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Custom Indexing for Nanopore Sequencing Platform

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We use this protocol and it's working

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Disclaimer

This protocol utilizes some public information shared by both Illumina and Oxford Nanopore on DNA sample indexing strategy, and has been created without consultation of either company. Recommendations herein, reflect the opinions and experiences of the authors.

Abstract

This indexing protocol details how to index greater than 96 samples using a combination of custom indices and the Native Barcoding Kit from Oxford Nanopore Technologies for sequencing on a Nanopore sequencer.

Protocol materials

Nuclease-free Water In 4 steps

GoTaq Green Master Mix **Promega Catalog #**M7122 Step 11

PCR 1 - Amplification PCR

1 PCR 1 can be any PCR reaction targeting any genetic DNA marker, but with primers that have been modified to include an overhang on the 5' end of the gene-specific priming site, which will be used subsequently for sample indexing.

Overhang that is compatible with Illumina sequencing chemistry:

Forward: 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG [gene-target] 3'
Reverse: 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACA [gene-target] 3'

Overhang that is compatible with Oxford Nanopore Technologies (ONT) barcoding kits:

Forward: 5' TTTCTGTTGGTGCTGATATTGC 3'
Reverse: 5' ACTTGCCTGTCGCTCTATCTTC 3'

The primary advantage of using the Illumina overhang is that after PCR1 the Illumina i5/i7 indices can used. BOTH overhangs allow for indexing with the custom indices described below and subsequent sequencing on an ONT platform.

2 Carry out PCR 1 with primers containing the previously mentioned overhangs. In most cases, the addition of overhangs gene-specific primers does not impact the chemistry or cycling conditions.

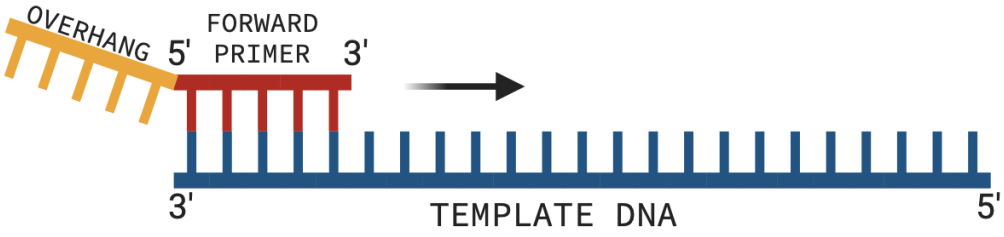


Figure 1. During PCR 1, primers bind to the gene-specific target , incoporating the overhang into amplified product.

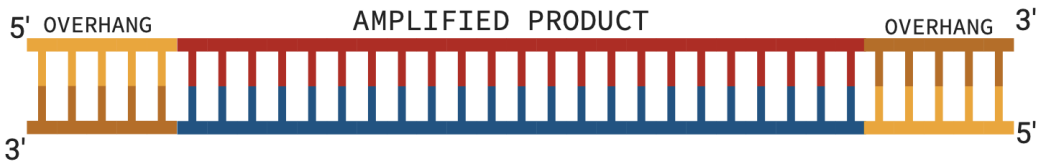


Figure 2. All amplified PCR products now have the overhang on either end. The length of the PCR product is increased by ~ 45 - 60 bp, including overhangs on each end.

Custom Indices

3 Based on the OVH chosen for PCR 1, the next step will involve attaching a unique oligo index to each sample. These indexing primers may be custom-ordered in individual tubes or in a 96-well plate format (recommended). The following steps assume indexing primers are ordered in a plate format. Below are provided Excel files of 96 indexing primers for the Illumina and ONT overhang, respectively. Files are pre-formated for ordering from Integrated DNA Technologies (IDT).

Download link **96 indexing primers for the Ilumina OVH**

- ONT-ILL_overhang_FwPlate_Vertical… 11KB
- ONT-ILL_overhang_RvPlate_Vertical… 10KB

Download link **96 indexing primers for the Nanopore OVH**

- ONT-ONT_overhang_FwPlate_Vertic… 11KB
- ONT-ONT_overhang_RvPlate_Vertic… 12KB

PCR 2 - Indexing PCR (Theoretical Overview)

4 In the Indexing PCR, custom index primers, which consist of the index + overhang, will use the overhang region from PCR 1 products as a primer binding site, attaching the custom and unique oligo index to the 5' end.

