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ONT Flongle FlowCell Sequening of cfDNA

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We are still developing and optimizing this protocol

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Abstract

This protocol is a custom adaptation for cfDNA sequencing using ONT Flongle Flow Cell, Watchmaker genomics library prep kit and STRECK tubes. It is meant for microbial cfDNA Sequencing.

Protocol materials

- Proteinase K **Thermo Fisher Scientific** **Catalog #**100005393
- SDS 20% solution **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**05030-500ML-F
- cfPure Lysis/Binding Buffer **BioChain** **Catalog #**K5011610-V2
- cfPure Magnetic Bead **BioChain** **Catalog #**K5011610-V2
- cfPure Wash Buffer **BioChain** **Catalog #**K5011610-V2
- cfPure Wash Buffer **BioChain** **Catalog #**K5011610-V2
- cfPure Elution Buffer **BioChain** **Catalog #**K5011610-V2
- ER/AT Buffer **Watchmaker Genomics** **Catalog #**7K0101-024
- ER/AT Enzyme Mix **Watchmaker Genomics** **Catalog #**7K0101-024
- Ligation Buffer **Watchmaker Genomics** **Catalog #**7K0101-024
- Ligation Enzyme **Watchmaker Genomics** **Catalog #**7K0101-024
- Ligation Adaptor (LA) **Oxford Nanopore Technologies**
- Ligation Sequencing Kit V14 **Oxford Nanopore Technologies** **Catalog #**SQK-LSK114
- AMPure XP Beads (AXP) **Oxford Nanopore Technologies** **Catalog #**SQK-RBK114.96
- Short Fragment Buffer (SFB) **Oxford Nanopore Technologies**
- Elution Buffer (EB) **Oxford Nanopore Technologies**
- Flow Cell Flush (FCF) **Oxford Nanopore Technologies** **Catalog #**SQK-RBK114.96
- Flow Cell Tether (FCT) **Oxford Nanopore Technologies** **Catalog #**SQK-RBK114.96
- Flongle Sequencing Expansion **Oxford Nanopore Technologies** **Catalog #**XP-FSE002

Plasma Isolation from STRECK Tube

30m

1 Cell-Free DNA BCT is a blood collection tube that stabilizes cell-free DNA for up to **14 days at 6 °C to 37 °C*** and CTCs for up to 7 days at 15 °C to 30 °C*. It is available as a CE marked in vitro diagnostic device or as a Research Use Only tube, depending on region of use.

2 300 rpm, 20°C, 00:20:00 , If the tubes were still at room temperature for > 24h you can skip 20m step.

Centrifuge the STRECK tube Sample

Expected result

Expect to see the fases, plasma, plaquetes and leucocytes, and hematites

3 5 mL Pippete the Plasma into a 15 mL Falcon tube

4 3000 rpm, 20°C, 00:10:00 10m

Expected result

Debris and Cells in the pellet

5 Sample Pippete the supernatant into a new Falcon 15 mL tube.

6 Freeze at -20 °C

Extract cfDNA from plasma isolated from STRECK Tubes using cfPure

46m 10s

7 Thaw the plasma On ice 1 hour

8 Add 30 µL Proteinase K **Thermo Fisher Scientific Catalog #**100005393 at 50 mg/mL to each tube

9 Add 50 µL SDS 20% solution **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**05030-500ML-F for every 1 mL of plasma.

10 Incubate at 56 °C for 00:20:00 in the water bath with low shake 20m

11 After incubation place tubes On ice for 00:05:00 to cool 5m Room temperature

12 Add 1.25 mL cfPure Lysis/Binding Buffer **BioChain Catalog #**K5011610-V2 for every 1 mL of Plasma

13 Add 25 µL of cfPure Magnetic Bead **BioChain Catalog #**K5011610-V2 for every 1 mL of Plasma.

Important: Mix well prior to adding. There should be no visible sedimentation at the bottom of the
solution after mixing.

