Molecular Biology Laboratory Manual V. 2025

ENVIRO-RESPONSIBLE

CLEAN TODAY • BRIGHTER TOMORROW

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Introduction

The following Laboratory Manual describes the working conditions, protocols and recipes for identifying Aquatic Invasive Species (AIS) in environmental samples using a genetic approach (eDNA) and the use of Oxford nanopore metabarcoding sequencing technology for the analysis of eDNA samples to support genomic diversity studies.

Results provided by this laboratory for the identification AIS correspond to the LEVEL 3 (Essential) according to the 5-level validation scale proposed by Thalinger et al. 2021 to aid in evaluating eDNA assays and appropriate interpretation of results. The analysis method is based on a Quantitative Polymeric Chain Reaction (qPCR) using amplification primers obtained from the literature. This laboratory intends to continue to improve its service by reaching Operational Level 5 and extending its service to the identification of other invasive species.

Oxford Nanopore Technologies' metabarcoding solutions for environmental samples are designed to provide high-throughput sequencing of environmental DNA (eDNA) samples. These technologies enable researchers to analyze biodiversity, ecosystem biomonitoring, and animal conservation. The MinION sequencer is a portable and affordable device that allows for real-time sequencing at the sample source, making it ideal for both in-field and lab-based analysis. The technology offers enhanced species identification and real-time data analysis, providing immediate access to results. This capability is particularly beneficial for environmental research, as it allows for rapid and accurate identification of microbial communities and their implications for biodiversity and the ecosystem.

Laboratory Safety Rules

All personnel working in this lab must abide by the following safety rules:

Before starting to work

- Ensure you are familiar with chemicals and their SDS and WHMIS regulations as per Ontario. Reg. 860
- Read all protocols and procedures included in this manual
- Check instruments are working properly, including verification/calibration if required
- Prepare all your waste containers. Solid waste composed of pipette tips, qPCR plates, empty containers and others are collected in small plastic bags and disposed of in regular recycling. Liquid waste from filtration contains 70% ethanol. This solution is stored and disposed of as chemical waste.
- Put on your PPE before commencing the work (lab coats, gloves and safety glasses). PPE must be removed before leaving the laboratory and when sitting at the office station.

At the workplace

- Wearing Personal Protective Equipment (PPE) while working in the lab is mandatory.
- It is essential to wear lab coats all the time in the lab.
- Gloves shall be worn while working with biological samples since some nucleases are commonly present in our hands and can destroy your material (DNA or RNA samples).
- Safety glasses are recommended to avoid eye exposure in case of spills or splashes.
- Different working benches separate the sample extraction area, the PCR - qPCR setup area, and amplification machine. Since the quantitative-PCR equipment (qPCR) is located in the same room, a PCR safety cabinet is used to prepare the reaction mix and handle the eDNA samples. In this way, we can separate the setup area from the amplification one, avoiding PCR products cross-contamination.
- Dedicated materials are placed on each bench to avoid cross-contamination. Never switch pipettes or tips between areas unless they have been previously decontaminated.
- Eppendorf tubes must be autoclaved before use.
- Use only barrier tips during your work.

Laboratory Process

Our laboratory works primarily with water, sediment, and soil environmental matrices. However, our procedures can be adapted to meet customer requirements.

The main steps on the process are described below:

1. Sample Receipt & Logging
2. Samples filtration (water only)
3. DNA Extraction (we use different kits depending on the matrix)
4. DNA quality (concentration and purity)
5. DNA electrophoresis (analysis for contaminants and degradation products)
6. qPCR (AIS custom primers) or Metabarcodes amplification (16S / 18S / ITS / COI) using qPCR Agilent 3500
7. Library Preparation (if sequencing)
8. Sequencing (Oxford technologies)
9. Bioinformatics & Taxonomic Assignment
10. Report Generation & Data Delivery

1) Sample Receipt & Logging

Environmental samples accepted and minimum requirements are indicated in the tables below.

Sample Matrix	Typical Applications	Accepted Formats	Minimum Amount Required	Notes
Water	Rivers, lakes, ponds, coastal waters, wastewater	Raw water in sterile bottles or pre-filtered membranes	250–1000 mL (unfiltered) OR 1–2 filter membranes (0.22–0.45 µm)	Larger volumes recommended for low-DNA environments
Sediment	Lake/riverbeds, estuarine/marine sediments	Wet or dry sediment in sterile tubes/Whirl-Paks	≥ 5 g (wet weight)	Ideal: 10–20 g for replicates & repeats
Soil	Forest/rangeland/agricultural soils	Surface/core soils in sterile containers	≥ 5 g	Avoid plant roots and macrofauna unless needed

1.2 Storage and transport requirements

Step	Recommended Conditions	Details
Short-term (<24 h)	4 °C on ice	Transport cooled in insulated container
Medium-term (<1 week)	4 °C for filters/sediments; –20 °C for water if unfiltered	Avoid thaw-freeze cycles
Long-term	–20 °C or –80 °C for all matrices	Filters may also be stored in DNA/RNA Shield, ethanol (95%), or Longmire's buffer
Containers (preferable)	Sterile 50 mL Falcon tubes, Whirl-Pak bags, cryotubes, or Nalgene bottles	No glass containers for freezing
Avoid	High temperatures, UV exposure, prolonged storage without preservative	

For sample submissions, the customer must complete the **Analysis Request Form (Appendix 1)**, providing all required customers and sample information to ensure accurate processing and traceability.

