the required amount, the reagent may not be dispensed to all the tubes with an electric pipette.)

KAPA HiFi HS ReadyMix	7.5 µL
Primer	0.88 µL each
Milli-Q 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 combination of the index sequences, the use of the rows and columns of a tube rack is described below. Since 8-strip tubes 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-strip tubes. A method is described of creating parallel sequences consisting of 32 libraries (samples) by differently combining eight types of forward primer (index sequences D501–D508) and four types of reverse primer (index sequences A701–A704). Also, as discussed in the beginning, in order to minimize the effect of carryover between runs (the library of the previous runs that remain in the microtubes inside MiSeq), a combination of index sequences should not be used continuously in a few subsequent runs (two to three 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. 5-2-2-1-2). By creating premixes containing primers in this way (rather than dispensing only one primer at a time), you may accurately adjust the reaction volume (15 µL in this case) even if the total amount of the PCR reaction is small.

4) Prepare 1.2 times the required volume of the required premix (premix containing KAPA + Milli-Q water only). Specifically, add 288 µL of Kapa ReadyMix (= 7.5 µL × 32 × 1.2) and 149 µL of Milli-Q water (= 3.88 µL × 32 × 1.2) to the above-mentioned 32 libraries. The combined volume of Kapa ReadyMix and Milli-Q water totals 437 µL.

5) Make 12 1.5 mL tubes available for eight types of forward primers and four types of reverse primers and fill in the necessary information on the cap.

6) Dispense 25.5 µL of the required pre-mix ([7.5 µL + 3.88 µL] × 4 × 1.12/2) to eight tubes containing above-mentioned eight types of forward primers (1.12 times the volume).

7) Dispense 51 µL of the required pre-mix ([7.5 µL + 3.88 µL] × 8 × 1.12/2) to four tubes containing the above-mentioned four types of reverse primer (1.12 times the volumes).

8) Dispense 3.94 µL (0.88 µL × 4 × 1.12) of the required eight types of forward primers to each tube in 6).

9) Dispense 7.88 µL (0.88 µL × 8 × 1.12) of the required four reverse primers to each tube of 7).

10) Dispense 6.57 μL each of the premix containing the forward primer in the horizontal direction.

11) Dispense 6.57 μL each of the premix containing reverse primer in the vertical direction.

12) Gently press caps into the 8-strip tube after dispensing, centrifuge with a small desktop centrifuge and shake off the solution toward the tube bottom.

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

14) Carefully remove the caps of the 8-strip tubes in a manner to keep the premix from splashing and add 1.86 μL each of diluted first PCR product to the specified tubes.

15) Firmly press the caps on the 8-strip tubes and gather the solution to the tube bottom with a desktop mini centrifuge.

16) In the 2nd PCR, perform shuttle PCR combining annealing and extension at the same temperature. Set the thermal cycler as follows.

First insertion of denaturation at 95˚C for 3 minutes
Denaturation at 98˚C for 20 seconds
Annealing plus extension at 72˚C for 15 seconds } 10 cycles
Final insertion of a 5-min extension at 72˚C

17) Set the 8-strip tube on the thermal cycler and start the 2nd PCR.

18) Once the 2nd PCR is completed, a unique combination of indices is added to each sample, so they can be mixed into a single tube. Collect the samples that you want to analyze together (for example, 10 samples taken in one survey) in one tube (Fig. 5-2-2-1-2).

5-2-2-2. Purification of 2nd PCR product using gel electrophoresis

The 2nd PCR product is a 1st PCR product (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 in addition to a target fish-derived product. In this section, we describe the method of excising only the 2nd PCR product (about 370 bp) derived from fish by gel electrophoresis using E-Gel (practically, collecting the target product from the well using a pipette).

1) Add 920 μL of Milli-Q water to 80 μL of molecular size marker (50 bp DNA Ladder) stock solution to dilute the stock solution.

2) Place the E-Gel iBase Power System (iBase) on the E-Gel Safe Imager E-Gel Real-time Transilluminator (Safe Imager), connect the iBase to the Safe Imager using the supplied cable, and turn on the switch.

3) Remove E-gel SizeSelect II 2% (gel enclosed in a plastic case, hereafter called “gel”) from the package and gently remove the two combs from the upper and lower wells (Fig. 5-2-2-2-1).

4) Insert the cassette into the iBase from its right side (Fig. 5-2-2-2-2). Press down on the left side of the cassette to secure the cassette. The iBase red light turns on when the gel is inserted correctly.

5) Mix the 2nd PCR product and the supplied Loading Buffer and load 20–25 μL of the mixture into the upper wells. Load 20–25 μL of the molecular weight marker into separate wells from

the wells for the sample. Load the same amount of Milli-Q water into unused wells (Fig. 5-2-2-2-3). Do not damage the gel or introduce bubbles into the wells.

