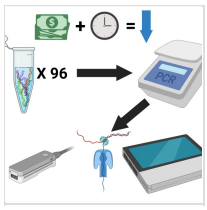

Nov 19, 2021

A streamlined protocol for high-throughput amplification-based analysis of DNA samples via nanopore sequencing (based on the 96-well PCR barcoding kit)

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Nanopore sequencing facilitates the rapid and cost-effective sequencing of long fragment DNA for a massive range of applications. When looking to holistically analyse low-yield DNA samples using nanopore sequencing, the optimal method is likely to involve the PCR Barcoding Kit. This effectively involves blunt end ligation of priming sites onto all extant DNA for holistic amplification to achieve yields suitable for nanopore sequencing. The currently available kits from nanopore facilitate the multiplexing of 96 samples in one sequencing run using this method, but the reagent costs are inherently multiplicative. This protocol is designed to streamline (in terms of cost, reagents and time) the process of sequencing up to 96 samples of genomic DNA through nanopore sequencing.

This protocol is best applied to large numbers of samples (up to 96). For smaller numbers of samples, consider the smaller "PCR Barcoding" kits provided by nanopore which similarly achieve holistic DNA amplification and sequencing, but without the need for additional adapter ligation. The protocol is best suited to samples with low DNA yields (100 ng input is recommended). If you can input 1000 ng of DNA from each of your samples, consider using the 96-well Ligation Kit from Oxford Nanopore which can similarly be streamlined in terms of cost and time, but avoids the amplification step.

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This protocol is designed specifically around the Oxford Nanopore Technologies PCR Barcoding Expansion 1-96 (EXP-PBC096) kit. The kit protocol deviates from the standard PCR barcoding kit protocol, existing as a hybrid between that and the ligation kit (SQK-LSK109). As such, both EXP-PBC096 and SQK-LSK109 will be required (alternative protocols are possible using SQK-LSK110).

This protocol is designed to streamline (in terms of both cost/reagents and time) the process of sequencing many (up to 96) samples of genomic DNA using nanopore sequencing. The process involves ligating priming sites onto the genomic DNA, priming and amplifying the full DNA strands and then ligating adapters onto the amplified products.

Equipment:
Qubit spectrophotometer (or alternative DNA concentration measurement system)
96-well plate magnetic rack
Thermocycler
MinION nanopore sequencer
Pipettes (P2, P20, P200, P1000, or otherwise overlapping range of volumes)

Consumables:
Plasticware
Ethanol
Ice
Oxford Nanopore Technologies PCR Barcoding Expansion 1-96 (EXP-PBC096)
Oxford Nanopore Technologies Ligation Sequencing Kit (SQK-LSK109)
Oxford Nanopore Technologies Flow Cell Priming Kit (EXP-FLP002)
Oxford Nanopore Technologies flongle and/or R9.4.1/R10.3 flow cell
SPRI beads
AMPure beads
Qubit BR dsDNA assay kit (HS dsDNA may be more applicable in some cases; alternative DNA concentration measurement methods are equally viable)
Nuclease-free water
NEB Ultra II End Prep reaction buffer and enzyme mix
NEB Blunt TA Ligase Master Mix
NEB 2X LongAmp Taq Master Mix
NEBNext FFPE DNA Repair buffer and enzyme mix
NEBNext Quick T4 DNA Ligase buffer and enzyme
10mM Tris-HCl pH 8.0 with 50 mM NaCl

Please review the COSSH documentation for all reagents to ensure safe use and what to do in the event of accidents.

This protocol is best applied to large numbers of samples (up to 96). For smaller numbers of samples, consider the smaller "PCR barcoding" kits provided by nanopore which similarly achieve holistic DNA amplification and sequencing, but without the need for adapter ligation at the end. The protocol is best suited to samples with low DNA yields (100 ng input is recommended). If you can input 1000 ng of DNA from each of your samples, consider using the 96-well ligation kit from Oxford Nanopore which can similarly be streamlined in terms of cost and time, but avoids the amplification step.

Prepare DNA

1 Extract and purify your DNA in a manner appropriate for the sample type, the hypothesis being tested and this protocol. Consider the length of DNA that you are ideally interested in and how the tissue/sample type might best be lysed/extracted from. This protocol will be most successful when using samples with low concentrations of contaminants/inhibitors.

1.1 Consider whether a size selection/clean/concentration would be appropriate for the samples. If targeting long fragments, clean the samples with a 0.4X

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SPRI Select bead clean, washing twice with 200 µL 70% ethanol and eluting into 12.5 µL nuclease-free water (the step-by-step protocol for bead cleans is given below in Step 7). If the samples are low concentration or contain a high concentration of inhibitors/contaminants, consider carrying out a 1X bead clean to concentrate them and remove inhibitors.