All customer and sample data are stored electronically and assigned **unique identifiers**. Customer details—including contact information, project references, and reporting preferences, are securely maintained and protected within the system.

The laboratory maintains strict **data integrity controls** to ensure all records are complete, accurate, and protected from unauthorized alteration. Data entry, processing, review, and reporting follow controlled workflows supported by audit trails, version control, and verification procedures.

Electronic records are safeguarded through **access-controlled systems**, user authentication, and role-based permissions. Routine backups and secure server infrastructure ensure the **protection, availability, and long-term preservation** of all client information. Data transmissions, including delivery of final results, are conducted through **secure, encrypted channels** in accordance with confidentiality and data protection standards.

Results are shared electronically according to the customer's reporting preferences and required formats.

Standard Protocols and Procedures

2. Environmental sample filtration

A Filtration apparatus MultiVac 610-MS-T, with 6-Branch Aluminum Manifold Vacuum Filtration System, AC 220V/50Hz is used to filter up to 500 mL of plankton samples preserved in 70% ethanol. Samples are passed through a 26 mm pore-size paper filter under vacuum pressure to collect any solid material or free DNA in the sample. During filtration, any residual from the sample bottle and the funnel are rinsed with regular tap water (the same used to prepare a negative extraction control sample).

1. Wearing gloves, remove the upper portion of the filtration funnel and place a 26 mm membrane filter on the bottom portion of each filter. Use clean forceps to remove filters from their box.
2. Ensure the filter covers the black filtration area.
3. Put back the filtration funnel. It has a magnet seal.
4. Check manifold valves are all in the vertical position (Closed)
5. Remove lids from funnels
6. Check the waste collection bottle is empty and there are no leaks on the drainage hose.
7. Turn on the vacuum pump.
8. Turn manifold valves to the horizontal position (Open)
9. Add a small amount of high-quality water to wet the filters.
10. Start adding samples, taking care not to overflow the funnel. Continue adding until all the sample bottles are emptied.
11. Rinse the bottle with high-quality water and add the rinse to the filtration funnel. Take care not to cross-contaminate samples if running more than one filter.
12. After filtration is complete, turn the manifold valves to the vertical position (Closed)
13. Turn off the vacuum pump and remove the upper portion of the apparatus.
14. Using two sets of sterile forceps, pick up the white filter membrane at opposite edges and roll the filter into a cylinder with the top side facing inward. Note: Do not tightly roll or fold the filter membrane.
15. Insert the filter into a **5 ml** PowerWater Bead Pro Tube.

16. Stored at -20 degrees Celsius until processing.
17. After each filtration, the funnels must be disinfected in a 10% bleach bath for at least ten minutes. After this time, filters must be rinsed with high- / distilled water and placed back onto the manifold ready for another filtration round.

3. DNA extraction (water)

DNA samples are extracted from the filters using a DNeasy PowerWater Kit (QIAGEN Cat. No. / ID: 14900-100-NF). The kit can isolate genomic DNA from filtered water samples, free from salts, metals, humic substances and other organic materials. The resulting DNA can be used in any downstream application, including qPCR, Sanger sequencing and next-generation sequencing. Viable DNA can be acquired from common filter membrane types, including 0.45 µm and 0.22 µm filter funnels. DNA is ready to use in a final 100 µl volume.

The DNeasy PowerWater Kit (cat. nos. 14900-50-NF and 14900-100-NF) can be stored at room temperature (15–25°C) until the expiry date printed on the box label. Further information DNeasy PowerWater Kit Handbook: www.qiagen.com/HB-2267 Safety Data Sheets: www.qiagen.com/safety

Notes Before starting Solution PW1 must be warmed to 55°C for 5–10 min to dissolve precipitates prior to use. Solution PW1 should be used while still warm.

If Solution PW3 has precipitated, heat to 55°C for 5–10 min to dissolve the precipitate. Shake to mix Solution PW4 before use.

Steps

1. Remove the 5 **ml** PowerWater Bead Pro Tube with the filter from the -20 C
2. Let it warm at room temperature
3. Add 1 ml of Solution PW1 to the PowerWater Bead Pro Tube. Note: For samples containing organisms that are difficult to lyse (e.g., fungi and algae) an additional heating step can be included. See Alternate Lysis Method in the Appendix and Troubleshooting Guide.
4. Secure the tube horizontally to a vortex adapter.
5. Vortex at maximum speed for 5 min. Centrifuge the tubes ≤4000 x g for 1 min at room temperature. (This centrifugation step is optional if a centrifuge with a 15 ml tube **rotor** is not available but will result in minor loss of supernatant). Heating can aid the lysis of some organisms (fungi, algae). After adding Solution PW1 (Step 5 of the protocol), heat the sample at 65°C for10 min. Resume protocol from step 6.
6. Transfer the supernatant to a clean 2 ml collection tube (provided). Draw up the supernatant using a 1 ml pipette tip by placing it down into the beads. Note: Placing the pipette tip down into the

beads is required. Pipette until you have removed all the supernatant. Expect to recover **600–650 µl** of supernatant.

