

**BioVendor
Group**

NGS

ENG

Instructions for Use:
fastGEN MSI Kit

Catalogue number:
RDNGS0019

For research use only



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R&D®**



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HISTORY OF CHANGES

Previous version	Current version
ENG.001.A	ENG.002.A
Adjustment of Table 1 in chapter 8.	

1. INTENDED USE

RDNGS0019 fastGEN MSI Kit is intended for rapid preparation of the sequencing library required to assess microsatellite instability in clinically relevant regions by next-generation sequencing (NGS) in general population. fastGEN MSI Kit allows analysis of microsatellite regions BAT-25, BAT-26, BAT-40, CAT-25, NR-21, NR-22, NR-24, NR-27, MONO-27, TGFBR1, D2S123 and D18S58.

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

1.1 Abbreviations

Ct	cycle threshold
DNA	deoxyribonucleic acid
FAM/SYBR	6-carboxyfluorescein/asymmetrical cyanine dye
FFPE	Formalin-fixed, paraffin-embedded samples
LoD	limit of detection
MSI	Microsatellite Instability
MSI-H	Microsatellite Instability – High
MSI-L	Microsatellite Instability – Low
MSS	Microsatellite Stability
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 microsatellite instability assessment in microsatellite regions.
- Kit contains **complete Master Mixes** including indexes supplied in a ready to use format and **sequencing primers**.
- fastGEN MSI Master Mix is supplied in **2 tubes** for each sample.
- The fastGEN MSI Kit is designed to evaluate deletions and insertions of microsatellite regions in 16 samples with a unique combination of indexes in a single sequencing run.
- In the procedure of fastGEN MSI Kit, simple **addition of isolated DNA** to the Master Mix, and analysis in a Real-Time PCR cyclers is required.

3. STORAGE, EXPIRATION

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

- fastGEN MSI Kit is delivered frozen at -20°C .
- After delivery, store the fastGEN MSI Kit at -20°C .
- **Protect kit components from light.**
- Avoid repeated freeze-thaw cycles of Master Mixes.
- Opened R2SP MSI and ISP MSI store the components at -20°C . Sequencing primers can be used up to 3 times.
- Do not use expired kits or components.

4. INTRODUCTION

Microsatellites are short repeating sequences of nucleotides that appear in repeats in DNA. Microsatellite instability (MSI) is defined by changes in microsatellite length, i.e. changes in the number of repetitions. These changes are caused by the inability of the DNA repair system to correct an error in the sequence, and the errors then accumulate in the genome. Insertions and deletions arise as a consequence of genomic instability as a concomitant of cancer, and therefore microsatellite instability is an important marker for the occurrence and monitoring of tumor development. Diseases associated with MSI-H status include Lynch syndrome and cancers of the colorectum, stomach, breast, prostate, gallbladder, ovary, endometrium, and pancreas. Microsatellite instability also serves as a predictive marker for cancer treatment with immunotherapy.

When MSI is detected, 3 different statuses can be determined - high instability (MSI-H), low instability (MSI-L) and microsatellite stability (MSS). These statuses are determined by bioinformatic analysis of clinically relevant regions.

Genetic screening based on the NGS method is highly sensitive, specific and suitable for diagnosis.

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

- a target sequence to assess microsatellite instability (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 MSI Kit is used to prepare a sample for microsatellite instability assessment of clinically relevant microsatellite regions by NGS. First, short amplicons are obtained by a single PCR with hybrid primers with tags, in which sequences with an average length of 240 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 MSI 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 MSI Kit components do not contain infectious material.
- Samples used for the fastGEN MSI 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 MSI Kit** is supplied in a ready to use format for the analysis of 16 samples, i.e. to perform 32 reactions (Table 1). Kit includes **specific Master Mixes** containing all the necessary reaction components and **sequencing primers** for microsatellite regions.

