INSTRUCTION

for use the set of reagents **BCR-ABL1 Mbcr RQ Kit (b2a2, b3a2 transcripts, p210) 48 tests** for detection and quantification of the mRNA of the chimeric gene *BCR-ABL1* (*Mbcr*) and the mRNA of the *ABL1* gene in clinical specimens of patients with chronic myeloid leukemia (CML) and acute lymphoblastic leukemia (ALL) utilizing polymerase chain reaction (PCR) and hybridization with fluorescent detection in "real time".

Compatible with thermocyclers: «iQ5» (Bio-Rad); «CFX96» (Bio-Rad); «ABIPrism» (ThermoFisher Scientific); «Lightcycler» (Roche): «Rotor-Gene» 3000/6000 (Corbett Research/Qiagen).

PURPOSE OF THE KIT

Chronic myeloid leukemia (CML) refers to a group of chronic myeloid diseases. More than 95% of cases are caused by chromosomal rearrangement between chromosomes 9 and 22 –t(9;22) (Philadelphia chromosome), characterized by a fusion between genes *BCR* and *ABL1*. The gene is transcribed by mRNA and has two different splice variants – b2a2 (40% of cases) and b3a2 (55% of cases). These variants encode a chimeric protein with a molecular mass of 210kDA (p210 transcripts, *Mbcr*), which has tyrosine kinase activity.

Likewise, e1a2 variant (p190 transcript, *mbcr*) can be expressed in 35% of patients with acute lymphoblastic leukemia.

The morbidity rate of CML is 1-2 people per 100,000 and approximately 20% of all affected are adults. The disease manifests itself in an increased number of mature and functionally active myeloid cells. However, ignoring the somewhat optimistic probability that the duration of the disease with treatment is 4-6 years, it is noted that in patients the disease progresses into the so-called accelerated phase, termed the "blast crisis". The advent of modern tyrosine inhibitor therapy significantly improved the prognosis of CML patients. The goal of this therapy is to attain 100% survivability of patients while also attaining no tumors with the protein marker BCR-ABL1.

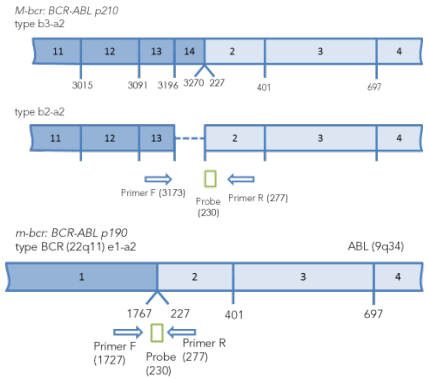

Figure 1. *The formation of chimeric transcripts BCR-ABL1 b3a2, b2a3 and e1a2. The places of binding of primers and probes for "real time" PCR are indicated on picture. The positions of the primers and probes are indicated relative to the 5 'end of the nucleotide sequences of the normal transcripts.*

Application of PCR in real time (Real-Time quantitative PCR, RQ-PCR) for assess the expression level of chimeric transcripts *BCR-ABL1* allows for the achievement of significantly higher sensitivity in regard to the detection of minimal residual disease, revealing one cancerous cell among 50,000 healthy ones.

More information related to diagnostic approaches, periodicity, research, and evaluating the prognosis of the disease is available on the website of the international organization European Leukemia Net (http://www.leukemia-net.org)

The reagent kit is designed to carry out the research in a quantitative format – 48 patient samples carried out in duplicate (144 PCR reactions, including controls).

Research Use Only Reagent

SET CONTENTS

The reagent set «**BCR-ABL1 Mbcr RQ Kit, 48 tests**» includes:

Reagent			Description	Volume (µL)	Count
DNA-calibrators	ABL1/BCR-ABL1 Mbcr	C1 ABL1 / BCR-ABL1 Mbcr	Clear, colorless liquid	50	1 test-tube
		C2 ABL1 / BCR-ABL1 Mbcr	Clear, colorless liquid	50	1 test-tube
		C3 ABL1 / BCR-ABL1 Mbcr	Clear, colorless liquid	50	1 test-tube
		C4 ABL1 / BCR-ABL1 Mbcr	Clear, colorless liquid	50	1 test-tube
		C5 ABL1 / BCR-ABL1 Mbcr	Clear, colorless liquid	50	1 test-tube
Synthetic oligonucleotides	BCR-ABL1 Mbcr	PrimerMix BCR-ABL1 Mbcr	Clear, colored liquid	150	1 test-tube
	ABL1	PrimerMix ABL1	Clear, colored liquid	150	1 test-tube
PCR Master-mix		PCR Mix	Clear, colorless liquid	750	2 test-tube
$MgCl_2$		MgCl2	Clear, colorless liquid	75	1 test-tube
MQ water		Water	Clear, colorless liquid	1125	1 test-tube