7. Centrifuge at 13,000 x g for 1 min at room temperature.
8. Avoid the pellet, transfer the supernatant to a clean 2 ml collection tube (provided).
9. Add 200 µl of Solution IRS and vortex briefly to mix. **Incubate at 2–8°C for 5 min**. (this step requires an ice bath)
10. Centrifuge the tubes at 13,000 x g for 1 min.
11. Avoid the pellet, transfer the supernatant to a clean 2 ml collection tube (provided).
12. Add 650 µl of Solution PW3 and vortex briefly to mix.
13. Load 650 µl of supernatant onto an MB Spin Column. Centrifuge at 13,000 x g for 1 min. Discard the flow-through. Repeat until all the supernatant has been processed.
14. Place the MB Spin Column Filter into a clean 2 ml collection tube (provided).
15. Add 650 µl of Solution PW4 (shake before use). Centrifuge at 13,000 x g for 1 min.
16. Discard the flow-through and add 650 µl of ethanol (provided) and centrifuge at 13,000 x g for 1 min.
17. Discard the flow-through and centrifuge again at 13,000 x g for 2 min.
18. Place the MB Spin Column into a clean 2 ml collection tube (provided).
19. Add **100 µl of Solution EB** to the center of the white filter membrane.
20. Centrifuge at 13,000 x g for 1 min.
21. Discard the MB Spin Column. The DNA is now ready for downstream applications.
22. For long-term preservation, store samples at -20C

3.1 DNA extractions (sediments and soils)

We use the Quick-DNA™ Fecal/Soil Microbe Miniprep Kit DNA from fecal, soil, and microbial samples.

Rapid method for the isolation of inhibitor-free, PCR-quality DNA (up to 25 µg/prep) from microbes including Gram-positive and Gramnegative bacteria, fungi, algae, protozoa, etc. in fecal and soil samples in as little as 20 minutes.

The Quick-DNA™ Fecal/Soil Microbe Miniprep Kit1 is designed for the simple, rapid isolation of inhibitor-free, PCR-quality DNA from a variety of fecal (including humans, birds, rats, mice, cattle, etc.) and soil (including clay, sandy, silty, peaty, chalky, and loamy soils) samples. The kit can be used to successfully isolate DNA from tough-to-lyse Gram-positive and Gram-negative bacteria, fungi, algae, protozoa, etc. that inhabit fecal and soil samples. The procedure is easy and can be completed in as little as 15 minutes: fecal samples (≤150 mg each) or soil samples (≤250 mg each) are added directly to a ZR BashingBead™ Lysis Tube (0.1 & 0.5 mm)2 and rapidly and efficiently lysed by bead beating without the use of organic denaturants or proteinases. Zymo-Spin™ Technology is then used to isolate

the DNA, which is subsequently filtered to remove humic acids/polyphenols that inhibit PCR. The DNA is ideal for downstream molecular-based applications including PCR, arrays, genotyping, etc

Steps

1. Add ≤ 250 mg of soil sample to a ZR BashingBead™ Lysis Tube (0.1 & 0.5 mm). Add 750 µl BashingBead™ Buffer to the tubel.
2. Secure ZR BashingBead™ Lysis Tube (0.1 & 0.5 mm) in a bead beater fitted with a 2 ml tube holder assembly and process using optimized beat beating conditions (speed and time) for your device. Recommended: Bead beat at max speed for 40 minutes using the Vortex Genie® 2 (S5001) with the Horizontal Microtube Adapter (S5001-7).
3. Centrifuge the ZR BashingBead™ Lysis Tube (0.1 & 0.5 mm) in a microcentrifuge at ≥ 10,000 x g for 1 minute.
4. Transfer up to 400 µl supernatant to a Zymo-Spin™ III-F Filter in a Collection Tube and centrifuge at 8,000 x g for 1 minute.
5. Add 1,200 µl of Genomic Lysis Buffer to the filtrate in the Collection Tube from Step 4. Mix well.
6. Transfer 800 µl of the mixture from Step 5 to a Zymo-Spin™ IICR Column4 in a Collection Tube and centrifuge at ≥ 10,000 x g for 1 minute.
7. Discard the flow through from the Collection Tube and repeat Step 6.
8. Add 200 µl DNA Pre-Wash Buffer to the Zymo-Spin™ IICR Column in a new Collection Tube and centrifuge at ≥ 10,000 x g for 1 minute.
9. Add 500 µl g-DNA Wash Buffer to the Zymo-Spin™ IICR Column and centrifuge at ≥ 10,000 x g for 1 minute.
10. Transfer the Zymo-Spin™ IICR Column to a clean 1.5 ml microcentrifuge tube and add 100 µl (50 µl minimum) DNA Elution Buffer directly to the column matrix. Centrifuge at ≥ 10,000 x g for 30 seconds to elute the DNA5,6.
11. Place a Zymo-Spin™ III-HRC Filter7,8 in a clean Collection Tube and add 600 µl Prep Solution. Centrifuge at 8,000 x g for 3 minutes.
12. Transfer the eluted DNA to a prepared Zymo-Spin™ III-HRC Filter in a clean 1.5 ml microcentrifuge tube and centrifuge at exactly 16,000 x g for 3 minutes.

