

IVD

For Professional Use Only

GUIDELINES

to AmpliSens[®] *Toxoplasma gondii*-FRT PCR kit for
qualitative detection of *Toxoplasma gondii* DNA in the
clinical material by the polymerase chain reaction (PCR)
with real-time hybridization-fluorescence detection

AmpliSens[®]



Ecoli Dx, s.r.o., Purkyňova 74/2
110 00 Praha 1, Czech Republic
Tel.: +420 325 209 912
Cell: +420 739 802 523



Federal Budget Institute of
Science “Central Research
Institute for Epidemiology”
3A Novogireevskaya Street
Moscow 111123 Russia

Not for use in the Russian Federation

TABLE OF CONTENTS

INTENDED USE	3
AMPLIFICATION AND DATA ANALYSIS USING Rotor-Gene 3000/6000 (Corbett Research, Australia) INSTRUMENTS	3
AMPLIFICATION AND DATA ANALYSIS USING iCycler iQ and iCycler iQ5 (Bio-Rad, USA) INSTRUMENTS	7
AMPLIFICATION AND DATA ANALYSIS USING Mx3000P, Mx3005P (Stratagene, USA) INSTRUMENTS.....	10

INTENDED USE

The guidelines describe the procedure of the use of **AmpliSens® *Toxoplasma gondii*-FRT** PCR kit for qualitative detection of *Toxoplasma gondii* DNA in the clinical material (white blood cells of whole peripheral blood, autopsy material, cerebrospinal fluid, amniotic fluid) by the polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection using the following instruments:

- Rotor-Gene 3000, Rotor-Gene 6000 (Corbett Research, Australia);
- iCycler iQ, iCycler iQ5 (Bio-Rad, USA);
- Mx3000P, Mx3005P (Stratagene, USA).

AMPLIFICATION AND DATA ANALYSIS USING Rotor-Gene 3000/6000 (Corbett Research, Australia) INSTRUMENTS

Insert the tubes into the rotor of the Rotor-Gene 3000/6000 instrument beginning from the first well (the rotor wells are numbered, the numbers are used for the further programming of the samples' order in the thermocycler). Insert the rotor into the instrument, close the lid.

NOTE: If some wells of the rotor remain unloaded, the rotor should be balanced. Place empty tubes into the empty wells. Do not use tubes from the previous tests. Well 1 must be filled with any test tube except for an empty one.

Hereinafter, all the terms corresponding to different instruments and software are indicated in the following order: for Rotor-Gene 3000 / for Rotor-Gene 6000.

Programming the thermocycler

1. Click the **New** button in the software main menu.
2. In the opened window, select the template **Advanced** and select **Dual Labeled Probe/Hydrolysis probes**. Click the **New** button.
3. In the opened window, select **36-Well Rotor** (or **72-Well Rotor**) and **No Domed 0.2 ml Tubes/Locking ring attached**. Click **Next**.
4. In the opened window, set the operator and select the reaction mixture volume: **Reaction volume – 25 µl**. For Rotor-Gene 6000, tick the **25 µl oil layer volume** option. Click **Next**.
5. In the opened window, set the temperature profile of the experiment. To do this click the **Edit profile** button and set the following parameters (see Table 1):

AmpliSens-1 amplification program

Step	Temperature, °C	Time	Fluorescence detection	Cycles
1	95	15 min	–	1
2	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
3	95	5 s	–	40
	60	20 s	FAM/Green, JOE/Yellow	
	72	15 s	–	

NOTE: Several tests with the use of AmpliSens PCR kits can be performed simultaneously within the same PCR run using the unified AmpliSens-1 amplification program (for example, together with the tests for detecting DNA of STI pathogens).

6. After setting up the temperature profile click the **OK** button.
7. In the **New Run Wizard** window, click the **Calibrate/Gain Optimisation** button.
 - Perform calibration in FAM/Green and JOE/Yellow channels (click the **Calibrate Acquiring/Optimise Acquiring** button);
 - Perform the calibration before the first detection (**Perform Calibration Before 1st Acquisition/Perform Optimisation Before 1st Acquisition**);
 - To set calibration of all channels, indicate **3** in the **Min Reading** column and **8** in the **Max Reading** column. Tick **Perform Calibration Before 1st Acquisition/Perform Optimisation Before 1st Acquisition**. Click the **Close** button.
8. Click the **Next** button and then **Start run** to start the amplification run.
9. Name the experiment and save it (the results of the experiment will be automatically saved in this file).
10. Enter the data into the grid of samples (it opens automatically after the amplification has been started). Enter the names/numbers of the tested samples in the **Name** column. Define Negative Control of Amplification as **NCA**, Positive Control of Amplification as **C+**. Set the type **Unknown** opposite all the test samples, the type **Positive Control** – for Positive Control of Amplification, the type **Negative Control** – for Negative Control of Amplification. Set the type **None** for the cells matching with the corresponding empty tubes.

