AmpliSens® Leucosis Quantum *M-bcr*-FRT PCR kit

CE IVD

For Professional Use Only

Instruction Manual

KEY TO SYMBOLS USED

REF	Catalogue number		Caution
LOT	Batch code		Contains sufficient for <n> tests
IVD	*In vitro* diagnostic medical device		Use-by Date
VER	Version		Consult instructions for use
	Temperature limit		Keep away from sunlight
	Manufacturer	C1, C2, C3, C4, C5	DNA calibrators
	Date of manufacture	C–	Negative control of extraction
EC REP	Authorized representative in the European Community	PC-1, PC-2	Positive controls of Extraction
	GHS02: Flame		GHS06: Skull and crossbones
	GHS05: Corrosion		GHS08: Health hazard
	GHS09: Environmental hazard		GHS07: Exclamation mark

1. INTENDED USE

AmpliSens® Leucosis Quantum *M-bcr*-FRT PCR kit is an in vitro nucleic acid amplification test for qualitative and quantitative detection of the *bcr-abl* chimeric gene (*M-bcr* variant) mRNA and *abl* gene mRNA in the clinical materials (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, confirmation of CML diagnosis, and monitoring of the minimal residual disease (MRD) and therapy efficiency.

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

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

NOTE: The results of RT PCR analysis are taken into account in complex diagnostics of disease.

2. PRINCIPLE OF PCR DETECTION

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

- total RNA extraction from peripheral blood cells and bone marrow aspirate (according to Homchinsky);
- reverse transcription reaction;
- amplification with real-time detection (two oligonucleotide mixes are used): amplification of mRNA fragment of the 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 an endogenous internal control and gene normalizer.

The results of *bcr-abl* cDNA amplification are detected in the channel for JOE fluorophore, the results of *abl* amplification are detected in the channel for JOE fluorophore as well.

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 the quantity of *bcr-abl* chimeric gene mRNA considering the quality and amount of clinical material (normalizing).

AmpliSens® Leucosis Quantum *M-bcr*-FRT PCR kit uses "hot-start", which greatly reduces the 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.

The results of amplification are registered in the following fluorescence channel:

Table 1

Channel for fluorophore	JOE	
PCR-mix-1-FRT	**PCR-mix-1-FRT *M-bcr-abl***	**PCR-mix-1-FRT *N-abl***
cDNA-target	*bcr-abl* cDNA	*abl* cDNA
Target gene	*bcr-abl* chimeric gene area	*abl* gene area

3. CONTENT

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

Form 1: RIBO-zol-D variant 100, **REVERTA-L** variant 100, **PCR kit** variant FRT, REF TR-O1(RG,iQ,Mx,A)-CE.

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

Reagent	Description	Volume, ml	Quantity
Solution A	transparent viscous yellow 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:

Reagent	Description	Volume, ml	Quantity
Negative Control (C–)	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
Glycogen 1%	colorless clear liquid	1.2	1 tube

Reagent kit is intended for RNA extraction from 120 samples (including controls).

REVERTA-L RT reagents kit variant 100 includes:

Reagent	Description	Volume, ml	Quantity
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

Reagent kit is intended for 120 reactions (including controls).

PCR kit variant FRT includes:

Reagent		Description	Volume, ml	Quantity
PCR-mix-1-FRT *M-bcr-abl*		clear liquid from colorless to light lilac colour	0.13	10 tubes
PCR-mix-1-FRT *N-abl*		clear liquid from colorless to light lilac colour	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	**C1 *bcr-abl / gus***	colorless clear liquid	0.045	5 tubes
	C2 *bcr-abl / gus*	colorless clear liquid	0.045	5 tubes
	C3 *bcr-abl / gus*	colorless clear liquid	0.045	5 tubes
	C4 *bcr-abl / gus*	colorless clear liquid	0.045	5 tubes
	C5 *bcr-abl / gus*	colorless clear liquid	0.045	5 tubes

PCR kit is intended for 360 reactions (180 reactions with each PCR-mix-1, including controls).

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 from 25 to 100 °C.
- Vacuum aspirator with flask for removing supernatant.
- Desktop centrifuge with a rotor for 2-ml reaction tubes.
- 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 the temperature from 2 to 8 °C.
- Deep-freezer with the temperature from minus 24 to minus 16 °C.
- Disposable powder-free gloves and laboratory coat.
- Container with disinfectant.

