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Uppsala Fungal Barcoding using ONT: DNA extraction -> Library preparation V1

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Protocol status: Working

We use this protocol and it's working

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Abstract

Abstract

This protocol was developed by Uppsala Svampklubb (fungal society), in cooperation with Evolutionary Biology Centre (EBC) at Uppsala University, as a cost efficient method to help amateurs with fungal species identification using DNA sequences.

The advantage of Nanopore technology is that over 2000 samples can be sequenced at the same time, thereby significantly lowering the cost per sample. The more that is sequenced at the same time, the cheaper it becomes per sample. The main challenge is to balance a high enough success rate with a low cost per sample, which is fairly optimized in this protocol, especially for agarics.

The protocol follows the process all the way from collecting fungi in the field to having a library of DNA ready for sequencing. It yields an 85% success rate for mushrooms and toadstools (fairly easy to sequence). Hydnoids, polypores and corticioids usually also works well. Ascomycetes are usually more difficult but works with some tweaking of the protocol.

Time required:

Time consumption is shown on a note in the fist step of each section. The table below summarizes the time consumption of the entire barcoding process; from collecting specimens to analysing and documenting DNA sequences.

Protocol section	Samples	hours	minutes/sample	time %
Fieldwork	96	14	9,0	18%
Collection handling	96	12	7,6	15%
Labwork - extraction, PCR, pooling, library prep	96	4,6	2,9	6%
Labwork - MinION sequencing	96	0,4	0,3	1%
Analysis, documentation	96	48	30,0	60%
Total	96	80	50	100%

Time consumption per 96 samples shown for the different sections of the process. Note that pre-lab work and analysis is significantly more time consuming per sample than the lab work.

Materials

Reagents

Mason & Botella (2020) extraction buffer:

(20 mM Tris (pH 8), 25 mM NaCl, 2.5 mM EDTA, 0.05% (wt/vol) SDS, and 2% (wt/vol) PVP-40).

-> Stored in room temperature

CITATION

Mason MG, Botella JR (2020). Rapid (30-second), equipment-free purification of nucleic acids using easy-to-make dipsticks..

LINK

<https://doi.org/10.1038/s41596-020-0392-7>

ONT Native Barcoding Kit 24 V14: (store.nanoporetech.com)

-> Stored in the freezer

 Molecular Water IBI Scientific Catalog #IB42130

 dNTP Mix 10 mM each Thermo Fisher Scientific Catalog ##R0191 (thermofisher.com)

-> Stored in the freezer

 Phusion Green High-Fidelity DNA Polymerase (2 U/µL) Thermo Fisher Catalog #F534L

(thermofisher.com): 4685 SEK. Enough for 51 PCR plates.

-> Stored in the freezer

 NEBNext Quick Ligation Module New England Biolabs Catalog #E6056S (neb.com):

4750 SEK. Enough for 100-200 barcodes.

-> Stored in the freezer

 NEBNext Ultra II End Repair/dA-Tailing Module - 24 rxns New England Biolabs Catalog #E7546S (neb.com):

3770 SEK. Enough for 96 barcodes.

-> Stored in the freezer

 Blunt/TA Ligase Master Mix New England Biolabs Catalog #E7373 in Kit E7370 or E7445 (neb.com)

Catalog #M0367S: 1500 SEK. Enough for 50 barcodes. We buy 2 at a time.

-> Stored in the freezer.

 Agencourt AmPure XP beads Contributed by users Catalog #A63880 (beckman.com):

4000 SEK. Enough for 36 barcodes. We buy 2 at a time.

-> Stored in the freezer.

Primers we use (see references for publications)

>Primer ITS1 (5'->3') White et al. 1990

TCCGTAGGTGAACCTGC

>Primer LR5 (5'->3') Hopple & Vilgalys 1994

TCCTGAGGGAAACTTCG

>Primer ITS4 (5'->3') White et al. 1990

TCCTCCGCTTATTGATATGC

>Primer ITS1-F_KY02 (5'->3') Toju et al. 2012

TAGAGGAAGTAAAAGTCGTA

Consumables

1.5 mL LowBind tubes - can be bought from e.g. Sarstedt or Eppendorf.

PCR 8-strip 0.2 mL (sarstedt.com): 2002 SEK. Enough for 40 PCR plates.

Pipette filter tip 2.5 µL (sarstedt.com): 1286 SEK. Enough for 20 PCR plates.

PCR plate without skirt (sarstedt.com): 1977 SEK. Enough for 100 PCR plates.

PCR lid strips (sarstedt.com): 492 SEK. Enough for 40 PCR plates.

Equipment

DNA LoBind tubes, 1.5 mL

NAME

Tubes

TYPE

Eppendorf

BRAND

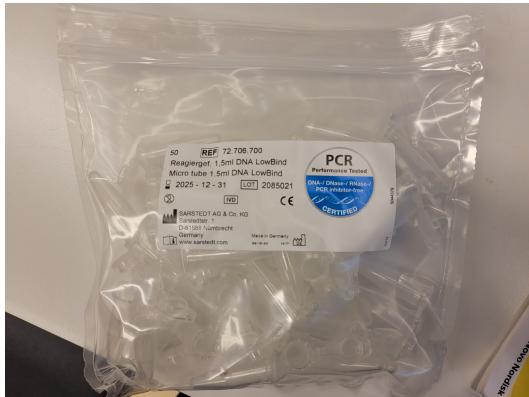
022431021

SKU

<https://online-shop.eppendorf.us/US-en/Laboratory-Consumables-44512/Tubes-44515/DNA-LoBind-Tubes-PF-LIN-K-56252.html>

1.5 mL

SPECIFICATIONS



The 1.5 mL LowBind tubes we use.

Equipment

Magnetic rack: ([Amazon](#)): 618 SEK

PCR tube rack x5 ([Amazon](#)) : 177 SEK

Further recommendations on equipment can be found in:

Protocol



NAME

Quick DNA Extraction for Fungal Barcoding (X-Amp)

CREATED BY

Stephen Douglas Russell

[PREVIEW](#)

Protocol



NAME

Preparing ONT-tagged Primers and Master Mix for Fungal DNA Barcoding

CREATED BY

Stephen Douglas Russell

[PREVIEW](#)

Protocol



NAME

ONT Post-PCR Pooling & Purification for Fungal Barcoding

CREATED BY

Stephen Douglas Russell

PREVIEW

Protocol materials

-  Short Fragment Buffer (SFB) **Oxford Nanopore Technologies** Step 75
-  NEBNext Quick Ligation Module **New England Biolabs Catalog #E6056S** Materials, Step 74
-  Phusion High Fidelity Master Mix with HF Buffer **Thermo Fisher Scientific Catalog #F531L** Step 11
-  dNTP Mix 10 mM each **Thermo Fisher Scientific Catalog ##R0191** Materials, Step 11
-  Elution Buffer (EB) **Oxford Nanopore Technologies** Step 75
-  NEBNext Ultra II End Repair/dA-Tailing Module - 24 rxns **New England Biolabs Catalog #E7546S** Materials, Step 37
-  Agencourt AMPure beads **Beckman Coulter** Step 21
-  Blunt/TA Ligase Master Mix **New England Biolabs Catalog #E7373** in Kit E7370 or E7445 Materials, Step 53
-  Agencourt AmPure XP beads **Catalog #A63880** Materials
-  Molecular Water **IBI Scientific Catalog #IB42130** Materials
-  Phusion Green High-Fidelity DNA Polymerase (2 U/µL) **Thermo Fisher Catalog #F534L** Materials

Fungal collecting, storing and documentation

1

Note

Time consumption: Field work and collection handling takes around 25-30 hours / 96 samples (1 PCR plate).

Before starting:

In order to maximize the chances that your barcoding is successful there are several things to consider when collecting, storing and documenting your fungal specimens. Collections coupled with a DNA sequence are also more valuable when donated to a herbarium (a voucher), in which case it is important to properly conserve them so that they meet the standards of the herbarium.

Some things to consider:

- When possible collect many fruit bodies in the field. Larger collections are better than small ones since material is constantly being used for research.
- Document the finding. Minimum parameters for herbarium donations are: date, region, locality and coordinates. The locality should be described as detailed as possible in case the coordinates are wrong. Specimens should preferably be determined to at least genus level.
- Specimens should be dried in 30-35 °C until they are completely dry. The optimal temperature depends on the type of fungus and other factors. DNA might be damaged if the temperature is too high, or the specimen is dried too quickly, or too slowly. If specimens are dried too slowly they also often get eaten by worms and insects. Room temperature is often sufficient for fungi with lower water content, e.g. many hard wood living fungi such as polypores and corticioids.
- Very large specimens should be sliced into several thinner pieces before drying in order to fit in herbarium sheets.
- If the fungus is an epiphyte on a fragile plant it is good to use a plant press or similar.
- Store the specimens as dark as possible as light exposure destroys DNA over time. Properly dried and stored collections lasts at least a few years before DNA deteriorates to a level that makes sequencing harder.
- Keep a database, e.g. an excel sheet, with all your data on the collected specimens. It is practical to assign personal collection numbers to your specimens which can be used to keep track of them when they enter the barcoding stage or are deposited to a herbarium.
- Report the finding to an observation database such as iNaturalist or Arportalen (in Sweden).

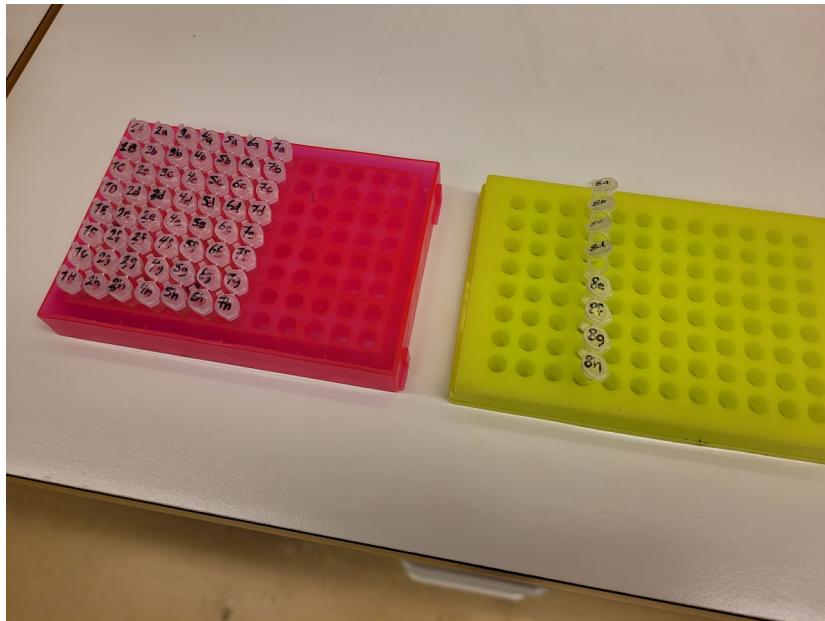
DNA extraction from dried fungi

2

Note

Time consumption: DNA extraction takes around 3-5 hours / 96 samples (1 PCR plate).

