

Product Data Sheet:

fastGEN CFTR 8-kit

Catalogue number:

RDNGS0006

For research use only!

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1. INTENDED USE

BioVendor fastGEN CFTR kit is intended for rapid preparation of the sequencing library required for *CFTR* gene genotyping by next-generation sequencing (NGS) using the Illumina® instrument. Sequencing data are analysed online by GENOVESA software, fastGEN module.

Abbreviations

CFTR	<i>cystic fibrosis transmembrane conductance regulator</i>
CF	cystic fibrosis
CNV	copy number variation
Ct	cycle threshold
DNA	deoxyribonucleic acid
FAM/SYBR	6-carboxyfluorescein/asymmetrical cyanine dye
LoD	limit of detection
NC	negative control
NGS	Next Generation Sequencing
PC	positive control
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction

Features

- **European Union: For research use only!**
- **Rest of the world: For research use only!**
- Total preparation time is less than 3 hours including **less than 30 minutes of hands-on time**.
- Technology is based on the **fast** and **robust single-step preparation** of sequencing libraries for *CFTR* gene genotyping.
- Kit contains **complete Master Mixes** including indexes supplied in a ready to use format and **sequencing primers**.
- FastGEN CFTR Master Mix is supplied for each tested sample in **4 tubes**.
- The FastGEN CFTR 8-kit is intended for mutation testing in *CFTR* gene of **8 samples** with the unique combination of indexes for one sequencing run.
- In the procedure of fastGEN CFTR kit, **simple addition of isolated DNA** to the Master Mix, and analysis in a Real-Time PCR cyclers is required.
- The input material for sequencing library preparation is DNA isolated from **whole blood, buccal swab, dried blood spots**.
- Genotyping with the fastGEN CFTR kit allows the analysis of all exons with adjacent exon/intron boundaries and 4 deep intronic regions (7, 11, 12 and 22). FastGEN CFTR detects polyT and TG repetitive regions in intron 9. The kit also enables the detection of CFTR dele2,3 using deletion-specific primers. Other large deletions are detected by quantitative CNV analysis.
- Sequencing data are analysed online in fastGEN module of GENOVESA software.

2. STORAGE, EXPIRATION

Store the kit at -20°C . Under these conditions, all components are stable until the expiration date (see label on the box).

- fastGEN CFTR kit is delivered frozen at -20°C .
- After delivery, store the fastGEN CFTR kit at -20°C .
- **Protect kit components from light.**
- Avoid repeated freeze-thaw cycles.
- Do not use expired kits or components.

DEMO

3. INTRODUCTION

Gene *CFTR* (*cystic fibrosis transmembrane conductance regulator*) is located on chromosome 7q31.2, includes 27 coding exons and its genome sequence is approximately 230 kb long. The gene encodes a protein of the same name. The protein functions as an ion channel on the cell surface and participates in maintaining the homeostasis of the ionic environment in the organism. Mutations in the *CFTR* gene lead to impaired function or protein formation and the manifestation of cystic fibrosis (CF). CF is the most common autosomal recessive disease in the Caucasian population. More than 2 000 variants in the *CFTR* gene have been discovered (including mutations associated with the development of CF, potentially pathogenic variants or variants with unclear pathogenicity). A critical factor in survival is the severity of lung disease. Treatment of patients with CF is palliative, but early initiation improves the clinical course of the disease and life expectancy.

Currently, genotyping methods based on the NGS sequencing technologies are available, and specific probes or primers for each acceptable mutation variant are not required, thus the risk of false-negative results is reduced. Screening based on NGS is highly sensitive and specific. It is suitable for the diagnosis of cystic fibrosis and for the determination of a carrier. In addition, information on mutational status is important to indicate the correct therapy.

The NGS genotyping is based on the preparation of a suitable double-stranded DNA construct (sequencing library), which must contain:

- a target sequence for genotyping (DNA locus)
- an adapter sequence for sequencing primers annealing
- the index sequence, unique per sample and run, which serves to identify the corresponding DNA sample (patient) and sequencing result, and thus allows parallel sequencing of multiple samples (typically more than ten per run)
- a sequence for binding the DNA construct to the surface of the sequencing flow cell

4. TEST PRINCIPLE

The fastGEN CFTR kit was developed for the determination of *CFTR* mutation status by NGS. First short amplicons are obtained by a single PCR with hybrid primers with tags, in which up to 330 bp length sequences are amplified. Then it is followed by high coverage sequencing. The use of short amplicons increases DNA amplifiability and diagnostic yield. Master Mixes are supplied as ready to use, thus the total time and the risk of error is reduced.

