MNF-BiOc-101: Practical Course/ Lectures in Biological Oceanography

Molecular Microbial Ecology
Oct 20-30th, 2025

Supervisors: Kristina Bayer, Jan Muschiol, Jutta Wiese, David K. Ngugi, Kerstin Petersen, Cordula Meyer, Osvaldina Fernandes Soares, Tanja Rahn, Andrea Hethke, Ute Hentschel Humeida

General safety lab rules

You have to consider and strictly follow the following safety procedures:

Safety procedure

You have to wear lab coats inside the lab, while working wear gloves and safety googles, if necessary.

Inform yourself about the next safety devices like fire-extinguisher, emergency showers, eye showers and first-aid kits.

Injuries have to be reported to the advisor immediately.

Never pipette by mouth!

When you eat and drink, please keep away from working space to avoid contamination.

Close tip boxes after use to avoid contamination.

Special care needs to take when working with flammable solutions (EtOH) and fire. Follow the supervisor's instructions strictly.

You've to disinfect your bench before and after work (70% EtOH).

Centrifuges must become cleaned and disinfect everyday (70% EtOH).

It's not allowed to centrifuge open vessels (danger of aerosols).

Before you leave the lab you've to disinfect your hands first, wash and then you may apply a hand cream.

In any case of questions arising, please ask the advisor.

4 groups of 4 or 5 students each

Date	Responsible Person/s	Location	groups	time	Contents
Mon 10/20	J. Muschiol J. Wiese T. Rahn A. Hethke K. Bayer	5-0.319	all	8-12am 2-4pm	•Introduction to the topic •Sampling (exp 1.1) •Filtration for DNA extraction (exp. 1.2) •Fixation (exp 1.3) •Plating (exp 3.1), fun plates
Tue 10/21	J. Muschiol K. Petersen O. Soares T. Rahn A. Hethke K. Bayer	5-0.319	Gr 1 + 2 Gr 3 + 4	8-12am	•DNA Extraction (exp 2.1) •Counting DAPI (exp 3.2) •Flow cytometry (exp 3.3)
	U. Hentschel	5-1.206	all	2-4pm	•Lecture: Microbial diversity I
Wed 10/16					
Thu 10/23	J. Muschiol K. Petersen O. Soares T. Rahn A. Hethke K. Bayer	5-0.319	Gr 1 + 2 Gr 3 + 4	8-12am	•Counting DAPI (exp 3.2) •Flow cytometry (exp 3.3) •DNA Extraction (exp 2.1)
	J. Muschiol	5-1.214	all	2-4pm	•Lecture: Molecular methods
Fri 10/24	J. Muschiol T. Rahn A. Hethke K. Bayer	5-0.319	all	8-12pm	•PCR and gel electrophoresis (exp 2.2)
	D. Needham	5-1.213	all	2-4pm	•Lecture: Microbial diversity II
Mon 10/27	David Ngugi A. Hethke	5-0.319	all	8-12am	•Introduction to next gen sequencing and Nanopore Sequencing •Library preparation and loading (exp 4)
	D. Ngugi	5-1.214	all	2-4pm	•Lecture: Composition analysis
Tue 10/28	J. Wiese T. Rahn A. Hethke K. Bayer	5-0.319	all	8-12am	•counting culturable bacteria (exp 3.1) •light microscopy •fun plates •summarize cultured vs. uncultured
	D. Needham	5-1.211	all	2-4pm	•Lecture: Viruses
Wed 10/29					
Thu 10/30	David Ngugi E. Borchert	5-3.228 - Terminal-room	all	8-12am	•Results and analysis Nanopore sequencing
	Bayer, Ngugi, Wiese	5-1.206	all	2-4pm	•Final discussion
Fri 10/31	Public holiday				

The “Great Plate Count Anomaly”

The phenomenon of the “Great Plate Count Anomaly” (Amann et al, 1995) describe the finding, that only approximately 1% out of the bacteria occurring in the environment are cultivable in the laboratory using common cultivation conditions and standard media. Especially the investigation of samples from marine habitats such as deep-sea habitats or macroorganisms, e.g. sponges, algae or corals, provides a rich resource of unknown bacteria.

The survival of microorganisms in the laboratory, as well as in nature, depends on their ability to grow under certain biological, chemical, and physical conditions. An understanding of these conditions enables us to obtain bacterial isolated, to characterize these isolates, and to differentiate between different types of bacteria. Media used in the laboratory for the cultivation of bacteria must supply all of the necessary nutrients required for cellular growth and maintenance of the organisms. A wide variety of culture media is employed by the bacteriologist for the isolation, growth, and maintenance of pure cultures as well as for the identification of bacteria according to their biochemical and physiological properties. A culture medium must supply suitable carbon and energy sources and other nutrients, sometimes including growth factors. It is important to note that one medium never supports the growth of all microorganisms. Accordingly, the elements required for the maintenance, growth, and reproduction of all organisms will be used by different organisms in different ways.

The determination of the “total” number of bacteria in environmental samples is performed with DAPI. It has to be emphasized that the method, how the cells were obtained from the sample, has a strong influence on the results. Furthermore, all cells are counted and is not possible to distinguish between living and dead cells.

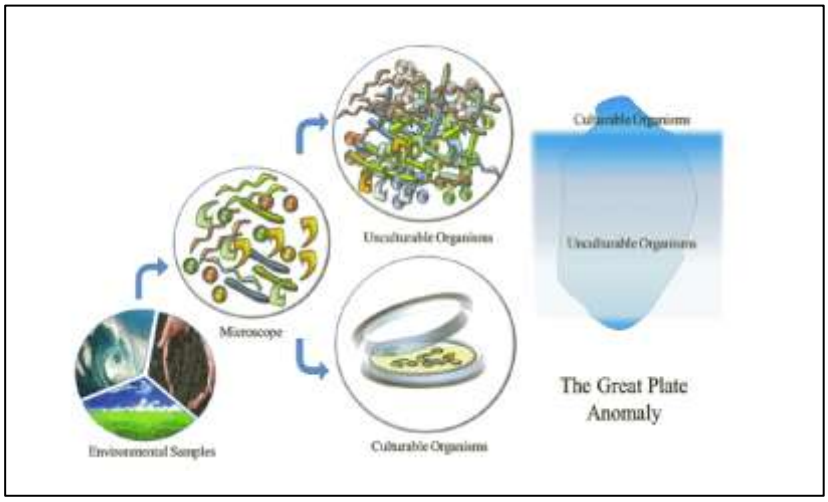


Figure 1: The great plate count anomaly describes the disparity between viable plate counts an direct microscopical evidence in samples from various habitats. (modified after Rajeev et al (2021)

The “Great Plate Count Anomaly” will be demonstrated on bacteria from sea water samples, i.e. the Kiel Fjord and the Schwentine River, by applying two different methods, a cultivation-dependent (growth experiments on various media differing in nutrient conditions) and a cultivation-independent approach (microscopic studies to determine the total number of bacterial cells using DAPI staining as well as flow cytometry). In addition to the determination of the number of the colony forming units (CFUs) and total bacterial counts, the number of bacteria showing an antibiotic activity against a test strain will be estimated.

References

Amann, R.I., Ludwig, W., & Schleifer, K.H. (1995). Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiology Reviews, 59:143-169.

Rodrigues, C. J., & de Carvalho, C. C. (2022). Cultivating marine bacteria under laboratory conditions: Overcoming the "unculturable" dogma. Frontiers in bioengineering and biotechnology, 10, 964589.

Isabel Sanz-Sáez, et al (2023), Top abundant deep ocean heterotrophic bacteria can be retrieved by cultivation, ISME Communications, Volume 3, Issue 1 92, https://doi.org/10.1038/s43705-023-00290-0

1.1 Sampling

During the practical course we want to compare the microbes from marine and estuary waters. Therefore, you are going to sample sea water from the Kiel fjord and river water from the Schwentine river. To sample from the Kiel fjord, you will be provided with sampling bottles already on Monday. Ideally you take the fjord samples on Tuesday morning before the practical starts. The Schwentine river samples will be taken on Tuesday morning during the course at the Wellingdorf ferry stop close to GEOMAR using a bucket.

CAUTION: Please be careful when sampling and make sure not to fall into the water. Ask a fellow student to join you in case something happens.

Sampling procedure

1. Flush the sampling device 3x with the water to be sampled. Fill the bottles to the top.
2. Take the sample by filling the bottles to the top leaving no air inside and close using the provided lid.

Are there other parameters you could/should record (metadata)?

Materials

Sterilized bottles
Bucket
Marker

1.2 Sample processing

➢ Filtration for DNA extraction (2.1)

One liter of seawater will be filtered using a specific filter unit. Genomic DNA will be extracted from these filters which will be stored in -80°C until usage. Listen to the supervisor how to handle the filter unit and prepare the filter for all three water samples per group.

Materials

- filtration unit
- filter (Supor-200; 0.2µm pore size, 47 mm, membrane filter, Pall 60300)
- plastic measuring cylinders, 1 L, pre-washed with 3.7 % HCl
- Eppendorf tubes, 1.6 mL
- forceps
- Ethanol-solution, 70 Vol.-% for cleaning

➢ Fixation for DAPI staining (3.2) and Flow Cytometry (3.3)

Materials

Glutaraldehyde (GA, 2.5%) **Attention TOXIC**
Falcon tubes (15 ml tubes and 5 ml cryo vials)
Pipettes and tips

Immediately after sampling the samples are fixed. As fixative glutaraldehyde, 2,5% is used, whereby a final concentration of 0.25% is aimed for (therefore add 1vol GA to 9 vol water sample). After 20 minutes incubation time in the dark, at room temperature, the fixed samples are flash frozen in liquid nitrogen and then stored at -80°C until analysis.

