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CFTR Long Read Sequencing Protocol using Oxford Nanopore Technology

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Nada EL Makhzen¹, Alexandre Bokhobza², Anne-Flore Hämmerli¹, Jean-Sébastien ougier¹, Hugues Abriel¹

¹Ion Channels and Channelopathies Laboratory, Institute for Biochemistry and Molecular Medicine, University of Bern, Bühlstrasse 28, 3012 Bern, Switzerland.;

²Department of Physiology, University of Bern, Switzerland.



Nada EL Makhzen

Ion Channels and Channelopathies Laboratory, Institute for B...



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We use this protocol and it's working

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Abstract

Cystic fibrosis (CF) is a genetic autosomal recessive disorder, commonly associated with European populations, with insufficient awareness in African populations. Whole-gene *CFTR* sequencing is effective for identifying diverse *CFTR* variants, facilitating accurate CF carrier screening in African populations. The proposed method uses long-read Oxford Nanopore Technologies to sequence the entire *CFTR* gene. This involves amplifying ~25Kb fragments of the *CFTR* gene using specially designed long-range PCR primer pairs, followed by barcoded library preparation and sequencing with ONT's R10.4 flow cells and the Mk1C device. This approach is especially advantageous for genetic laboratories with limited resources, due to its cost-effectiveness and relative ease of implementation.

Guidelines

Blood collection for this protocol requires prior approval by the users' Institutional Ethics Board or equivalent ethics committee.

1) DNA Extraction using puregene blood kit (Cat.#158023)

1

Processing

1ml blood samples:



Things to do before starting:

- Work with fresh blood samples collected in an EDTA tube (if working with frozen blood, thaw the sample quickly in a 37°C water bath with mild agitation and store it on ice before beginning the procedure).
- Preheat the water bath to 65°C for use in step 4.
- Optional: Preheat the water bath to 37°C for use in step 3.

1-1) Procedure

- 2
 - Dispense 3 ml of RBC Lysis Solution into a 15 ml falcon centrifuge tube.
 - Add 1 ml whole blood, and mix by inverting 10 times.
 - Incubate for 5 min at room temperature (15–25°C). Invert at least once during the incubation.
 - Centrifuge for 2 min at 2000x g to pellet the white blood cells.
 - Carefully discard the supernatant by pipetting or pouring, leaving approximately 70 µl of the residual liquid and the white blood cell pellet.
 - Vortex the tube vigorously to resuspend the pellet in the residual liquid. Vortexing greatly facilitates cell lysis in the next step.

Note

The pellet should be completely dispersed after vortexing.

- 3
 - Add 1 ml Cell Lysis Solution, and pipet up and down to lyse the cells or vortex vigorously for 10 sec.

Note

Usually, no incubation is required; however, if cell clumps are visible, incubate at 37°C until the solution is homogeneous.



- Optional: If RNA-free DNA is required, add 5 μ l, RNase A Solution, and mix by inverting 25 times.

Incubate for 15 min at 37°C.

Then incubate for 3 min, on ice to quickly cool the sample.

- Add 300 μ l, Protein Precipitation Solution, and vortex vigorously for 20 sec. at high speed.
- Centrifuge for 5 min at 2000 x g

Note

The precipitated proteins should form a tight, dark brown pellet. If the protein pellet is not tight, incubate on ice for 5 min and repeat the centrifugation.

- Pipet 1 ml isopropanol into a clean 15 ml tube and add the supernatant from the previous step by pouring it carefully.

Note

Be sure that the protein pellet is not dislodged during pouring.

- 4
 - Mix by inverting gently 50 times (Do not vortex) until the DNA is visible as threads or a clump.
 - Centrifuge for 3 min at 2000 x g (The DNA may be visible as a small white pellet).
 - Carefully discard the supernatant, and drain the tube by inverting on a clean piece of absorbent paper, taking care that the pellet remains in the tube.
 - Add 1 ml of 70% ethanol and invert several times to wash the DNA pellet.
 - Centrifuge for 1 min at 2000 x g
 - Carefully discard the supernatant. Drain the tube on a clean piece of absorbent paper, taking care that the pellet remains in the tube. Air dry the pellet for 5-10 min.

