

Non-destructively barcoding hundreds of freshwater macroinvertebrates with a MinION

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1 Works for me

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ABSTRACT

This project aimed to optimize protocols needed to produce CO1 barcodes for 1000s of African freshwater macroinvertebrates, from many different orders, in the most cost-efficient way possible. Since many of these specimens represent undescribed or poorly-known taxa we also wanted to utilize a non-destructive method of DNA extraction. To do so, we modified the methods detailed by Srivathsan et al. (2021). Here we present the protocol from specimen preparation and DNA extraction to sequence generation. In addition to the methods outlined by Srivathsan et al. (2021) we also pulled together the protocols from Oxford Nanopore Technologies and other vendors. We have added some tips and comments to these procedures that we found helpful in the process. We used these protocols to produce barcodes for hundreds of freshwater macroinvertebrates that were collected in Gabon. This project was funded by the National Science Foundation's Research Experience for Post-Baccalaureate Students (REPS) program (DEB #1920116).

Srivathsan A, Lee L, Katoh K, Hartop E, Kutty SN, Wong J, Yeo D, Meier R (2021). ONTbarcoder and MinION barcodes aid biodiversity discovery and identification by everyone, for everyone.. BMC biology.
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KEYWORDS

Barcode, Freshwater macroinvertebrates, non-destructive, biodiversity, CO1, Nanopore, Flongle

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IMAGE ATTRIBUTION

Ray C. Schmidt

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64798

GUIDELINES

These protocols are modified from those described in Srivathsan et al 2021.

Srivathsan A, Lee L, Katoh K, Hartop E, Kutty SN, Wong J, Yeo D, Meier R (2021). ONTbarcoder and MinION barcodes aid biodiversity discovery and identification by everyone, for everyone.. BMC biology.
<https://doi.org/10.1186/s12915-021-01141-x>

MATERIALS TEXT

MinION
Sequencer
Oxford Nanopore Technologies MinION 1B / MinION 1C

Flongle adaptor
Oxford Nanopore Technologies ADP-FLG001

 [QuickExtract DNA Extraction](#)

Solution Lucigen Catalog #QE09050 In 2 steps

 [NEBNext Ultra II End Prep Reaction Buffer New England](#)

Biolabs Catalog #E7647 Step 4.2

 [NEBNext Ultra II End Prep Enzyme Mix New England](#)

Biolabs Catalog #E7646 Step 4.2

 [AMPure XP Beckman](#)

Coulter Catalog #A63880 In 5 steps

 [Adapter Mix \(AMX\) Oxford Nanopore Technologies](#) Step 5.1

 [NEBNext Quick T4 DNA Ligase New England Biolabs](#) Step 5.1

 [Ligation Buffer \(LNB\) Oxford Nanopore Technologies](#) Step 5.1

 [Elution Buffer \(EB\) Oxford Nanopore Technologies](#) In 2 steps

 [Short Fragment Buffer \(SFB\) Oxford Nanopore Technologies](#) In 3 steps

 [FB \(Flush Buffer\) Oxford Nanopore](#)

Technologies Catalog #EXP-FLP002 Step 7.1

 [FLT \(Flush Tether\) Oxford Nanopore](#)

Technologies Catalog #EXP-FLP002 Step 7.1

 [Sequencing Buffer II \(SBII\) Oxford Nanopore Technologies](#) Step 7.2

 [Loading Beads II \(LBII\) Oxford Nanopore Technologies](#) Step 7.2

 [2.0X Taq RED Master Mix Genesee](#)

Scientific Catalog #42-138 Step 2.1

 [QuantiFluor ONE dsDNA Dye Promega](#) Step 3.18

T100 thermocycler
BioRad 1861096

blueGel
MiniPCR QP-1500-01

Quantas Fluorometer
Fluorometer
Promega E6150

Specimen preparation and DNA extraction 20m

1

Specimen preparation and DNA extraction

Specimen preparation 20m

1.1 Fill two Petri dishes with ddH₂O

1.2 Remove specimen/s from ethanol and place in one dish of ddH₂O for 10m 00:10:00

You may need to work under a microscope depending on what type of specimens you are working with.