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

- 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), iCycler iQ (Bio-Rad, USA), Mx3000P (Stratagene, USA), ABIPrism (Applied Biosystems, USA).
- For Rotor-Gene: 0.2-ml disposable flat-cap non-strip polypropylene microtubes for PCR (for example, Axygen, USA) for a 36-well rotor or 0.1-ml microtubes (Corbett Research, Australia) for a 72-well rotor.
  For iCycler iQ: 0.2-ml disposable domed polypropylene PCR microtubes (for example, Axygen, USA), domed strip tubes or a 96-well PCR plate equipped with heat-sealing optically transparent films (Bio-Rad, USA).
  For Mx3000P: 0.2-ml disposable domed strip/non-strip polypropylene PCR microtubes (for example, Axygen, USA) for a 36-well rotor or plates for PCR equipped with heat-sealing optically transparent films (Bio-Rad, USA).
- Refrigerator with the temperature from 2 to 8 °C.
- Deep-freezer with the temperature from minus 24 to minus 16 °C.
- Reservoir for used tips.

5. GENERAL PRECAUTIONS

The user should always pay attention to the following:

- Use sterile pipette tips with aerosol barriers and use a new tip for every procedure.
- Store all extracted positive material (specimens, controls and amplicons) away from all other reagents and add it to the reaction mix in a distantly separated facility.
- Thaw all components thoroughly at room temperature before starting an assay.
- When thawed, mix the components and centrifuge briefly.
- Use disposable protective gloves and laboratory cloths, and protect eyes while samples and reagents handling. Thoroughly wash hands afterwards.
- 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 specimens and unused reagents in accordance with local regulations.
- Samples should be considered potentially infectious and handled in biological cabinet in compliance with appropriate biosafety practices.
- Clean and disinfect all samples or reagents spills using a disinfectant, such as 0.5 % sodium hypochlorite or another suitable disinfectant.
- Avoid samples and reagents contact with the skin, eyes, and mucous membranes. If these solutions come into contact, rinse the injured area immediately with water and seek medical advice immediately.
- Safety Data Sheets (SDS) are available on request.
- Use of this product should be limited to personnel trained in DNA amplification techniques.
- Workflow in the laboratory must be one-directional, beginning in the Extraction Area and moving to the Amplification and Detection Area. Do not return samples, equipment and reagents in the area where the previous step was performed.

Solution A **Danger**	Phenol EC No 203-632-7 CAS No 108-95-2 H301: Toxic if swallowed. H311: Toxic in contact with skin. H314: Causes severe skin burns and eye damage. H331: Toxic if inhaled. H341: Suspected of causing genetic defects. H373: May cause damage to organs through prolonged or repeated exposure. H411: Toxic to aquatic life with long lasting effects. P201: Obtain special instructions before use. P260: Do not breathe vapours. P264: Wash your hands thoroughly after handling. P303+ P361+ P353: IF ON SKIN (or hair): Take off immediately all contaminated clothing. Rinse skin with water. P405: Store locked up. P501: Dispose of contents in accordance with national regulation.
Solution B **Danger**	Contains substance: chloroform H302: Harmful if swallowed. H315: Causes skin irritation. P319: Causes serious eye irritation. H331: Toxic if inhaled. H336: May cause drowsiness or dizziness. H351: Suspected of causing cancer. H361d: Suspected of damaging the unborn child. H372: Causes damage to organs through prolonged or repeated exposure. P261: Avoid breathing dust/fume/ gas/mist/vapours/spray. P280: Wear protective gloves/protective clothing/eye protection/face protection P305 + P351 + P338: IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses if present and easy to do – continue rinsing. P311: Call a POISON CENTER or a doctor. P501: Dispose of contents in accordance with national regulation.
Solution C **Danger**	Isopropanol EC No 200-661-7 CAS No 67-63-0 H225: Highly flammable liquid and vapour. H319: Causes serious eye irritation. H336: May cause drowsiness or dizziness P210: Keep away from heat, hot surfaces, sparks, open flames and other ignition sources. No smoking. P261: Avoid breathing vapours. P264: Wash your hand thoroughly after handling. P305+P351+P338: IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses if present and easy to do – continue rinsing. P403+P233: Store in a well ventilated place. Keep container tightly closed. P501: Dispose of contents in accordance with national regulation.
Solution D **Danger**	Contains substance: guanidine thiocyanate H302: Harmful if swallowed. H312: Harmful in contact with skin. H314: Causes severe skin burns and eye damage H317: May cause an allergic skin reaction H332: Harmful if inhaled. H412: Harmful to aquatic life with long lasting effects. EUH032: Contact with acids liberates very toxic gas. P260: Do not breathe vapours. P264: Wash your hands thoroughly after handling. P273: Avoid release to the environment. P302+P352: IF ON SKIN: Wash with plenty of water. P501: Dispose of contents in accordance with national regulation.
Solution E **Danger**	Contains substance: acetic acid H226: Flammable liquid and vapour H314: Causes severe skin burns and eye damage P210: Keep away from heat, hot surfaces, sparks, open flames and other ignition sources. No smoking. P280: Wear protective gloves/protective clothing/eye protection/face protection. P305 + P351 + P338: IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses if present and easy to do – continue rinsing. P310: Immediately call a POISON CENTER or doctor. P405: Store locked up. P501: Dispose of contents in accordance with national regulation.
Washing Solution 3 **Warning**	Contains substance: isopropyl alcohol H226: Flammable liquid and vapour. H319: Causes serious eye irritation. H336: May cause drowsiness or dizziness P210: Keep away from heat, hot surfaces, sparks, open flames and other ignition sources. No smoking. P261: Avoid breathing vapours. P264: Wash your hand thoroughly after handling. P305+P351+P338: IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses if present and easy to do – continue rinsing. P403+P233: Store in a well ventilated place. Keep container tightly closed. P501: Dispose of contents in accordance with national regulation.