The filtered DNA is now suitable for PCR and other downstream applications

4. DNA quality concentration and purity

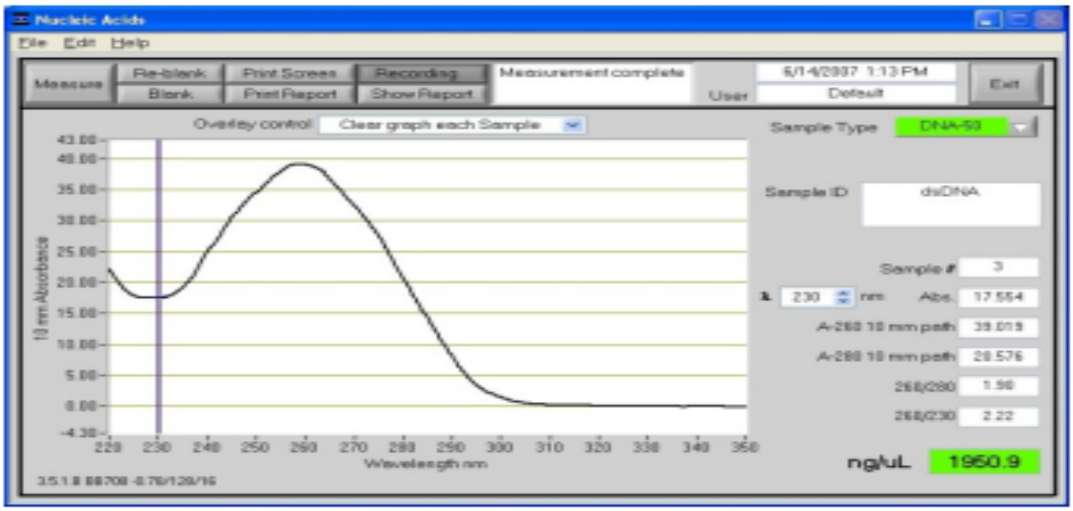
DNA solution concentration is measured using a NanoDrop ND-1000. The purity of the DNA solution is assessed by measuring the ratios A260/A280.

The NanoDrop ND-1000 is a full spectrum (220-750nm) spectrophotometer that provides a simple and robust instrument for quantification and evaluation of purity of samples, such as proteins and nucleic acids. The technology is based on a patented sample retention system which allows for the analysis of sample sizes as small as 0.5ul, without the need for awkward cuvettes or capillaries. In addition, there is no need for sample dilution in most cases and the high absorbance capability of the instrument is reported to be 50 times that of traditional spectrophotometers.

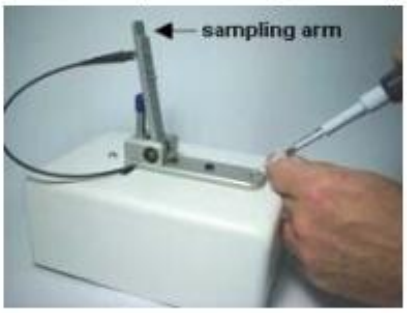
Procedure

1. Turn on the spectrophotometer at least 15 min before use to warm up the lamp.
2. Put on gloves
3. Remove samples from -20C (if they are in long term preservation)
4. Place samples on ice to thaw.
5. Open the NanoDrop application
6. In the Main Menu With the sampling arm in the down position, start the operating software by selecting the following path: Start Æ Programs Æ NanoDrop Æ ND-1000
7. Click on the Nucleic Acid option

8. The Nucleic Acid Module will show up



9. Check Sample Type is DNA-50 (this is the default mode and will measure double stranded DNA concentration)
10. With the sampling arm open, use an automatic pipette with a filter tip and load 1uL of a blank sample (**Use the solution EB from the extraction kit**) onto the lower measurement pedestal.



11. After loading the sample, lower the sampling arm into the ‘down’ position.
12. Click on the ‘Blank’ (F3) button.
13. When the measurement is complete, open the sampling arm and wipe the blanking buffer from both upper and lower pedestals using a laboratory wipe.
14. Analyze an aliquot of the blanking solution as though it were a sample. This is done using the ‘Measure’ button (F1). The result should be a spectrum with a relatively flat baseline. Wipe the blank from both measurement pedestal surfaces and repeat the process until the spectrum is flat.
15. To start measuring samples click on the Start Report / Recording option. The user can log measurement results in a report table and print them to the desired printer. To initiate this feature, select the ‘Start Report’ button. The default setting has the Recording feature activated. Refer to Data Viewer in Section 14 for additional details. Note: To override this feature, click on the ‘Recording’ button. Once de-selected, the button will read Start Report.
16. Type the sample name in the Sample ID box
17. With the sampling arm open, use an automatic pipette with a filter tip and load 1uL of the sample onto the lower measurement pedestal.
18. After loading the sample, lower the sampling arm into the ‘down’ position.

