Is it possible to automatically monitor organisms in Norwegian waters?

Et bilde som inneholder person, klær, innendørs, vindu

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Automatisk generert beskrivelse

Stockholm Junior Water Prize 2023

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# Preface and help received

First, we would like to thank the University of South-Eastern Norway (USN) for lending us the BioMEMS laboratories, which contained everything we needed to carry out this project. Thanks to Frank Karlsen (Professor of Micro- and Nanotechnology, USN), Lars E. Roseng (Associate Professor, USN), and Karoline Krogstad (Department Engineer and Laboratory Manager, USN) for good guidance, especially when we tested the new and unknown FORDETECT technology. We would also like to thank our teachers at Horten High School, Lars Kristian Asbjørnsen and Jan Kåre Trandem Qvam, who suggested we enter the "Norwegian Junior Water Prize", and then the "Stockholm Junior Water Prize".

# Summary

In 2019, the Norwegian Environment Agency requested the development of a new technology that detects eDNA in Norwegian rivers and waterways automatically (Miljødirektoratet, 2023). The request has been issued to gain insight into which species exist in the water, in order to regulate unwanted species and map original species. The revolutionary FORDETECT technology has been developed to contribute to this. The technology is Norwegian and has been developed by institutes, universities, and companies across Europe for almost 22 years. FORDETECT is a lab-on-chip technology that uses molecular biological methods to detect microscopic eDNA/eRNA residues in water samples. Our project aims to compare the automatic FORDETECT technology with two manual laboratory methods, PCR and LAMP. This was conducted by detecting eDNA from the highly endangered eel in water samples obtained from our local water, “Borrevannet”. The samples were filtered and extracted. After close examination of the results from PCR and LAMP, in addition to FORDETECT, we conclude that the FORDETECT technology can become much more efficient and economically sustainable than traditional manual laboratory methods. However, the technology is still in an early development phase, which means it has not yet reached its full potential. Nevertheless, we see the great advantages this technology can provide for monitoring biological activity, and strongly encourage further development of FORDETECT. In the near future, this technology can ensure and maintain a high quality of life for aquatic creatures all over the world. This is done by preventing the spread of unwanted and harmful organisms to a significant extent.

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# Purpose

The Norwegian Environment Agency has issued a request for new and modern technology that can automatically detect eDNA in Norwegian rivers and water, but also in the whole world. The purpose is, amongst others, to monitor waterborne harmful organisms. It is therefore critical to develop technology that can monitor and map the species that live there in order to preserve ecosystems and increase the quality of life of species in Norwegian waters. Horten Commune has drawn up clear targets for the long-term use and management of water bodies which will ensure that natural values are protected (Horten Kommune, 2022). For example, they want to preserve the eel Anguilla Anguilla, which is highly endangered (as of 2023) in Borrevannet.

USN is one of the participants in the Norwegian Environment Agency's project, and they have locally developed a lab-on-a-chip technology called FORDETECT. This technology can eventually be used to automatically monitor eDNA in water in a more efficient and less resource-intensive way than today's traditional manual methods. This project aims to compare manual and time-consuming molecular biological methods such as LAMP and PCR with the new automatic technology FORDETECT, to investigate whether it is realistic to automatically monitor eDNA in lakes and watercourses (Littlefair, et al., 2022). The flourishing of unwanted species can be detected faster and more sustainably with FORDETECT so that measures can be put in place to ensure effective regulation before it affects the original species in the water. The main hypothesis for this project is, therefore; can the FORDETECT technology contribute to an increase in quality of life for organisms in water?

# Equipments

## Filtration

* Electric pump with two hoses
* Deionized (DI) water
* Filter (Sterivex filter, Whatman filter paper or AcroCap)

## Manual eDNA extraction

* Stand for microcentrifuge tubes
* Magnetic stand
* Incubator
* 1.5 ml microcentrifuge tubes
* 1.5 ml RNase/DNase free tubes
* Micropipettes
* Sterile pipette tips with filter (volume: 50 – 1000 µL)
* Gloves
* Filtered water samples
* The kit «NUCLISENS magnetic Extraction Reagents (ref. 200293) » developed by Biomerieux in Lyon, France.