NOTE: Samples indicated as **None** won't be analysed.

Data analysis

The obtained results (fluorescence accumulation curves for two channels) are analyzed by the instrument software. Accumulation of the amplified product of STI-87 DNA (IC) region is detected in the FAM/Green channel, and accumulation of the amplified product of *Toxoplasma gondii* DNA (C+) is detected in the JOE/Yellow channel.

The results are interpreted according to the crossing (or not-crossing) of the fluorescence curve with the threshold line set at the specific level, that corresponds to the presence (or absence) of the *Ct* (threshold cycle) value of the DNA sample in the corresponding column of the results table.

Amplification data analysis for *Toxoplasma gondii* DNA (JOE/Yellow channel):

1. Activate the **Analysis** button in the menu, select the **Quantitation** analysis mode and activate the **Cycling A. JOE/Cycling A. Yellow, Show** buttons.
2. Cancel the automatic choice of the threshold line level **Threshold**.
3. Activate the **Dynamic tube** and **Slope Correct** buttons in the main window menu (**Quantitation analysis**).
4. In the **CT Calculation** menu (in the right part of the window), indicate the threshold line level **Threshold = 0.03**.
5. Choose the parameter **More settings/Outlier Removal** and set **10 %** for the value of negative samples threshold (**NTC/Threshold**).
6. In the results grid (**Quant. Results** window), *Ct* values will appear.
7. *Ct* values should be absent for the Negative Control of Extraction – Negative Control (C–).
8. *Ct* values should be absent for the Negative Control of Amplification (NCA) – DNA-buffer (NCA).
9. *Ct* values should not exceed **30 cycles** for the Positive Control of Amplification – Positive Control DNA *Toxoplasma gondii* and STI (C+*T.gondii* and STI).
10. Samples are considered positive if *Ct* values for them do not exceed **40 cycles**. If the *Ct* value exceeds this threshold, the result is considered **equivocal**. It is necessary to repeat the analysis twice. If a reproducible positive *Ct* value is determined twice, the sample is considered **positive**. If irreproducible values are obtained in two repeats, the result is considered equivocal.

Amplification data analysis for the Internal Control (FAM/Green channel):

1. Activate the **Analysis** button in the menu, select the **Quantitation** analysis mode and activate the **Cycling A. FAM/Cycling A. Green, Show** buttons.

2. Cancel the automatic choice of the threshold line level **Threshold**.
3. Activate the **Dynamic tube** and **Slope Correct** buttons in the main window menu (**Quantitation analysis**).
4. In the **CT Calculation** menu (in the right part of the window), indicate the threshold line level **Threshold = 0.03**.
5. Choose the parameter **More settings/Outlier Removal** and set **10 %** for the value of negative samples threshold (**NTC/Threshold**).
6. In the results grid (**Quantitation Results** window), *Ct* values should appear for each test sample and they should not exceed **30** cycles. The samples are considered negative if the *Ct* values determined in the FAM/Green channel (IC STI-87 detection) do not exceed **30** cycles and *Ct* values are absent in the JOE/Yellow channel (*Toxoplasma gondii* DNA detection). The result is considered invalid if the *Ct* value for IC STI-87 is absent or exceeds **30** and *Ct* value for *Toxoplasma gondii* DNA is absent or exceeds **40**. In this case, the analysis should be repeated for this sample starting from the extraction stage.
7. *Ct* values should not exceed **30** cycles for the Negative Control of Extraction – Negative Control (C–).
8. *Ct* values should be absent for the Negative Control of Amplification (NCA) – DNA-buffer.
9. *Ct* values should not exceed **30** cycles for the Positive Control of Amplification – Positive Control DNA *Toxoplasma gondii* and STI (C+*T.gondii* and STI).