Start with laying out (96) 0.2 mL-tubes (12X 8-strips) with individual caps on a 96-well PCR plate rack. Label the tubes with their row and column number in the plate, i.e. a combination of columns 1-12 and row A-H (1A-12H). Also label each 8-strip with the plate number. It is convenient to use two plate racks, one as your current workspace and one with your completed strips.



Some labeled 8-strips ready for extraction.

3 Assign your fungal specimens to a row and column number in your excel data sheet.

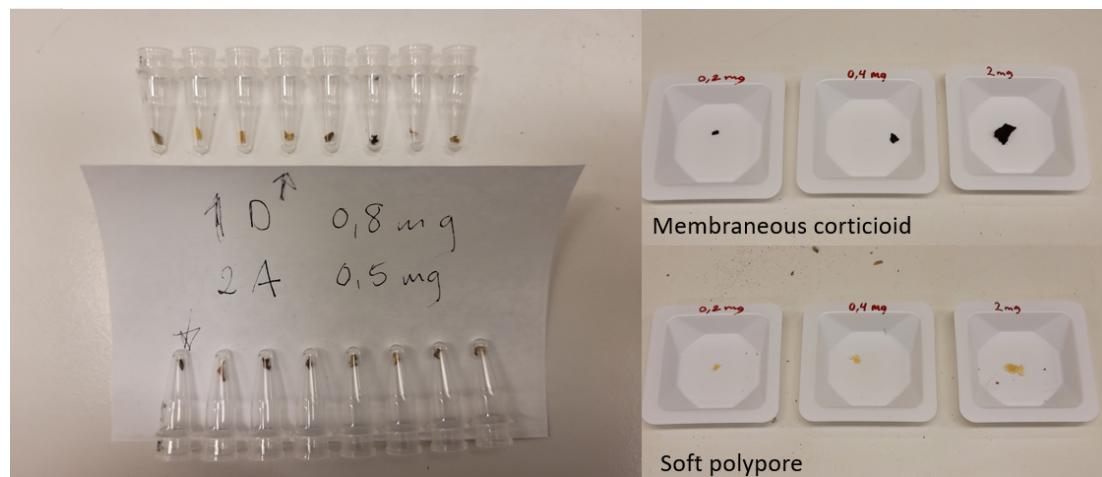
It is very important that you keep track of which collection belongs which tube. Without this information you will later have no way of knowing which DNA sequence belong to which collection.

4 Put the specimens in numerical order on your work bench with regards to the pairing of your personal collection numbers with the row-column (number-letter) combination on the 96 tubes. The collection assigned to tube 1A should be placed first in order etc. for efficient DNA extraction.

5 Consider performing step  go to step #6 before this step.

Put a very small piece of tissue in each tube. Usually  0.2 mg or 1-2 mm² is well enough for this manual and one can often use even smaller amounts. The amount of tissue can be changed for different species if needed but keep in mind that one may also need to change the amount of buffer when doing so in order to maintain optimal dilution. If you are new to this protocol it is a good idea to calibrate your weight/mass perception of different fungal species beforehand using a high resolution lab scale. Later, when doing this at home, you will then be better at estimating amounts. It is often preferable to choose tissue from the fertile part of the fungus, i.e. the hymenium containing spores.

Dried tissue can be broken off from the fungus using e.g. a scalpel or tweezers and a white paper. Choose a piece that is close to the desired size and put it in the tube. Toothpicks are very useful for putting small pieces of tissue into the tubes. Between specimens, wipe the scalpel or tweezer with paper. If you want to be extra careful, dip it in ethanol before wiping.



Different amounts of tissue for calibration. The left picture shows 0.8 vs. 0.5 mg of tissue in 0.2 mL-tubes, which can often be considered too much depending on dilution.



Tissue size to weight reference for a toadstool.

6 Pipette  10 μL of Mason & Botella (2020) extraction buffer (**20 mM Tris (pH 8), 25 mM NaCl, 2.5 mM EDTA, 0.05% (wt/vol) SDS, and 2% (wt/vol) PVP-40**) in each tube with tissue using a dispensing pipette or multipipette. This step can be done in the lab both before and after placing the tissue in the tubes. We recommend that it is done beforehand since the tissue is easier to stick in the tubes when there is liquid inside. Static electricity can otherwise make it difficult to handle small pieces of tissue in plastic tubes.

CITATION

Mason MG, Botella JR (2020). Rapid (30-second), equipment-free purification of nucleic acids using easy-to-make dipsticks..

LINK

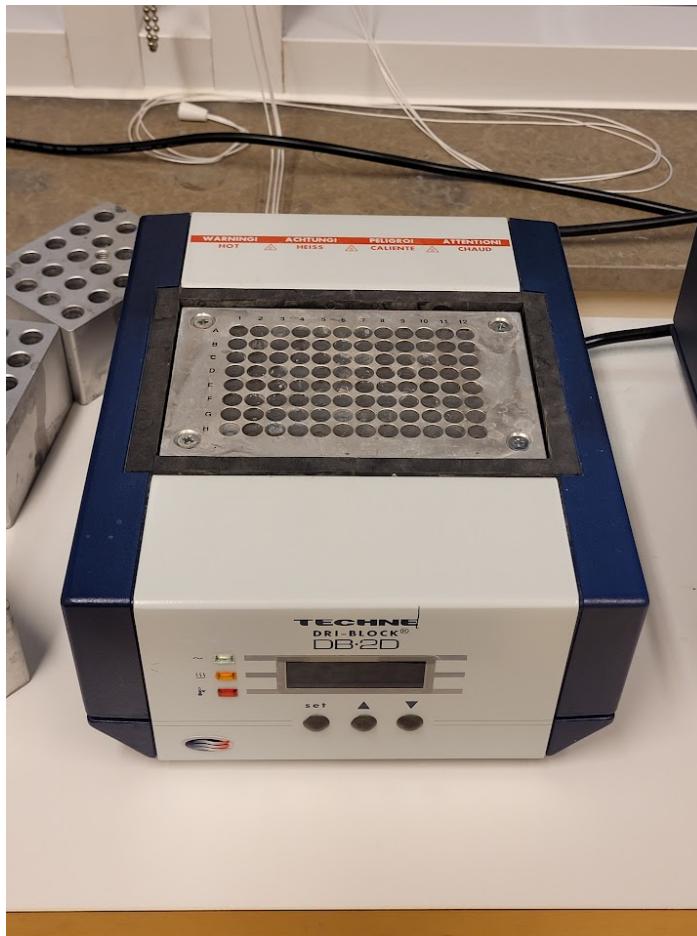
<https://doi.org/10.1038/s41596-020-0392-7>



Many steps during the extraction can be made easier using a dispensing pipette.

- 7 Spin down the strips in a mini centrifuge for 10 seconds to ensure that all the tissue and liquid is in the bottom. Make sure that the tissue is in the fluid and that no large air bubbles are left. Heat the tubes with the tissue for 01:00:00 to 01:30:00 at 80 °C on a dry heat block or in a thermocycler. The heat will break down the cell walls, releasing DNA.

2h 30m



Dry heat block for cell lysis.

- 8 Add 190 µL of nuclease-free water to the strips in order to dilute the samples.

-> The 1:50 dilution have proven to be sufficient for most mushrooms and toadstools. However, different genera often require different dilutions depending on the amount of DNA and PCR inhibitors present in the material. Strongly pigmented fungi often require a higher dilution. What method works the best for certain fungal groups is highly experience based. In some cases even a 1:1000 dilution may work better. In this step we have diluted the sample to 1:20. Later, in the PCR protocol, we also reduced the initial template volume from 1.4 to 0.5 in order to achieve a ~1:50 dilution. If you think that a higher dilution (>1:60) is necessary, you will need to use an extra set of tubes and pipette tips in this step (dilute further in new tubes).

- 9 **Save point:** The tubes with diluted DNA will be used in the following section (PCR protocol) and can be stored in the fridge overnight. For long term storage both the tubes with tissue and dilution can be stored in the freezer without degrading the DNA. The DNA will only degrade after repeated freezing and thawing which should not be a problem here.

PCR Protocol

Note

Time consumption: The PCR protocol, not including the making of a primer plate nor the optional gel electrophoresis, takes around 40 minutes / 96 samples (1 PCR plate). There is also a waiting time of 1.5 hours during the PCR run.

We will start this section by making a primer plate. The primer plate will cover the primer use for over 10 PCR plates and therefore only needs to be made once in a while. The instructions are based on the ITS1 forward primer (White et al. 1990) and LR5 reverse primer (Hopple & Vilgalys 1994) with 8 and 12 tags respectively. We have, however, also started using the ITS1-F_KY02 forward primer (Toju et al. 2012) for difficult ascomycetes and the ITS4 reverse primer (White et al. 1990) for old material.

- First time using the primers: Start with thawing your newly purchased primers. Add nuclease-free water to each of the primer tubes so that the resulting volume has a concentration of [M] 100 micromolar (μM). As each tube contains a different amount (moles) of primer, you will have to calculate the volume of water you need to put in each tube individually. See the picture below for an example.
- Vortex the tubes for a few seconds and spin down. You may want to repeat this step to make sure that the primer is evenly mixed.