## Dilution of positive control

* Kit (VWR, Norway)
* Bio-Masher
* Lysis buffer

## PCR and LAMP

* PCR Kit (Eurogentec, Belgium)
* Warmstart LAMP Kit (Bio Nordica, Norway):
* RNase-free water

## FORDETECT

* FORDETECT hardware and software
* Lab-on-chip board
* The program: ASILOG (Qiagen, Germany)
* The program: FLDigital
* The program: DNA example tool (Mectro, Norway)

# Method

## Collection of water samples

Et bilde som inneholder kart

Automatisk generert beskrivelseThe water samples were collected from Borrevannet in Horten commune September 11, 2022. The containers, 1-1,5 L bottles, got cleaned with spring water before use.

The samples were collected from different locations in the following time (see figure 1):

* Stream (VD, 1): 13:00
* Dock (BH, 2): 14:20
* Reeds (SS, 3): 14:50
* Scout cabin (SH, 4): 15:10

When the containers got filled with water, they got marked with two letters and a number according to place and sample number. The samples were then transported to USN, where they were stored until filtering. This was to prevent eventual degradation of DNA. A total of 43 water samples were collected in this project.

Figure 1: topographic image of the Borrevannet, with the sampling locations marked with numbers. 1:VD, 2:BH, 3:SS, 4:SH. Photo: Statens kraftverk (NLOD)

## Filtering

**Water samples:**

The water samples taken from Borrevannet were filtered to extract the desired eDNA residues from the water. The filtration started with two water hoses cleaned with DI water and connected to an electric pump. At the end of one of the water hoses, a microfilter (< 1 micrometre; Sterivex, Whatman or AcroCap filter) was fitted and the other water hose was placed in a water sample. The pump was turned on and the water was slowly filtered through the filter. It took between 5min-20min per sample, depending on water quality and filter. When no more water came through the filter, and the pump wore out, the filtration of the sample was stopped. The filter was removed from the hose and marked with name, date, and filter number. Other useful data, such as time, amount of ml filtered and any problems experienced, were noted. The process was repeated for all water samples.

Sample No. 1 – 7:   
Filtered with Sterivex

Sample No. 8 – 18: Filtered with Whatman

Sample No. 19 – 43: Filtered with AcroCap

**Positive control - Buccal mucus and surface mucus from eel:**

To conclude whether the results from the water samples were positive or negative, positive controls were needed which we knew contained real eel DNA. For this, buccal mucus and surface mucus from a live Anguilla Anguilla were used under the Norwegian forest (Norske Skog ASA) approval system in Halden. 500µL of methanol was added to 1ml of sample material with surface mucus and buccal mucus. 1500 µL of mucus dissolved in methanol was injected into each RNase-free microcentrifuge tube and centrifuged for 2 minutes. Then 500 µL methanol was removed from the top layer of the liquid and as much lysis buffer as possible, approximately 1 mL, added. The positive controls were placed in a deep freezer (-148°C), as they were not to be extracted immediately.

**Positive Control - Tissue sample:**

In addition, two positive controls were made from eel tissue. A small piece (approx. a pinhead) of eel tissue was removed from a dead eel (after approval from the State Administrator in Vestfold) and placed in a tube belonging to a biomasher kit. The eel was collected and killed by NINA after approval from authorities. Subsequently, lysis buffer (approx. 1 mL) was added and the tissue sample was mashed using the kit. The samples were then extracted and placed in a deep freezer (-148°C).

## Ordering oligomers

To find the most suitable housekeeping gene in eels, we used the National Library of Medicine's PubMed® database and the Blastn application. The RNA polymerase sequences polr2j and polr1h were considered most prominent by the application as the eel's cells must have these RNA polymerase sequences to survive. We searched for "Anguilla Anguilla RNA polymerase I subunit H (polr1h), mRNA" and "Anguilla Anguilla RNA polymerase II subunit J (polr2j), mRNA" in the Nucleotide database MeSH, in NCBI. To find out which primer components should be combined, we used the application beacon design for PCR, and LAMP designer for LAMP. The fasta sequence for this limited part of the genome was copied into the application beacon design and LAMP designer. The result was proposals for primers for both PCR and LAMP. They also showed how they were built. These primers were given the abbreviated names PII (polr2j) and PIH (polr1h) and consisted of different oligomers. The oligomers were to be synthesized to be able to detect the RNA polymerase sequences PII and PIH. They were ordered from Eurofins through Professor Frank Karlsen, and on arrival diluted according to a predetermined protocol.