Troubleshooting

1. If any *Ct* value is determined for the Negative Control of amplification (NCA) in the FAM/Green and/or JOE/Yellow channels, it indicates the contamination of reagents or samples. In this case, the results of analysis for all samples are invalid. The analysis for all samples should be repeated and measures for detecting and elimination of contamination source must be taken.
2. If the *Ct* value is absent for the Positive Control of amplification (C+) in the JOE/Yellow channel, the results of analysis for all samples are invalid. PCR should be repeated for all samples.
3. If *Ct* values in the FAM/Green channel (IC) are absent in clinical samples, it indicates improper DNA extraction. For these samples, analysis should be repeated starting from the DNA extraction stage. If the *Ct* value of IC exceeds 30 and *Ct* value of *Toxoplasma gondii* DNA exceeds 40 for the test sample, analysis should be repeated starting from

the DNA extraction stage. High *Ct* values may be obtained due to the loss of DNA during extraction or presence of inhibitors.

AMPLIFICATION AND DATA ANALYSIS USING iCycler iQ and iCycler iQ5 (Bio-Rad, USA) INSTRUMENTS

1. Turn on the instrument and start the iQ5 software.

NOTE: The lamp is to be warmed up during 15 min before starting the experiment.

2. Insert the tubes, strips (part of plate) or plate into the reaction module of the thermocycler and program the instrument.

NOTE: Monitor the tubes. There must not be drops left on the walls of the tubes as falling drops during the amplification process may lead to the signal failure and complicate the results analysis. Don't turn the tubes (strips) upside down while inserting them into the instrument.

Program the thermocycler according to manufacturer's manual

1. Click the **Create New** button in the **Workshop** module to create a new protocol.
2. Set the amplification parameters in the opened window (see Table 2):

Table 2

AmpliSens-1 amplification program

Step	Temperature, °C	Time	Fluorescence detection	Cycle repeats
1	95	15 min	–	1
2	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
3	95	5 s	–	40
	60	30 s	FAM, JOE/HEX	
	72	15 s	–	

NOTE: Several tests with the use of AmpliSens PCR kits can be performed simultaneously within the same PCR run using the unified AmpliSens-1 amplification program (for example, together with the tests for detecting DNA of STI pathogens).

3. Name the new protocol and save it.
4. Create a new plate of samples (**Plate Setup**). Set the scheme of the tubes order in the plate.
5. In the opened window mark all clinical samples as **Unknown**, positive controls as «+», negative controls as «–». For all samples set the fluorescence measurement in 2 channels: HEX-530 and FAM-490.
6. Name the scheme of the tubes order and save it.
7. Click the **Run** button (for iCycler iQ5) or the **Run with selected protocol** button (for

iCycler iQ). Set the volume of the sample as **25 µl**. For iCycler iQ, select **Experimental Plate** to determine the well factor. For iCycler iQ5, both the mode with measurement of well factors by experimental tubes and the mode with fixed well factors (recommended) can be used. Click the **Begin Run** button and save the experiment.

8. Place tubes into the reaction module of the thermocycler and program the instrument.

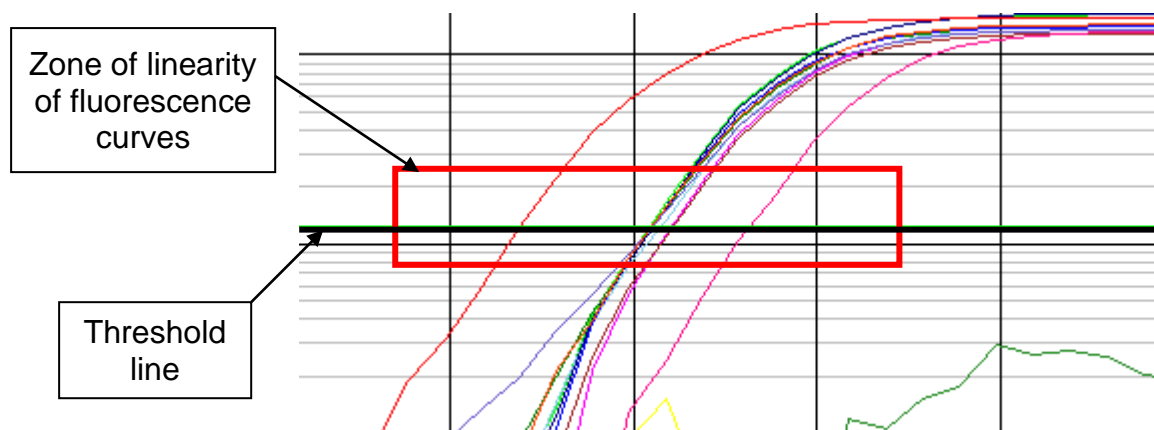
Data analysis

The obtained results (fluorescence accumulation curves for two channels) are analyzed by the instrument software. Accumulation of the amplified product of STI-87 DNA (IC) region is detected in the FAM channel, and accumulation of the amplified product of *Toxoplasma gondii* DNA (C+) is detected in the JOE/HEX channel.

