BioVendor Group NGS

ENG

Instructions for Use:
fastGEN Food Intolerance Kit

Catalogue number:
RDNGS0018

For research use only

BioVendor R&D®

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CONTENT

HISTORY OF CHANGES

Previous version	Current version
ENG.002.A	ENG.003.A
Changed the kit name throughout the documentation.	
Modification of the list of measured regions (removal of rs370793608).	
In chapter 12., added information on limit of detection.	
In chapter 12.1.3, the optional melting curve measurement step has been changed to recommended.	
In chapters 12.3.4 and 16., the value of paired-end reads has been changed to 50,000 per sample.	
In chapters 12.3.5, 12.3.6, 12.3.7 and 12.3.8, the volumes of sequencing primers have been changed.	
In chapter 16., the information on evaluating Tm values obtained from the melting curve in the qPCR step has been added.	

1. INTENDED PURPOSE

RDNGS0018 fastGEN Food Intolerance Kit is intended for rapid preparation of the sequencing library required for *ALDOB* (rs118204429, rs764826805, rs387906225, rs1800546, rs76917243, rs78340951, rs77718928), *AOC1* (rs10156191, rs1049742, rs2268999, rs1049793, rs2052129) and *MCM6* (rs4988235, rs182549) genes genotyping by next-generation sequencing (NGS) in general population.

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

1.1 Abbreviations

ALDOB	gene encoding Aldolase B
AOC1	gene encoding Amine oxidase copper-containing 1
Ct	cycle threshold
DNA	deoxyribonucleic acid
FAM/SYBR	6-carboxyfluorescein/asymmetrical cyanine dye
LoD	limit of detection
MCM6	gene encoding Minichromosome maintenance complex component 6
NC	negative control
NGS	Next Generation Sequencing
PC	positive control
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction
RFU	relative fluorescence units
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 *ALDOB, AOC1* and *MCM6* genotyping.
- Kit contains **complete Master Mixes** including indexes supplied in a ready to use format and **sequencing primers**.
- The fastGEN Food Intolerance Kit is designed for *ALDOB, AOC1* and *MCM6* genes genotyping in 16 samples with a unique combination of indexes in a single sequencing run.
- In the procedure of fastGEN Food Intolerance 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 Food Intolerance Kit is delivered frozen at –20 °C.
- After delivery, store the fastGEN Food Intolerance Kit at –20 °C.
- **Protect kit components from light.**
- Avoid repeated freeze-thaw cycles of Master Mixes.
- Opened R2SP Food Intolerance and ISP Food Intolerance store the components at −20 °C. Sequencing primers can be used up to 3 times.
- Do not use expired kits or components.

4. INTRODUCTION

Food intolerances can cause a range of unpleasant symptoms, including digestive problems, skin rashes, headaches, and other health problems. Early detection and correct diagnosis of these intolerances are crucial to improving the quality of life of affected individuals and to designing an appropriate dietary regimen.

Fructose intolerance is the body's inability to properly metabolize fructose, a sugar found in many fruits and some sweeteners. There are two main forms: congenital fructose intolerance, caused by a genetic deficiency of the enzyme aldolase B, and fructose malabsorption, where there is a problem with fructose transport in the intestine. Genetic variants examined: **rs118204429**, **rs387906225**, **rs1800546**, **rs76917243**, **rs78340951**, **rs77718928**, **rs764826805**.

Histamine intolerance occurs when the body cannot effectively break down histamine, a substance naturally occurring in certain foods. Histamine is a biogenic amine that is involved in several physiological processes, including inflammatory reactions. Genetic variants examined: **rs10156191**, **rs1049742**, **rs2268999**, **rs1049793**, **rs2052129**.

Lactose intolerance is the inability to digest lactose, a sugar found in milk and dairy products. This condition is caused by a deficiency of the enzyme lactase, which is necessary for the breakdown of lactose into glucose and galactose. The *MCM6* (minichromosome maintenance complex component 6) gene plays a key role in regulating the expression of the *LCT* gene (lactase), thus in the ability to digest lactose. Genetic variants examined: **rs4988235**, **rs182549**.Genetic screening based on the NGS method is highly sensitive, specific and suitable for diagnosis.