# Manual extraction of DNA/RNA

The kit used for the extraction of the samples was "NUCLISENS magnetic Extraction Reagents (ref 200292)” from Biomerieux in Lyon, France. However, before extraction could take place, the filters that were stored in the refrigerator had to first be thawed. Gloves were worn to avoid contamination. After the filters reached room temperature, 500 µL of lysis buffer was added to each filter. The filters were then placed on a bench to be incubated at room temperature for 5 minutes (see figure 2). After this, the lysed liquid was pumped out of the filters using a syringe and transferred into 1.5 ml microcentrifuge tubes. Each tube was assembled in a rack together with 50 µL silica magnetic beads from the kit (see figure 3). The tubes were incubated at room temperature for 2 minutes while gently shaken. The tubes were then placed in a magnetic stand and the lysed liquid was extracted from the tube with a micropipette with a corresponding pipette tip (see figure 4). The tubes were removed from the magnetic stand and 400 µL of wash buffer 1 was added. Wash buffer 1 was incubated for approximately 2 min, while the tube was gently shaken. The tubes were placed in the magnetic rack and wash buffer 1 was removed using a micropipette. The same process was carried out two more times, but now once with 500 µL wash buffer 2 and finally with 400 µL wash buffer 3. 100 µL elution buffer was added to each tube. The tubes with the elution buffer and magnetic beads with the offered genetic material were placed in an incubator at 65°C for 2 min (see figure 4). Then the tubes were placed in a magnetic rack for the last time. The elution buffer with genetic material was removed from the tubes and transferred to freshly prepared RNase-free tubes. Half of the fully extracted liquid was stored in a freezer (-147°C) for later experiments.

Figure 3: magnetic beads, Photo: Private

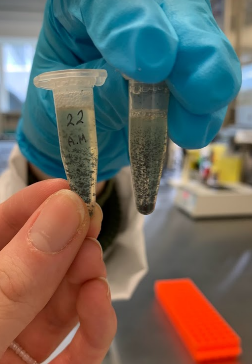


Figure 4: Incubator, Photo: Private

Figure 2: addition of lysis buffer, Photo: Private



Figure4: magnetic stand, Photo: Private

## Mastermix for LAMP and PCR

The same procedure was followed for both LAMP and PCR. First, a calculation was made of how many samples were to be tested. At the same time, an outline was set up in the software StepOne™ of which wells should contain what. A total of 52 samples were to be tested. Mastermix was therefore made for 55 reactions, to have some extra for pipetting wastage. To mix the Mastermix components, all the necessary reagents were thawed while the rest were put on dry ice. This was to avoid degradation of eDNA before it was desired. Mastermix components were mixed in a RNase-free tube according to the tables (see figures 5 and 6). 24 µL of Mastermix was added to the wells marked in Step One and 1 µL of extracted DNA from each sample was added to each well. In the negative extraction control, 1 µL of RNase-free water was added, and in the negative control, 25 µL of RNase-free water was added. Between each time a sample was pipetted over to its place in the wells, the other samples were covered with paper to avoid contamination. Finally, the entire board was covered with parafilm and placed in the StepOnePlus™ machine. When using the StepOne™ software, it was chosen to run either PCR or LAMP as the target.

|  |  |  |  |
| --- | --- | --- | --- |
| Components | 25µL reaction | 10x reaction | 55x reaction |
| WarmStart LAMP 2X Master Mix | 12,50µL | 125,0µL | 687,5µL |
| LAMP Primer AA PII | 2,500µL | 25,00µL | 137,5µL |
| LAMP fluorescent dye | 0,500µL | 5,000µL | 27,50µL |
| Extracted DNA | 1,000µL | 10 \* 1,000µL | 55 \* 1,000µL |
| RNase-free H2O | 8,500µL | 85,00µL | 467,5µL |
| Total: | 25,00µL | 250,0µL | 1375µL |

Figure 5: Table for mixing ratios for Mastermix, LAMP

|  |  |  |  |
| --- | --- | --- | --- |
| Components | 25µL reaction | 10x reaction | 55x reaction |
| Takyon™ ROX Probe 2X Mastermix | 10,00µL | 100,0µL | 550,0µL |
| *Anguilla Anguilla* A PCR primer | 4,000µL | 40,00µL | 137,5µL |
| PCR fluorescent dye | 0,500µL | 5,000µL | 27,50µL |
| Extracted DNA | 2,500µL | 10 \* 2,500µL | 55 \* 2,500µL |
| RNase-free H2O | 10,50µL | 105,0µL | 577,5µL |
| Total: | 25,00µL | 250,0µL | 1375µL |