The results are interpreted according to the crossing (or not-crossing) of the fluorescence curve with the threshold line set at the specific level, that corresponds to the presence (or absence) of the *Ct* (threshold cycle) value of the DNA sample in the corresponding column of the results table.

Data processing

1. Start the software and open the saved file: select **Data file** in the **Workshop** module and select the data file. Pass on **Data Analysis** mode.
2. View data separately for each channel.
3. Check the correctness of threshold line automatic choice for each channel. The threshold line is to cross only with sigmoid curves describing the accumulation of the signal detecting positive samples and controls. The threshold line is not to cross the base line. If it happens, the threshold level should be raised. To do this, click the **Log View** and set (with the left mouse button) the threshold line at the level where the fluorescence curves have a linear character and do not cross with the curves of the negative samples (see the figure below).



In the results grid (**Quant. Results** window), *Ct* values for the analyzed channel will

appear.

4. To analyze the results, click the **PCR quant** button (for iCycler iQ) or activate the **Results** button (it is situated under the buttons with the fluorophores' names) (for iCycler iQ5).

Amplification data analysis for *Toxoplasma gondii* DNA (HEX-530 channel):

1. *Ct* values for *Toxoplasma gondii* DNA will appear in the results grid (**Quant. Results** window).
2. *Ct* values should be absent for the Negative Control of Extraction – Negative Control (C–).
3. *Ct* values should be absent for the Negative Control of Amplification (NCA) – DNA-buffer.
4. *Ct* values should not exceed **30** cycles for the Positive Control of Amplification – Positive Control DNA *Toxoplasma gondii* and STI (C+*T.gondii* and STI).
5. Samples are considered positive if *Ct* values do not exceed **40 cycles**. If the *Ct* value exceeds this threshold, the result is considered **equivocal**. It is necessary to repeat the analysis twice. If a reproducible positive *Ct* value is determined twice, the sample is considered **positive**. If irreproducible values are obtained in two repeats, the result is considered equivocal.

Amplification data analysis for the Internal Control (FAM-490 channel):

1. In the results grid (**Quantitation Results** window), *Ct* values should appear for each test sample and they should not exceed **30** cycles. The samples are considered negative if the *Ct* values determined in the FAM-490 channel (IC STI-87 detection) do not exceed **30** cycles and *Ct* values are absent in the HEX-530 channel (*Toxoplasma gondii* DNA detection). The result is considered invalid if the *Ct* value for IC STI-87 is absent or exceeds **30** and *Ct* value for *Toxoplasma gondii* DNA is absent or exceeds **40**. In this case, the analysis should be repeated for this sample starting from the extraction stage.
2. *Ct* values should not exceed **30** cycles for the Negative Control of Extraction – Negative Control (C–).
3. *Ct* values should be absent for the Negative Control of Amplification (NCA) – DNA-buffer (NCA).
4. *Ct* values should not exceed **30 cycles** for the Positive Control of Amplification – Positive Control DNA *Toxoplasma gondii* and STI (C+*T.gondii* and STI).

Troubleshooting

1. If any *Ct* value is determined for the Negative Control of amplification (NCA) in the HEX-530 and/or FAM-490 channels, it indicates the contamination of reagents or samples. In this case, the results of analysis for all samples are invalid. The analysis for all samples should be repeated and measures for detecting and elimination of contamination source must be taken.
2. If the *Ct* value is absent for the Positive Control of amplification (C+), the results of analysis for all samples are invalid. PCR should be repeated for all samples.
3. If *Ct* values in the FAM-490 channel (IC) are absent in clinical samples, it indicates improper DNA extraction. For these samples, analysis should be repeated starting from the DNA extraction stage. If the *Ct* value of IC exceeds 30 and *Ct* value of *Toxoplasma gondii* DNA exceeds 40 for the test sample, analysis should be repeated starting from the DNA extraction stage. High *Ct* values may be obtained due to the loss of DNA during extraction or presence of inhibitors.