⚠ Some components of this kit contain sodium azide as a preservative. Do not use metal tubing for reagent transfer.

6. SAMPLING AND HANDLING

NOTE: 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/DNA extraction kits from:

- peripheral blood cells;
- bone marrow aspirate cells.

Peripheral blood cells.

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 h from the time of blood taking (only if blood was stored at 2–6 °C). Remove all white cells (white pellicle on the surface of packed red blood cells) up to the sample volume of 200 µl, immediately transfer into a tube with 800 µl of Solution D (provided with the **RIBO-zol-D** extraction kit), and stir. This sample can be stored at ≤ –68 °C for 1 year.
- Add 7.0 ml of **Hemolytic** (not provided with the kit) to the tube that contains 2.5 ml of whole blood, stir, and centrifuge at 3,000 rpm for 5 min. Remove the supernatant (do not disturb the pellet). Add 800 µl of Solution D (provided with the RIBO-zol-D extraction kit) to the tube with the pellet and stir. This sample can be stored at ≤ –68 °C for 1 year.

Variant 2. *Blood with RNA stabilizer.* Blood (2.5 ml) should be collected into a tube that contains an RNA stabilizer (for example, *PAXgene, PreAnalytix*). Peripheral blood should be obtained in the morning after overnight fasting. 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 and at 4 °C for 4 days.

Bone marrow aspirate cells.

In case of quantitative analysis, immediately after puncture, transfer 200 µl of bone marrow aspirate to a tube with 800 µl of Solution D (provided with the RIBO-zol-D extraction kit) and stir. Centrifuge the tubes at 5,000 rpm for 5 min. In case the pellet has formed, transfer the supernatant to a new tube. Use the supernatant for further use. Divide the obtained lysate into two equal parts. To do this, transfer 400 µl of lysate to each clear 1.5 ml tube.

In case of qualitative analysis, immediately after puncture, transfer 100 µl of bone marrow aspirate to a tube with 400 µl of Solution D (provided with the RIBO-zol-D extraction kit) and

stir. Centrifuge the tubes at 5,000 rpm for 5 min. In case the pellet has formed, transfer the supernatant to a new tube. Use the supernatant for further use.
Lysed samples can be stored at the temperature from minus 24 to minus 16 °C for 1 month and at the temperature not more than minus 68 °C for 1 year.

7. WORKING CONDITIONS

AmpliSens® Leucosis Quantum *M-bcr*-FRT PCR kit should be used at 18–25 °C.

8. PROTOCOL

RECOMMENDED ANALYSIS FORMAT

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

Analysis format	***Quantitative***		***Screening (qualitative)***	
	One panel *(36-Well Rotor)*	Two panel *(72-Well Rotor)*	One panel *(36-Well Rotor)*	Two panel *(72-Well Rotor)*
Number of samples to be tested	*5 samples*	*11 samples*	*10 samples*	*22 samples*
RNA extraction	*12 extraction procedures* 5 clinical samples in two replicates, low Positive Control (PC-2), and Negative Control in one replicate	*24 extraction procedures* 11 clinical samples in two replicates, low Positive Control (PC-2), and Negative Control in one replicate	*12 extraction procedures* 10 clinical samples, low Positive Control (PC-2), and Negative Control in one replicate	*24 extraction procedures* 22 clinical samples, low Positive Control (PC-2), and Negative Control in one replicate
PCR with reverse transcription (RT-PCR)	*18 reactions with PCR-mix-1 M-bcr-abl* *18 reactions with PCR-mix-1 N-abl* 12 extracted samples and 1 PCR of C– with each mix; 5 and 5 DNA calibrators in one replicate (depending on the mix)	*36 reactions with PCR-mix-1 M-bcr-abl* *36 reactions with PCR-mix-1 N-abl* 24 extracted samples and 2 PCR of C– with each mix; 5 or 5 DNA calibrators in two replicates (depending on the mix).	*14 reactions with PCR-mix-1 M-bcr-abl* *14 reactions with PCR-mix-1 N-abl* 12 extracted samples, PCR of C– and DNA calibrator C3 for *N-abl* mix; and PCR of C– and DNA calibrator C5 for *M-bcr-abl* mix (each in one replicate).	*26 reactions per PCR-mix-1 M-bcr-abl* *26 reactions with PCR-mix-1 N-abl* 24 extracted samples, PCR of C–, and DNA calibrators C3 for *N-abl* mix; and PCR of C– and DNA calibrator C5 for *M-bcr-abl* mix (each in one replicate).

