RUO

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®

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

For research use only. Not for diagnostic procedures

TABLE OF CONTENTS

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 polymerase chain reaction (PCR) with 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).

Correspondence of names of fluorophores and detection channels

Channel for the fluorophore	Detection channel name for different instrument models[1]
FAM	FAM/Green
JOE	JOE/HEX/R6GYellow/Cy3

[1] The detection channels names in each section of the guidelines are specified in accordance with the described instrument.

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 6.1 and higher 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/Q.

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 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. Turn on the instrument, run the Rotor-Gene software.
2. Insert the tubes or strips into the rotor of the Rotor-Gene 3000/6000/Q instrument (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: Well 1 must be filled with any test tube except for an empty one.

3. Program the instrument according to the *Instruction Manual* given by the manufacturer of the instrument.

Creating the template for the run

1. Press the ***New*** button in the main program menu. To create the template select the ***Advanced*** tab in the opened window ***New run***.
2. Select the ***TwoStep/Hidrolysis Probes*** template in the tab for edition and click the ***New*** button.
3. In the opened window, select ***36-Well Rotor (or 72-Well Rotor)*** and tick the ***No Domed 0.2 ml Tubes/Locking ring*** attached. Click the ***Next*** button.
4. In the opened window enter the operator name, select the reaction volume – **25 µl**. Tick the ***15 µl oil layer volume***. Click the ***Next*** button.
5. In the ***New Run Wizard*** window set the temperature profile of the experiment. To do this click the ***Edit profile*** button and set the amplification program:

Amplification program

Step	*Temperature, °C*	*Time*	*Fluorescence detection*	*Number of 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	–	

6. After setting up the temperature profile, click the ***OK*** button.
7. Click the ***Calibrate/Gain Optimisation…*** button in the ***New Run Wizard*** window. In the opened window:
   a) for signal measurement optimisation for the selected channels set calibration from 5Fl to 10Fl for the FAM/Green, JOE/Yellow channels. To do this, click the ***Calibrate Acquiring/Optimise Acquiring*** button. In the opened window for first channel (***Auto Gain Optimisation Channel Settings/Auto Gain Calibration Channel Settings***) indicate the values of minimum and maximum signal in the ***Target Sample Range*** line. Click the ***OK*** button. The window for the next channel will open automatically. The selected values for all the channels can be checked in the ***Min Reading, Max Reading*** boxes.
   b) perform the calibration in the selected channels before the first detection (tick the ***Perform Calibration Before 1st Acquisition/ Perform Optimisation Before 1st Acquisition*** option). Click the ***Close*** button.
8. Click the ***Next*** button. For saving the programmed template it is necessary to click the ***Save Template*** button and enter the template file name, corresponding to the amplification program. Save the file into a proposed folder: ***Templates\Quick Start Templates***; close the ***New Run Wizard*** window***.*** After that the programmed template will appear in the template list in the ***New Run*** window.

Using the ready template for the run

1. Click the ***Next*** button in the software main menu. In the opened ***New Run*** window select the ***Advanced*** tab. Then select the **AmpliSens** template (which is programmed as described in the “Creating the template for the run” section) in the template list.
2. In the opened window select the ***36-Well Rotor (or 72-Well Rotor)*** and tick the ***No Domed 0,2ml Tubes / Locking Ring Attached*** option. Click the ***Next*** button.
3. In the opened window check that the reaction volume is **25 µl** and the ***15 µl oil layer volume*** option is activated. Click the ***Next*** button.

4. In the next window the correctness of the amplification program and signal level auto-optimisation parameters can be checked. Go to the next window clicking the ***Next*** button. Start the amplification by the ***Start run*** button. Herewith, the rotor with the samples should be already fixed and the lid should be closed. Name the experiment and save it to the disc (the results of the experiment will be automatically saved in this file).
5. Enter the data into the grid of the samples (it opens automatically after the amplification has been started). Enter the names/numbers of the test samples in the ***Name*** column. Define the Negative control of amplification as NCA, the Positive control of amplification as C+. Set the type ***Unknown*** opposite all the test samples, the type ***Positive control*** – for the Positive control of amplification, the type ***Negative control*** – for the Negative control of amplification. Set the type ***Standard*** for DNA-calibrators C1 and C2 and enter the values from the *Important product information bulletin* enclosed to the given lot of the PCR kit into the ***Given conc.*** column. Set the type ***None*** for the cells matching with the corresponding empty tubes. Click the ***Finish/OK*** button.

NOTE: Data for samples indicated as ***None*** won't be analysed.

Note – To edit the table of samples before the start it is needed previously to select the ***Edit Samples Before Run Started*** option in the ***User preferences*** submenu of the ***File*** menu.

Data analysis

The obtained results are analyzed by the Rotor-Gene software. The results are interpreted according to the crossing (or not-crossing) of the S-shaped (sigmoid) 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 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 ***Threshold***.
3. Select the ***Linear scale***.
4. Activate the ***Dynamic tube*** and the ***Slope Correct*** buttons in the menu of the main window (***Quantitation analysis***).
5. In the ***CT Calculation*** menu (in the right part of the window), indicate the threshold level ***Threshold* = 0.03** in the ***Threshold*** box.

