

Introducing DNA Sequencing to the Next Generation on a Research Vessel Sailing the Bering Sea Through a Storm

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Appendix 1 – Workshop slides presentation

Slide Deck on figshare at: [10.6084/m9.figshare.8085422](https://doi.org/10.6084/m9.figshare.8085422)

Appendix 2 - Analysis questions for participants

1. What is the yield of data generated by your run?
2. What is the amount of data that has been classified?
3. How many genus and species have been classified?
4. What seems to be the most abundant classified organism?
5. Using the phylogenetic tree provided, can you tell if the classified prokaryotes are closely related?
6. Can you tell if the classified species are marine?
7. How look the communities of different depths compared to each other?

Appendix 3 - List of materials

For instructors in charge of supervising the collection and processing of samples, before the workshop:

Sample collection and filtering:

- Sample containers (survival water bags or Nalgene bottles; 2L minimum; adjust quantity to the amount of sampling intended),
- 1 peristaltic pump and tubings,
- 1 plastic cylinder of 2L for each line of the peristaltic pump,
- Millipore Mixed Cellulose GSWP 0.22 µM filters (adjust the quantity to the amount of sampling intended),
- sterile tweezers,
- 5 ml tubes (adjust the quantity to the amount of sampling intended),

DNA extraction:

- Lysozyme (10 mg/ml; 100 µl per sample),
- Mutanolysin (25 KU/ml; 12 µl per sample),
- Lysostaphin (4000 U/ml; 6 µl per sample),
- QiaGen DNeasy PowerWater extraction kit (adjust quantity to the amount of sampling intended),
- RNase A (4 µl per sample),
- 1 spinner,
- 1 bench centrifuge,
- 1 vortex,

DNA clean-up and quality control:

- Eppendorf LoBind microcentrifugation tubes (1.5 ml),
- MagBio High Prep PCR magnetic beads,
- 1 magnetic tube rack,
- 2 heat blocks,
- 1 Qbit device and reagents (dsDNA HS assay kit),
- Nuclease-free water,

DNA sequencing:

- Oxford Nanopore Technology Flow cell (1 for each group of participants; optional: one for the instructor),
- Oxford Nanopore Technology RAD004 kit (6 reactions/kit; adjust quantity to the amount of library preparation intended),
- Oxford Nanopore MinION (1 for each group of participants; optional: one for the instructor),
- SSD hard-drive laptop (1 for each group of participants; optional: one for the instructor),

Misc.:

- permanent markers,
- gloves,
- disposal containers,
- pipettor set (P1000, P200, P20 or P10; one set for each group of participants),
- Tips,
- 1 timer,
- 1 projector,
- erasable board and markers,
- hardcopy of taxonomic books and research papers related to expected communities (optional: if no internet),

Back-up:

- DNA extracts,
- fastq files dataset.

For each group of participants during the workshop:

- 1 Oxford Nanopore MinION,
- 1 SSD hard-drive laptops,
- parafilm,
- 1 used flow cells,
- 1 new flow cells,
- gloves,
- 1 set of pipettors (P1000, P200, P20 or P10),
- tips,
- 1 disposal container,
- 1 tube rack (for 1.5ml tubes; 1 for each group of participants).

Appendix 4 - Faculty instructions

LAPTOP PREPARATION

- Laptop (SSD 1Tb) + power adapter,
- set the latest version of MinKNOW (the offline version of the software is available upon request to ONT support if the activity is taking place in a location devoid on internet connection),
- make sure enough space is available on hard drives (~ 500Gb),
- proceed to updates if using the online version of MinKNOW (for Microsoft OS),
- set R studio,
- set 'Pavian' R package,

PROTOCOLS FOR INSTRUCTORS

Considering the unpredictable conditions inherent to field situations, we advise instructors to prepare multiple sampling plans. We describe here the steps for the sample collection proceeded in this field case. However, we also set backup plans i.e, seawater transect and sediments processing, with the corresponding material, in case a CTD cast was not accomplished during this transit.

1- Sample collection:

- Wear gloves,
- Rinse collection bottles or water bags including caps, 3 times with the water to collect,
- Sample 2L of water or more and mark the collection bottles or water bags,
- Collect required field metadata,
- Bring the samples back to the lab and store them at 4°C or on ice until filtering.

2- Sample filtering:

- Assemble the filtering unit,
- Filter water samples through a 0.22 µm filter until clogging,
- Upon completion of filtering, roll filters with a pair of sterile tweezers and place each one of them individually in a sterile 5 ml tube,
- Store filters at +4°C if DNA extraction is planned within the next 24 hours. Otherwise, freeze samples at -20°C.

3- DNA extraction (an adaptation of Qiagen Powerwater DNeasy extraction protocol for MARC3 project):

- The following enzymes were added to 1 ml of PW1 in the 5 ml tube with the filter and incubated at 37 °C for 1 hour.

NOTE: Solution PW1 must be warmed to 55 °C to dissolve precipitates prior to use.

Solution PW1 should be used while still warm.