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 reagent is used. **Two panels** are calculated for the same reagents in a double volume: **two tubes** of each reagent are used.

CONTROLS

Positive controls of Extraction (PC-1 and PC-2) are quantitatively described fragments of *bcr-abl* mRNA protected by the capsule of an RNA phage. These controls make it possible to assess the quality of all test stages as well as reagent workability. For test assessment, the specified concentrations of control samples should be compared with those obtained during the test. Positive Control PC-2 (low concentration) should be performed each time when samples are treated. Positive Control PC-1 (high concentration) should be performed once (at the beginning of the analysis).

DNA calibrators (C1, C2, C3, C4, and **C5)** are quantitatively characterized plasmid specimens carrying cDNA of a *bcr-abl* chimeric fragment and an *abl* gene normalizer fragment. DNA calibrators are used to construct a calibration curve for both PCR-mixes (*M-bcr-abl* and *N-abl*) as well as Positive Controls of Amplification.

Negative Control of Extraction (C–) is a sample that initially does not contain *bcr-abl* and *abl* RNA and that was subjected to all procedures of sample treatment. Negative Control allows assessment of the quality and purity of test performance as well as data validity.

8.1. RNA Extraction

8.1. RNA Extraction

Volume of clinical material for RNA isolation is **150–200 µl**.

In case of quantitative test format, RNA extraction and RT-PCR for each sample are performed in duplicate.

In case of screening (qualitative) 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. Treatment with Hemolytic

   The blood sample should be washed with Hemolytic if leukocyte pellicle cannot be removed. In case of quantitative analysis, add **7.0 ml** of **Hemolytic** and **2.5 ml** of the **whole blood** to a 10-ml tube (individual for each sample). In case of qualitative analysis, add **3.5 ml** of **Hemolytic** and **1.25 ml** of the **whole blood** to a 10-ml tube (individual for each sample). Stir on vortex, and then centrifuge at 3,000 rpm for 5 min. Discard supernatant making sure that the pellet is not disturbed.

   Add **400 µl** of **Solution D** in case of qualitative analysis or **800 µl** of **Solution D** in case of quantitative analysis to the tube with the pellet. Thus lysed sample can be stored at the temperature from minus 24 to minus 16 °C for 1 month and at the temperature not more than minus 68 °C for 1 year.

   NOTE: Divide the prepared lysate into two equal parts: transfer 400 µl of the lysate to two clean 1.5-ml tubes in case of quantitative analysis.

   b. Treatment of the leukocyte pellicle (without Hemolytic):

   In case of qualitative analysis, take the required number of 1.5-ml tubes. Add **400 µl** of **Solution D** and **100 µl** of **leukocytes** (collected within 48 h from the blood taking time if blood samples were stored at 2–6 °C), mix. In case of quantitative analysis, take the required number of 1.5-ml tubes. Add **800 µl** of **Solution D** and **200 µl** of **leukocytes** (collected within 48 h from the blood taking time if blood samples were stored at 2–6 °C), mix. Thus lysed sample can be stored at the temperature from minus 24 to minus 16 °C for 1 month and at the temperature not more than minus 68 °C for 1 year.

   NOTE: Divide the prepared sample into two equal parts: transfer 400-450 µl of the lysate to two clean 1.5-ml tubes in case of quantitative analysis.

   Variant 2. Blood with RNA stabilizer

   Divide the blood sample into two equal parts **in case of quantitative analysis**: transfer 4.5 ml of the sample to two new 5-ml tubes. Transfer 4.5 ml of the sample to a new 5-ml tube **in case of qualitative analysis.**

   Centrifuge the tubes at 3,500–5,000 g for 10 min. Discard the supernatant making sure that the pellet is not disturbed. Add 4 ml of mQ water to the tube with the pellet and resuspend it on vortex. The presence of some insoluble debris is allowed. Centrifuge at 3,500-5,000 g for 10 min and discard the supernatant completely.

   Add **400 µl** of **Solution D** to each tube with the pellet.

   Thus lysed sample can be stored at the temperature from minus 24 to minus 16 °C for 1 month and at the temperature not more than minus 68 °C for 1 year.

   NOTE: 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 out 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 (C–),
   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 (C–).