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

5. TEST PRINCIPLE

The fastGEN Food Intolerance Kit is used to prepare a sample for NGS testing for genetic variants associated with fructose (*ALDOB*), histamine (*AOC1*) and lactose (*MCM6*) intolerance. First, short amplicons are obtained by a single PCR with hybrid primers with tags, in which sequences 243 bp in average length 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 Food Intolerance 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 Food Intolerance Kit components do not contain infectious material.
- Samples used for the fastGEN Food Intolerance 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 Food Intolerance Kit** is supplied in a ready to use format for the analysis of 16 samples, i.e. to perform 16 reactions (Table 1). Kit includes **specific Master Mixes** containing all the necessary reaction components and **sequencing primers** for *ALDOB, AOC1* and *MCM6* genes.

fastGEN Food Intolerance Kit components	Index sequences	Volume per 1 tube (µl)	Number of tubes	State
Food Intolerance Master Mix i732	CCTATTAC	18	1	ready to use
Food Intolerance Master Mix i733	GAGGATAC	18	1	ready to use
Food Intolerance Master Mix i734	AGCTCGAC	18	1	ready to use
Food Intolerance Master Mix i735	GTATACCA	18	1	ready to use
Food Intolerance Master Mix i737	CTTGAGAA	18	1	ready to use
Food Intolerance Master Mix i738	ATCCTCAA	18	1	ready to use
Food Intolerance Master Mix i742	TTGGCGTT	18	1	ready to use
Food Intolerance Master Mix i745	GCAGGCTT	18	1	ready to use
Food Intolerance Master Mix i755	ACGCGGAT	18	1	ready to use
Food Intolerance Master Mix i756	CAATCAAT	18	1	ready to use
Food Intolerance Master Mix i758	ATTAGCTG	18	1	ready to use
Food Intolerance Master Mix i759	CGAATATG	18	1	ready to use
Food Intolerance Master Mix i762	CATCATGG	18	1	ready to use
Food Intolerance Master Mix i763	CGGTCCGG	18	1	ready to use
Food Intolerance Master Mix i765	CCAGCGCG	18	1	ready to use
Food Intolerance Master Mix i766	GGTAAGCG	18	1	ready to use
R2SP Food Intolerance		55	1	to be diluted
ISP Food Intolerance		55	1	to be diluted

Table 1: fastGEN Food Intolerance Kit components

9. RECOMMENDED MATERIAL (NOT SUPPLIED)

9.1 Chemicals

- Examined DNA
- Standardized sample containing the required variants of the examined *ALDOB, AOC1* and *MCM6* genes (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
- 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 or must be diluted.

10.1 fastGEN Food Intolerance Kit: Master Mix

For *ALDOB, AOC1* and *MCM6* genes genotyping let the appropriate number of fastGEN Food Intolerance Kit Master Mixes tubes thaw and keep them cool until use.

10.2 Sequencing primers

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

- 1 tube: R2SP Food Intolerance
- 1 tube: ISP Food Intolerance

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 13. 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 Food Intolerance 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 *ALDOB, AOC1* and *MCM6* genotyping in one sequencing run.

