

ENG

Instructions for Use:
fastGEN POLE/CTNNB1 Cancer kit

Catalogue number:
RDNGS0010

For research use only!

 **BioVendor
R&D[®]**



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HISTORY OF CHANGES

Previous version	Current version
ENG.001.A	ENG.002.A
Chapter 12.3.2 removed.	
Links to supporting documents on the web have been removed from the IFU.	

1. INTENDED USE

RDNGS0010 BioVendor fastGEN POLE/CTNNB1 Cancer kit is intended for rapid preparation of the sequencing library required for *POLE* (DNA polymerase epsilon, catalytic subunit; exons 9, 11, 13, 14) and *CTNNB1* (β -catenin 1, exons 3,7 and 8) gene genotyping by next-generation sequencing (NGS) on Illumina® instrument.

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

1.1 Abbreviations

Ct	cycle threshold
<i>CTNNB1</i>	Catenin Beta 1
DNA	deoxyribonucleic acid
FAM/SYBR	6-carboxyfluorescein/asymmetrical cyanine dye
FFPE	Formalin-fixed, paraffin-embedded samples
LoD	limit of detection
NC	negative control
NGS	Next Generation Sequencing
PC	positive control
PCR	polymerase chain reaction
<i>POLE</i>	DNA polymerase epsilon catalytic subunit
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 tumour markers (*POLE* and *CTNNB1*) genotyping.
- Kit contains **complete Master Mixes** including indexes supplied in a ready to use format and **sequencing primers**.
- The fastGEN *POLE/CTNNB1* Cancer kit is designed for *POLE* and *CTNNB1* gene genotyping in 16 samples with a unique combination of indexes in a single sequencing run.
- In the procedure of fastGEN *POLE/CTNNB1* Cancer kit, simple **addition of isolated DNA** to the Master Mix, and analysis in a Real-Time PCR cycler is required.

3. STORAGE, EXPIRATION

Store the kit at $-20\text{ }^{\circ}\text{C}$. Under these conditions, all components are stable until the expiration date (see label on the box).

- fastGEN *POLE/CTNNB1* Cancer kit is delivered frozen at $-20\text{ }^{\circ}\text{C}$.
- After delivery, store the fastGEN *POLE/CTNNB1* Cancer kit at $-20\text{ }^{\circ}\text{C}$.
- **Protect kit components from light.**
- Avoid repeated freeze-thaw cycles.
- Do not use expired kits or components.

4. INTRODUCTION

Mutations in the *POLE* or *CTNNB1* gene have been identified in approximately 3% of tumours, including endometrial, lung, colorectal or hepatocellular carcinomas [1]. Only those tumours that have at least one of the defined hot-spot pathogenic mutations (variants) in the exonuclease domain of *POLE* gene in exons 9–14 should be assessed as *POLE*-ultramutated tumours. The list of these variants represents the current situation and may further change with the development of scientific knowledge [2, 3].

The *CTNNB1* gene encodes the protein β -catenin, which is part of the Wnt signaling pathway. In many tumours, the Wnt signaling pathway is deregulated, β -catenin accumulates in the cytoplasm and moves to the nucleus where it functions together with Tcf/Lef as a transcription factor. As a result of β -catenin accumulation, many other proto-oncogenes are activated, e.g. c-myc or cyclin D1 [4].

Personalized therapy can provide an opportunity for patients with various types of cancer to prolong and improve their quality of life. To effectively set up the therapy, it is necessary to distinguish patients who will benefit from treatment from patients for whom the therapy is not adequate. Appropriate predictive molecular markers can increase the overall efficiency and reduce the financial costs of therapy [5, 6].

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 POLE/CTNNB1 Cancer kit is developed for the determination of clinically relevant *POLE* and *CTNNB1* mutation status by NGS. First, short amplicons are obtained by a single PCR with hybrid primers with tags, in which sequences up to 258 bp in 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 POLE/CTNNB1 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 POLE/CTNNB1 Cancer kit components do not contain infectious material.
- Samples used for the fastGEN POLE/CTNNB1 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.