In the procedure of the fastGEN CFTR kit, only the addition of isolated DNA to a specific Master Mix and amplification in Real-Time PCR thermocycler is required.

Sequencing data are analysed online in fastGEN module of GENOVESA software, which is a part of a complex solution.

5. PRECAUTIONS

- **For professional use only.**
- fastGEN CFTR kit components do not contain infectious material.
- Samples used for the fastGEN CFTR kit should be treated as potentially infectious and standard safety precautions must be followed.
- Do not drink, eat, or smoke in areas where biological material is handled.

6. TECHNICAL HINTS

- The kit is intended for use by professional users in an adequate laboratory environment.
- Before and after each test, the working environment must be decontaminated with standard RNase, DNase disinfectant. Working in an unsuitable environment can lead to contamination of the kit components.
- Aliquotation and repeated thawing is not recommended. Multiple thawing cycles can negatively affect the quality of the test.
- Thaw the individual components right before use. Minimize the time reagents are at room temperature. The use of cooling racks is recommended.
- Vortex and centrifuge reagents gently before use.
- Perform the qPCR preparation and post-amplification steps in separated laboratory areas.
- Avoid the contamination of samples and reagents. For this purpose, use disposable tips for each sample and reagent.
- Do not mix reagents with different lot numbers.
- Dispose of the used and unused material in accordance with the legislation.

7. REAGENT SUPPLIED

The **fastGEN CFTR 8-kit** is supplied in a ready to use format for the analysis of 8 samples, i.e., 32 reactions for the *CFTR* gene (Table 1). Kit includes **specific Master Mixes** containing all the necessary reaction components and **sequencing primers** for *CFTR* gene.

fastGEN CFTR 8-kit components	Index sequences	Volume per 1 tube (µl)	Number of tubes	State
CFTR Master Mix <u>i701 (A-D)</u>	TAAGGCGA	18	4	ready to use
CFTR Master Mix <u>i721 (A-D)</u>	TACGCTGC	18	4	ready to use
CFTR Master Mix <u>i722 (A-D)</u>	ATGCGCAG	18	4	ready to use
CFTR Master Mix <u>i723 (A-D)</u>	TAGCGCTC	18	4	ready to use
CFTR Master Mix <u>i724 (A-D)</u>	ACTGAGCG	18	4	ready to use
CFTR Master Mix <u>i726 (A-D)</u>	CCTAAGAC	18	4	ready to use
CFTR Master Mix <u>i727 (A-D)</u>	CGATCAGT	18	4	ready to use
CFTR Master Mix <u>i728 (A-D)</u>	TGCAGCTA	18	4	ready to use
R1SP		12	1	to be diluted
R2SP CFTR		47	1	to be diluted
ISP CFTR		47	1	to be diluted

Table 1: fastGEN CFTR 8-kit components.

8. RECOMMENDED MATERIAL (NOT SUPPLIED)

Chemicals

- Examined DNA
- Standardized sample containing the required variants of the examined *CFTR* gene (suitable as a **positive control**)
- Water for molecular biology (Nuclease Free Water, also suitable as a **negative control**)
- Illumina sequencing kit
- Qubit® dsDNA HS Assay Kit (Life Technologies)
- NaOH (p.a.)
- Tween 20
- Purification kit for DNA pool
- Commercially available surface decontamination solutions

Equipment

- 0.2 ml tubes and 1.5 – 2 ml tubes appropriate for nucleic acids (RNase + DNase free, low binding nucleic acid tubes)
- PCR tubes/strips/plates for use in a Real-Time PCR thermocycler (appropriate for working with nucleic acids)
- Adhesive PCR seals
- Racks for tubes
- Cooling racks/refrigerator/freezer/box with ice
- Single-use sheets suitable for optical instruments
- Pipette tips with filters
- Protective equipment (gloves, clothes)

Instruments

- Automatic pipettes for 0.2 – 1 000 µl volumes
- Real-Time PCR thermocycler
- Flowbox/PCR box
- Fluorometer
- Vortex, combi-spin (centrifuge and vortex), centrifuge
- Illumina® MiSeq™ sequencing machine

9. PREPARATION OF REAGENTS

Prepare the appropriate number of tubes with Master Mixes needed for testing.