DNA calibrators (C1, C2, C3, C4, C5) – are quantitatively characterized by their plasmid mounted cDNA insert portion of *BCR-ABL1 Mbcr* fusion gene or a portion of the *ABL1* or *GUSB* gene normalizer. PCR was used to construct a calibration curves for BCR-ABL1 Mbcr and ABL1 as well as the positive PCR controls.

The results of the amplification of *BCR-ABL1 Mbcr* cDNA were detected on Green/FAM channel fluorescence, *ABL1* amplification results were detected on channel Yellow/JOE/HEX/R6G.

RESEARCH IMPLEMENTATION

When receiving the kit, distribute the reagents for storage in accordance with the temperature regime:

- The reaction mixture (PCRMix), oligonucleotides (PrimerMix), MgCl2 should be stored at a temperature of -20 ° C
- DNA calibrators (C1 ... C5) should be stored at + 4 ° C (shelf life- 4 months).

1. Before starting work, defrost PCRMix, PrimerMix and MgCl2 at room temperature (for 15-30 minutes). Before using the mixtures, make sure that the ice crystals have completely melted;
2. Thoroughly mix all components of the reaction, precipitate the drops with a desktop centrifuge - 10 seconds at 1000 g (quick spin);
3. Mix the PCR reactions in PCR micro tubes. Consumption of reagents per reaction:

Table 1. Consumption of reagents per reaction

Reagent	PCR-reaction, volume, (µL)		
	For 1 reaction	For 26 reactions (10 patient samples)	For 26 rections (10 patient samples taking into account the possible error of the pipettes)
PrimerMix BCR-ABL1 Mbcr	1	26	28
PrimerMix ABL1	1	26	28
PCR Mix	10	260	280
MgCl2	0,5	13	14
Water	7,5 (up to 20 µL)	195	210

NB! For each patient, it is recommended that the study be performed in duplicate. In each series of studies, it is recommended to place an additional control tube without adding a DNA matrix (NTC control).

4. Using a pipette tip with an aerosol barrier, add 5 µL of the cDNA sample to the tube with the reaction mixture;
5. It is necessary to place 5 control samples – calibrators. To accomplish this, add 5 DNA calibrators (5 µL each, C1, C2, C3, C4, C5) to 5 different test tubes with PCR reaction;
6. Place the tubes in a thermal cycler;
7. Program the thermal cycler to perform amplification and the detection of fluorescent signals (**the total reaction volume is 25 µl**):

Table 2. Amplification program for «iQ iCycler» («Bio-Rad», USA), «CFX96» (Bio-Rad), «Quantstudio» (Applied Biosystem, USA)

Stage	Temperature, °C	Time	Fluorescence detection	Repeats
Hold	95	10 min	-	1
Cycling	95	15 s	-	50
	60	60 s	FAM/JOE/HEX	

Table 3. *Amplification program for «Rotor-Gene» 3000/6000, 72-well rotor («Corbett Research», Australia)*

Stage	Temperature, °C	Time	Fluorescence detection	Repeats
Hold	95	10 min	-	1
Cycling	95	10 s	-	47
	60	50 s	Green/Yellow	

ANALYSIS AND RESULTS

The received data relating to the fluorescent signal curves are analyzed using the "real-time" PCR software tool that is found in the device instructions.

The one tubes register the accumulation of amplification products of the cDNA of *BCR-ABL1 Mbcr* and the cDNA of the normalized *ABL1* gene.

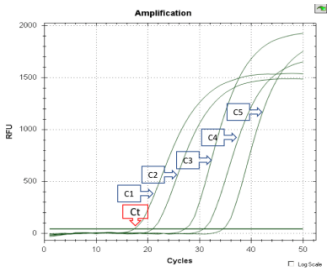

Figure 3. Accumulation of the PCR product

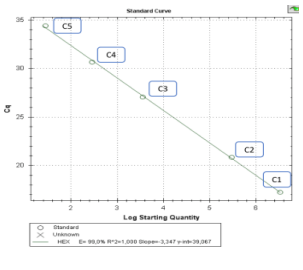

Figure 4. Calibration curve of DNA calibrators

Based on the "CT" threshold cycle values (the intersection of the fluorescence curve with the fixed level threshold line) and based on the set of calibrators values (check the attached supplementary sheet) occurs automatically build a calibration curve and calculation of values for cDNA copies of the *BCR-ABL1 Mbcr* gene and the *ABL1* gene in PCR sample (see the appropriate real-time thermal cycler guide).