Invitrogen Custom Primers
Certificate of Analysis

UPPSALA UNIVERSITET
Order Number: 026060 02
Order Date: 11/03/2024

Primer 1:

Primer Name:	ITS1FT0ju	Primer Number:	N6612A01 (A01)
Researcher:	PatrickFritzson	Primer Length:	21
Sequence (5' to 3')	TAG AGG AAG TAA AAG TCG TAA	Scale of Synthesis:	25n mol
Molecular Weight (μg/μmole):	6552.2	μg per OD:	24.4
Micromolar Extinction Coeff(OD/μmole)	268.4	nmoles per OD:	3.7
Purity	Desalting	OD's	6.50
Tm (1 M Na ⁺)	63	μg's*	158.68
Tm (50 mM Na ⁺)	41	nmoles	24.3
% GC	33	Coupling Eff.	99%
Notes:			

Primer 2:

Primer Name:	ITS1F051	Primer Number:	N6612A02 (A02)
Researcher:	PatrickFritzson	Primer Length:	21
Sequence (5' to 3')	GGT AGT CAG ACG ATG CGT CA	Scale of Synthesis:	25n mol
Molecular Weight (μg/μmole):	13100.4	μg per OD:	23.1
Micromolar Extinction Coeff(OD/μmole)	503.6	nmoles per OD:	3.7
Purity	Desalting	OD's	6.50
Tm (1 M Na ⁺)	83	μg's*	158.68
Tm (50 mM Na ⁺)	61	nmoles	24.3
% GC	43	Coupling Eff.	99%
Notes:			

Primer 3:

Primer Name:	ITS1F052	Primer Number:	N6612A03 (A03)
Researcher:	PatrickFritzson	Primer Length:	42
Sequence (5' to 3')	GGT AGC TAT ACA TGA CTC TGC TAG AGG AAG TAA AAG TCG TAA	Scale of Synthesis:	25n mol
Molecular Weight (μg/μmole):	13035.4	μg per OD:	26.2
Micromolar Extinction Coeff(OD/μmole)	496.7	nmoles per OD:	2.0
Purity	Desalting	OD's	11.80
Tm (1 M Na ⁺)	82	μg's*	309.68
Tm (50 mM Na ⁺)	60	nmoles	23.7
% GC	40	Coupling Eff.	99%
Notes:			

FOR LABORATORY RESEARCH USE ONLY.
CAUTION: Not for diagnostic use. The safety and efficacy of this product in diagnostic or other clinical uses has not been established.

invitrogen
by Thermo Fisher Scientific

Using the nanomole quantity - to reconstitute to a given concentration, convert the nmole figure to umole, and then divide by the desired concentration in umole/litre. For example, to make a 100 umole primer stock solution, assuming 24nmole yield:
 $24\text{nmole} \times 1\text{umole}/1000\text{nmole} = 0.024\text{ umole}$
 $0.024\text{umole}/100\text{umole/litre} = 0.00024\text{ L}$
 $0.00024\text{ L} \times 1000\text{mL/L} = 0.24\text{ml or } 240\text{μL}$

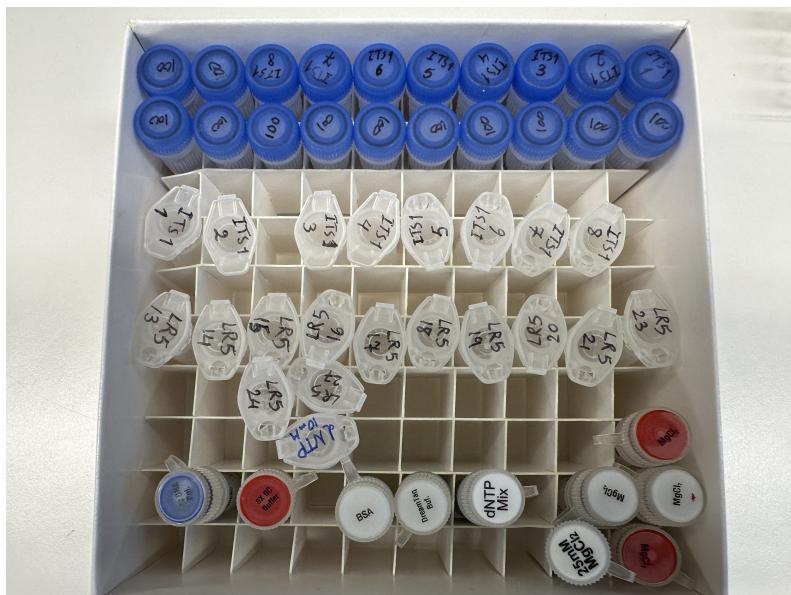
* Other supporting information available on-line.

0.024umole/100umole/litre = 0.00024 L
0.00024 L × 1000 = 0.24 L

The custom primer certificate should come with instructions on how to create a 100 μM solution. In this case add water (in μL) x10 the nmole quantity highlighted in red for each primer, i.e. if the quantity is 24 nmole, add 240 μL of water to the tube.

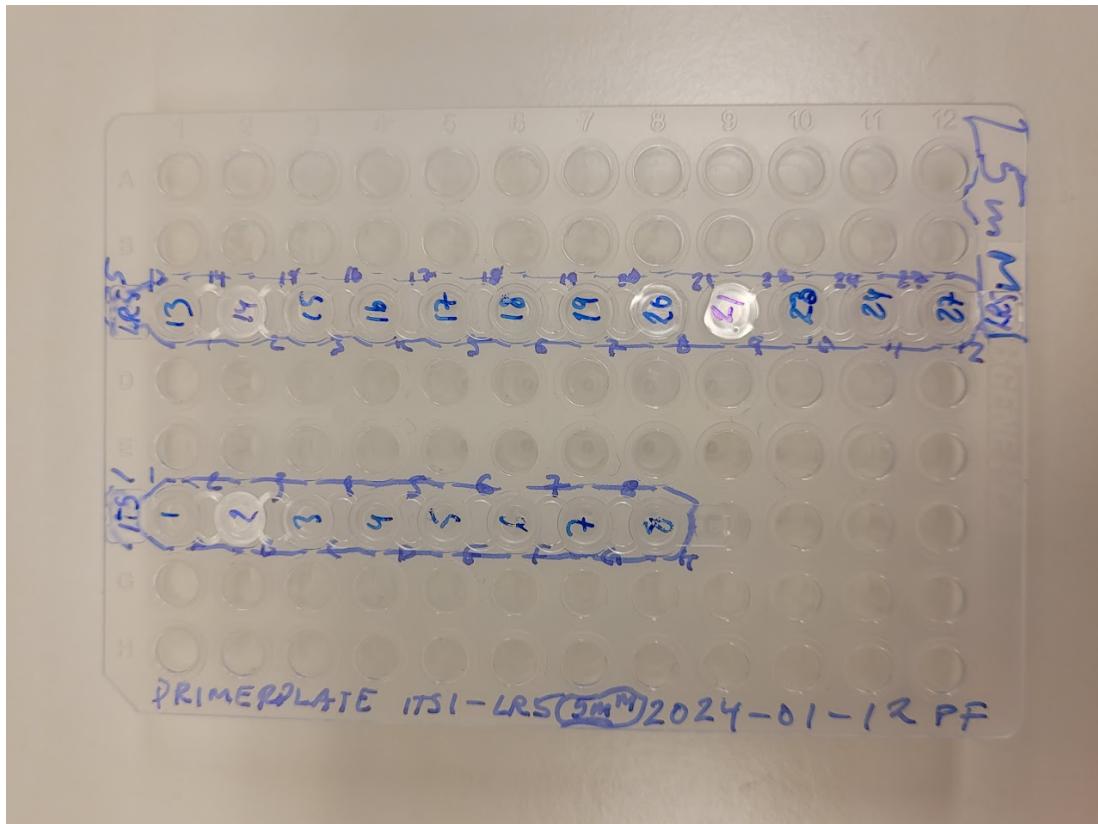
- Prepare (20) 1.5 mL tubes; 12 for the LR5 reverse primers and 8 for the ITS1 forward primers. Label them according to the tag number.

- Make a 1:20 dilution ($[M]$ 5 micromolar (μM)) with nuclease-free water in the new tubes by pipetting a volume of choice from the 100 μM tubes, e.g. $\text{50 } \mu\text{L}$ of primer + $\text{950 } \mu\text{L}$ of water. Your primer setup should look something like the picture below.
- Vortex the 1.5 mL tubes for a few seconds and spin down.



The blue tubes are the purchased primers; 12 LR5 reverse primers with different tags (labeled "100" here for 100 μM) and 8 ITS1 forward primers with different tags. The tubes below them are the 1.5 mL tubes we just filled with 5 μM primer solution.

- Prepare an empty PCR plate (a plate with 96 cells).
- Place an 8-strip lid horizontally over one of the rows. Cut an 8-strip lid in half to a 4-strip and place it next to the 8-strip to fill out the row with lids. Label the 12 lids with the LR5 reverse primer number (tag) you will use in each cell.
- On another row, place a new 8-strip lid. Label the 8 lids with the ITS1 forward primer number (tag) you will use in each cell.
- Transfer $\text{150 } \mu\text{L}$ of $[M]$ 5 micromolar (μM) primer solution from the 1.5 mL tubes to the corresponding cell on the primer plate.
- Label the primer plate with primer information, date and concentration. It should now look something like the picture below.



A finished primer plate. The ITS1 forward primer with 8 different tags is in the bottom row while the LR5 reverse primer with 12 different tags is in the top row.

11 For the PCR protocol in this manual we use a PHUSION mastermix:

 Phusion High Fidelity Master Mix with HF Buffer **Thermo Fisher Scientific Catalog #F531L**

and dNTP:s from:

 dNTP Mix 10 mM each **Thermo Fisher Scientific Catalog ##R0191**

Start with preparing the mastermix. The amount of mastermix you will need depends on how many PCR plates you prepare on the occasion. Use the table below to calculate how much of each reagent you will need. We recommend that you prepare a bit more mastermix than is needed on paper since some will get stuck in the tube and pipette. 7% or 9% extra solution have been proven to be sufficient for us. **Important:** Wait with adding the polymerase to just before you are going to add mastermix to the PCR plate

( go to step #15). The mix with the other reagents can sit on ice until then.

