BioVendor Group NGS

ENG

Instructions for Use:
fastGEN BCR::ABL1 Cancer Kit

Catalogue number:
RDNGS0011

For research use only

BioVendor R&D®

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

CONTENT

HISTORY OF CHANGES

Previous version	Current version
ENG.001.A	ENG.002.B
Entire document, tables and figures renumbered.	
Chapter 1, added Table 1.	
Chapter 3, added information on repeated freezing of sequencing primers and Master Mixes Major and Minor.	
Chapter 9.1., corrected recommended kit for RNA to cDNA transcription.	
Chapter 11.1.1, added recommended kit for RNA to cDNA transcription.	
Chapter 11.1.2, inserted new chapter – rest renumbered.	
Chapter 11.1.4, inserted figure 2 with comment.	
Chapter 12, updated figure 3.	
Chapter 13, completely revised.	
Chapter 14, added information on the accuracy of the detection limit.	
Chapter 15, added full company name.	
Chapter 16, question 5, added information on sequenced NC.	
Chapter 16, question 6, specific specification of tested kits for RNA to cDNA transcription.	

1. INTENDED USE

RDNGS0011 fastGEN BCR::ABL1 Cancer Kit is intended for rapid preparation of the sequencing library required for *BCR::ABL1* fusion gene genotyping by next-generation sequencing (NGS). Genotyping with the fastGEN BCR::ABL1 Cancer Kit allows analysis of mutations in the kinase domain of the *BCR::ABL1* fusion gene. Regions of interest are listed in Table 1.

The input material for the sequencing library preparation is cDNA containing the *BCR::ABL1* fusion gene.

Region designation	Region specification	Position of the region according to GRCh38/hg38		
		Chromosome	Start	Stop
ABL1 exon 4	Part of an exon	chr9	130 862 881	130 863 035
ABL1 exon 5	Entire exon	chr9	130 872 130	130 872 213
ABL1 exon 6-a	Part of an exon	chr9	130 872 861	130 872 931
ABL1 exon 6-b	Part of an exon	chr9	130 872 971	130 873 037
ABL1 exon 7-a	Part of an exon	chr9	130 874 869	130 874 987
ABL1 exon 7-b	Part of an exon	chr9	130 875 031	130 875 052
ABL1 exon 8	Entire exon	chr9	130 878 416	130 878 567
ABL1 exon 9	Entire exon	chr9	130 880 069	130 880 157
ABL1 exon 10	Part of an exon	chr9	130 880 501	130 880 571

Table 1: Regions targeted by the fastGEN BCR::ABL1 Cancer Kit

1.1 Abbreviations

ABL1	ABL Proto-Oncogene 1, Non-Receptor Tyrosine Kinase
ATP	adenosine triphosphate
BCR	BCR Activator of RhoGEF and GTPase
BCR::ABL1	fusion gene *BCR::ABL1*
cDNA	complementary deoxyribonucleic acid
Ct	cycle threshold
CML	chronic myeloid leukemia
DNA	deoxyribonucleic acid
ELFO	electrophoresis
FAM/SYBR	6-carboxyfluorescein/asymmetrical cyanine dye
IS	International Scale
KD	kinase domain
LoD	limit of detection
NC	negative control
NGS	Next Generation Sequencing
NTC	no template control
PC	positive control
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic Acid
RT	reverse transcription
TKI	tyrosine kinase inhibitors
Tm	melting temperature
VAF	Variant Allele Frequency

2. FEATURES

- **For research use only.**
- The input material for fastGEN analysis is **cDNA.**
- Total preparation time is less than 5 hours including less than 60 minutes of hands-on time.
- Technology is based on the preamplification of the *BCR::ABL1* fusion gene followed by **fast and robust single-step preparation** of sequencing libraries to genotype the kinase domain of this gene.
- Kit contains **complete Master Mixes** in a ready to use format: **Master Mix for *BCR::ABL1* fusion gene preamplification**, a **Master Mix for fastGEN** (including indexes and **sequencing primers**.
- Master Mix for preamplification of transcript variant **major** is supplied in **2 tubes** for 16 samples and Master Mix for preamplification of transcript variant **minor** is suplied in **1 tube** for 5 samples.
- Master Mix for fastGEN is supplied in **2 tubes** for each sample (Master Mix A and Master Mix B).
- The fastGEN BCR::ABL1 Cancer Kit is designed for genotyping in the kinase domain of the *BCR::ABL1* fusion gene in 16 samples with a unique combination of indexes in a single sequencing run.
- Library preparation using the fastGEN BCR::ABL1 Cancer kit requires only the **addition of cDNA to the qPCR preamplification reaction**, followed by the addition of its **product** to a specific **fastGEN Master Mix** and analysis using a Real-Time PCR cycler. The Master Mix for preamplification is supplied with the kit.

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 BCR::ABL1 Cancer Kit is delivered frozen at −20 °C.
- After delivery, store the fastGEN BCR::ABL1 Cancer Kit at −20 °C.
- **Protect kit components from light.**
- Avoid repeated freeze-thaw cycles of Master Mixes.
- Sequencing primers can be used up to 3 times. Master Mixes Major and Minor can be used up to 5 times.
- Do not use expired kits or components.