The obtained values are used to calculate the normalized concentration of *BCR-ABL1 Mbcr* copies in the test and control samples, the steps for which are laid out here:

1. Calculate the ratio for all samples:

The number of copies of cDNA *BCR-ABL1 Mbcr /ABL1* cDNA copies

2 Calculate the mean value of the *BCR-ABL1 Mbcr /ABL1* ratio of sample concentrations for the two repeats. Multiply the result by 100.

QUALITY CONTROL CRITERIA

Criteria	Valid values/results
Deviation count between repeats	≤ 2 (with an average Ct >36) ≤ 1,5 (with an average Ct ≤36)
Slope The Standard Curve (Slope)	between -3,0 **и** -3,9 (A PCR effeciency of 100% Slope= -3,32)
Determination coefficient (R2) for the standard curve	> 0.98
Minimum standard dilution of C5 or **C**4 (For the *ABL1* gene)	It must be detected and included in the standard curve
Quality control of the patient samples given by the number of copies of the *ABL1* gene for the reaction (*ABL1* copy number)	*ABL1* copy number>10,000 for optimal sensitivity
Negative controls without the added matrix (NTC) for *BCR-ABL1 Mbcr* **и** *ABL1*	Not to be detected

IMPORTANT! Results are not valid if:

1. The concentration value of *ABL1* (normalizer gene) is less than 10,000 copies per reaction: the sample is not valid; a reinvestigation of the sample is required from the first phase of the analysis. In the case of the same results you must rerun the test and to repeat sampling of the biological material.
2. There is a fourfold difference of copies concentrations relation (*BCR-ABL1 Mbcr /ABL1*) in the duplicate for one sample (first repeat *BCR-ABL1 Mbcr /ABL1*) / (second repeat *BCR-ABL1 Mbcr /ABL1*) > 4 or < 0,25*
   *With the exception of samples for which the number of measured copies of *BCR-ABL1* is less than 25
3. The determination coefficient R^2 in the construction of the calibration curve is less than 0.95: reinvestigate all samples from the first stage of analysis
4. The control indicates the presence of contamination in the samples or reagents. In this case, the analysis results of all samples are considered invalid. This requires a repeat of the analysis of samples, and the taking measures to identify and eliminate the source of the contamination.

REFERENCE INFORMATION

More information can be found in the following studies:

1. GabertJ,Beillard E et al. Standardization and quality control studies of real-time quantitative reverse transcriptase polymerase chain reaction of fusion gene transcripts for residual disease detection in leukemia – a Europe Against Cancer program. Leukemia. 2003 Dec;17(12):2318-57.
2. Baccarani, M. et al. (2009) Chronic myeloid leukemia: an update of concepts and management recommendations of European LeukemiaNet. J. Clin. Oncol. 27, 6041.
3. Branford, S. et al. (2006) Rationale for the recommendations for harmonizing current methodology for detecting BCR-ABL transcripts in patients with chronic myeloid leukaemia. Leukemia 20, 1925.
4. Hughes, T. et al. (2006) Monitoring CML patients responding to treatment with tyrosine kinase inhibitors: review and recommendations for harmonizing current methodology for detecting BCR-ABL transcripts and kinase domain mutations and for expressing results. Blood 108, 28.
5. van der Velden, V.H., Hochhaus, A., Cazzaniga, G., Szczepanski, T., Gabert, J., and van Dongen, J.J. (2003) Detection of minimal residual disease in hematologic malignancies by real-time quantitative PCR: principles, approaches, and laboratory aspects. Leukemia 17, 1013.
6. Gabert, J. et al. (2003) Standardization and quality control studies of 'real-time' quantitative reverse transcriptase polymerase chain reaction of fusion gene transcripts for residual disease detection in leukemia - a Europe Against Cancer program. Leukemia 17, 2318.
7. Beillard, E. et al. (2003) Evaluation of candidate control genes for diagnosis and residual disease detection in leukemic patients using 'real- time' quantitative reverse-transcriptase polymerase chain reaction (RQ- PCR) - a Europe against cancer program. Leukemia 17, 2474.