6) Fill all the middle wells (collection wells) with Milli-Q water. Note that if you do not fill the collection wells with Milli-Q water, electrophoresis will not proceed successfully.

7) Place the amber filter over the iBase device (Fig. 5-2-2-2-4) and press the mode button several times to select the Run SizeSelect 2% (No. 9) mode. Provisionally set the electrophoresis time to 15 minutes. Press "Start run" to start the gel protocol.

8) Before you become familiar with the E-Gel run, monitor the run periodically by activating the "Back light" button. The molecular size marker specified in this manual is a good reference for a target band with approximately 370 bp because the marker lights brightly at 350 bp than at other sizes.

9) After 15 minutes, a flashing red light and beeping signal the end of the run. Activate the "Back light" button to check the target band position. If the target band has not reached to the reference line, continue electrophoresis for few minutes.

10) Once the target band reaches the reference line, pause the electrophoresis.

11) Since Milli-Q water in the collection well decreases in volume during the electrophoresis over time, add the reduced amount.

12) Restart electrophoresis. Stop electrophoresis once the whole band is completely entered into collection wells.

13) Recover the target band from the collection well using a pipette and collect the band into one 1.5 mL tube per library (Fig. 5-2-2-2-5). Avoid piercing the agarose during collection.

Note: In many cases, a sufficient amount 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 such a case, you may repeat a sequence of the following procedures: fill the collection wells with Milli-Q 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. A sufficiently high concentration of the 2nd PCR product may be obtained by concentrating the product using a column.

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

This section describes the method for measuring the concentration of a library excised from E-Gel.

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) As many tubes as the calibration Standards #1 and #2 plus libraries are required to measure the concentration of Qubit.

3) When the temperature of the kit returns to room temperature, prepare as many 200 µL premixes, which contain 1 µL of Qubit Reagent and 199 µL of Qubit Buffer, as needed (Figs. 5-2-2-3-2, 3). If measurement of the concentration is required for only one library, a total of three libraries, including two calibration standards, are required. 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 add 2 µL of the library (Fig. 5-2-2-3-5). Close the lids, vortex them for 2–3 seconds, and centrifuge 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) On the Home screen of the Qubit 2.0 Fluorometer, press "DNA" from the screen and then select "dsDNA High Sensitivity." A prompt will be displayed asking whether you want to calibrate using the calibration standard. Measure Standards #1 and #2 mentioned in 4) in that order according to Qubit instructions (Figs. 5-2-2-3-8/9).

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. It is advisable to repeat the concentration measurement of the same sample until the measured value becomes stable.

8) Qubit displays the measured value (ng/mL) after 100-fold dilution of the template (Fig. 5-2-2-3-10). When the value is less than 10.0 ng/mL, it may be necessary to increase the concentration by subjecting the same library to E-gel electrophoresis using several wells and concentrate the recovered products using a spin column.

5-2-2-4. Index information

The table shown below is an index sequence (8 bases) widely used in metabarcoding (A501–508/A701–712 and D501–508/D701–712, Illumina Inc.). When you order a custom primer, insert the i5 sequence into the forward primer and the i7 sequence into the reverse primer without modifying them. The table below shows the sequences of eight bases in the direction of 5′ to 3′. However, when you fill in the sample sheet for the MiSeq run, note that the i7 sequence converted to a reverse complement sequence should be filled in. If you want to use an index other than this, you may pick an appropriate one from the index array listed in Hamady et al. (2008). As you have more index sequences available, more libraries can be analyzed simultaneously. In addition, by using a different index for each run, you can avoid cross-contamination between runs (contamination in the microtubes inside MiSeq by the residual library carried over from the last run). In addition, you may share a MiSeq run from across several different laboratories by using different combinations of index sequences, leading to savings in research costs.

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

The latest information on Illumina-accredited index sequences is available on the following site (as of June 2018).

https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/experiment-design/illumina-adapter-sequences-1000000002694-06.pdf

Name	i5 bases	Name	i7 bases	Name	i5 bases	Name	i7 bases
A501	TGAACCTT	A701	ATCACGAC	D501	TATAGCCT	D701	ATTACTCG
A502	TGCTAAGT	A702	ACAGTGGT	D502	ATAGAGGC	D702	TCCGGAGA
A503	TGTTCTCT	A703	CAGATCCA	D503	CCTATCCT	D703	CGCTCATT
A504	TAAGACAC	A704	ACAAACGG	D504	GGCTCTGA	D704	GAGATTCC