4. INTRODUCTION

Molecular monitoring of patients with chronic myeloid leukemia (CML) is a key part of the treatment protocol in the era of tyrosine kinase inhibitors (TKIs). CML is characterized by the presence of the *BCR::ABL1* fusion gene, which arises as a result of the t(9;22)(q34;q11.2) translocation. The *BCR::ABL1* fusion gene produces a pathological, constitutively activated tyrosine kinase, Bcr-Abl, which is responsible for uncontrolled hematopoietic cell proliferation and reduced response to proapoptotic signals.

The tyrosine kinase activity of Bcr-Abl can be inhibited by TKIs that target the ATP binding site in the kinase domain (KD) and stabilize the inactive conformation of Bcr-Abl, thereby reducing the percentage of pathological cells and increasing the overall survival of patients.

Despite the success of these drugs targeting the ATP binding site, patients suffer from poor initial response or loss of response due to the development of resistance to the drug administered. Resistance to tyrosine kinase inhibitors occurs in approximately 13 % of patients [1]. Over 90 different mutations have now been described, with the most significant ones being T315, Y253, E255, M351, G250, F359 and H396 [2].

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 BCR::ABL1 Cancer Kit is developed for the determination of mutation status in the kinase domain of fusion gene *BCR::ABL1* by NGS. First, short amplicons are obtained by a single PCR with hybrid primers with tags, in which sequences up to 315 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 BCR::ABL1 Cancer Kit, only the addition of cDNA to the qPCR preamplification reaction is required, followed by the addition of its product to a specific fastGEN Master Mix and analysis using a Real-Time PCR thermocycler.

Master Mix for preamplification is supplied with a kit.

Sequencing data are analysed online in the 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 BCR::ABL1 Cancer Kit components do not contain infectious material.
- Samples used for the fastGEN BCR::ABL1 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 BCR::ABL1 Cancer Kit** is supplied in a ready to use format for the analysis of 16 samples. Kit includes **preamplification Master Mixes** for **major transcript** variant (20 reactions) and **minor transcript** variant (5 reaction) of *BCR::ABL1* fusion gene, **specific fastGEN Master Mixes** and **sequencing primers** for the *BCR::ABL1* fusion gene. Master Mixes contain all the necessary reaction components.

fastGEN BCR::ABL1 Cancer Kit components	Index sequences	Volume per 1 tube (µl)	Number of tubes	State
Master Mix Major		460	**2**	**ready to use**
Master Mix Minor		230	**1**	**ready to use**
BCR::ABL1 Master Mix i730 (A-B)	AGACGCGC	18	**2**	**ready to use**
BCR::ABL1 Master Mix i731 (A-B)	CATGGACC	18	**2**	**ready to use**
BCR::ABL1 Master Mix i741 (A-B)	CGTTGGTT	18	**2**	**ready to use**
BCR::ABL1 Master Mix i743 (A-B)	GACCAGTT	18	**2**	**ready to use**
BCR::ABL1 Master Mix i744 (A-B)	AAGTTCTT	18	**2**	**ready to use**
BCR::ABL1 Master Mix i746 (A-B)	TCTCTATT	18	**2**	**ready to use**
BCR::ABL1 Master Mix i747 (A-B)	CTACTGGT	18	**2**	**ready to use**
BCR::ABL1 Master Mix i748 (A-B)	AATACGGT	18	**2**	**ready to use**
BCR::ABL1 Master Mix i751 (A-B)	CCGGAAGT	18	**2**	**ready to use**
BCR::ABL1 Master Mix i753 (A-B)	GCTTCTCT	18	**2**	**ready to use**
BCR::ABL1 Master Mix i754 (A-B)	AGCGATCT	18	**2**	**ready to use**
BCR::ABL1 Master Mix i757 (A-B)	GTACCTTG	18	**2**	**ready to use**
BCR::ABL1 Master Mix i761 (A-B)	ATGGTTGG	18	**2**	**ready to use**
BCR::ABL1 Master Mix i764 (A-B)	TTCTTGCG	18	**2**	**ready to use**
BCR::ABL1 Master Mix i767 (A-B)	GAGCTACG	18	**2**	**ready to use**
BCR::ABL1 Master Mix i768 (A-B)	GACTGCAG	18	**2**	**ready to use**
R2SP BCR::ABL1 Cancer		35	**1**	**to be diluted**
ISP BCR::ABL1 Cancer		35	**1**	**to be diluted**

Table 2: fastGEN BCR::ABL1 Cancer Kit components.