Note

The pellet might be loose and easily dislodged. Avoid over-drying the DNA pellet, as the DNA will be difficult to dissolve.

- Add 100 μ l DNA Hydration Solution and vortex for 5 sec. at medium speed to mix.
- Incubate at 65°C for 1h to dissolve the DNA.

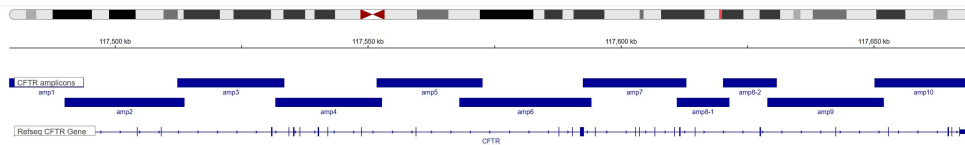
- Incubate at room temperature overnight with gentle shaking. Ensure that the tube cap is tightly closed to avoid leakage. Samples can then be centrifuged briefly and transferred to a storage tube (1.5 ml DNA LoBind Tube).

Note

DNA should be stored at -20°C for long-term use.

2) Amplification (PCR using takara Ex Premier™ DNA Polymerase Dye plus (cat.#RR371A))

5



11 overlapping PCR products to cover the whole CFTR gene

	A	B	C	D
		Sequence (5'→3') Forward	Sequence (5'→3') Reverse	Primer pair size
	CFTR Primer pair 1	AAGTGAAGTTCCTGCTTTG CTTTGGGTAAG	TGGGTTCTTGGTGGCATT TTAACCCCTTTT	22.803 kb
	CFTR Primer pair 2	GTCCCCATTCCAGCCATTT GTATCCTTAGA	AGTCCCTAGATAGCCAAT CACAGGAAGAGA	23.74 kb
	CFTR Primer pair 3	CAGCAGCCAGATAGATGCA ACATTGTCTTT	CACAATCCCTTCTTCAAG GCGACCTAGTAT	21.181 kb
	CFTR Primer pair 4	AAGAGACCAAATTGCCGA GGCATCATTTAG	GTCCAAGATAGTGGGAAA AGCAAGAAGCAA	21.115 kb

A	B	C	D
CFTR Primer pair 5	TAGAATTTTGCATCACTGG ATGCCCTTGAC	AATAAGTGTGGTAGGAGC AGAGGGAACAAG	20.963 kb
CFTR Primer pair 6	GTAGAATGAAATGAGCCTG GACCCAAGAGA	ATCTGGATTTGAACCCAA CCTCCACCATT	26.089 kb
CFTR Primer pair 7	AGATCTCCTCCTCAGTCAC ACAGA	GGAGCTGTTAAGGGAGAC AAGGAA	20.492 kb
CFTR Primer pair 8.1	GTGTTGGAGCTGCCATTTTC G	TATGGCCACAGGACACA TC	10.41 kb
CFTR Primer pair 8.2	GTCTGTGGTAAGAGCCCT GC	CCTTTCCCACCCACTGAG AC	10.635 kb
CFTR Primer pair 9	TGCCACCCCTTTGCTTTG CT	TACTTAGTTCTCTGTGCC TGGGGTGAATTG	23.059 kb
CFTR Primer pair 10	CTTTGATTGCTGAATGCCA ATGGATGGGTA	GACAGGGACAAGTAAAGC CCCAAATCAAG	22.251 kb

CFTR Primer Pairs

6

- Prepare 11 PCR tubes per patient **on ice**, using the following mix:

A	B
Reagent	Volume
Takara Ex Premier™ DNA Polymerase Dye plus (cat.#RR371A)	12.5 µl
Nuclease free water	10.5 µl
Forward primer at 10 µM	0.5 µl
Reverse primer at 10 µM	0.5 µl
gDNA (100ng/µl)	1 µl
Total	25 µl

**Note**

Pipette up and down, and if needed spin down briefly.