A505	CTAATCGA	A705	ACCCAGCA	D505	AGGCGAAG	D705	ATTCAGAA
A506	CTAGAACA	A706	AACCCCTC	D506	TAATCTTA	D706	GAATTCGT
A507	TAAGTTCC	A707	CCCAACCT	D507	CAGGACGT	D707	CTGAAGCT
A508	TAGACCTA	A708	CACCACAC	D508	GTACTGAC	D708	TAATGCGC
		A709	GAAACCCA			D709	CGGCTATG
		A710	TGTGACCA			D710	TCCGCGAA
		A711	AGGGTCAA			D711	TCTCGCGC
		A712	AGGAGTGG			D712	AGCGATAG

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 successfully sequencing with this method is to prepare a good library that contains exclusively 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 (0.1 ng/μL) and the quantified 1st PCR products are used as templates for the 2nd PCR. In addition, since maintenance of MiSeq is very important in order to successfully perform runs as well, information related to maintenance is listed first.

Reagents and consumables required for MiSeq maintenance

- Tween 20 (P7949, Sigma Aldrich Japan GK)
- Milli-Q water
- 50 mL freestanding centrifuge tubes
- 500 mL washing bottles
- 0.01% sodium hypochlorite solution (NaOCl)
- Tubes for sodium hypochlorite solution (PN: 15021932; personally, obtained from Illumina Inc.)
- Rubber gloves (powder-free)

Laboratory instruments, reagents, and consumables for sequencing

- MiSeq (Illumina Inc.)
- MiSeq Reagent Kit v2 (300 cycles) (MS-102-2002, Illumina Inc.): Use this product when a total of 15 million reads are required. If fewer reads are sufficient, use MiSeq Reagent Micro Kit v2 (MS-103-1002 for approximately 4 million reads) or MiSeq Reagent Nano Kit v2 (MS-103-1001 for approximately 1 million reads).
- Libraries with known concentrations
- 0.2 N NaOH (prepared for each experiment from stock with 2 N or more)
- PhiX Control v3 (FC-110-3001, Illumina Inc.)
- 99.5% ethanol with molecular biology grade (Wako Pure Chemical Industries, Ltd.)
- Milli-Q 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.)
• Micropipette P-1000, P-200, P-100, P-20, P-2 (E.g., Pipetman, Gilson Company Inc.)
• Thawing bat for reagent kit (Approximately 373 W × 273 D × 63 H) (e.g., PB-2, ASONE Corp.)
• Rubber gloves (powder-free)

The MiSeq Reagent Kit includes 1) an HT1 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. Rarely, the suppliers may mistakenly deliver the four components in the frozen state. Use of the kits is discouraged because PR2 bottles and flow cells deteriorate once they are frozen.

5-2-3-1. Maintenance of MiSeq

The MiSeq platform contains extensively deployed microtubes, which transfer libraries and reagents to flow cells where sequencing reactions occur. You should keep these microtubes clean so that the flow paths are not affected by the last run. It is one of the major factors in a successful run.

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 is performed. 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

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 diluted to 10% may be purchased and used. Wear rubber gloves and 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 Milli-Q 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 Milli-Q 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. Maintenance and standby washes require replacement of the wash solution as described below. Therefore, wash the wash bottle just used up during the wash with tap water and rinse it with Milli-Q before you prepare a 0.5% Tween 20 wash solution again.

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 Milli-Q 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) Preparation of wash: Make a wash cartridge available, and insert a sodium hypochlorite solution tube in port 17 (Fig. 5-2-3-1-15; available with manual and additional software from Illumina support) (Fig. 5-2-3-1-16) and pour 1 mL of the sodium hypochlorite solution adjusted to 0.01%. 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 containing a checkbox asking whether the sodium hypochlorite solution is used slightly below the center.

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 order to avoid the problems of an interrupted run, it is recommended that the MiSeq main unit be restarted every time a new run is performed.

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. Unlike other experiments (especially PCR), there is little risk of contamination at this stage, but be sure to wear rubber gloves during the experiment and expose pipettes and tubes to a UV sterilizer light bulb for approximately 20 minutes in advance to decontaminate them.

1) Thaw the frozen components: Put the frozen reagent cartridge and HT1 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). Let the cartridge and tube immersed in deionized water for approximately 1 hour to thaw the reagents inside the kit.

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. It is important to prepare a fresh 0.2 N NaOH solution by diluting from the 2 N NaOH solution (stockable) with Milli-Q water for every run. Since the amount to be used is very small, it is advisable to use a 1.5 mL tube to prepare 1 mL. You can prepare a sufficient amount without making errors during dilution. Store the prepared 0.2 N NaOH solution in the refrigerator before use.

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. If the saving format

is CSV, sample sheets can be edited with a spreadsheet, such as Excel. Therefore, it is more efficient to generate a base file with IEM and re-edit it with a separate 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 technique 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.