fastGEN MSI Kit components	Index P7 sequences	Index P5 sequences	Volume per 1 tube (µl)	Number of tubes	State
MSI Master Mix A29 (A-B)	CATCATGG	TCGTCGGC	18	2	ready to use
MSI Master Mix A30 (A-B)	CGGTCCGG	TCGTCGGC	18	2	ready to use
MSI Master Mix A31 (A-B)	CCAGCGCG	TCGTCGGC	18	2	ready to use
MSI Master Mix A32(A-B)	GGTAAGCG	TCGTCGGC	18	2	ready to use
MSI Master Mix B29 (A-B)	CATCATGG	CTCAGACG	18	2	ready to use
MSI Master Mix B30 (A-B)	CGGTCCGG	CTCAGACG	18	2	ready to use
MSI Master Mix B31 (A-B)	CCAGCGCG	CTCAGACG	18	2	ready to use
MSI Master Mix B32(A-B)	GGTAAGCG	CTCAGACG	18	2	ready to use
MSI Master Mix C29 (A-B)	CATCATGG	GAGCTCGT	18	2	ready to use
MSI Master Mix C30 (A-B)	CGGTCCGG	GAGCTCGT	18	2	ready to use
MSI Master Mix C31 (A-B)	CCAGCGCG	GAGCTCGT	18	2	ready to use
MSI Master Mix C32(A-B)	GGTAAGCG	GAGCTCGT	18	2	ready to use
MSI Master Mix D29 (A-B)	CATCATGG	TCTGAGTA	18	2	ready to use
MSI Master Mix D30 (A-B)	CGGTCCGG	TCTGAGTA	18	2	ready to use
MSI Master Mix D31 (A-B)	CCAGCGCG	TCTGAGTA	18	2	ready to use
MSI Master Mix D32(A-B)	GGTAAGCG	TCTGAGTA	18	2	ready to use
R2SP MSI			85	1	to be diluted
ISP MSI			85	1	to be diluted

Table 1: fastGEN MSI Kit components

9. RECOMMENDED MATERIAL (NOT SUPPLIED)

9.1 Chemicals

- Examined DNA
- Standardized sample containing the required instable microsatellite 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 or must be diluted.

10.1 fastGEN MSI Kit: Master Mix

For microsatellite regions assessment let the appropriate number of MSI 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 MSI
- 1 tube: ISP MSI

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 MSI 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	>20 ng/µl	5 x	1 µl DNA + 4 µl H ₂ O
B	1–20 ng/µl	No dilution	5 µl DNA
C	<1 ng/µl	No dilution	5 µl DNA in duplicates

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 MSI 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 kit is designed to process 16 samples for microsatellite regions assessment in one sequencing run. **The analysis of one sample is carried out in two separate reactions.**