AMPLIFICATION AND DATA ANALYSIS USING Mx3000P, Mx3005P (Stratagene, USA) INSTRUMENTS

1. Switch on the instrument and start the Stratagene Mx3000P software.
2. Select **Quantitative PCR (Multiple Standards)** in the **New Experiment Options** window and check the **Turn lamp on for warm-up** box.

NOTE: The lamp is to be warmed up during 15 min before starting the experiment.

3. Insert the tubes into the instrument and close the lid.
4. Select **Optics Configuration** in the **Options** menu and in the **Dye Assignment** tab set JOE/HEX parameter next to the **HEX/JOE filter set** item, parameter FAM next to **FAM filter set**.

NOTE: Don't turn the strips/plate upside down while inserting them into the instrument.

5. Lock the fixing arm and the door of the instrument
6. In the **New Experiment Options** window, select the **Quantitative PCR (Multiple Standards)** item and tick **Turn lamp on for warm-up**.
7. Set fluorescence detection parameters in the **Plate Setup** menu. To do this:
 - Select all wells with the test tubes or strips (hold the Ctrl button down and select the necessary region with the mouse);
 - Mark all selected wells as **Unknown** in the **Well type** window. Tick **FAM** and **JOE** for **Collect fluorescence data** option. Name each sample in the **Well Information** window by double clicking with the mouse. *It is possible to name the samples during*

the amplification or to return to **Plate Setup** menu after amplification ends.

8. Set the parameters for the fluorescence detection of the tubes in the **Plate Setup** tab. Select all the wells with test tubes (hold the Ctrl button and select necessary region by mouse). Click the **Unknown** button in the **Well type** menu, and tick **FAM** and **JOE** in the **Collect fluorescence data** tab. Name tubes in the **Well Information** window by double clicking with the mouse on each well: define Positive Control as «+» and Negative Control as «-».
9. Set the amplification program in the **Thermal Profile Setup** tab. To do this:

Using the template file for setting the amplification program (is recommended).

Click the **Import...** button which is to the right of the depicted thermocycling profile. Proceed to the folder containing previous experimental file and open it. The needed thermocycling profile will appear in the **Thermal Profile** window.

Individual programming

1. After setting all necessary values and parameters, select all wells with the test tubes once again. Proceed to the **Thermal Profile Setup** menu and set the amplification program specified in Table 3.

Table 3

AmpliSens-1 amplification program

Step	Temperature, °C	Time	Fluorescence detection	Cycle repeats
1	95	15 min	–	1
2	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
3	95	5 s	–	40
	60	30 s	FAM, JOE/HEX	
	72	15 s	–	

NOTE: Several tests with the use of AmpliSens PCR kits can be performed simultaneously within the same PCR run using the unified AmpliSens-1 amplification program (for example, together with the tests for detecting DNA of STI pathogens).

2. To set detection parameter of fluorescent signal at desired temperature, select the **All points** option for **Data collection by marker dragging** parameter and move it by mouse from right side to the shelf with desired temperature.
3. Select **Run** and **Start** and name the experiment file.

Data analysis

The obtained results (fluorescence accumulation curves for two channels) are analyzed by the instrument software. Accumulation of the amplified product of STI-87 DNA (IC) region is detected in the FAM channel, and accumulation of the amplified product of *Toxoplasma gondii* DNA (C+) is detected in the JOE/HEX channel.

The results are interpreted according to the crossing (or not-crossing) of the fluorescence curve with the threshold line set at the specific level, that corresponds to the presence (or absence) of the *Ct* (threshold cycle) value of the DNA sample in the corresponding column of the results table.

Data processing

1. Proceed to the **Analysis** mode (select the corresponding button on the toolbar).
2. Make sure that all samples in the **Analysis Selection/Setup** tab are active (wells corresponding to samples should have another color). Otherwise, select all tested samples holding the Ctrl button down and marking the necessary region with the mouse.
3. Proceed to the **Results** tab.
4. Make sure that both HEX and FAM channels are active (**HEX** and **FAM** buttons are pressed in the **Dyes Shown** field at the foot of the window).
5. Select the **Threshold fluorescence** field and make sure that tick marks are put against two fluorescence channels: JOE/HEX and FAM. Check the correctness of the automatically chosen threshold line. Normally, is to cross only with sigmoid¹ curves describing the accumulation of the signal detecting positive samples and controls. The threshold line is not to cross the base line. If it happens, the threshold level should be raised.