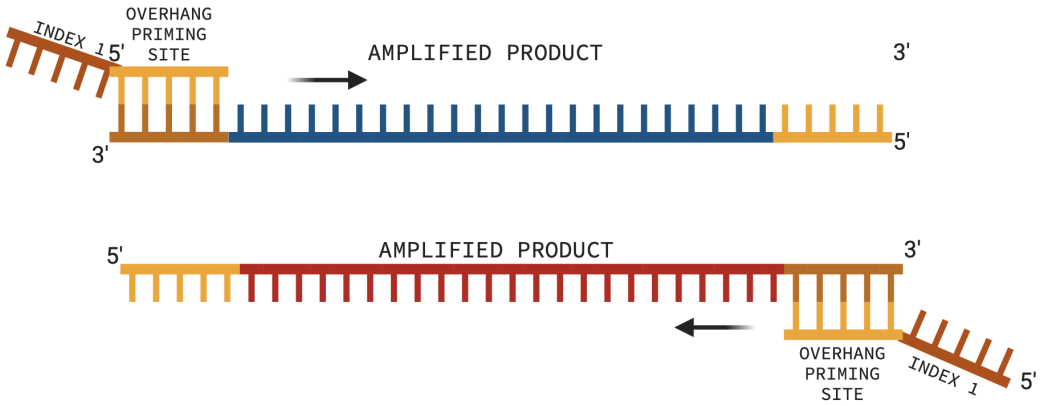


Figure 3. Index 1 is attaching to the top and bottom strands of the PCR 1 product.

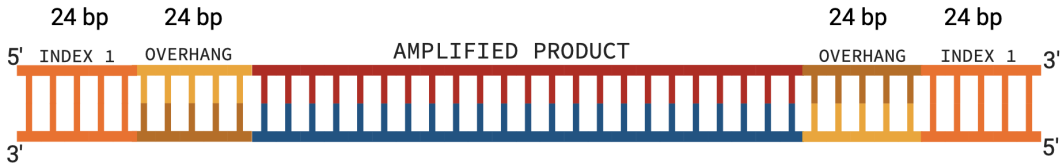


Figure 4. At the end of the indexing PCR, each product has ~96 extra bases, 48 bp on each end consisting of an index + overhang. Note: we give the same index to both ends of the amplified product, and in this case, that is Index #1.

Our custom index set matches the ONT indices, and consists of 96 indices that ONT sequencing software Minknow can recognize during real-time sequencing, basecalling and demultiplexing.