Note: You need to prepare fixation for both downstream applications

2.1 DNA extraction, Quality control (NanoDrop), PCR and Gel electrophoresis

DNA extraction from Filter protocol

General remarks before you start: **Try to avoid any possible contamination!**
Clean the surface/ instruments you're using with ethanol, 70 Vol.-%.
Wear gloves at all times.
Leave space in between samples.

The goal of DNA extraction is to break the DNA out of the cells with as little damage to the DNA as possible, then isolate the DNA from the rest of the broken-up cells.
There are various DNA extraction protocols. The protocol we are going to use in this practical is based on the Qiagen DNeasy Plant kit, which has been extended by a mechanical disruption step with small glass beads and a protein digestion step.

Due to lack of time during this practical we have shortened the protein digestion step from 1h to 15min (step 12).

Materials

- chemicals:
  - Qiagen extraction kit: DNeasy Plant Mini Kit (50) (Qiagen 69104)
  - Qiagen Proteinase K 10 mL (Qiagen 19133)
  - TE-buffer: TE buffer (1x) pH 8.0 for molecular biology, 1 L
  - Ethanol (96-100%)
- instruments:
  - Thermo Scientific Precision universal water bath 5L
  - Biospec Mini-Beadbeater-24
  - Heathrow Scientific Sprout mini-centrifuge
  - *Eppendorf Centrifuge 5424R, cooled, Rotor FA-45-24-11*
  - rotating incubator: Thermo Scientific Shake ′n′ stack Hybridisation oven
  - Thermo Scientific heating block plus block
  - Scientific Industries Vortex-Genie 2
  - ice machine
  - full liquid nitrogen container
  - fridge
  - -80°C freezer + 2"- cryo boxes

- further required materials:
    - pipettes and pipette tips
    - serological pipettes 50 mL
    - racks for water bath, for 2 mL tubes
    - microcentrifuge tubes (1.5 mL)
    - Lab soakers versi Dry
    - BD needle 21 Gauge 1,5 inch
    - whirl paks: Whirl-Pak sample bags 75x185 mm

Protocol

Preparation

1. Mix two sizes of glass beads (0.1 and 0.5 mm) in a 1:1 ratio in a 60 ml syringe, aliquot roughly 100 µl into 2 ml screw cap tubes and autoclave.

*Note: keep the cap loose while autoclaving

2. Turn on water bath, set temp to 65°C.
3. Turn on incubator, set temp to 55°C
4. Before step 23 (in DNeasy kit) pre-heat TE buffer to 65 °C in water bath or heat block.
5. Set up an ice bucket.

Freeze Fracture

6. Fill the liquid nitrogen dewar
7. Add 400 µl AP1 (from Qiagen kit) to your sample filter and transfer it into a 2 ml screw cap tube with glass beads.
8. Add 400 µl AP1 (from Qiagen kit). Incubate tubes in liquid nitrogen until frozen solid and then transfer to water bath (65°C). Repeat this freeze-thaw step 3 times.

*Note: make sure the top/cap of the tubes is NOT immersed in the water bath by arraying the tubes in a foam floater and transferring the entire floater back and forth between nitrogen and water bath. This helps prevent sample contamination.

Bead Beat

9. Transfer tubes directly from the last thaw step to bead beater. Bead beat samples for 2 min.

*Note: blot off water from the tubes carefully with a kim wipe.

*Note: Make sure tube holder is tightly screwed onto the bead beater and machine doesn't move off the bench due to vibration.

10. Remove from bead beater and pulse-centrifuge tubes to reduce foam.

Proteinase-K Treatment

11. Add 45 µl of proteinase-K to each tube and invert to mix well. Put tubes in a whirlpack or zip lock bag.
12. Put bags into incubator (55°C) and incubate for 15 minutes.

Rest of (Modified) Qiagen Plant kit protocol

13. Remove tubes from incubator. Add 4 µl of RNase A stock solution (Qiagen) to each tube and vortex vigorously.
14. Incubate tubes at 65°C on the heating block for 10 min. Vortex tubes 2- or 3-times during incubation.
15. With sterile needles or tips remove filters

*Note: Try to squeeze liquid out of the filters as much as possible. Needles need to go into a yellow sharps box.

16. Add 130 µl P3 to lysate and vortex. Incubate tubes on ice for 10 min.
17. Centrifuge at max. speed for 5 min. to pellet precipitates and beads.
18. Apply supernatant from above to QIAshredder Mini Spin Column (purple) placed in a 2 ml collection tube and centrifuge at max. speed for 2 min.
19. Transfer flow-through from above to new 2 ml tube. If a debris pellet has formed, avoid disturbing it while transferring. Steps 17 and 18 may need to be done twice if volume from step 16 is larger than 650 µl. Flow-through from repeat should be added to same 2 ml tube as the flow-through from the first spin.
20. Add 1.5 volumes of Buffer AW1 mix (as outlined in Qiagen kit) to the cleared lysate. Mix by pipetting.

*Note: be sure ethanol has been added to the buffer before adding to lysate!

21. Apply 650 µl of the mixture from above to DNeasy Mini Spin (white) column sitting in a 2 ml collection tube. Centrifuge for 1 min. at 6,000 x g. Discard flow-through. Repeat with remaining sample using same collection tube until all sample has been run through the column
22. Place DNeasy spin column in a new collection tube from kit, and add 500 µl of Buffer AW2 to the DNeasy Spin Column and centrifuge 1 min. at 6,000 x g. Discard flow-through and reuse collection tube in next step.
23. Add 500 µl Buffer AW2 to the DNeasy Spin Column and centrifuge 2 min. at max. speed to dry the membrane.
24. Transfer the spin column to a sterile 1.5 ml microcentrifuge tube, being careful not to allow bottom of column to come in contact with liquid in collection tube. Apply 25 µl of pre-heated TE directly to the DNeasy Spin Column membrane. Incubate at room temp for 5 min.
    Centrifuge for 5 min. at 6,000 x g to elute. Repeat with a second 25 µl aliquot of TE so that final total volume is 50 µl.
25. Aliquot and store DNA extract at -80°C.

Quality control using the NanoDrop: The NanoDrop is a spectrophotometer that gives you quantitative (concentration) and qualitative (purity) information about your extracted DNA.

principle of measurement: The absorbance of the sample at different wavelengths is measured.

DNA and RNA absorb light at 260 nm. Main contaminants are organic or carbohydrate compounds (230 nm), phenols (270 nm) and proteins/enzymes (280 nm).

To note the purity of the nucleic acid the 260 nm/280 nm and the 260 nm/230 nm ratio are determined:

260/280 Ratio Sample Consistency	260/230 Ratio Sample Consistency
1.3 < 50 % contaminants	< 2.0 Carbohydrate, Phenol, Guanidine, Glycogen contaminants
1.5 50 % nucleic acid & 50 % contaminants	
1.8 pure DNA	**2.0 - 2.2 pure nucleic acid**
2 pure RNA / Phenol contamination	> 2.2 bad blank or wrong blank solution

It's a direct but non-specific measurement.