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. When several libraries of different sample sizes are sequenced at a time, the mixing ratios have to be calculated from the sample sizes (excluding blanks), and the amounts of different libraries should be 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. Denatured DNA has a concentration of 2 nM at this point in time.

5) Adjust to 20 pM: Add 990 μL of HT1 Buffer to the denatured DNA and adjust it to 20 pM (100-fold dilution).

6) 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 specified by the vendor. You may add 360 μL of the library and 240 μL of HT1 buffer to attain a final concentration of 12 pM. Note that if the library is of low quality (i.e. the library contains DNA shorter than intended length of around 370 bp), the final concentration adjusted to 12 pM will result in over-clustering and eventually become a run failure.

7) PhiX spike-in: In order to improve signal separation between clusters on the flow cell, add PhiX with good base balance as a spike-in up to 10% to 25%. PhiX control v3 (Illumina Inc.) is a library based on known sequences derived from a bacteriophage adjusted to 4 nM. 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 HT1 buffer. Then, extract 60 μL from the above 600 μL library and add 36 μL of PhiX adjusted to 20 pM and 24 μL of the HT1 buffer.

With the above procedure, a library of 600 μL adjusted to 12 pM is successfully created. Then, you have just to inject the adjusted library into the reagent cartridge and execute the final procedure for starting the run.

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.

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. Invert and stir the reagent cartridge about 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 make a hole in the foil at the library injection port (marked by the orange color).

3) Use a 1200 μL or 1000 μL pipette tip to insert 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.

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

5) When the SEQUENCE button, a confirmation button, is pressed with MiSeq connected to the Internet, a button will be displayed asking whether to use BaseSpace, cloud environment provided by Illumina Inc. (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. The flow cell is stored in a container in which the cell is immersed in the buffer. You should wash the flow cell thoroughly to remove the buffer before setting it in MiSeq. If the cell is not sufficiently washed, the remaining buffer may be deposited on the flow cell during sequencing, and data may not be read. Wear rubber gloves and rinse the glass and polyethylene parts of the flow cell thoroughly with Milli-Q water before setting 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 upper latch in order to prevent the latch 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 is being 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 before 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) Look at the lower left of the MCS screen to check 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) Insert the reagent cartridge filled with the library into the reagent chiller. Note that the reagent chiller door will not close unless the cartridge is pushed in the chiller to the end.

17) Close the reagent chiller door. Look at the lower left of the MCS screen to check if the ID (RFID) on the reagent cartridge is being read successfully.

18) If the reading process is ongoing successfully, 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.

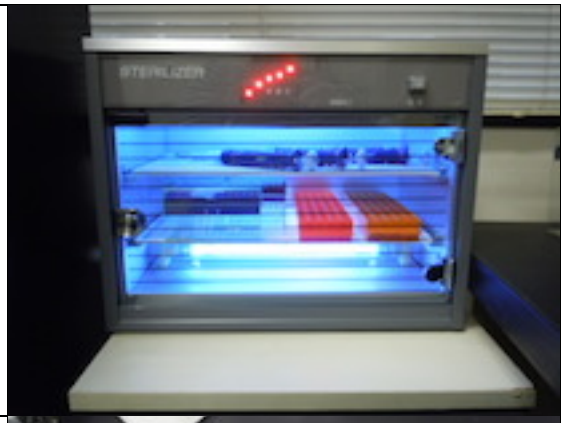
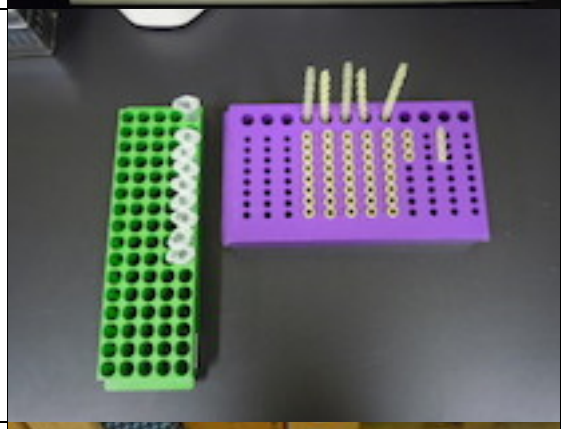
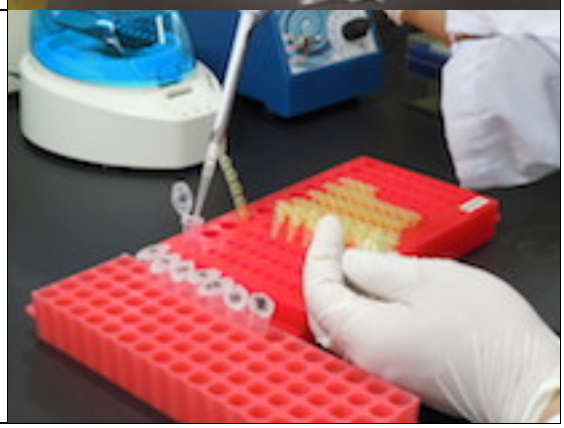
Fig. 5-2-1-1-1 Micropipettes, filter tips, tubes, and tube racks are being sterilized with ultraviolet rays using a UV sterilizer light bulb. You should build the habit of sterilize instruments before experiment to reduce the risk of cross contamination	
Fig. 5-2-1-1-2 The 1st PCR is set up to perform PCR eight times by using one 8-strip tube for the same sample. The picture provided at right shows five samples, including a total of three blanks; one filtration, one extraction, and one 1st PCR blank.	
Fig. 5-2-1-2-1 GeneRead Size Selection Kit and additionally ordered 2.0 mL collection tubes.	
Fig. 5-2-1-2-2 The 1st PCR products from eight PCR replicates are being collected in a single 1.5 mL tube.	