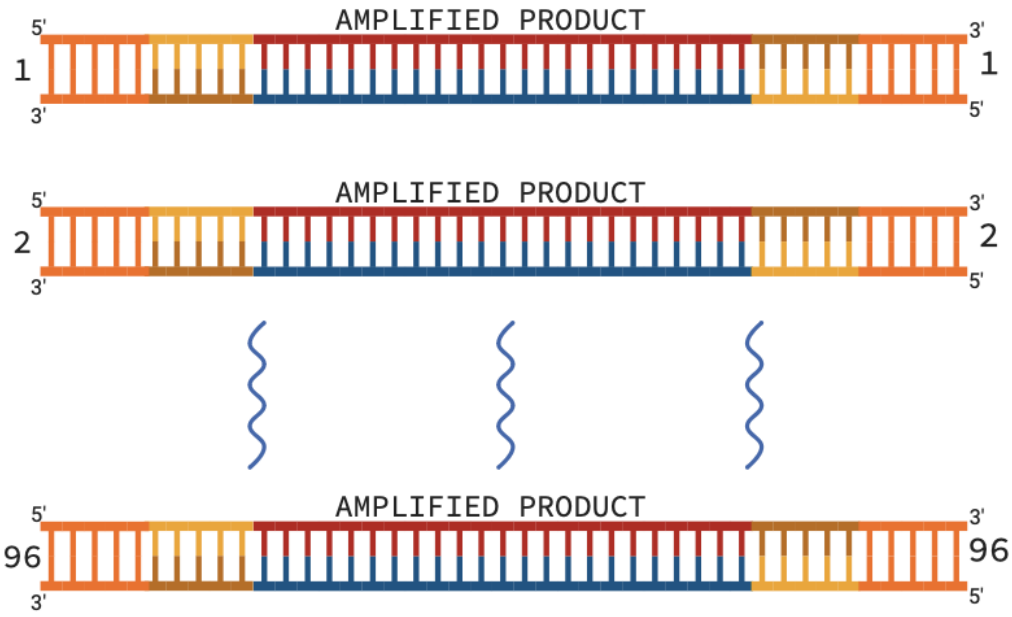


Figure 5. If you index a full plate, you will now have 96 wells of product, with indices 1 to 96 attached to them.

go to step #13 If you are curious how to index more than 96 samples

Preparing Indexing Primers

5 Regardless of the overhang chosen (Illumina or ONT), the following steps outline how to prepare the indexing plate upon arrival, assuming 96 forward indices and 96 reverse indices, each in their own plate.

5.1 Spin down both plates, since they contain lyophilized materials.

5.2 Refer to the oligo documentation, and dilute each oligo to [M] 100 micromolar (μM) .

For eg: If you have 25 nm in the well, add 250 μL of Nuclease-free Water **Contributed by users** to get [M] 100 micromolar (μM)

5.3 Combine 50 µL of the Forward and Reverse primers each to a new plate, mixing by pipetting up and down. Ensure the forward and reverse for each index number into the same well, across all 96 wells.

Note

Be careful to identify exactly which order your original primers arrived, going 12 across or 8 down.

We recommend making any intermediate primer plates to be 8 down. i.e. Index 1 in position A1 and Index 8 in H1.

5.4 Add 100 µL of Nuclease-free Water **Contributed by users** to each well, mix by pipetting up and down. This will have been a 1:4 dilution, arriving at 25uM concentration of each primer in a final volume of 200 µL, but in a single plate with Forward and Reverse combined for each index. This is an interim indexing plate with primer concentrations of 25uM.

6 From the interim indexing plate (25uM), prepare a 1:20 dilution to arrive at a 1.25uM indexing primer plate.

6.1 Add 5 µL from the interim indexing plate (25uM) to a new plate.

6.2 Add 95 µL of Nuclease-free Water **Contributed by users** to the new plate and mix by pipetting up and down.

6.3 This is now the final indexing plate (1.25uM) at a total volume of 100 µL. Store at -20 °C

Note

Don't make high volumes of this plate since contamination from overusing the same plate can result in index swapping. Feel free to set the volume of this dilution as befits your workflow and the frequency with which you will use the plate.

Do: seal the plate with piercable aluminum plate seals, and wipe down the plate before use. Seal it shut thoroughly after use with a new plate seal. Never peel off the seal, as this will cause aerosols and risk mixing your indices between wells.

Run the Indexing PCR

7 Before carrying out the indexing PCR, **evaluate the PCR 1 product on an electrophoresis gel**.

- If your amplified product is clean to begin with, proceed to step 12.
- If the product has a primer dimer, or other unintended bands, it is recommend to do a bead-based cleanup to remove any unwanted products. Confirm that the cleanup worked, by visualizing a few samples on a gel, then proceed to step 12.

Note

If the clean-up step is skipped, the subsequent indexing step may create indexed primer dimer, and indexed non-specific bands. All of these may negatively impact the efficiency of the indexing PCR.

8 **Prepare a 1:10 Dilution of PCR products to a volume of** 20 µL **:**

- In a clean microcentrifuge tube or in a fresh 96-well plate, add 18 µL of water.
- Then, carefully add 2 µL of PCR product to the tube/well.
- Do this for each sample.

9 **Mix:**

- Gently pipette up and down to mix the solution thoroughly. Alternatively pulse vortex for five seconds.
- Spin down.
- This creates a 1:10 dilution of the PCR product.

