For *in Vitro* Diagnostic Use

AmpliSens® Leucosis Quantum M-bcr-FRT PCR kit

Instruction Manual

AmpliSens®

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1. INTENDED USE.

AmpliSens® Leucosis Quantum M-bcr-FRT PCR kit is an in vitro nucleic acid amplification test for qualitative detection and quantitation of mRNA of bcr-abl chimeric gene (variant of M-bcr) and mRNA of abl gene in the clinical material (peripheral blood, bone marrow) by using real-time hybridization-fluorescence detection.

AmpliSens® Leucosis Quantum M-bcr-FRT PCR kit can be used for screening detection of CML (chronic myelogenous leukemia) associated with M-bcr-abl chromosomal rearrangement, for confirming of CML diagnosis, for monitoring of minimal residual disease (MRD), and therapy efficiency.

AmpliSens® Leucosis Quantum M-bcr-FRT PCR kit is intended for one of the listed below formats:

- Quantitative analysis: 50 clinical samples in two repeats.
- Qualitative analysis (screening): 100 clinical samples (120 RNA extractions, 120 reverse transcription reactions, and 360 PCR, including controls).

2. PRINCIPLE OF PCR DETECTION.

Bcr-abl gene mRNA detection in the clinical material is based on:

- total RNA extraction from peripheral blood cells, bone marrow punctate (Homchinsky method);
- reverse transcription reaction;
- amplification with real time detection (two oligonucleotide mixes are applied): amplification of mRNA fragment of chimeric M-bcr-abl (p210) gene, that conform to fragment of bcr and abl (b2a2 and b3a2) genes linkage, and mRNA fragment of abl gene splicing site (recommended by Europe Against Cancer (EAC) group) as endogenous internal control and gene-normalizer.

Results of bcr-abl cDNA amplification are registered on JOE/Yellow/HEX fluorescent channel, results of abl amplification are detected on JOE/Yellow/HEX channel.

Using of endogenous internal control allows not only monitoring of main stages of the test (sampling and handling, RNA extraction, reverse transcription, and cDNA amplification), but also precise calculation of bcr-abl chimeric gene mRNA quantity considering quality and amount of clinical material (normalizing).

AmpliSens® Leucosis Quantum M-bcr-FRT PCR kit uses "hot-start", which greatly reduces frequency of nonspecifically primed reactions. "Hot-start" is guaranteed by using chemically modified polymerase (TaqF). Chemically modified polymerase (TaqF) is activated by heating at 95 °C for 15 min.

3. CONTENT.

AmpliSens® Leucosis Quantum M-bcr-FRT PCR kit is produced in 1 form:

AmpliSens® Leucosis Quantum M-bcr-FRT **form 1** is consist of "RIBO-zol-D" variant 100, "REVERTA-L"

variant 100, AmpliSens® Leucosis Quantum M-bcr-FRT PCR kit variant FRT, REF TR-01(RG,iQ,Mx,A)-CE.

RIBO-zol-D nucleic acid extraction kit variant 100 includes:

Reagent	*Description*	*Volume (ml)*	*Amount*
Solution A	colorless, clear liquid	48	1 vial
Solution B	colorless, clear liquid	10	2 vials
Solution C	colorless, clear liquid	48	1 vial
Solution D	colorless, clear liquid	48	1 vial
Solution E	colorless, clear liquid	1.5	4 tubes
Washing Solution 3	colorless, clear liquid	100	1 vial
RNA-eluent bcr-abl	colorless, clear liquid	0.4	10 tubes

Additionally provided reagents:

Negative Control	colorless, clear liquid	1.6	2 tubes
tRNA 1 µg/µl	colorless, clear liquid	0.06	5 tubes
PC-1 bcr-abl-rec	colorless, clear liquid	0.03	1 tube
PC-2 bcr-abl-rec	colorless, clear liquid	0.03	5 tubes

REVERTA-L RT reagents kit variant 100 includes:

Reagent	*Description*	*Volume (ml)*	*Amount*
RT-G-mix-1	colorless, clear liquid	0.01	10 tubes
RT-mix	colorless, clear liquid	0.125	10 tubes
Revertase (MMlv)	colorless, clear liquid	0.06	1 tube
DNA-buffer	colorless, clear liquid	1.2	2 tubes

AmpliSens® Leucosis Quantum M-bcr-FRT PCR kit variant FRT includes:

Reagent		*Description*	*Volume (ml)*	*Amount*
PCR-mix-1- FRT M-bcr-abl		colorless, clear liquid	0.13	10 tubes
PCR-mix-1- FRT N-abl		colorless, clear liquid	0.13	10 tubes
PCR-buffer-FRT		colorless, clear liquid	0.3	10 tubes
Polymerase (TaqF)		colorless, clear liquid	0.02	10 tubes
DNA-buffer		colorless, clear liquid	1.2	1 tube
DNA-calibrators	**K1 bcr-abl / gus**	colorless, clear liquid	0.045	5 tubes
	K2 bcr-abl / gus	colorless, clear liquid	0.045	5 tubes
	K3 bcr-abl / gus	colorless, clear liquid	0.045	5 tubes
	K4 bcr-abl / gus	colorless, clear liquid	0.045	5 tubes
	K5 bcr-abl / gus	colorless, clear liquid	0.045	5 tubes