9. RECOMMENDED MATERIAL (NOT SUPPLIED)

9.1 Chemicals

- Examined cDNA
- Standardized sample containing the required variants of the examined *BCR::ABL1* fusion gene (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
- **Reverse transcription kit** (recommended kit: **SuperScript™ IV First-Strand Synthesis System**, Invitrogen™; Catalog number: 18091050, Thermo Fischer Scientific)
- 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 tube of preamplification Master Mix and the appropriate number of tubes with fastGEN 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 Preamplification Master Mix Major / Minor

To amplify the *BCR::ABL1* fusion gene cDNA, let the preamplification Master Mix tube thaw. Select Master Mix Major or Master Mix Minor depending on the analysis of transcript variant. Keep the component cool until use.

10.2 fastGEN BCR::ABL1 Cancer Kit Master Mix

For kinase domain of *BCR::ABL1* fusion gene genotyping let the appropriate number of BCR::ABL1 Master Mixes (A and also B) tubes thaw and keep them cool until use.

10.3 Sequencing primers

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

- 1 tube: R2SP BCR::ABL1 Cancer
- 1 tube: ISP BCR::ABL1 Cancer

11. PREPARATION OF SAMPLES

11.1.1 Reverse transcription of RNA

Firstly, every RNA sample has to be transcribed into cDNA. **The reverse transcription kit is not supplied with the kit.** We recommend **using the SuperScript™ IV First-Strand Synthesis System.**

Recommendation:

– For **samples with low IS value** (IS < 1 %) it is recommended to perform reverse transcription reaction with maximum possible input of RNA according to the RT kit manufacturer's instructions.

11.1.2 Quality control of input cDNA

After reverse transcription of RNA into cDNA, verify the sufficient length of the resulting fragments by electrophoresis or other suitable analytical method. **The reagents necessary for this verification are not provided with the kit.** The optimal length of the fragments after transcription is at least 5000 bp; fragments shorter than 1700 bp are not suitable for sample analysis with the fastGEN BCR::ABL1 Cancer Kit.

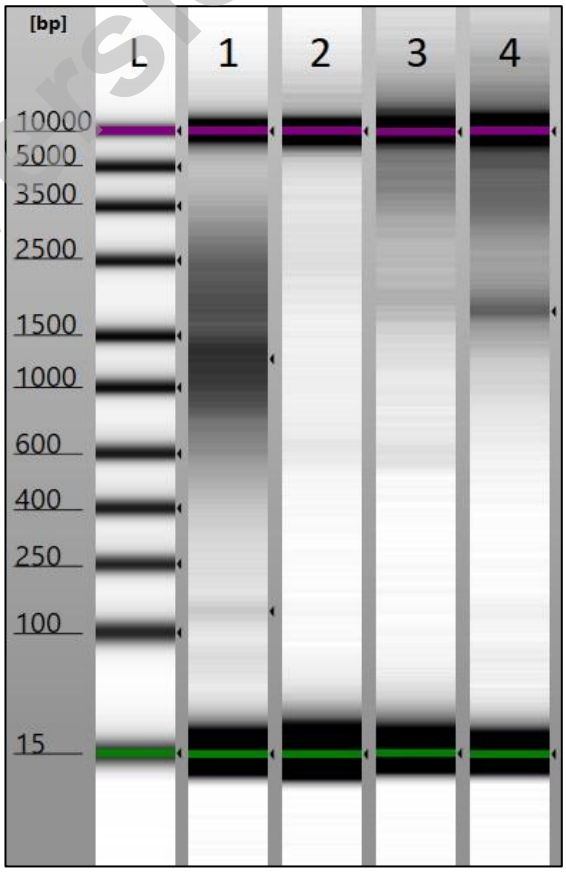


Figure 1: Display of the result of the cDNA fragment size verification. A size marker is placed at position L to determine the length of the nucleic acid fragments in the sample. The sample at position 1 is unsuitable because most fragments are approximately 1000–1500 bp in length. The sample at position 2 shows low signal intensity but contains cDNA fragments with predominantly longer lengths. This sample can be analysed, however, it is necessary to verify the size of the pre-amplification products by electrophoresis (see Chapter 11.1.3). Samples at positions 3 and 4 meet the required criteria. The sample at position 3 contains cDNA fragments exceeding 2500 bp in length. The sample at position 4 contains cDNA fragments exceeding 3500 bp in length, but also a significant number of fragments with a size slightly exceeding 1500 bp.

11.1.3 Preparation of preamplification

Work at the appropriate PCR box.

- The analysis of one sample includes preamplification qPCR and two separate fastGEN qPCRs.
- Mark the PCR plate or the PCR strip, vortex and centrifuge cDNA samples shortly.
- Select preamplification **Master Mix Major** or **Master Mix Minor** depending on the analysis of transcript variant.
- Briefly vortex and centrifuge the preamplification Master Mix when thawed.
- Add **45 µl of preamplification Master Mix** and **5µl of cDNA** into PCR plate or strip.
- The total volume per PCR reaction is **50 µl**.
- Close the tubes, vortex gently and spin down (15 s; 280x g).

Recommendation:

- Perform the preamplification reaction in strip or self-sealing tubes to prevent cross-contamination during pipetting of the fastGEN reaction.
- For **samples with low IS value** (IS < 1 %) we recommend perform the reaction in duplicate.
- 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 BCR::ABL1 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.