10 **Blanks and Controls:**

Since each well in the plate has a different index, it is hard to create a negative control for the whole process. Instead, we recommend using at least one or preferably two spots on the plate for the following:

1. a negative control (water instead of template + the assigned index to that well)

2. a PCR1 negative control simply treated like another sample and included in the indexing PCR
3. a PCR1 positive control simply treated like another sample and included in the indexing PCR

11 **Prepare master mix:**

Ingredients:

1. GoTaq Green Master Mix **Promega Catalog #**M7122
2. Nuclease-free Water **Contributed by users**
3. Indexing Plate (2.5uM) from Step 6

	A	B	C	D	E
	Reaction size	15			
	Template DNA (diluted)	2 uL			
	Cocktail volume	10.6 uL			
	Primer from indexing plate	2.4 uL			
	Number of samples	96			
		Starting [ ]	Ending [ ]	1 x (uL)	105.6 x (96+10% buffer)
	Go-Taq Green	2	1	7.5	792
	H20			4.3	454.1

Master Mix Recipe and the PCR Plan

12 Run the PCR as follows: 5m 10s

1. Initial denaturation at 95 °C for 00:03:00
2. 15 cycles of:
   - Denaturation at 95 °C for 00:00:15
   - Annealing at 55 °C for 00:00:15
   - Extension at 72 °C for 00:00:40
3. Final extension at 72 °C for 00:01:00

4. A hold at 10 °C indefinitely

Note

Modify the extension steps as needed based on the length of the target fragment. Generally add a minute for every additional 1000 bases.

Expected result

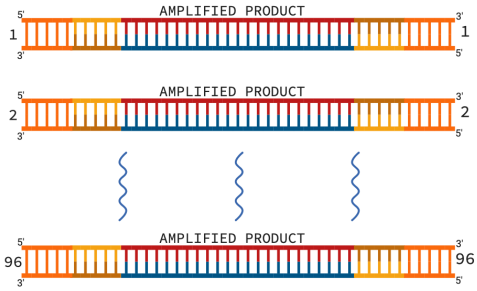
Most of the input PCR product should be indexed. In the downstream analysis, if many reads remain unclassified (i.e. cannot be demultiplexed), it may be necessary to return to step 9 and dilute further dilute the PCR 1 product. This requires optimization for each experiment to some extent, because everyone begins with a different amplification success in PCR 1.

Dual Indexing >96 samples (Theoretical Overview)

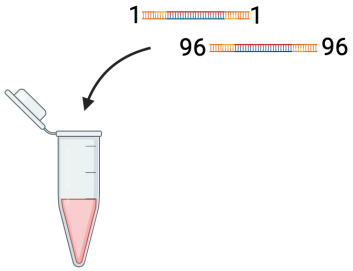
13 Indexing more than 96 samples is possible with an extended protocol. Broadly speaking, this can be achieved as follows:

Pooling 96+ amplicons on a single nanopore sequencing run

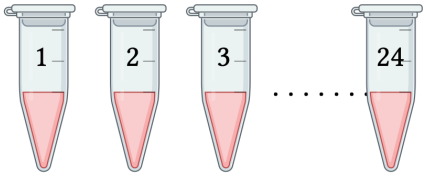
1. Add an index to each PCR product up to 96 products



2. Normalise products into an equimolar pool.



3. Make up to 24 such pools



4. Ligate a native barcode (1 to 24) to each of the pools using the NBD kit from ONT

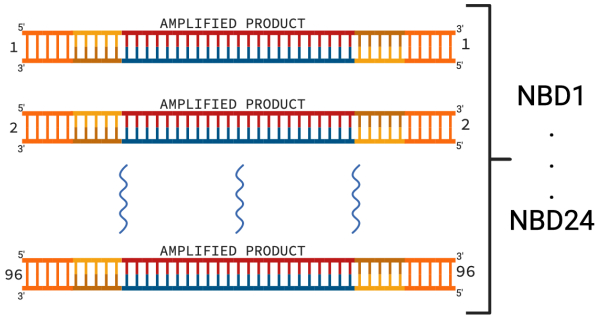


Figure 6. Protocol to take several plates of 96 PCR-indexed samples and dual index them with outer Native Barcodes from Oxford Nanopore Technologies' NBD114.24 kit. This will allow for a total of 2,304 indexed samples in a single pool.

