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High-throughput nanopore sequencing of cell-free DNA

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We use this protocol and it's working

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Abstract

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Epigenetic characterization of cell-free DNA **(cfDNA)** is an emerging approach for detecting and characterizing diseases such as cancer. We developed a strategy using nanopore-based single-molecule sequencing to measure cfDNA methylomes. This approach generated up to 200 million reads for a single cfDNA sample from cancer patients, an order of magnitude improvement over existing nanopore sequencing methods. We developed a single-molecule classifier to determine whether individual reads originated from a tumor or immune cells. Leveraging methylomes of matched tumors and immune cells, we characterized cfDNA methylomes of cancer patients for longitudinal monitoring during treatment.

Guidelines

This protocol assumes that you are sequencing multiple cfDNA samples on the Oxford Nanopore Technologies' PromethION system.

This protocol also assumes you have an existing method of extracting cfDNA from plasma.

Protocol materials

- KAPA HyperPrep Kit (PCR-free) **Roche** **Catalog #**KK8505
- Agencourt AmPure XP beads **Catalog #**A63880
- Mag-Bind® TotalPure NGS **Omega Biotek** **Catalog #**M1378-01
- Native Barcoding Expansion 96 **Oxford Nanopore Technologies** **Catalog #**EXP-NBD196
- Oxford Nanopore Ligation Sequencing Kit **Oxford Nanopore Technologies** **Catalog #**SQK-LSK110
- Qubit® dsDNA HS Assay Kit **Thermo Fisher Scientific** **Catalog #**Q32854
- E-Gel™ EX Agarose Gels 2% **Catalog #**G401002

Multiplexed cfDNA library preparation (barcode ligation)

1 25 µL of each Sample (usually this is half of the extracted volume, in case of reaction failure) is diluted with 25 µL of water in a PCR strip tube or microtiter plate.

2 A master mix of end-repair and a-tailing mix KAPA HyperPrep Kit (PCR-free) **Roche Catalog #**KK8505 according to vendor instructions. Briefly, 7 µL of end-repair buffer and 3 µL of end-repair enzyme is combined together to create a master mix. Use 10-20% overage.

3 Add 10 µL of master mix to each cfDNA sample to obtain 60 µL total volume. Pipet mix.

4 Incubate at 20 °C 30 minutes followed by 65 °C 30 minutes .

5 Add 5 µL water, 30 µL ligation buffer from KAPA HyperPrep Kit (PCR-free) **Roche Catalog #**KK8505 , and 5 µL of a sample barcode from Native Barcoding Expansion 96 **Oxford Nanopore Technologies Catalog #**EXP-NBD196 to each well. Add 10 µL ligation enzyme from KAPA HyperPrep Kit (PCR-free) **Roche Catalog #**KK8505 . Mix thoroughly.

6 Place samples in a thermocycler and incubate at 20 °C 4.5 hours followed by 4 °C overnight .

Ligation cleanup

30m

7 Add 88 µL of Agencourt AmPure XP beads **Catalog #**A63880 to each well and mix thoroughly. You can use any off-brand beads. We use Mag-Bind® TotalPure NGS **Omega Biotek Catalog #**M1378-01 . Incubate for 00:05:00 .

5m

8 Pool all samples together into a 50ml centrifuge tube. Magnetize using a 20m

Equipment	
Dynamag-50 Separation Magnet	NAME
Magnet	TYPE
Thermo Fisher Scientific	BRAND
12302D	SKU

for at least 00:20:00 . This may take much longer depending on the number of samples. The supernatant should be completely clear with no haziness.

9 Aspirate out the supernatant with a 50ml serological pipet, taking care to not disturb the beads. Wash the beads twice with 80% ethanol using a serological pipet by slowly pipetting the ethanol down the side of the centrifuge tube without disturbing the beads.

10 Pulse centrifuge the 50ml tube and magnetize. Remove any residual ethanol. Repeat this step three times.

11 Elute in 600 µL 10mM Tris-HCl pH 8.0 buffer. Close the centrifuge tube tightly and vortex to resuspend the beads. Incubate for 00:05:00 for full elution. Magnetize the beads and remove the elution buffer. Store in a fresh 1.5ml microcentrifuge tube. 5m

12 Perform a second bead cleanup by adding 900 µL Ampure XP beads to the pooled samples. Incubate for 00:05:00 . 5m

13 Place on a 5m

Equipment	
DynaMag-2	NAME
Magnet	TYPE
Invitrogen	BRAND
12321D	SKU
https://www.thermofisher.com/order/catalog/product/12321D#/12321D	LINK

and magnetize for at least 00:05:00 . Remove the supernatant and wash twice with 80% ethanol. Remove any residual ethanol by pulse centrifugation and magnetizing twice.

14 Elute in 50 µL 10mM Tris-HCl pH 8.0 buffer. Incubate for at least 00:05:00 for full elution. Magnetize and remove elution buffer and place in new PCR strip tube. 5m

Nanopore adapter ligation 1h 35m

15 Add 7 µL of end repair buffer and 3 µL end repair enzyme to the pooled sample. Incubate at 20 °C 30 minutes followed by 65 °C 30 minutes .

16 Add 30 µL ligation buffer from KAPA HyperPrep Kit (PCR-free) **Roche Catalog #**KK8505 , and 10 µL of AMX-F adapter from Oxford Nanopore Ligation Sequencing Kit **Oxford Nanopore Technologies Catalog #**SQK-LSK110 . Add 10 µL ligation enzyme from KAPA HyperPrep Kit (PCR-free) **Roche Catalog #**KK8505 . Mix thoroughly.

17 Incubate at room temperature for at least 01:30:00 . 1h 30m

18 Add 88 µL of Agencourt AmPure XP beads **Catalog #**A63880 to the ligation reaction. Incubate for 00:05:00 . Magnetize the beads and discard the supernatant. 5m

19 Add 200 µL of SFB wash buffer from Oxford Nanopore Ligation Sequencing Kit **Oxford Nanopore Technologies Catalog #**SQK-LSK110 . **DO NOT USE ETHANOL!!!** Remove the tube from the magnet, cap it, and flick it gently with a pencil to resuspend the beads. Pulse centrifuge, magnetize, and repeat this step one more time.

20 Resuspend in 25 µL of EB buffer from Oxford Nanopore Ligation Sequencing Kit **Oxford Nanopore Technologies Catalog #**SQK-LSK110 .

21 Quantify the libraries using Qubit® dsDNA HS Assay Kit **Thermo Fisher Scientific Catalog #**Q32854 with an approximate size of 330bp (you can check the size range with E-Gel™ EX Agarose Gels 2% **Catalog #**G401002 but it's not super critical). Load 150fmol of library per PromethION flow cell using standard loading protocols.

Protocol references

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