3. Add **40 µl** of **Solution E** to the tubes with samples lysed in Solution D. Stir on vortex and centrifuge the tubes to sediment drops.
4. Add **400 µl** of **Solution A to** the tubes with the solution. Stir on vortex and centrifuge the tubes to sediment drops.
5. Add **130 µl** of **Solution B** to the tubes with the solution. Stir on vortex for 1-2 min (the color of the solution may vary from milky to milk-and-coffee, which depends on the amount of erythrocytes in the sample).
6. Incubate the tubes in a freezer at not more than –16 °C for 10 min.
7. Centrifuge the tubes at 13,000–16,000 rpm for 10 min. The solution will be separated into two phase: the bottom phase that contains proteins and DNA and the top (aqueous) phase that contains RNA.
8. While samples are centrifuged, collect new 1.5 ml tubes (the number of tubes should correspond to the number of samples plus two controls) and add **400 µl** of **Solution C** and **10 µl** of **glycogen 1%** per each tube.

   NOTE: Add **10 µl** of **tRNA 1µg/µl** to the tubes with Solution C intended for extraction of PC-2 (or PC-1) and C–.

9. After the samples were centrifuged, remove the supernatant (about 400 µl) using tips with aerosol barrier and transfer it to the tubes with Solution C. Transfer the top phase of the Control samples (PC-1, PC-2 and Negative Control) to the tubes with Solution C, tRNA and glycogen 1%.
10. Stir the tubes on vortex, centrifuge to remove drops and incubate in a freezer at –16 °C for 20 min.
11. Centrifuge the tubes at 14,000–16,000 rpm for 10 min. Carefully remove and discard the supernatant using a vacuum aspirator and a new tip for each sample. Make sure that the pellet is not disturbed. If the pellet is not visually detected, do not touch tube walls and leave ~20 µl of the liquid on the tube bottom while removing the supernatant.
12. Incubate the vial with **Washing Solution 3** in a freezer at the temperature not more than 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.

The supernatant contains purified RNA and can be used for reverse transcription and PCR.
RNA samples can be stored at the temperature not more than minus 68 °C for 1 year.

8.2. Reverse transcription

Total reaction volume – **25 µl**, volume of RNA sample - **15 µl**.

NOTE: Use only disposable sterile RNase-free, DNase-free plastic consumables in work with RNA.

NOTE: **RNA-eluent *brc-abl*** contains components required for reverse transcription. RNA diluted in other RNA eluents should not be used.

1. Prepare required number of 0.2 ml microtubes.
2. Prepare ready-to-use reagent mix for 12 reactions. To do this, add **5 µl** of **RT-G-mix-1** to the tube containing **RT-mix**, mix on vortex, sediment the drops tube's cap.
3. Add **6 µl** of **Revertase (MMlv)** into the tube with reagent mix, mix on vortex, sediment the drops tube's cap.
4. Add **10 µl** of ready-to-use reagent mix into each microtube.
5. Using tip with aerosol barrier add **15 µl** of **RNA-sample** to the tube with ready-to-use reagent mix. Carefully mix.
6. Place the test tubes into the thermocycler with the next program:

Step	Temperature	Time
1	50 °C	15 min
2	95 °C	3 min

cDNA samples can be stored at ≤ –16 °C for a week or at ≤ –68 °C for a year.

8.3. Preparing the PCR

The total reaction volume is **25 µl**, the volume of cDNA sample is **10 µl**.

8.3.1. Preparing tubes for PCR

1. Prepare the required number of PCR tubes (0.1- or 0.2-ml). Tubes should be prepared 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*). The following samples should be included in calculation:
   - Negative Controls (one for each PCR-mix-1-FRT);
   - DNA standards for quantitative format (5 for PCR-mix-1-FRT *M-bcr-abl* and 5 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 the reaction mixtures for **one panel** as follows:
   - **PCR-buffer-FRT** and **polymerase (TaqF)**. Transfer 0.02 ml of polymerase (TaqF) (one tube) to the tube that contains PCR-buffer-FRT (0.3 ml) and carefully stir on vortex (avoid foaming).
   - Add **145 µl** prepared mix of PCR-buffer-FRT and polymerase (TaqF) to the tube that contains **PCR-mix-1-FRT *M-bcr-abl***. Mix on vortex and sediment drops.
   - Add **145 µl** prepared mix of PCR-buffer-FRT and polymerase (TaqF) to the tube that contains **PCR-mix-1-FRT *N-abl***. Mix on vortex and sediment drops.

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

3. If another number of samples should be prepared, mix the reagents in the following proportion (per one reaction):
   - 7.0 µl of PCR-mix-1-FRT;
   - 7.5 µl of PCR-buffer-FRT;
   - 0.5 µl of polymerase (TaqF).