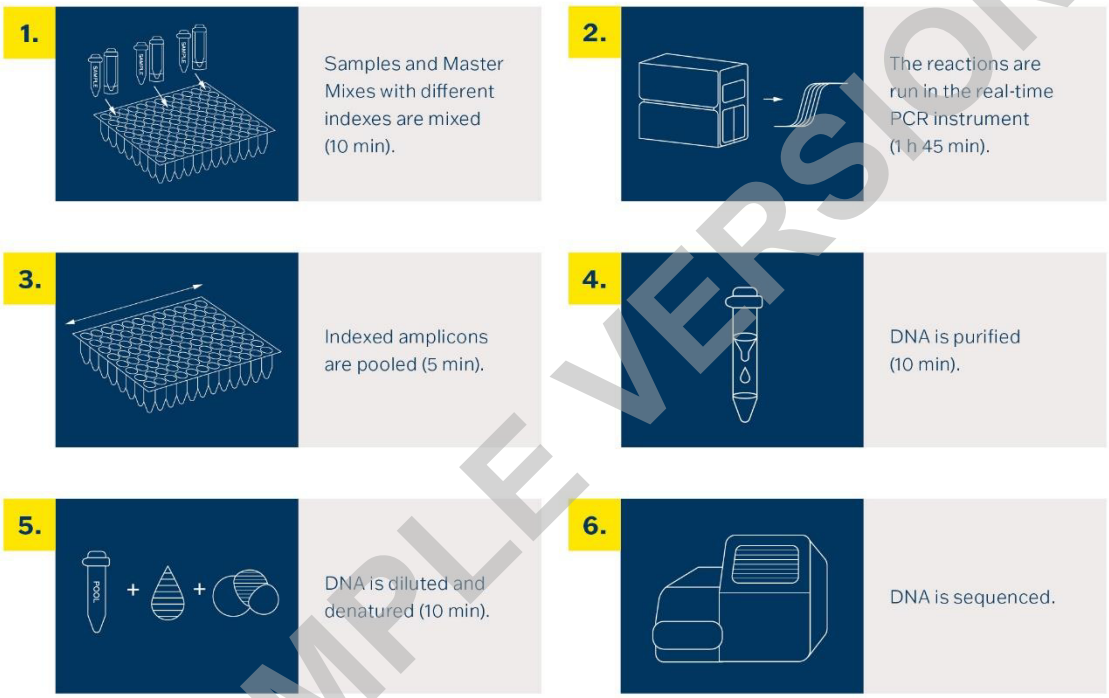

Figure 1: Workflow of genotyping using the fastGEN kit

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 *ALDOB, AOC1* and *MCM6* genes 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 2 µl. You get a DNA pool in a volume of 32 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 > 33, 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:

$$c[nM] = \frac{\rho i \left[\frac{ng}{\mu l}\right] \times 10^6}{(660 \times 243)}$$

  - ρi is the DNA mass concentration
  - **243 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 Sequencing primers preparation

The sequencing library prepared with the fastGEN Food Intolerance Kit is suitable for use on all Illumina® sequencing machines. Dilute custom R2SP and ISP sequencing primers with HT1 buffer or Illumina® sequencing primers according to the sequencing machine used, vortex and centrifuge briefly. If mixing fastGEN libraries with other libraries requiring Illumina sequencing primers, use the appropriate Illumina sequencing primer instead of HT1 buffer for dilution. **For Read 1, use Illumina® sequencing primers.** Indicate the use of custom positions in the SampleSheet.

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.

50,000 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.

Sequencing primers preparation:

- Remove the Illumina® sequencing primers for Read 1 from position 12 into a clean tube (use clean Pasteur pipette)
- Index sequencing primers (ISP): 12 µl ISP Food Intolerance + 588 µl HT1
- Read2 sequencing primers (R2SP): 12 µl R2SP Food Intolerance + 588 µl HT1

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: Illumina® sequencing primers for Read 1 taken from position 12

Position 19: ISP diluted in HT1

Position 20: R2SP diluted in HT1

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 0.2 M NaOH for 5 min at room temperature. Add 5 µl of 200 mM TrisHCl. 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. 150 µl DNA 5 pM pool + 319 µl HT1). The dilution should correspond to the optimal raw sequencing density values in the long term.

Sequencing primers preparation:

- Remove the Illumina® sequencing primers for Read 1 from position 24 into a clean tube
- Index sequencing primers (ISP): 9.8 µl ISP Food Intolerance + 810.2 µl HT1 or Illumina® sequencing primers (position 28)
- Read2 sequencing primers (R2SP): 7.4 µl R2SP Food Intolerance + 602.6 µl HT1 or Illumina® sequencing primers (position 25)

Pipette 500 µl of the diluted 1.4 pM or 1.6 pM DNA library and the total volume of the diluted sequencing primers into the sequencing cartridge into positions 13–16 in the following order:
Position 16: DNA library in HT1
Position 15: Illumina® sequencing primers for Read 1 taken from position 24
Position 13: diluted ISP
Position 14: diluted R2SP

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.