Do not use components after the expiration date marked on the label.

Reagents are supplied as ready to use or must be diluted.

fastGEN CFTR: Master Mix

For *CFTR* gene genotyping let the appropriate number of CFTR kit Master Mixes tubes thaw and keep them cool until use.

Sequencing primers

Before sequencing library denaturation, let primers thaw and keep them cool until use:

- 1 tube: R1SP
- 1 tube: R2SP CFTR
- 1 tube: ISP CFTR

Recommended:

It is recommended to add the positive control (PC, standardized sample containing the required variants of target genes, not supplied in the kit) and the negative control (NC) into each run using the fastGEN CFTR kit to assess the proper preparation and to eliminate the risk of contamination. In case of non-compliance, false positive or negative results cannot be ruled out.

Handle the positive control with care and **add it as the last one**. Improper handling may result in contamination of the test and false positive results. If contamination is suspected, repeat the test.

10. PREPARATION OF SAMPLES

Work at the appropriate PCR box

- The input material for sequencing library preparation is DNA isolated from whole blood, buccal swab, dried blood spots.
- Optimal results are achieved when **10 ng** of input DNA is used in the PCR reaction. The kit is validated to use 2-50 ng of input DNA.
- High concentration DNA should be diluted to a concentration of 2 ng/μl.
- Using highly concentrated DNA can lead to PCR inhibition and/or incorrect results. Do not dilute samples with **very low DNA concentrations** but include them in the analysis in a duplicate (add 5 μl of DNA into tubes with two different CFTR Master Mixes).
- Add **5 μl DNA** into each reaction.
- The sample diluted to an appropriate concentration is **prepared for analysis**. Proceed to chapter 11. Assay Procedure.

Recommended:

Positive control (PC)

PC is not included in the kit. Analysis of positive control is recommended for each run.

Prepare the PC similarly to DNA samples.

Precaution: Do not open the tube with positive control during PCR preparation. Pipette the PC as the last one. Inappropriate manipulation can result in contamination and false-positive results. If contamination is suspected, repeat the test.

11. ASSAY PROCEDURE

Using the NGS technology, multiple DNA segments are sequenced with coverage of thousands of reads per sample. Genotyping with the fastGEN CFTR kit allows the analysis of all exons with adjacent exon/intron boundaries and 4 deep intronic regions (7, 11, 12 and 22). FastGEN CFTR detects polyT and TG repetitive regions in intron 9. The kit also enables the detection of CFTR dele2,3 using deletion-specific primers.

The kit is designed to process 8 samples for *CFTR* genotyping in one sequencing run. **One sample is analyzed in four separate reactions.**