4. ADDITIONAL REQUIREMENTS.

For use in the Extraction Area:

- Laminar box.
- Thermostatic bath or dry block for tubes with controlled temperature and capability to incubate at temperature between 25 and 100 °C.
- Vacuum aspirator with flask for removing supernatant.
- Desktop microcentrifuge with rotor for 2 ml reaction tubes (RCF max. 16,000 rpm).
- Vortex mixer.
- Pipettes (adjustable).
- Disposable 1.5 ml volume polypropylene sterile screw-on or tightly closing tubes.
- Tube racks.
- Sterile pipette tips with aerosol barriers up to 200 µl and 1000 µl.
- Refrigerator with temperature between 2 and 8 °C.
- Deep-freezer with temperature not more than minus16 °C.
- Disposable powder-free gloves and laboratory coat.
- Container with disinfectant.

For use in the Reverse Transcription, Amplification and Detection Area:

- Disposable powder-free gloves and laboratory coat.
- Pipettes (adjustable).
- Sterile tips for micropipettes (up to 200 µl).
- Sterile pipette tips with aerosol barriers (up to 200 µl).
- Tip and tube racks.
- Thermostatic bath or dry block for tubes with controlled temperature and capability to incubate at temperature between 25 °C and 100 °C.
- Vortex mixer.
- PCR box.
- Personal thermocyclers (for example, Rotor-Gene 3000/6000 (Corbett Research, Australia), iQ iCycler (Bio-Rad, USA), Mx3000P (Stratagene, USA), ABIPrism (Applied Biosystem, USA).
- For Rotor-Gene: disposable polypropylene undomed and unsrtipe 0.2 ml microtubes for PCR (for example, "Axygen", USA) for 36-well rotor or 0.1 ml microtubes (Corbett Research, Australia) for 72-well rotor.
  For iQ iCycler: disposable polypropylene domed 0.2 ml PCR microtubes (for example, "Axygen", USA), stripe domed tubes or 96-wells PCR plate equipped with heat sealing optically transparent films (Bio-Rad, USA).
  For Mx3000P: disposable polypropylene domed and stripe/unsrtipe 0.2 ml PCR microtubes (for example, "Axygen", USA) for 36-well rotor or plate for PCR equipped with heat sealing optically transparent films (Bio-Rad, USA).
- Refrigerator with temperature between 2 and 8 °C.
- Deep-freezer with temperature not more than minus 16 °C.
- Waste bin for used tips.

5. GENERAL PRECAUTIONS.

The user should always pay attention to the following:

- Use sterile pipette tips with aerosol barriers and use new tip for every procedure.
- Store and handle amplicons away from all other reagents.
- Thaw all components thoroughly at room temperature before starting detection.
- When thawed, mix the components and centrifuge briefly.
- Use disposable gloves, laboratory coats, protect eyes while samples and reagents handling. Thoroughly wash hands afterward.

- Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas.
- Do not use a kit after its expiration date.
- Dispose of all samples and unused reagents in compliance with local authorities requirements.
- Samples should be considered potentially infectious and handled in a biological cabinet in accordance with appropriate biosafety practices.
- Clean and disinfect all sample or reagent spills using a disinfectant such as 0.5 % sodium hypochlorite, or other suitable disinfectant.
- Avoid contact with the skin, eyes and mucosa. If skin, eyes and mucosa contact immediately flush with water, seek medical attention.
- Material Safety Data Sheets (MSDS) are available on request.
- Use of this product should be limited to personnel trained in the techniques of DNA amplification.
- The laboratory process must be one directional, it should begin in the Extraction Area move to the Amplification and Detection Area. Do not return samples, equipment and reagents to the area in which the previous step was performed.

Hazard symbols	Solution	Hazards
T, C	**Solution A**	Contains phenol: toxic (T), corrosive (C) Risk and safety phrases:* R23/24/25-34-48/20/21/22-68 S24/25-26-28-36/37/39-45
Xn	**Solution B**	Contains chlorophorm: harmful (Xn) Risk and safety phrases:* R22-38-40-48/20/22 S36/37 Contains isoamyl alcohol: harmful (Xn). Risk and safety phrases:* R10-20-37-66, S46
Xi, F	**Solution C**	Contains 2-propanol: highly flammable (F), irritant (Xi) Risk and safety phrases:* R 11-36-67 S 7-16-24/25-26
Xn	**Solution D**	Contains guanidine thiocyanate. harmful (Xn). Risk and safety phrases:* R20/21/22-32 S13-26-36-46
F	**Washing Solution 3**	Contains ethanol: flammable (F) Risk and safety phrases:* R11 S2-7-16

