CE
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Instructions For Use

Cystic Fibrosis Transmembrane Conductance Regulator Gene (*CFTR*) Library Prep Kit for Illumina®

NGS Library Prep by Reverse Complement PCR

Version: *2*
REF: *IFU-CFTR*
Revision Date: *2022-10-05*

Description of the symbols used on the labels

Symbol	Description
	Manufacturer
	Use-by date
	Lot number
	Reference number
	Upper limit of temperature for storage
	Temperature limit for storage
	Consult the instructions for use
	Contains sufficient for <*n*> tests
	CE-mark
	*In vitro* diagnostic medical device
	The box contains 2 index plates
	Matrix code containing the reference number, lot number and use-by date

Product and Company Information

Product name:	Cystic Fibrosis Transmembrane Conductance Regulator Gene (*CFTR*) Library Prep Kit for Illumina®
REF:	RC-CFTR096-I
Product use:	For *In Vitro* Diagnostic use
Company:	NimaGen B.V. Hogelandseweg 88 6545 AB Nijmegen The Netherlands
Telephone:	+31 (0)24 820 02 41
Email:	info@nimagen.com

Product Use

The intended purpose of this assay is NGS library preparation for Illumina® sequencing of the human CFTR gene coding exons, including a minimum of 20 bases upstream and downstream of each exon; to aid in the diagnosis of (the congenital predisposition to) cystic fibrosis by detecting sequence variants. This assay also includes 2 intronic hotspot mutations.

The assay is designed for use with DNA extracted from peripheral blood. Specimens should have an absorbance ratio (260/280) of ~1.8, regardless of the extraction method used.

The assay provides reagents for Multiplex Amplicon-based NGS library preparation and is for professional use only.

The kit is based on the patented Reverse Complement PCR technology, providing a safe, robust and simple workflow, combining multiplex amplification in a single reaction, decreasing the risk of PCR contamination and sample swapping.

Kit Content: RC-CFTR096-I (-20 °C storage)

Description	Content
RC-PCR Probe Panel A (REF: PM-CFTR-A)	Tube 24 µL
RC-PCR Probe Panel B (REF: PM-CFTR-B)	Tube 24 µL
2x Master Mix (Hot Start HiFi) (REF: MMHS096)	2 Tubes 1.15 mL
Probe Dilution Buffer (REF: RC-PDB)	Tube 500 µL

To be ordered separately: IDX096-U01D-I or IDX096-U02D-I (max 25 °C storage)

Description	Content
IDX* Primer Plates, dehydrated, coloured, 96 Unique Dual, 10 bp Indexes for Illumina®	2x 96-well plate, with Alphanumeric Coding
Caps	2x 12 Domed 8-Cap Strips

**IDX plates are semi-skirted, ABI style PCR Plates, breakable per 8 tubes, containing dehydrated and stabilized Unique Dual Index primer pairs in each well and a red tracking dye, ready to use.*

The reagents do not contain any tissues or substances from human or animal origin.

Needed, but not included

Description	Vendor
Adjustable Pipette Set (P2, P10, P20, P100, P200, P1000)	Multiple Vendors
TapeStation, Bioanalyzer System, or equivalent, incl. consumables, or optional: agarose gel system	Agilent® or other
Ethanol absolute, mol. biol. grade	Multiple Vendors
AMPureXP™ Bead Solution (or similar)	Beckman Coulter
General plasticware, DNAse free (1.5 mL tubes, pipette tips w/filter)	Multiple Vendors
Ice or tabletop cooling block	Multiple Vendors
Mini Spinner for 1.5 mL tubes and 8-well PCR strips	Multiple Vendors
Magnetic Stand for 1.5 mL Eppendorf tubes	Multiple Vendors
PCR Grade Water	Multiple Vendors
Qubit™ Fluorometer including High Sensitivity consumables	Thermo Fisher
Thermocycler with heated lid (0.2 mL standard PCR tubes) compatible with semi-skirted ABI style PCR plates and option for ramp rate programming of 0.1 °C/sec (or 2% of max).	Multiple Vendors
The next five items are only necessary when sequencing in-house (not needed in case of sending samples to a core facility)	
NaOH solution (2 N)	Multiple Vendors
Tris/HCl (200 mM), pH 7	Multiple Vendors
Low TE (10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA)	Multiple Vendors
Illumina® NGS Sequencing instrument	Illumina®
Illumina® Sequencing Reagent kit (300 (2x151) bp run)	Illumina®

Procedure

General precaution

Use a Pre-PCR environment for setting up the RC-PCR. Pooling, cleaning and library preparation should be performed in a Post-PCR environment.