   When calculating, include the 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+**7**) * 7.0 µl of PCR-mix-1-FRT *N-abl* (N+**7**) * 7.5 µl of PCR-buffer-FRT (N+7) * 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 *N-abl* (N+**3**) * 7.5 µl of PCR-buffer-FRT (N+**3**) * 0.5 µl polymerase (TaqF)
7 = 5 DNA-standards + 1 Negative Control + 1 extra	7 = 5 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. Add **15 µl** of the prepared ***M-bcr-abl* reaction mix** to each PCR microtube intended for detection of the *M-bcr-abl* transcript and **15 µl** of prepared ***N-abl* reaction mix** to each PCR microtube intended for detection of the *abl* gene normalizer.
5. Using tips with aerosol barrier, add **10 µl** of the **cDNA sample** obtained from clinical or control samples at the stage of reverse transcription to the tube with the *M-bcr-abl* reaction mix and then to the tube with the *N-abl* reaction mix.
6. Carry out the control amplification reactions (regardless the number (one or two) of examined panels):

Quantitative test format

Prepare 5 control samples – calibrators for the *M-bcr-abl* reaction mix. Add **10 µl** of each **DNA calibrator (C1, C2, C3, C4,** and **C5)** to the corresponding tube.

Prepare 5 control samples – calibrators for the *N-abl* reaction mix. Add **10 µl** of each **DNA calibrator (C1, C2, C3, C4,** and **C5)** to the corresponding tube.

Qualitative (screening) test format

Prepare the Positive Control of Amplification. Add **10 µl of DNA calibrator C3 *bcr-abl / gus*** to the tube with the *M-bcr-abl* reaction mix and to the tube with the *N-abl* reaction mix.

8.4.2. Amplification

1. Create a temperature profile on your instrument (see Tables 2, 3).

Table 2

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

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

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

Table 3

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

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

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

2. Insert tubes into the reaction module of the device.
3. Run the amplification program with fluorescence detection.
4. Analyze results after the amplification program is completed.

9. DATA ANALYSIS

The results are interpreted by the software of the used instrument by the crossing (or not-crossing) of the fluorescence curve with the threshold line.

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 *abl* gene normalizer / 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 the threshold line, which are recorded for the tubes with PCR-mix-1-FRT *M-bcr-abl*, indicates the presence of *bcr-abl* mRNA transcript in the sample, i.e., a **positive result**.

The absence of a positive signal in PCR-mix-1-FRT *M-bcr-abl* along with a valid signal value of the gene normalizer for PCR-mix-1-FRT *N-abl* indicates a **negative result**.

The gene normalizer signal value is considered to be valid if the Ct value (the crossing of the fluorescence curve with the specified threshold line) of the sample with PCR-mix-1-FRT *N-abl* is less than the Ct value for the Positive Control (DNA calibrator C3 *bcr-abl / gus*).

Quantitative test format

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

The obtained data are used for estimation of the normalized concentration of *M-bcr-abl* RNA of clinical and control samples as described below:

1. Calculate the following ratio for all samples:
   Number of *M-bcr-abl* cDNA copies / number of *N-abl* cDNA copies.
2. Calculate the mean *M-bcr-abl* / *abl* concentration ratio for samples analyzed in duplicate.

10. TROUBLESHOOTING

Qualitative (screening) test format

Results are irrelevant:

1. If the gene normalizer signal is invalid. The sample analysis is to be repeated from the first step of analysis. If an invalid result is registered again, perform analysis once again starting from the material sampling step.
2. If the *Ct* value for the Negative Control is present in the result grid, it means that reagents or samples are contaminated. Analysis must be repeated and measures to detect and eliminate the source of contamination are to be taken.

Quantitative test format

Results are irrelevant:

1. If the concentration of *abl* (gene normalizer) is less than 10,000 copies per reaction, the result of analysis is considered to be invalid. The analysis of the sample should be repeated starting from the first step of analysis. If an invalid result is registered again, perform analysis once again starting from the material sampling step.
2. If the *M-bcr-abl*/*N-abl* concentration ratio for a sample analyzed in duplicate differs more than four times. That is,
   (repeat 1 of *M-bcr-abl*/*N-abl*) / (repeat 2 of *M-bcr-abl*/*N-abl*) > 4 or < 0.25, except for the samples for which the estimated number of *M-bcr-abl* copies is less than 25.
3. If the correlation coefficient R^2 for the calibration curve is less than 0.98, analysis of all samples should be repeated starting from the first step of the test.
4. If the calculated concentrations of Positive Control-1/Positive Control-2 do not fall into the range specified in the Important Product Information Bulletin, analysis of all samples should be repeated starting from the first step of the test.
5. If a Ct value for the Negative Control is present in the result grid, it means that reagents or samples are contaminated. 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 encounter problems, please contact our Authorized representative in the European Community.