Example Version

8. REAGENT SUPPLIED

The **fastGEN POLE/CTNNB1 Cancer kit** is supplied in a ready to use format for the analysis of 16 samples (Table 1). Kit includes **specific Master Mixes** containing all the necessary reaction components and **sequencing primers** for *POLE* and *CTNNB1* gene.

fastGEN POLE/CTNNB1 Cancer kit components	Index sequences	Volume per 1 tube (µl)	Number of tubes	State
POLE/CTNNB1 Master Mix i730	AGACGCGC	18	1	ready to use
POLE/CTNNB1 Master Mix i731	CATGGACC	18	1	ready to use
POLE/CTNNB1 Master Mix i741	CGTTGGTT	18	1	ready to use
POLE/CTNNB1 Master Mix i743	GACCAGTT	18	1	ready to use
POLE/CTNNB1 Master Mix i744	AAGTTCTT	18	1	ready to use
POLE/CTNNB1 Master Mix i746	TCTCTATT	18	1	ready to use
POLE/CTNNB1 Master Mix i747	CTACTGGT	18	1	ready to use
POLE/CTNNB1 Master Mix i748	AATACGGT	18	1	ready to use
POLE/CTNNB1 Master Mix i751	CCGGAAGT	18	1	ready to use
POLE/CTNNB1 Master Mix i753	GCTTCTCT	18	1	ready to use
POLE/CTNNB1 Master Mix i754	AGCGATCT	18	1	ready to use
POLE/CTNNB1 Master Mix i757	GTACCTTG	18	1	ready to use
POLE/CTNNB1 Master Mix i761	ATGGTTGG	18	1	ready to use
POLE/CTNNB1 Master Mix i764	TTCTTGCG	18	1	ready to use
POLE/CTNNB1 Master Mix i767	GAGCTACG	18	1	ready to use
POLE/CTNNB1 Master Mix i768	GACTGCAG	18	1	ready to use
R2SP POLE/CTNNB1 Cancer		47	1	to be diluted
ISP POLE/CTNNB1 Cancer		47	1	to be diluted

Table 1: fastGEN POLE/CTNNB1 Cancer kit components.

9. RECOMMENDED MATERIAL (NOT SUPPLIED)

9.1 Chemicals

- Examined DNA
- Standardized sample containing the required variants of the examined *POLE* and *CTNNB1* gene (suitable as a **positive control**)
- Water for molecular biology (Nuclease Free Water, also suitable as a **negative control**)
- Illumina sequencing kit
- Qubit® dsDNA HS Assay Kit (Life Technologies)
- NaOH (p.a.)
- Tween 20
- 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
- Illumina®, Inc. 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 POLE/CTNNB1 Cancer kit: Master Mix

For *POLE* and *CTNNB1* gene genotyping let the appropriate number of POLE/CTNNB1 Cancer 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 POLE/CTNNB1 Cancer
- 1 tube: ISP POLE/CTNNB1 Cancer

Example Version

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 POLE/CTNNB1 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 POLE/CTNNB1 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 5 % can be detected.

The kit is designed to process 16 samples for *POLE* and *CTNNB1* genotyping in one sequencing run.

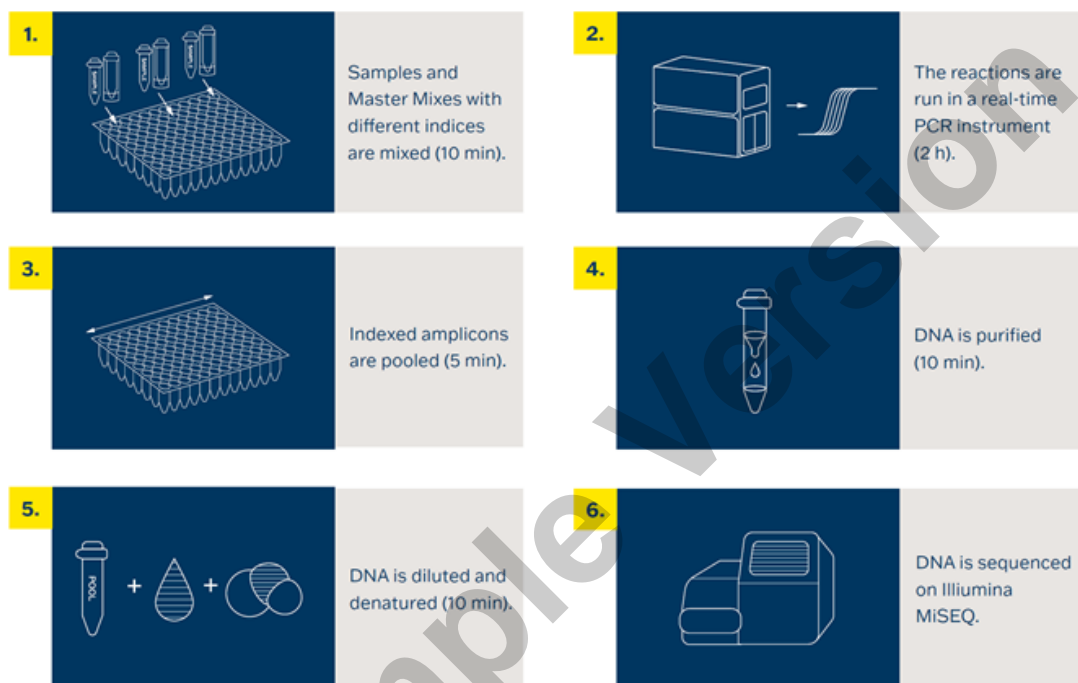


Figure 1: Workflow of genotyping using the fastGEN kit.