*R68 Possible risk of irreversible effects.
R10 Flammable.
R11 Highly flammable.
R20 Harmful by inhalation.
R22 Harmful if swallowed.
R32 Contact with acids liberates very toxic gas.
R34 Causes burns.
R36 Irritating to eyes.
R37 Irritating to the respiratory system.
R38 Irritating to the skin.
R40 Limited evidence of a carcinogenic effect.
R66 Repeated exposure may cause skin dryness or cracking.
R67 Vapours may cause drowsiness and dizziness.
R20/21/22 Harmful by inhalation, in contact with skin and if swallowed.
R23/24/25/ Toxic by inhalation, in contact with skin and if swallowed.
R48/20/21/22 Harmful: danger of serious damage to health by prolonged exposure through inhalation, and in contact with skin and if swallowed.
R48/20/22 Harmful: danger of serious damage to health by prolonged exposure through inhalation and if

swallowed.
S2 Keep out of the reach of children.
S7 Keep container tightly closed.
S13 Keep away from food, drink and animal feeding stuffs.
S16 Keep away from sources of ignition. No smoking.
S26 In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.
S28 After contact with skin, wash immediately with plenty of (to be specified by the manufacturer).
S36 Wear suitable protective clothing.
S45 In case of accident or if you feel unwell, seek medical advice immediate.
S46 If swallowed, seek medical advice immediately and show this container or label.
S24/25 Avoid contact with skin and eyes.
S36/37 Wear suitable protective clothing and gloves.
S36/37/39 Wear suitable protective clothing, gloves and eye/face protection.

Some components of this kit contain Sodium Azide as a preservative. Do not use metal tubing for reagent transfer.

6. SAMPLING AND HANDLING.

Obtaining samples of biological materials for PCR-analysis, transportation and storage is described in manufacturer's handbook [3]. It is recommended that this handbook is read before starting work.

AmpliSens® Leucosis Quantum M-bcr-FRT PCR kit is intended for analysis of RNA extracted with RNA isolation kits from:

- *peripheral blood cells.*
- *bone marrow punctate cells.*

6.1. *Peripheral blood cells.*

6.1.1. Variant 1. *Blood with EDTA.* Blood should be collected in a tube with 6 % EDTA solution (1:20). Peripheral blood should be obtained in the morning on an empty stomach. After the tube is filled and sealed it should be inverted several times to ensure proper mixing. Cell isolation:

- centrifuge the tubes at 800-1600 rpm for 20 min at room temperature within 48 hours from the time of blood taking (only if blood was stored at 2-6 °C). Remove all white cells (white pellicle on surface of packed red blood cells) up to the sample volume of 200 µl, immediately transfer into the tube with 800 µl of Solution D (provided with "RIBO-zol-D" extraction kit), and stir. This sample can be stored at not more than minus 68 °C for 1 year.
- in the tube that contains 2.5 ml of whole blood add 7.0 ml of Hemolitic, **REF** 137 (it is not provided with the kit), stir, and centrifuge at 3,000 rpm for 5 min. Remove supernatant (do not disturb the pellet). Into the tube with the pellet add 800 µl of Solution D (provided with "RIBO-zol-D" extraction kit) and stir. This sample can be stored at not more than minus 68 °C for 1 year.

6.1.2. Variant 2. *Blood with RNA-stabilizer.* Blood (2.5 ml) should be collected in a tube that contains RNA-stabilizer (for example*, PAXgene, PreAnalytix*). Peripheral blood should be obtained in the morning on

an empty stomach. After the tube is filled and sealed it should be inverted several times to ensure proper mixing. This sample can be stored at 25 °C for 2 days, at 4 °C for 4 days.

6.2. *Bone marrow punctate cells.* Immediately after sampling transfer 200 µl of bone marrow punctate in the tube with 800 µl of Solution D (provided with "RIBO-zol-D") and stir. Centrifuge the tubes at 5,000 rpm for 5 min. In case the pellet has formed, transfer supernatant in a clean tube. Use supernatant for RNA extraction.

Samples can be stored at not more than minus 16 °C for 1 month, at not more than minus 68 °C for 1 year.

7. PROTOCOL.

RECOMMENDED ANALYSIS FORMAT.

Due to the fact that prepared for PCR with reverse transcription (RT-PCR) mix must be used as soon as possible we recommend the test planning with minimal waste of reagents. The table listed below helps to plan the test.

Analysis format	***Quantitative***		***Screening (qualitative)***	
	One plate *(36-Well Rotor)*	Two plates *(72-Well Rotor)*	One plate *(36-Well Rotor)*	Two plates *(72-Well Rotor)*
Number of samples to be tested	*5 samples*	*11 samples*	*10 samples*	*22 samples*
RNA extraction	*12 extractions* 5 clinical samples per two repeats, low Positive Control (PC-2), and Negative Control per one repeat.	*24 extractions* 11 clinical samples per two repeats, low Positive Control (PC-2), and Negative Control per one repeat.	*12 extractions* 10 clinical samples per two repeats, low Positive Control (PC-2), and Negative Control per one repeat.	*24 extractions* 22 clinical samples per two repeats, low Positive Control (PC-2), and Negative Control per one repeat.
PCR with reverse transcription (RT-PCR)	*18 reactions per PCR-mix-1 bcr-abl* *16 reactions per PCR-mix-1 N-abl* 12 extracted samples and 1 PCR C- per each mix; 5 and 3 DNA-calibrators per one repeat (depend on the mix), correspondingly.	*36 reactions per PCR-mix-1 bcr-abl* *32 reactions per PCR-mix-1 N-abl* 24 extracted samples and 2 PCR C- per each mix; 5 or 3 DNA-calibrators per two repeats (depend on the mix).	*14 reactions per PCR-mix-1 bcr-abl* *14 reactions per PCR-mix-1 N-abl* 12 extracted samples, PCR C-, and K3 DNA-calibrators per *N-abl* mix; and PCR C–, K5 DNA-calibrator per M-bcr-abl mix each in one repeat.	*26 reactions per PCR-mix-1 bcr-abl* *26 reactions per PCR-mix-1 N-abl* 24 extracted samples, PCR C-, and K3 DNA-calibrators per *N-abl* mix; and PCR C–, K5 DNA-calibrator per M-bcr-abl mix each in one repeat.