Figure 6: Table for mixing ratios for Mastermix, PCR

Detection of Anguilla Anguilla with FORDETECT

“Borrevann” sample number 21 was taken out of the freezer (-148°C) and thawed to room temperature as it showed the earliest growth in the LAMP test. At the same time, all the units on the FORDETECT instrument were calibrated to the LOC cassette to be used with the program "DNA example tool". All the necessary auto scripts were obtained from the task manager in the same program. The FORDETECT cassette was removed from the instrument and 50 µL silica beads were added to the EX-chamber. When this was done, the lid of the EX-chamber was screwed on tightly and ensured that the chamber was tight. In addition, 2.5 µL primer mix and 0.5 µL fluorescent dye (from the LAMP kit) were added to each of the 8 micro reaction chambers in the cassette and incubated. The entire micro-RX area was then covered with PCR film. The cassette was then placed back into the instrument to test each valve individually. When the filtered and lysed sample was thawed, 500 µL of it was added to V4. In addition, 400 µL wash buffer 1 was added to V, 400 µL wash buffer 2 to V2, 400 µL wash buffer 3 to V3 and 150 µL LAMP WarmStart elution buffer to V8.

Only after all the preparations had been completed did the actual extraction of the sample begin. This was done by running pre-made auto scripts that move the liquid back and forth between desired chambers in the FORDETECT cassette. Finally, the eluted water sample was transferred to the reaction chamber RX. Here, one of the eight reaction chambers was selected with the most even distribution of the sample (RX7 – FORDETECT cassette). The detector on the FORDETECT instrument, ESElog, which detects changes in fluorescent dye was therefore calibrated to stay above chamber RX7. The FLDigital software was started, and raw data was collected. The curves were presented in FLDigital’s digital spreadsheet.

# Results and observations

The samples are named with the letters B, P and N, which stand for “Borrevann” sample (B), positive control (P), and negative control (N). In addition, they have a corresponding sample number. The tables are divided by filter type and for negative and positive controls.

## LAMP results

**Sample 1-7, filtered with Sterivex:**

Et bilde som inneholder line, Plottdiagram, kvittering

Automatisk generert beskrivelse

B4

B2

B7

B6

B5

B3

B1

The most clearly positive sample filtered with Sterivex is B7 as one can observe an early increase rate at around Ct-50. This means that the eel Anguilla Anguilla has been detected in Borrevannet even though it was not seen while this project was being carried out. B5 and B6 also showed a clear positive increase, but not as early as B7. B1 and B3 have an even later increase above CT-60, which means they are very likely to be negative. B2 and B4 are clearly negative.

**Sample 8-18, filtered with WhatMan:**

Et bilde som inneholder line, Plottdiagram, skjermbilde

Automatisk generert beskrivelse

B15

B11

B13

B16

B18

B8

B9

B10

B12

B14

B17

In samples 8 to 18 filtered with WhatMan, there are far more positive samples with an early increase before Ct-60 (samples B8, B9, B10, B11, B13, B14, B16, and B17). Samples B12, B15 and B17, on the other hand, are clearly negative.

**Sample 19-24, filtered with AcroCap:**

Et bilde som inneholder line, Plottdiagram, Font

Automatisk generert beskrivelse

B22

B21

B19

B23

B24

B20

The rest of the samples (19-43) were filtered with the AcroCap filter, where only the first 6 samples have graphs after some errors with the StepOnePlus™ machine. Only B21 and B22 are clearly positive with a positive increase before Ct-50, while B19, B20, B23, and B24 are clearly negative.

**Positive controls 1-3:**

Et bilde som inneholder line, Plottdiagram, tekst, kvittering

Automatisk generert beskrivelse

P1

P2

P3

All the positive controls experienced a certain rise, but only P2 can be considered clearly positive, with a distinct increase before Ct-60.

**Negative controls 1-4:**

Et bilde som inneholder tekst, line, kvittering, Plottdiagram

Automatisk generert beskrivelse

N3

N4

N1

N2

All negative controls are clearly negative.

## PCR results

Et bilde som inneholder tekst

Automatisk generert beskrivelseThe results of the PCR analysis showed that the primers that were made did not work. As a result, the graphs did not show any sensible curves; for example, the graph had a very low CT value, which does not match what a typical PCR graph looks like. Therefore, the results cannot be used for further analysis and discussion.