Fig. 5-2-1-2-3 Supplement the 1st PCR product with four times the amount of SB1 and pipet it to the tubes.	
Fig. 5-2-1-2-4 Place spin columns and write the necessary information on the caps.	
Fig. 5-2-1-2-5 Pour the mixed 1st PCR product + SB1 solution into the columns.	
Fig. 5-2-1-2-6 Centrifuge the columns for 1 minute at 20,000 g.	

Fig. 5-2-1-2-7 Transfer the columns to new collection tubes.	
Fig. 5-2-1-2-8 Discard used collection tubes and waste liquid.	
Fig. 5-2-1-2-9 Pipet 700 µL of 80% ethanol into the columns.	
Fig. 5-2-1-2-10 Centrifuge at 20,000 g for 1 minute.	

Fig. 5-2-1-2-11 Transfer the columns to 1.5 mL tubes.	
Fig. 5-2-1-2-12 Pipet 90 µL of the TE buffer onto the column membrane, let stand for 1 minute, and centrifuge at 20,000 g for 1 minute.	
Fig. 5-2-1-2-13 Supplement the collected solution (PCR product obtained after the first purification) with four times the amount of SB1 and pipet the solution.	
Fig. 5-2-1-2-14 Add the mixed solution into the columns.	

Fig. 5-2-1-2-15 After the second purification, 17 µL of the EB buffer is being added onto the column membrane. Let the columns stand for 1 minute and then centrifuge them at 20,000 g for 1 minute.	
Fig. 5-2-1-2-16 The purified first PCR product is recovered in a 1.5 mL tube (low DNA bind).	
Fig. 5-2-1-2-17 Freeze and store at –20˚C.	
Fig. 5-2-1-3-1 Kit for TapeStation 2200. High Sensitivity D1000 Screen Tape (left) and reagent (right).	

Fig. 5-2-1-3-2 Boot up the computer supplied with the TapeStation 2200.	
Fig. 5-2-1-3-3-3 Set ScreenTape and the required number of pipette tips.	
Fig. 5-2-1-3-4 Dispense 2 µL of the buffer using an electric micropipette.	
Fig. 5-2-1-3-5 Add 2 µL of the ladder into the first tube.	

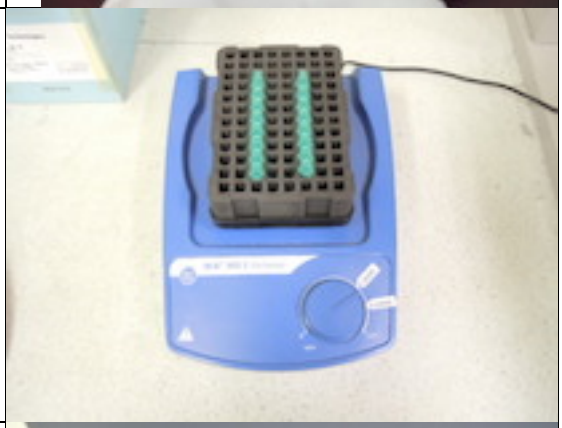
Fig. 5-2-1-3-6 Add 2 μL of purified and concentrated first PCR product.	
Fig. 5-2-1-3-7 After stirring at 2000 rpm for 1 minute, collect the liquid at the bottom of the tube with a desktop mini centrifuge.	
Fig. 5-2-1-3-8 Gently open the caps of the 8-strip tubes in a manner to prevent the liquid from scattering from inside, and set it in the TapeStation 2200 cassette.	
Fig. 5-2-1-3-9 All the tubes are set in the TapeStation 2200.	

