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mcfDNA Sequencing from blood samples

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We use this protocol and it's working

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Disclaimer

This protocol corresponds to the protocol "multiplex sequencing of human cfDNA from blood using SQK-NBD114.24" provided by Oxford Nanopore.

Abstract

This protocol describes the preparation, library construction, and sequencing of human cell-free DNA (cfDNA) starting from blood plasma using the Oxford Nanopore Ligation Sequencing Kit V14 (SQK-NBD114.24) on GridION devices. The workflow comprises cfDNA extraction, DNA repair and end-prep, barcode and adapter ligation, and sequencing, followed by basecalling and downstream bioinformatic analysis. In our study, this workflow was applied to microbial cell-free DNA (mcfDNA) sequencing for rapid and sensitive pathogen detection in clinical samples.

Guidelines

Sample Collection & Handling:

- **Tube Type:** This protocol is optimized for **Streck Cell-Free DNA BCT®** tubes to minimize the lysis of human white blood cells. If using standard EDTA tubes, samples must be processed to plasma immediately (within 2–4 hours) to prevent genomic DNA contamination.
- **Temperature:** Do **not** freeze whole blood collected in Streck tubes; store and transport at room temperature (6 °C to 37 °C).
- **Contamination Control:** Microbial cell-free DNA (mcfDNA) analysis is highly sensitive to environmental contamination. Use aerosol-resistant (filter) pipette tips and nuclease-free water throughout the procedure.

Safety warnings

Biohazard / Biosafety Level 2:
Human blood and plasma should be treated as potentially infectious. Handle samples in a Class II biological safety cabinet (BSC) and wear appropriate personal protective equipment (PPE), including gloves, lab coat, and eye protection. Dispose of all waste in biohazard containers.

Ethics statement

Human Subjects Research:
Blood collection for this protocol requires prior approval by the users' Institutional Ethics Board or equivalent ethics committee. Ensure informed consent is obtained from all participants prior to sample collection in accordance with the Declaration of Helsinki.

Before start

Kit & Device Compatibility:
This protocol utilizes the **QIAamp MinElute ccfDNA Midi Kit** for extraction and the **Ligation Sequencing Kit V14 (SQK-NBD114.24)** for library prep.
Note on Volumes: While based on PromethION documentation, this specific protocol is adapted for **GridION/MinION** flow cells. Ensure priming and loading volumes in the final steps are appropriate for the specific flow cell type used (e.g., FLO-MIN114).

cfDNA extraction from patient blood

1h 15m 30s

1 Basis of this protocol: **Kit for Isolation of mcfDNA**

2 **Draw approximately** 10 mL Blood **patient into two 10 mL Streck Cell-Free DNA BCT ® tubes (Streck, USA) each and follow with step 2 and 3 as soon as possible.** 4m

3 Centrifuge at 1900 x g for 10 minutes and distribute the supernatant into **2 ml Reaction tubes.** 1900 x g, 4°C, 00:10:00 10m

4 Centrifuge the plasma at 16000 x g for 10 minutes and collect the supernatant of each sample into one 15 ml tube.
The plasma can now be stored for up to a week at 4 °C until further processing.
16000 x g, 4°C, 00:10:00 10m

5 To 1 ml Plasma add: 3m

	Reagent	Volume [µl]
	Magentic Bead Suspension	30
	Proteinase K	55
	Bead Binding Buffer	150
	Total	1235

6 Optional: Spin down for 30 seconds at 200 x g in a swing out rotor centrifuge to remove any solution in the cap. 30s
200 x g, Room temperature, 00:00:30

7 Place tube into a magnetic rack for 00:02:00 or until the supernatant is clear before discarding the supernatant. 2m

8 Remove from magnetic rack and resuspend the beads in 200 µL Bead Elution Buffer . Pipette mix to rinse beads from the wall. 2m

9 Transfer the whole mixture into a 2 ml Bead Elution Tube. 1m

10 Incubate the Bead Elution Tube for 00:05:00 on a tube rotator mixer (Hula mixer) at room temperature. 5m

11 Place the Bead Elution Tube on a magnetic rack and let it stand for ≥ 00:01:00 until the solution is clear. 2m

12 Transfer the supernatant containing cfDNA into a new Bead Elution tube. **Discard** the **bead pellet**. 1m

13 Add 300 µL ACB Buffer to the Bead Elution tube containing the supernatant. Vortex to mix. Briefly centrifuge the tube to remove drops from inside the lid. 3m