1.2 Consider running the samples in duplicate/triplicate for best results. Ideally, these would use different barcodes downstream for post-sequencing comparison of results between samples to ascertain consistency.

Ligation of barcode adapters 3h

2 10m
Before beginning the ligation process, it would be ideal to quantify the concentration of your samples using Qubit high-sensitivity/broad-range (depending on application) dsDNA assays or similar, but this can be omitted if necessary.

3 15m
In a new 0.2 ml tube (or 96-well plate), set up the following reaction for each sample:

A	B
Component	**Volume**
DNA in nuclease-free water	12.5 µL
Ultra II End Prep Reaction Buffer	1.75 µL
Ultra II End Prep Enzyme Mix	0.75 µL
Total	**15 µL**

3.1 Consider including negative and positive controls alongside your samples which can be sequenced for later quality control.

4 16m
Incubate the samples as follows:

A	B
Temperature	**Time**
25 °C	10 mins
65 °C	5 mins
4 °C	1 min

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15m

5 Get your barcode adapter ligation reaction ready **in the order given**:

A	B
Component	**Volume**
End-prepped DNA (from last step)	15 µL
BCA (Barcode Adapter)	8 µL
Blunt TA Ligase Master Mix	10 µL
Total	33 µL

31m

6 Incubate the samples as follows:

A	B
Temperature	**Time**
Room temperature (~20 °C)	20 mins
70 °C	10 mins
On ice/4 °C	1 min

1h 30m

7 Clean the adapter-ligated DNA with 0.4X AMPure beads (13.2 µL), washing twice with 200 µL 70% ethanol. Resuspend in 26 µL nuclease-free water, leaving 1 µL behind to prevent bead uptake.

7.1 If unfamiliar with bead cleaning, it will be described once here which can be referred to for subsequent cleans. Stay vigilant regarding the volume/ratio of beads to add, the solution used to wash the beads (i.e., ethanol vs. SFB/LFB) and the resuspension volume.

First, resuspend the beads in solution, ensuring they are at room temperature.

7.2 Add the required volume of beads (e.g., 0.4X clean of 33 µL sample requires an addition of 13.2 µL bead solution) and mix by gentle flicking and inversion (slower) or pipetting (risks fragmentation).

7.3 Incubate the solution for 5 mins (unless not physically mixed, in which case

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incubate for at least 10 mins). The solution can be incubated on a mixer to ensure thorough mixing.

7.4 Prepare the cleaning solution (e.g., make up fresh 70 % ethanol or thaw LFB/SFB).

7.5 Place the sample on a magnet for 5 mins to separate the beads from the rest of the solution.

7.6 Keeping the sample on the magnet, pipette off the supernatant and wash the beads with the cleaning solution (e.g., 200 µL 70 % ethanol) without disturbing the pelleted beads.

7.7 Incubate for 30 seconds and remove the ethanol supernatant by pipetting without disturbing the pelleted beads. Wash the beads with a further round of the cleaning solution (e.g., 200 µL 70 % ethanol) without disturbing the pelleted beads.

7.8 Pipette off any residual cleaning solution (briefly spinning down first can help, but ensure the sample is back on the magnet before attempting to remove any solution). Briefly air dry the beads (waiting for the beads to lose their glossy shimmer and become matte - this is usually ~30-120 seconds for SPRI and AMPure depending on the efficiency with which cleaning solution is removed).

7.9 Resuspend the pellet with the required resuspension volume (e.g., 25 µL nuclease-free water) and incubate for 5 mins.

7.10 Pellet the beads on a magnet until the solution is clear and colourless, and the beads are fully separated.

7.11 Remove and retain the required volume of resuspended DNA in a new tube.

Amplification of libraries 5h 15m

8 LongAmp Hot Start Taq 2X Master Mix, Barcode Primer 1 (BP 01). 25m

Set up the following reaction:

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A	B
Volume	**Reagent**
10 µl	Adapter-ligated DNA
14 µl	Nuclease-free water
1 µl	Barcode Primer (BC01-96, one per reaction)
25 µl	2X LongAmp Taq Master Mix
Total: 50 µl	

9 Mix the reaction by gentle flicking and inversion, and incubate as follows: 3h 15m

A	B	C
Temperature	**Time**	**Cycles**
95 °C	3 mins	1
95 °C	0:15 mins	18
62 °C	0:15 mins	
65 °C	V mins	
65 °C	6 mins	1
4 °C	-	-

The extension length (V) depends on the expected DNA length. Approximately 50 seconds per kbp is recommended (e.g., 16:40 for expected fragment sizes of ~20 kbp). Sizes can be pre-determined using gel electrophoresis or digital analysis tools such as TapeStation or Qiaxcel.

