BioVendor Group NGS

ENG

Instructions for Use:
fastGEN MPN Cancer Kit

Catalogue number:
RDNGS0021

For research use only

BioVendor R&D®

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CONTENT

HISTORY OF CHANGES

Previous version	Current version
	ENG.001.A
New Document	

1. INTENDED PURPOSE

RDNGS0021 fastGEN MPN Cancer Kit is intended for rapid preparation of the sequencing library required for selected targeted regions of *JAK2*, *MPL* and *CALR* genes genotyping by next-generation sequencing (NGS) in general population. Precise specification of selected targeted regions is listed in Table 1. The input material for the sequencing library preparation is isolated DNA. The reagent kits are intended for professional use in the laboratory.

Region designation	Region specification	Position of the region according to GRCh38/hg38		
		Chromosome	Start	Stop
JAK2 exon 12	entire exon	chr9	5069888	5070066
JAK2 exon 13	entire exon	chr9	5072453	5072676
JAK2 exon 14	entire exon	chr9	5073676	5073801
JAK2 exon 16	part of an exon	chr9	5078330	5078432
MPL exon 4	part of an exon	chr1	43339466	43339579
MPL exon 10	part of an exon	chr1	43349253	43349346
MPL exon 12	part of an exon	chr1	43352616	43352719
CALR exon 9	entire exon	chr19	12943706	12943930

Table 1: Specification of targeted regions of fastGEN MPN Cancer Kit.

1.1 Abbreviations

CALR	Calreticulin
Ct	Cycle Threshold
DNA	Deoxyribonucleic Acid
FAM/SYBR	6-carboxyfluorescein/Asymmetrical Cyanine Dye
JAK2	Janus Kinase 2
LoD	Limit of Detection
MPL	MPL proto-oncogene, thrombopoietin receptor
MPN	Myeloproliferative Neoplasms
NC	Negative Control
NGS	Next Generation Sequencing
PC	Positive Control
PCR	Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction

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 *JAK2*, *MPL* and *CALR* genotyping.
- Kit contains **complete Master Mixes** including indexes supplied in a ready to use format.
- The fastGEN MPN Cancer Kit is designed for *JAK2*, *MPL* and *CALR* gene genotyping in 16 samples with a unique combination of indexes in a single sequencing run.
- In the procedure of fastGEN MPN Cancer 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 MPN Cancer Kit is delivered frozen at −20 °C.
- After delivery, store the fastGEN MPN Cancer 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

Myeloproliferative neoplasias (MPN) are a group of hematological diseases characterized by increased proliferation of blood elements in the bone marrow. These diseases include polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF). The pathogenesis of MPNs is closely linked to activating somatic mutations in the *JAK2*, *MPL* and *CALR* genes that lead to constitutive activation of the JAK-STAT signaling pathway.

Molecular diagnosis of mutations in *JAK2*, *MPL* and *CALR* genes is essential for correct classification of MPN according to WHO classification, differential diagnosis, monitoring of minimal residual disease and selection of targeted therapy. Knowledge of the mutational profile also has significant prognostic and predictive potential, which increasingly influences personalized medicine. Genetic screening based on NGS is highly sensitive, specific and suitable for diagnosis [1–3].

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 MPN Cancer Kit is designed for the preparation of NGS libraries to enable analysis of mutational profiles in the targeted regions of *JAK2*, *MPL* and *CALR* genes. First, short amplicons are obtained by a single PCR with hybrid primers with tags, in which sequences with an average length 297 bp 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 MPN Cancer 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 MPN Cancer Kit components do not contain infectious material.
- Samples used for the fastGEN MPN Cancer 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 MPN Cancer Kit is supplied in a ready to use format for the analysis of 16 samples, i.e. to perform 32 reactions (Table 2). Kit includes specific Master Mixes containing all the necessary reaction components for *JAK2*, *MPL* and *CALR* genes.

Kit components	Index P7 sequences	Index P5 sequences	Volume per 1 tube (µl)	Number of tubes	State
MPN Master Mix B17 (A-B)	CCTATTAC	CTCAGACG	18	**1**	**ready to use**
MPN Master Mix B18 (A-B)	GAGGATAC	CTCAGACG	18	**1**	**ready to use**
MPN Master Mix B19 (A-B)	AGCTCGAC	CTCAGACG	18	**1**	**ready to use**
MPN Master Mix B20 (A-B)	GTATACCA	CTCAGACG	18	**1**	**ready to use**
MPN Master Mix C17 (A-B)	CCTATTAC	GAGCTCGT	18	**1**	**ready to use**
MPN Master Mix C18 (A-B)	GAGGATAC	GAGCTCGT	18	**1**	**ready to use**
MPN Master Mix C19 (A-B)	AGCTCGAC	GAGCTCGT	18	**1**	**ready to use**
MPN Master Mix C20 (A-B)	GTATACCA	GAGCTCGT	18	**1**	**ready to use**
MPN Master Mix D17 (A-B)	CCTATTAC	TCTGAGTA	18	**1**	**ready to use**
MPN Master Mix D18 (A-B)	GAGGATAC	TCTGAGTA	18	**1**	**ready to use**
MPN Master Mix D19 (A-B)	AGCTCGAC	TCTGAGTA	18	**1**	**ready to use**
MPN Master Mix D20 (A-B)	GTATACCA	TCTGAGTA	18	**1**	**ready to use**
MPN Master Mix E17 (A-B)	CCTATTAC	CAAGTTAT	18	**1**	**ready to use**
MPN Master Mix E18 (A-B)	GAGGATAC	CAAGTTAT	18	**1**	**ready to use**
MPN Master Mix E19 (A-B)	AGCTCGAC	CAAGTTAT	18	**1**	**ready to use**
MPN Master Mix E20 (A-B)	GTATACCA	CAAGTTAT	18	**1**	**ready to use**