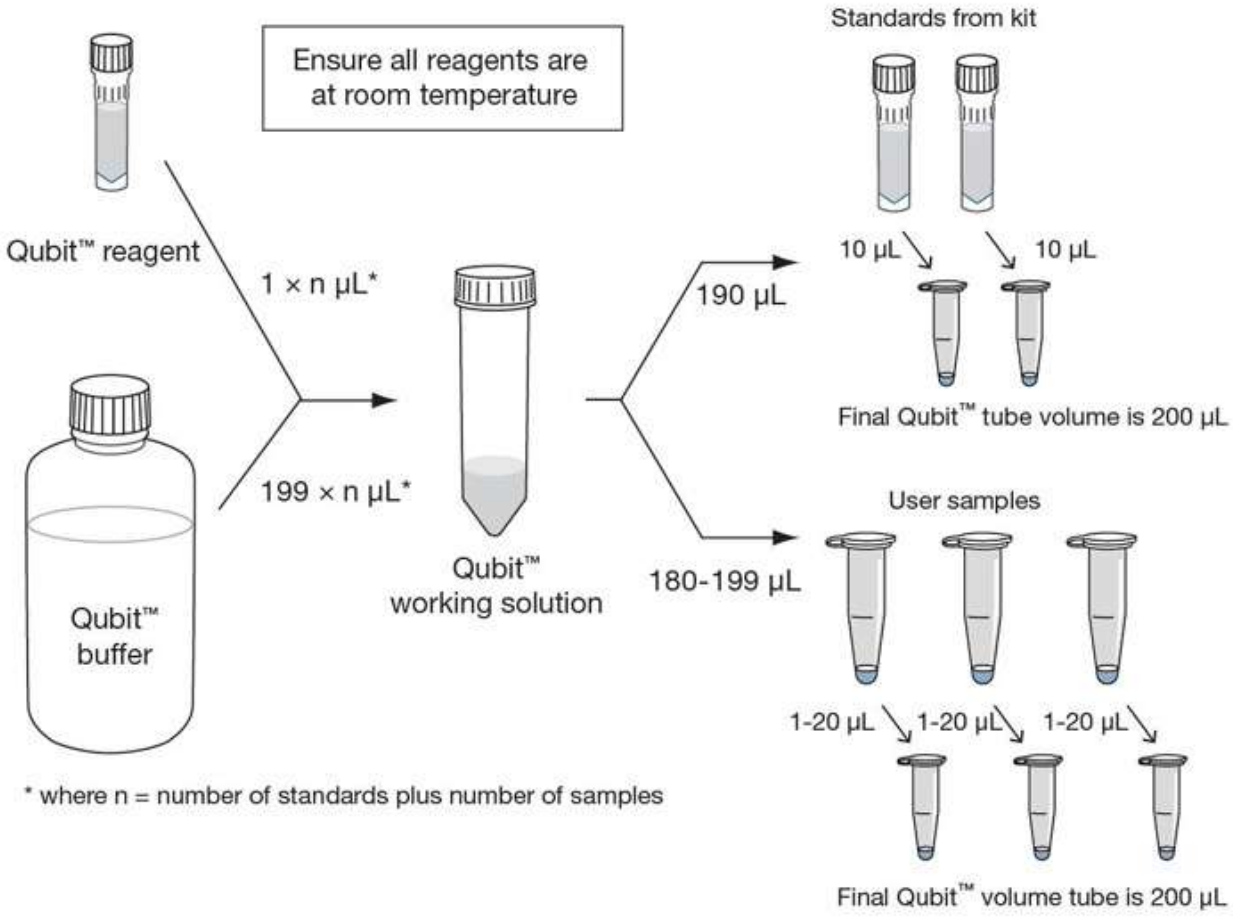
References

http://www.nanodrop.com/Library/T042-NanoDrop-Spectrophotometers-Nucleic-Acid-Purity-Ratios.pdf
http://www.nanodrop

Quantity control using the Qubit: The measurement of DNA concentrations using the Qubit, also gives you quantitative information (but not qualitative). The measurements rely on intercalating dyes and the results are supposed to be more precise.

Procedure:

– **bring all Qubit reagents at room temperature** (10 min before the PCR is done)
– students need to already prepare the reagent **for 4 samples, two standards, plus one extra (for two groups each).**
– You will need 2 ml Eppi!



Protocol for Quantification of DNA using the HS Qubit reagent

2.2 PCR (polymerase chain reaction) to amplify genes of interest

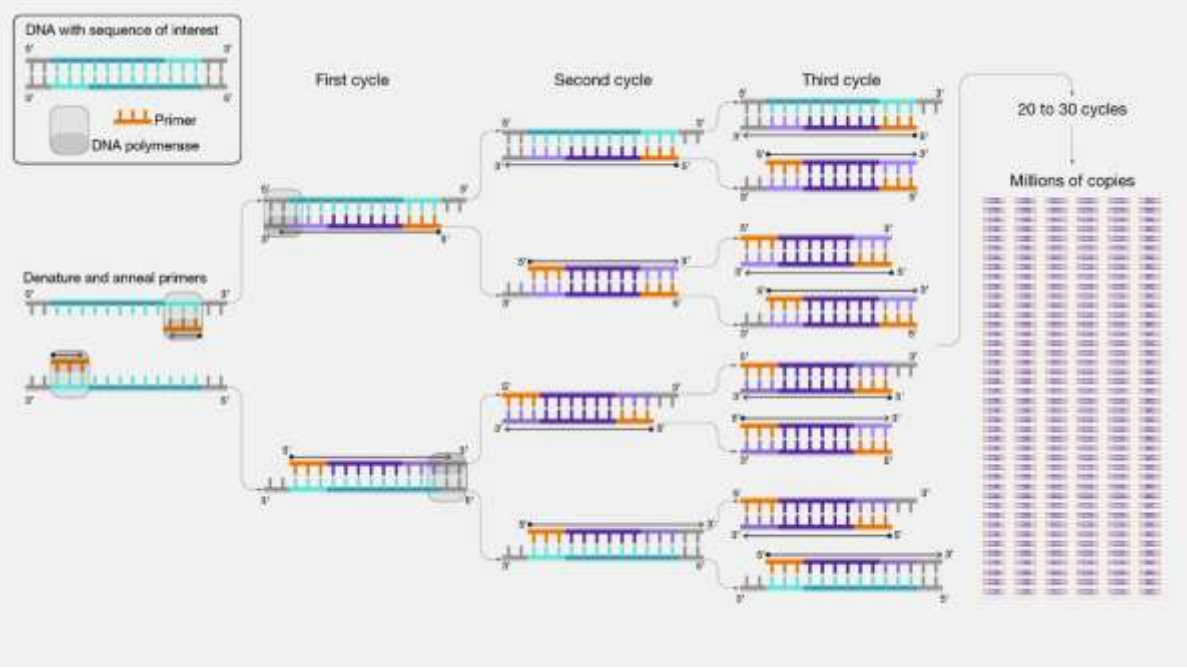
Main points:

- specific small pieces of DNA can be amplified by PCR to identify organisms in a sample
- the 16S rRNA gene is used to identify prokaryotes and some types of phytoplankton

Theory

Specific DNA sequences can be used as a kind of fingerprint to identify organisms. DNA is comprised of sequences called genes that code for specific structures. One gene that is important in identifying microscopic organisms is the 16S rRNA gene, which encodes for a part of the ribosome of prokaryotes. This gene has been shown to change very little over evolutionary time, so it can be used to tell how related different organisms are to each other and distinguish one kind of bacterium or phytoplankton from another.

Once we have extracted the DNA, we can make a bunch of copies of the 16S rRNA genes using a process called polymerase chain reaction (PCR). We end up with the 16S rRNA gene copies from many different organisms found in the water sample. This includes prokaryotic organisms (mostly bacteria) and also phytoplankton, because they contain plastids, which are organelles derived from cyanobacteria that still have bacterial DNA. We can then sequence these genes to see what kind of bacteria and phytoplankton we have in the sample.



The principle of a PCR.

Materials

- chemicals
    - 27F forward primer
    - 1492R reverse primer
    - nuclease-free water
    - PCR tubes
    - 1.5 mL or 2 mL tubes for making master mix
    - 10x Dream Taq Green Buffer (Thermo Fisher)
    - dNTP Mix 10mM each (Thermo Fisher)
    - Dream Taq DNA Polymerase 5 U/µL (Thermo Fisher)

- instruments:
    - Pipettes and tips
    - PCR tubes
    - Micro Spin
    - Vortex
    - Thermocycler
    - A bucket of crushed ice

PCR conditions	**Specifications**
27F Forward primer	5'-GAG TTT GAT CCT GGC TCA G-3'
1492R Reverse primer	5'-GGT TAC CTT GTT ACG ACT T-3'
Annealing Temp./Time	56 °C/1,5 min.
Template	Undiluted sample, 10 ng Optimum
Template size	~ 1500 bp

To set up parallel reactions and to minimize the possibility of pipetting errors, prepare a PCR master mix by mixing water, buffer, dNTPs, primers and DreamTaq DNA Polymerase. Prepare enough master mix for the number of reactions **plus one extra.** Aliquot the master mix into individual PCR tubes and then add template DNA. One well without DNA serves as negative control.

- Gently vortex and briefly centrifuge all solutions after thawing.
- Place a thin-walled PCR tube on ice and add the components for single 50 µL reaction:

dH_2O	38.75 µl
10x Dream Taq Green Buffer*	5 µl
dNTP Mix 10 mM each	1 µl
Forward primer 10 mM	2 µl
Reverse primer 10 mM	2 µl
Dream Taq DNA Polymerase 5 U	0.25 µl
Template DNA 10 pg-1 µg	1 µl

- Place the reactions in a thermal cycler. Perform PCR using recommended thermal cycling conditions:

Step	Temperature, °C	Time	No. of Cycles
Initial denaturation	95	3 min.	1
Denaturation	95	30 sec.	
Annealing	56	30 sec.	29x
Extension	72	1,5 min.	
Final Extension	72	5 min.	1

Agarose gel electrophoresis

Main points:

- agarose gel electrophoresis is a way to determine if the PCR was successful
- we can tell if the fragments are the correct size based on how far the DNA fragments travel down the gel
- the green dye helps us to track the DNA during the run

Theory

Since DNA is a negatively charged molecule, it will migrate toward a positive charge. We use that principle in agarose gel electrophoresis. We add the DNA from the PCR reaction at one end of the agarose gel, and set up an electrical field so that the other end of the gel is positively charged. This makes the DNA move from one end of the gel to the other. Small pieces of DNA move faster through the gel than large pieces, and we use this to determine the size of the DNA fragments we got from our PCR. We do this by adding a DNA ladder to the gel. The DNA ladder contains a mixture of DNA fragments of known length that separate based on size. We see where our DNA fragments end up on the gel and compare that to the ladder to estimate the size of our DNA fragments. The 16S rRNA gene is about 1500 base pairs long.

Materials

- chemicals:
    - agarose, e.g. peq GOLD Universal Agarose
    - TAE buffer, pH 8
    - DNA-size marker/ladder: Track-it 1Kb ladder
    - GelGreen Nucleic Acid Gel Stain 10000x in water
- instruments:
    - electrophoresis tray assembly, combs, and tank
    - measuring cylinder, Erlenmeyer flask and a magnetic stirring bar
    - magnetic stirring plate
    - microwave
    - Pipettes and tips
    - gel dock, UV-transilluminator, image capture device

Check the appearance and size of your amplified gene via 1% Agarose gel.