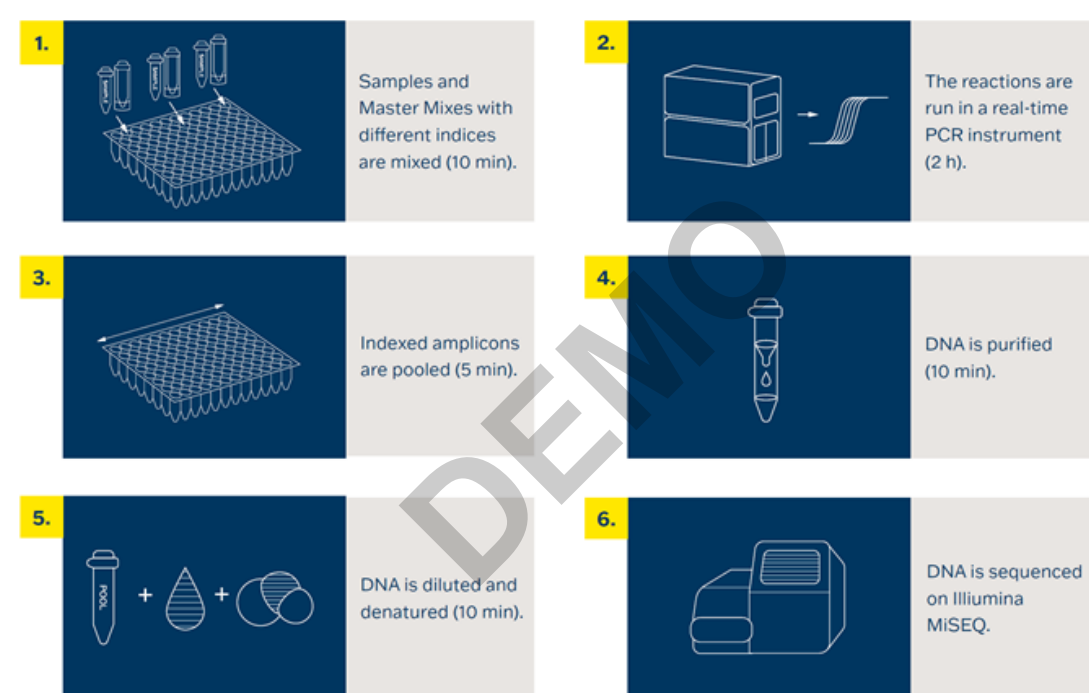


Figure 1: Workflow of genotyping using the fastGEN kit.

11.1 DNA library preparation

11.1.1 Preparation of examined DNA

Use the PCR box.

- Prepare samples.
- Vortex and centrifuge DNA samples shortly.
- Add **5 µl** of DNA of the appropriate concentration to Master Mixes A–D (see chapter 10) into the PCR plate or strip. Recommended concentration of isolated DNA is 2 ng/µl.
- Recommended:
 - Include positive (PC) and negative (NC) control.
 - Add **5 µl of positive control DNA with appropriate concentration** for Master Mixes A–D (see chapter 10).
 - Add **5 µl of molecular grade water** as a negative control for Master Mixes A–D.

11.1.2 Preparation of Master Mixes

Use the PCR box in the pre-PCR room.

- Mark the PCR plate or the strip.
- Briefly vortex and centrifuge the Master Mixes when thawed.
- Add **15 µl** of Master Mix A–D to each sample or control consecutively into 4 wells.
- The total volume per PCR reaction is **20 µl**.
- Use only **one** Master Mix per position.
- The number of samples analysed simultaneously in one run is 8, including controls.
- Master Mixes have to be opened one by one right before being added into the sample. Close the tube with Master Mix immediately after use. Do not open tubes with various Master Mixes simultaneously to avoid cross-contamination.
- Seal the plate or close the tubes, vortex gently and spin down (15 s; 280x g).

11.1.3 qPCR

Set the cycling conditions according to Table 2.

Step	Time	Temperature	
Denaturation	2 min	95 °C	
Amplification	15 s	95 °C	40 cycles
	30 s	62 °C	
	30 s	72 °C*	
Final elongation	5 min	72 °C	
Melting curve acquisition		60 °C → 95 °C	

* FAM/SYBR signal detection

Table 2: qPCR amplification program.

- Set sample names into qPCR software.
- Start the run.
- Export the qPCR data and perform an amplification check. Save the Ct values for possible later control.
- Store the PCR products at 4 °C for further use. For long-term storage, store at –20 °C.

11.2 Sequencing machine preparation

- During the qPCR run, perform “maintenance wash” of the sequencing machine, and thaw the cartridge.

11.3 Pooling the amplicons, purification and quantification

Use the appropriate box in the post-PCR room and **keep amplicons and DNA pool on ice the whole time, with the exception of denaturation step.**

11.3.1 Pooling

- Centrifuge plates/strips briefly after the qPCR run.
- For genotyping **CFTR** gene in one library:
 - Mix the individual amplicons of all samples into one DNA pool in the same ratio.

Example: For 8 samples, mix the individual amplicons in an amount of 3 µl. You get a DNA pool in a volume of 96 µl.

- The final volume of the DNA pool should follow recommendation from the user manual of the purification kit.

Recommended: If the Ct of the sample is > 31 or even > 34, double or triple the volume of the sample added into the final DNA pool.

- Use a new 1.5 ml tube for DNA pool purification.
- Store the plate/strip with samples in the freezer in case of repeated purification.

11.3.2 DNA pool purification

- Follow instructions from the user manual of the purification kit.
- Store the purified DNA pool according to the user manual of the purification kit.

11.3.3 DNA pool quantification

- Assess the mass concentration of the purified DNA pool fluorometrically.
- Recommended DNA pool mass concentration is approximately 40–80 ng/μl; the minimum concentration is 10 ng/μl.
- Assess the DNA pool molarity (molar concentration) according to the equation:

$$c[nM] = \frac{\rho_i \left[\frac{ng}{\mu l} \right] \times 10^6}{(660 \times 330)}$$

- ρ_i is the DNA mass concentration
- 330 is the average DNA molecule length (bp) after indexing
- 660 g/mol is the average molar mass of 1 base pair (bp)

11.4 Preparation for sequencing run

11.4.1 Run assignment

To assign the run, use the SampleSheet „fastGEN-sample-sheet.xlsx“ from www.biovendor.com and edit it (date, run ID, sample identification using the indexes).