If you are removing specimens from a jar, be sure to top off that jar with ethanol when you are finished.

1.3 Remove specimen/s from water, and rinse again in the second dish for 5m
⌚00:05:00

1.4 Place specimen/s on a paper towel to dry for about 5m ⌚00:05:00

Arranging the specimens in rows of eight makes the next steps easier.

DNA extraction 20m

1.5 Put 10 µL
[QuickExtract DNA Extraction](#)
[Solution Lucigen Catalog #QE09050](#) in each well of a 96-well plate

I have found that working in rows of three is the most effective and reduces the likelihood to make mistakes

1.6 Place one rinsed and dried specimen in each well head-first

Be sure the head is fully submerged. If the specimen is too large to fit in the well, remove a leg and place that in the quick extract.

When working with legs, be sure to keep one side of the specimen intact

This was the work flow I found to be most effective: remove 24 specimens at

a time and place them in the water to soak. Fill three rows of the plate with

 QuickExtract DNA Extraction

Solution Lucigen Catalog #QE09050

Remove specimens individually from the water and place in 3 rows of 8 to dry out (they do not need to dry for long). Place each specimen in a well. Put clean water in both dishes (trying to prevent ethanol from being a PCR inhibitor). Repeat this process until the plate is full.

1.7 Cover with TempPlate sealing foil or reusable TempPlate pressure-fit sealing mat

1.8 Place the covered 96-well plate in the thermal cycler

Seal plate as well as you can when heating,

 QuickExtract DNA Extraction

Solution Lucigen Catalog #QE09050

will

start to evaporate.

1.9 Heat at  65 °C for  00:18:00 ,  98 °C for  00:02:00 20m

1.10 After heating, remove the

 QuickExtract DNA Extraction

Solution Lucigen Catalog #QE09050

from

each well and transfer to a clean 96-well plate

- Be very careful to not touch specimens when removing

 QuickExtract DNA Extraction

Solution Lucigen Catalog #QE09050

from the wells containing the specimens. Some soft-bodied specimens may disintegrate and others may be very fragile and should not be touched

- Some hard-bodied specimens tend to soak up the

QuickExtract DNA Extraction

Solution Lucigen Catalog #QE09050

If you go to remove the

QuickExtract DNA Extraction

Solution Lucigen Catalog #QE09050

from that well and there isn't any liquid in the well, add **10 µL** of ddH₂O, pipette up and down, and remove this as your extract.

- Move slowly when doing this, it is easy to get mixed up on which well you are on when dealing with a full 96 well plate

DNA Dilution

1.11 Put **10 µL** of ddH₂O in each well of a 96-well plate

1.12 Put **1 µL** of DNA extraction in each coinciding well

- We have found that the concentration of the quick extract DNA extract can range anywhere between about 1ng/mL to about 50ng/mL. Diluting the DNA 1:10 has been the most successful generalized protocol (about 75% PCR success)
- Seal the dilution plates well when storing, they will evaporate if not

PCR Amplification

2 PCR Amplification

PCR Setup

2.1

Combine ddH₂O,

2.0X Taq RED Master Mix Genesee

Scientific Catalog #42-138

, and

forward primer in a tube

A	B
	Per 20µL Reaction
ddH2O	7
2x Buffer-APEX red	10
Primer F	0.5
Primer R	0.5-not in master mix
DNA Template	2-not in master mix

We have 12 forward primers and 24 reverse primers (purchased from Integrated DNA Technologies, Inc.) so I typically work with 24 wells at a time. I make the master mix for 25x to make sure I have enough master mix to distribute into each tube.