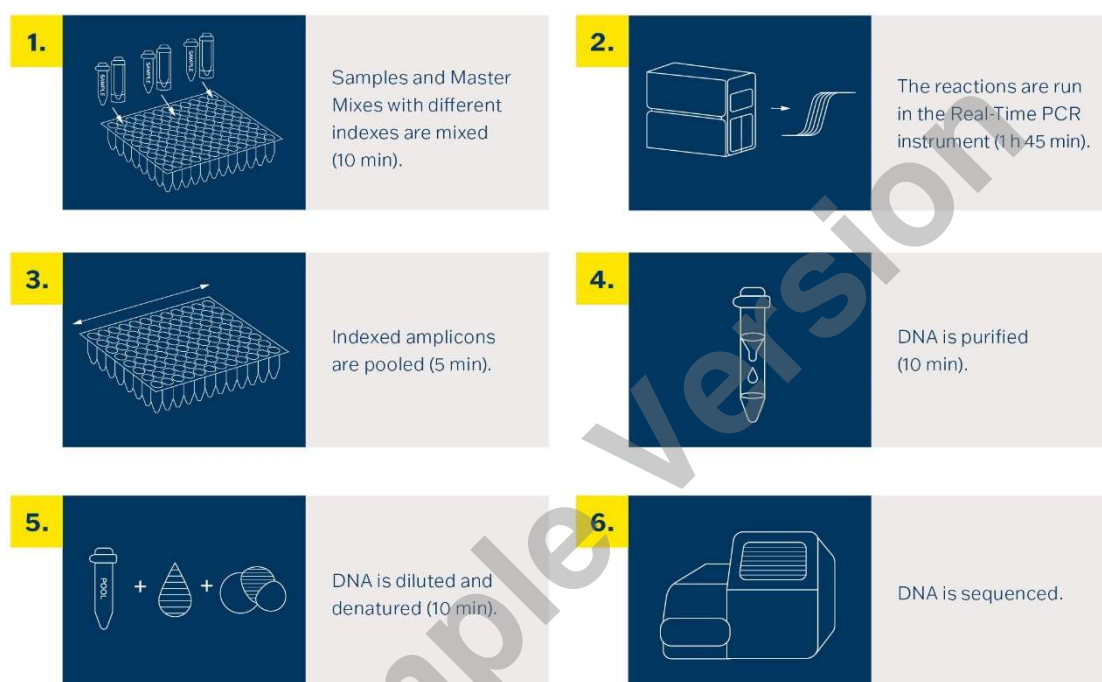


Figure 1: Workflow of genotyping using the fastGEN Kit

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 Mixes A-B of one index (see chapter 11).
 - Add **5 µl water for molecular biology** as a negative control for Master Mixes 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 µl** 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 A and B.
- 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 assessment of microsatellite regions 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 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 > 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.
- Assess the DNA pool molarity (molar concentration) according to the equation:

$$c[nM] = \frac{\rho_i \left[\frac{ng}{\mu l} \right] \times 10^6}{(660 \times 240)}$$

- ρ_i is the DNA mass concentration
- **240 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 MSI 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.

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.

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): 19.5 µl ISP MSI + 581 µl HT1
- Read2 sequencing primers (R2SP): 19.5 µl R2SP MSI + 581 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): 16 µl ISP MSI + 804 µl HT1 or Illumina® sequencing primers (position 28)
- Read2 sequencing primers (R2SP): 11.9 µl R2SP MSI + 598 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): 39 µl ISP MSI + 1 961 µl Illumina® sequencing primers (position 22)
- Read2 sequencing primers (R2SP): 29.3 µl R2SP MSI + 1 471 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): 39 µl ISP MSI + 1 961 µl Illumina® sequencing primers (position 22)
- Read2 sequencing primers (R2SP): 39 µl R2SP MSI + 1 961 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 150 µl; 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 MSI Extra Sequencing Primers RDNSP0019A 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): 68 µl ISP MSI + 3 432 µl Illumina® sequencing primers (position 23)

- Index sequencing primers (ISP; S4): 100 µl ISP MSI + 4 900 µl Illumina® sequencing primers (position 23)
- Read2 sequencing primers (R2SP; SP, S1, S2): 39 µl R2SP MSI + 1 961 µl Illumina® sequencing primers (position 13)
- Read2 sequencing primers (R2SP; S4): 68 µl R2SP MSI + 3 432 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

All microsatellite regions should be sequenced at least 100x.

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 Microsatellite instability assessment

Result of microsatellite instability assessment is considered as **positive** (MSI-H status) if **more than 30% of detected regions are instable**.

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

Result of microsatellite instability assessment is considered as **negative** (MSS status) if **less than 15% of detected regions are instable**.

MSI-L status is also considered as clinically **negative** result. MSI-L status is defined as number of detected instable regions in range **15–30%**.

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 ($Ct_{PC} + 3 \leq Ct_{NC}$).
- After the sequencing data evaluation, positive control is evaluated as MSI-H.

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 PN 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 MSI Kit was validated on DNA samples isolated from FFPE tumour tissue.
- 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 MSI Kit is designed for rapid preparation of the sequencing library required for microsatellite instability assessment of clinically relevant microsatellite regions using the NGS technology.
- Variants are detected only in mentioned microsatellite regions, variants in other regions are not detectable by the fastGEN MSI 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.
- Microsatellite region BAT-26 should be sequenced at least 30x.

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 MSI 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 clinical samples with a known mutation status. The results of microsatellite instability assessment 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 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 CLC Genomics Workbench or MSIsensor-pro 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 (MSI status). Further, verify technical settings and check if a manual error has occurred. If the difference between PC and NC is less than 3 Ct, include the sample in the DNA pool for sequencing as well. Reads in the analysed region should not show up during sequencing with a standard NC. In case of ambiguity, contact customer support.

