IVD

For Professional Use Only

GUIDELINES

to AmpliSens® *Yersinia pestis*-FRT PCR kit

for detection of *Yersinia pestis* DNA in human biological material (blood; bubo aspirate, vesicle aspirate, pustule aspirate, carbuncle aspirate; sputum; oropharyngeal swabs; urine; faeces; lymph nodes; liver, spleen, lungs, adrenal, and brain tissues; as well as pathologically modified tissues and organs), animal material (blood, faeces, parenchymal organs, brain tissues, and pathologically changed tissues and organs), fleas, ticks, bird pellets and soil using real-time fluorescence-hybridization detection

AmpliSens®

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INTENDED USE

The guidelines describe the procedure of using **AmpliSens® *Yersinia pestis*-FRT** PCR kit for detection of *Yersinia pestis* DNA in human biological material (blood; bubo aspirate, vesicle aspirate, pustule aspirate, carbuncle aspirate; sputum; oropharyngeal swabs; urine; faeces; lymph nodes; liver, spleen, lungs, adrenal, and brain tissues; as well as pathologically modified tissues and organs), animal material (blood, faeces, parenchymal organs, brain tissues, and pathologically changed tissues and organs), fleas, ticks, bird pellets and soil by the real-time fluorescence-hybridization detection using the following instruments:

- Rotor-Gene 3000, Rotor-Gene 6000 (Corbett Research, Australia),
- Rotor-Gene Q (Qiagen, Germany),
- iCycler iQ, iCycler iQ5 (Bio-Rad, USA).

AMPLIFICATION AND DATA ANALYSIS USING Rotor-Gene 3000/6000 (Corbett Research, Australia) and Rotor-Gene Q (Qiagen, Germany)

When working with Rotor-Gene 3000 one should use the Rotor-Gene Version 6 software and the Rotor-Gene 6000 versions 1.7 (build 67) software or higher for Rotor-Gene 6000 and Rotor-Gene Q instruments.

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

Carry out the sample pretreatment and reaction mixture preparation stages according to the PCR kit *Instruction Manual*. When carrying out the amplification it is recommended to use thin-walled PCR tubes (0.2 ml) with flat caps (e.g. Axygen, USA), or Rotor-Gene PCR tubes (0.1 ml) with caps from the four-pieces-strips (e.g. Corbett Research, Australia; QIAGEN, Germany) (detection through the bottom of the tube).

Programming the thermocycler

1. Switch the instrument on.
2. Insert the tubes into the carousel of the Rotor-Gene 3000/6000/Q instrument (the carousel cells are numbered, the numbers are used for the further programming of the samples' position in the thermocycler). Program the instrument.
3. Press the ***New*** button in the main program menu.
4. In the newly opened window, select the template of the experiment start-up ***Advanced*** and mark ***Dual Labeled Probe/Hydrolysis probes***. Press the ***New*** button.

5. In the newly opened window, select ***36-Well Rotor*** and ***No Domed 0.2 ml Tubes/Locking ring*** attached. Click ***Next***.
6. In the newly opened window, set the operator and select the reaction mixture volume: ***Reaction volume*** – **25 µl**. For Rotor-Gene 6000, put a checkmark next to ***15 µl oil layer volume***. Click ***Next***.
7. In the newly opened window, set the temperature profile of the experiment by pressing the ***Edit profile*** button and set the following parameters (see Table 1):

Table 1

Amplification program

Step	***Temperature, °C***	***Time***	***Fluorescence detection***	***Cycles***
Hold	95	15 min	–	1
Cycling 1	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
Cycling 2	95	5 s	–	40
	60	30 s	FAM/Green, JOE/Yellow	
	72	15 s	–	

8. Press the ***OK*** button.

9. Select the ***Calibrate/Gain Optimisation*** button in the ***New Run Wizard*** window:
   - perform the calibration in FAM/Green, JOE/Yellow and channels (activate the ***Calibrate Acquiring/Optimise Acquiring*** button);
   - perform the calibration in FAM/Green, JOE/Yellow channels before the first detection (activate the ***Perform Calibration Before 1st Acquisition/ Perform Optimisation Before 1st Acquisition*** button);
   - to set channels calibration, indicate **5 FI** in the ***Min Reading*** box and **10 FI** in the ***Max Reading*** box (activate ***Edit…,*** the window ***Auto gain calibration channel settings***).
10. Press the ***Next*** button, start the amplification program by activating the ***Start Run*** button***,*** and name the experiment.
11. Name the experiment and save it on the hard drive (the results of the experiment will be automatically saved in this file).
12. Insert the data into the samples table (opens automatically after the amplification starts). Set names/numbers of tested and control samples in the ***Name*** column. For empty wells, indicate ***None***.