	25x
H2O	175
2x Buffer-APEX red	250
Primer F	12.5
Primer R	0.5-not in mastermix
DNA Template	2-not in mastermix

 **Primer List.xlsx**

2.2 Distribute **17.5 µL** of master mix into each tube of a strip tube

Work on an ice block because this setup takes anywhere from **00:10:00** to **00:20:00**

2.3 Distribute DNA template and reverse primer into each tube

Workflow: in a 1.5 ml tube, combine the 25x amount of the water,

2.0X Taq RED Master Mix Genesee

Scientific Catalog #42-138

, and

forward primer that you are using. Vortex and spin down. In three 8-tube strip tubes, distribute **17.5 µL** of the mastermix. Each separate tube gets

0.5 µL of a unique reverse primer. Each separate tube gets **2 µL** of a unique specimen's diluted DNA. Add the DNA and reverse primers in order to be able to keep track more easily.

2.4 Thermal Cycler Protocol: 94 °C for 00:01:00, then 94 °C for 00:00:30, 42 °C for 00:01:00, 72 °C for 00:01:30, go back to step 2 39x, 72 °C for 00:07:00, infinite hold at 10 °C 11m

T100 Thermal Cycler

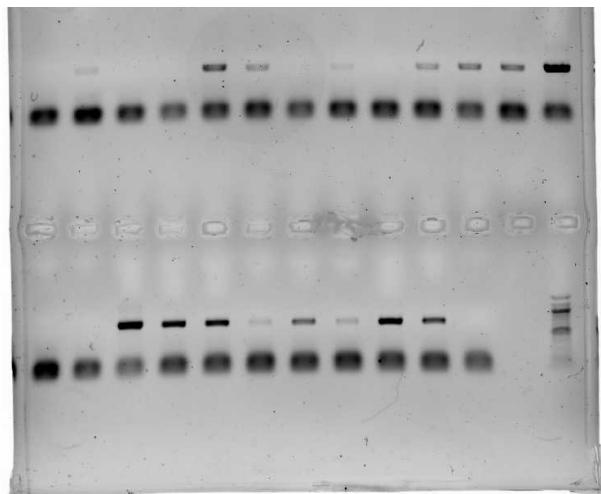
Thermal Cycler

BioRad 1861096

Gel Electrophoresis

2.5 Run a gel on at least 6 specimens from each master mix batch. Including a subset of the reaction on the gel allows you to confirm that there were no issues with the PCR master mix and also estimates how many reactions were successful.





Running a subset of reaction on a gel to confirm successful amplification, allows estimation success rate (e.g. ~66%)

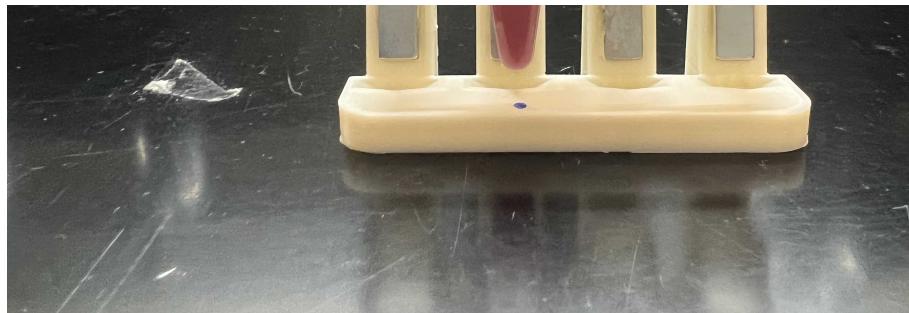
Pooling and Cleaning

3 Pooling and cleaning

3.1 Take **4 µL** from each PCR-This will yield approximately **1.152 mL** of PCR product

3.2 Take **250 µL** from the pooled PCR





Unpurified pooled PCR reactions

3.3

 AMPure XP Beckman

Add  125 μ L of Coulter Catalog #A63880 and pipette up and down to mix (Followed manufacturer protocol but listed here).