- 7
- Amplify using the following thermocycler conditions:

	A	B	C
	Steps	Temperature	Time
	Initial Denaturation	94°C	1 min
	For 27cycles (Annealing and extension in one step)	98°C	10 sec.
		68°C	13 min
	Hold	4°C	∞

Thermocycler condition for PCR 1, 2, 3, 4, 5, 6, 7, 9 and 10

	A	B	C
	Steps	Temperature	Time
	Initial denaturation	94°C	1 min
	For 27 cycles (Annealing and extension in one step)	98°C	10 sec.
		68°C	10 min
	Hold	4°C	∞

Thermocycler condition for PCR 8-1 and 8-2

3) DNA visualization in agarose gel electrophoresis

- 8
- Prepare a 0.4% Tris Acetate EDTA (TAE) agarose gel.
 - Load 5 µl of each amplified product.

Note

A ladder with a 1 kb size needs to be added to one of the wells as a reference for analysis.

- Run the gel at 160V for 30 min.
- Visualize the DNA using a gel imaging device.

Note

Some unspecific band products may appear during amplification and be visible in the agarose gel; however, if their intensity remains below the target range, it is considered acceptable, since the next section steps will help get rid of these bands.

4) PCR product Purification

- 9
 - Transfer each PCR-amplified product to a DNA LoBind Tube (11 tubes per patient).
 - Resuspend the AMPure Beads (*Beckman coulter, Cat.#A63881*) by vortexing, and add 1.8 volume of AMPure beads for 1 volume of PCR product.
 - Mix the solution by pipetting up and down, be gentle, and avoid making bubbles.
 - Incubate for 5 min at RT, and prepare fresh 80% ethanol during this time.
 - Place on a magnetic rack for at least 2 min until the liquid becomes clear.
 - Remove the supernatant without disturbing the beads.
 - Add 200 µl of freshly prepared 80% ethanol without disturbing the pellet.
 - Remove ethanol.
 - Repeat the previous step.
 - Eliminate residual ethanol (use a new pipette tip to aspirate ethanol in the bottom of the tube).
 - Remove from the magnet and elute with 40 µl of Elution Buffer (10 mM Tris pH8.0 + 50 mM NaCl). Incubate for 2 min at RT.
 - Place on a magnetic rack for 1 min (until the eluate is clear).
 - Aspirate and keep eluate (35-37 µl) without taking the beads.
 - Remove and retain the eluate (keep on ice).

Note

- DNA can be kept at 4°C overnight.
- Quantify the DNA.

5) Sequencing using Rapid Barcoding Kit V14 (SQK-RBK114.24)

10 **For each PCR-amplified purified product (11 amplicons for each individual) input, transfer the following quantity depending on the number of individuals:**

- 50 ng gDNA per PCR- amplified purified product for >4 individuals.
- 200 ng gDNA per PCR- amplified purified product for <4 individuals.

Note

Sometimes low quality obtained during purification makes it difficult to get 50ng gDNA per PCR-amplified purified product; in such cases, <50ng gDNA input per PCR-amplified purified products might be a solution; just be sure to have the same quantity input for all individuals.

Prepare the DNA in nuclease-free water:

- Transfer the gDNA into 0.2 ml thin-walled PCR tubes (11 tubes per individual).
- Adjust the volume to 10 µl with nuclease-free water.
- Pipette mix the tubes 10-15 times to avoid unwanted shearing.
- Spin down briefly in a microfuge.

11 ■ **In each 0.2 ml thin-walled PCR tube:**

A	B
Reagent	Volume per Sample
gDNA	10 µl
Rapid Barcodes (RB01-24) one for each patient	1 µl
Total	11 µl

Note

Pipette up and down to ensure the components are mixed.