19. Click on the Measure button and wait for the measurement complete message shows on the main menu
20. When the measurement is complete, open the sampling arm and wipe the sample from both upper and lower pedestals using a **laboratory wipe.**
21. Repeat steps 15 to 19 for each replicate and sample you are going to measure.
22. After completing all the measurements, use the Print report option to save the results.
23. Close the arm and close the program. Do not turn off the computer if you are planning to use it the next day.
24. Return samples to -20°C

DNA yields will vary depending on the type of water, sample location, and time of year. Examples of expected yields are provided as a reference. Due to diversity of water sample types, yields may fall outside of the examples provided.

Table 1. Water sample types

Type of water sample	Sample volume (ml)	DNA yield (ng/µl)
Saltwater bay	100	40–72
Freshwater lake	100	15–25
Lagoon	20–100	3–38
Ocean coastal	100	3–11
Sewage influent	50	95
Treated effluent	50	18

A260/280 readings are one measure of DNA purity. For samples with low biomass, which would lead to low DNA yields (<20 ng/µl), this ratio may fall below 1.5. However, this ratio is not an indicator of amplification ability or DNA integrity. Ethanol precipitation followed by resuspension in a smaller volume to concentrate the DNA may help to improve the A260/230 ratio.

Concentration of DNA samples

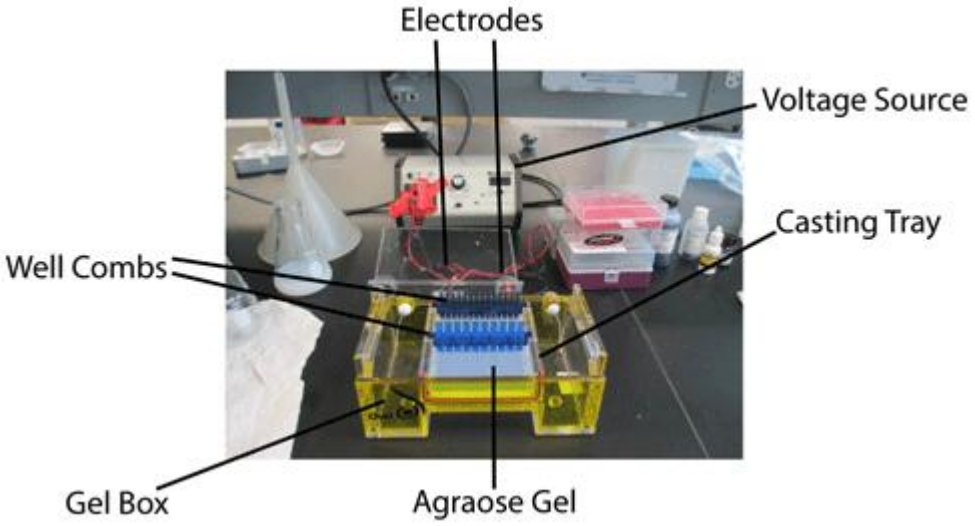
The final volume of eluted DNA will be 100 µl. The DNA may be concentrated by adding 5 µl of 3 M NaCl and inverting 3–5 times to mix. Next, add 200 µl of 100% cold ethanol and invert 3–5 times to mix. Centrifuge at 10,000 x g for 5 min at room temperature (15–25°C). Decant all liquid. Briefly dry residual ethanol in a speed vac or ambient air. Avoid over-drying the pellet or resuspension may be difficult. Resuspend precipitated DNA in the desired volume of 10 mM Tris (Solution EB).

5. DNA electrophoresis

Gel electrophoresis is the standard lab procedure for separating DNA by size (e.g., length in base pairs) for visualization and purification. Electrophoresis uses an electrical field to move the negatively charged

DNA through an agarose gel matrix toward a positive electrode. Shorter DNA fragments migrate through the gel more quickly than longer ones. Thus, you can determine the approximate length of a DNA fragment by running it on an agarose gel alongside a DNA ladder (a collection of DNA fragments of known lengths).

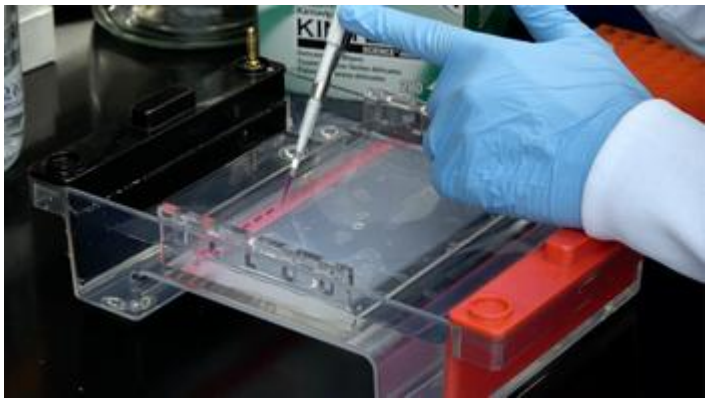
Pouring a Standard 1% Agarose Gel:

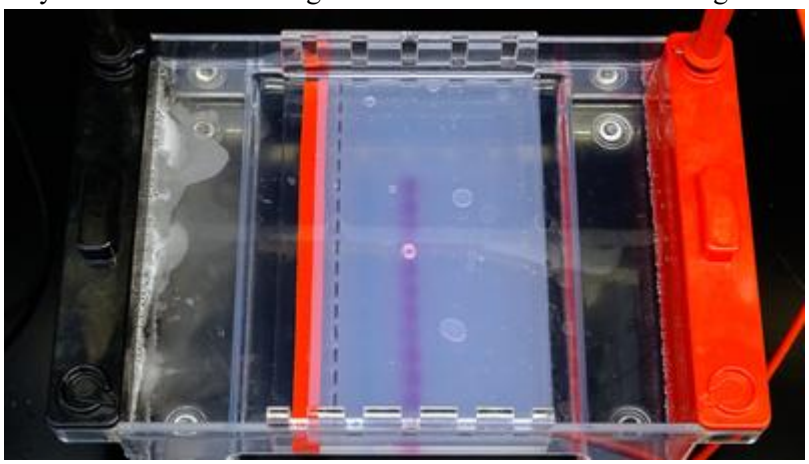
1. Measure 1 g of agarose.
   Pro-Tip
   Agarose gels are commonly used in concentrations of 0.7% to 2% depending on the size of bands needed to be separated. Simply adjust the mass of agarose in a given volume to make gels of other agarose concentrations (e.g., 2 g of agarose in 100 mL of TAE will make a 2% gel).
2. Mix agarose powder with 100 mL 1xTAE (or 1x TBE) in a microwavable flask.
3. 

4. TBE can be used instead of TAE, labs usually use one or the other, but there is very little difference between the two.
   Note: *Make sure to use the same buffer as the one in the gel box (do not mix different buffers and do not use water).*
5. Microwave for 1-3 min until the agarose is completely dissolved (but do not over boil the solution, as some of the buffer will evaporate and thus alter the final percentage of agarose in the gel. Many people prefer to microwave in pulses, swirling the flask occasionally as the solution heats up.).
   Caution
   HOT! Be careful stirring, eruptive boiling can occur.
6. Let agarose solution cool down to about 50 °C (about when you can comfortably keep your hand on the flask), for about 5 mins.
7. *Optional*: Add SYBR green or RedDye to the agarose solution to a final concentration of 0.2-0.5 µg/mL (usually about 2-3 µl of lab stock solution per 100 mL gel). Dyes bind to the DNA and allow you to visualize the DNA under ultraviolet (UV) light.
8. Pour the agarose into a gel tray with the well comb in place. Pour slowly to avoid bubbles which will disrupt the gel. Any bubbles can be pushed away from the well comb or towards the sides/edges of the gel with a pipette tip.

9. Place newly poured gel at 4 °C for 10-15 mins OR let sit at room temperature for 20-30 mins, until it has completely solidified. If you are in a hurry, the gel will set more quickly if you place the gel tray at 4 °C earlier so that it is already cold when the gel is poured into it.

Loading Samples and Running an Agarose Gel:



1. Add loading buffer to each of your DNA samples.
   Note: *Loading buffer serves two purposes: 1) it provides a visible dye that helps with gel loading and allows you to gauge how far the DNA has migrated; 2) it contains a high percentage of glycerol that increases the density of your DNA sample causing it settle to the bottom of the gel well, instead of diffusing in the buffer.*
2. Once solidified, place the agarose gel into the gel box (electrophoresis unit).
3. Fill gel box with 1xTAE (or TBE) until the gel is covered.
4. Carefully load a molecular weight ladder into the first lane of the gel.
5. 
6. ***Note:*** *When loading the sample in the well, maintain positive pressure on the sample to prevent bubbles or buffer from entering the tip. Place the very top of the tip of the pipette into the buffer just above the well. Very slowly and steadily, push the sample out and watch as the sample fills the well. After all of the sample is unloaded, push the pipettor to the second stop and carefully raise the pipette straight out of the buffer.*
7. Carefully load your samples into the additional wells of the gel.

8. Run the gel at 80-150 V until the dye line is approximately 75-80% of the way down the gel. A typical run time is about 1-1.5 hours, depending on the gel concentration and voltage.
   Note: *Black is negative, red is positive. The DNA is negatively charged and will run towards the positive electrode.* ***Always Run to Red.***
9. Turn OFF power, disconnect the electrodes from the power source, and then carefully remove the gel from the gel box.
10. Using any device that has UV light, visualize your DNA fragments. The fragments of DNA are usually referred to as 'bands' due to their appearance on the gel.
    Note: *When using UV light, protect your skin by wearing safety goggles or a face shield, gloves and a lab coat.*

Analyzing Your Gel:

Using the DNA ladder in the first lane as a guide (the manufacturer's instructions will tell you the size of each band), you can infer the size of the DNA in your sample lanes. For more details on doing diagnostic digests and how to interpret them, please see the Diagnostic Digest page.

Tips and FAQ

How do you get a better resolution of bands?

A few simple ways to increase the resolution (crispness) of your DNA bands include a) running the gel at a lower voltage for a longer period; b) using a wider/thinner gel comb; or c) loading less DNA into the well. Another method for visualizing very short DNA fragments is polyacrylamide gel electrophoresis (PAGE), which is typically used to separate 5 - 500 bp fragments.

How do you get better separation of bands?