We used 0.5 X AMPure beads

3.4

Let sit for  00:10:00 at room temperature

10m

3.5

Let sit on magnetic rack for  00:02:00

2m

Keep the tube open, jolting the tube when closing and opening the lid can disturb the pellet

3.6

Remove supernatant, leaving about  5 μ L on the bottom of the tube

3.7

Add  250 μ L of freshly made 70% ethanol and pipette gently up and down

3.8 Let sit on rack for **00:00:30** 30s

3.9 Remove all ethanol

3.10 Add another **250 µL** of 70% ethanol and let sit for **00:00:30** 30s

3.11 Remove all ethanol

3.12 Let dry for **00:05:00** to **00:10:00** with the cap open so the ethanol can evaporate 15m

3.13 Add **40 µL** of ddH₂O and elute the pellet (remove from the rack)

3.14 Let sit for **00:02:00** 2m

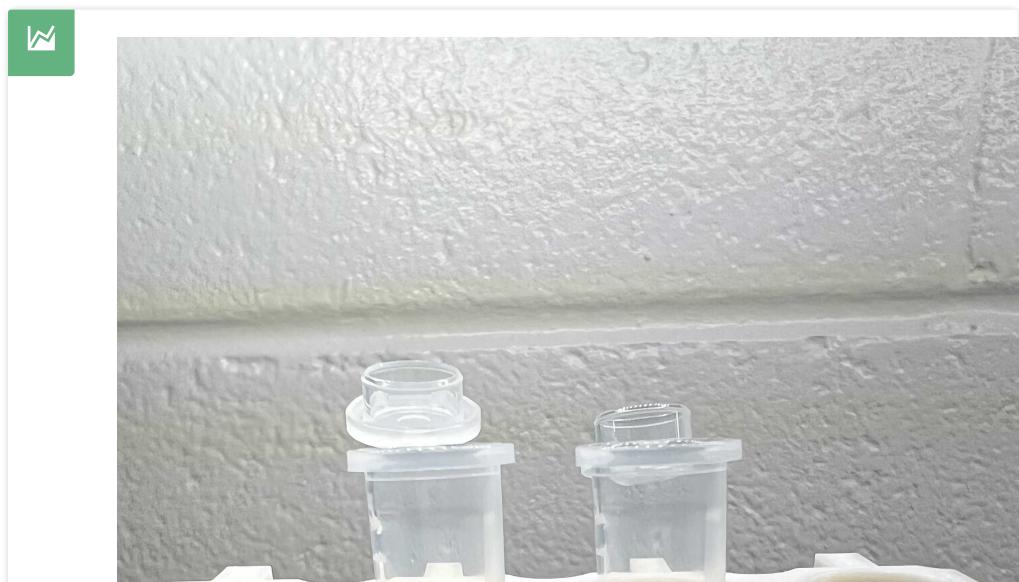
3.15 Put back on the rack and let pellet form