Reagent	Vol (µL)			
	1 cell	1 plate	+7%	+9%
ddH2O	7.64	733	785	800
10 mM dNTP	0.42	40	43	44
Phusion HF buffer	2.8	269	288	293
Phusion DNA polymerase	0.14	13.4	14.3	14.6
SUM	11	1056	1130	1151

Reagent volumes when preparing PHUSION mastermix. The volumes are shown for 1 cell, 1 plate and for 1 plate with an additional 7% and 9% solution. We use Milli-Q water instead of double distilled water (ddH2O).

Prepare a tube that can hold the volume of mastermix that you will need. Pipette the reagents into the tube. Gently tilt the tube 180 degrees back and forth to mix the reagents. The mastermix is now ready for use.

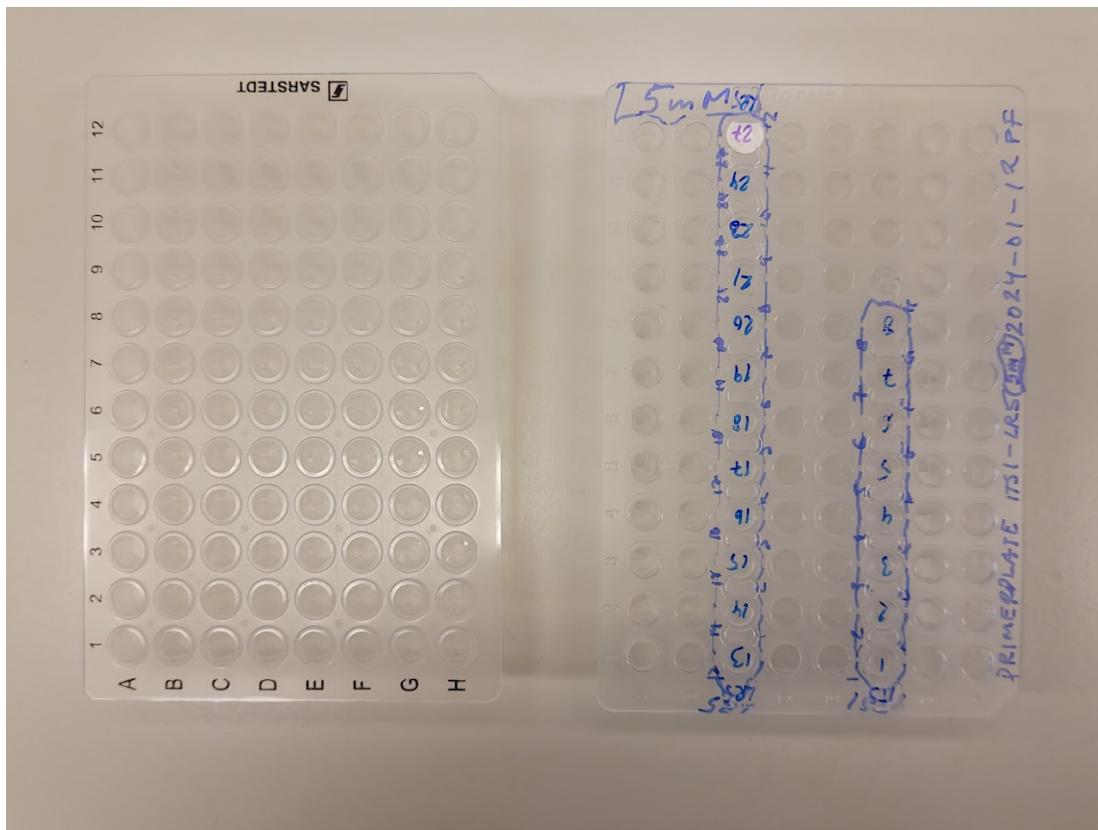


A pack of Phusion master mix includes several reagents including DMSO, which come in separate tubes. We only use the two shown above; the phusion DNA polymerase and the green HF buffer.

- 12 Prepare an empty PCR plate (a plate with 96 cells). Add  1.4 µL of the LR5 reverse primer to each cell using a multichannel pipette.



-> Be sure to align the primer plate and the PCR plate (columns) to avoid mistakes. As the multichannel pipette can only transfer primers to 8 columns (cells) at a time, pipetting the 12 LR5 reverse primers must be done in two rounds; one with 8 pipette tips and one with 4. One can use the same pipette tips within the same round (same primers) but **NOT** between rounds (different primers).



The PCR plate (left) and primer plate (right) horizontally aligned by columns 1-12. In this step primers are pipetted from the left blue lane on the primer plate to the corresponding columns on the PCR plate.

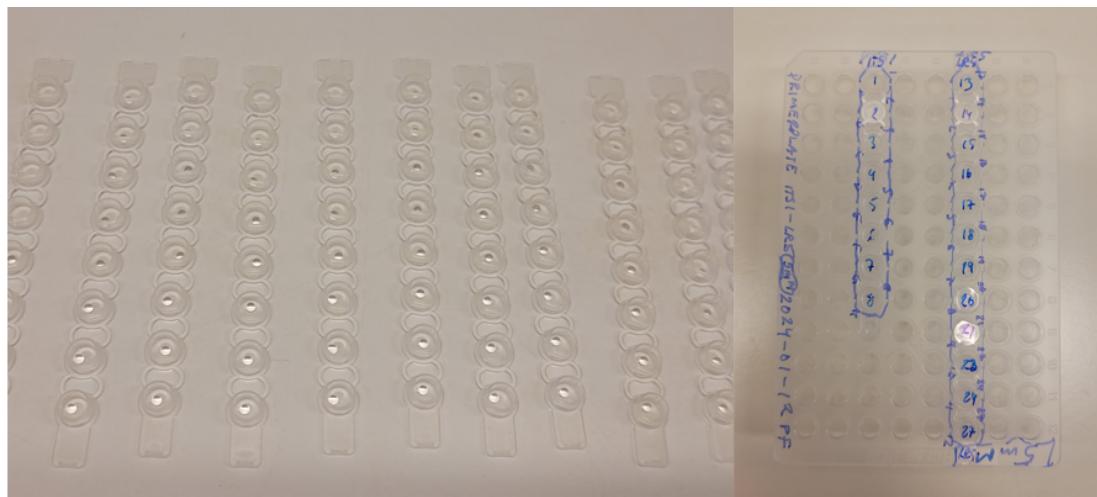
- 13 Vortex the 8-strips containing your DNA template for a few seconds to ensure even concentration of DNA. Then add $\text{PCR} \quad 0.5 \mu\text{L}$ of DNA template to each cell in the PCR plate using a multichannel pipette. Make sure that the position of each specimen stays the same (1A-12H).

-> We reduced the initial template volume (1.4) to 0.5 in order to reach a 1:50 dilution without having to use an extra set of tubes and pipettes in PCR go to step #8

- 14 Place your 12 PCR plate lids (8-strip lids) upside down on the workbench. Prepare the next primer in your primer pair (FÖRKLARING) by pipetting $\text{PCR} \quad 1.4 \mu\text{L}$ of the primer into the PCR plate lid using a multichannel pipette. This will ensure that primers do not come into contact with

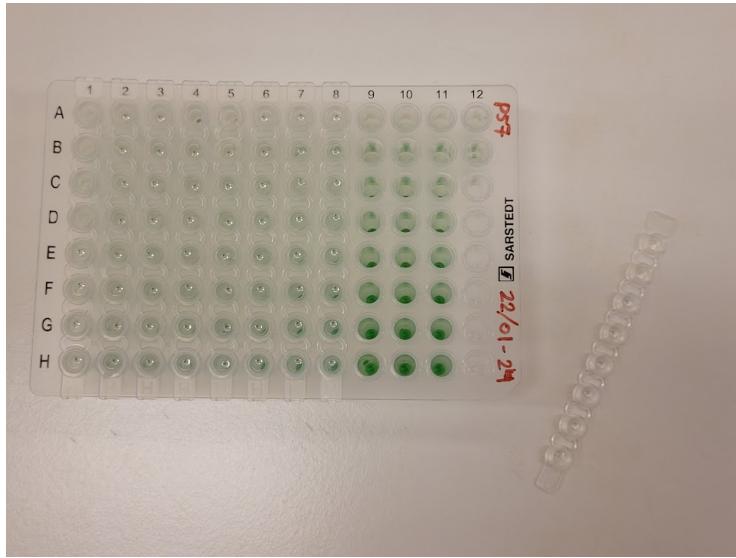
each other in the plate cells while you prepare the PCR plate. This is important as primers attach to each other over time rendering them useless.

Important: It is important that the master mix is ready and that you immediately proceed to the next step after performing this one. The small primer drops are prone to evaporate quickly so make sure that they are not left out for too long.



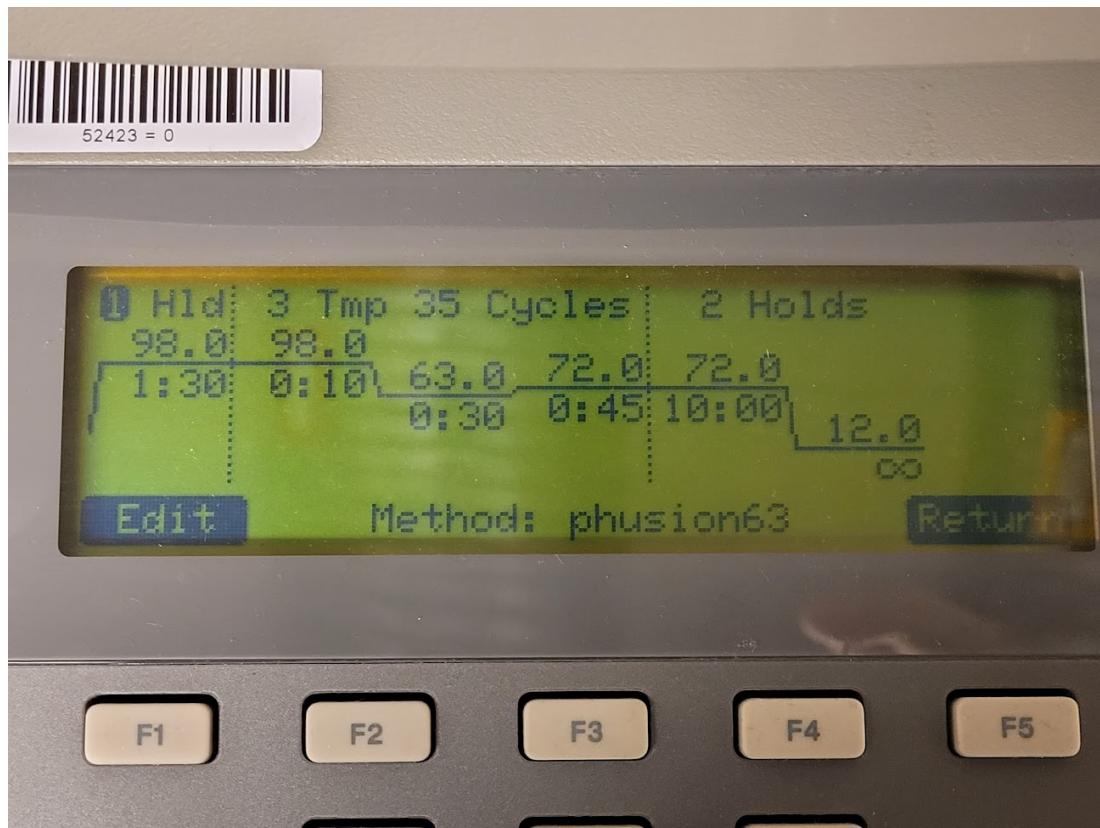
PCR plate lids placed upside down with a 1,4 µL primer drop (left) and primer plate (right) vertically aligned by rows 1-8 based on the left blue lane containing the 8 ITS1 forward primers. This alignment does not only make it easier, it is also important for ensuring that each primer goes into the correct cells.