One panel is calculated for the following reagents: "REVERTA-L" kit (RT-mix, RT-G-mix-1), PCR kit (PCR-mix-1-FRT M-bcr-abl, PCR-mix-1-FRT N-abl, PCR-buffer-FRT, Polymerase (TaqF), **one tube** of each. **Two panels** are calculated for the same reagents in a double volume: **two tubes** of each.

CONTROLS.

Positive controls of Extraction (PC-1 and PC-2) are quantitatively described fragments of bcr-abl mRNA protected by capsule of RNA-containing phag. These controls permit to assess quality of all test stages as well as reagent workability. For test assessment specified in quality passport concentrations of control samples should be compared with those obtained during the test. It is necessary to make assessment of Positive Control PC-2 (low concentration) whenever samples are treated. Positive Control PC-1 (high concentration) should be tested once (at the beginning of the analysis).

DNA-calibrators (K1, K2, K3, K4, K5) are quantitatively described plasmids specimen, containing insertion of bcr-abl chimera fragment cDNA and abl gene-normalizer fragment. DNA-calibrators are used for construction of PCR calibration curve for both PCR-mixes (M-bcr-abl and N-abl), and as a Positive Controls of Amplification.

Negative Control of Extraction (C-) is a sample that initially doesn't contain bcr-abl and abl RNA but undergone sample treatment. Negative Control allows assessment of quality and purity of test performance as well as data validity.

7.1. RNA Isolation.

Volume of clinical material for RNA isolation is **150-200 ml**.

In case of qualitative test format, RNA extraction and RT-PCR are performed in two repeats for each sample.

In case of screening (quantitative) test format, RNA extraction is performed from half of collected clinical material, while the other part should be stored at minus 16 °C if further test is required.

1. Lysis.

Variant1. Blood with EDTA.

a. Hemolitic treatment.

The blood sample should be washed with Hemolytic if leucocyte pellicle cannot be removed. In a 10 ml tube (separate for each sample) add **7.0 ml** of **Hemolitic** and **2.5 ml** of the **whole blood**. Stir on vortex then centrifuge at 3,000 rpm for 5 min. Remove and discard supernatant, ensure that the pellet is not disturbed.

Into the tube that contains the pellet add **800 µl** of **Solution D**. Obtained sample can be stored at not more than minus 16 °C for 1 month, at not more than minus 68 °C for 1 year.

Divide prepared sample in 2 equal parts: transfer 400-450 µl of lysate in two clean 1.5 ml tubes.

b. Treatment of leukocyte pellicle (without Hemolitic):

Collect required number of 1.5 ml tubes. Add **800 µl** of **Solution D**. Transfer **200 µl** of **leucocytes** (within 48 hours from the time of blood taking if blood samples were stored at 2-6 °C). Prepared sample can be stored at not more than minus 16 °C for 1 month, at not more than minus 68 °C for 1 year.

Divide prepared sample in 2 equal parts: transfer 400-450 µl of lysate in two clean 1.5 ml tubes.

Variant 2. Blood with RNA-stabilizer.

Divide blood sample in 2 equal parts: transfer 4.5 ml of the sample in two clean 5 ml tubes.

Centrifuge the tubes at 3,500-5,000 g for 10 min. Remove and discard supernatant (ensure that the pellet is not disturbed). Into the tube with the pellet add 4 ml of mQ water. Stir on vortex until pellet is resuspended. Presence of some insoluble debris is permitted. Centrifuge at 3,500-5,000 g for 10 min. Remove supernatant completely. Add **400 µl** of **Solution D** into each tube with the pellet.

The pellet does not dissolve completely after addition of **Solution D**.
The pellet will dissolve after addition of **Solution E** and **Solution A**.

2. Carry the control reactions:

PC-1(or PC-2)	Into the tube for Positive Control of Extraction add: 400 µl of Solution D, 50 µl of Negative Control, 10 µl of PC-2 bcr-abl-rec (or PC-1 bcr-abl-rec).
C-	Into the tube for Negative Control of Extraction add: 400 µl of Solution D, 50 µl of Negative Control.

3. In the tubes with lysed in Solution D samples add **40 µl** of **Solution E**. Stir on vortex. Centrifuge the tubes to remove drops.
4. Add **400 µl** of **Solution A** in the tubes with the solution. Stir on vortex. Centrifuge tubes to remove drops.
5. In the tubes with the solution add **130 µl** of **Solution B**. Stir on vortex for 1-2 min (color of solution can vary from milky to milk-and-coffee that depends on the amount of erythrocytes in the sample).
6. Incubate the tubes in a freezer at not more than minus 16 °C for 10 min.
7. Centrifuge the tubes at 13,000-16,000 rpm for 10 min. The solution will separate in two phase: bottom phase that contains proteins and DNA and top phase (aqueous) that contains RNA.
8. While samples are centrifuged, collect clean tubes (the number of tubes should correspond to the number of samples plus two controls) and add **400 µl** of **Solution C**.

