

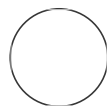


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High-throughput nanopore sequencing of cell-free DNA

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ABSTRACT

<https://www.biorxiv.org/content/10.1101/2022.06.22.497080v1>

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Protocol status: Working
We use this protocol and it's working

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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.

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 Contributed by users Catalog #A63880 to each well and mix thoroughly. You can use any off-brand beads. We use

5m

 Mag-Bind® TotalPure NGS **Omega Biotek Catalog #M1378-01** . Incubate for

 00:05:00 .

- 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 μ L 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.
- 12 Perform a second bead cleanup by adding  900 μ L Ampure XP beads to the pooled samples. Incubate for  00:05:00 .
- 13 Place on a

5m

5m

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 μ L 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 μ L of end repair buffer and  3 μ L end repair enzyme to the pooled sample. Incubate at  20 °C 30 minutes followed by  65 °C 30 minutes .

- 16 Add  30 μ L ligation buffer from  KAPA HyperPrep Kit (PCR-free) **Roche Catalog #KK8505** , and  10 μ L of AMX-F adapter from  Oxford Nanopore Ligation Sequencing Kit **Oxford Nanopore Technologies Catalog #SQK LSK110** . Add  10 μ L 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 **Contributed by users Catalog #A63880** to the ligation reaction. Incubate for  00:05:00 . Magnetize the beads and discard the supernatant.
- 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% **Contributed by users Catalog #G401002** but it's not super critical). Load 150fmol of library per PromethION flow cell using standard loading protocols.