- 15 Add the Phusion DNA polymerase to the mastermix. It is now ready to be added to the PCR plate.
- 16 Add  11 µL of the mastermix you prepared in step  go to step #11 to each cell on the plate using a dispenser pipette. Be careful when using the dispenser pipette! Make sure not to get air bubbles in the liquid which will cause the pipette to dispense uneven amounts between cells. The same will happen if one tries to use all the liquid in the pipette before refilling it. Always make sure to refill the pipette before it almost runs out so that this does not happen.
- 17 Carefully place the lids containing the second primer on the PCR plate. The cells now contain  14.3 µL of mastermix, template and primers and the plate is ready for PCR. Before going to the thermocycler (PCR machine), vortex the plate for 5-7 seconds and then centrifuge it up to 4000 rpm.



An almost ready PCR plate with green phusion mastermix and primers in the lids. By vortexing and centrifuging the plate, the primers in the lids will mix with the other reagents.

- 18 Set the PCR-machine according to the picture below. Annealing temperature should be  63 °C and the number of cycles should be 35. Set the reaction volume to 14 µL. Start the PCR machine. The program will take around  01:30:00 .



The PCR program used in this protocol. Note that the annealing temperature depends on the primers used.

Optional: If you want to check if the PCR was successful you can perform gel electrophoresis on the entire PCR plate. We strongly recommend this in your early experiments as many things can go wrong during the extraction and PCR process. From performing electrophoresis you will also know the amplification success rate in your plate which will be useful when calculating the amount of DNA you have in later stages.

- 19 **Save point:** The primer plate should be stored in the freezer. The PCR plate can after amplification be stored in the fridge for short term storage (a few days) and in the freezer for long term storage. **IMPORTANT:** The PCR plate can not be stored before amplification as the primers will malfunction.

Post PCR pooling & cleaning

20

Note

Time consumption: Pooling and cleaning takes 1-2 hours depending on how many PCR plates you work with.

This section, along with the "End-prep", "Native barcode ligation" and "Adapter ligation and clean-up" sections will go smoother if the needed tubes are prepared and labeled beforehand. Keep in mind that one tube for each PCR plate is needed, until the end of the "Native barcode ligation" section when DNA from all the plates is pooled together (right most tubes in the picture below). It is a good idea to label the tubes with plate number and barcode number, especially the latter (barcodes are explained in step [go to step #55](#)).

Tube guide

- > X stands for the number of PCR plates you want to sequence
- > all tubes with contents from a single PCR plate should be labeled with plate number and barcode number
- > When performing Qubit, 2 additional tubes to the samples are always needed for the standards

Post PCR pooling & cleaning

- (X) 0.2 mL 8-strips
- (X) 1.5 mL LowBind tubes -> for pooling
- (X) 1.5 mL LowBind tubes -> for cleaning
- (X) 1.5 mL LowBind tubes -> cleaned
- (X+2) Qubit tubes

End-prep

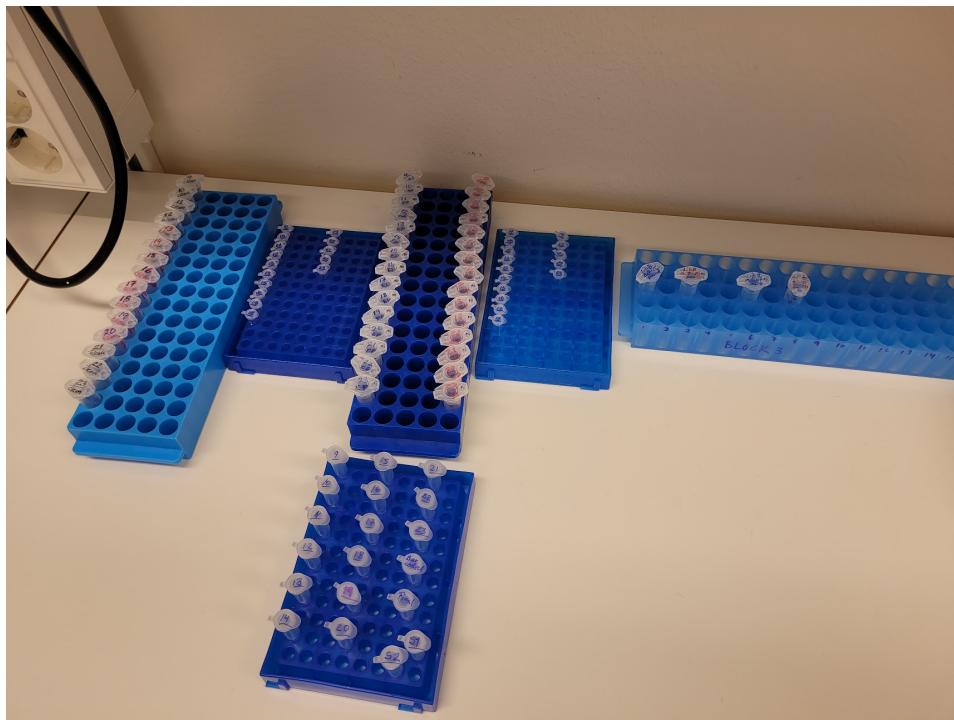
- (X) 0.2 mL thin-walled tubes -> for end-prep
- (X) 1.5 mL LowBind tubes -> for cleaning
- (X) 1.5 mL LowBind tubes -> cleaned
- (X+2) Qubit tubes

Native barcode ligation

- (X) 0.2 mL thin-walled tubes -> for barcoding
- (1) 1.5 mL LowBind tube -> for pooling & cleaning
- (1) 1.5 mL LowBind tube -> cleaned
- (3) Qubit tubes

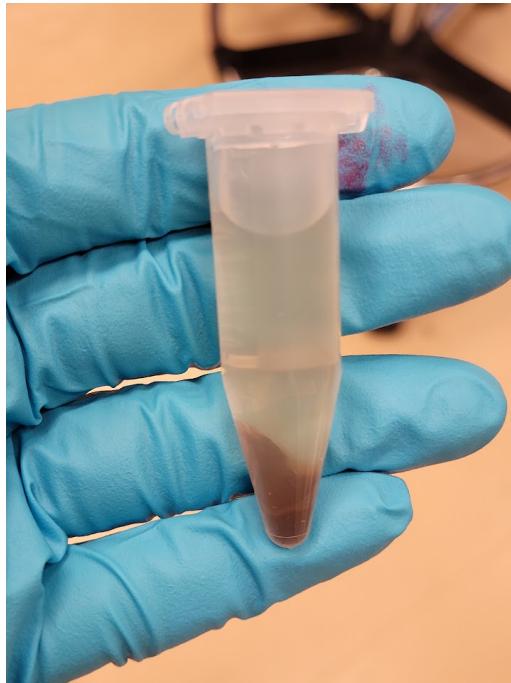
Adapter ligation & clean-up

- (1) 1.5mL LowBind tube -> for adapter ligation & cleaning
- (1) 1.5 mL LowBind tube -> cleaned library
- (3) Qubit tubes
- (1) 1.5 mL LowBind tube -> finished library



Several series of 1.5 mL Lowbind tubes, 0.2 mL thin-walled tubes and Qubit tubes prepared beforehand. The tubes in the vertical rows each correspond to a PCR plate. In the final stages of this protocol, only one tube is needed for each step since the plates are pooled together at the end of the "Native barcode ligation" section. OBS: this picture does not show ALL the required tubes listed above.

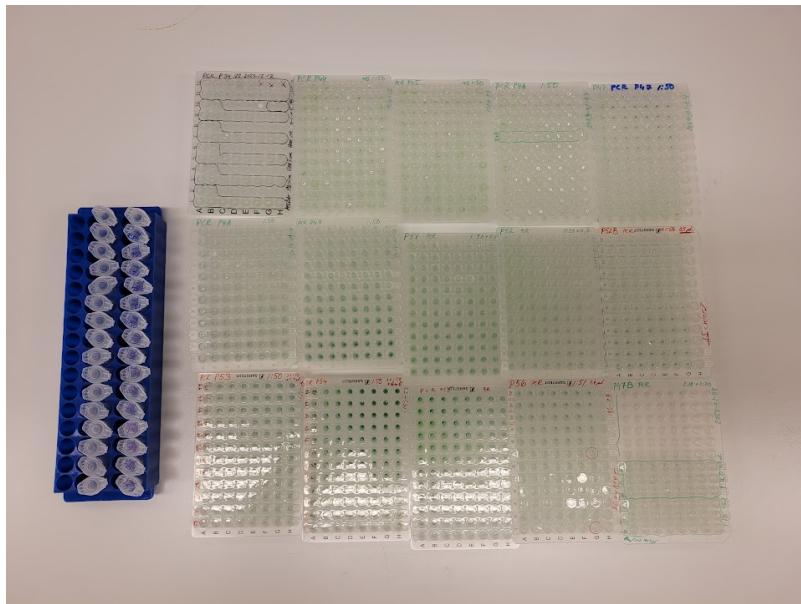
- 21 The  Agencourt AMPure beads **Beckman Coulter** magnetic beads, which are stored in the fridge, should be brought to room temperature. Initially the beads are in the bottom of the bottle and need to be suspended by shaking it.