11.4.2 Purified DNA pool dilution

- Dilute the DNA pool (sequencing library) with molecular grade water to a final concentration of **2 nM**.
- The final library concentration should be between 1.6 nM – 2.4 nM.
- Verify the concentration of diluted DNA pool by Qubit HS or other techniques. Use at least 10 μl of diluted DNA pool.
- Dilution should correspond to the raw sequencing density of 1 000–1 200 K/mm². In the case of non-optimal cluster density (>1 200 K/mm²), it is recommended to decrease the final pool concentration to 1.8 nM and increase to 2.2 nM if the raw density is <1 000 K/mm². To facilitate the dilution, use the tool “fastGEN-pool-dilution.xlsx”, available at www.biovendor.com
- Otherwise, the DNA pool has to be diluted repeatedly, and concentration has to be assessed again.

11.4.3 DNA pool denaturation

- Prepare fresh 0.2 M NaOH solution: 480 µl H₂O + 20 µl 5 M NaOH.
- Mix **10 µl of diluted DNA pool with 10 µl of freshly prepared 0.2 M NaOH (1:1)**. It is necessary to use fresh NaOH solution.
- Incubate for **5 min at room temperature**.

11.4.4 Sequencing primers dilution

- Prepare new tubes for sequencing primers dilution. During denaturation, dilute sequencing primers:
 - **Read1 sequencing primers (R1SP):** 3 µl R1SP + 597 µl HT1
 - **Index sequencing primers (ISP):** 44 µl ISP CFTR + 556 µl HT1
 - **Read2 sequencing primers (R2SP):** 44 µl R2SP CFTR + 556 µl HT1

11.4.5 Denatured DNA pool dilution

Dilute the denatured DNA pool with cooled HT1 solution from the refrigerator to a final concentration of 10 pM (e.g., 10 µl DNA pool + 990 µl HT1). **The obtained DNA pool is ready to be used in the sequencing cartridge.** Keep the DNA pool in the refrigerator before sequencing.

11.4.6 Sequencing cartridge preparation

- Check that the cartridge is completely thawed and turn it over 3x to mix the content.
- Pipette 600 µl of the diluted 10 pM DNA library and diluted sequencing primers into the sequencing cartridge into positions 17-20 in the following order:
 - Position 17: DNA library in HT1
 - Position 18: R1SP diluted in HT1
 - Position 19: ISP diluted in HT1
 - Position 20: R2SP diluted in HT1
- Prepare the sequencing flowcell according to the appropriate user manual.
- Run the sequencing program (Illumina software). Follow the manufacturer's recommendation for machine usage.

12. RESULTS EVALUATION

For sequencing raw data interpretation, use the fastGEN module of the GENOVESA software, which is available at www.biovendor.com

GENOVESA fastGEN module

fastGEN module is the cloud, all-in-one solution for sequencing raw data analysis (FASTQ files) with technical and application support provided in the English language.

Software enables:

- Advanced quality control of raw sequencing data
- Automated warnings for insufficiently covered regions
- Simple filtration of relevant variants
- Monthly updates of annotation databases
- Customization
- Saving patient's data and variants into the internal database
- One-click report generation

12.1 CFTR genotyping

CFTR genotyping result is considered as positive (mutation detected), if a variant in the *CFTR* gene **was detected with a frequency corresponding to the heterozygous or homozygous state.**

In the case of a positive finding of a mutation in the *CFTR* gene with a frequency < 15 %, we recommend retesting in duplicate or verifying results with another method. Deletions found by CNV analysis should be verified by another method.

Genotyping of samples with extremely low DNA concentration (<0,4 ng/μl; fluorometrically) is valid if the results of both replicates processed with different Master Mixes match.

12.2 Negative result

If none of the variants is detected or the frequency is lower than the threshold, genotyping result is negative (no mutation is detected).

12.3 PC and NC interpretation

The inclusion of positive and negative control for each run of the test (a group of samples measured simultaneously) is recommended to verify that the DNA library preparation has been performed correctly and to avoid technical issues.