1. Thermocycling program

Temp	Duration	Ramping rate (from previous step)	Cycles
98 °C	2 minutes	N/A	1 x
98 °C	10 seconds	Max	1 x
58 °C	10 minutes	**0.1 °C/sec (or 2% of max)**	
72 °C	1 minute	Max	
95 °C	10 seconds	Max	2 x
80 °C	1 second	Max	
58 °C	90 **minutes**	**0.1 °C/sec (or 2% of max)**	
72 °C	30 seconds	Max	
95 °C	10 seconds	Max	34 x
80 °C	1 second	Max	
58 °C	2 minutes	**0.5 °C/sec (or 10% of max)**	
72 °C	30 seconds	Max	

Heated lid at 105 °C.

Double Check: Depending on the instrument, this protocol takes 6-7 hours to complete.

2. Reverse Complement PCR

The target specific RC-probes will be transformed into the functional, tailed and indexed PCR primers, followed by multiplex DNA amplification of the target regions.

2.1 Thaw on ice:

- RC-PCR Probe Panel A (Black cap)
- RC-PCR Probe Panel B (Red cap)
- Probe Dilution Buffer (Blue cap)
- 2x HiFi Master Mix (White cap)

Note: The Master Mix contains isostabilizers and may not freeze completely, even when stored at -15 °C to -25 °C. The Master Mix may contain precipitates when thawed at +2 °C to +8 °C. Always ensure that the Master Mix is fully thawed and thoroughly mixed before use.

2.2. Take two identical IDX PCR plates and cut off the number of strips needed. Mark the plates with 'A' and 'B'.

Note: Register the indexes used (IDX set/strip-column number and well position for each sample).

Note: For each sample, two PCR reactions are needed (Panel A and Panel B). Always use the same well position for the same sample, in order to generate identical indexes for each sample in both panels .

Note: Make sure to use the strips or plates in the right orientation.

2.3. Prepare in a fresh 1.5 mL Eppendorf tube the Probe-Polymerase premix panel A, by combining and mixing:

- 0.2 µL RC-PCR Probe Panel A per reaction (Black cap)
- 1.8 µL Probe Dilution Buffer per reaction (Blue cap)
- 4 µL Molecular Grade Water
- 10 µL Master Mix per reaction (White cap)

2.4. Prepare in a fresh 1.5 mL Eppendorf tube the Probe-Polymerase premix panel B, by combining and mixing:

- 0.2 µL RC-PCR Probe Panel B per reaction (Red cap)
- 1.8 µL Probe Dilution Buffer per reaction (Blue cap)
- 4 µL Molecular Grade Water
- 10 µL Master Mix per reaction (White cap)

Example: 24 samples + 10% extra volume*

- **Probe-Polymerase premix:**
  - **5.28 µL RC-PCR Probe Panel**
  - **47.52 µL Probe Dilution Buffer**
  - **105.6 µL Molecular Grade Water**
  - **264 µL Master Mix**

*** It is recommended to allow for a 10% excess when preparing the Probe-Polymerase premix to correct for any pipetting loss. The kit contains extra reagent for this.**

2.5. Add to each tube of plate A: 16 µL of Probe-Polymerase premix Panel A (from 2.3).

2.6. Add to each tube of plate B: 16 µL of Probe-Polymerase premix Panel B (from 2.4).

2.7. Add to each well 4 µL of DNA (5 ng/µL).

2.8. Close the tube strips carefully with caps and mix by flicking. Check for a homogeneous pink coloured reaction mix and then spin shortly.

2.9. Start the RC-PCR program in the thermal cycler(s) and place the samples in the cycler when the block is between 60 °C and 98 °C. Then close the lid.

