BioVendor Group NGS

ENG

Instructions for Use:
fastGEN Crohn's & Celiac Disease Kit

Catalogue number:
RDNGS0022

For research use only

BioVendor R&D®

BioVendor – Laboratorní medicína s.r.o.
Karásek 1767/1, 621 00 Brno, Czech Republic
+420 549 124 185
info@biovendor.com
sales@biovendor.com
www.biovendor.com

CONTENT

HISTORY OF CHANGES

Previous version	Current version
ENG.001.A	ENG.001.B
Change of variant nomenclature	

1. INTENDED PURPOSE

RDNGS0022 fastGEN Crohn's & Celiac Disease Kit is designed for the rapid preparation of sequencing libraries required for genotyping the rs2395182, rs7775228, rs4713586, rs2187668, rs7454108, rs1004819, rs10889677, rs2066844, rs2066845, and rs2066847 using next-generation sequencing (NGS). These regions are associated with the HLA-DQ2 and HLA-DQ8 haplotypes linked to a predisposition to celiac disease and with the *IL23R* and *NOD2* genes associated with a predisposition to Crohn's disease.

The input material for preparing the sequencing library is isolated DNA.

1.1 Abbreviations

Ct	Cycle threshold
DNA	Deoxyribonucleic acid
FAM/SYBR	6-carboxyfluorescein/asymmetrical cyanine dye
HLA	Human Leukocyte Antigen
IL23R	Interleukin-23 receptor
LoD	Limit of Detection
NC	Negative Control
NGS	Next Generation Sequencing
NOD2	Nucleotide-binding oligomerization domain-containing protein 2
PC	Positive Control
PCR	Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction
SNP	Single-Nucleotide Polymorphism
VAF	Variant Allele Frequency

2. FEATURES

- **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 genotyping selected regions relevant to determining predisposition to celiac and Crohn's disease.
- Kit contains **complete Master Mixes** including indexes supplied in a ready to use format.
- The fastGEN Crohn's & Celiac Disease Kit is designed to test a total of 10 selected regions across 16 samples with a unique combination of indexes in a single sequencing run.
- In the procedure of fastGEN Crohn's & Celiac Disease Kit**, simple addition of isolated DNA** to the Master Mix, and analysis in a Real-Time PCR cycler is required.

3. STORAGE

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

- fastGEN Crohn's & Celiac Disease Kit is delivered frozen at −20 °C.
- After delivery, store the fastGEN Crohn's & Celiac Disease Kit at −20 °C.
- **Protect kit components from light.**
- Avoid repeated freeze-thaw cycles of Master Mixes.
- Do not use expired kits or components.

4. INTRODUCTION

Crohn's disease is a chronic inflammatory disease of the digestive tract classified as an idiopathic intestinal inflammation.

Mutations in the ***NOD2*** gene are considered one of the most significant genetic risk factors. The variants under investigation – **rs2066844**, **rs2066845**, and **rs2066847** are among the best-known mutations in the *NOD2* gene.

Another risk gene is ***IL23R***, which is associated with immune regulation and autophagy – the process by which damaged cells and microorganisms are eliminated. The genetic variants being tested are **rs1004819** and **rs10889677**.

Celiac disease is a chronic autoimmune disorder in which the immune system reacts to gluten. In genetically predisposed individuals, gluten intake leads to damage to the lining of the small intestine, which impairs nutrient absorption.

HLA-DQ2.5, **HLA-DQ2.2**, and **HLA-DQ8** are genetic variants of the HLA class II system that are significantly associated with the development of celiac disease. The most significant genetic risk factor is HLA-DQ2.5, which is present in approximately 90–95% of patients with celiac disease. HLA-DQ8 is present in a smaller proportion of patients, while HLA-DQ2.2 is associated with a lower risk but, in combination with other risk variants, may also contribute to the development of the disease. The genetic variants examined are **rs2395182**, **rs7775228**, and **rs4713586** for the HLA-DQ2.2 haplotype; **rs2187668** for HLA-DQ2.5; and **rs7454108** for HLA-DQ8.

Genetic screening based on the NGS method is highly sensitive and specific.