In the tubes with Solution C for PC-2 (or PC-1) and C- add **10 µl of tRNA 1µg/µl**.

9. After the samples have been centrifuged, remove supernatant (about 400 µl) using tips with aerosol barrier and transfer it in the tubes with Solution C. Transfer top phase of the Control samples (PC-1 or PC-2 and NC) into the tubes with Solution C and tRNA.
10. Stir the tubes on vortex, centrifuge to remove drops and incubate in a freezer at minus 16 °C for 20 min.

11. Centrifuge the tubes at 14,000-16,000 rpm for 10 min. Carefully remove and discard supernatant using vacuum aspirator and separate tip for each sample. Ensure that the pellet isn't disturbed. If the pellet is not visually detected, do not touch tube walls and leave at the tube bottom about 20 µl of the liquid while removing the supernatant.
12. Incubate the vial with **Washing Solution 3** in a freezer at minus 16 °C while centrifuging the tubes with the samples.
13. Add **800 µl** of cold **Washing Solution 3** into the tubes with the pellet. Resuspend the pellet. Stir on vortex, then centrifuge at 14,000-16,000 rpm for 10 min. Remove and discard supernatant, trying not to disturb the pellet.
14. Incubate the tubes with the pellet at 56 °C for 5-7 min (for predrying). Ensure that tubes are open.
15. Add **30 µl** of **RNA-eluent bcr-abl** then incubate at 56 °C for 2-3 min.

    Supernatant contains purified RNA and can be used for reverse transcription and PCR.

    RNA samples can be stored at not more than minus 68 °C for 1 year.

7.2. Reverse transcription.

It's recommended to use the following kit for complementary DNA (cDNA) synthesis from RNA:

- "REVERTA-L", containing RT-G-mix, **REF** K3-4-100-CE.

Carry the reverse transcription reaction according to the manufacturer's instructions.

RNA-eluent brc-abl contains essential for reverse transcription components.
RNA diluted in other eluents should not be used.

After addition of RNA samples to the tubes with reaction mix place the test tubes into thermocycler and incubate at 50 °C for 15 minutes, then at 95 °C for 3 minutes. Do not dilute cDNA samples.

7.3. Preparing the PCR.

Total reaction volume is **25 µl**, volume of cDNA sample is **10 µl**.

7.3.1. Preparing tubes for PCR.

1. Collect the required number of PCR tubes (0.1 or 0.2 ml). Calculation of microtubes should be done taking into account that each sample is to be analyzed with two PCR-mixes (PCR-mix-1-FRT M-bcr-abl and PCR-mix-1-FRT N-abl). Moreover, following samples should be included in calculation:

- Negative Controls (one for each PCR-mix-1-FRT);
- DNA-standards for quantitative format (5 ones for PCR-mix-1-FRT M-bcr-abl and 3 ones for PCR-mix-1-FRT N-abl);
- Positive Control for qualitative format (one for each PCR-mix-1-FRT).

If N is a required number of the tubes:

in case of **quantitave test format:**
N = number of samples of cDNA *2 + 10

in case of **screening (qualitative) test format:**
N = number of samples of cDNA *2 + 4

2. Prepare reaction mixes for **one panel** as follows:

- **PCR-buffer-FRT** and **polymerase (TaqF)**. Transfer 0.02 ml of polymerase (TaqF) (one tube) into the tube that contains PCR-buffer-FRT (0.3 ml) and carefully stir on vortex (avoid foam forming).
- into the tube that contains **PCR-mix-1-FRT M-bcr-abl** add **145 µl** prepared mix of PCR-buffer-FRT and polymerase (TaqF). Mix on vortex, sediment drops.
- into the tube that contains **PCR-mix-1-FRT N-abl** add **145 µl** prepared mix of PCR-buffer-FRT and polymerase (TaqF). Mix on vortex, sediment drops.

In case of **two panels**, mixes should be prepared in double volume.

3. In case of another number of samples each PCR requires:

- 7.0 µl of PCR- mix-1-FRT;
- 7.5 µl of PCR-buffer-FRT;
- 0.5 µl of polymerase (TaqF).

When calculating include reagents for one extra reaction. For analysis of N cDNA samples mix:

Quantitative test format		***Qualitative (screening) test format***	
Mix for detection of M-bcr-abl	Mix for detection of N-abl	Mix for detection of M-bcr-abl	Mix for detection of N-abl
(N+**7**) * 7.0 µl PCR-mix-1-FRT M-bcr-abl (N+**7**) * 7.5 µl of PCR-buffer-FRT (N+**7**) * 0.5 µl of polymerase (TaqF)	(N+**5**) * 7.0 µl of PCR-mix-1-FRT M-bcr-abl (N+**5**) * 7.5 µl of PCR-buffer-FRT (N+**5**) * 0.5 µl of polymerase (TaqF)	(N+**3**) * 7.0 µl of PCR-mix-1-FRT M-bcr-abl (N+**3**) * 7.5 µl of PCR-buffer-FRT (N+**3**) * 0.5 µl of polymerase (TaqF)	(N+**3**) * 7.0 µl PCR-mix-1-FRT M-bcr-abl (N+**3**) * 7.5 µl of PCR-buffer-FRT (N+**3**) * 0.5 µl полимеразы (TaqF)
7 = 5 DNA-standards + 1 Negative Control + 1 extra	5 = 3 DNA-standards + 1 Negative Control + 1 extra	3 = 1 Positive Control + 1 Negative Control + 1 extra	3 = 1 Positive Control + 1 Negative Control + 1 extra