11.1.4 Preamplification reaction

– 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	30 s	98 °C	
Amplification	5 s	98 °C	40 cycles
	60 s	72 °C*	
Final elongation	2 min	72 °C	
Melting curve acquisition		72 °C → 95 °C	
Hold	∞	4 °C	

Table 3: qPCR preamplification program

– Set sample names into qPCR software.
– Start the run.
– Export the qPCR data and perform a preamplification check. Save the Ct and Tm values for possible later control.
– Store the PCR products at 4 °C for further use. For long-term storage, store at −20 °C.

Recommendation:

After cDNA preamplification, verify again the sufficient length of the resulting fragments using electrophoresis or another suitable analytical method. The adequate length of the amplicons is approximately 1650 bp for the e13a2 break (major) and the e1a2 break (minor). In the case of the e14a2 break, the size of the amplicons is larger (approximately 1725 bp).

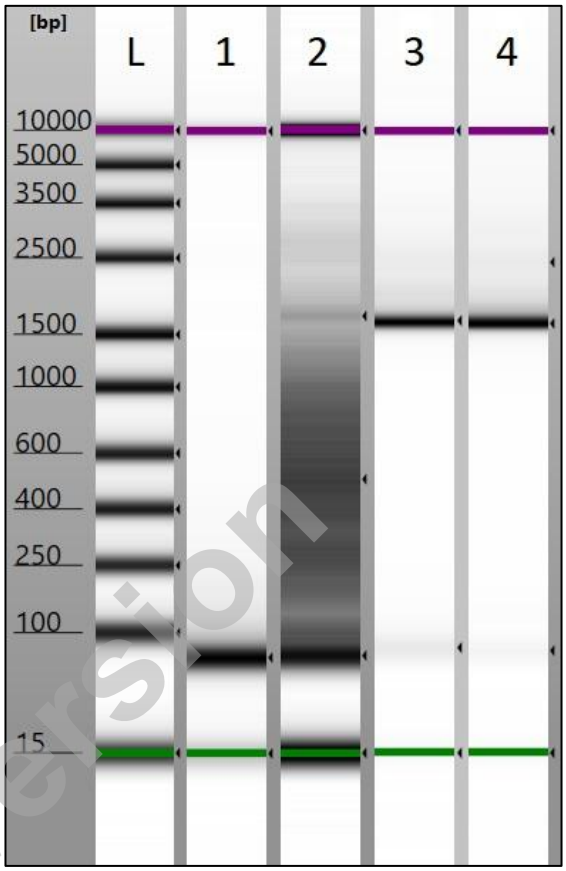


Figure 2: Display of the result of verification of the size of the amplicons after preamplification. A size marker is placed at position L to determine the length of the nucleic acid fragments in the sample. The sample at position 1 is the amplified cDNA from position 1 in Figure 2. The absence of amplicons of a specific length of 1650 bp or longer can be observed. It is not possible to proceed to the fastGEN reaction with such a sample. For samples at positions 2 to 4, specific amplicons of a length of approximately 1650 bp can be observed. With these samples, the fastGEN reaction can be continued.

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 genotyping of the KD of the *BCR::ABL1* fusion gene in one sequencing run. **The analysis of one sample includes preamplification qPCR and two separate fastGEN qPCRs.**