Sequencing primers preparation (Mid Output):

- Remove the Illumina® sequencing primers for Read 1 from position 20 into a clean tube
- Index sequencing primers (ISP): 24 µl ISP Food Intolerance + 1976 µl Illumina® sequencing primers (position 22)

– Read2 sequencing primers (R2SP): 18 µl R2SP Food Intolerance + 1482 µl Illumina® sequencing primers (position 21)

Sequencing primers preparation (High Output):

– Remove the Illumina® sequencing primers for Read 1 from position 20 into a clean tube
– Index sequencing primers (ISP): 24 µl ISP Food Intolerance + 1976 µl Illumina® sequencing primers (position 22)
– Read2 sequencing primers (R2SP): 24 µl R2SP Food Intolerance + 1976 µl Illumina® sequencing primers (position 21)

Pipette 1 300 µl of the diluted 1.5 pM or 1.8 pM DNA library and the total volume of the diluted sequencing primers into the sequencing cartridge into positions 7–10 in the following order:
Position 10: DNA library in HT1
Position 7: Illumina® sequencing primers for Read 1 taken from position 20
Position 9: diluted ISP
Position 8: diluted R2SP

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).

Sequencing primers preparation (for sufficient sequencing primers for S4 NovaSeq fastGEN Food Intolerance Extra Sequencing Primers RDNSP0018A must be purchased):

– Remove the Illumina® sequencing primers for Read 1 from position 24 into a clean tube
– Index sequencing primers (ISP; SP, S1, S2): 42 µl ISP Food Intolerance + 3 458 Illumina® sequencing primers (position 23)
– Index sequencing primers (ISP; S4): 60 µl ISP Food Intolerance + 4 940 µl Illumina® sequencing primers (position 23)
– Read2 sequencing primers (R2SP; SP, S1, S2): 24 µl R2SP Food Intolerance + 1 976 µl Illumina® sequencing primers (position 13)
– Read2 sequencing primers (R2SP; S4): 42 µl R2SP Food Intolerance + 3 458 µl Illumina® sequencing primers (position 13)

Pipette 150 µl (SP, S1), 225 µl (S2), 465 µl (S4) of the diluted, denatured and neutralized DNA library and the total volume of the diluted sequencing primers into the sequencing cartridge into positions 5–8 in the following order:

Position 8: DNA library in HT1

Position 5: Illumina® sequencing primers for Read 1 taken from position 24 (2 000 µl – SP, S1, S2; 3 500 µl – S4)

Position 7: diluted ISP

Position 6: diluted R2SP

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 *ALDOB*, *AOC1* and *MCM6* genotyping

A genotyping result for ***ALDOB***, ***AOC1*** and ***MCM6*** is considered positive if a variant has been detected with a frequency of ≥ 30 % in the regions of rs118204429, rs1825555, rs76917243, rs78340951, rs77718928 in the *ALDOB* gene, regions rs10156191, rs1049742, rs2268999, rs1049793, rs2052129 in the *AOC1* gene and regions rs4988235, rs182549 in the *MCM6* gene.

ATTENTION! Since lactose intolerance in the regions rs4988235, rs182549 in the *MCM6* gene is manifested in the allele listed in the reference genome GRCh38, the occurrence of the variant allele indicates a healthy individual. In a patient suffering from lactose intolerance, 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 replicates processed with different Master Mixes match.

List of variants and their evaluation

Gene	Region (dbSNP)	HGVSc [GRCh38] (orientation according to the gene)	Reference allele in the report (orientation ref. genome = plus)	Enzyme-reducing allele (orientation ref. genome = plus)	Intolerance variant (orientation ref. genome = plus)
ALDOB	**rs118204429**	c.178C>T / C>A	G	A / T	GA / AA /GT / TT
ALDOB	**rs764826805**	c.324+1G>A	C	T	CT / TT
ALDOB	**rs387906225**	c.360_363del / dup	TTTG	del / dup TTTG*	dup. or del. in area delTTTG / dupTTTG*
ALDOB	**rs1800546**	c.448G>C / G>A	C	G / T	CG / GG / CT / TT
ALDOB	**rs76917243**	c.524C>A	G	T	GT / TT
ALDOB	**rs78340951**	c.1005C>G / C>T	G	C / A	CG / CC / GA / AA
ALDOB	**rs77718928**	c.1013C>T / C>G / C>A	G	A / C / T	GA / AA / CG / CC / GT / TT
AOC1	**rs10156191**	c.47C>T	C	T	CT / TT
AOC1	**rs1049742**	c.995C>T	C	T	CT / TT
AOC1	**rs2268999**	c.-16-578A>T	A	T	AT / TT
AOC1	**rs1049793**	c.1933C>G / C>T	C	G / T	CG / GG / CT / TT
AOC1	**rs2052129**	g.150851884G>A / G>T (GRCh38.p14 chr 7)	G	A / T	GT / TT / GA / AA
MCM6	**rs4988235**	c.1917+326C>T / C>G / C>A	G	G	GG
MCM6	**rs182549**	c.1362+117G>A	C	C	CC