NOTE: Data for samples indicated as ***None*** will not be analysed.

Data analysis

Data are analysed by the real-time PCR instrument software. The interpretation of results is based on the crossing or not crossing of the threshold line by the fluorescence curve that corresponds to the presence or absence of the threshold cycle (Ct) value in the corresponding column of the results grid.

Amplification data analysis for FAM/Green channel (Internal Control STI-87 (IC)):

1. Activate the button ***Analysis*** in the menu, select the mode of the analysis ***Quantitation***, activate the buttons ***Cycling A. FAM/Cycling A. Green***, ***Show***.
2. Cancel the automatic choice of the threshold line level in the main open window by activating the ***Threshold*** button.
3. Activate the ***Dynamic tube*** and the ***Slope Correct*** buttons in the menu of the main window (***Quantitation analysis***).
4. In the ***CT Calculation*** menu (in the right part of the window), indicate the threshold level ***Threshold* = 0.03**, exclude cycles until **5**.
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) one will be able to see the *Ct* values.

Amplification data analysis for JOE/Yellow channel (*Yersinia pestis* DNA):

1. Activate the button ***Analysis*** in the menu, select the mode of the analysis ***Quantitation***, activate the buttons ***Cycling A. JOE/Cycling A. Yellow***, ***Show***.
2. Cancel the automatic choice of the threshold line level in the main open window by activating the ***Threshold*** button.
3. Activate the ***Dynamic tube*** and the ***Slope Correct*** buttons in the menu of the main window (***Quantitation analysis***).
4. In the ***CT Calculation*** menu (in the right part of the window), indicate the threshold level ***Threshold* = 0.03**, exclude cycles until **5**.
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) one will be able to see the *Ct* values.

NOTE: If the fluorescence curves in all channels do not correspond to exponential growth, set the threshold value for negative samples (***NTC threshold***) as **15 %**.

Interpretation of results for control samples

The result of the analysis is considered reliable only if the results obtained for Positive and Negative Controls of amplification as well as for the Negative Control of extraction

are correct (seeTable 2).

Table 2

Results for controls of PCR-analysis (Rotor-Gene 3000/6000/Q)

Control	Stage for control	Signal in channel (*Ct* values)	
		FAM/Green	**JOE/Yellow**
		IC detection	***Y.pestis* detection**
C–	DNA extraction	< 24	Absent
NCA	PCR	Absent	Absent
C+$_{Y.pestis\ /\ STI}$	PCR	< 27	< 23

Interpretation of results for clinical samples

- *Yersinia pestis* DNA is **detected** in a sample if its *Ct* value detected in the result grid in the JOE/Yellow channel is less than the boundary *Ct* value, specified in the *Important Product Information Bulletin*. Moreover, the fluorescence curve of the sample should intercept the threshold line in the area of typical exponential growth of fluorescence.
- *Y.pestis* DNA is **not detected** in a sample if its *Ct* value defined in the result grid in the FAM/Green channel is less than the specified boundary value (see Table 3), whereas the *Ct* value in the JOE/Yellow channel is not defined.
- The result is **invalid** if the *Ct* value of a sample in the JOE/Yellow channel is absent whereas the *Ct* value in the FAM/Green channel is also absent or is greater than the specified boundary *Ct* value. Repeat the PCR test for such a sample.

Table 3

Results for test samples (Rotor-Gene 3000/6000/Q)

PCR-mix-1	Signal in channel (Ct values)	
	FAM/Green	**JOE/Yellow**
PCR-mix-1-FRT *Yersinia pestis*	IC detection	*Y.pestis* detection
	< 27	< 38

Results of analysis are not taken into account in the following cases:

1. If the negative result is obtained for all channels (except NCA), PCR should be repeated. If the same result is obtained again, analysis should be repeated from the extraction stage. Negative result for NCA in all channels is normal.
2. If the *Ct* value of the Positive Control of Amplification (C+$_{Y.pestis\ /\ STI}$) detected in the JOE/Yellow channel is absent or greater than the boundary value, PCR should be repeated for all samples in which specific DNA was not detected.
3. If the *Ct* value of the Negative Control of Extraction (C–) in the JOE/Yellow channel and/or Negative Control of Amplification (NCA) in any channel is detected, the PCR should be repeated for all samples in which DNA was detected.