Safe stopping point after RC-PCR

3. Pool, Purify and Sequence

After the PCR, the samples have been amplified and tagged with sample specific indexes and sequencing tails. From this point, PCR products can be pooled together in a single tube, purified by a bead purification to remove primers, primer-dimers and salt.

Note: Before pooling, optionally check 3 µL of the unpurified PCR products on agarose (2%).

3.1. Bring the AMPureXP™ beads solution to Room Temperature.

3.2. Perform steps 3.3 to 3.7 for both Panel A and Panel B individually.

3.3. Combine 5 µL RC-PCR products from all the reaction wells from Panel A/B (except negative controls) in a 1.5 mL Eppendorf tube.

3.4. Mix well and transfer 40 µL of the pool to a new 1.5 mL Eppendorf tube.

3.5. Add 60 µL Low TE buffer or molecular grade water to the tube (total volume is now 100 µL).

3.6. Beads purification:

a. Vortex the beads thoroughly to resuspend.
b. Add 100 µL beads solution to the 100 µL pool (from step 3.5) and mix well immediately by pipetting up and down 5 times.
c. Incubate for 5 minutes, off magnet.
d. Place the tube on magnet for 3 minutes or for the solution to be fully cleared.

2x Ethanol wash

e. Remove and discard all liquid carefully without disturbing the beads.
f. Add 200 µL (freshly prepared) 75% ethanol, without disturbing the beads.
g. Wait for 1 minute.

h. Repeat steps **e.**, **f.** and **g.** for a second ethanol wash step.

i. Carefully remove all liquid without leaving traces of ethanol. (Optionally a quick spin can be performed, then place the tube back on magnet and remove excess ethanol)
j. Dry with open cap for 2-3 minutes at Room Temperature.
Do not over-dry.

3.7. Elution:

a. On Magnet: Add 100 µL Low TE buffer to the tube.
b. Off Magnet: Re-suspend the beads by flicking or short vortexing.
c. Incubate for 2 minutes, off magnet.
d. Put the tube on magnet and wait for 1-3 minutes or for the solution to be fully cleared.
e. Carefully bring 90 µL of the clear solution to a new 1.5 mL Eppendorf tube, making sure not to transfer any of the beads.

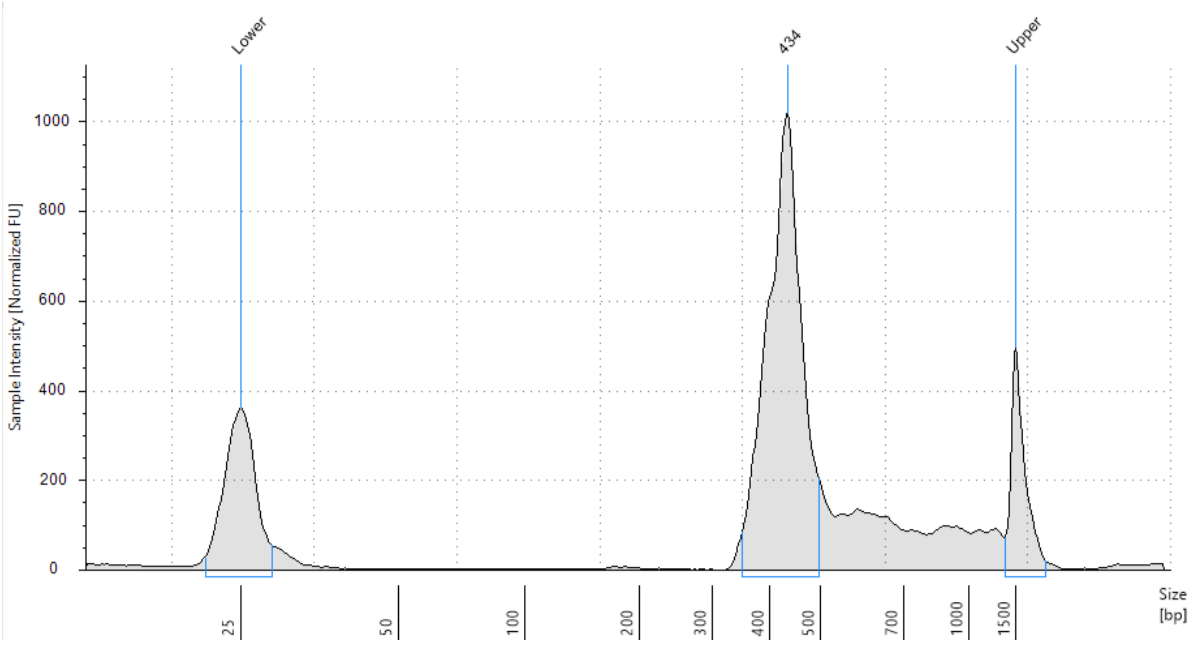
The libraries are now ready for quantification and qualification.