Positive control must meet the following criteria:

- In the qPCR amplification step, the Ct of PC is at least 3 Ct lower than NC ($Ct_{PC} + 3 \leq Ct_{NC}$).
- After the sequencing data evaluation, frequencies of *CFTR* gene variants are as expected.

Negative control must meet the following criteria:

- In the qPCR amplification step, the NC is not detected, or the Ct value is at least 3 Ct higher than the sample/PC with the highest Ct.

If PC or NC does not meet any of the parameters, analysis was not performed correctly, and it is necessary to interpret the effect on results. You can contact the application specialists at www.biovendor.com.

For more information see chapter 15. FAQ

13. KIT LIMITATIONS

- The fastGEN CFTR 8- kit was validated on DNA samples isolated from whole blood and dried blood spots.
- The result of genotyping is affected by the quality of the sample. Proper collection, transport, DNA isolation, and sample storage are crucial for test performance.
- Genotyping results should be interpreted by a healthcare professional.
- The fastGEN CFTR kit is designed for rapid preparation of the sequencing library required for the *CFTR* gene genotyping using the NGS technology. Variants in other genes than *CFTR* are not detectable.
- A negative result does not exclude mutations below the detection limit of the method.
- Rare sequence variants in the primer region may affect the functionality of individual fastGEN primers and may lead to reduced amplification efficiency of a given amplicon.
- Analysis of polyT and TG repeats may be negatively affected by lower sequencing coverage of the respective region (<200x).
- The fastGEN CFTR kit is unable to distinguish CFTR dele2 from CFTR dele2ins182.
- *CFTR* exon 10 is not included in the CNV analysis.

All instructions in this document should be followed when performing the test. Otherwise, the quality and reliability of the results can be affected.

14. KIT CHARACTERISTICS

Analytical sensitivity and specificity of the BioVendor fastGEN CFTR 8-kit was determined. The LoD for kit was determined, and the crossreactivity of primers was verified (*in silico*). The repeatability and robustness of the method were tested. The diagnostic accuracy (sensitivity) of the tests was determined based on the analysis of clinical and reference samples with a known mutation status. The results of *CFTR* genotyping were correct in all samples, including repeated measurements (sensitivity 100 %).

15. FAQ

1. How many samples can be sequenced in one run?

It is necessary to obtain 200 000 pair-end reads per sample. The MiSeq Reagent kit v2 Nano, which has 2 million paired end reads, is sufficient for up to 8 samples. The MiSeq Reagent kit v2 Micro, which has 8 million paired end reads, is 75 % full when sequencing 24 samples.

2. Is it possible to use a different tool for data analysis?

Yes. It is possible to use Illumina® Experiment Manager.

3. Which sequencing machine is appropriate for sample analysis by fastGEN kits?

Illumina® brand sequencing machines should be used to sequence the fastGEN sequencing libraries.

4. Is it possible to combine several kits for genotyping?

Yes, it is possible to combine all fastGEN kits. If you mix several pools, follow the recommendation „fastGEN-pool-mix.docx”, which is available at www.biovendor.com, or contact the application specialists.

5. How should the results be interpreted if PC or NC does not meet quality criteria?

There can be several reasons for the non-standard results of PC and NC. We recommend the PC verification (targeted genes and their variants must contain mutations). Further, verify technical settings and check if a manual error has occurred. In case of ambiguity, contact customer support.

16. REFERENCES

For more references see our websites www.biovendor.com.

[1] Richards, C. S., Bradley, L. A., Amos, J., Allitto, B., Grody, W. W., Maddalena, A., ... & Watson, M. S. (2002). Standards and guidelines for CFTR mutation testing. *Genetics in Medicine*, 4(5), 379-391.




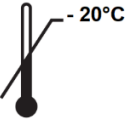



[2] Deignan, J. L., Astbury, C., Cutting, G. R., Del Gaudio, D., Gregg, A. R., Grody, W. W., ... & Richards, S. (2020). CFTR variant testing: a technical standard of the American College of Medical Genetics and Genomics (ACMG). *Genetics in Medicine*, 22(8), 1288-1295.

[3] <https://www.klubcf.cz>

[4] <https://cftr2.org/>

DEMO

17. EXPLANATION OF SYMBOLS

	Catalogue number
	Batch code
	Use by date
	Upper limit temperature
	Manufacturer
	Read electronic instructions for use - eIFU
	The content is sufficient for 8 tests

DEMO



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