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

Carry out the sample pretreatment and reaction mixture preparation stages according to the PCR kit *Instruction Manual*. When carrying out the amplification it is recommended to use thin-walled PCR tubes (0.2 ml) with round or optically transparent flat caps, or tubes (0.1 ml) with transparent caps from the eight-pieces-strips (e.g. Axygen, USA) (detection through the cap of the tube).

1. Switch on the instrument and the power supply unit of the optical part of the instrument.

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

2. Start the program iCycler iQ/iQ5.
3. Insert the tubes or strips into the reaction module of the amplifier (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 strips upside down while inserting them into the instrument.

Program the thermocycler only according to the *Instruction Manual* given by the manufacturer of the instrument:

1. Set the plate setup (set the order of the tubes in the reaction chamber and the detection of fluorescent signal).

– For **iCycler iQ5** click the ***Create New*** or ***Edit*** buttons in the ***Selected Plate Setup*** window of the ***Workshop*** module. One can edit the plate setup in the ***Whole Plate loading*** mode. In the ***Select and load Fluorophores*** option, set fluorescence measurement for all tubes in FAM and JOE channels. Set the reaction volume ***Sample Volume*** – **25 µl**, type of the caps ***Seal Type*** – ***Domed Cap****,* and the type of tubes ***Vessel Type – Tubes****.* Save the plate setup by pressing the ***Save&Exit Plate Editing*** button.

– For **iCycler iQ** select the setup of the samples' position in the reaction module by choosing the ***Samples: Whole Plate Loading*** option in the ***Edit Plate Setup*** window of the ***Workshop*** module. Name each sample in the ***Sample Identifier*** window. In the ***Select and load Fluorophores*** option, set fluorescence measurement for all tubes in **FAM-490** and **JOE-530** channels. Save the plate setup: enter the file name (with .pts extension) in the ***Plate Setup Filename*** window and press the ***Save this plate setup*** button (at the top of the display). The

previously used ***Plate Setup*** can be edited: in the ***Library*** window, open ***View Plate Setup***, select the required ***Plate Setup*** (file with .pts extension), and press the ***Edit*** button on the right side. The edited file should be saved before use. Press the ***Run with selected protocol*** button to set the use of the selected plate setup.

2. Set the amplification program (see Table 4).

Table 4

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	30 s	FAM-490/FAM, JOE-530/JOE	
	72	15 s	–	

– For the **iCycler iQ5** instrument, in order to create a protocol, press the ***Create New*** or ***Edit*** button in the ***Selected Protocol*** window of the ***Workshop*** module. Set the amplification parameters and save the protocol by pressing the ***Save&Exit Protocol Editing*** button. For further experiments, the file with this program can be selected in the ***Protocol*** block (protocol files are saved in the ***Users*** folder by default).

– For the **iCycler iQ** instrument, create the amplification program: select the ***Edit Protocol*** option of ***Workshop*** module. Set the amplification parameters in the bottom window (cycle repeats, time, and temperature), in the right window specify the scanning step for the fluorescent signal: ***Cycle 3*** – ***Step 2***. Save the protocol, name the file in the ***Protocol Filename*** window (with .tmo extension), and press the ***Save this protocol*** button (in the upper part of the display). For further experiments, the file with this program can be selected in the ***View Protocol*** tab in the ***Library*** module. Press the ***Run with selected plate setup*** button after selecting or editing the required program to start it.

3. Insert the prepared tubes into the reaction module according to the selected plate setup.

– For the **iCycler iQ5** instrument, before starting the program, ensure that the protocol *(**Selected Protocol***) and the plate setup *(**Selected Plate Setup**)* were selected correctly. Press the ***Run*** button to start the program. For the well factor measurement, select the ***Use Persistent Well Factors*** variant. For amplification

use the same type of plastic as for calibration. Press the ***Begin Run*** button, name the experiment (results of the experiment will be saved in this file automatically), and press the ***OK*** button. Select the type of the caps ***Seal Type*** – ***Domed Cap****,* and the type of the tubes ***Vessel Type – Tubes***.