AMPure bead sediment which needs to be suspended by shaking, vortexing, tapping the bottle etc.

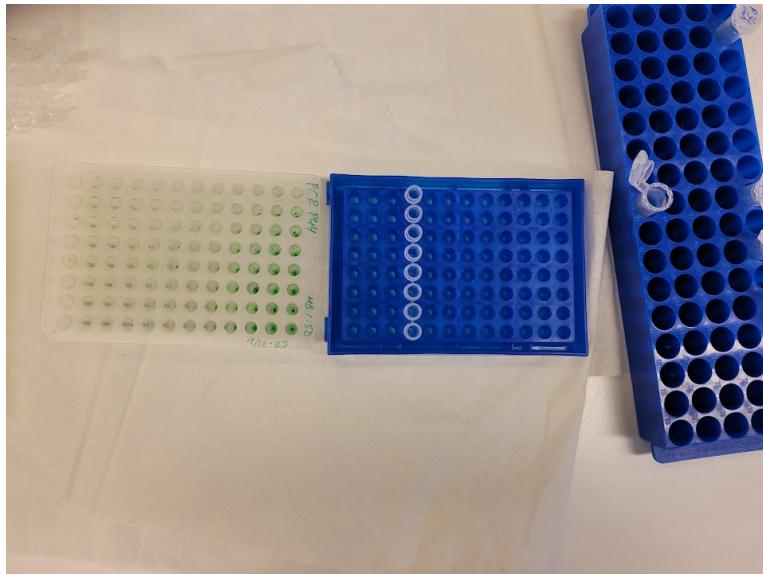
- 22 At this point you should have a number of ready PCR plates with amplicons. The following steps are done for every plate. Quickly centrifuge the PCR plate so that there is enough amplicons in the bottom of the cells.

Start by pooling  3 μL from each cell on the PCR plate into a new 0.2 mL 8-strip using a multichannel pipette. This can be done without changing the pipette tips between columns since they will be pooled together anyway. After this, every tube in the 8-strip should contain the contents from 12 cells in its corresponding row in the PCR plate ($12 \times 3 = 36 \mu\text{L}$). Keep the PCR plate with the remaining amplicons in case something goes wrong later.



Ready PCR plates with amplicons that will be pooled into their corresponding tube on the left.

- 23 Pool the contents of the 8-strip into a single 1.5 mL LowBind tube with a pipette. The tube will then contain $96 \times 3 = 288 \mu\text{L}$ of amplicon solution, i.e. the contents from an entire PCR plate (unless the plate was not completely filled). After this step you should have an equal amount of 1.5 mL tubes to PCR plates.



Amplicons are first pooled into an 8-strip using a multichannel pipette. The contents of the 8-strip are then pooled into a 1.5 mL tube.

24 The following instructions are modified from: [AMPure XP Protocol](#).

In this step you will transfer X µL from the amplicon pool to a new 1.5 mL LowBind tube. The volume you transfer depend on how much DNA there was in your PCR plate. A PCR plate with a low success rate (fewer cells with successful amplification = less total DNA) of around 30% may require 300 µL, while a high success rate may require 100 µL. If you performed gel electrophoresis on the plate you will know the success rate. Otherwise it can often be estimated by the proportions of easy vs. difficult fungi in the plate. Mushrooms, toadstools etc are relatively easy while wood-living fungi, hydnoids and many ascomycetes are examples of more difficult groups, often with lower success rates in this protocol. Estimating which species are easy vs. difficult requires some experience.

IMPORTANT: Save both the pool tubes and the LowBind tubes containing your pooled amplicons. Make sure to properly label them!

- 25 **Save point:** Store the pool tubes in the fridge until you have successfully completed the protocol. If something goes wrong during the rest of the protocol, the pool tubes are your lifeline and you can start over from this point. When the protocol is completed, they can be stored in the freezer.
- 26 The magnetic beads should have been brought to room temperature and are ready to use. Shake the magnetic bead bottle to resuspend any magnetic particles that may have settled. Then add bead solution to the LowBind tube with amplicons. The amount you add should be **1,8X** the amount of amplicons you transferred in the previous step. Pipette the mix up and down in the tube 10 times to thoroughly mix it and the incubate for  00:05:00 at room temperature. DNA is now stuck to the beads.
- 27 Spin down the tube for  00:00:02 in a mini centrifuge. Place the tube on a magnetic rack/magnetic separator (see materials section) and let the beads separate from the solution during  00:02:00. When all the beads are stuck to the magnet, proceed to the next step.



Magnetic rack/separator with the pooled amplicons and bead solution in the process of separating.

- 28 Remove the solution using a pipette and discard it. Be careful not to come in contact with the beads. It is not important that 100% of the solution is removed in this step. Instead, be careful not to suck beads into the pipette.



Beads are stuck to the magnet and the solution is ready to be removed.

- 29 It is now time to wash the beads with 70% ethanol. Use an ethanol volume that is at least the total volume of the sample and the bead solution you removed in the previous step, and a minimum of  200 μL . Pipette the ethanol into the tubes and incubate for  00:00:30. Then remove and discard the ethanol.

Important: Freshly prepare the ethanol yourself by mixing >99% ethanol and nuclease-free water in 0.7/0.3 proportions.

- 30 Repeat the previous step  go to step #29 . In this step it is important to remove **all** the ethanol. Let the tubes dry for  00:00:30 to evaporate small traces of ethanol. Be careful not to dry the beads any longer since they will crack and clump together making it hard to elute them.
- 31 Remove the tubes from the magnets and add  30 µL of molecular water. Pipette mix at least 10 time, making sure that the beads are homogenously eluted in the water. Incubate for  00:02:00 .
- 32 Place the tubes back on the magnets for  00:01:00 to separate the beads. Transfer the eluate to a new 1.5 mL LowBind tube. **OBS: the eluate contains the DNA and must not be discarded!**



The beads are separated from the eluate, which is ready for transfer to a new tube.

- 33 Make sure that the eluate contains enough DNA for the End-prep section  go to step #38 . This can be measured using a Qubit fluorometer.



Quantifying DNA using a Qubit fluorometer.

- 34 **Save point:** Tubes with cleaned amplicons can be stored in the fridge until the start of the next section.

End-prep

35

Note

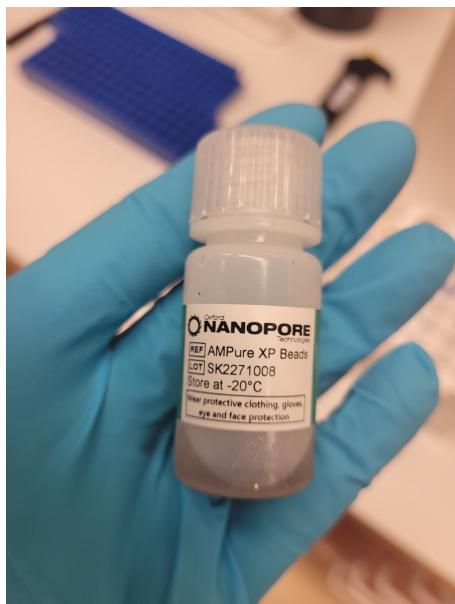
Time consumption: End-prep takes around 2-3 hours depending on how many samples you work with.

This section, along with the "Native barcode ligation" and "Adapter ligation and clean-up" sections require reagents from the ONT Native Barcoding Kit 24 V14, and the following protocol is modified from the manuals provided with the kit: [Native barcoding protocol](#)



Native Barcoding Kit 24 V14, and some tubes with the final product ready for nanopore sequencing.

36 Thaw the AMPure XP beads at room temperature.

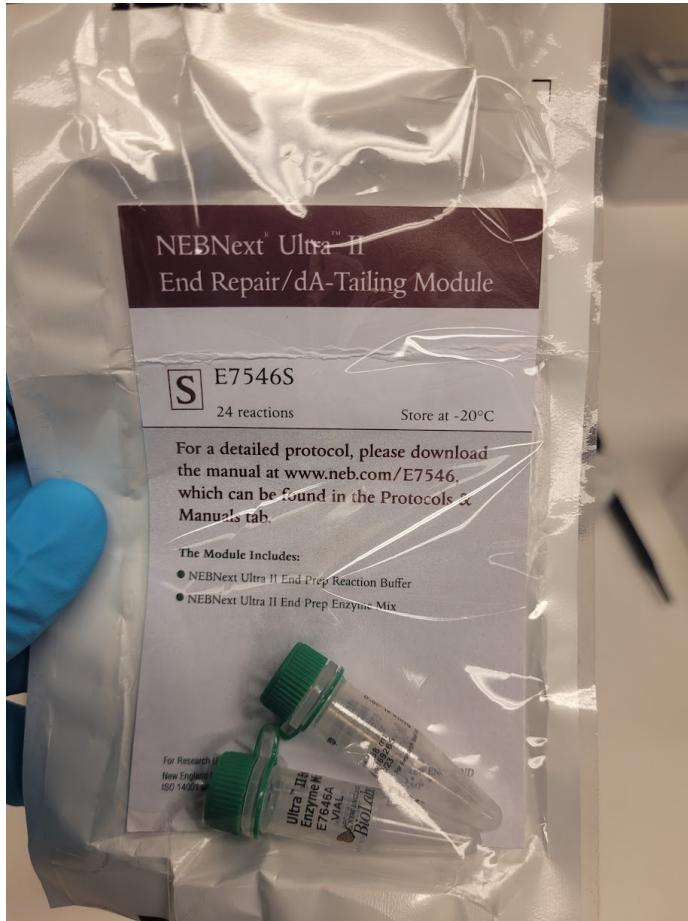


AMPure XP bottle.

37 Thaw the Ultra II reagents on ice. Flick or invert the tubes to ensure that they are well mixed. **Do not vortex the Enzyme mix!** Spin down the tubes before opening. The Reaction buffer should be brought to room temperature, then pipette mixed several times. Contrary to the enzyme mix, it should then be vortexed for at least 30 seconds before usage. Make sure that potential precipitate in the bottom of the tube is solubilized.