Note

You may also index up to 96 plates if you use the NBD114.96 kit. This will allow for a total of 9,216 samples in a single pool.

Making Equimolar Sample Pools

14 **Pooling samples in an equimolar fashion can be done in two ways.**

1. With access to a plate reader, a fluorometer, and sufficient supplies, measure the concentration of dsDNA from the Indexing PCR products (just after Step 14). This is an expensive proposition for many, and time consuming with only a single-channel Quantus or Qubit Fluorometer. If you choose this route, go to step #14.3
2. Alternatively, normalize by estimating concentration using brightness of bands as seen on a 2% agarose gel. In this case, the success of normalization will not be 100%, but may be sufficient and is affordable. If you choose this route, go to step #14.1

14.1 Normalizing using gel images can be done across many samples if the following criteria are met:

1. Will be carried out by an experienced laboratory scientist that can load gels quickly without errors, since band brightness is sensitive to manual loading errors
2. Utilize a gel rig that can accommodate 48 or more samples at once, ideally a plate at a time. This makes the process more reliable, since all samples will be evaluated on 1 or 2 gel images, reducing variation across gels images.

14.2 Run a 1.5% or 2% agarose gel for each sample with the appropriate sized ladder. Use multiple combs on the gel, but ensure the target band is clear. Load gels quickly without delays, to ensure all samples on the gel are comparable. If flushing of a well occurs, note which one and rerun the gel.

Once the gel has been photographed, annotate it and identify the strongest, intermediate and weakest bands. To normalize, we advise carrying forward 3 uL, 6 uL and 8 uL for the strong, intermediate and weak bands, respectively. Carry forward negative control blanks as weak bands.

14.3 If individually quantifying all samples, normalize each indexed product into a single equimolar pool.

Preparing Outer Index Ligation

15 Once pools of 96 indexed samples are ready, follow the NBD114.24 ligation protocol by ONT, as listed on the Nanopore Community webpage. However, some further steps should be taken.

15.1 Pools are likely to be well above 100 uL in volume, and DNA concentration may be too diluted for direct use, we would recommend a quality check as follows:

1. Take 3 uL of each pool and run it on a gel. Check for primer dimers and unwanted bands.
2. If there are no unwanted bands, and only the target band, quantify the pool.
3. If the pool is sufficiently concentrated to give the appropriate amount of material for the NBD114.24 protocol (200fmol per pool), proceed directly to that protocol.
4. If the pool is not concentrated enough, OR there are unwanted bands in step 1, perform a bead cleanup and concentration of half of the pool.

15.2 Bead cleanup - if necessary:

1. Clean up half the pool, saving the other half as a backup in case of errors downstream
2. Choose a sample:bead ratio based on the PCR product size that will remove unwanted bands, and perform a final elution into ~30uL
3. Take 1 uL and add 9uL to make a 1:10 dilution with water.
4. From this 10uL, run 2 uL on a gel to confirm the cleanup went as planned.
5. Also quantify it on a flourometer and multiply by 10 to get the concentration of the original cleaned up pool.
6. If not enough DNA, return to to bead cleanup and either a) cleanup the supernatant from the first step which contains the DNA or b) attempt another clean up on the second half of your pool.
7. If the gel looks good, and the quantification indicates sufficient DNA, proceed to the NBD protocol.

Outer Index Ligation

16 The protocol for the Native Barcoding Kit (114.24 or 114.96) is on the Oxford Nanopore Community website, with detailed instructions on how to complete it. Here are a few lessons we have learned to improve the process:

1. Pay attention to the bead cleanup ratios, they matter a LOT. Do not blindly follow the protocols online because those are generally intended for long reads. For shorter amplicons, run tests to see what fits best. Here a few suggestions, but always pre-evaluate beads to make sure they function as desired:

	A	B
	Band size	Cleanup ratios
	100-300 bp	2.0 - 2.5X for end prep, and 1.2X for adapter ligation
	500 - 1500 bp	1x for both

	A	B
	> 1.5 kb	0.8x for end prep and 0.4x for adapter ligation

2.

Protocol references

References for figures:

Created in BioREnder. Erkenswick Watsa, M. (2024) BioRender.com/x00t095

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