14 Adapt the Falcon 15 mL tubes by attaching 2 mL eppendorf using tape

15 Vortex or 3000 rpm, 00:10:00 on 10m

Equipment

Digital Vortex-Genie 2	NAME
Vortex	TYPE
Scientific Industries, Inc	BRAND
SI-A236	SKU
https://www.scientificindustries.com/digital-vortex-genie-2.html	LINK

at Room temperature

16 Place tube(s) into a magnet stand 00:03:00 to 00:05:00 or until solution clears. 3m

17 While keeping the tube(s) on the magnet stand, remove supernatant. Be careful not to remove magnetic particles

18 Keep tube(s) on magnet stand for 00:01:00 , and remove residual supernatant 1m

19 **First Wash**

19.1 Add 1000 µL of cfPure Wash Buffer **BioChain Catalog #**K5011610-V2 to lysis/binding tube(s)

19.2 Resuspend beads by vortexing 00:00:10 or pipetting up and down 10 times 10s

19.3 Transfer magnetic particle suspension into 1.5 ml micro tube(s) on magnet stand

19.4 Allow beads to attach to magnet stand for 10-30 seconds

19.5 Pipette supernatant from 1.5 ml tube(s) and use the supernatant to wash the lysis/binding tube(s)

19.6 Transfer the rest of the magnetic particles in lysis/binding tube(s) to the 1.5 ml tube(s)

19.7 Keep tube(s) on magnet stand for 10-30 seconds or until solution is clear

19.8 Remove as much buffer as possible using a 1000 µl pipette. Tap magnet stand on bench 5 times and remove remaining wash buffer with 200 µl pipette

19.9 Transfer tube(s) to non-magnetic rack and add 1000 µL of cfPure Wash Buffer **BioChain Catalog #**K5011610-V2

19.10 Resuspend beads by vortexing for 20 seconds or pipetting up and down 10 times

19.11 Place tube(s) on magnet stand for 10-30 seconds. Remove as much buffer as possible using a 1000 µl pipette

20 **Second Wash**

20.1 Transfer tube(s) to non-magnetic rack and add 1000 µL of **80% EtOH**

20.2 Resuspend beads by vortexing for 20 seconds or pipetting up and down 10 times

20.3 Place on magnet stand for 10-30 seconds or until solution clears

20.4 Remove as much buffer as possible using a 1000 µl pipette. Remove remaining EtOH with 200 µl pipette

20.5 Transfer tube(s) to non-magnetic rack and add 1000 µL of **80% EtOH**

20.6 Resuspend beads by vortexing for 20 seconds or pipetting up and down 10 times

20.7 Place on magnet stand for 10-20 seconds. Remove as much EtOH as possible using a 1000 µl pipette and leave cap open. Remove remaining EtOH with 200 µl pipette. Leave tube(s) open on magnet stand for 00:02:00 2m

21 **Elution Step**

21.1 Transfer microtube(s) to non-magnetic rack and add 50 µL of cfPure Elution Buffer **BioChain Catalog #**K5011610-V2 and resuspend the beads.
Important: A minimum of 20 µl of cfPure Elution Buffer per ml of plasma is recommended to elute DNA to
ensure optimal yields

21.2 Vortex or shake tube(s) vigorously for 00:05:00 5m

21.3 Centrifuge tube(s) briefly. And Place tube(s) on magnetic rack for 10 to 30 seconds.

21.4 Transfer elute into a new 1.5 ml tube(s

Library Preparation for ONT using WatchMaker

32m

22 Thaw On ice all reagents and samples

23 **End Repair A-tailing**

23.1 Prepare End Repair and A-tailing (ER/AT) master mix.

7 µL ER/AT Buffer **Watchmaker Genomics Catalog #**7K0101-024
3 µL ER/AT Enzyme Mix **Watchmaker Genomics Catalog #**7K0101-024 for every sample

Component	Volume (µL)
ER/AT Buffer	7
ER/AT Enzyme Mix	3

23.2 Mix with pipette up and down

23.3 Prepare a 200 µL eppendorf with 25 µL of H_2O + 25 µL of cfDNA Sample

23.4 Add 10 µL of ER/AT master mix into 50 µL Sample

Component	Volume (µL)
Input DNA	50
End repair and A-tailing master mix	10

23.5 Place the tubes in the thermocycler and initiate the end repair and A-tailing program

Step	Temperature	Time
Lid temperature	85°C[1]	N/A
End repair	20°C	30 min
A-tailing	65°C	30 min
HOLD	4°C	HOLD

[1]A heated lid is required for this incubation. If possible, set the temperature of the lid at 85°C, instead of the usual ~105°C.