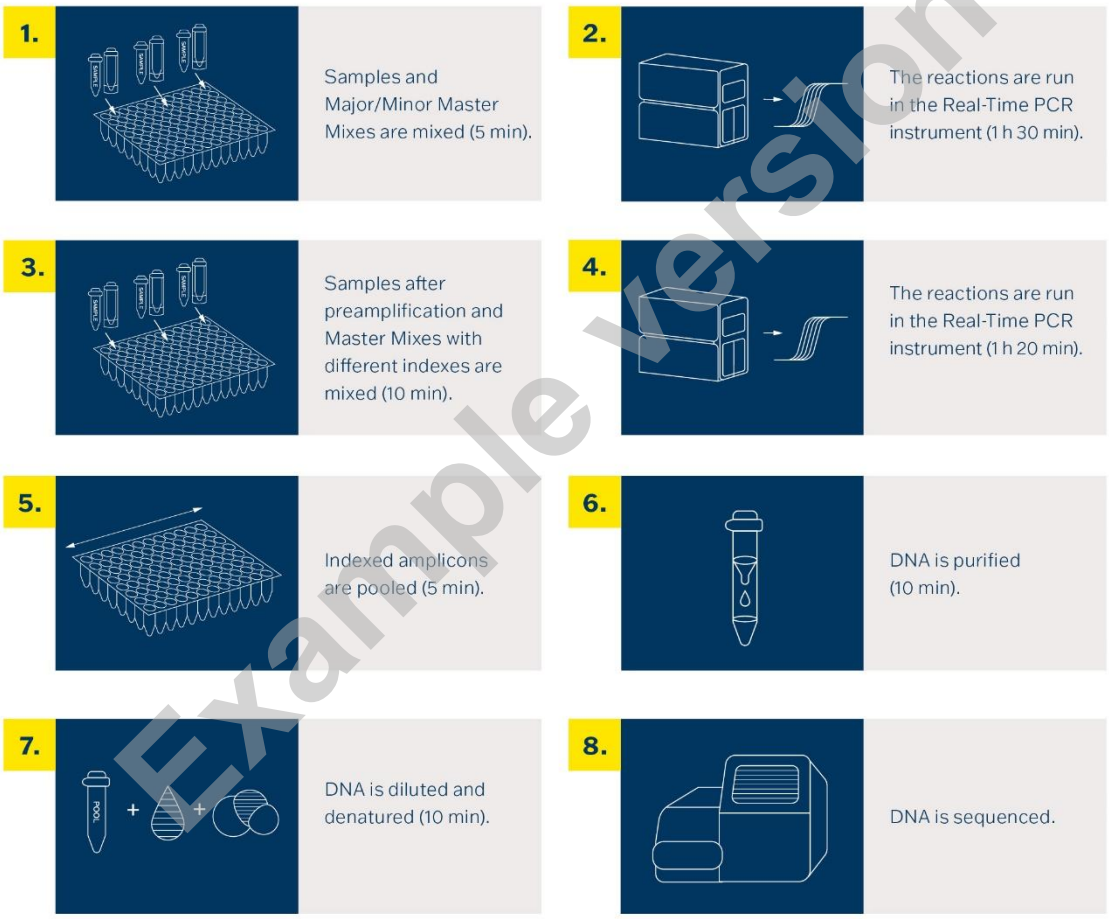


Figure 3: Workflow of genotyping using the fastGEN kit

12.1 DNA library preparation

12.1.1 Preparation of examined cDNA

Use the PCR box.

- Prepare samples.
- Vortex and centrifuge aplified cDNA samples shortly.
- Pipette **5 µl of a preamplification product** into the PCR plate or strip for Master Mixes A-B of one index (see Chapter 11).

Recommendation:

- Include the positive (PC) and negative (NC) preamplification products in the test sample pool.
- Pipette **5 µl of the positive control (PC) preamplification PCR product** for the A-B Master Mixes of the given index (see Chapter 11).
- Pipette **5 µl of the negative control (NC) preamplification PCR product** for the A-B Master Mixes of the given index (see Chapter 11).
- Pipette **5 µl of water for molecular biology** as a negative control **(NTC)** for the A-B Master Mixes of the given index.

12.1.2 Preparation of Master Mixes

Use the PCR box in the post-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 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	20 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

- Set sample names into qPCR software.
- Start the run.
- Export the qPCR data and perform an amplification check. Save the Ct and Tm values for possible later control.
- Check the correct setting of the baseline threshold for Ct determination. If a highly concentrated sample enters the fastGEN qPCR, its signal could be above or below the automatically set threshold from the beginning of the reaction, and will be flagged as negative by the evaluation software. Therefore, it is necessary to check the amplification curve as well as the Tm values obtained from the melting curve, see Figure 4.
- Store the PCR products at 4 °C for further use. For long-term storage, store at −20 °C.

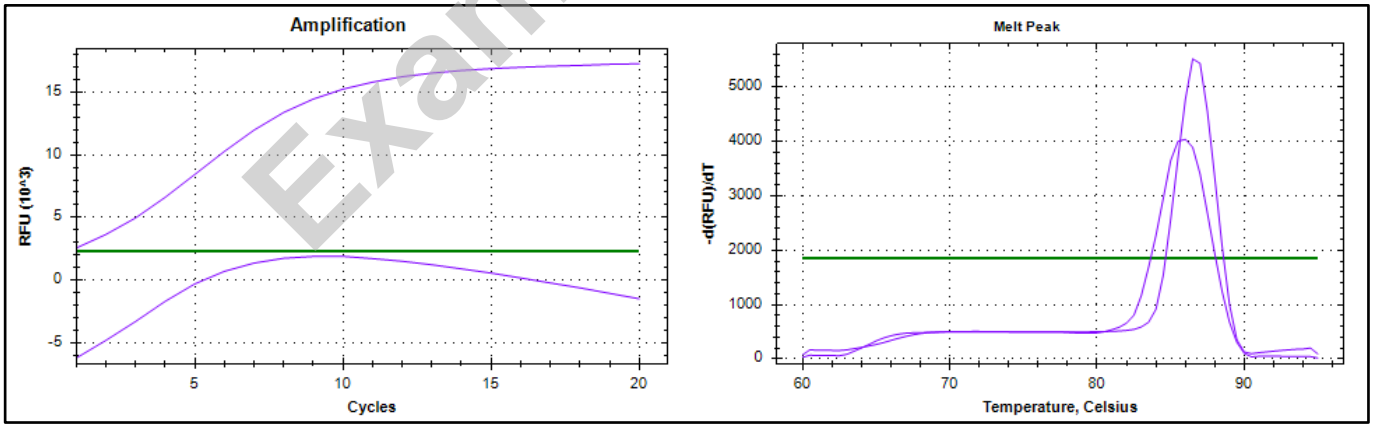


Figure 4: Representation of highly concentrated samples (shown in purple) in fastGEN reaction above and below the automatic threshold (shown in green). Both Ct values were evaluated as "N/A", but the specific "peak" Tm (86 ± 1.5 °C) can be clearly distinguished on the melting temperature plot.