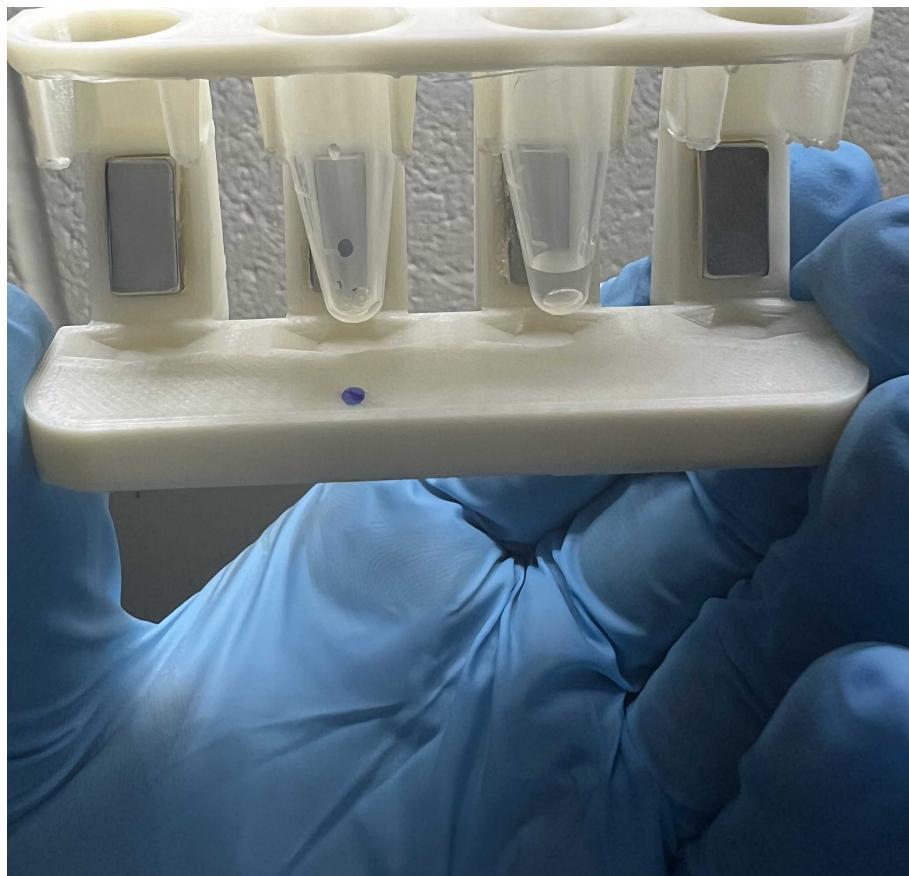




pelleted PCR product

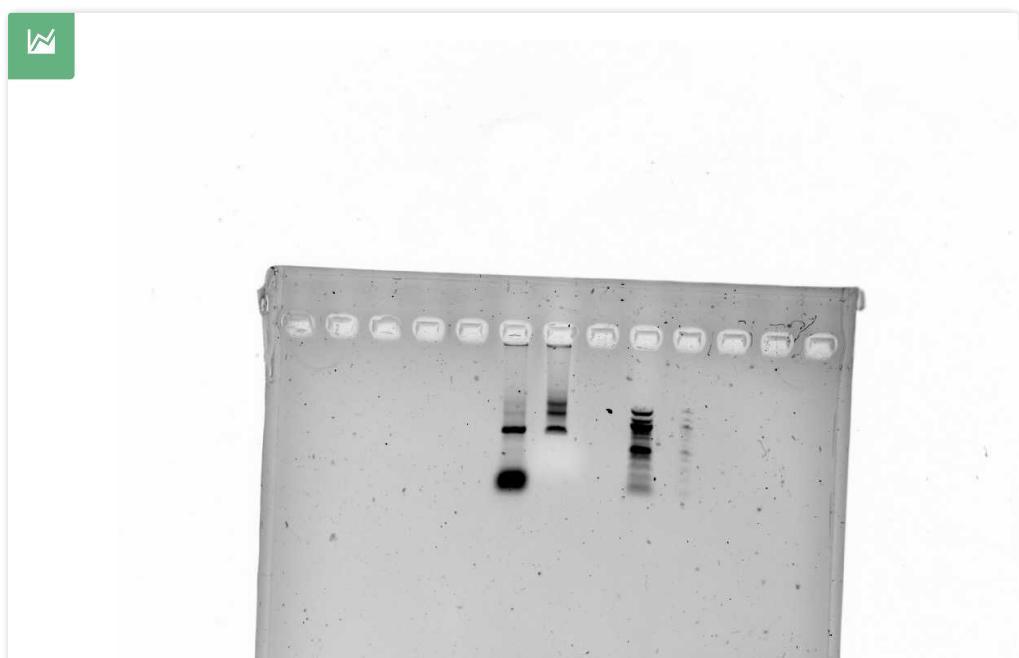
3.16 Remove liquid away from pellet-->this is our cleaned product





purified PCR product on right

3.17 Run in a gel compared to uncleaned product to evaluate success





First column is uncleaned product, second column is cleaned product, third column is 1 kb marker

- The clean was successful if the cleaned product does not have any bands below the Co1 band