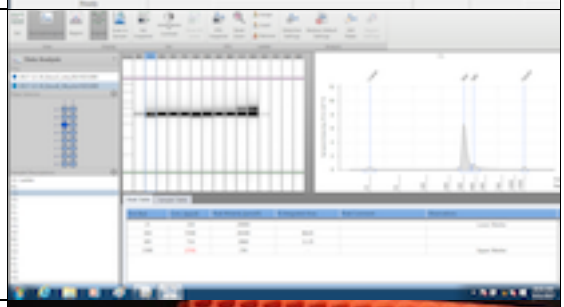
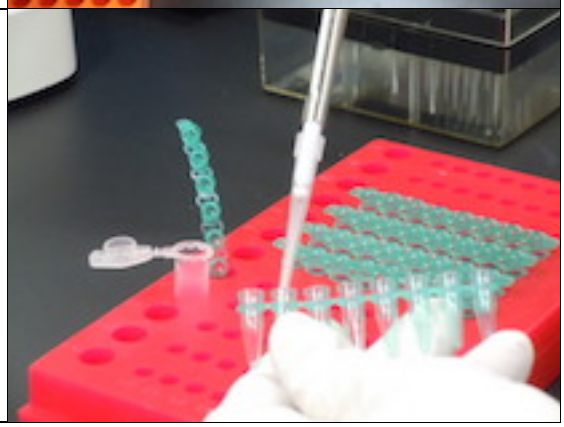
Fig. 5-2-1-3-10 Specify on the screen the arrangement of the ladder and the PCR product to be measured on the tubes using the 8-strip tube image in the left portion of the screen.	
Fig. 5-2-1-3-11 The target band (about 310 bp) is displayed along with the pherogram.	
Fig. 5-2-2-1-1 When the number of samples is 40.	
Fig. 5-2-2-1-2 Collect the reaction solution in a single tube.	

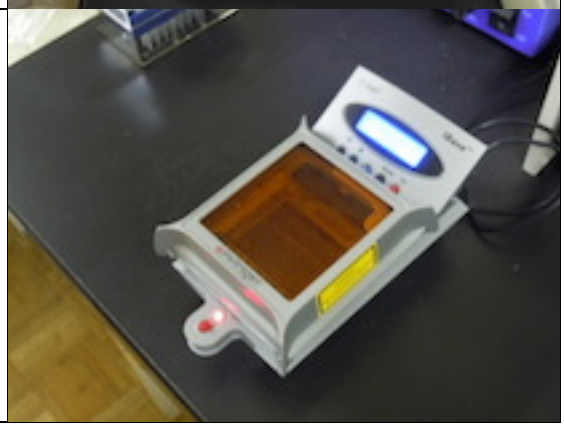
Fig. 5-2-2-2-1 Carefully remove the comb.	
Fig. 5-2-2-2-2 Insert the cartridge from the right side of the gel into the iBase.	
Fig. 5-2-2-2-3 Load the product.	
Fig. 5-2-2-2-4 Set the observation plate unit (unit with an orange filter) on the iBase.	

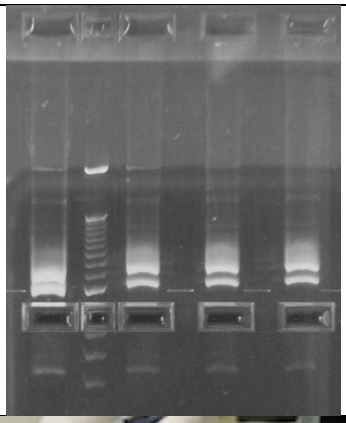
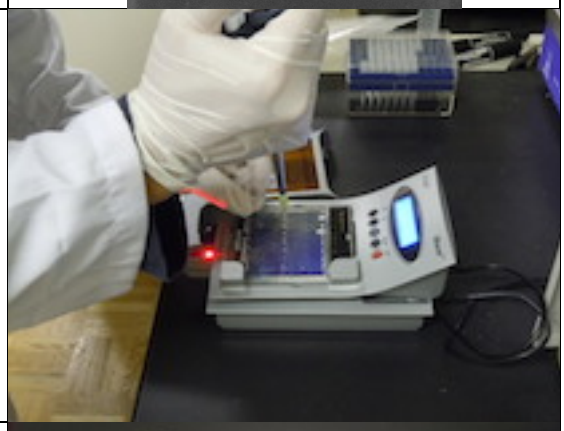
Fig. 5-2-2-2-5 The target product (about 370 bp) reaches the reference line. The second from the left is the ladder (size marker), and the bright ladder reaching the reference line indicates 350 bp. The target product (370 bp) is slightly above it. The band with the smear is further above it. The larger band is a non-specific product, so be careful not to put it in the collection well.	
Fig. 5-2-2-2-5 The electrophoresed product is being collected.	
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 Buffer in a new tube.	