If you have similar sized bands that are running too close together, you can adjust the agarose percentage of the gel to get better separation. A higher percentage agarose gel will help resolve smaller bands from each other, and a lower percentage gel will help separate larger bands.

10% Rule

For each sample you want to load on a gel, make 10% more volume than needed because several microliters can be lost in pipetting. For example, if you want to load 1.0 µg in 10 µL, make 1.1 µg in 11 µL.

5. Quantitative PCR (qPCR)

Quantitative PCR qPCR is done using FastStart SYBR Green Master and following the manufacturer's instructions.

FastStart™ SYBR® Green Master is a ready-to-use hot start reaction mix without ROX for quantitative polymerase chain reaction (qPCR) and reverse transcription (RT)-qPCR on real-time PCR systems other than the LightCycler® instruments. This master mix simplifies the preparation of reactions for DNA detection and analysis. In combination with a real-time PCR instrument, suitable PCR primers, and a hydrolysis probe, FastStart™ TaqMan® Probe Master allows very sensitive detection and quantification of defined DNA sequences.

Controls

To detect DNA contamination, always include a negative control in each run. To prepare this control, replace template DNA with water, PCR Grade.

Primers

All primers stocks are stored at -20 C at a final concentration of 100 uM in the TE buffer. Working primer combinations (forward and reverse primers) are stored in separated boxes at a final concentration 0.3 µM each.

Preparation of PCR Master Mix

1. Master mix are prepared inside the PCR cabinet
2. Before using the cabinet, clean the surface with 70% ethanol and turn on the UV light
3. Wait for 30 min, until the UV light turns off automatically.
4. Remove the PCR primer, eDNA samples, and SYBR Green Master mix reagents from the freezer
5. Thaw the solutions and, for maximum recovery of the contents, briefly spin vials in a microcentrifuge before opening.
6. Mix solutions carefully by pipetting them up and down, then store on ice

For each 50 µl reaction, prepare the following reaction mix:
1. Prepare enough master mix to run all samples in duplicate.
To prepare the PCR mix for more than one reaction, multiply the amounts in the “Volume” column by z, where z = the number of reactions to be run + one additional reaction.

a) Be sure to include duplicate No template Negative Controls (NTC) replacing a eDNA template with Molecular Biology Grade (MOBI) water.
b) Select the appropriate table below based on the qPCR reagent selected.

c) Calculate the amount of reagents to mix. Add 10% volume to allow for pipetting error.

d) Mix well, avoiding bubbles.

In a 1.5 ml reaction tube on ice, prepare the PCR mix for one 50 µl reaction by adding the following components in the order listed below:

Reagent	Volume[1] [50 µl reaction]	Final Conc.
FastStart Universal SYBR Green Master (ROX)	25 µl	1x
Forward Primer (30 µM)	0.5 µl	300 nM
Reverse Primer (30 µM)	0.5 µl	300 nM
Water, PCR Grade	19.0 µl	
Total Volume	45 µl	

2. Setup reactions:

1. Prepare the tubes or microplates for PCR (e.g., seal tubes with optical tube caps or the plate with self-adhesive foil).
2. For NTC reactions, add 5 µL of water to the reaction tube.
3. For experimental reactions, add 5 µL of cDNA solution to the reaction tube.
4. Centrifuge all tubes briefly. Visually confirm that all tubes or wells contain samples at the bottom at the correct volume.
5. Carefully aliquot 45 µL of template master mix into each qPCR tube or plate well.
6. Mix reactions well and spin if needed.
7. Cap tubes or seal the PCR plate and label (according to instrument requirements). (Make sure the labelling does not obscure the instrument excitation/detection light path.)
8. Run samples as per the instrument manufacturer's recommendations. Examples of standard and fast cycling have been included below.
9. Runs are completed using a Strategene 3005P qPCR thermocycler. Each run consisted of an activation step at 95°C for 10 min and 40 amplification cycles (95°C-15sec, 58°C-60°C-15sec, 72°C -30sec) followed by a dissociation profile to detect the target's dissociation temperature (Tm).

Cycles	Analysis Mode	Target Temperature	Hold Time	Remarks
1 (optional)	None	50°C	2 min	Only if UNG has been added for carryover prevention.
1	None	95°C	10 min	Activation of FastStart Taq DNA Polymerase
40	None	95°C	15 s (10 s[1])	Amplification and real-time analysis
	Quantification	primer-dependent (typically +58 to +60°C)	1 min (30 s[1])	

6. Metabarcoding of eDNA Samples Using Oxford MinION

To generate sequencing-ready libraries we follow the official instructions supplied with commercial kits or the Oxford Nanopore Technologies (ONT) library preparation kits and flow cells; those documents must always be followed alongside this procedure and are attached to the main laboratory binder.

The Oxford Nanopore MinION is a compact, portable DNA and RNA sequencer that enables real-time, long-read sequencing directly from your laptop. It’s ideal for fieldwork, rapid diagnostics, and decentralized genomics.