- Incubate the tubes as follows:

	A	B
	<i>Temperature</i>	<i>Time</i>
	30°C	2 min
	80°C	2 min

Note

After incubation, briefly put the tubes on ice to cool.

12

- **Pool all barcoded samples in a 1.5 ml Eppendorf DNA LoBind tube.**

13

- Resuspend the AMPure XP Beads (*Beckman coulter, Cat.#A63881*) by vortexing.
- Add an equal volume of resuspended AMPure XP Beads to the entire pooled barcoded sample from step 10, mix by pipetting up and down, and avoid making bubbles.

	A	B
	<i>Volume per sample</i>	<i>Volume of AMPure XP Beads to be added</i>
	11 µl	11 µl

- Incubate for 5 min at room temperature, and prepare at least 3 ml of fresh 80% ethanol during this time.
- Place on a magnetic rack for at least 2 min until the liquid becomes clear.
- Remove supernatant without taking beads.
- Add 1.5 ml of 80% of freshly prepared ethanol without disturbing the pellet.
- Remove ethanol.
- Repeat the previous step.
- Eliminate residual ethanol (use a new pipette tip to aspirate ethanol in the bottom of the tube).
- Remove from the magnet and elute with 15 µl of Elution Buffer (EB). Incubate for 10 min at room temperature.
- Place on the magnet for at least 2 min until the eluate becomes clear.



- Aspirate and keep eluate 15 µl without taking the beads into a clean Eppendorf DNA LoBind tube.

Note

Quantify 1 µl of the eluted sample.

- 14
- Transfer 11 µl of the sample into a clean 1.5 ml Eppendorf DNA LoBind tube.

Note

Transfer a maximum of 800 ng of the DNA library.

- In a fresh 1.5 ml Eppendorf DNA LoBind tube, dilute the Rapid Adapter (RA) as follows and pipette mix:

	A	B
	Reagent	Volume
	Rapid Adapter (RA)	1.5 µl
	Adapter Buffer (ADB)	3.5 µl
	Total	5 µl

- Add 1 µl of the diluted Rapid Adapter (RA) to the barcoded DNA.
- Mix gently by flicking the tube, and spin down.
- Incubate the reaction for 5 min at RT.

Note

After the incubation, store your sample on ice, until ready to load.

6) Library Loading on R10.4.1 flow cell (FLO-MIN114) using Flow Cell Priming Kit (EXP-FLP004)

15



- Perform a flow cell check to determine the number of available pores (for the flow cell, the number of pores should be >800 pores).

6) Library Loading on R10.4.1 flow cell (FLO-MIN114) using Flow Cell Priming Kit (EXP-FLP004)

16 **Material:** - Using Flow Cell Priming Kit (EXP-FLP004), thaw the sequencing Buffer (SB), library Beads (LIB), Flow cell tether (FCT), and one tube of Flow cell Flush (FCF) at room temperature. Mix by vortexing and spin down.

- Prepare the flow cell priming mix, using the following reagents:

A	B
<i>Reagents</i>	<i>Volume per flow cell</i>
FCF	1,170 µl
FCT	30 µl
Final volume in FCF tube	1,200 µl

- 17
- Open the priming pore (1) of the flow cell as displayed in the picture and:
 - Set a P1000 pipette to 200 µl.
 - Insert the tip into the priming port.
 - Turn the wheel until the dial shows 220-230 µl, or until you can see a small volume of buffer entering the pipette tip.