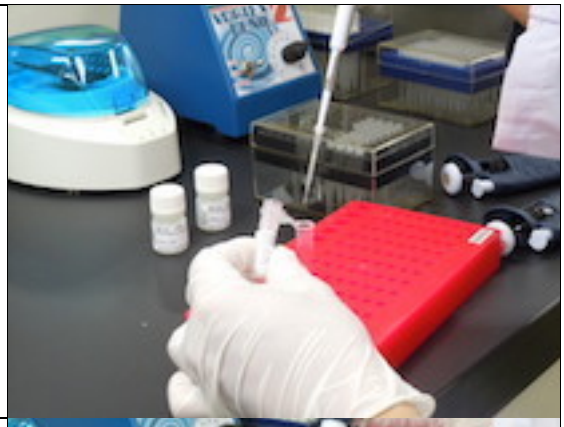
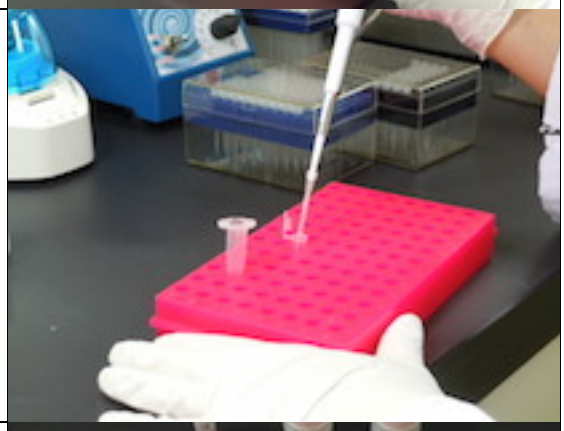
Fig. 5-2-2-3-3 Add the Qubit Reagent.	
Fig. 5-2-2-3-4 Add 10 µL each of standard #1 and #2 to tubes separately.	
Fig. 5-2-2-3-5 Add 2 µL of the library to the tubes.	
Fig. 5-2-2-3-6 Let stand at room temperature for 2 minutes. If this procedure is omitted, no stable measurements will be obtained.	

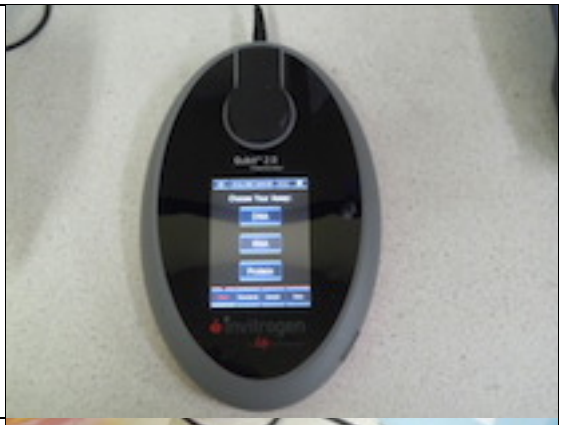
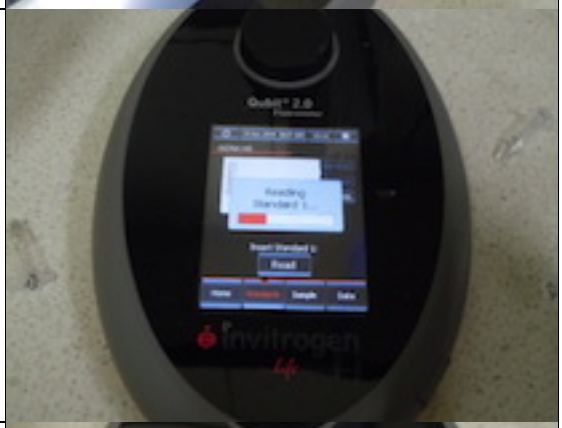
Fig. 5-2-2-3-7 Start the Qubit 2.0 Fluorometer by touching its screen.	
Fig. 5-2-2-3-8 Measure Standards #1 and #2 in that order according to the instructions from Qubit.	
Fig. 5-2-2-3-9 Qubit measures the concentration of the sample.	
Fig. 5-2-2-3-10 The measured value (ng/mL) of the template diluted 100 times is displayed.	

Fig. 5-2-3-A Frozen components; HT1 tube (right front) and reagent cartridge (left front). If this box is refrigerated and the tube and cartridge are thawed when the box is received, submit a complaint to the supplier and ask to send a replacement.	
Fig. 5-2-3-B Refrigerated components; PR2 bottle (center) and flow cell (right). If the components are frozen when they are received, submit a complaint to the supplier and ask to send a replacement.	
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.	