14 Pipet the supernatant–ACB-Buffer mixture from the previous step into a QIAamp UCP MinElute column. 1m

15 Centrifuge for 1 min at 6,000 x g.
6000 x g, Room temperature, 00:01:00 1m

16 Place the QIAamp UCP MinElute column into a clean 2 ml collection tube. **Discard** the **flow-through**. 1m

17 Add 500 µL ACW2-Buffer to the QIAamp UCP MinElute column and centrifuge for 1 min at 6000 x g.
6000 x g, Room temperature, 00:01:00 1m

18 Place the QIAamp UCP MinElute column into a clean 2 ml collection tube. **Discard** the **flow-through**. 1m

19 Centrifuge the QIAamp UCP MinElute column at 20,000 x g for 3 min.
20000 x g, Room temperature, 00:03:00 3m

20 Place the QIAamp UCP MinElute column into a new 1.5 ml elution tube. **Discard** the **2 ml collection tube**. 1m

21 Open the lid of the tube and incubate the assembly in a shaker for microcentrifuge tubes at 56°C for 3 min to dry the membrane completely.
00:03:00 56 °C 3m

22 Carefully pipet 23 µL ultra-clean water into the centre of the membrane. Close the lid and incubate at Room temperature for 00:15:00 . 15m

23 Centrifuge at 20,000 x g for 1 min to elute the DNA.
20000 x g, Room temperature, 00:01:00 1m

24 To maximise yield from the elution: Aspirate the eluate from the previous step and reload it onto the centre of the membrane. **For this, carefully take off the QIAamp UCP MinElute column and after reloading, place it in the same 1.5 ml elution tube.** Close the lid and incubate 00:01:00 at Room temperature . 1m

25 Centrifuge at 20,000 x g for 1 min to elute the DNA.
20000 x g, Room temperature, 00:01:00 1m

26 Quantify 1 µL eluted sample using a Qubit fluorometer. Therefore add 1 µL eluted sample to 199 µL Qubit-Solution into a Qubit-Tube. 2m

27 **The DNA can now be stored for up to a week at 4 °C until further processing.**

28 **Continue with the protocol when enough samples are collected so that the total amount of DNA for one GridION Flowcell is between 500 ng and 1000 ng to not load one Flowcell with a molar mass of more than 2000 fmol.**

DNA repair and end-prep 2h 23m

29 Prepare the NEBNext FFPEDNA Repair Buffer v2, NEBNext FFPE DNA Repair Mix v2 and according to the protocol and place on ice. 2m

30 In each of the 0.2 ml thin-walled PCR tube containing your cfDNA samples, mix the following: 10m

	Reagent	Volume [µl]
	cfDNA from the previous step	20
	NEBNext FFPE DNA Repair Buffer v2	3
	NEBNext FFPE DNA Repair Mix v2	0.9
	Total	**23.9**

Use 20 µl of the DNA eluted in Step 30. Do not normalise samples by diluting with nuclease free water.

31 Thoroughly mix the reaction by gently pipetting and briefly spinning down. 2m

32 Using a thermal cycler with a heated lid set to 50 °C, incubate the reaction at 37 °C for 00:15:00 and hold at 4 °C. 15m

33 Remove the reaction from the thermal cycler and place the tube on ice. 30s

34 Keeping the tubes on ice, add 0.9 µL NEBNext Thermolabile Proteinase K directly to each of the repaired reaction mixtures. 2m

35 Mix by pipetting **10 times**, followed by spinning down quickly to collect all liquid from the sides of the tube. 2m

36 Using a thermal cycler with a heated lid set to 75 °C, incubate at 37 °C for 00:15:00 and 65 °C for 00:05:00, then hold at 4 °C. 20m

37 Remove the reaction from the thermal cycler and place the tube on ice. 30s

38 Keeping the tube on ice, add 1.3 µL NEBNext Ultra II End Prep Enzyme Mix directly to the reaction mixture for a total volume of 26.1 µl. 2m

39 Mix by pipetting 10 times, followed by spinning down quickly to collect all liquid from the sides of the tube. 2m

40 Using a thermal cycler with a heated lid set to 75 °C, incubate at 20 °C for 00:30:00 and 65 °C for 00:30:00, then hold at 4 °C. 1h