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 KD of *BCR::ABL1* fusion 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 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.
  - Recommendation 1: If the Ct of the sample is “N/A”, check the run evaluation settings to verify that the reaction is not oversaturated (the signal of sample would be above the threshold value from the beginning of the reaction). However, if such sample does not show a specific Tm value according to melting curve analysis (approx. 86 +/-1,5 °C) see Figure 5, discard the sample from the sequencing.

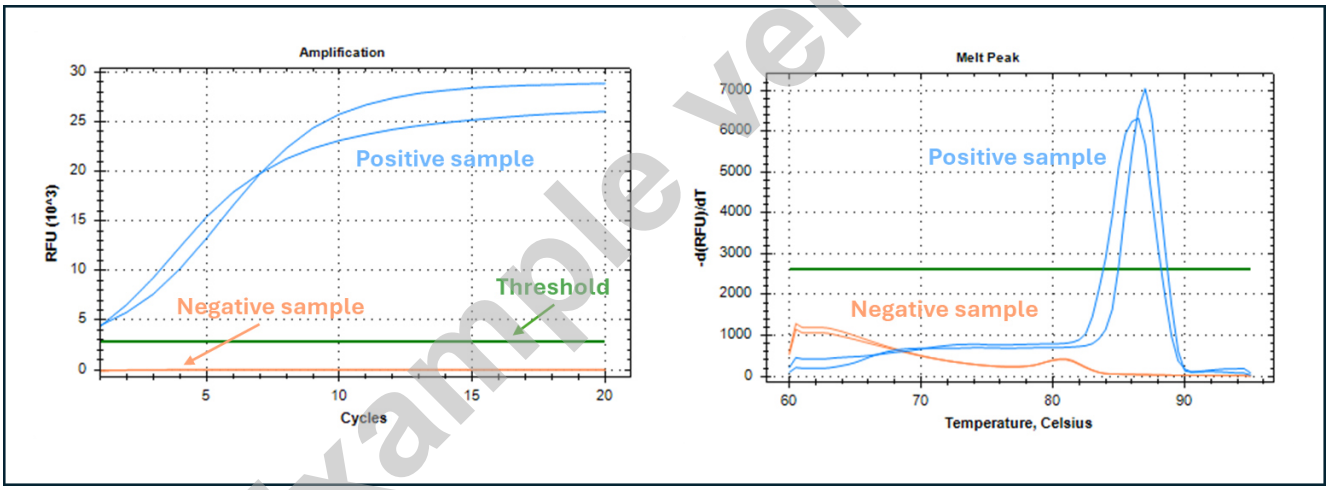


Figure 5: Schematic of amplification and Melt Peak; negative samples are highlighted in orange, positive samples with Ct = "N/A" in blue.

  - Recommendation 2: If the Ct of the sample is > 10, double the volume of the sample added into the final DNA pool.
  - 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:

$$c[nM] = \frac{\rho i \left[\frac{ng}{\mu l}\right] \times 10^6}{(660 \times 315)}$$

  - ρi is the DNA mass concentration
  - **315 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 BCR::ABL1 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, 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): 7.5 µl ISP BCR::ABL1 Cancer + 592.5 µl HT1
- Read2 sequencing primers (R2SP): 7.5 R2SP BCR::ABL1 Cancer + 592.5 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): 6.2 µl ISP BCR::ABL1 Cancer + 813.8 µl HT1 or Illumina® sequencing primers (position 28)
- Read2 sequencing primers (R2SP): 4.6 µl R2SP BCR::ABL1 Cancer + 605.4 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): 15 µl ISP BCR::ABL1 Cancer + 1 985 µl Illumina® sequencing primers (position 22)

– Read2 sequencing primers (R2SP): 11.3 µl R2SP BCR::ABL1 Cancer + 1 488.7 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): 15 µl ISP BCR::ABL1 Cancer + 1 985 µl Illumina® sequencing primers (position 22)
– Read2 sequencing primers (R2SP): 15 µl R2SP BCR::ABL1 Cancer + 1 985 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 0.1–1 % 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 BRC::ABL1 Extra Sequencing Primers RDNSP0011A 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): 26.3 µl ISP BCR::ABL1 Cancer + 3 473.7 µl Illumina® sequencing primers (position 23)
– Index sequencing primers (ISP; S4): 37.5 µl ISP BCR::ABL1 Cancer + 4 962.5 µl Illumina® sequencing primers (position 23)
– Read2 sequencing primers (R2SP; SP, S1, S2): 15 µl R2SP BCR::ABL1 Cancer + 1 985 µl Illumina® sequencing primers (position 13)
– Read2 sequencing primers (R2SP; S4): 26.3 µl R2SP BCR::ABL1 Cancer + 3 473.7 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 Genotyping of the kinase domain of *BCR::ABL1* fusion gene

KD of *BCR::ABL1* genotyping result is considered as positive (mutation detected) if a variant in the KD of *BCR::ABL1* fusion gene was detected with a frequency ≥ 5 % and at the same time the measured frequency is higher than the calculated cut-off value for the given reaction.