3.8. Determine the final concentration of the libraries by a double Qubit (HS) measurement according to manufacturer's manual.

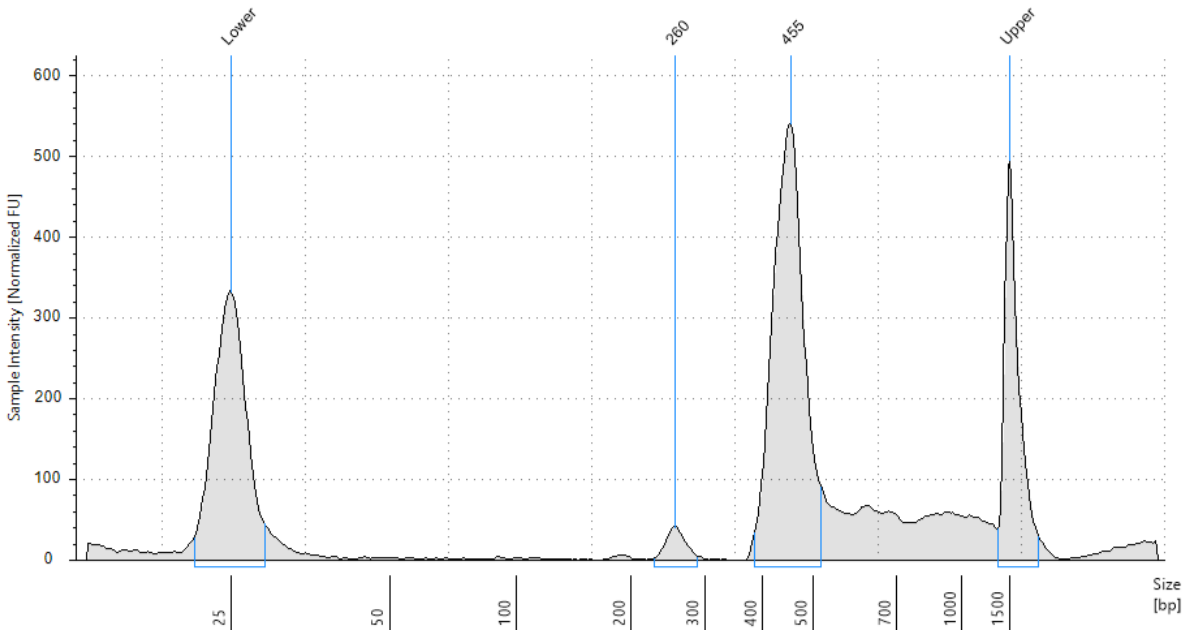
3.9. Verify the libraries on TapeStation or Bioanalyzer, according to the manufacturer's protocol. If needed, dilute the panels. Example: For TapeStation High Sensitivity kit, dilute to ~2 ng/µL.

Example of clean libraries on TapeStation:

Panel A



Panel B



3.10. Perform sequencing on an Illumina® NGS platform, according to the manufacturer's manual.

a. Read at a minimum of 151-8-8-151.

b. The Bed. File is available at: https://nimagen.com/gfx/Human_Genetics/CFTR_customers.txt

c. Using a read depth of 32 000 per sample will, on average, result in a minimum read depth of 100 reads per amplicon.

Note: For technical assistance contact our technical support at techsupport@nimagen.com.

Apply local regulations regarding waste disposal.

Legal Notices:

RC-PCR is patent protected (PCT/GB2016/050558, WO2016146968A1) and exclusively licensed to NimaGen B.V. Nijmegen

Qubit is a trademark of Thermo Fisher Scientific Inc.

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