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

- a target sequence for genotyping (a DNA segment)
- an adapter sequence for the binding of sequencing primers
- an index sequence that is unique to the sample in a given run, used to match the obtained results with the corresponding DNA sample (patient), thereby enabling parallel sequencing of multiple samples in a single run
- a sequence for binding the DNA construct to the surface of the flow cell

5. TEST PRINCIPLE

The fastGEN Crohn's & Celiac Disease Kit is used to prepare samples for testing of genetic variants associated with a predisposition to Crohn's disease (**rs2066844, rs2066845, rs2066847, rs1004819, rs10889677**) and a predisposition to celiac disease (**rs2395182, rs7775228, rs4713586, rs2187668, rs7454108**) using NGS. The assay utilizes short amplicons obtained via a single polymerase chain reaction with tagged hybrid primers, amplifying segments with an average length of 283 base pairs, followed by high-coverage sequencing. The use of short amplicons increases DNA amplifiability and 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 Crohn's & Celiac Disease 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.

6. PRECAUTIONS

- **For professional use only, by trained personnel in an adequate laboratory environment.**
- fastGEN Crohn's & Celiac Disease Kit components do not contain infectious material.
- Samples used for the fastGEN Crohn's & Celiac Disease 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.

7. TECHNICAL HINTS

- Before and after each test, the working environment must be decontaminated with appropriate RNase and DNase removers as well as standard disinfectants. Working in an unsuitable environment can lead to contamination of the kit components.
- Aliquotation and repeated thawing of Master Mixes 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. Work on ice or use cooling racks.
- 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.

8. REAGENT SUPPLIED

The **fastGEN Crohn's & Celiac Disease Kit** is supplied in a ready to use format for the analysis of 16 samples (Table 1). Kit includes **specific Master Mixes** containing all the necessary reaction components.

Kit components	Index P7 sequences	Index P5 sequences	Volume per 1 tube (µl)	Number of tubes	State
Master Mix B21	CTTGAGAA	CTCAGACG	18	**1**	**ready to use**
Master Mix B22	ATCCTCAA	CTCAGACG	18	**1**	**ready to use**
Master Mix B23	TTGGCGTT	CTCAGACG	18	**1**	**ready to use**
Master Mix B24	GCAGGCTT	CTCAGACG	18	**1**	**ready to use**
Master Mix C21	CTTGAGAA	GAGCTCGT	18	**1**	**ready to use**
Master Mix C22	ATCCTCAA	GAGCTCGT	18	**1**	**ready to use**
Master Mix C23	TTGGCGTT	GAGCTCGT	18	**1**	**ready to use**
Master Mix C24	GCAGGCTT	GAGCTCGT	18	**1**	**ready to use**
Master Mix D21	CTTGAGAA	TCTGAGTA	18	**1**	**ready to use**
Master Mix D22	ATCCTCAA	TCTGAGTA	18	**1**	**ready to use**
Master Mix D23	TTGGCGTT	TCTGAGTA	18	**1**	**ready to use**
Master Mix D24	GCAGGCTT	TCTGAGTA	18	**1**	**ready to use**
Master Mix E21	CTTGAGAA	CAAGTTAT	18	**1**	**ready to use**
Master Mix E22	ATCCTCAA	CAAGTTAT	18	**1**	**ready to use**
Master Mix E23	TTGGCGTT	CAAGTTAT	18	**1**	**ready to use**
Master Mix E24	GCAGGCTT	CAAGTTAT	18	**1**	**ready to use**

Table 1: fastGEN Crohn's & Celiac Disease Kit components

9. RECOMMENDED MATERIAL (NOT SUPPLIED)

9.1 Chemicals

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

9.2 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, thin plastic Pasteur pipette
- Protective equipment (gloves, clothes)

9.3 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
- Sequencing machine

10. 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.

10.1 fastGEN Crohn's & Celiac Disease Kit: Master Mix

To genotype the target regions, let the appropriate number of MPN Master Mixes tubes thaw and keep them cool until use.

11. PREPARATION OF SAMPLES

Work at the appropriate PCR box

- The input material for sequencing library preparation is isolated DNA.
- Assess the appropriate dilution according to the DNA concentration, see Table 2.
- 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 duplicates (add 5 µl of DNA into tubes with two different Master Mixes).
- Add **5 µl DNA** prepared according to Table 2 into each reaction.
- The sample diluted to an appropriate concentration is **prepared for analysis**. Proceed to chapter 12. Assay procedure.