Figure 7: PCR results, Photo: Private

## Graphical user interface, application Description automatically generatedFORDETECT results

Our task was to demonstrate that the FORDETECT technology worked. We ran the lysed sample after filtration from Borrevannet (B21). This water sample had been tested in the laboratory in advance to be positive for eel. A successful test with the automatic functions on the FORDETECT instrument and cassette was carried out including purification of RNA/DNA and automatic amplification and detection. Figure 8 shows the successful automatic detection of Anguilla Anguilla RNA polymerase. From a background of 700 photons to a total result of 1000, the figure illustrates the start of a typical positive result.

Figure 8: FORDETECT results, Photo: Private

# Discussion

## LAMP

Samples 1-7 were the first samples filtered and dissolved in a lysis buffer. It is also with these samples that one can observe several increases after Ct-60. This can be explained by the fact that when a sample becomes positive after Ct-60, there may be an increased probability of errors and possibly the formation of a false positive or negative primer dimer. It is also possible that the samples contained eDNA, but that the high Ct value is due to degradation of the genetic material. This may be because the first seven samples were kept in a freezer with a lysis buffer for a long time (91 days, September16 – December 16, 2022) before being tested with LAMP. In the long period between extraction and testing, eDNA degradation may have occurred. This is especially true as they have been thawed to room temperature several times. With FORDETECT, which in the future may automatically extract samples, such waiting periods can be avoided. This ensures clear results.

Samples 8-18 and 19-25 showed more positive results relatively early. It may come from the shorter time between extraction and LAMP running, compared to samples 1-7.

**Other LAMP tests:**

It is pertinent to mention that several LAMP tests were carried out, but not all had usable results. A recurring problem was that all samples were positive, as well as both of the negative controls, which made it impossible to draw any conclusions. The main theory why the results were like this at the time was that the samples were not sufficiently covered during pipetting. Which led to aerosols with eDNA contaminating the samples along the way. It is also likely that the RNase-free water added to each sample was already contaminated.

# FORDETECT

FORDETECT is a prototype technology that has not yet been standardized. One of the challenges was getting the cassette valves to work every time. Another challenge was to ensure that all reaction chambers were filled consistently. This led to a repair period through the autumn and winter of 2022. The cassette we were going to use was also not ready from Germany or the USN before Christmas. Therefore, we only had the opportunity to test one sample with FORDETECT (sample B21), which gives a generally poor basis for comparison with the manual amplification methods PCR and LAMP.

Fortunately, the testing of sample B21 went almost flawlessly. From the amount of data collected during the testing, the graph shows the start of a positive sample. One of the main problems with the test was the Rx chamber temperature. As we know, both LAMP and PCR require a high temperature to successfully detect the gene sequence we want. At high temperatures, on the other hand, liquids can evaporate, and this happened with sample B21 so that it did not rise above 1000. As you can see on the graph for sample B21, the rise is abruptly interrupted after only a few minutes. This is because the liquid in the chamber evaporated. Nevertheless, the ESElog detector managed to record the breaking point between the relative photon value of 700 and the relative photon value of 1000. This is the breaking point that we should have to register positive samples. When the FORDETECT cassette is completely standardized and produced in its entirety with injection moulding and not including milled areas, evaporation will not occur. This is because injection moulding supports laser bonding and makes the entire reaction area tight.

# Conclusion

In this project, we have detected eDNA/eRNA from eels in water using both manual molecular biological methods such as LAMP and PCR, and the prototype for the revolutionary and automatic lab-on-chip FORDETECT technology. It was not possible for us to compare FORDETECT well enough against the traditional amplification methods used in the laboratory with the StepOnePlus™ machine. This is because there were not enough cassettes ready for the automatic FORDETECT machine. FORDETECT will be trial produced and prepared for real validation in the coming months. Therefore, we clearly see the opportunities this technology represents for society when it is completed as a product. In this project, we have used today's most accurate and efficient methods for detecting DNA and eDNA and believe that this will ensure that we preserve the quality of life of species in water all over the world. Being able to place machines that can automatically search for species or pests in water can both give us more understanding of the ecosystem, but also what measures may need to be taken to preserve the quality of life. During the project, we also learned that although the manual methods for detecting eDNA give accurate results, it takes time before the sample is taken until it is transported to the lab and then examined manually there. This means that when FORDETECT has been fully developed, it can be used in the field for automatic monitoring of watercourses "on-site in real-time". This will be far more financially sustainable compared to the use of manual methods such as PCR and LAMP where results are available after a week. This is a significant reason for using FORDETECT rather than the manual amplification methods.

FORDETECT has a high potential within several aspects of life, especially environmental monitoring. We are looking forward to following the development of this innovative technology along with the societal, economic, and environmental benefits this technology will have for the future.

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