Key Features of the MinION

- Portable design: Weighs ~100 g and fits in your palm; connects via USB.
- Real-time sequencing: View data as it's generated, enabling rapid decision-making.
- Long-read capability: Reads from 20 bp to over 4 Mb, ideal for structural variant detection and genome assembly.
- Flexible throughput: Up to 48 Gb per flow cell depending on run conditions.
- Simple workflow: Library prep in as little as 10 minutes.
- Temperature control: Operates reliably across 10–35°C environments.

Models

MinION Mk1B: USB-powered, connected to a laptop for sequencing and data transfer.

Sequencing protocols

6.1 Amplicon sequencing from DNA using SQK-RBK114 (.24 or .96)

This is the fastest and simplest protocol to sequence amplicon DNA.

This protocol describes how to carry out rapid barcoding of amplicon DNA using the Rapid Barcoding Kit 24. (Nanopore store: Rapid Barcoding Kit 24 V14). The Rapid Barcoding Kit 24 V14 (SQK-RBK114.24) contains enough reagents for **six reactions**.

The protocol:

- Is for multiplexing up to 96 single species amplicon samples
- Is optimised for 500 bp to 5 kb amplicons
- Has a library preparation time of ~60 minutes
- Has a high yield
- Includes fragmentation
- Is compatible with R10.4.1 flow cells

6.2 Rapid sequencing DNA V14 - barcoding (SQK-RBK114.24 or SQK-RBK114.96)

This protocol describes how to carry out rapid barcoding of genomic DNA using the Rapid Barcoding Kit 24. (Nanopore store: Rapid Barcoding Kit 24 V14).

This protocol:

- Uses genomic DNA
- Offers multiplexing of 1-96 samples
- Has a library preparation time of ~60 minutes
- Has a high yield
- Includes fragmentation
- It is compatible with R10.4.1 flow cells

Appendix 1 Enviro Responsible - Analysis Request Form

Customer Information

Company/Organization: _____

Contact Person: _____

Position/Title: _____

Address: _____

Email: _____

Phone Number: _____

Purchase Order / Reference Number: _____

Sample Information

Sample Description / Matrix: _____

Geographic Location / Collection Site: _____

Date of Sampling: _____

Number of Samples Submitted: _____

Storage / Handling Requirements: _____

Requested Analyses

☐ PCR

☐ qPCR

☐ eDNA Analysis

Metabarcoding:

- ☐ COI (Animal Barcode)
- ☐ 16S (Bacterial/Prokaryotic Community Profiling)

☐ 18S (Eukaryotic Community Profiling)

☐ ITS (Fungal Barcode)

☐ AIS Identification: _____

☐ AIS Quantification: _____

☐ Other (specify): _____

Reporting Requirements

Preferred Report Format (PDF, Excel, etc.): _____

Delivery Method (Email, Secure Portal, Other): _____

Additional Notes or Instructions: _____

Data Protection & Security Acknowledgement

By submitting this form, the customer acknowledges that all information and materials will be handled according to the laboratory's data integrity, confidentiality, and security protocols.

Customer Signature: _____ Date: _____

Appendix 2 Metabarcoding groups

Community	Common marker	Typical amplicon (PGM-friendly)	Notes
Bacteria/Archaea	**16S rRNA V4** (515F/806R)	~250 bp	Gold standard for microbes; great for soil/sediment/water.
Eukaryotes (broad)	**18S rRNA V9** (1391f/EukBr)	~130 bp	Broad euk coverage; short (good for degraded eDNA).
Fungi	**ITS2** (fITS7/ITS4 or gITS7/ITS4)	~200–300 bp	Use UNITE db.
Metazoans (animals)	**COI** ("Leray" mlCOIintF/jgHCO2198)	~313 bp	Works with PGM **400-bp chemistry**; or pick a shorter COI mini-barcode (≈150–220 bp) for 200-bp chemistry.
Fish/vertebrates (water eDNA)	**12S MiFish**	~170 bp	Very popular for aquatic vertebrates.

Target Group	Primer Name	Gene Region	Forward Primer (5′→3′)	Reverse Primer (5′→3′)	Reference
Vertebrates	MiFish-U/E	12S rRNA	GTCGGTAAAACTCGTGCCAGC	CATAGTGGGGTATCTAATCCCAGTTTG	Miya et al. 2015
Invertebrates	mlCOIintF / HCO2198	COI	GGWACWGGWTGAACWGTWTAYCCYCC	TAAACTTCAGGGTGACCAAAAAATCA	Leray et al. 2013
Fungi	ITS1F / ITS2	ITS1	CTTGGTCATTTAGAGGAAGTAA	GCTGCGTTCTTCATCGATGC	Gardes & Bruns 1993
Plants	rbcL-aF / rbcL-aR	rbcL	ATGTCACCACAAACAGAGACTAAAGC	GTAAAATCAAGTCCACCRCG	Kress et al. 2009

Appendix 3. Metabarcoding primers

Overhanging primers

Forward: 5′ TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-[primer]

Reverse: 5′ GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-[primer]

PCR Amplification conditions

PCR mix: 25 µL reactions with 1× buffer, 0.2 µM primers, 1.25 U polymerase (e.g., Platinum Taq), and 2 µL DNA.

Thermocycling:

Initial denaturation: 95°C for 3 min

35 cycles: 95°C 30s, 50–55°C 30s, 72°C 45s

Final extension: 72°C for 5 min