Amplification data analysis for *Toxoplasma gondii* DNA (JOE/HEX channel):

1. *Ct* values for *Toxoplasma gondii* DNA will appear in the results grid.
2. *Ct* values should be absent for the Negative Control of Extraction – Negative Control (C–).
3. *Ct* values should be absent for the Negative Control of Amplification (NCA) – DNA-buffer.
4. *Ct* values should not exceed **30** cycles for the Positive Control of Amplification – Positive Control DNA *Toxoplasma gondii* and STI (C+_{*T.gondii*} and STI).
5. Samples are considered positive if *Ct* values do not exceed **40 cycles**. If the *Ct* value

¹ Curves of signal accumulation are displayed linear by default. To change curve from linear to logarithmic, click with the left mouse button on one of axis area (X or Y) and indicate **Scale** next to the **Log** item in **Graph properties** window (Y axis).

exceeds this threshold, the result is considered **equivocal**. It is necessary to repeat the analysis twice. If a reproducible positive *Ct* value is determined twice, the sample is considered **positive**. If irreproducible values are obtained in two repeats, the result is considered equivocal.


Amplification data analysis for the Internal Control (FAM channel):

1. In the results grid (**Quantitation Results** window), *Ct* values should appear for each test sample and they should not exceed **30** cycles. The samples are considered negative if the *Ct* values determined in the FAM channel (IC STI-87 detection) do not exceed **30** cycles and *Ct* values are absent in the JOE/HEX channel (*Toxoplasma gondii* DNA detection). The result is considered invalid if the *Ct* value for IC STI-87 is absent or exceeds **30** and *Ct* value for *Toxoplasma gondii* DNA is absent or exceeds **40**. In this case, the analysis should be repeated for this sample starting from the extraction stage.
2. *Ct* values should not exceed **30** cycles for the Negative Control of Extraction – Negative Control (C–).
3. *Ct* values should be absent for the Negative Control of Amplification (NCA) – DNA-buffer (NCA).
4. *Ct* values should not exceed **30 cycles** for the Positive Control of Amplification – Positive Control DNA *Toxoplasma gondii* and STI (C+*T.gondii* and STI).

Troubleshooting

1. If any *Ct* value is determined for the Negative Control of amplification (NCA) in the JOE/HEX and/or FAM channels, it indicates the contamination of reagents or samples. In this case, the results of analysis for all samples are invalid. The analysis for all samples should be repeated and measures for detecting and elimination of contamination source must be taken.
2. If the *Ct* values in the FAM and JOE/HEX channels are absent for the Positive Control of amplification (C+), the results of analysis for all samples are invalid. PCR should be repeated for all samples.
3. If *Ct* values in the FAM channel (IC) are absent in clinical samples, it indicates improper DNA extraction. For these samples, analysis should be repeated starting from the DNA extraction stage. If the *Ct* value of IC exceeds 30 and *Ct* value of *Toxoplasma gondii* DNA exceeds 40 for the test sample, analysis should be repeated starting from the DNA extraction stage. High *Ct* values may be obtained due to the loss of DNA during extraction or presence of inhibitors.

List of Changes Made in the Instruction Manual

VER	Location of changes	Essence of changes
23.06.11 LA	Cover page	The name of Institute was changed to Federal Budget Institute of Science “Central Research Institute for Epidemiology”
31.08.15 ME	Text	Corrections according to the template
	Footer	REF R-P1(RG,iQ,Mx)-CE-B was deleted
	Amplification and data analysis using Rotor-Gene 3000/6000 (Corbett Research, Australia) instruments; Amplification and data analysis using iCycler iQ and iCycler iQ5 (Bio-Rad, USA) instruments; Amplification and data analysis using Mx3000P, Mx3005P (Stratagene, USA) instruments	The amplification data analysis was supplemented with the description of negative and invalid results
28.12.15 PM	Intended use	The clinical material was specified
27.02.20 PM	Front page	The phrase “Not for use in the Russian Federation” was added
25.12.20 EM	Through the text	The symbol  was changed to NOTE:
01.03.21 KK	—	The name, address and contact information for Authorized representative in the European Community was changed