12.1 DNA library preparation

12.1.1 Preparation of examined DNA

Use the PCR box.

- Prepare samples.
- Vortex and centrifuge DNA samples shortly.
- Add **5 µl of DNA** of the appropriate concentration (see chapter 11) into the PCR plate or strip.
- Recommendation:
 - Include positive (PC) and negative (NC) control.
 - Add **5 µl of positive control DNA** with 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.

- 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 *POLE* and *CTNNB1* gene in one library:
 - Mix the individual amplicons of all samples into one DNA pool in the same ratio.
Example: For 16 samples, mix the individual amplicons in an amount of 3 μ l. You get a DNA pool in a volume of 48 μ l.
 - 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:

$$c[nM] = \frac{\rho_i \left[\frac{ng}{\mu l} \right] \times 10^6}{(660 \times 258)}$$

- ρ_i is the DNA mass concentration
- **258 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 POLE/CTNNB1 Cancer 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

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.

70,000 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 nM–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): 10.5 µl ISP POLE/CTNNB1 Cancer + 589.5 µl HT1
- Read2 sequencing primers (R2SP): 10.5 µl R2SP POLE/CTNNB1 Cancer + 589.5 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 nM–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): 8.6 µl ISP POLE/CTNNB1 Cancer + 811.4 µl HT1 or Illumina® sequencing primers (position 28)
- Read2 sequencing primers (R2SP): 6.4 µl R2SP POLE/CTNNB1 Cancer + 603.6 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 nM–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): 21 µl ISP POLE/CTNNB1 Cancer + 1 979 µl Illumina® sequencing primers (position 22)
- Read2 sequencing primers (R2SP): 16 µl R2SP POLE/CTNNB1 Cancer + 1 484 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): 21 µl ISP POLE/CTNNB1 Cancer + 1 979 µl Illumina® sequencing primers (position 22)
- Read2 sequencing primers (R2SP): 21 µl R2SP POLE/CTNNB1 Cancer + 1 979 µl Illumina® sequencing primers (position 21)

Pipette 1 300 µl of the diluted 1.5 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 nM–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 POLE/CTNNB1 Extra Sequencing Primers RDNSP0010A 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): 37 µl ISP POLE/CTNNB1 Cancer + 3 463 µl Illumina® sequencing primers (position 23)
- Index sequencing primers (ISP; S4): 52.5 µl ISP POLE/CTNNB1 Cancer + 4 947.5 µl Illumina® sequencing primers (position 23)
- Read2 sequencing primers (R2SP; SP, S1, S2): 21 µl R2SP POLE/CTNNB1 Cancer + 1 979 µl Illumina® sequencing primers (position 13)
- Read2 sequencing primers (R2SP; S4): 37 µl R2SP POLE/CTNNB1 Cancer + 3 463 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.

Example Version

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 *POLE* and *CTNNB1* genotyping

POLE and *CTNNB1* genotyping result is considered as positive (mutation detected), if a variant in the *POLE* and/or *CTNNB1* gene was detected with a frequency $\geq 5\%$.

If the *POLE* and/or *CTNNB1* variants are detected with a frequency from 1–5 %, 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 ($Ct_{PC} + 3 \leq Ct_{NC}$).
- After the sequencing data evaluation, frequencies of *POLE* and *CTNNB1* 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 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 POLE/CTNNB1 Cancer 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 POLE/CTNNB1 Cancer kit is designed for rapid preparation of the sequencing library required for the *POLE* and *CTNNB1* gene genotyping using the NGS technology. Variants in other genes than *POLE* and *CTNNB1* are not detectable by the fastGEN POLE/CTNNB1 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.

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 BioVendor fastGEN POLE/CTNNB1 Cancer kit 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 *POLE* and *CTNNB1* 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 70 000 pair-end reads per sample. The MiSeq Reagent kit v2 Nano, which has 2 million paired end reads, is 56 % 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?




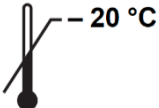



There can be several reasons for the non-standard results of PC and NC. We recommend the PC verification (targeted genes and their variants must contain mutations). Further, verify technical settings and check if a manual error has occurred. In case of ambiguity, contact customer support.

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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