3.18 Quantify cleaned PCR with

Quantas Fluorometer
Fluorometer
Promega E6150

Qubit Fluorometer (Quantas™ Fluorometer protocol listed below)

1. Mix **1 µL** of DNA sample with **200 µL** of **QuantiFluor ONE dsDNA Dye Promega** in a 0.5ml PCR tube.
2. Vortex
3. Place tube into the tube holder and close the lid
4. Vortex sample again and repeat to be sure you got an accurate reading

Library preparation

10m

4 Library preparation

Make sure to check flow cells before starting this step. There is a two-week lead time for Flongle flow cells so plan accordingly.

4.1 Dilute DNA-->concentration of DNA should be 100-200fmol amplicon DNA

The protocol listed below is provided by Oxford Nanopore Technologies, modified based on the recommendations given in Srivathsan et al. 2021.

Srivathsan A, Lee L, Katoh K, Hartop E, Kutty SN, Wong J, Yeo D, Meier R (2021). ONTbarcoder and MinION barcodes aid biodiversity discovery and identification by everyone, for everyone.. BMC biology. <https://doi.org/10.1186/s12915-021-01141-x>

 [amplicon-sqk-lsk109_protocol.pdf](#)

- When the concentration of our pooled and cleaned product was **[M]41 ng/µL**, we did **21 µL** of water and **1.5 µL** of DNA.
- When the concentration of the pooled and cleaned product was about **[M]80 ng/µL** and about **[M]94 ng/µL**, we did **21.5 µL** of water, **1 µL** of DNA

4.2 Combine in a strip-tube: **22.5 µL** of diluted DNA, **3.5 µL** of



Biolabs Catalog #E7647

, **1.5 µL** of



Biolabs Catalog #E7646

, and **2.5 µL** of H₂O

4.3 Heat at **20 °C** for **00:05:00**, **65 °C** for **00:05:00**

10m

4.4 Transfer mix into new 1.5 ml tube

4.5  **AMPure XP Beckman**

Vortex **Coulter Catalog #A63880**

4.6  **AMPure XP Beckman**

Add **30 µL** of **Coulter Catalog #A63880** to the mixture

4.7 Incubate on Hula mixer for **00:05:00**

5m

4.8 Spin down, place on magnet until clear

4.9 Keeping the tube on the magnet, pipette off supernatant

4.10 Add **100 µL** of 70% ethanol (do not touch pellet)

4.11 Remove ethanol

4.12 Add **100 µL** of 70% ethanol, take off magnet rack and spin down, put back on magnetic rack

4.13 Remove ethanol, allow to dry for about **00:00:30**

30s

4.14 Add **30.5 µL** of ddH₂O, remove from magnetic rack and resuspend pellet

4.15 Incubate off rack for 00:02:00

2m

4.16 Place back on magnetic rack until clear

4.17 Remove liquid and put in clean tube

Library preparation: Ligation

10m

5 Ligation

5.1

Adapter Mix (AMX) Oxford Nanopore

Spin down Technologies

and

NEBNext Quick T4 DNA Ligase New England

Biolabs

and put on ice.

Thaw and vortex

Ligation Buffer (LNB) Oxford Nanopore

Technologies

and put on

ice, thaw and mix

Elution Buffer (EB) Oxford Nanopore

Technologies

(EB) and put on

ice, thaw

Short Fragment Buffer (SFB) Oxford Nanopore

Technologies

(SFB),

vortex, put on ice

5.2 Combine in a new tube in this order: **30 µL** of DNA from previous step, **12.5 µL** of LNB, **5 µL** of **NEBNext Quick T4 DNA Ligase New England Biolabs**, and **2.5 µL** of AMX

5.3 Flick and spin mixture, incubate at room temp for **00:10:00** 10m

5.4 **AMPure XP Beckman**
Vortex **Coulter Catalog #A63880**

5.5 **AMPure XP Beckman**
Add **20 µL** of **Coulter Catalog #A63880** to mixture and flick tube