- For the **iCycler iQ** instrument, before starting the program, ensure that the name of the protocol and the plate setup were selected correct in the ***Run Prep*** window. For the well factor measurement, select the ***Persistent Plate*** variant in the ***Select well factor source*** menu*.* Set the reaction mix volume in the window ***Sample Volume*** – **25 µl**. Press the ***Begin Run*** button to start the program, name the experiment (results of the experiment will be saved in this file automatically), and press the ***OK*** button*.*

After finishing, start data analysis.

Data analysis

The results are interpreted according to the presence (or absence) of the intercept between the S-shaped fluorescence curve and the threshold line (set in the middle of the linear fragment of fluorescence growth of the positive control in the log scale) and shown as the presence (or absence) of the *Ct* (threshold cycle) value in the results grid.

Data processing

- For the **iCycler iQ** instrument, activate the ***View Post-Run Data*** window in the ***Library*** module. In the ***Data Files*** window, select the required file with data of analysis and press the ***Analyse Data*** button. In the ***PCR Quantification*** option (***Select a Reporter*** menu), select the icon of the corresponding channel. The ***PCR Base Line Subtracted Curve Fit*** data analysis mode should be selected (by default). In the ***Threshold Cycle Calculation*** menu, select manual setting the threshold line and automatic baseline calculation. Select the ***Auto Calculated*** in the ***Baseline Cycles*** submenu, select ***User Defined*** in the ***Threshold Position*** submenu. To set the threshold line level, click on it and drag it by the cursor holding the left mouse button down. Press the ***Recalculate Threshold Cycles*** button. ***Ct*** values will appear in the results grid.
- For the **iCycler iQ5** instrument, select the required file with data of analysis (in the ***Data File*** window of the ***Workshop*** module*)* and press the ***Analyze*** button. Select data for the corresponding channel in the module window. Data analysis mode ***PCR Base Line Subtracted Curve Fit*** should be selected (by default).

Amplification data analysis for IC DNA:

1. Press the ***FAM*** button in the ***Data Analysis*** menu.
2. Select the ***Baseline Threshold*** option by pressing the right mouse button on the plot of fluorescence curves accumulation.
3. Select ***User Defined***, ***Select all***, ***Edit Range*** and set ***Start Cycle = 2***, ***Ending Cycle = 20*** in the ***Base Line Cycles*** menu. Select ***User Defined*** and set ***Threshold position = 50*** in the ***Crossing Threshold*** menu. Press the ***OK*** button.
4. In the results grid (***Results*** window), *Ct* values will appear.

Amplification data analysis for *Yersinia pestis* DNA:

1. Press the ***JOE*** button in the ***Data Analysis*** menu.
2. Select the ***Baseline Threshold*** option by pressing the right mouse button on the plot of fluorescence curves accumulation.
3. Select ***User Defined***, ***Select all***, ***Edit Range*** and set ***Start Cycle = 2***, ***Ending Cycle = 10*** in the ***Base Line Cycles*** menu. Select ***User Defined*** and set ***Threshold position = 100*** in the ***Crossing Threshold*** menu. Press the ***OK*** button.
4. In the results grid (***Results*** window), *Ct* values will appear.

Interpretation of results for control samples

The result of the analysis is considered reliable only if the results obtained for positive and negative controls of amplification as well as for the positive and negative controls of DNA extraction are correct (see Table 5).

Table 5

Results for controls (for iCycler iQ and iCycler iQ5 instruments)

Control	Stage for control	Signal in channel (*Ct* values)	
		FAM-490/FAM	JOE-530/JOE / HEX
		IC detection	*Y.pestis* detection
C–	DNA extraction	< 28	Absent
NCA	PCR	Absent	Absent
C+*Y.pestis* / STI	PCR	< 31	< 30

Interpretation of results for clinical samples

- *Y.pestis* DNA is **detected** in a sample if its *Ct* value is defined in the results grid in the JOE-530/JOE/HEX channel as less than the specified boundary *Ct* value (see Table 6). Herewith, the fluorescence curve should intercept the threshold line in the area of characteristic exponential fluorescence growth.
- *Y.pestis* DNA is **not detected** in a sample if its *Ct* value is defined in the result grid in the FAM-490/FAM channel as less than the specified boundary *Ct* value (see Table 6), whereas the *Ct* value in the JOE-530/JOE/HEX channel is not defined.