The cut-off value of the mutation frequency must be calculated for each sample or reaction separately according to the formula:

$$\boldsymbol{LoD_{VAF}}[\%] = \frac{\mathbf{50\,000}}{(\boldsymbol{N_{ABL1}} \times \boldsymbol{IS}[\%])}$$

- **N_{ABL1}** is the total number of molecules containing the ABL1 domain (i.e. molecules containing *BCR::ABL1* and molecules containing "non-fusion" *ABL1*) in 5 µl of sample entering the preamplification reaction.
- **IS** is the IS value measured for the given sample (if it is, for example, 0.14%, enter the value 0.14 in the formula).

Using the formula – example no. 1: In 5 µl of cDNA sample, 25,000 molecules containing the ABL1 domain were measured, and the IS value was measured and calculated as 0.12%. After substituting into the formula, we get a value of 16.67%. This means the detected mutations in this

sample are considered positive in the case of VAF ≥ 16.67%. For any mutation with a lower VAF, it cannot be determined with certainty that it is error-free.

Using the formula – example no. 2: In 5 µl of cDNA sample, 14,000 molecules containing the ABL1 domain were measured, and the IS value was measured and calculated as 0.017%. After substituting into the formula, we get a value of 210.08%. This value exceeds 100% and therefore no mutation with any VAF value can be determined with certainty. This is because not enough molecules containing the *BCR::ABL1* gene enter the reaction.

You can see the application of the formula to the selected parameters in the example Table 5.

VAF cut-off value for mutation detection	**IS value for the analysed sample**							
Minimal amount of ABL1	**1,00 %**	**0,60 %**	**0,20 %**	**0,10 %**	**0,060 %**	**0,020 %**	**0,010 %**	**0,006 %**
6 250	8,00 %	13,33 %	40,00 %	80,00 %	*N/A*	*N/A*	*N/A*	*N/A*
12 500	4,00 %	6,67 %	20,00 %	40,00 %	66,67 %	*N/A*	*N/A*	*N/A*
25 000	> 2,00 %	3,33 %	10,00 %	20,00 %	33,33 %	100,00 %	*N/A*	*N/A*
50 000	> 2,00 %	> 2,00 %	5,00 %	10,00 %	16,67 %	50,00 %	100,00 %	*N/A*
100 000	> 2,00 %	> 2,00 %	2,50 %	5,00 %	8,33 %	25,00 %	50,00 %	83,33 %
200 000	> 2,00 %	> 2,00 %	> 2,00 %	2,50 %	4,17 %	12,50 %	25,00 %	41,67 %
400 000	> 2,00 %	> 2,00 %	> 2,00 %	> 2,00 %	2,08 %	6,25 %	12,50 %	20,83 %

Table 5: Overview of VAF limit values for correct mutation detection for given sample input parameters (application of the formula from page 25). N/A = "not applicable"

In the case of the accurately measured concentration of *BCR::ABL1* fusion gene molecules in the cDNA sample, an alternative formula can also be used:

$$\boldsymbol{LoD_{VAF}}[\%] = \frac{\mathbf{500}}{\boldsymbol{N_{BCR::ABL1}}}$$

– $\mathbf{N_{BCR::ABL1}}$ is the total number of *BCR::ABL1* molecules in 5 µl of sample entering the preamplification reaction.

If variants in the KD of the *BCR::ABL1* fusion gene are detected with a frequency from 1–5 %, the result is valid only if the of both replicates processed with different Master Mixes match (i.e. by measuring the sample in duplicate, on two indexes). Or we recommend retesting or verifying results with another method.

Genotyping of **samples with extremely low cDNA concentration or low IS or VAF values** 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:

- After **the preamplification step**, it is verified (e.g. using ELFO) that the sample contains amplicons of approximately 1650 bp or more in the case of breaks other than b2a2 (major) and e1a2 (minor).
- In the **fastGEN qPCR step**, the Ct of PC is at least 3 Ct lower than NC (Ct_{PC} +3 ≤ Ct_{NC}).
  Note: If the sample does not show amplification (i.e. it shows a Ct value = “N/A”), but at the same time the melting temperature of the PCR products corresponds to a specific value (see Chapter 12.2.1, Figure 5), it may be an oversaturated reaction. Check the run evaluation settings and possibly try a different threshold calculation setting for determining Ct. However, if such a sample does not show a specific Tm value (86 ± 1.5 °C) according to the melting curve analysis, see Figure 5, the reaction was unsuccessful.
- After the sequencing data evaluation, frequencies of *BCR::ABL1* fusion gene variants are as expected.

13.3.2 Negative control must meet the following criteria:

- In the **fastGEN qPCR 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.
- After evaluation of the sequencing data, it shows the presence of the *BCR::ABL1* fusion gene variants or no specific products suitable for analysis are found.