Reagents:

 NEBNext Ultra II End Repair/dA-Tailing Module - 24 rxns **New England Biolabs Catalog #E7546S**



Ultra II End Prep Enzyme Mix and Reaction Buffer.

- 38 Using a maximum of $\text{12.5 } \mu\text{L}$, transfer 200 fmol of DNA (195 ng for 1.5 kb amplicons) from the eluate in step [#32](#) to new 0.2 mL thin-walled PCR tubes. If less than $\text{12.5 } \mu\text{L}$ is needed, make up the rest with nuclease-free water.

=> In this step you need to know approximately how much DNA your cleaned samples contain.

- 39 Add $\text{1.75 } \mu\text{L}$ of Ultra II End-prep Reaction buffer to the new tubes. Pipette mix 10-20 times.

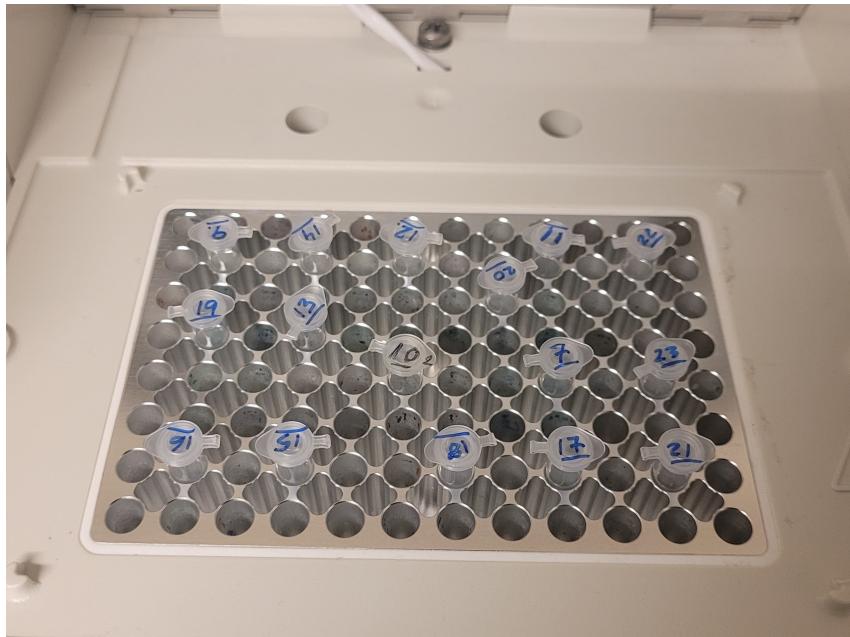
Then:

Add  0.75 μ L of Ultra II End-prep Enzyme Mix to the tubes. Pipette mix 10-20 times.

=> The easiest way to do this is to make a master mix with the Ultra II buffer and enzyme mix. Then adding 2.5 μ L to each tube.

40 The total volume in the tubes should now be  15 μ L. Mix by pipetting and spin down in a mini-centrifuge.

41 Incubate in a thermal cycler at  20 °C for  00:05:00 followed by  65 °C for  00:05:00 .



Thin-walled tubes with End-prep reagents placed in a thermocycler.

42 Transfer each sample into a 1.5 mL LowBind tube.

43 Resuspend the AMPure XP beads by vortexing. Then add  15 μ L to each tube and mix by flicking the tube. Incubate on a rotator mixer for  00:05:00 at room temp. If you don't have access to a rotator mixer, hold the tubes on a rack and wave them around in a circular motion.

- 44 Prepare a sufficient volume of 80% freshly prepared ethanol in nuclease-free water for step
 go to step #46 .
- 45 Spin down the samples and place them on the magnetic rack until the eluate is clear. Pipette off the eluate and discard it.
- 46 Carefully wash the beads with  200 µL of fresh 80% ethanol. Then remove the ethanol using a pipette and discard it. If you disturb the beads, wait for them to gather at the magnet again before removing the ethanol.
- 47 Repeat the previous step  go to step #46 . Briefly spin down the tubes and put them back on the magnets. Pipette off any residual ethanol. Let the tubes dry for  00:00:30 . **Do not wait longer than this since the the beads will crack and clump together!**
- 48 Remove the tubes from the magnets and resuspend the beads in  10 µL nuclease-free water. Pipette mix until the beads are homogenously suspended. Spin down and incubate for  00:02:00 at room temp.
- 49 Place the tubes back on the magnets until the eluate is clear.
- 50 Pipette  10 µL of the clear eluate into clean 1.5 mL LowBind tubes. **OBS: The clear eluate contains DNA and must not be discarded!**
- 51 It is a good idea to check if there is sufficient DNA concentration in each sample using a Qubit fluorometer.
- 52 **Save point:** End-prepped DNA can be stored in the fridge until the start of the next section.

Native barcode ligation

53

Note

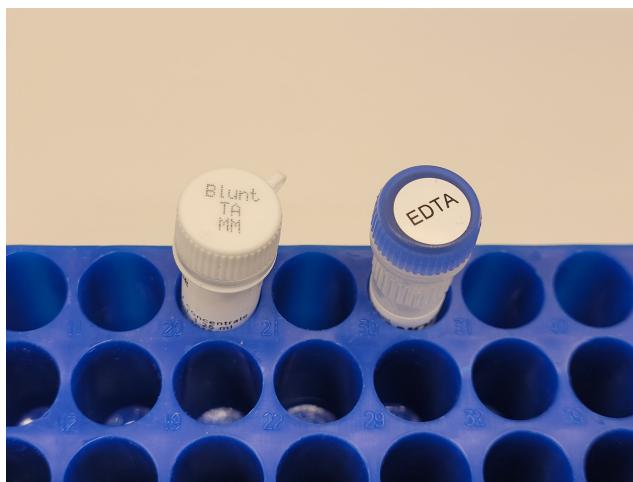
Time consumption: Barcode ligation takes around 2-3 hours.

Thaw the Blunt/TA Master mix at room temp. Spin down the tube for  00:00:05 . Mix it by pipetting 10 times.

Reagent:

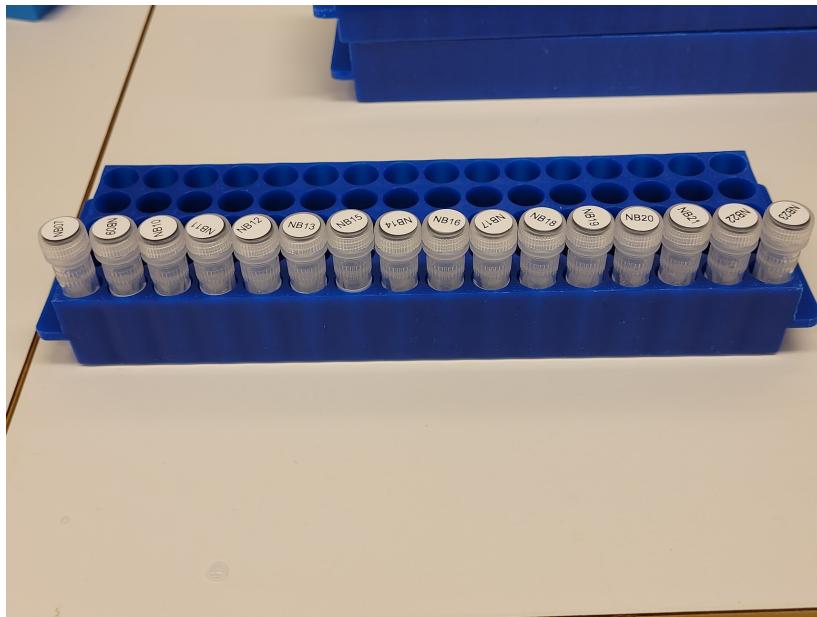
 Blunt/TA Ligase Master Mix **New England Biolabs Catalog #E7373** in Kit E7370 or E7445

- 54 Thaw the EDTA (with the blue cap) at room temp and mix by vortexing. Then spin down and place on ice.



The Blunt/TA Master mix and EDTA tubes.

- 55 Thaw the number of native barcodes (NB01-24) required for your samples (one per sample) at room temp. Individually mix them by pipetting, spin down and place them on ice. The barcode number for each sample (PCR plate) should be carefully noted in your excel data sheet. Only use one barcode per sample.



Native barcodes thawing at room temp. The number of barcodes you need is the same as PCR plates (16 in this picture).

- 56 If not already done: Prepare a clean 0.2 mL thin-walled PCR tube for every sample and label it with the barcode number.
- 57 Add $\text{7.5 } \mu\text{L}$ of End-prepped DNA from step [go to step #50](#) to each corresponding tube.
- 58 Add $\text{2.5 } \mu\text{L}$ of the corresponding barcode to each tube. Pipette mix 10-20 times.
- 59 Add $\text{10 } \mu\text{L}$ of Blunt/TA Ligase Master Mix. Pipette mix 10-20 times. Spin down briefly.
- 60 Incubate for 00:20:00 at room temp.
- 61 Add $\text{4 } \mu\text{L}$ of EDTA (with the blue cap) to each tube. Mix thoroughly by pipetting and spin down briefly.
=> EDTA is added to stop the barcode reaction.
- 62 **Pool all** the barcoded samples in a 1.5 mL LowBind tube. The total pooled volume should now be $24 \mu\text{L} \times \text{number of barcodes/samples}$.

- 63 Resuspend the AMPure XP Beads by vortexing. Then add 0.4X bead solution to the pooled sample and mix by pipetting. Incubate on a rotator mixer for  00:10:00 at room temp.
- 64 Prepare  2 mL of fresh 80% ethanol in nuclease-free water for use in step  [go to step #66](#) and forward.
- 65 Spin down the pooled sample and place it on the magnetic rack for  00:05:00 until the eluate is clear from beads. Pipette off the eluate and discard.
- 66 Wash the beads with  700 µL of 80% ethanol without disturbing the beads. If the beads are disturbed, wait until they have gathered on the magnet before proceeding. Pipette off the ethanol and discard.
- 67 Repeat the previous step.  [go to step #66](#)
- 68 Spin down and place the tube back on the magnet. Pipette off residual ethanol. Allow to dry for not more than  00:00:30.
- 69 Remove the tube from the rack and resuspend the beads in  35 µL nuclease-free water by gently flicking the tube.
- 70 Incubate for  00:10:00 at  37 °C. We typically use a dry heat block. Every 2 minutes, flick the tube for  00:00:10 to encourage DNA elution.
- 71 Place the tube back on the magnetic rack until the eluate is clear. Then remove and **KEEP**  35 µL of eluate in a new 1.5 mL LowBind tube.
- 72 It is a good idea to check if there is sufficient DNA concentration in the eluate using a Qubit fluorometer. In that case use 1 µL of the sample, using more can result in the DNA concentration being too high for the fluorometer to read.