	Qubit HS concentration	Dilution	Dilution
A	>10 ng/µl	5 x	1 µl DNA + 4 µl H_2O
B	1–10 ng/µl	No dilution	5 µl DNA

Table 2: Appropriate DNA dilution

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 Crohn's & Celiac Disease 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. Prepare the PC similarly to DNA samples.

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.

12. ASSAY PROCEDURE

Using the NGS technology, multiple DNA segments are sequenced with coverage of thousands of reads per sample. Therefore, the method is highly sensitive. The minimal DNA input is 5 ng of DNA.

The kit is designed to process 16 samples for the detection of genetic variants associated with a predisposition to Crohn's disease and a predisposition to celiac disease in one sequencing run.

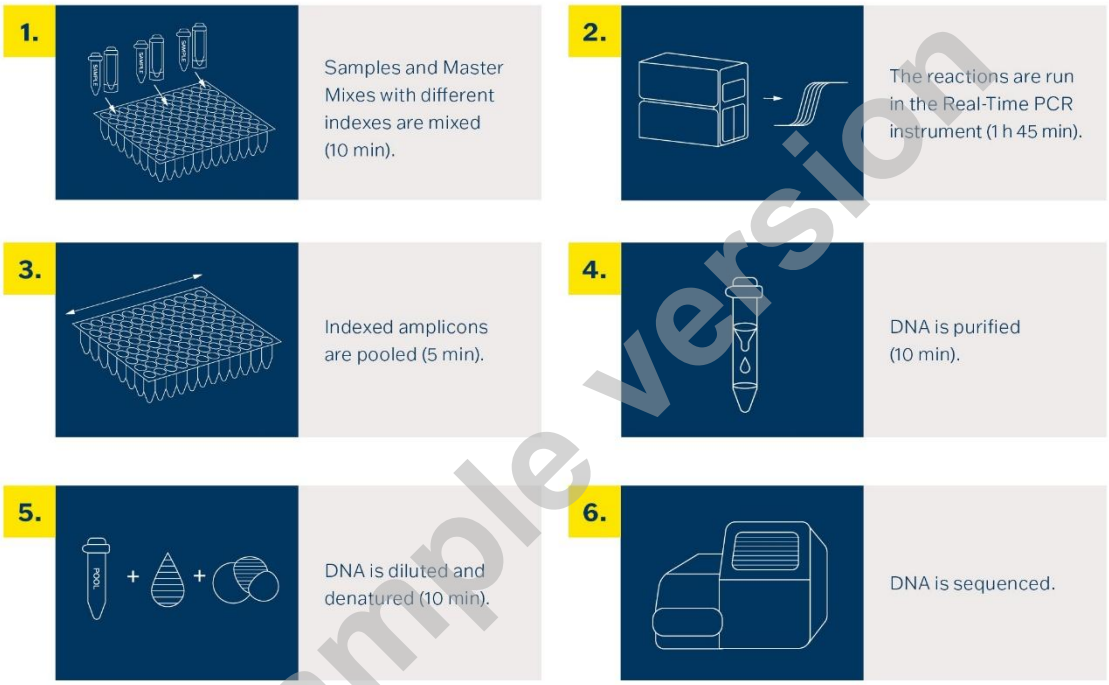

Figure 1: Workflow of genotyping using the fastGEN kit

12.1 DNA library preparation

12.1.1 Preparation of examined DNA

Use the PCR box.

- Prepare samples.
- Vortex and centrifuge DNA samples shortly.
- Pipette **5 µl of a DNA** sample of the appropriate concentration into the PCR plate or strip (see Chapter 11).
- Recommendation:
  - Include positive (PC) and negative (NC) control.
  - Add **5 µl of positive control DNA** of appropriate concentration (see Chapter 11).
  - Add **5 µl water for molecular biology** as a negative control.

12.1.2 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 to each sample or control.
- 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 16, 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).

12.1.3 qPCR

Set the cycling conditions according to Table 3.

Signal detection takes place in an **amplification cycle***, in the **FAM/SYBR/Green channel**.

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	
Hold	∞	4 °C	

Table 3: qPCR amplification program († optional step)

- 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.

12.2 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**.