- Mix the Agarose with 200 ml 1xTAE buffer in a 300 ml Erlenmeyer flask
- Heat up the solution with the microwave until the Agarose is completely dissolved
- Add a magnetic stir bar let it mix and cool down
- Add Gelgreen (1000x) with a final concentration of 0,5x and mix
- Carefully rinse the Agarose solution into a gel tray inclusive comb
- After the gel is cured it can be transferred into the electrophoresis chamber and cover it with 1xTAE running buffer
- Pipette DNA size Ladder and your samples into the slots and start the electrophoresis with the power supply with constant 200 V for 20 min.
- Check the results using blue light table and/ or a gel doc and document it by taking a photo

Literature:

Dojka, MA, Harris, K, Pace NR. Expanding the known diversity and environmental distribution of an uncultured phylogenetic division of bacteria. Appl. Environ. Microbiol. 2000;66:1617-1621.

More useful links about the strength and impact of 16S rRNA sequencing:

http://www.earthmicrobiome.org/
http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0093827
https://bmcmircobiol.biomedcentral.com/articles/10.1186/s12866-015-0450-4
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC523561/

Experiment 3: Cultivable versus not-cultivable bacteria from marine samples

3.1 Cultivation experiments

Materials

- Sterile Eppendorf cups (2 ml)
- Eddings (water proof)
- Sterile saline (9 g NaCl per liter)
- Eppendorf pipettes (100 µl, 1000 µl)
- Sterile Eppendorf tips (100 µl, 1000 µl)
- Sterile disposable spatulaIncubation chamber (28 °C)
- Sterile disposable inoculation loops
- Parafilm, shears
- Nutrient agar plates (two different media):

1) Low nutrient seawater medium (SM)

NH_4Cl	0.53 g
Tropic Marine Sea Salt	15 g
Bacto-Agar	15 g
aq. deion.	1000 ml
pH = 7.7	

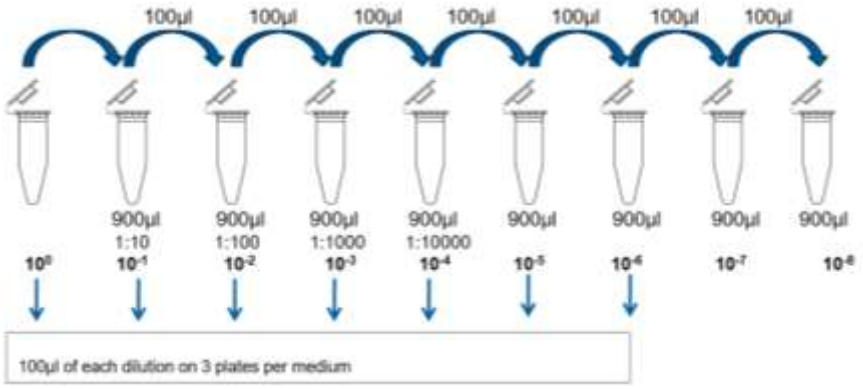
2) Marine Broth (MB)

Yeast Extract (BD, 212750)	1 g
Bacto peptone (BD, 211820)	5 g
Tropic Marine Sea Salt	15 g
Bacto-Agar	15 g
aq. deion.	1000 ml
pH = 7.7	

Preparing serial dilutions

Serial dilutions from the seawater samples will be prepared as follows:

- Number the Eppendorf tubes with 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}.
- Transfer 900 µl sterile saline to each Eppendorf cup.
- Add 100 µl of the water sample (10^0) to 900 µl sterile saline and **vortex** to get 10^{-1}.
- Add 100 µl of the dilution 10^{-1} to 900 µl sterile saline and **vortex** to get 10^{-2}.
- Continue until the dilution 10^{-4}.



Inoculation of the nutrient agar plates

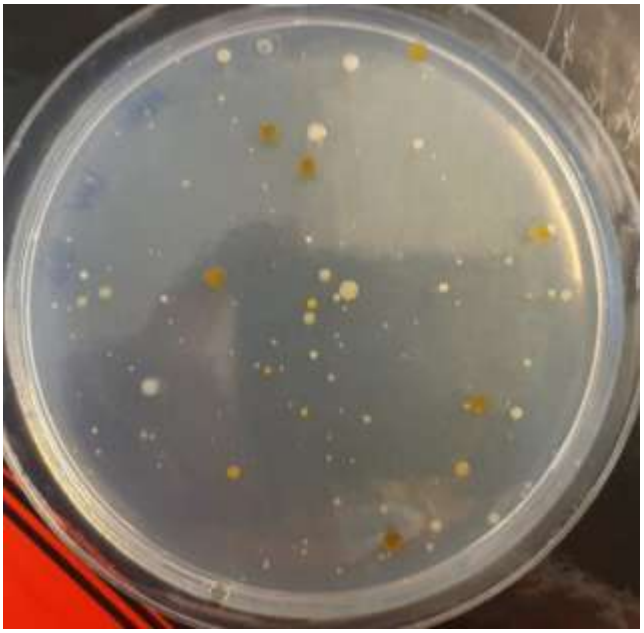
- Before inoculation, important information is written on the bottom of the plates, close to the rim: name of the sample, name of the medium, dilution step, date of inoculation, temperature of incubation
- Transfer 100 µl of each dilution onto three plates of each medium (triplicates), beginning from the highest dilution (10^{-4}).
- Distribute the suspension over the agar plates.
- As negative control 100 µl of the sterile saline are plated as triplicates on both media.
- Seal the plates with parafilm, place them into plastic bags (head first) labelled with your group number.
- Incubate at 28°C for 7 days.

Evaluation of the nutrient agar plates

- Determine the number of "colony forming units" (CFU) from each plate.
- Calculate the number of CFUs per ml seawater sample by considering the dilution steps.
- Describe the morphotypes of the colonies. Is there a specific morphotype dominant?

Discussion

- Compare the number of the CFU/ml sample and the colony diversity regarding the different media and the various seawater samples.
- Use your results to calculate the proportion of culturable microbes from the total cell number derived from the both cultivation-independent experiments.
- How could you get pure cultures from single colonies?
- Discuss the advantages and disadvantages of a cultivation-dependent approach possible reasons of differences in numbers and diversity.



Colonies on a nutrient plate.

3.2 Microbial cell counting (Microscopy)

DAPI, is a fluorescent stain that binds strongly to adenine–thymine rich regions in DNA. It is used extensively in fluorescence microscopy, especially when researchers are interested in the whole microbial community of a given sample (compared to cultivation approaches). It's very easy to handle, the procedure is easy and time efficient. As DAPI can pass through an intact cell membrane, it can be used to stain both live and fixed cells, though it passes through the membrane less efficiently in live cells and therefore the effectiveness of the stain is lower. As it is a DNA binding compound, it is likely to have some low-level mutagenic properties and care should be taken in its handling and disposal.

Materials

Microscope
Filtration unit and filter
Sterile saline (9 g NaCl per liter)
70 % ethanol
Pipettes and tips
Embedding medium incl DAPI (4′,6-diamidino-2-phenylindole)

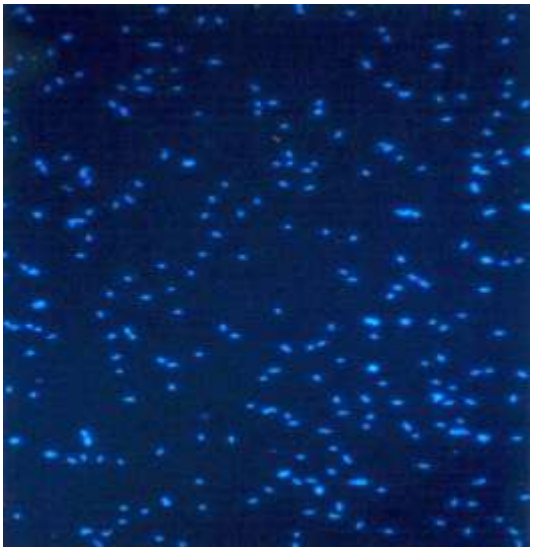
Experimental procedure

- Assembly filtration unit incl filter
- Add some sterile saline
- Add 500µl of your water sample, mix really good and gently apply vacuum to filter
- Wash filter with some 70% EtOH
- Release the vacuum, remove the filter and carefully apply it to a slide (labeled)
- Add embedding medium (less is more) and apply a clean cover slip
- Store in the dark until microscopical analysis

Analysis

Using a fluorescence microscope each group will count DAPI stained cells (at least 10 grid areas at different positions on the filter) and calculate the total number of cells per ml sea water for each sample type. To do so you may need some additional information:

Grid size: 122 µm
Filter area: 16,2 mm



DAPI stained microbes under the microscope

Use your results to calculate the proportion of culturable microbes from the total number and discuss advantages and disadvantages of this approach.

3.3 Microbial cell counting (Flow cytometry)

Materials

glutaraldehyde (GA, 2.5%) **Attention TOXIC**
cryotubes (5 ml)
liquid nitrogen
-80°C freezer
pipettes and tips
sample tubes

Sample preparation for further analysis (already done the first day)

Immediately after sampling the samples are fixed. As fixative glutaraldehyde, 2,5% is used, whereby a final concentration of 0.25% is aimed for (therefore add 1vol GA to 9 vol water sample). After 20 minutes incubation time in the dark, at room temperature, the fixed samples are flash frozen in liquid nitrogen and then stored at -80°C until analysis.

What is flow cytometry? What are fields of application?