4. Into each PCR microtube, intended for detection of M-bcr-abl transcript, add **15 µl** of prepared **M-bcr-abl reaction mix**. Into each PCR microtube, intended for detection of abl gene-normalizer, add **15 µl** of prepared **N-abl reaction mix**.
5. Using tips with aerosol barrier add **10 µl** of **cDNA sample**, obtained from clinical or control samples at the stage of reverse transcription in the tube with M-bcr-abl reaction mix and then in the tube with N-abl reaction mix.
6. Carry the **control amplification reactions** (regardless the number (one or two) of examined panels):

Quantitative test format

Prepare 5 control samples – calibrators for M-bcr-abl reaction mix. Add **10 µl** of each **DNA-calibrators (K1, K2, K3, K4, K5)** in corresponding tube.

Prepare 3 control samples – calibrators for N-abl reaction mix. Add **10 µl** of each **Positive Control DNA-calibrators (K1, K2, K3)** in corresponding tube.

Qualitative (screening) test format

Prepare positive control of amplification. Add **10 µl of PC DNA-calibrator (K3)** both in the tube with M-bcr-abl reaction mix as well as in the tube with N-abl reaction mix.

7.4.2. Amplification.

1. Place the tubes into the thermocycler.
2. Run the following program on the thermocycler (see table 1, 2).

Table 1

Amplification program for
"Rotor-Gene" 3000/6000 (Corbett Research, Australia)

Step	*Temperature, °C*	*Time*	*Fluorescence detection*	*Repeats*
Hold	95	15 min	–	1
Cycling	95	15 sec	–	45
	60	45 sec	JOE/ Yellow	

- Perform calibration before first acquisition;
- Perform calibration parameters for JOE/Yellow channels in the range of 3FI-5FI.

Table 2

Amplification program for "iQ iCycler", "iQ5" (Bio-Rad, USA); "Mx3000P", "Mx3005P" (Stratagene, USA); "ABIPrism" 7x00 (Applied Biosystem, USA)

Step	*Temperature, °C*	*Time*	*Fluorescence detection*	*Repeats*
1	95	15 мин	–	1
2	95	20 с	–	47
	60	55 с	HEX	

When programming "ABIPrism" 7x00 detection system, set ROX reference dye.

3. Proceed to data analysis by the end of amplification program.

8. DATA ANALYSIS.

Obtained data (curves of fluorescent signal accumulation) are analyzed by the software of used instrument for real-time PCR in accordance with the device instruction manual. Accumulation of M-bcr-abl cDNA fragment amplification product (positive control) is registered in the tubes with RCR-mix-1-FRT M-bcr-abl, while accumulation of gene-normalizer/abl internal control cDNA amplification product is registered in the tubes with RCR-mix-1-FRT N-abl.

Qualitative (screening) test format

Sigmoid curves of fluorescent signal accumulation that cross threshold line, that is registered in the tubes with PCR-mix-1-FRT M-bcr-abl indicates presence of mRNA transcript of bcr-abl in the sample, i.e. **positive result**.

Absence of positive signal in PCR-mix-1-FRT N-abl while gene-normalizer signal value is valid indicates **negative result**.

Gene-normalizer signal value is considered to be valid if Ct (crossing of fluorescence curve with specified threshold line) value of the sample with PCR-mix-1-FRT N-abl is less than Ct value of Positive Control (DNA-calibrator of Positive Control BCR-ABL-rec K3).

Quantitative test format

Construction of calibration curve and calculation of bcr-abl and N-abl cDNA copies in the sample are performed automatically on the basis of Ct values and specified calibrators values first for M-bcr-abl mix, then for N-abl mix (specified calibrators concentrations are identical for both mixes).

Obtained data are used for estimation of normalized concentration of M-bcr-abl RNA of clinical and control samples as listed below:
Calculate following ratio for all samples:
Number of cDNA M-bcr-abl copies / number of cDNA N-abl copies.
Calculate average value of M-bcr-abl/abl concentrations ratio in two repeats.

9. TROUBLESHOOTING.

Qualitative (screening) test format

Results are irrelevant:
If gene-normalizer signal is invalid. The sample analysis is to be repeated from the first step of analysis. If invalid result is registered again, perform addition analysis starting from the sampling.

If Ct value for the Negative Control is present in result grid it means that reagents or samples contamination has taken place. Test analysis must be repeated and measures to detect and eliminate the source of contamination are to be taken.

Quantitative test format

Results are irrelevant:
If concentration of abl (gen-normalizer) is less than 10,000 copies per reaction. The sample is considered to be invalid. The sample analysis is to be repeated starting from the first step of analysis. If invalid result is registered again, perform addition analysis starting from the sampling.
If difference of M-bcr-abl/N-abl concentrations ratio for the sample in two repeats is more than four times over. That is,
(repeat 1 of M-bcr-abl/N-abl) / (repeat 2 of M-bcr-abl/N-abl) > 4 or < 0.25,
excluding samples for which estimated number of M-bcr-abl copies is less than 25.
If correlation coefficient R^2 is less than 0.98 while calibration curve constructing, analysis for all samples are to be repeated starting from the first step of the test.
If calculated concentrations of Positive Control-1/Positive Control-2 do not fall into the range specified in the Important product information bulletin, analysis for all samples should be repeated starting from the first step of the test.
If Ct value for the Negative Control is present in result grid it means that reagents or samples contamination has taken place. Test analysis must be repeated and measures to detect and eliminate the source of contamination are to be taken.