23.6 Proceed immediately to Adapter Ligation after the program has finished and the samples have returned to 4 °C

24 Adapter Ligation

24.1 Prepare the Ligation master mix as follows:

25 µL Ligation Buffer **Watchmaker Genomics Catalog #**7K0101-024

5 µL Ligation Enzyme **Watchmaker Genomics Catalog #**7K0101-024

Component	Volume (µL)
Ligation Buffer	25
Ligation Enzyme	5

24.2 Add the Ligation master mix 30 µL to the Sample 60 µL and 3 µL of Ligation Adaptor (LA) **Oxford Nanopore Technologies** from the

Ligation Sequencing Kit V14 **Oxford Nanopore Technologies Catalog #**SQK-LSK114

Component	Volume (µL)
End repair and A-tailed DNA	60
Adapter	5
Ligation master mix	30

Here it says 5 µL but we use **3µL**

24.3 Place the sample tubes in the thermocycler and initiate the Ligation incubation program 15m

Step	Temperature	Time
Lid temperature	OFF	N/A
Ligation	20°C	15 min[1]

[1]Ligation time may be extended to a maximum of 16 hours. Library quality decreases with overnight ligation.

00:15:00

24.4 Once the program has completed, proceed immediately to Post-ligation Cleanup

25 **Post-Ligation Cleanup**

25.1 Thaw SPRI beads

AMPure XP Beads (AXP) **Oxford Nanopore Technologies Catalog #**SQK-RBK114.96

On ice to Room temperature

25.2 Add 93 µL of
AMPure XP Beads (AXP) **Oxford Nanopore Technologies Catalog #**SQK-RBK114.96
to the 93 µL Sample (from thermocycler)

NOTE: SPRI-to-sample bead ratios may be optimized for different applications or adapter configurations. A ratio of 0.8X is recommended as a starting point when using high-quality DNA and full-length adapters. Then 'stubby' adapters are used, the SPRI bead ratio may be increased to 1X (95 µL) to improve performance.

25.3 Transfer the mix to a new 1.5 mL eppendorf tube. Incubate the library-bead mixtures at Room temperature for at least 00:05:00 to maximize library recovery. 5m

25.4 Place sample tubes on a magnet for at least 00:05:00 , or until all beads have been collected on the tube wall and the solution is clear 5m

25.5 Carefully remove and discard the supernatant from each tube

25.6 Add 200 µL of Short Fragment Buffer (SFB) **Oxford Nanopore Technologies** ,taking care to not disturb the bead pellet on the tube wall.
IMPORTANT: Do not use ethanol, use SFB for the cfDNA

25.7 Incubate tubes at room temperature for at least 30 sec without removing the tube from the magnet
or disturbing the bead pellet.

25.8 Repeat 25.6

25.9 Allow remaining Short Fragment Buffer (SFB) **Oxford Nanopore Technologies** to evaporate by allowing the pellets to air dry for 00:03:00 3m

25.10 Remove sample tubes from the magnet and carefully resuspend each bead pellet in 22 µL of Elution Buffer (EB) **Oxford Nanopore Technologies** . Pipetting carefully will
minimize bubbling and allow for greater library recovery.

25.11 Incubate tubes at r Room temperature for at least 00:02:00 before placing back on the magnet. 2m

25.12 Leave sample tubes on the magnet for at least 00:02:00 , or until all beads have collected on the tube wall and the solution is clear 2m

25.13 Carefully transfer 22 µL of each library-containing supernatant to a new, labeled tube. If desired, library amplification may be carried out in the same tube in the presence of SPRI beads (See Library Amplification for reaction setup and Appendix A for details regarding PCR in the presence of beads).

25.14 Samples can be stored at 4°C for up to 1 week and at -20°C for up to 1 month

ONT Flongle FlowCell Sequencing

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Equipment

MinION Mk1C	NAME
Sequencer	TYPE
Oxford Nanopore Technology	BRAND
MIN-101C	SKU
https://store.nanoporetech.com/minion-mk1c.html	LINK

Equipment

Flongle Flow Cell (R10.4.1)	NAME
Flow cell	TYPE
Oxford Nanopore Technologies	BRAND
FLO-FLG114	SKU
https://store.nanoporetech.com/flongle-flow-cell-pack-r10-4-1.html	LINK

27 Prepare Flush Cell Buffer

117 µL

Flow Cell Flush (FCF) **Oxford Nanopore Technologies Catalog #**SQK-RBK114.96

3 µL

Flow Cell Tether (FCT) **Oxford Nanopore Technologies Catalog #**SQK-RBK114.96

28 Prepare library for load

Reagent	Volume
Sequencing Buffer (SB)	15 µL
Library Beads (LIB)	10 µL
3 - 20 fMol of the DNA library	5 µL
Total	30 µL

from

Flongle Sequencing Expansion **Oxford Nanopore Technologies Catalog #**XP-FSE002

29 Load the Flongle Flow Cell after FloCell check