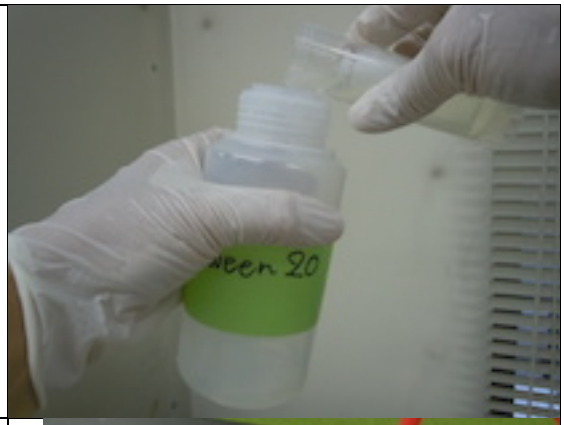
Fig. 5-2-3-1-3 Place 25 mL of 10% Tween 20 solution into a 500 mL wash bottle.	
Fig. 5-2-3-1-4 Inject the wash solution into the wash cartridge using the wash bottle.	
Fig. 5-2-3-1-5 After the wash solution is injected into the cartridge, transfer the remaining wash solution to a wash bottle (about 350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

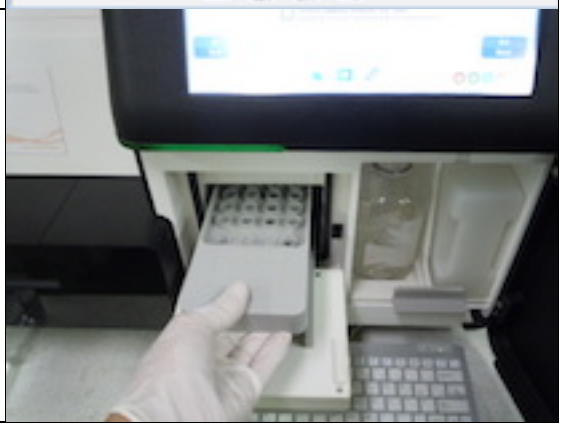
Fig. 5-2-3-1-7 Press the **Perform Wash** button. Three different wash types are displayed.	
Fig. 5-2-3-1-8 Check that the used flow cell is set in the flow cell compartment.	
Fig. 5-2-3-1-9 An image depicting the reagent cartridge and the bottle being taken out is displayed on the screen.	
Fig. 5-2-3-1-10 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 Sodium hypochlorite solution tube (Illumina Customer Support will supply the tubes free of charge on demand).	
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 Insert the HT1 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 figure 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 and bring the reagents down to the tube bottom.	
Fig. 5-2-3-4-2 First, 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 displayed.	

Reference

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

Suggested citations

Whole manual

The eDNA Society (2019) Environmental DNA Sampling and Experiment Manual Version 2.1. Otsu, Japan.

Chapter 2

Seino, S. and Minamoto, T. (2019) Selection of sampling sites. Pages 6–10 *in* Environmental DNA sampling and experimental manual version 2.1. Ed. by eDNA Methods Standardization Committee, The eDNA Society, Otsu, Japan.

Chapter 3

Miya, M. and Sado, T. (2019) Water sampling and on-site filtration using a filter cartridge. Pages 12–22 *in* Environmental DNA sampling and experimental manual version 2.1. Ed. by eDNA Methods Standardization Committee, The eDNA Society, Otsu, Japan.

Minamoto, T. (2019) Water sampling and filtration using glass fiber filters in the laboratory. Pages 23-29 *in* Environmental DNA sampling and experimental manual version 2.1. Ed. by eDNA Methods Standardization Committee, The eDNA Society, Otsu, Japan.

Chapter 4

Miya, M. and Sado, T. (2019) DNA extraction from a filter cartridge. Pages 31–42 in Environmental DNA sampling and experimental manual version 2.1. Ed. by eDNA Methods Standardization Committee, The eDNA Society, Otsu, Japan.

Minamoto, T. (2019) DNA extraction from glass fiber filters. Pages 43–51 in Environmental DNA sampling and experimental manual version 2.1. Ed. by eDNA Methods Standardization Committee, The eDNA Society, Otsu, Japan.

Chapter 5

Minamoto, T. (2019) Single species detection and quantification of eDNA. Pages 52–54 in Environmental DNA sampling and experimental manual version 2.1. Ed. by eDNA Methods Standardization Committee, The eDNA Society, Otsu, Japan.

Miya, M. and Sado, T. (2019) Multiple species detection using MiFish primers. Pages 55–92 in Environmental DNA sampling and experimental manual version 2.1. Ed. by eDNA Methods Standardization Committee, The eDNA Society, Otsu, Japan.

Acknowledgements

The English version of the manual was produced with the support of Northwest Pacific Region Environmental Cooperation Center (NPEC).

Environmental DNA Sampling and Experiment Manual Version 2.1
(Published: April 25, 2019)