If you have any further questions or if you encounter problems, please contact our Authorized representative in the European Community.

10. STABILITY AND STORAGE.

All components of the **REVERTA-L** and **AmpliSens® Leucosis Quantum M-bcr-FRT PCR kit variant FRT** (except for PCR-buffer-FRT, DNA-buffer and DNA-calibrators) are to be stored at the temperature not more than minus 16 °C, when not in use. All components of the **RIBO-zol-D** (except for RNA-eluent bcr-abl and tRNA) are to be stored at the temperature between 2 and 8 °C, when not in use. All components are to be stable until labeled expiration date.

PCR-buffer-FRT, DNA-buffer and DNA-calibrators are to be stored at the temperature between 2 and 8 °C.
RNA-eluent bcr-abl and tRNA are to be stored at the temperature not more than minus 16 °C.

11. SPECIFICATIONS.

11.1. Sensitivity.

Estimation of analytical sensitivity of **AmpliSens® Leucosis Quantum M-bcr-FRT** PCR kit was performed by using control RNA-comprising phage preparations: b3a2 (contains 13 and 14 exon bcr and 2 exon abl) and b2a2 (contains 13 exon bcr and 2 exon abl) with specified concentrations. RNA extraction and real-time RT-PCR were performed for 2x diluted control phage preparations in the presence of 10^7 leucocytes per extraction.

Table 3

Variant of mRNA	Sensitivity, copies of mRNA per extraction	Sensitivity, copies of mRNA per ml
b2a2	24 (19.5 – 28.5)	237 (189 – 282)
b3a2	48 (37.5 – 52.5)	474 (378 – 525)

Sensitivity (copies of mRNA per extraction) is a number of control phages particles that should be added in extraction to ensure 100 % positive test result in the presence of 10^7 leucocytes. Sensitivity value is a dilution of control phage that can be reproducibly detected as positive in 12 repeats from 12. This value represents minimum number of detected mRNA copies in half of leucocytes of peripheral blood sample or half of bone marrow sample. Therefore, 2.5 ml of blood sample specificity should be 20-30 mRNA copies per 1 ml (according to test protocol, analysis runs in 2 repeats, therefore RNA is extracted from leucocytes of 1.25 ml of blood sample).

Sensitivity measured in mRNA copies per ml is the sensitivity translated on 1 ml (reasoning from the fact that extraction is performed for 0.1 ml of a sample). This sensitivity is valid, for example, for analysis of the whole blood without isolation of leucocytes.

The claimed analytical features of **AmpliSens® Leucosis Quantum M-bcr-FRT** PCR kit are guaranteed only when additional reagents kits “RIBO-zol-D” and “REVERTA-L” (manufactured by Federal State Institution of Science Central Research Institute of Epidemiology) are used.

11.2. Specificity.

Specificity of **AmpliSens® Leucosis Quantum M-bcr-FRT** PCR kit is assured by selection of specific primers and probes, as well as the selection of strict reaction conditions. The primers and probes were checked for possible homologies to all in gene banks published sequences by sequence comparison analysis. Specificity of **AmpliSens® Leucosis Quantum M-bcr-FRT** PCR kit was confirmed in laboratory clinical trials.

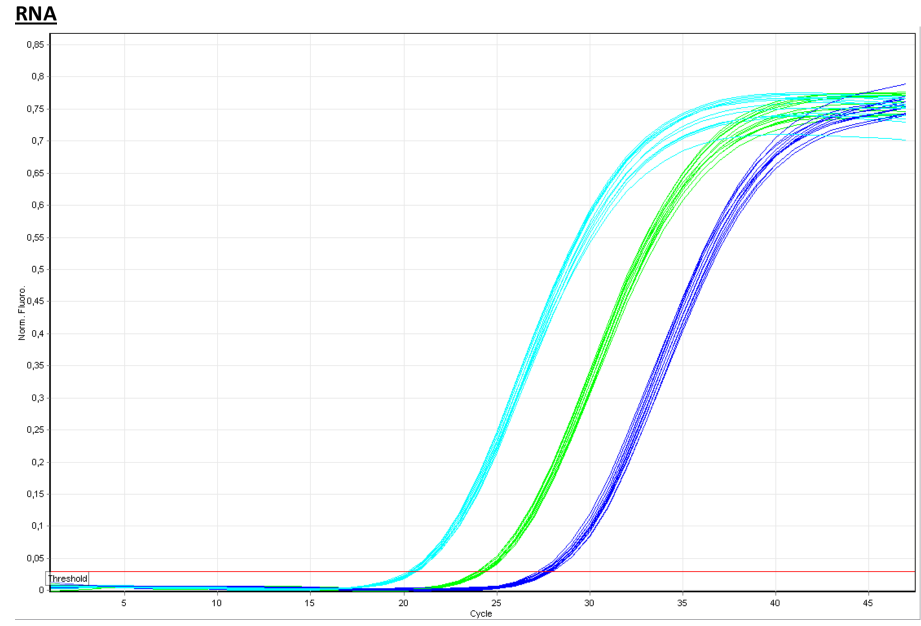
Specificity was estimated on 240 peripheral blood samples taken from healthy donors. Valid signal of internal control (gene-normalizer abl) was detected for all samples whereas signal of bcr-abl was not registered.