Note

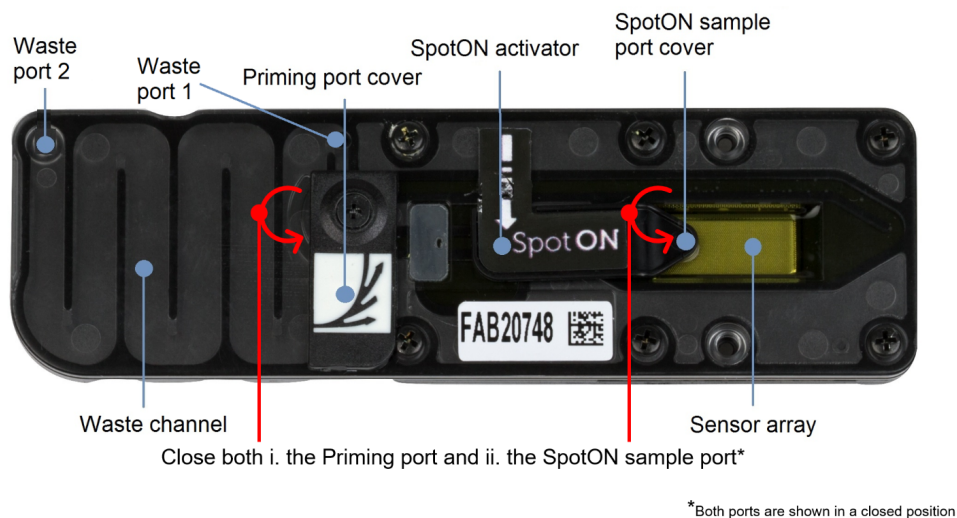
Visually check that there is a continuous buffer from the priming port across the sensor array.

- Load 800 μ l of priming mix in the priming port (1) of the flow cell, avoiding the introduction of air bubbles. Wait for 5 min.
- Prepare the library:
 - Mix the LIB component in a low DNA binding tube.
 - In a new tube, prepare the library for loading as follows:

A	B
<i>Reagent</i>	<i>Volume per flow cell</i>
SB	37,5 μ l

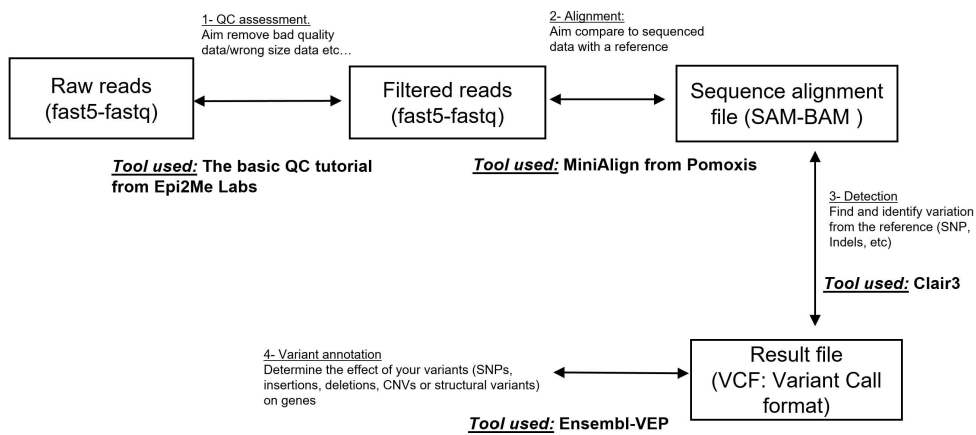
A	B
LIB (mixed immediately before use)	25,5 μ l
DNA library	12 μ l
Total	75 μl

- Lift the SpotOn sample port cover (2).
- Load 200 μ l of priming mix via the priming port (1).
- Mix the prepared library by pipetting up and down (do not make bubbles!) and load 75 μ l on the **SpotON sample port (2)** (different port!) drop by drop on top of the pore.
- Put back the SpotOn sample port cover and close the priming pore.
- Start sequencing.



7) Data Analysis

- 18
 - Stop sequencing, after a maximum of 24h (it depends on how many individuals are sequenced), in our case, sequencing was stopped with an estimated N50 of ~7kb and with ~ 2.3M of generated reads.
- 19
 - Data Analysis can be done using EPI2Me tools from Oxford Nanopore, or through a bioinformatic analysis pipeline using tools as described in the figure below:

Nanopore bio-informatics analysis.

- 20
- Aligned reads and detected variants can be visualised using integrative genomics viewer (IGV) (<https://igv.org/app/>)