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 BCR::ABL1 Cancer Kit was validated on RNA samples isolated from peripheral blood or bone marrow. Reverse transcription kit is not included.
- To determine the exact VAF detection limit for the measured sample, it is necessary to know the IS value and the concentration of ABL1 domains or the *BCR::ABL1* fusion gene in the given sample.
- The result of genotyping is affected by the quality of the sample. Proper collection, transport, RNA isolation, reverse transcription to cDNA and sample storage are crucial for test performance.
- Genotyping results should be interpreted by a healthcare professional.
- The fastGEN BCR::ABL1 Cancer Kit is designed for rapid preparation of the sequencing library required for the KD of the *BCR::ABL1* fusion gene genotyping using the NGS technology. Variants in other genes than *BCR::ABL1* are not detectable by the fastGEN BCR::ABL1 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 6). While the fastGEN BCR::ABL1 Cancer Kit assay can be used for the detection of additional mutations within the targeted regions of the KD of the *BCR::ABL1* fusion gene (as outlined in Table 6), their identification is not formally validated by the manufacturer.
- End-users are responsible for the validation of fastGEN BCR::ABL1 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 BCR::ABL1 Cancer 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 method is highly sensitive and enables the detection of mutations at a frequency of 5 % in samples with a very low number of copies of the fusion gene (≥ 0.1 % IS). 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 and synthetic samples with a known mutation status. The genotyping results of the KD of the *BCR::ABL1* were correct in all samples, including repeated measurements (sensitivity and specificity 100 %).

As part of the analytical characterization of the fastGEN BCR::ABL1 Cancer Kit, mutations in the KD of the *BCR::ABL1* were validated. Summary of validated mutations is provided in Table 6.

Gene	Mutation
ABL1 NM_005157.6	c.730A>G p.(Met244Val)
	c.749G>A p.(Gly250Glu)
	c.757T>C p.(Tyr253His)
	c.763G>A p.(Glu255Gly)
	c.764A>T p.(Glu255Val)
	c.895G>T p.(Val299Leu)
	c.933C>A p.(Phe311Leu)
	c.944C>T p.(Thr315Ile)
	c.1075T>G p.(Phe359Val)
	c.1187A>C p.(His396Pro)
	c.1376A>G p.(Glu459Gly)
	c.1439C>T p.(Pro480Leu)
	c.1520A>G p.(Glu507Gly)
	c.1526A>G p.(Glu509Gly)

Table 6: List of validated mutations in the KD of the BCR::ABL1 fusion gene

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 (and is 40 % full). 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. We recommend the PC verification (must contain the *BCR::ABL1* fusion gene with 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. **Which kit should be used for reverse transcription?**

   In the development of the fastGEN BCR::ABL1 Cancer Kit, the best results were achieved using the reverse transcription kit: SuperScript™ IV First-Strand Synthesis System, Invitrogen™; Catalog No.: 18091050, Thermo Fischer Scientific. However, the fastGEN BCR::ABL1 Cancer Kit has also been successfully tested using the High-Capacity cDNA Reverse Transcription Kit, Applied Biosystems™, Catalog No.: 4374966, Thermo Fischer Scientific.

7. **What is the range of the analysis area?**

   The fastGEN BCR::ABL1 Cancer Kit allows analysis of mutations in KD of the *BCR::ABL1* fusion gene in major and minor transcript variants. Targeted regions are listed in Table 1.

8. **What to do if samples have an invalid Ct value in the fastGEN reaction, even though they show a fluorescent signal?**

   Samples or positive controls may show “N/A” Ct values in the fastGEN reaction. However, if the fluorescence signal of such a sample reaches higher RFU values than the background of the qPCR run and the melting temperature curve shows specific Tm values (86 ± 1,5°C), this is an “oversaturation” of the reaction with the input sample. The signal of such a sample is already from the beginning of the reaction above the threshold of the qPCR run and can be flagged as negative by the evaluation software. Using regression instead of threshold can help to evaluate such samples, but it is still necessary to check the melting curve of these samples as well.

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

   It is possible to purchase the related product fastGEN BCR::ABL1 Extra Sequencing Primers RDNSP0011A.

10. **Is it possible to order preamplification Master Mixes separately?**

    Yes, it is possible to purchase the related product fastGEN BCR::ABL1 Extra Preamplification Master Mix for major and minor break RDNSP0011B.

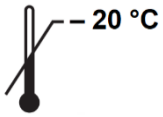
17. REFERENCES

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

[1] Zhou, T., Medeiros, L.J. & Hu, S. Chronic Myeloid Leukemia: Beyond BCR-ABL1. Curr Hematol Malig Rep 13, 435–445 (2018). https://doi.org/10.1007/s11899-018-0474-6.

[2] Braun, T. P., Eide Ch. A., Druker, B. J., Response and Resistance to BCR-ABL1-Targeted Therapies. Cancer Cell 37, 530-542 (2020). https://doi.org/10.1016/j.ccell.2020.03.006.

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®

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