11.3. Reproducibility.

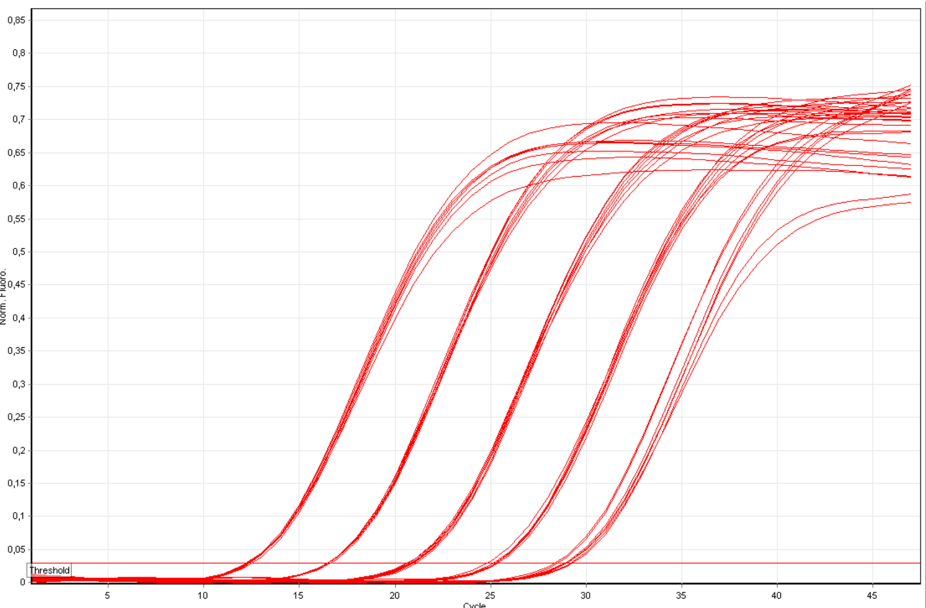
Table 4

	Concentration, copies/ml	n	Average Ct	Standard deviation Ct	CV%
RNA	$8.91 * 10^5$	12	20.51	0.15	0.73
	$8.91 * 10^4$	12	24.27	0.17	0.70
	$8.91 * 10^3$	12	27.72	0.24	0.87
DNA	$1.82 * 10^7$	7	12.40	0.10	0.83
	$7.94 * 10^6$	7	16.58	0.05	0.30
	$4.57 * 10^5$	7	20.93	0.15	0.01
	$3.16 * 10^4$	7	25.26	0.18	0.71
	$3.02 * 10^3$	7	28.93	0.33	1.14

RNA



DNA



Estimation of mRNA concentration measurement error (if using DNA-plasmids as standards) and b3a2 mRNA concentration measurement error (if using b2a2 as standards).

Due to the fact that the efficiency of amplification of plasmid DNA and cDNA after reverse reaction distinguish as well as efficiency of amplification of fragment b2a2 and b3a2 (because of length difference) small distortion of measured concentration can take place.

Efficiency of PCR on b3a2 and b2a2 variants of mRNA and cDNA preparations were determined for estimation of concentration measurement error.

Table 5

Target	Reaction efficiency	Anticipated concentration measurement error for point of $5*10^3$ copies/ml, times (lg difference)
DNA b2a2	0.930±0.020	1
RNA b2a2	0.910±0.010	1.104 (0.043 lg)
RNA b3a2	0.855±0.025	1.901 (0.279 lg)

Precision of bcr-abl RNA concentration measurement in vitro in accordance with DNA standards.

Table 6

Concentration of RNA phage, detected by independent method		**Phage type (repeats)**	**Result of concentration measurement by this reagents kit in reference to DNA-standards**			**Error, lg difference**
particle/ml	**particle lg/ml**		**Average, lg particle/ml**	**Standard deviation**	**CV%**	
$1.77 * 10^6$	6.25	b2a2 (5)	6.37	0.05	0.77	-0.12
$2.53 * 10^4$	4.40	b2a2 (5)	4.46	0.05	1.22	-0.06
$1.58 * 10^6$	6.20	b3a2 (5)	6.09	0.10	1.57	0.11
$2.79 * 10^4$	4.45	b3a2 (5)	4.09	0.09	2.19	0.36

12. REFERENCES.

1. Hughes T, Deininger M et al. 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. 2006 Jul 1; 108(1):28-37.
2. Gabert J, 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.
3. Handbook “Sampling, transportation, storage of clinical material for PCR diagnostics”, developed by Federal State Institution of Science Central Research Institute of Epidemiology of Federal Service for Surveillance on Consumers” Rights Protection and Human Well-Being, Moscow, 2008.

13. QUALITY CONTROL.

In compliance with Federal State Institution of Science “Central Research Institute of Epidemiology” ISO 13485 – certified Quality Management System, each lot of **AmpliSens® Leucosis Quantum M-bcr-FRT** PCR kit has been tested against predetermined specifications to ensure consistent product quality.

14. EXPLANATION OF SYMBOLS.