Table 2: fastGEN MPN Cancer Kit components.

9. RECOMMENDED MATERIAL (NOT SUPPLIED)

9.1 Chemicals

- Examined DNA
- Standardized sample containing the required variants of the examined *JAK2*, *MPL* and *CALR* genes (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 MPN Cancer Kit: Master Mix

For *JAK2*, *MPL* and *CALR* gene genotyping 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 3.
- 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 MPN Master Mixes).
- Add **5 µl DNA** prepared according to Table 3 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	>20 ng/µl	5 x	1 µl DNA + 4 µl H2O
B	1–20 ng/µl	No dilution	5 µl DNA
C	<1 ng/µl	No dilution	5 µl DNA in duplicates

Table 3: 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 MPN Cancer 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 and somatic mutations with frequency from 1 % can be detected. The minimal DNA input is 5 ng of DNA.

The kit is designed to process 16 samples for *JAK2*, *MPL* and *CALR* targeted regions genotyping in one sequencing run.

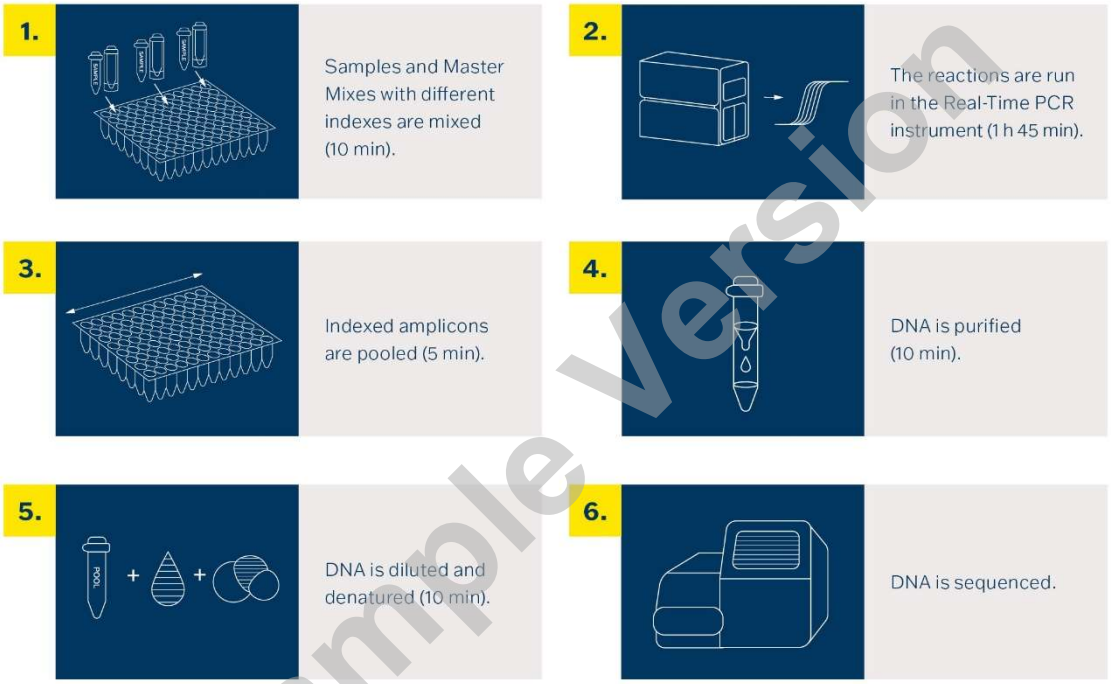


Figure 1: Workflow of genotyping using the fastGEN kit.

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 for Master Mixes A-B of one index (see Chapter 11).
- Recommendation:
  - Include positive (PC) and negative (NC) control.
  - Add **5 µl of positive control DNA** of appropriate concentration for Master Mix A-B of one index (see Chapter 11).
  - Add **5 µl water for molecular biology** as a negative control for Master Mix A-B of one index.

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 µ**l of Master Mix A-B to each sample or control.
- The total volume per PCR reaction is **20 µl**.
- Use only **one** Master Mix per position. Use separate position for Master Mix.
- 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 4.