Take forward the sample to the "Adapter ligation and clean-up" section.
- 73 **Save point:** If the protocol is too long to be completed in one go, the barcoded sample can be stored at  4 °C overnight.

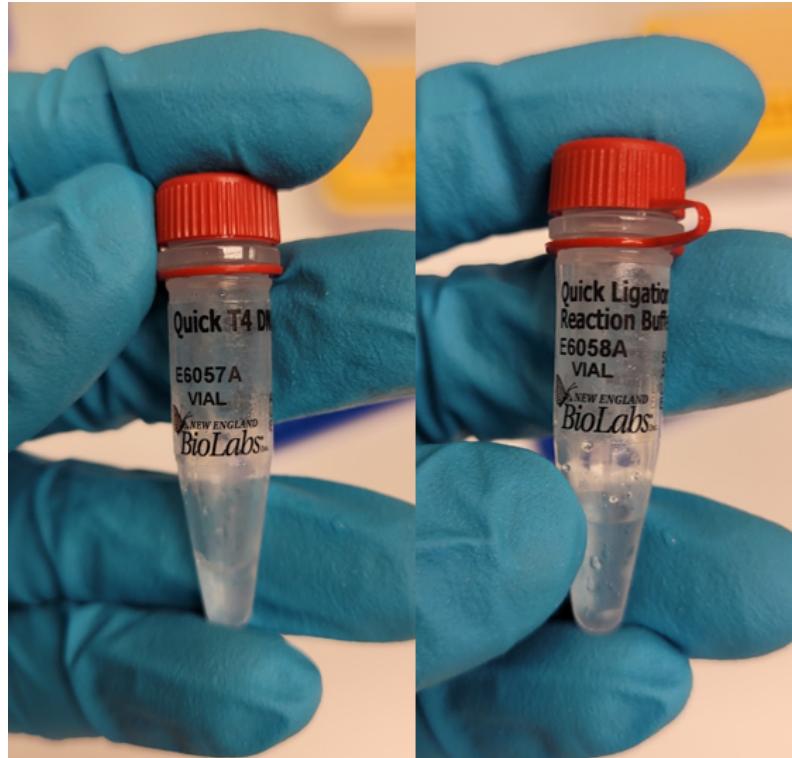
Adapter ligation and clean-up

74

Note

Time consumption: Adapter ligation takes around 2-3 hours.

1. Thaw the NEBNext Quick Ligation Reaction Buffer (5X) and Quick T4 DNA Ligase at room temperature.
2. Then spin down the two tubes for  00:00:05 .
3. Ensure that each tube is fully mixed by performing 10 full volume pipette mixes.
4. When the Quick Ligation Reaction Buffer is sufficiently pipette mixed (no precipitate can be seen), vortex the tube for several seconds to ensure thorough mixing. Do **NOT** vortex the Quick T4 DNA Ligase.
5. Place on ice.

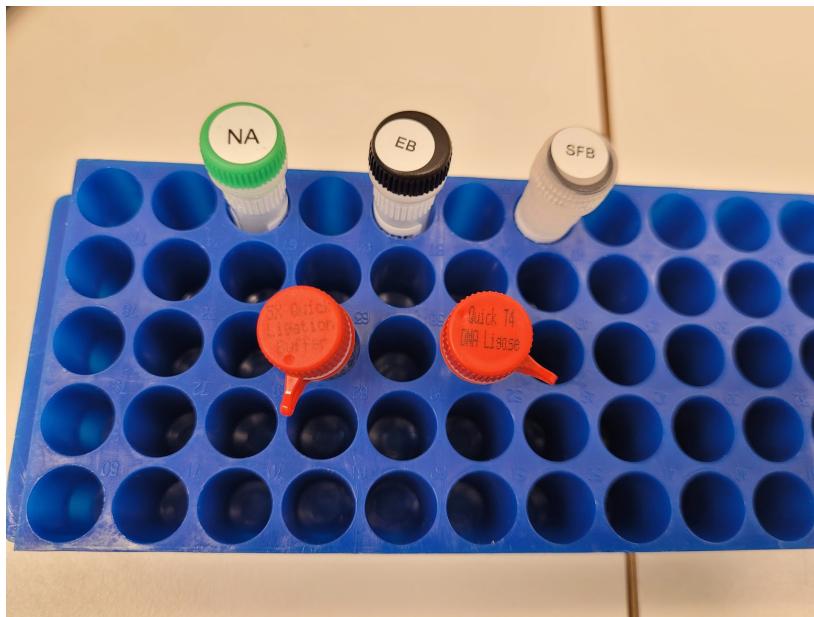


The NEBNext Quick Ligation Reaction Buffer (5X) (right) and Quick T4 DNA Ligase (left).

Reagents:

 NEBNext Quick Ligation Module New England Biolabs Catalog #E6056S

- 75 - Spin down the Native Adapter (NA), pipette mix and place on ice.
- Thaw the Elution Buffer (EB) at room temperature and mix by vortexing. Then spin down and place on ice.
- Thaw the Short Fragment Buffer (SFB) at room temperature and mix by vortexing. Then spin down and place on ice.



The Native Adapter (NA; green cap), Elution Buffer (EB; black cap) and Short Fragment Buffer (SFB) used in this section. Below are also the NEBNext Quick Ligation Module reagents.

Reagents:

 Short Fragment Buffer (SFB) Oxford Nanopore Technologies

 Elution Buffer (EB) Oxford Nanopore Technologies

- 76 Add  30 μ L of the pooled, barcoded sample to a new 1.5 mL LowBind tube.
- 77 Add  5 μ L of the Native Adapter (NA) and pipette mix 10 - 20 times.

- 78 Add $\text{10 } \mu\text{L}$ of the NEBNext Quick Ligation Reaction Buffer (5X) and pipette mix 10 - 20 times.
- 79 Add $\text{5 } \mu\text{L}$ of the Quick T4 DNA Ligase and pipette mix 10 - 20 times. The LowBind tube should now contain a total of $\text{50 } \mu\text{L}$.
- 80 Thoroughly mix the reaction by gently pipetting at least 10 times and briefly spinning down. Then incubate for $00:20:00$ at room temperature.
- 81 Resuspend the AMPure XP Beads by vortexing. Then add $\text{20 } \mu\text{L}$ of beads to the reaction and mix by pipetting. Incubate on a Rotator mixer for $00:10:00$ at room temp.
- 82 Spin down the sample and place on the magnetic rack. When the beads are stuck to the magnet and the eluate is clear, pipette off eluate and discard.
- 83 Wash the beads with $\text{125 } \mu\text{L}$ of Short Fragment Buffer (SFB). Flick the the tube to resuspend the beads in the buffer, then briefly spin down. Then return to the magnetic rack and wait for the beads to stick to the magnet. Pipette off the eluate and discard.

Do **NOT** use ethanol here as it will ruin the sequencing reaction.



Clear eluate with beads sticking to the magnet.



- 84 Repeat the previous step. [go to step #83](#)
- 85 Spin down the tube and place it back on the magnet. Pipette off any residual supernatant.
- 86 Remove the tube and resuspend the beads in $\text{15 } \mu\text{L}$ of Elution Buffer (EB). Spin down and incubate for $00:10:00$ at 37°C . We typically use a dry heat block. Every 2 minutes, flick the tube for $00:00:10$ to encourage DNA elution.
- 87 Place the tube back on the magnet for at least $00:01:00$, until the eluate is clear.
- 88 Remove and **KEEP** $15 \mu\text{L}$ of eluate, which now contains the DNA library, in a 1.5 mL LowBind tube. The beads can be disposed of.
- 89 Quantify $1 \mu\text{L}$ of the sample using a Qubit fluorometer. Depending on how many PCR plates were pooled into the sample, and the success rate (percentage of cells with successful amplification) of the PCR plates, the Qubit value may end up around $50-100 \text{ ng}/\mu\text{L}$.
- 90 Make up the library to $12 \mu\text{L}$ at 35-50 fmol for simplex reads. This may require using nuclease-free water and a new 1.5 mL LowBind tube.
- 91 **Save point:** The prepared library is used for loading on the flowcell of the nanopore device. Store the library at 4°C for short term or repeated use. For long term (more than 3 months) or single use store the library at -80°C in LowBind tubes.

Protocol references

AMPure XP Protocol

Native barcoding protocol

(White et al. 1990)

White, T.J., Bruns, T., Lee, S. & Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J. & White, T.J. (eds) PCR Protocols: a Guide to Methods and Applications. pp. 315-322. Academic Press, New York.

(Hopple & Vilgalys 1994)

Hopple, J. S., & Vilgalys, R. (1994). Phylogenetic Relationships among Coprinoid Taxa and Allies Based on Data from Restriction Site Mapping of Nuclear rDNA. *Mycologia*, 86(1), 96–107. <https://doi.org/10.2307/3760723>

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Toju, H., Tanabe, A.S., Yamamoto, S., & Sato, H. (2012). High-Coverage ITS Primers for the DNA-Based Identification of Ascomycetes and Basidiomycetes in Environmental Samples. *PLOS ONE*, 7(7), e40863.

<https://doi.org/10.1371/journal.pone.0040863>

Citations

Mason MG, Botella JR. Rapid (30-second), equipment-free purification of nucleic acids using easy-to-make dipsticks.

<https://doi.org/10.1038/s41596-020-0392-7>

Step 6

Mason MG, Botella JR. Rapid (30-second), equipment-free purification of nucleic acids using easy-to-make dipsticks.

<https://doi.org/10.1038/s41596-020-0392-7>