41 Resuspend the AMPure XP Beads (AXP) by vortexing. 1m

42 Transfer each cfDNA sample into a separate clean 1.5 ml Eppendorf DNA LoBind tube. 2m

43 Add 80 µL resuspenden AMPure XP Beads (AXP) to each end-prep reaction and mix by flicking the tube. 2m

44 Incubate on a Hula mixer (rotator mixer) for 00:05:00 at room temperature. 5m

45 Prepare 5 mL 80 % fresh ethanol in nuclease-free water. 1m

46 **Spin down** the sample and pellet on a magnet until supernatant is clear and colourless. Keep the tube on the magnet, and pipette off the supernatant. 1m

47 Keep the tube on the magnet and wash the beads with 200 µL 80 % Ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard. 1m
Note If the pellet was disturbed, wait for beads to pellet again before removing the ethanol.

48 Repeat the previous step. 1m

49 **Spin down** and place the tube back on the magnet. Pipette off any residual ethanol. Allow to dry for 00:00:30 , but do not dry the pellet to the point of cracking. 2m

50 For each sample, remove the tube from the magnetic rack and resuspend the pellet in 10 µL nuclease-free water by flicking. 2m

51 Incubate for 00:02:00 at Room temperature . 2m

52 **Pellet** the beads **on a magnet** until the eluate is clear and colourless, for at least 00:01:00 . 2m

53 For each sample, remove and retain 10 µL Eluate into a separate clean 1.5 ml Eppendorf DNA LoBind tube. 1m

54 Quantify 1 µL eluted sample using a Qubit fluorometer according to Step 26. 2m

Native barcode ligation 1h 23m

55 Prepare the Blunt/TA Ligase Master Mix according to the protocol and place on ice. 2m

55.1 Thaw the EDTA at Room temperature and mix by vortexing. Then spin down and place on ice. 2m

55.2 Thaw the Native Barcodes (NB01-24) at Room temperature . Briefly spin down, individually mix the barcodes required for your number of samples by pipetting, and place them on ice. 3m

56 Select a unique barcode for each sample to be run together on the same flow cell.
The number of samples barcoded and combined in one experiment depends on the total amount of DNA as described in Step 28.

57 In the 0.2 ml PCR-tubes containing your normalised sample inputs, add the reagents in the following order for each sample: 5m

	Reagents	Volume [µl]
	End-prepped DNA	7.5
	Native Barcode (NB01-24)	2.5
	Blunt/TA Ligase Master Mix	10
	Total	**20**

Use 7.5 µl of the DNA eluted in Step 53. Do not normalise samples by diluting with nuclease free water.

58 Thoroughly mix the reaction by gently pipetting and briefly spinning down. 2m

59 Incubate for 00:20:00 at Room temperature . 20m

60 Add the following volume of EDTA to each well and mix thoroughly by pipetting and spin down briefly. 5m

	EDTA cap colour	Volume [µl]
	clear cap EDTA	2 µl
	blue cap EDTA	4 µl

61 Pool all the barcoded samples in a 1.5 ml Eppendorf DNA LoBind tube.

2m

62 Resuspend the AMPure XP Beads (AXP) by vortexing. 1m

63 Add 1.2X AMPure XP Beads (AXP) to the pooled reaction, and mix by pipetting. 1m
The total volume of the pooled suspension is dependent on your number of samples. Multiply your volume by 1.2 to get the right volume of AMPure XP Beads.

64 Incubate on a Hula mixer for 00:10:00 at Room temperature . 10m

65 Prepare 2 mL fresh 80% ethanol in nuclease-free water. 2m

66 **Spin down** the sample and pellet on a magnet for 00:05:00 . 6m
Pipette off the supernatant.

67 Keep the tube on the magnetic rack and wash the beads with 700 µL 80 % Ethanol 2m
without disturbing the pellet. Remove the ethanol using a pipette and discard.

68 Repeat the previous step. 2m

69 **Spin down** and place the tube back on the magnetic rack. Pipette off any residual 2m
ethanol. Allow the pellet to dry for ~30 seconds, but do not dry the pellet to the point of cracking.

70 Remove the tube from the magnetic rack and resuspend the pellet in 1m
32 µL nuclease-free water by gently flicking.

71 Incubate for 00:10:00 at 37 °C . Every 00:02:00 , agitate the sample by 11m
gently flicking for 00:00:10 to encourage DNA elution.