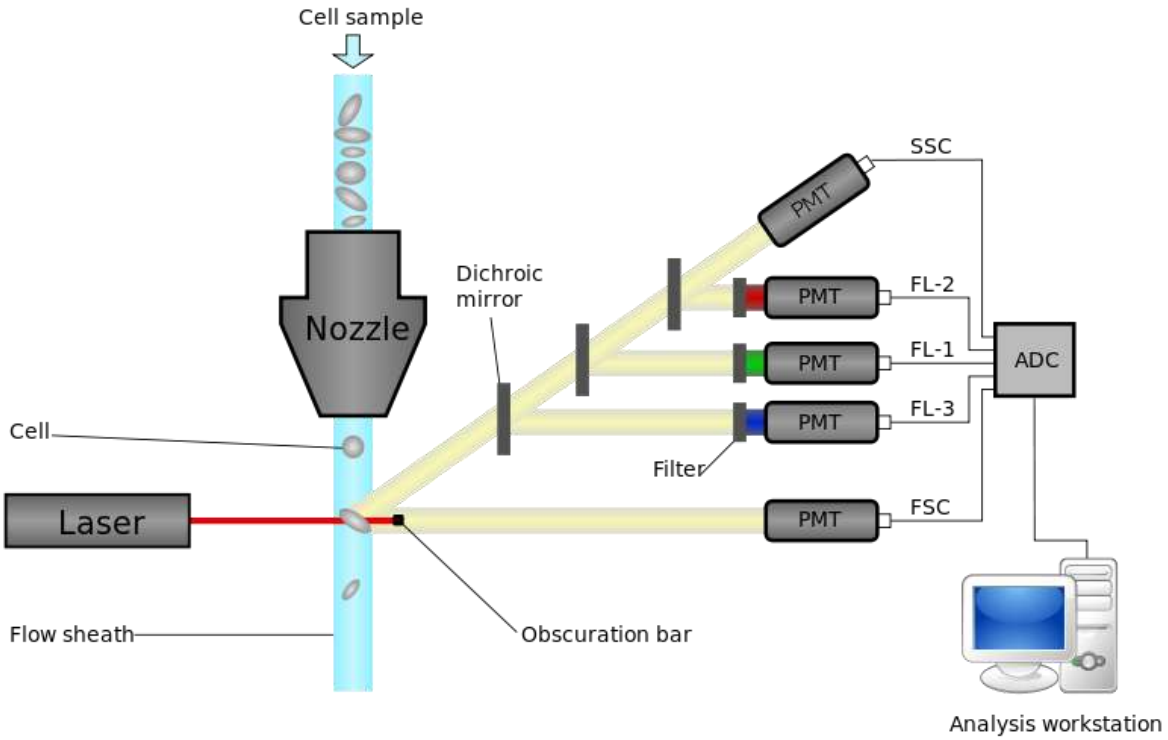
Flow cytometry is a method for analysis of particles and/or single cells in suspension based on scattered light and fluorescence properties. It was first used in the 1950s.
There are numerous potential applications of this method, e.g. in medicine it is routinely used in hematology, infectiology and immunology. In marine biology it can be used for example to count plankton/bacteria cells in order to characterize community structure.
The great advantages of flow cytometry over microscopy are that it is very fast and that it is able to measure a large number of parameters and samples.

The method

Flow cytometry (FCM - also known as FACS, stands for Fluorescence activated cell sorting) uses sophisticated technology that makes use of the principles of light scattering by particles crossing a beam of light, and excitation and fluorescence emission of fluorochromes attached to specific molecules or expressed by cells, to identify, analyze, and/or sort different populations of cells.

The process begins with a population of single cells, or particles, suspended in a medium, injected into a stable stream that forces cells to travel one by one to be interrogated by the flow cytometer. Each particle passes through one or more beams of laser light. Scattered light and fluorescence emission provide information about the particle's properties. Information is gathered from the manner in which a particle scatters light or by the light emitted by fluorochromes attached to, or contained in, the particle. Light scattered in the forward direction of a laser beam is focused by a confocal lens and detected by a light detector which converts it into an electrical signal that is digitalized to generate a parameter known as Forward Scatter (FSC). The FSC signal will give information about the size and shape of the cell, and information can also be gathered by a side confocal lens and detected by a detector reading side scattered light. The Side Scatter (SSC) signal gives information about the granularity of the cell. As FSC and SSC are unique for each type of particle, the combination of the two can help identify different types of cells.

Several optical detectors called photomultiplier tubes (PMT) are used in a flow cytometer to read fluorescence. These read the light emitted from the particle crossing the laser beams that excite the attached fluorochromes. Different fluorochromes will emit light at different wavelengths and these are split into specific colors by optical filters and sent to PMTs.



Experimental procedure

A. FCM Sample Fixation

1. Add 25% glutaraldehyde to your sample in a ratio of 1:10 (0.25% final concentration) and gently vortex to mix.

2. Aliquot 1 mL into 3 cryovials (i.e triplicate samples).

3. Incubate at room temperature in the dark for 20 minutes.

5. OPTIONAL: Flash freeze in liquid nitrogen.

6. OPTIONAL: Store at -80°C until analysis.

B. Bacteria staining and enumeration.

1. If needed, thaw sample in dark and protect it from light until analysis on the cytometer.

2. Prepare a 1.7 mL microcentrifuge tube for staining by adding 2.5 µL of 100X SYBR Green I nucleic acid stain. *NOTE: This can be pre-aliquoted and kept in the dark at room temperature.*

3. Add 497.5 µL of sample to the staining tube (0.5X SYBR Green I final concentration) and mix by pipetting and gentle vortexing.

4. Incubate the stained sample for 15 minutes in the dark at room temperature.

5. After incubation, transfer 490 µL of the stained sample to a 5 mL round bottom tube.

6. Add 10 µL 3° YG beads to the 5 mL tube.

7. Run sample immediately on flow cytometer equipped with 488 nm laser, triggering on green fluorescence (520±35 nm bandpass filter). Make sure that the voltage for the Forward Scatter (FSC) is high enough to get the whole bacteria population on scale. Run for 2-4 min at a flow rate of ~25 µL/min so that approx. 10,000 bacteria events are enumerated.

C. Reagents and Solutions.

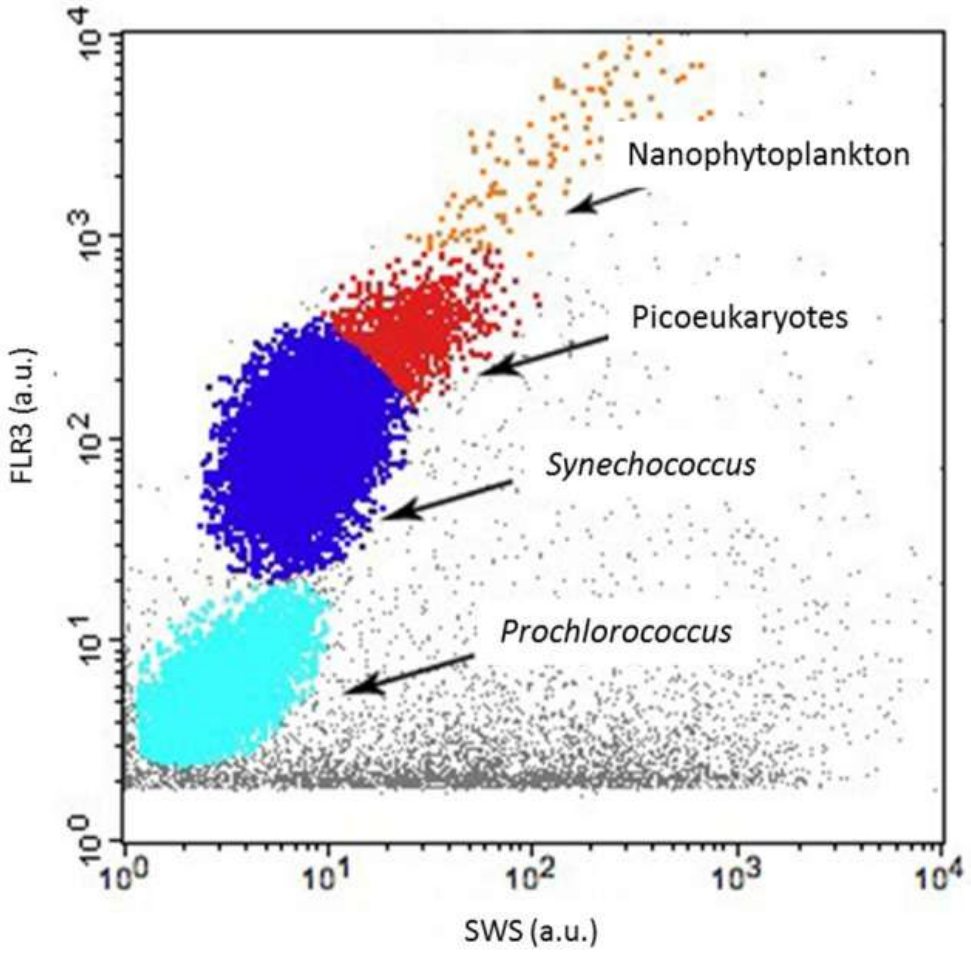
1. 25% EM-grade glutaraldehyde: stored at 4°C and protected from light.

2. SYBR Green I working stock: Dilute concentrated 10,000X SYBR stock (Thermo Fisher # S7563 or S7567) to 100X in molecular grade DMSO (Sigma D8418) (i.e., 10 µL concentrated SYBR in 990 µL DMSO). Prepare small aliquots (30-50 µL) and discard after thawing. Staining efficiency decreases from freeze/thaw cycles (stable for 2-3 thaws). Briefly spin stock solution (20,000 x *g*) to reduce noise levels.