5.6 Incubate at room temp on Hula mixer for **00:05:00** 5m

5.7 Spin sample, put on magnetic rack, let pellet form, pipette off supernatant

5.8 Add **125 µL** of **Short Fragment Buffer (SFB) Oxford Nanopore Technologies**, flick tube to resuspend, spin down, return tube to magnetic rack, let pellet form, remove supernatant

5.9 Add **125 µL** of **Short Fragment Buffer (SFB) Oxford Nanopore Technologies**, flick tube to resuspend, spin down, return tube to magnetic rack, let pellet form, remove supernatant

I have found that the pellet does not stick to the magnet as strongly during this step as it does in previous steps, making it harder to pipette off the supernatant. Pushing the tube as close to the magnet as you can and tilting the rack while pipetting the supernatant off helps.

5.10 Allow to dry for about **⌚00:00:30**

30s

5.11 Add **15 µL** of **⊗ Elution Buffer (EB) Oxford Nanopore Technologies**, spin down and resuspend, incubate at room temp for **⌚00:10:00**

10m

5.12 Place on magnetic rack and allow pellet to form, remove liquid and transfer to a clean tube

I repeat this step twice to be sure it is fully cleaned

5.13 Quantify

Following same quantifying protocol as previously listed

5.14 Dilute so the concentration of the DNA library is 3-20fmol

When the concentration of the DNA library was **[M]1.2 ng/µL**, we diluted

3 µL DNA: **2 µL**

⊗ Elution Buffer (EB) Oxford Nanopore Technologies

When the concentration of the DNA library was **[M]1.4 ng/µL**, we diluted

2.9 µL DNA: **2.2 µL**

⊗ Elution Buffer (EB) Oxford Nanopore Technologies

Setting Nanopore Parameters

10m

6 Setting Nanopore Sequencing Parameters

- 6.1 We largely followed the default settings for the sequencing run. We set the sequencing kit to SQK-LSK-109, run time of 17 hours, and changed the FastQ so that it didn't compress the files.

Loading flow cell

10m

7 Loading flow cell

This is modified from

https://community.nanoporetech.com/docs/prepare/library_prep_protocols/gDNA-sqk-lsk109/v/gde_9063_v109_revam_25may2022/priming-and-loading-the-sp?devices=flongle

- 7.1 Priming buffer: mix **117 µL** of

FB	(Flush	Buffer) Oxford	Nanopore
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Technologies Catalog #EXP-FLP002

and **3 µL** of

FLT	(Flush	Tether) Oxford	Nanopore
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Technologies Catalog #EXP-FLP002

in a tube

- 7.2 Mix **15 µL** of **10 µL**

Sequencing Buffer II (SBII) Oxford Nanopore Technologies	of
---	----

Loading Beads II (LBII) Oxford Nanopore Technologies	and 5 µL of
---	--------------------

DNA library in a separate tube

7.3 Insert **120 µL** of the priming buffer into the flow cell

7.4 Insert **30 µL** of prepped DNA library into flow cell

Demultiplexing 10m

8 Demultiplexing

Full description of these procedures and the different options can be found here:

<https://github.com/asrivathsan/ONTbarcoder>

Creating Demultiplexing file 10m

- 8.1
- Create an excel sheet with 5 columns
 - First column: sample/specimen ID
 - Second column: forward tag
 - Third column: reverse tag
 - Fourth column: forward primer sequence

- When filling in sheet, be careful to not insert extra spaces or punctuation
- Columns 4 and 5 should be the same throughout the whole sheet
- If you extracted and amplified 288 specimens, you should have 288 rows

8.2 Save as a .txt file

 [Example demultiplexing text file.txt](#)

8.3 Merge resulting Fastq files

We used MergeFasta to do this.

<http://www.dnabaser.com/download/Merge%20Fasta/index.html>

8.4 Run ONTBarcoder <https://github.com/asrivathsan/ONTbarcoder> using default parameters.

8.5 View resulting CO1 sequences for further analyses.