72 **Pellet** the beads on a **magnetic rack** until the eluate is clear and colourless. 1m

73 Remove and retain 32 µL eluate into a clean 1.5 ml Eppendorf DNA LoBind tube. 1m

74 Quantify 1 µl of eluted sample using a Qubit fluorometer according to step 26. 2m

Adapter ligation and clean-up 55m 5s

75 Prepare the NEBNext Quick Ligation Reaction Module (NEBNext Quick Ligation Reaction Buffer + Quick T4 DNA Ligase) according to the manufacturer's instructions, and place on ice. 2m
Note: Do NOT vortex the Quick T4 DNA Ligase.

75.1 Thaw the Native Adaper (NA) and Elution Buffer (EB) at Room temperature and place on ice. 2m

75.2 Spin down the reagent tubes for 00:00:05 . 5s

76 In a 1.5 ml Eppendorf LoBind tube, mix in the following order: 10m
Between each addition, pipette mix 10 - 20 times.

	Reagents	Volume (µl)
	Pooled barcoded sample	30
	Native Adapter (NA)	5
	NEBNext Quick Ligation Reaction Buffer (5X)	10
	Quick T4 DNA Ligase	5
	Total	**50**

77 Thoroughly mix the reaction by gently pipetting and briefly spinning down. 2m

78 Incubate the reaction for 00:20:00 at Room temperature . 20m

79 Resuspend the AMPure XP Beads (AXP) by vortexing. 1m

80 Add 60 µL (1.2x) resuspended AMPure XP Beads (AXP) to the reaction and mix by pipetting. 2m

81 Incubate on a Hula mixer for 00:10:00 at Room temperature . 10m

82 **Spin down** the sample and **pellet** on the **magnetic rack**. Keep the tube on the magnet and **pipette off** the **supernatant**. 3m

83 Quantify 1 µl of eluted sample using a Qubit fluorometer according to step 26. 3m

84 **Note:** The total amount of DNA should now be ≤430 ng to not exceed a molar mass of 2000 fmol with a DNA fragment length of 350 bp.

Priming and loading the GridION FlowCell 24m 40s

85 Thaw the Flow Cell Flush (FCF), BSA (50 mg/ml), Flow Cell Tether (FCT), Sequencing Buffer (SB) and Library Beads (LIB) at Room temperature . Place on ice. 4m

86 **Before: Perform a Flowcell-Check** 10m

86.1 Place your Flowcell in the GridIon-Device.

86.2 Open the MinKnow-Software and click on "Start". Go to "Flow Cell Check" and choose the position of your Flowcell and click "Start".

87 Prepare the Flowcell priming mix at room temperature. Mix by inverting. 2m

	Reagents	Volume [µl]
	Flow Cell Flush (FCF)	1.170
	BSA (50 mg/ml)	5
	Flow Cell Tether (FCT)	30
	Total	**1.205**

88 To open the priming port of the flow cell, slide the port cover clockwise. 10s

89 Set a P1000 pipette to 200 µl. Insert the tip into the priming port. Turn the wheel of the pipette until the dial shows ~220-240 µl. 1m

90 Load 800 µL priming mix into the flow cell by using the priming port. 2m
→ Dial the wheel of the pipette until a small drop of priming mix is visible on the pipette tip.
Incubate 00:05:00 . While Incubation proceed with step 93.

91 Prepare the library in a new 1,5 ml Eppendorf tube. Thoroughly mix the Library Beads by pipetting. 3m

	Reagents	Volume [µl]
	Sequencing Buffer (SB)	37.5
	Library Beads (LIB)	25.5
	DNA library	12
	Total	**75**

92 Gently open the SpotON sample port. 10s

93 Load 200 µL priming mix into the **priming port**. 1m

94 Mix the library by gently pipetting. In a **dropwise** fashin load 75 µL library into the flow cell via the SpotON port. 1m

95 Gently close the SpotON and priming port. Place the flow cell into the sequencing device. Start Sequencing. 20s

Protocol references

Oxford Nanopore Technologies. (2025). *Ligation sequencing V14 – Human cfDNA multiplex (SQK-NBD114.24)*. https://nanoporetech.com/document/ligation-sequencing-v14-human-cfdna-multiplex-sqk-nbd114-24