12.2.1 Pooling

- Centrifuge plates/strips briefly after the qPCR run.
- For genotyping regions associated with a predisposition to Crohn's disease and a predisposition to celiac disease in one library:
  - Mix the individual amplicons of all samples into one DNA pool in the same ratio.
  - Example: For 16 samples, mix the individual amplicons in an amount of 3 µl. You get a DNA pool in a volume of 48 µl.
  - The final volume of the DNA pool should follow recommendations from the user manual of the purification kit.
  - Recommended: If the Ct of the sample is > 31, double the volume, or even > 34, triple the volume of the sample added into the final DNA pool. If the Ct of the sample is > 36, do not add it to the DNA pool and discard it from the sequencing.
- 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.

12.2.2 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.

12.2.3 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.
- Asses the DNA pool molarity (molar concentration) according to the equation:

$$c[nM] = \frac{\rho i \left[\frac{ng}{\mu l}\right] \times 10^6}{(660 \times 283)}$$

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

12.3 Preparation for sequencing run

12.3.1 Sequencing machine preparation

Before using the sequencing machine, preferably during the qPCR run, wash the sequencing machine (maintenance wash) and thaw the sequencing cartridge. Power cycle the sequencing machine.

12.3.2 Library Preparation

The sequencing library prepared using the fastGEN Crohn's & Celiac Disease Kit is suitable for use on all Illumina® sequencers and utilizes Illumina® sequencing primers.

12.3.3 DNA pool dilution and denaturation

Dilute the purified DNA pool to the desired concentration as recommended by Illumina® and according to the sequencing machine being used.

Perform denaturation of the appropriately diluted DNA pool using NaOH. It is necessary to use fresh NaOH solution. Dilute the denatured DNA pool with chilled HT1 buffer from the refrigerator to the final concentration. Keep the DNA pool in the refrigerator before sequencing.

12.3.4 Sequencing cartridge preparation, starting the sequencing program

Check that the cartridge is completely thawed and turn it over 3x to mix the content. Prepare the flow cell according to the manufacturer's instructions and run the sequencing program (Illumina® software). Follow the instrument manufacturer's instructions.

75,000 paired-end reads are required per sample. When setting up the run, specify a read length of 151 (paired-end read) and an index size of 8 bp.

12.3.5 MiSeq recommendations

The concentration of the diluted DNA pool must be in the range of 1.6–2.4 nM. Denature 5 µl of the DNA pool with 5 µl of freshly prepared 0.2 M NaOH for 5 min at room temperature. Dilute the denatured DNA pool with chilled HT1 buffer to a final concentration of 10 pM (e.g. 10 µl DNA pool + 990 µl HT1). The dilution should correspond to the optimal raw sequencing density values in the long term.

Pipette 600 µl of the diluted 10 pM DNA library into the sequencing cartridge into position 17.

12.3.6 MiniSeq recommendations

The concentration of the diluted DNA pool must be in the range of 0.8–1.2 nM. Denature 5 µl of the DNA pool with 5 µl of freshly prepared 0.1 M NaOH for 5 min at room temperature. Add 5 µl of 200 mM Tris-HCl. Dilute the denatured DNA pool with 985 µl chilled HT1 buffer to a concentration of 5 pM. Then dilute the 5 pM DNA pool with chilled HT1 to a final concentration of 1.4 pM (e.g. 150 µl DNA 5 pM pool + 385 µl HT1) or 1.6 pM (e.g. 170 µl DNA 5 pM pool + 361 µl HT1). The dilution should correspond to the optimal raw sequencing density values in the long term.

Pipette 500 µl of the diluted 1.4 pM or 1.6 pM DNA library into the sequencing cartridge into positions 16.

12.3.7 NextSeq 500/550 recommendations

The concentration of the diluted DNA pool must be in the range of 3.6–4.4 nM. Combine the fastGEN DNA pool to the diluted pool of another sequencing library. Denature 5 µl of total DNA pool with 5 µl of freshly prepared 0.2 M NaOH for 5 min at room temperature. Add 5 µl of 200 mM Tris-HCl. Dilute the denatured DNA pool with 985 µl of chilled HT1 buffer to a concentration of 20 pM. Dilute the 20 pM DNA pool with chilled HT1 to a final concentration of 1.5 pM (e.g. 100 µl 20 pM DNA pool + 1 233 µl HT1) for Mid Output or 1.8 pM (e.g. 120 µl 20 pM DNA pool + 1 213 µl HT1) for High Output. The dilution should correspond to the optimal raw sequencing density values in the long term.