11. TRANSPORTATION

AmpliSens® Leucosis Quantum *M-bcr*-FRT PCR kit should be transported at 2–8 **°**C for no longer than 5 days.

12. STABILITY AND STORAGE

All components of the **REVERTA-L** and **PCR kit** variant FRT (except for PCR-buffer-FRT, DNA-buffer and DNA calibrators) are to be stored at temperature from minus 24 to 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 2–8 °C when not in use. All components of the **AmpliSens® Leucosis Quantum *M-bcr*-FRT** PCR kit are stable until the expiration date on the label. The shelf life of reagents before and after the first use is the same, unless otherwise stated.

NOTE: PCR-buffer-FRT, DNA-buffer, and DNA calibrators are to be stored at 2–8 °C.

NOTE: RNA-eluent *bcr-abl* and tRNA are to be stored at temperature from minus 24 to minus 16 °C when not in use.

NOTE: PCR-mix-1-FRT *N-abl,* PCR-mix-1-FRT *M-bcr-abl* are to be kept away from light.

13. SPECIFICATIONS

13.1. Sensitivity

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

Table 4

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

The sensitivity (mRNA copies per extraction procedure) is the number of control phage particles that should be added during the extraction procedure to ensure 100 % positive test result in the presence of 10^7 leukocytes. The sensitivity value is the dilution of the control phage that can be reproducibly detected as positive in 12 of 12 replicates. This value represents the minimum detectable number of mRNA copies in one-half of a peripheral blood leukocyte sample or one-half of a bone marrow sample. Therefore, the detection sensitivity during the treatment of 2.5-ml blood sample is 20–30 mRNA copies per 1 ml (according to the test protocol, analysis is performed in duplicate; therefore, RNA is extracted from leukocytes of 1.25 ml of a whole-blood sample).

The sensitivity expressed as the number of mRNA copies per 1 ml is the sensitivity recalculated per 1 ml (assuming 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 leukocytes.

13.2. Specificity

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

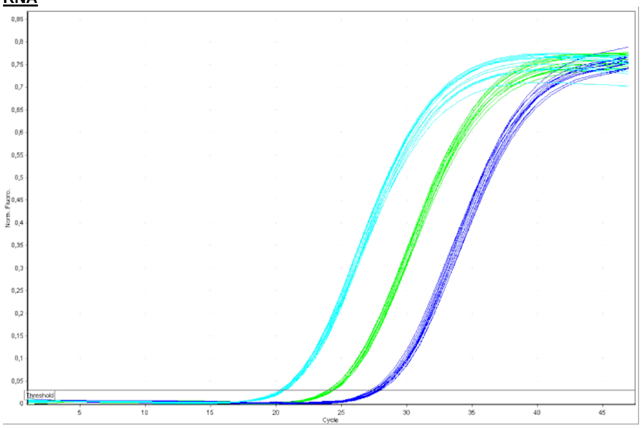
Specificity was estimated for 240 peripheral blood samples taken from healthy subjects. Valid signal of the internal control (gene normalizer *abl*) was detected for all samples whereas the signal of *bcr-abl* was not detected.

13.3. Reproducibility

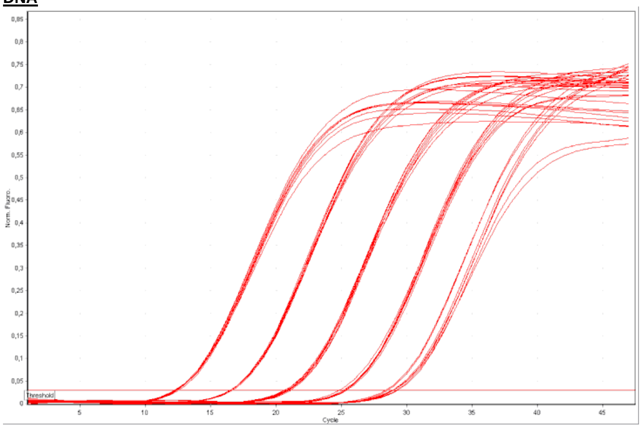
Table 5

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

RNA



DNA



Estimation of mRNA concentration measurement error (with DNA plasmids used as standards) and *b3a2* mRNA concentration measurement error (if using b2a2 as standards)

Since the efficiencies of amplification of plasmid DNA and cDNA after reverse reaction somewhat differ and the efficiencies of amplification of fragments *b2a2* and *b3a2* (because of length difference) differ as well, there may be a small bias in the measured concentrations. The efficiencies of PCR in *b3a2* and *b2a2* variants of mRNA and cDNA preparations were determined to estimate the concentration measurement error.

Table 6

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

Accuracy of bcr-abl RNA concentration measurement in vitro using DNA standards

Table 7

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, log difference
particle/ml	particle log/ml		Mean, log 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

14. 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, and 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.