10 Measure the concentration of your samples using Qubit high-sensitivity/broad-range (depending on expected concentration) dsDNA assays (or similar). 1h

11 Pool your barcoded samples together based on concentration to achieve approximate equimolarity. 15m

11.1 Divide each concentration by the largest concentration across all samples to determine the volume of each to add to achieve approximate equimolarity.

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12 Clean the adapter-ligated DNA with 1X AMPure beads, washing twice with 200 µL 70% ethanol. Resuspend in 50 µL nuclease-free water, taking 48 µL out (leaving 2 µL behind to prevent bead uptake), and using 1 µL to measure concentration. 20m

Ligation of adapters 1h 20m

13 Get your adapter ligation reaction ready **in the order given**: 10m

A	B
Component	**Volume**
DNA	47 µL
DNA CS	1 µL
NEBNext FFPE DNA Repair Buffer	3.5 µL
NEBNext FFPE DNA Repair Mix	2 µL
Ultra II End-prep reaction buffer	3.5
Ultra II End-prep enzyme mix	3
Total	**60 µL**

14 Incubate the sample as follows: 10m

A	B
Temperature	**Time**
20 °C	5 mins
65 °C	5 mins

15 Clean the sample with 1X AMPure beads, washing twice with 200 µL 70% ethanol. Resuspend in 61 µL nuclease-free water, taking 60 µL out (leaving 1 µL behind to prevent bead uptake). 20m

You can theoretically skip this clean, but if you do, **do not incubate for longer than 10 mins after the next step**.

16 Get your ligation reaction ready in the order given: 10m

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A	B
Component	**Volume**
DNA	60 µL
Adapter Mix AMX	5 µL
Ligation Buffer LNB (or T4 buffer)	25 µL
NEBNext Quick T4 DNA Ligase	10 µL
Total	**100 µL**

17 Incubate at room temperature (~20 °C) for 10 mins. 10m

18 Clean the sample with 1X AMPure beads, washing twice with either **LFB** or **SFB** (**DO NOT CLEAN WITH ETHANOL**; SFB for target fragments less than 3 kbp in length, otherwise use LFB). Resuspend in 17 µL elution buffer (EB, or 10mM Tris-HCl pH 8.0 + 50mM NaCl), leaving 1 µL of it behind and measuring the concentration of a further 1 µL using Qubit or similar. 20m

Target input should be:
5-50 fmol for R9.4.1 flow cell
25-75 fmol for R10.3 flow cells
3-20 fmol for flongle flow cells

Loading of library (flongle) 30m

19 If looking to check the quality of the DNA for sequencing, or only looking to generate relatively small amounts of sequencing data, consider sequencing on a flongle. 30m

Allow the flongle to reach room temperature for 20-30 mins ahead of loading of the library. Insert the flongle into the MinION sequencer and check the number of active pores available. Visually check the flongle for air bubbles.

Thaw the sequencing buffer (SQB), loading beads (LB), flush tether (FLT) and flush buffer (FB) at room temperature and vortex all but the FLT briefly and spin them down.

Put together the following flush solution:

Volume	Reagent
3 µl	Flush Tether (FLT)
117 µl	Flush Buffer (FB)
Total: 120 µl	

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Of this, add 100 µL to the flongle via the priming port, with caution to avoid the introduction of air bubbles.

Ensure the beads of the LB are suspended by vortexing again immediately before pipetting, then assemble the following solution:

Volume	Reagent
10 µl	Loading Beads (LB)
15 µl	Sequencing Buffer (SQB)
5 µl	DNA library
Total: 30 µl	

Gently mix the library by gentle flicking and inversion immediately before loading 30 µL via the priming port. Begin sequencing using the default settings for 24 hours.

Loading of library (full flow cell) 30m

20 Allow the flow cell to reach room temperature for 20-30 mins ahead of loading of the library. 30m
Insert the flow cell into the MinION sequencer and check the number of active pores available. Visually check the flow cell for air bubbles. Removing a small (<20 ul) amount of yellow buffer to purge any air.

Thaw the sequencing buffer (SQB), loading beads (LB), flush tether (FLT) and ***a full tube of*** flush buffer (FB) at room temperature, briefly vortex all but the FLT and spin them down. To the full FB tube, add 30 µl FLT, and mix by gentle flicking and inversion. Of this, add 800 µl to the flow cell via the priming port, with caution to avoid the introduction of air bubbles.

Ensure the beads of the LB are suspended by vortexing again immediately before pipetting, then assemble the following solution:

A	B
Volume	**Reagent**
25.5 µl	Loading Beads (LB)
34 µl	Sequencing Buffer (SQB)
4.5 µl	Nuclease-free water
10 µl	DNA library
Total: 75 µl	

This was gently mix the library by gentle flicking and inversion, load 75 µl via the SpotON sample port in a dropwise manner, ensuring each drop flows into the port before adding the next. The flow cell can then begin sequencing using the default settings.

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