6. Analysed area

fastGEN MSI Kit allows analysis of 12 microsatellite regions – BAT-25, BAT-26, BAT-40, CAT-25, NR-21, NR-22, NR-24, NR-27, MONO-27, TGFBR1, D2S123 and D18S58. Microsatellite instability assessment of region D5S346 is possible but not contained in evaluation software GENOVESA and therefore not declared by the manufacturer.

7. Number of all detected regions

If 6 or less regions are detected, we recommend repeating the analysis or verification by different method.

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

It is possible to purchase the related product fastGEN MSI Extra Sequencing Primers RDNSP0019A.

9. What is the length of detected microsatellite regions?

Microsatellite region	Detected length of microsatellite regions*	Repetitive unit
BAT-40	36	T
MONO-27	27	T
BAT-26	27	A
D2S123	21	AC
NR-24	23	T
TGFBRII	10	A
BAT-25	24	T
CAT-25	25	T
NR-27	26	A
NR-22	21	T
NR-21	21	A
D18S58	18	GT

Table 4: Length of repetitive units detected in microsatellite regions contained in fastGEN MSI Kit (* median detected in stable samples)

10. How is unstable and stable microsatellite regions evaluated?

Instability is defined as a change in the number of repetitive units manifested by deletion or insertion. The length of patient's microsatellite regions is compared to the length of regions in baseline. The region is interpreted by the algorithm based on the values forming the baseline.

11. What is baseline?

Baseline is value of stable repetitive units displayed in graph. It is made from stable samples of colorectal carcinoma and endometrial carcinoma.

12. How to interpret visualisation of microsatellite regions?

In the graph, the length of the microsatellite region is marked on the X-axis for a particular repetitive unit (listed in the table in question 9). The Y-axis shows the normalized DNA copy number of a particular region. The shape of the stable microsatellite region follows the shape of the stable reference - the baseline. An unstable region is characterised by a shift on the X-axis, i.e. shortening or lengthening of the microsatellite region.

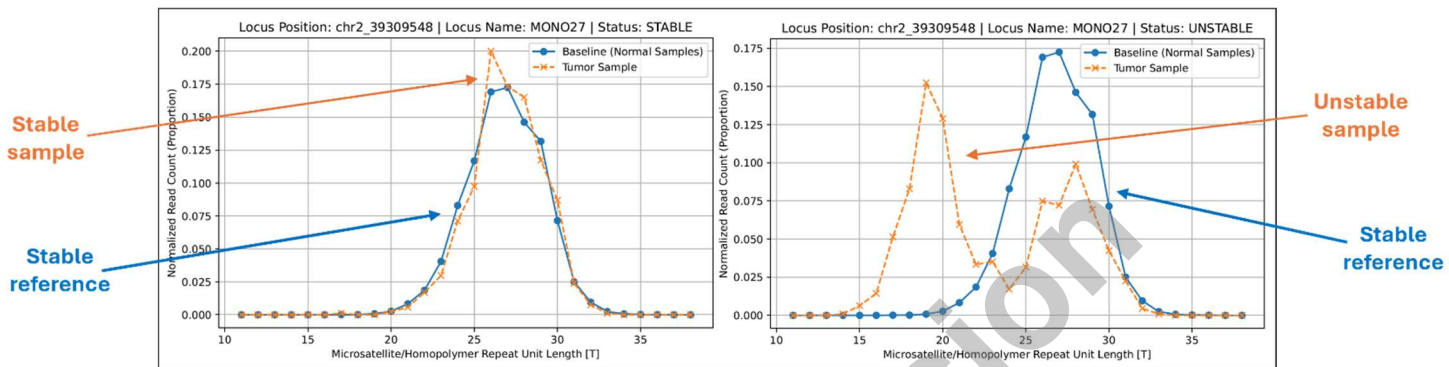


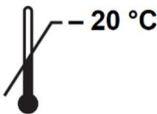
Figure 2: Visualisation of stable and unstable region MONO-27

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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