3. Fluorescent beads: YG 0.75 µm beads (Polysciences #17153): Prepare 2° stock by diluting 1 drop in 10 mL MilliQ or 1x PBS. Then prepare a 3° stock (working stock) by diluting 20-50 uL of 2° stock in 10 mL MilliQ or 1x PBS depending on desired bead concentration.

How to interpret the results

When a cell is interrogated by the flow cytometer, data on its FSC, SSC, and fluorescence are plotted as a single data point on a graph called a cytogram. The data points cluster together according to size and fluorescence. Different groups of phytoplankton have characteristic sizes (which determine FSC and SSC) and photosynthetic pigments (which determine fluorescence), so they show up as distinct clusters of data points. We can count the data points in each cluster to determine how many representatives from each group are in the water sample.



Plot of red fluorescence vs. sideward/side scatter (SWS or SSC) where different groups of phytoplankton are highlighted in various colors (Thyssen et al 2014).

Tasks

- **Calculate the cell counts for your samples considering the several dilution steps during sample preparation.**
- **Compare your results to those from your culturing experiment, to DAPI cell counts and discuss advantages and disadvantages.**

References:

https://www.thermofisher.com/de/de/home/life-science/cell-analysis/cell-analysis-learning-center/molecular-probes-school-of-fluorescence/flow-cytometry-basics.html

Shapiro, Howard M.: Practical Flow Cytometry – 4th ed., John Wiley & Sons, 2003

Thyssen, M., Grégori, G. J., Grisoni, J. M., Pedrotti, M. L., Mousseau, L., Artigas, L. F., ... & Denis, M. J. (2014). Onset of the spring bloom in the northwestern Mediterranean Sea: influence of environmental pulse events on the in situ hourly-scale dynamics of the phytoplankton community structure. *Frontiers in microbiology*, *5*, 387.

Just in case there is some free time while waiting for experiments running…

Additional experiments

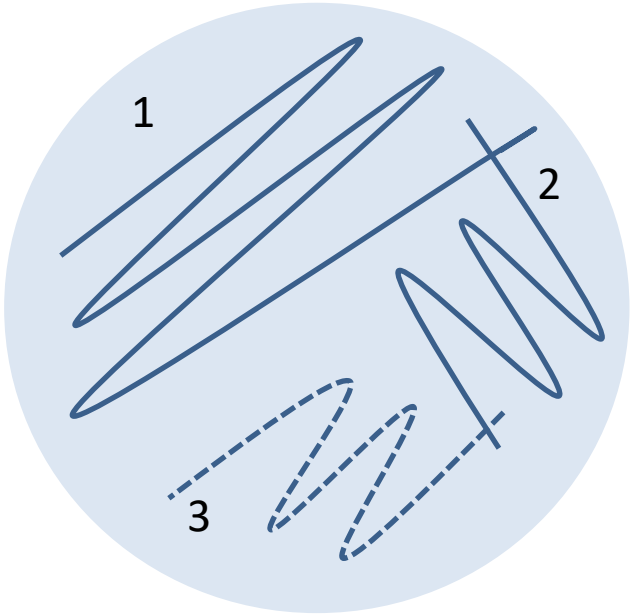
1. Obtaining pure cultures from colonies (streak plates)

Materials

- Plates from the cultivation experiments
- Media plates (SW, MB)
- Sterile disposable inoculation loops

Experimental procedure

- A small amount of cell material from a colony is taken using the inoculating loop.
- Spread the cell material out in one direction from the initial site of inoculation. This is done by moving the loop from side to side, passing through the initial site.
- Spread the bacteria from the last streak. Repeat this step a second time.



Evaluation and discussion

- Was it possible to gain single colonies?
- Why is it important for further steps such as storage of isolates, identification, growth experiments, and/or screening for targets representing biotechnological applications to work with pure cultures?

2. Laying-up nutrient agar plates

This experiment is aimed at the detection of possible contaminants from the ambience while performing the experimental steps, such as preparing the serial dilutions and plating.

Materials

- **Nutrient agar plates**

Experimental procedure

- Place the plates with open lid for 5 minutes on your working area on the laboratory bench and e.g. on the window bench in and outside the laboratory.
- Put the plates in a plastic bag and incubate them at 28°C.

Evaluation and discussion

- Which morphotypes of the colonies could be found?
- Are there differences from those morphotypes derived from the seawater samples?

3. Light microscopy

Materials

- Nutrient agar plates with bacterial strains, such as *Bacillus subtilis*, *E. coli*, *Staphylococcus lentus*, , isolates obtained from the breadcrumb sponge *Halichondria panicea* collected at the Kiel Fjord (e.g. *Enterovibrio* sp., *Vibrio* sp., *Streptomyces* sp.), and isolates from the Schwentine estuary (e.g. *Pseudoalteromonas* sp., *Flavobacterium* sp.)
- Sterile disposable inoculation loops
- Microscopic slides and cover slips
- Immersion oil (for 100x objective)
- Light microscope
- Binocular

Experimental procedure

- Place a drop of sterile saline on a microscopic slide.
- A small amount of cell material from a bacterial colony is suspended in the drop.
- Cover with the cover slide.
- Place a drop of immersion oil on the cover slide when using 100x objective.

Evaluation

- Describing the cell morphology, e.g. cell form, cell size, and motility.

Experiment 4: Community profiling via 16S rRNA gene sequencing with Nanopore

Introduction to Oxford Nanopore technology

In this part of the course, you will profile the composition of the microbial community in the aquatic samples based on 16S rRNA gene sequences from DNA extracts and sequencing using the Oxford Nanopore technology. Our aim is to identify the taxa and composition of the microbes present in the water samples and compare the community structure and composition between the two different aquatic samples you collected in the Fjord and the Schwentine River.

The Oxford Nanopore sequencer is a third-generation sequencing technique used in the sequencing of DNA (and RNA) without the need for PCR amplification or labelling of the DNA. It is a sequencing approach, enabling rapid processing of samples in real time. Due to the presence of highly conserved (adequate for universal primers and phylogenetic signal), the 16S rRNA gene can be used for bacterial profiling from diverse sample types. This targeted approach focuses sequencing on informative regions, helping you characterize the composition of your samples without sequencing unnecessary parts of the genome. This makes your experiments faster and more cost-effective. The Microbial Amplicon Barcoding Kit 24 V14 includes 16S primers designed to amplify the full-length 16S rRNA gene for microbial identification. This process is called library preparation, and includes PCR amplification of the 16S rRNA genes in a sample, barcoding of each sample with a special sequence tag, and attaching adapters that allow each DNA to be sequenced.

Finally, the DNA library is mixed with an ionic solution to prepare it for sequencing. The DNA liquid is then pipetted directly into the MinION sequencer. Inside the Nanopore sequencer, an enzyme unwinds the DNA double helix and passes one of the DNA strands through a nanopore, a microscopic channel in the center of a protein molecule placed on a membrane. An ionic current is applied, causing ions to move through the nanopore, generating a measured current for each DNA base (A, T, G, or C), which is translated into DNA sequence (a text file with letters of A, T, G and C). The resulting raw sequence data is analyzed by specialist software into microbial genomes. After sequencing, we shall bioinformatic analysis of the community in the samples using the EPI2ME 16S workflow (wf-16s) to classify 16S amplicons from your samples.

Schematic diagram of nanopore sequencing workflow for 16S rRNA gene microbial profiling [adopted from Bertolo et al. 2024].

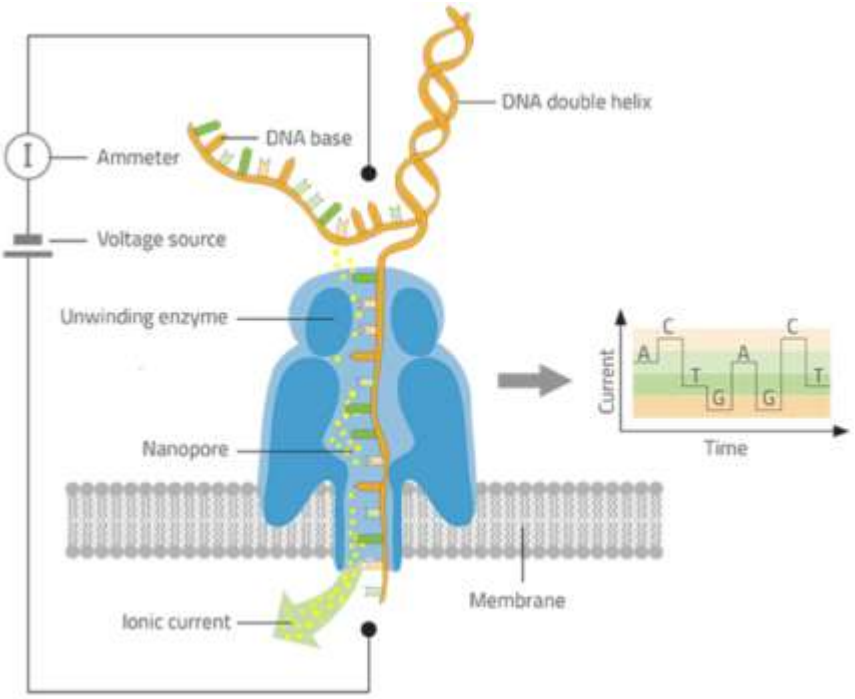


Illustration of the working principle of an Oxford Nanopore DNA Sequencer. A strand of DNA is passed through a nanopore, and an ionic current is measured and translated into DNA sequence.