*Table 4: List of reference and possible variants for selected regions (*to distinguish duplications and deletions, the surrounding nucleotides are also included in the analysis).*

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 *ALDOB, AOC1* and *MCM6* genes 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 Food Intolerance Kit was validated on DNA samples isolated from buccal swab, blood samples or synthetic DNA.
- 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 Food Intolerance Kit is designed for rapid preparation of the sequencing library required for the *ALDOB, AOC1* and *MCM6* genes genotyping using the NGS technology. Variants in other genes than *ALDOB, AOC1* and *MCM6* respectively, or other SNPs that are listed above are not detectable by the fastGEN fastGEN Food Intolerance Kit.
- 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.

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 of the fastGEN Food Intolerance Kit by BioVendor – Laboratorní medicína s.r.o. company was determined. The LoD for kit was determined, and the cross-reactivity of primers was verified (*in silico*). The repeatability and robustness of the method were tested using a series of identical samples in two independent experiments with a defined change of conditions. The diagnostic accuracy (sensitivity and specificity) of the tests was determined based on the analysis of clinical samples with a known mutation status. The results of *ALDOB, AOC1* and *MCM6* genotyping were correct in all samples, including repeated measurements (sensitivity and specificity 100 %).

16. FAQ

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

   It is necessary to obtain 50,000 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 (40% of its capacity). The MiSeq Reagent kit v2 Micro, which has 8 mil paired-end reads, is 10% 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. In the case of including a step for the melting curve of PCR products at the end of the qPCR amplification protocol (see Chapter 12.1.3), the melting curve has a specific progression with two peaks and a Tm1 value is at least 1 °C higher than the Tm value of NC (if measured); however, the Tm2 value has a significantly weaker signal and may therefore be below the threshold value for evaluation (see Figure 2).

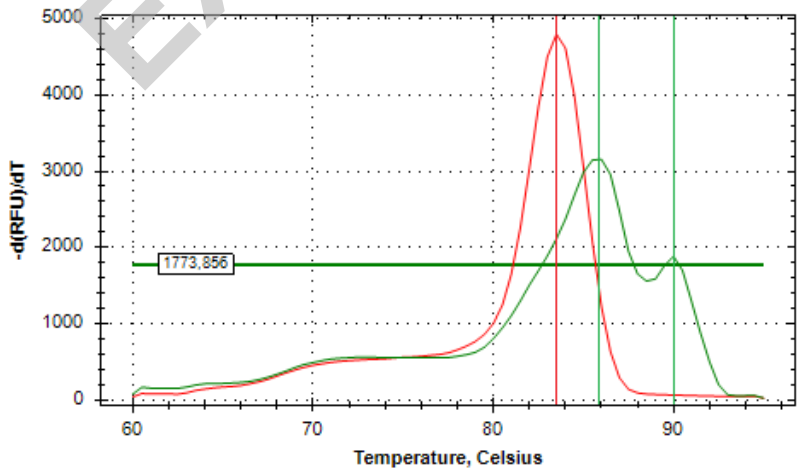

Figure 2: Graph of Tm values measured from the melting curve (PC = green, NC = red).

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 analysed region should not show up during sequencing with a standard NC. In case of ambiguity, contact customer support.

6. **What to do if all the sequencing primers are used up?**

   It is possible to purchase the related product fastGEN Food Intolerance Extra Sequencing Primers RDNSP0018A.

17. REFERENCES

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

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[3] COMAS-BASTÉ, Oriol, et al. Histamine intolerance: The current state of the art. Biomolecules, 2020, 10.8: 1181.

[4] MAINTZ, L., et al. Association of single nucleotide polymorphisms in the diamine oxidase gene with diamine oxidase serum activities. Allergy, 2011, 66.7: 893-902.

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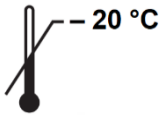
18. EXPLANATION OF SYMBOLS

	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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