— The result is **invalid** if the *Ct* value of a sample in the JOE-530/JOE/HEX channel is absent and the *Ct* value in the FAM-490/FAM channel is also absent or is greater than the specified boundary *Ct* value. In such cases, repeat the PCR analysis for corresponding sample.

Table 6

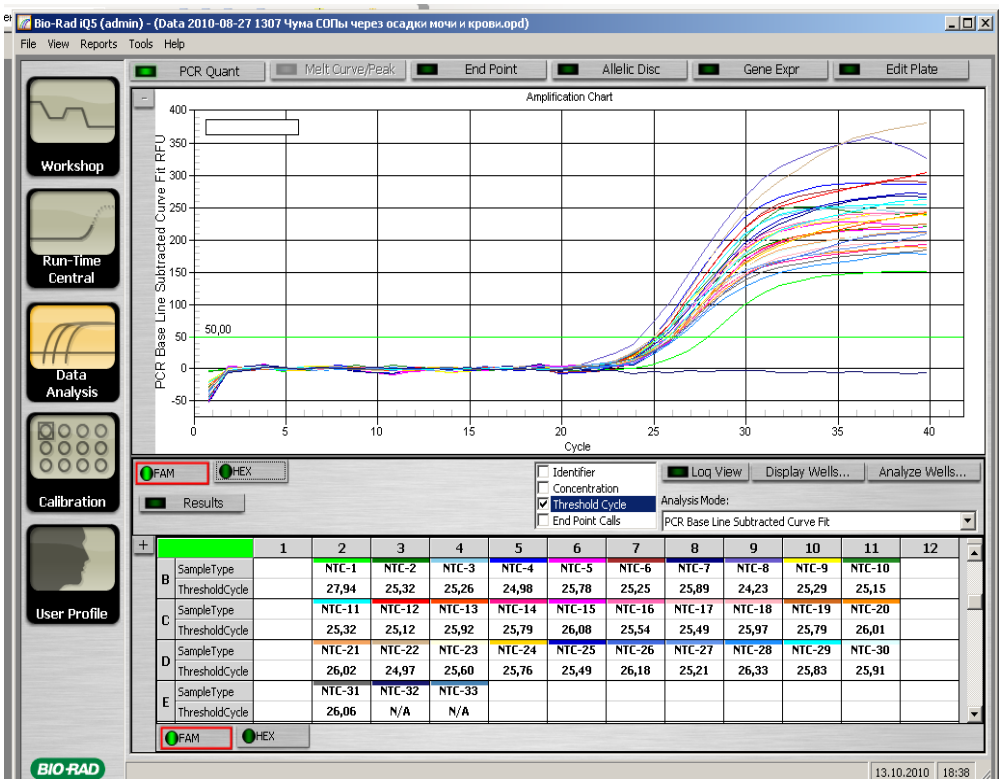
Results for test samples (iCycler iQ and iCycler iQ5 instruments)

PCR-mix-1	**Signal in channel (*Ct* values)**	
	FAM-490/FAM	**JOE-530/JOE / HEX**
PCR-mix-1-FRT *Yersinia pestis*	IC detection	*Y.pestis* detection
	< 31	< 39

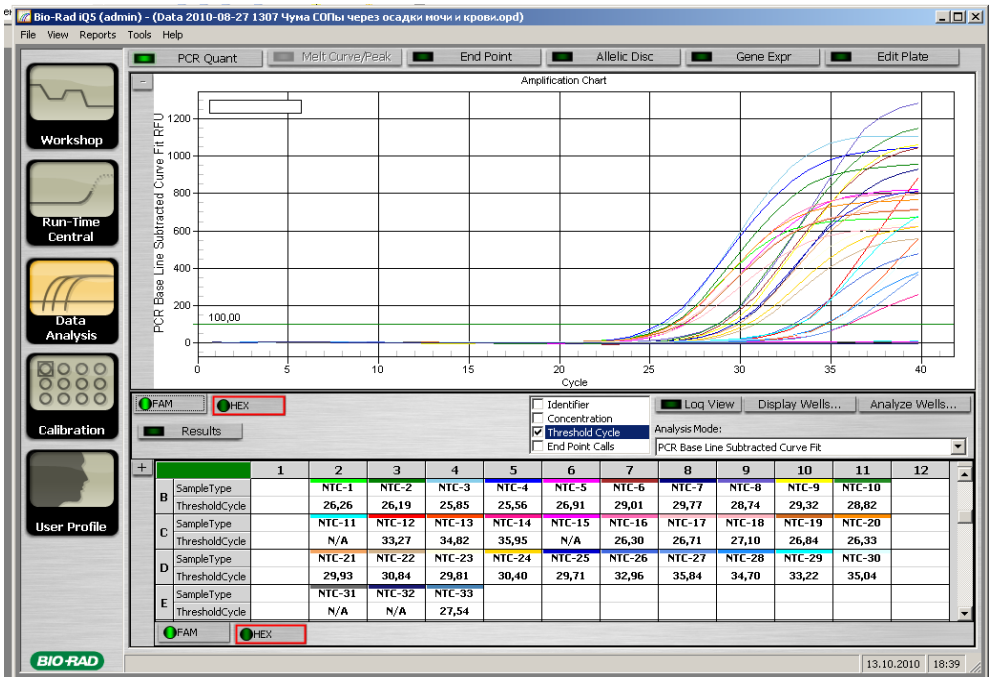
Results of analysis are not taken into account in the following cases:

1. If the negative result is obtained for all channels (except NCA), PCR should be repeated. If the same result is obtained again, analysis should be repeated from the extraction stage. Negative result for NCA is normal.
2. If the *Ct* value of the Positive Control of Amplification (C+$_{Y.pestis\ /\ STI}$) detected in the JOE-530/JOE/HEX channel is absent or is greater than the boundary *Ct* value, PCR should be repeated for all samples in which specific DNA was not detected.
3. If the *Ct* value of the Negative Control of Extraction (C–) in the JOE-530/JOE/HEX channel and/or Negative Control of Amplification (NCA) in any channel is detected, the PCR should be repeated for all samples in which DNA was detected.

Example of amplification with the use of iCycler iQ5 instrument



		1	2	3	4	5	6	7	8	9	10	11	12
B	SampleType		NTC-1	NTC-2	NTC-3	NTC-4	NTC-5	NTC-6	NTC-7	NTC-8	NTC-9	NTC-10	
	ThresholdCycle		27,94	25,32	25,26	24,98	25,78	25,25	25,89	24,23	25,29	25,15	
C	SampleType		NTC-11	NTC-12	NTC-13	NTC-14	NTC-15	NTC-16	NTC-17	NTC-18	NTC-19	NTC-20	
	ThresholdCycle		25,32	25,12	25,92	25,79	26,08	25,54	25,49	25,97	25,79	26,01	
D	SampleType		NTC-21	NTC-22	NTC-23	NTC-24	NTC-25	NTC-26	NTC-27	NTC-28	NTC-29	NTC-30	
	ThresholdCycle		26,02	24,97	25,60	25,76	25,49	26,18	25,21	26,33	25,83	25,91	
E	SampleType		NTC-31	NTC-32	NTC-33								
	ThresholdCycle		26,06	N/A	N/A								



		1	2	3	4	5	6	7	8	9	10	11	12
B	SampleType		NTC-1	NTC-2	NTC-3	NTC-4	NTC-5	NTC-6	NTC-7	NTC-8	NTC-9	NTC-10	
	ThresholdCycle		26,26	26,19	25,85	25,56	26,91	29,01	29,77	28,74	29,32	28,82	
C	SampleType		NTC-11	NTC-12	NTC-13	NTC-14	NTC-15	NTC-16	NTC-17	NTC-18	NTC-19	NTC-20	
	ThresholdCycle		N/A	33,27	34,82	35,95	N/A	26,30	26,71	27,10	26,84	26,33	
D	SampleType		NTC-21	NTC-22	NTC-23	NTC-24	NTC-25	NTC-26	NTC-27	NTC-28	NTC-29	NTC-30	
	ThresholdCycle		29,93	30,84	29,81	30,40	29,71	32,96	35,84	34,70	33,22	35,04	
E	SampleType		NTC-31	NTC-32	NTC-33								
	ThresholdCycle		N/A	N/A	27,54								

List of Changes Made in the Instruction Manual

VER	Location of changes	Essence of changes
28.12.20 MA	Through the text	The symbol  was changed to NOTE:
29.12.20 MA	Cover page	The phrase “Not for use in the Russian Federation” was added
12.03.21 MM	Front page	The name, address and contact information for Authorized representative in the European Community was changed