15. QUALITY CONTROL

In compliance with Federal Budget Institute of Science "Central Research Institute for 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.

List of Changes Made in the Instruction Manual

VER	Location of changes	Essence of changes
13.12.10	8. Protocol, table	Sentence "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" was changed for "18 reactions with PCR-mix-1 *M-bcr-abl* 18 reactions with PCR-mix-1 *N-abl* 12 extracted samples and 1 PCR C- per each mix; 5 and 5 DNA calibrators in one replicate (depending on the mix)."
		Sentence "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)." was changed for "36 reactions with PCR-mix-1 *M-bcr-abl* 36 reactions with PCR-mix-1 *N-abl* 24 extracted samples and 2 PCR C– per each mix; 5 or 5 DNA calibrators in two replicates (depending on the mix)."
	8. Protocol, Recommended analysis format	"PCR-mix-1-FRT bcr-abl" was changed to "PCR-mix-1-FRT M-bcr-abl" at the table.
	8.3.1. Preparing tubes for PCR	The number of DNA calibrators for PCR-mix-1 *M-bcr-abl* was changed from 3 to 5.
		In table, sentence "5 = 3 DNA-standards + 1 Negative Control + 1 extra" was changed for "7 = 5 DNA-standards + 1 Negative Control + 1 extra"
		The name of PCR-mix-1-FRT was changed from "PCR-mix-1-FRT M-bcr-abl" to "PCR-mix-1-FRT N-abl" at columns Mix for detection of N-abl in the table of item 3
		"PC DNA calibrator (K3)" was changed to "DNA calibrator C3" at the table of item 6
	Through the text	Corrections through the text
	Cover page	The phrase "For Professional Use Only" was added
	Content	New sections "Working Conditions" and "Transportation" were added
		The "Explanation of Symbols" section was renamed to "Key to Symbols Used"
	Stability and Storage	The information about the shelf life of open reagents was added
	Key to Symbols Used	The explanation of symbols was corrected
29.11.11 LA	Throughout the text	Reagent glycogen (in RIBO-zol-D) was added
		The procedure of extraction in the presence of glycogen was described
15.11.12 IvI	Intended use	Clarified the reagent kit in the qualitative analysis is designed for the study in one repetition
	RNA Extraction	Procedure description is completed. Solution D volumes is clarified in the case study in a qualitative analysis and in the case study in a quantitative analysis
		Lyzate volume was changed from 400-450 µl to 400 µl for treatment with Hemolytic
02.12.13 ME	RNA Extraction	Volume of clinical material for RNA isolation was changed from 150–200 ml to 150–200 µl
21.03.14 ME	3. Content, text	The name of the Negative control reagent was corrected to Negative control (C–)
16.02.15 ME	9. Data analysis	For qualitative (screening) test format the phrase "The absence of a positive signal in PCR-mix-1-FRT *N-abl* along with a valid signal value for the gene normalizer indicates a negative result" was changed to "The absence of a positive signal in PCR-mix-1-FRT *M-bcr-abl* along with a valid signal value of the gene normalizer for PCR-mix-1-FRT *N-abl* indicates a negative result". The positive control name was corrected from "DNA calibrator of Positive Control BCR-ABL-rec C3" to "DNA calibrator C3 *bcr-abl / gus"* in description of valid signal of gene normalizer
01.04.15 ME	5. General precautions	Information about hazards was corrected
15.05.15 ME	5. General precautions	Corrections in accordance with the template
	6. Sampling and handling	Differences in the procedure of bone marrow aspirate cells sampling for qualitative and quantitative analysis was described
	8. Protocol	The phrase "in two replicates" was deleted for screening analysis (RNA extraction)
	8.1. RNA Extraction	Differences in the procedure of blood with EDTA treatment for qualitative and quantitative analysis was described
	8.2. Reverse transcription	The procedure was described completely
	13.1. Sensitivity	The phrase "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 Budget Institute of Science "Central Research Institute for Epidemiology") are used" was deleted
18.01.18 PM	5. General precautions, 14. Key to symbols used	Information about hazards was rewritten according to the Regulation 1272/2008/EC.
	3. Content	The color of the reagents was specified
19.09.19 PM	Through the text	The text formatting was changed
	Key to symbols used	Information about hazards was added according to the Regulation 1272/2008/EC.
02.06.20 MM	Footer	The phrase "Not for use in the Russian Federation" was added
	2. Principle of PCR detection	The table with cDNA-target was added
24.09.20 MM	3. Content	The number of reactions for REVERTA-L kit was corrected
16.03.21 MA	—	The name, address and contact information for Authorized representative in the European Community was changed
25.01.22 MM	Through the text	The reference number of Hemolitic was deleted
01.06.22 MM	2. Principle of PCR detection	The table with cDNA-targets was clarified (FAM fluorophore was deleted)

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