Technology:

The method used for the detection of expression level of *BCR-ABL1 Mbcr* gene from clinical material is based on the amplification and detection in "real time" with two types of oligonucleotides: the amplification plot of the mRNA chimeric gene *BCR-ABL1 Mbcr* (p210), the respective portion of cross-linking the genes *BCR* and *ABL1* (b2a2 and b3a2) and the mRNA splicing region gene fragment *ABL1* (working group recommended "Europe Against Cancer", EAC), are used as an endogenous internal control gene and normalizer. The detection of amplification products was performed in "real time" using TaqMan technology.

Using endogenous internal controls allows for the control of the main steps of the analysis (sampling, transport, storage, isolation of RNA, and the reverse transcription reaction amplification of RNA and cDNA directly) and also allows for the accurate counting of mRNA of the fusion gene *BCR-ABL1*.

Collection and storage of samples

1. Sample with EDTA. The material is to be placed in a test tube with 6% EDTA solution. The closed tube is inverted several times.

For cell selection:

а) The test tube with blood or bone marrow is to be centrifuged for 20 minutes at 800-1600 RPM at room temperature within 48 hours of sample collection (whole blood stored at 2 to 6°C). All of the white blood cells (white film on the surface of red cell supernatant) are to be selected carefully (total volume of 200 μL of the sample, the capture of red blood cells and plasma are acceptable) and are to be immediately placed in a lysis solution from TriZ Reagent (Cat. No. IG-TRZ-100, Inogene, Russia). This sample may be stored prior to treatment at a temperature no higher than -68 °C for the period of 1 year.

б) The blood or bone marrow samples are to be treated with a lysis reagent to lyse erythrocytes in erythroid cells. To accomplish this, 2.5 mL of whole blood is added to 7.0 mL of reagent, stirred, and then centrifuged for 5 minutes at 3,000 RPM. The supernatant is to be removed without disturbing the precipitate. Subsequently, RNA isolation is performed according to the manufacturer's instructions – using the lysing reagent with the column method of separation (QIAamp RNA Blood Mini Kit (50) (Cat No. 52304, Qiagen, Germany) **или** TriZ Reagent.

2. The sample with the RNA stabilizer. The patient sample material with a volume of 2.5 mL is placed in a test tube with RNA stabilizer (e.g., PAXgene PreAnalytiX). The closed tube is then inverted several times. The sample can be stored for two days at a temperature of 25°C or for four days at 4°C.

ADDITIONAL MATERIALS AND EQUIPMENT REQUIRED FOR PCR ANALYSIS

Equipment:

1. Thermocycler («iQ5» (Bio-Rad); «CFX96» (Bio-Rad); «ABIPrism» (ThermoFisher Scientific); «Lightcycler» (Roche): «Rotor-Gene» 3000/6000 (Corbett Research/Qiagen).
2. Thermostat for tubes, such as "Eppendorf", which can handle temperatures ranging from 25 to 100 °C;
3. A separate set of automatic variable volume pipettes;
4. A refrigerator with a range of 2 to 8 °C and a freezer with a temperature of -16 °C or lower for the reagents of the DNA extraction.

Reagents for RNA stabilization:

- Blood RNA stabilizer (Cat No. IG-RSB-100, Inogene, Russia)
- PAXgene Blood RNA Tubes (Cat No. 764114, Qiagen, Germany)

Reagents for RNA isolation:

- QIAamp RNA Blood Mini Kit (50) (Cat No. 52304, Qiagen, Germany)
- TriZ reagent Kit (Cat No. IG-TRZK-60, Inogene, Russia)

When preparing the samples using the reagents for the stabilization of RNA it could possibly be recommended that the following set is used: PAXgene Blood RNA Kit (Cat No. 762174, Qiagen, Germany) or TriZ reagent Kit (Cat No. IG-TRZK-60, Inogene, Russia)

Reagents used for the reverse transcription reaction:

We recommend that the reverse transcription reaction is carried out with these kits:

- RevertAid First Strand cDNA Synthesis Kit (Cat No. K1621, ThermoFisher Scientific)
- SuperScript III (Cat No. 18080051, ThermoFisher Scientific)
- ReverZyme Kit (Cat No. IG-RT-1, Inogene, Russia)

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