Enzyme (stock concentration)	Volume (µl)	Final concentration
Lysozyme (10 mg/ml)	100	0.892 mg/ml
Mutanolysin (25 KU/ml)	12	0.267 KU/ml
Lysostaphin (4000 U/ml)	6	21.42 U/ml

<http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0033865>

- Recover the lysate (~800 ul) in a fresh tube provided with the kit,
- Centrifuge the lysate at 13,000 x g for 1 minute,
- Avoiding the pellet, transfer the supernatant in a clean 2 ml Collection Tube (provided),
- Add 4 µl RNase A (100 mg/ml) and leave at room temperature for 2 minutes,
- Add 200 µl of IRS and vortex briefly to mix. Incubate at 4°C for 5 minutes,
- Centrifuge the tubes at 13,000 x g for 1 minute,
- Avoiding the pellet, transfer the supernatant in a clean 2 ml Collection Tube (provided),
- Add 650 µl of Solution PW3 and vortex briefly to mix. Note: Check Solution PW3 for precipitation prior to use. Warm if necessary (heat to 55 °C). Solution PW3 can be used while still warm,
- Load 650 µl of supernatant onto a Spin Filter and centrifuge at 13,000 x g for 1 minute. Discard the flow through and repeat until all the supernatant has been loaded onto the Spin Filter. Note: A total of three loads for each sample processed are required,
- Place the Spin Filter basket into a clean 2 ml Collection Tube (provided),
- Shake to mix Solution PW4 before use. Add 650 µl of Solution PW4 and centrifuge at 13,000 x g for 1 minute,

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- Discard the flow through and add 650 μ l of Solution PW5 (ethanol) and centrifuge at 13,000 x g for 1 minute,
- Discard the flow through and centrifuge again at 13,000 x g for 2 minutes to remove residual wash,
- Place the Spin Filter basket into a clean 2 ml Collection Tube (provided),
- Add 50 μ l of Solution EB (10 mM Tris-HCl pH 8.5) to the center of the white filter membrane,
- Centrifuge at 13,000 x g for 1 minute,
- Recover the eluted nucleic acid,
- Discard the Spin Filter basket. The DNA is now ready for any downstream application.

4- DNA clean-up and concentration measurement:

- Prepare 500 μ l of 80% EtOH for each sample,
- Turn on heat block set at 37°C,
- Add magnetic beads to the sample for a x0.6 (v/v) ration of beads to sample,
- Incubate 5 minutes at room temperature,
- Set the sample on a magnetic rack and wait for the beads to have formed a pellet on the tube wall (supernatant should be clear),
- Remove supernatant carefully, do not disturb the bead pellet,
- Add gently 200 μ l of 80% ethanol making sure that the bead pellet is not disturbed and is completely immersed,
- Remove Ethanol,
- Proceed to another rinsing,
- Spin the sample briefly and replace the tube on a magnetic rack,
- Remove the leftover of ethanol,
- Let the bead pellet dry until it is not shinnying anymore but before the appearance of cracks,
- Add 10 μ l of nuclease-free water,
- Resuspend the pellet by finger flicking,

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- Incubate the sample at 37°C for 5 to 10 minutes,
- Place the tube on a magnetic rack and wait for the bead pellet to have formed on the tube wall,
- Collect the DNA extract,
- Measure the concentration of 1 µl of each DNA extract with Qbit,
- Store DNA extracts at +4°C if sequencing is planned within the next 24 hours. Otherwise, freeze samples at -20 °C.

LAB PREPARATION BEFORE THE WORKSHOP

For DNA sequencing with Oxford Nanopore RAD004 kit:

- Set projector and workshop presentation on instructor's laptop, 2 heat blocks (30°C and 80°C, turned ON) and timer.
- For each group of participants, prepare 400 ng of DNA in 7.5 µl final (use nuclease-free water for dilution if needed),
- Get the new flow cells at room temperature (kept by instructors),
- Assemble the library loading mix (to be labeled 'LLM') for each station:
 - 34 µl Sequencing Buffer (SQB)
 - 25.5 µl Loading beads (LB), mixed immediately before use
 - 4.5 µl nuclease-free water
- For each group of participants, set:
 - 1 laptop,
 - 1 MinION (possibility: without CTC cheap),
 - 1 set of pipettors (P1000, P200, P20 or P10),
 - tips,
 - 1 old flow cell,
 - DNA sample,
 - 4 ml of NFW in 5 ml tubes (for pipetting training with parafilm),
 - parafilm,
 - gloves,
 - 1 disposal container,
 - 1 tube rack.

BIOINFORMATICS DURING THE ACTIVITY

- Collect passed fastq files into one folder for each station on a USB drive,
- Import fastq files onto Linux server,
- Open Terminal screen,
- Concatenate fastq files of each station into one:

```
cd station1/  
cat *.fastq > station1.fastq
```
- Classify reads with Kraken and a database:

```
kraken --db whereisyourdb --threads 4 --output station1.out station1.fastq
```
- Generate a classification report:

```
kraken-report --db whereisyourdb station1.out > station1.report
```
- Collect classification reports of each station onto USB drive
- Import reports to each station
- Next steps will be proceeded by the participants (instructions figure in the workshop presentation).