Literature

Wang Y, et al. (2021). *Nanopore Sequencing technology, bioinformatics and applications*. Nature Biotechnology 39:1348–1365.

Curry DK, et al. (2022). *Emu: species-level microbial community profiling of full-length 16S rRNA Oxford Nanopore Sequencing data*. Nature Methods 19:845–853.

Zhang T, et al. (2023). *The newest Oxford Nanopore R10.4.1 full-length 16S rRNA sequencing enables the accurate resolution of species-level microbial community profiling*. Applied and Environmental Microbiology 89:e00605-23.

Bertolo A, et al. (2024). *Optimized bacterial community characterization through full-length 16S rRNA gene sequencing utilizing MinION nanopore technology*. BMC Microbiology 24:58.

Aja-Macaya P, et al. (2025). *Nanopore full-length 16S rRNA gene sequencing increases species resolution in bacterial biomarker discovery*. Scientific Report 15:26486.

Monday, Oct. 27th

Seminar: Introduction to Oxford Nanopore sequencing (08.00 – 8.30):

Full-length 16S rRNA gene sequencing using Nanopore MINION (9.00–12.00)

The DNA samples from the water samples are used to generate sequencing libraries using a rapid sequencing DNA and 16S barcoding kit (SQK-NBD114.24). A unique barcode will be linked to the PCR products of each sample, enabling multiplexing of samples. The sequencing libraries are prepared according to the manufacturer's instructions. *The protocol is provided below*. **Unless otherwise mentioned, please avoid vortexing of the samples** to prevent further shearing of DNA.

Materials:

- 10 ng high molecular weight genomic DNA for Nanopore Sequencing
- 16S Barcoding Kit 24 V14 (SQK-16S114.24).

Consumables:

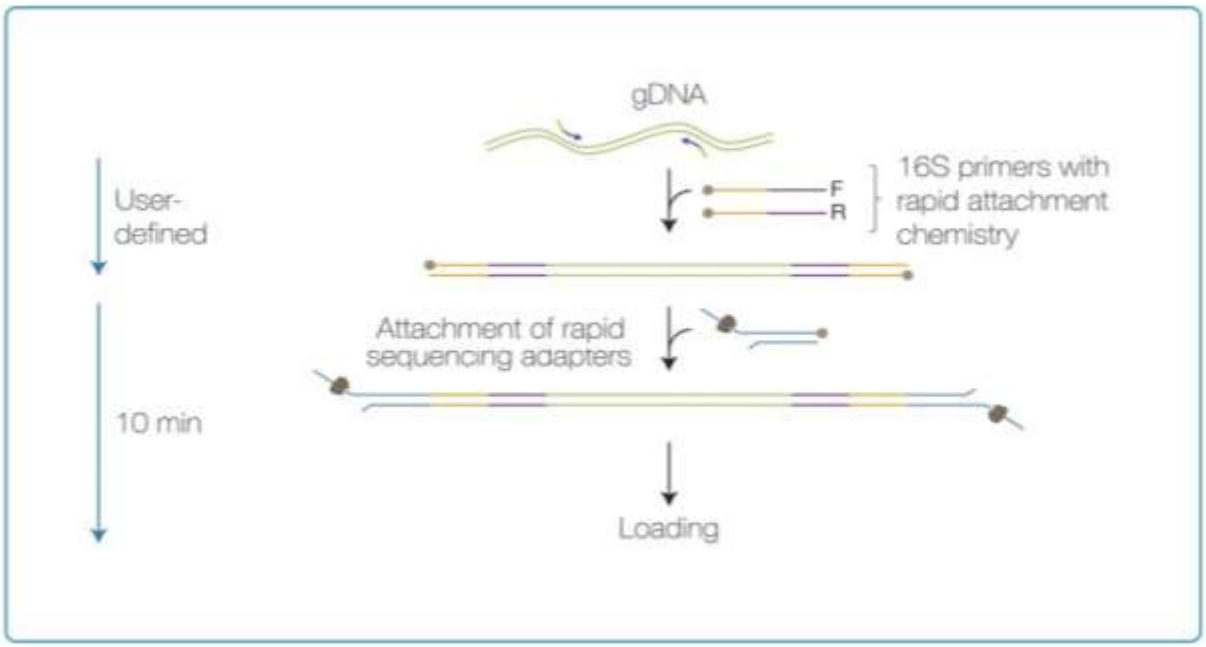
- Flow Cell (R10.4.1, with accuracy of 99.99%)
- LongAmp Hot Start Taq 2× Max Mix
- Bovine Serum Albumin (BSA) (50 mg/ml)
- Qubit dsDNA HS Assay Kit
- Freshly prepared 80% Ethanol in nuclease-free water
- Nuclease-free water
- 1.5 ml Eppendorf DNA LoBind tubes
- 0.2 ml PCR tubes
- 0.2 ml thin-walled PCR tubes
- Qubit Assay Tubes

Equipment:

- MinION Instrument
- Thermal cycler
- Magnetic rack
- Qubit fluorometer
- Pipettes
- Pipette tips
- Ice Box
- PCR Machine
- Microfuge
- Vortex
- Hula Mixer

Library preparation

Amplification and barcoding of 24 samples (4 samples from the practical course and 20 from a different project)



Library preparation step	Process	Time	Stop option
16S barcoded PCR amplification	Amplify the 16S gene using barcodes supplied in the kit	10 minutes + PCR	4°C overnight
Barcoded sample pooling and bead clean-up	Quantify and pool the barcoded samples and perform a library clean-up using beads	15 minutes	4°C short-term storage or for repeated use, such as re-loading your flow cell. -80°C for single-use long-term storage.
Adapter ligation	Attach the rapid sequencing adapters to the to the DNA ends.	5 minutes	We strongly recommend sequencing your library as soon as it is adapted.
Priming and loading the flow cell	Prime the flow cell and load the prepared DNA library for sequencing	5 minutes	

An overview of the key steps used for library preparation.

Procedures:

Important: There are four **Steps A to D**. Each group will do one step and the next group takes over, so that all groups participate.

Step (A). Amplification of the 16S rRNA genes using PCR

1). Remove a 96-well plate containing 16S barcodes from the freezer and decide which barcode you will use for the samples.

– Thaws the barcodes at room temperature.
– Briefly centrifuge barcodes in a microfuge to make sure the liquid is at the bottom of the tubes and place on ice
– Thaw the LongAmp Hot Start Taq 2× Master Mix, spin down briefly, mix well by pipetting and place on ice

2). Prepare the DNA in nuclease-free water:

– Transfer **10 ng of each genomic DNA** samples into a 0.2 ml thin-walled PCR tube
– Adjust the volume to **15 µl** with nuclease-free water
– **Mix thoroughly by flicking** avoiding unwanted shearing
– Spin down briefly in a microfuge

3). In each 0.2 ml thin-walled PCR tube containing a sample to be tested, prepare the following mixture (**total volume of 40 µl**):

Reagent	**Volume**
10 ng input DNA (from previous step)	15 µl
LongAmp Hot Start Taq 2× Master Mix	25 µl
Total	**40 µl**

– Ensure the components are thoroughly **mixed by pipetting and spinning down briefly**

4). Using clean pipette tips, carefully pierce the foil surface of the required barcodes.

– use a new tip for each barcode to avoid cross-contamination
– make a note of which barcode numbers will be run for each sample

5). Using a multi-channel pipette, mix 16S barcode by **pipetting up and down 10 times**.

– **transfer 10 µl** of each 16S barcode into the respective sample-containing tubes
– mix gently to minimize introducing air bubble to the reactions

6). **Amplify** using the following cycling conditions (under the program "16S_NanoAmplicon")

– takes 1.45 hours

Cycle step	**Temperature**	**Time**	**No. of cycles**
Initial denaturation	95 °C	1 min	1
Denaturation	95 °C	20 secs	25
Annealing	55 °C	30 secs	
Extension	65 °C	2 min	
Final extension	65 °C	5 min	1
Hold	4 °C	infinite	

7). Thaw reagents at room temperature, spin down briefly using a microfuge and mix by pipetting as indicated below (once thawed keep all reagents on ice):

Reagent	**1. Thaw at room temperature**	**2. Briefly spin down**	**3. Mix well by pipetting or vortexing**
Rapid Adapter (RA)	not frozen	yes	pipette
Adapter Buffer (ADB)	yes	yes	vortex or pipette
AMPure XP Beads (AXP)	yes	yes	*immediately before use*
Elution Buffer (EB)	yes	yes	vortex or pipette
EDTA	yes	yes	vortex or pipette

– once thawed keep all reagents on ice!!