Pipette 1 300 µl of the diluted 1.5 pM or 1.8 pM DNA library into position 10.

12.3.8 NovaSeq reagent kit v1.5 SP, S1, S2, S4 recommendations

The concentration of the diluted DNA pool must be in the range of 1–2 nM. Add the fastGEN DNA pool to the diluted pool of another sequencing library. Typically, the fastGEN library requires

0.2–1 % of the sequencing capacity of the NovaSEQ SP kit. The dilution and proportion can be adjusted to achieve optimal values of raw sequencing density and reads per sample. Denature the total DNA pool (SP/S1 100 µl; S2 150µl; S4 310 µl) with freshly prepared 0.2 M NaOH (SP/S1 25 µl; S2 37 µl; S4 77 µl) for 8 min at room temperature. Add 400 mM Tris-HCl (SP/S1 25 µl; S2 38 µl; S4 78 µl).

Pipette 150 µl (SP, S1), 225 µl (S2), 465 µl (S4) of the diluted, denatured and neutralized DNA library into position 8.

Note: If you mix several DNA libraries contact the application specialists.

13. 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

13.1 Genotyping of variants

For variants **rs10889677**, **rs2066844**, **rs2066845**, and **rs2066847**, the result is considered positive if the variant has been detected with a frequency of ≥ 14%.

The **rs1004819** variant is considered positive if it has been detected with a frequency < 86%.

For variants **rs2187668** (HLA-DQ2.5) and **rs7454108** (HLA-DQ8), the result is considered positive if the variant has been detected at a frequency of ≥ 14%.

The variants rs2395182, rs7775228, and rs4713586 are inherited together and jointly determine whether an HLA-DQ2.2 predisposition arises (Table 4). The HLA-DQ2.2 haplotype is positive under the following conditions:

- For variants **rs2395182** and **rs7775228**, the result is considered positive if the variant has been detected at a frequency of ≥ 14%
- For variant **rs4713586**, the result is considered positive if the variant has been detected at a frequency of < 86%

ATTENTION! Since variants rs4713586 and rs1004819 are classified as positive for the allele listed in the reference genome GRCh38, the occurrence of the variant allele indicates a healthy individual. For a patient with a homozygous positive variant, the VAF value will be < 1% and therefore it is not listed in GENOVESA in the list of variants!

Genotyping of **samples with extremely low DNA concentration** is valid if the results of both duplicates processed with different Master Mixes match.

List of variants and their evaluation:

Haplotype	dbSNP	Ref. allele	Alt. allele	Posit. allele	Genotyping result for VAF < 13.99%	Analysis results		Genotyping result for VAF 14–85.99%	Analysis results		Genotyping result for VAF > 86%	Analysis results	
HLA-DQ2.2	rs2395182	G	T	T	homozygote GG	negative	HLA-DQ2.2 negative	heterozygote GT	positive	HLA-DQ2.2 positive	homozygote TT	positive	HLA-DQ2.2 negative
	rs7775228	T	C	C	homozygote TT	negative		heterozygote TC	positive		homozygote CC	positive	
	rs4713586	A	G	A	homozygote AA	positive		heterozygote AG	positive		homozygote GG	negative	
HLA-DQ2.5	rs2187668	C	T	T	homozygote CC	negative	HLA-DQ2.5 negative	heterozygote CT	positive	HLA-DQ2.5 positive	homozygote TT	positive	HLA-DQ2.5 positive
HLA-DQ8	rs7454108	T	C	C	homozygote TT	negative	HLA-DQ8 negative	heterozygote TC	positive	HLA-DQ8 positive	homozygote CC	positive	HLA-DQ8 positive

Table 4: Evaluation of variants for predisposition to celiac disease

Gene	dbSNP	Ref. allele	Alt. allele	Posit. allele	Genotyping result for VAF < 13.99%	Analysis results	Genotyping result for VAF 14–85.99%	Analysis results	Genotyping result for VAF > 86%	Analysis results
IL23R	rs1004819	G	A	G	homozygote GG	positive	heterozygote GA	positive	homozygote AA	negative
IL23R	rs10889677	C	A	A	homozygote CC	negative	heterozygote CA	positive	homozygote AA	positive
NOD2	rs2066844	C	T	T	homozygote CC	negative	heterozygote CT	positive	homozygote TT	positive
NOD2	rs2066845	G	C	C	homozygote GG	negative	heterozygote GC	positive	homozygote CC	positive
NOD2	rs2066847	G	ins C	ins C	homozygote GG	negative	heterozygous insertion	positive	homozygous insertion	positive

Table 5: Evaluation of variants for predisposition to Crohn's Disease

13.2 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).