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 4: 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 *JAK*, *MPL* and *CALR* 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 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:

$$c[nM] = \frac{\rho i \left[\frac{ng}{\mu l}\right] \times 10^6}{(660 \times 297)}$$

  - ρi is the DNA mass concentration
  - 297 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 MPN Cancer 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.

100,000 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 *JAK2*/*MPL*/*CALR* genotyping

JAK2, *MPL* and *CALR* genotyping result is considered as positive (mutation detected) if a variant in the *JAK2*, *MPL* or *CALR* genes was detected with a **frequency ≥ 1 %. The minimal input of DNA is 5 ng**.

If the *JAK2* variants are detected with a frequency from 0.5–1 %, we recommend retesting in duplicate or verifying results with another method.

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

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 *JAK2, MPL* and *CALR* gene 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 NC 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 MPN Cancer Kit was validated on DNA samples isolated from peripheral blood, synthetic DNA controls and reference standards.
- The test result is affected by the quality of the sample. The correct procedure for collection, transport, DNA isolation and storage of samples is important for the examination. The quality of the sample (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 MPN Cancer Kit is designed for rapid preparation of the sequencing library required for the *JAK2*, *MPL* and *CALR* gene genotyping using the NGS technology. Variants in other genes or other than selected targeted regions of *JAK2*, *MPL* and *CALR* (as listed in Table 1) are not detectable by the fastGEN MPN Cancer 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.
- The manufacturer confirms only the detection of variants listed in the analytical specifications of the kit (Table 5). While the fastGEN MPN Cancer Kit assay can be used for the detection of additional mutations within the targeted regions of the *JAK2*, *MPL* and *CALR* genes (as outlined in Table 1), their identification is not formally validated by the manufacturer.
- Detection of concurrent *JAK2* c.1691G>A and c.1711G>A mutations is not possible using the fastGEN MPN Cancer Kit.
- End-users are responsible for the validation of fastGEN MPN Cancer Kit in combination with other products and instruments (e.g. isolation kit, sequencing machine, data evaluation software) when integrating them into the diagnostic process.

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 MPN Cancer Kit by BioVendor – Laboratorní medicína a.s. 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 DNA isolated from clinical samples (peripheral blood, synthetic controls and reference standards) with a known mutation status. The results of *JAK2*, *MPL* and *CALR* genotyping were correct in all samples, including repeated measurements (sensitivity and specificity 100 %).

As part of the analytical characterization of the fastGEN MPN Cancer Kit, mutations in the *JAK2*, *MPL* and *CALR* genes were validated. Summary of validated mutations is provided in Table 5.

Gene	Mutation
JAK2 NM_004972.4	c.1849G>T p.(Val617Phe)
	c.1624_1629del p.(Asn542_Glu543del)
	c.1627_1632del p.(Glu543_Asp544del)
MPL NM_005373.3	c.1544G>T p.(Trp515Leu)
	c.1543T>C p.(Trp515Arg)
	c.1502T>C p.(Val501Ala)
CALR NM_004343.4	c.1099_1150del p.(Leu367Thrfs*46)
	c.1176_1184del p.(Asp392_Asp394del)
	c.1116_1146del p.(Asp373Argfs*47)
	c.1103_1136del p.(Lys368Argfs*51)
	c.1154_1155insTTGTC p.(lys385Asnfs*47)

Table 5: List of validated mutations in the JAK2, MPL and CALR genes.

16. FAQ

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

   It is necessary to obtain 100,000 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. The MiSeq Reagent kit v2 Micro, which has 8 mil paired-end reads, is 20 % 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.

17. REFERENCES

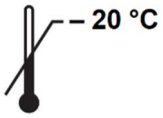
For more references see our websites www.biovendor.com.

[1] Barbui T, Thiele J, Gisslinger H, Kvasnicka HM, Vannucchi AM, Guglielmelli P, Orazi A, Tefferi A. The 2016 WHO classification and diagnostic criteria for myeloproliferative neoplasms: document summary and in-depth discussion. Blood Cancer J. 2018 Feb 9;8(2):15. doi: 10.1038/s41408-018-0054-y. PMID: 29426921; PMCID: PMC5807384.

[2] Maslah N, Verger E, Schlageter MH, Miclea JM, Kiladjian JJ, Giraudier S, Chomienne C, Cassinat B. Next-generation sequencing for JAK2 mutation testing: advantages and pitfalls. Ann Hematol. 2019 Jan;98(1):111-118. doi: 10.1007/s00277-018-3499-y. Epub 2018 Sep 26. PMID: 30259120.

[3] Pizzi M, Croci GA, Ruggeri M, Tabano S, Dei Tos AP, Sabattini E, Gianelli U. The Classification of Myeloproliferative Neoplasms: Rationale, Historical Background and Future Perspectives with Focus on Unclassifiable Cases. Cancers (Basel). 2021 Nov 12;13(22):5666. doi: 10.3390/cancers13225666. PMID: 34830822; PMCID: PMC8616346.

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

BioVendor R&D®

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