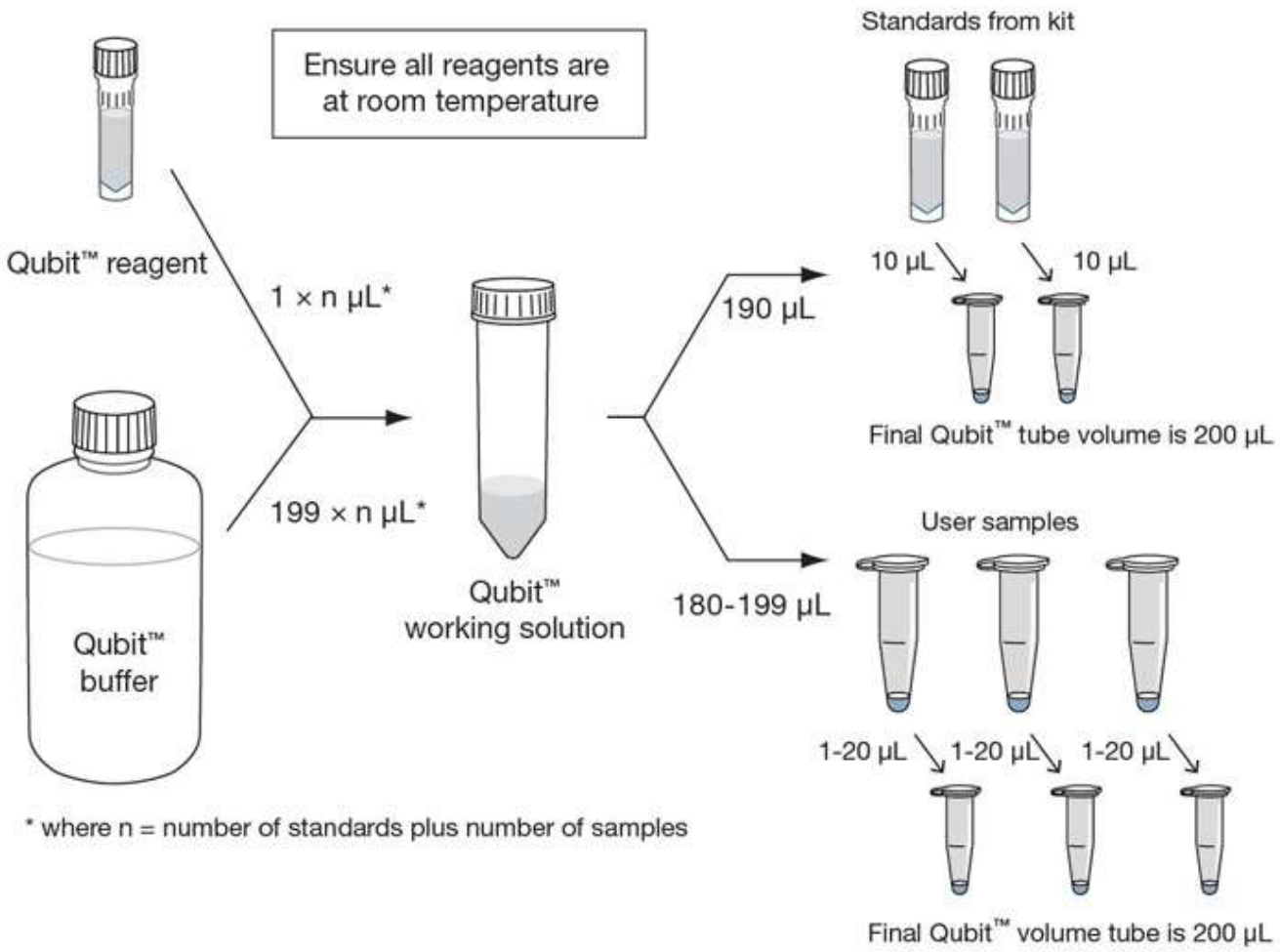
8). **Add 4 µl of EDTA to each barcoded sample**, mix thoroughly by pipetting and spin down briefly

– *EDTA (ethylenediaminetetraacetic acid) is added at this step to stop the reaction*
– *it is a chelating agent that binds to metal ions such as calcium, lead and iron.*

9). **Incubate for 5 minutes** at room temperature

10). **Quantify 1 µl** of each barcoded sample using a Qubit fluorometer for QC check

Important:
– **bring all Qubit reagents at room temperature** (10 min before the PCR is done)
– students need to already prepare the reagent **for 8 samples plus two standards, and one extra (for all groups).**
– You will need 5 ml eppi!



Protocol for Quantification of DNA using the HS Qubit reagent

11). Pool all barcoded samples in **equimolar ratios** in a 1.5 ml Eppendorf DNA LoBind tube

Important:

– pool the samples based on the total amount of DNA in the sample with the lowest DNA concentration

– *For example, if the smallest has 20 ng total DNA, then for the others you will need max 20 ng to have equimolar ratios.*

– note samples may vary in concentration after step 6!
– so, volumes added to the pool will be different

Step (B). Clean up the pooled PCR

12). Resuspend the AMPure XP Beads (AXP) by vortexing

13). To the pool of barcoded samples, **add a 0.6× volume ratio** of the resuspended AMpure XP Beads (AXP) and mix by pipetting:

– *Example volumes for reference*

Volume of barcoded sample pool (µl):	**37.5**	**75**	**150**	**300**	**600**
Volume of AMPure XP Beads (µl):	22.5	45	90	180	360

14). **Incubate** on a Hula mixer (rotator mixer) **for 5 minutes at room temperature**

15). **Prepare 2 ml of fresh 80% Ethanol** in nuclease-free water

– to be done 10 minutes in advance

16). Briefly spin down the sample and pellet on a magnetic rack until supernatant is clear and colorless.

– keep the tube on the magnetic rack, and pipette off the supernatant.

17). Keep the tube on the magnetic rack and wash the beads with **1 ml freshly prepared 80%** Ethanol without disturbing the pellet.

– remove the ethanol using a pipette and discard.

18). **Repeat the previous step 18** (then proceed to step 20)

19). Spin down and place the tube back on the magnet.

– pipette off any residual ethanol
– **allow to dry for ~30 seconds**, but do not dry the pellet to the point of cracking!

20). Remove from the magnetic rack

– resuspend the pellet by pipetting in **15 µl Elution Buffer** (EB)

– spin down and incubate for **5 minutes at room temperature**

21). **Pellet the beads on a magnetic** until the eluate is clear and colorless, **for at least 1 minute**

22). Pipette **15 µl of eluate** into a clean 1.5-ml Eppendorf DNA LoBind tube

– the elute contains the DNA library

– dispose of the pelleted beads

23). **Quantify 1 µl** of the eluted sample using a Qubit fluorometer

– **follow instructions in step 10**, but consider the low number samples here!!

Step (C). Hybridize Nanopore sequencing adapters to the barcoded amplicons

24). **Transfer 50 fmol** of your eluted sample into a clean 1.5 ml Eppendorf DNA LoBind tube

– **make volume to 11 µl** with Elution Buffer (EB).

25). In a fresh 1.5 ml Eppendorf DNA LoBind tube, dilute the Rapid Adapter (RA) as follows and pipette mix:

Reagent	**Volume**
Rapid Adapter (RA)	1.5 µl
Adapter Buffer (ADB)	3.5 µl
Total	**5.0 µl**

26). **Add 1 µl** of the diluted Rapid Adapter (RA) to the barcoded DNA.

27). Mix gently by flicking the tube, and spin down.

28). **Incubate the reaction for 5 minutes** at room temperature

– the prepared library is used for loading into the MinION flow cell
– store the library on ice until ready to load

Step (D). Priming and loading the MinION Flow Cell

– Realtime sequencing is performed with a MinION device (Mk1B, ONT) using the software MinKNOW v25.03.09

29). Please watch the "Priming and loading your flow cell" video first

– https://community.nanoporetech.com/nanopore_learning/lessons/priming-and-loading-your-flow-cell

30). Thaw the following reagents at room temperature (**10 minutes in advance**):

– Sequencing Buffer (SB)
– Library Beads (LIB)
– Flow Cell Teather (FCT)
– Flow Cell Flush (FCF)

– mix by vortexing
– then spin down and store on ice

31). Prepare the flow cell **priming mix** with BSA in a fresh 1.5 ml Eppendorf DNA LoBind tube

Reagent	Volume per flow cell
Flow Cell Flush (FCF)	1,170 µl
Bovine Serum Albumin (BSA) at 50 mg/ml	5 µl
Flow Cell Teather (FCT)	30 µl
Final total volume in tube	**1,205 µl**

– mix by inverting the tube and pipette mix at room temperature

32). Follow the instructions on **checking the Flow Cell** and **priming as per video.**

– this part will be done by the instructor.

33). Thoroughly mix the contents of the Library Beads (LIB) by pipetting

Important:
– These beads settle very quickly
– They should be mixed immediately before use!

34). In **a new 1.5 ml Eppendorf** DNA LoBind tube, **prepare the library** for loading as follows:

Reagent	Volume per flow cell
Sequencing buffer (SB)	37.5 µl
Library Beads (LIB) immediately mixed before use	5 µl
DNA library	12 µl
Final total volume in tube	**75 µl**

35). Complete the flow cell priming:

– this part will be done by the instructor.
– gently lift the SpotON sample port cover
– **load 200 µl of the priming mix into the flow cell priming port** (NOT the SpotON sample port)
– avoid introducing air bubbles.

36). **Mix the prepared library** gently by pipetting up and down just prior to loading

The next four parts will be done by the instructor.

37). **Load 75 µl of the prepared library** to the flow cell via the SpotON sample port **in a dropwise manner**

– Ensure each drop flows into the port before adding the next

38). Gently replace the SpotON sample cover back and close the priming port

39) **Install the light shield** on your flow cell as soon as the library has been loaded

40). Now you are **ready to start sequencing** with the advice of the instructors!

– set up an experiment on the MinKNOW software and start data acquisition.

– the sequencing will run for three days; thereafter we can analyse the data.

Thursday, Oct. 30th

Bioinformatic analysis of the Oxford Nanopore sequence data (08.00 – 12.00):

After the sequencing is completed, we shall use the raw data (either *.bam or *.fastq files), as input for the **software EPI2ME from Nanopore** to analyze the data in the computer room.

Each group will be assigned one computer and the instruction for data analysis will be given in real-time.