13.3 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.

13.3.1 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 ≤ Ct_{NC}).
– After the sequencing data evaluation, frequencies of variants are as expected.

13.3.2 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 the difference between PC and NC is less than 3 Ct, include the sample in the DNA pool for sequencing as well.

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 16. FAQ.

14. KIT LIMITATIONS

- The fastGEN Crohn's & Celiac Disease Kit is validated for DNA isolated from buccal swabs, peripheral blood, synthetic DNA controls, and reference standards. The result is influenced by sample quality. Proper procedures for sample collection, transport, DNA isolation, and storage are critical for the test. Sample quality (DNA integrity) affects its amplifiability. The user of the kit is responsible for the quality of the samples.
- Genotyping results should be interpreted by a healthcare professional.
- The fastGEN Crohn's & Celiac Disease Kit is designed for the rapid preparation of sequencing libraries required for genotyping the rs2395182, rs7775228, rs4713586, rs2187668, and rs7454108 in celiac disease, as well as the rs1004819 and rs10889677 regions in the *IL23R* gene and the rs2066844, rs2066845, rs2066847 in the *NOD2* gene for Crohn's disease using NGS technology. Sequence variants in other regions cannot be detected by the fastGEN Crohn's & Celiac Disease Kit.
- A negative result does not exclude mutations below the detection limit of the method.
- End-users are responsible for the validation of fastGEN Crohn's & Celiac Disease Kit in combination with other products and instruments (e.g. isolation kit, sequencing machine, data evaluation software) when integrating them into the diagnostic process.
- In the fastGEN Crohn's & Celiac Disease Kit, the primer regions contain sequence variants such as rs113035896, rs2858326, and rs116178934, which may affect the functionality of individual fastGEN primers and may lead to reduced amplification efficiency of a given amplicon. The functionality of fastGEN primers may also be affected by the presence of rare sequence variants.

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

15. KIT CHARACTERISTICS

Analytical sensitivity and specificity parameters were determined by evaluating data as part of the analytical characterization of the fastGEN Crohn's & Celiac Disease Kit from BioVendor – Laboratorní medicína s.r.o. The method's limit of detection was established for the kit, and the cross-reactivity of the primers was verified (in silico). The method's reproducibility and robustness were tested on a series of identical samples in two independent experiments with defined changes in conditions. The fastGEN Crohn's & Celiac Disease Kit was validated on DNA isolated from buccal swabs, peripheral blood, synthetic DNA controls, and reference standards.

16. FAQ

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

   It is necessary to obtain 75,000 paired-end reads per sample. The MiSeq Reagent kit v2 Nano, which has 2 million paired-end reads, is sufficient for up to 16 samples (60% of its capacity). The MiSeq Reagent kit v2 Micro, which has 8 mil paired-end reads, is 15% full when sequencing 16 samples.

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

   Yes, it is possible to use Local Run Manager or BaseSpace Sequencing Hub for secondary analysis.

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, 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. Reads in the targeted region should not show up during sequencing of NC. In case of ambiguity, contact customer support.

6. **A well-known insertion, c.2938dup (p.Leu980fs), has been described in the *NOD2* gene and causes a frameshift mutation. Can this variant be detected using the kit?**

   Yes, this variant, also known as c.3020insC (p.Leu1007fs), is detectable with the fastGEN Crohn's & Celiac Disease Kit. In 2017, its annotation in the dbSNP database was updated, and the variant rs5743293 was merged with other related variants into a single consolidated dbSNP record, rs2066847.

17. REFERENCES

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

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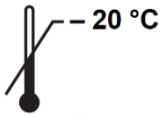
18. EXPLANATION OF SYMBOLS

	Catalogue number
	Batch code
	Use by date
	Upper limit temperature
	Manufacturer
 www.biovendor.com	Read electronic instructions for use – eIFU
	The content is sufficient for 16 tests

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BioVendor – Laboratorní medicína s.r.o.
Karásek 1767/1, 621 00 Brno, Czech Republic
+420 549 124 185
info@biovendor.com
sales@biovendor.com
www.biovendor.com