6. Set ***5*** in the ***Eliminate cycles before:*** menu (in the right part of the window).
7. Choose the parameter ***More settings/Outlier Removal*** and set **10 %** for the value of negative samples threshold (***NTC/Threshold***).
8. In the results grid (***Quantitation Results*** window) one will be able to see the *Ct* values.

Results analysis in the JOE/Yellow channel is carried out similarly to results analysis in the FAM/Green channel in accordance with the settings in the table below.

Channel	*Threshold*	*Dynamic tube*	*Slope Correct*	*More Settings/Outlier Removal*	*Eliminate Cycles before*
FAM/Green	0.03	on	on	10%	5
JOE/Yellow	0.03	on	on	10%	5

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 %**.

Results interpretation

The result of the PCR analysis is considered reliable only if the results for the controls of the amplification and the extraction are correct in accordance with the table of assessment of results for controls (see the *Instruction manual*) and boundary values specified in the *Important Product Information Bulletin* enclosed to the PCR kit.

The interpretation of the test samples is to be carried out in accordance with the *Instruction Manual* and the *Important Product Information Bulletin* enclosed to the PCR kit.

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 domed or flat optically transparent caps, or tubes (0.2 ml) with transparent caps from the eight-pieces-strips (e.g. Axygen, USA) (detection through the cap of the tube).

Programming the thermocycler

1. Turn on the instrument and the power supply unit of the optical block 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 according to the *Instruction Manual* given by the manufacturer of 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.

Creating the template for the run

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 1).

Table 1

Amplification program

Step	***Temperature, °C***	***Time***	***Fluorescence detection***	***Number of 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.

Using the ready template for the run

The test parameters and the plate setup set earlier can be used for the further runs. To do this:

- select the needed file with the run in the upper left window of the ***Workshop*** module;
- click the ***Edit*** button in the ***Selected Plate Setup*** area of the ***Workshop*** module and edit the plate setup (the files of protocols are saved in the ***SampleFiles*** folder by default);
- click the ***Edit*** button in the ***Selected Protocol*** area of the ***Workshop*** module and check the correctness of the selected protocol (the files of protocols are saved in the ***SampleFiles*** folder by default).

Data analysis

The obtained results are analyzed by the iCycler iQ5 / iQ software. The results are interpreted according to the crossing (or not-crossing) of the S-shaped (sigmoid) fluorescence curve with the threshold line set at the specific level, that corresponds to the presence (or absence) of the *Ct* (threshold cycle) value in the corresponding column of the results table.

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:

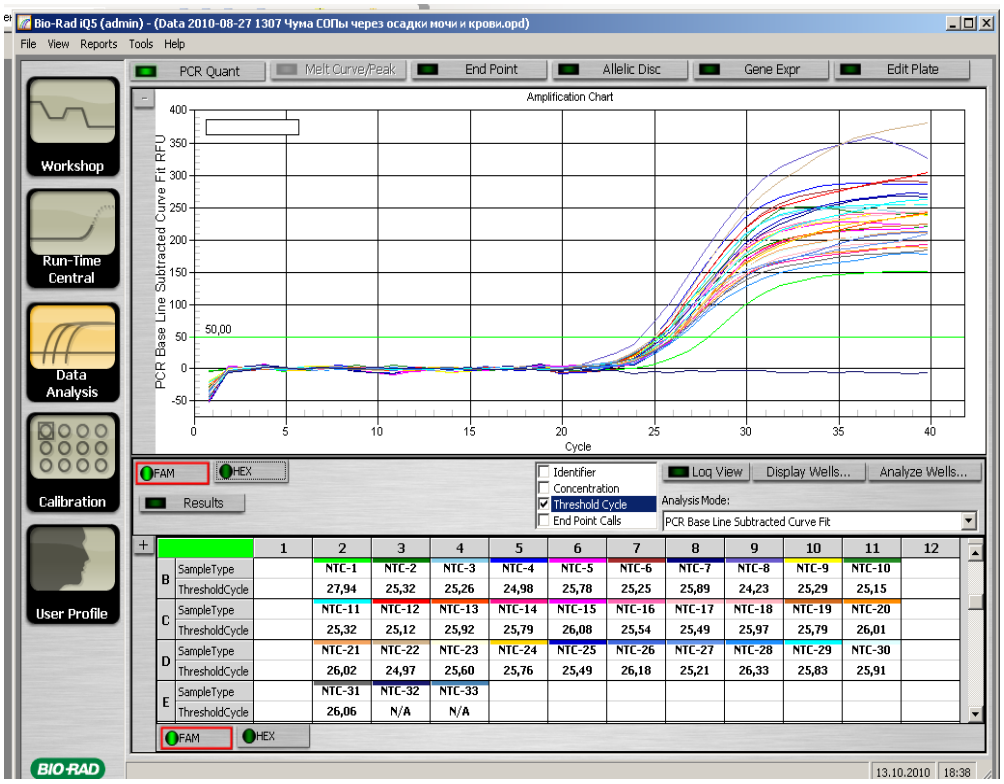
1. Press the ***JOE*** button in the ***Data Analysis*** menu.
2. Select the ***Baseline Threshold*** option by pressing the right mouse button on the plot of fluorescence curves accumulation.
3. Select ***User Defined***, ***Select all***, ***Edit Range*** and set ***Start Cycle = 2***, ***Ending Cycle = 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.

Results interpretation

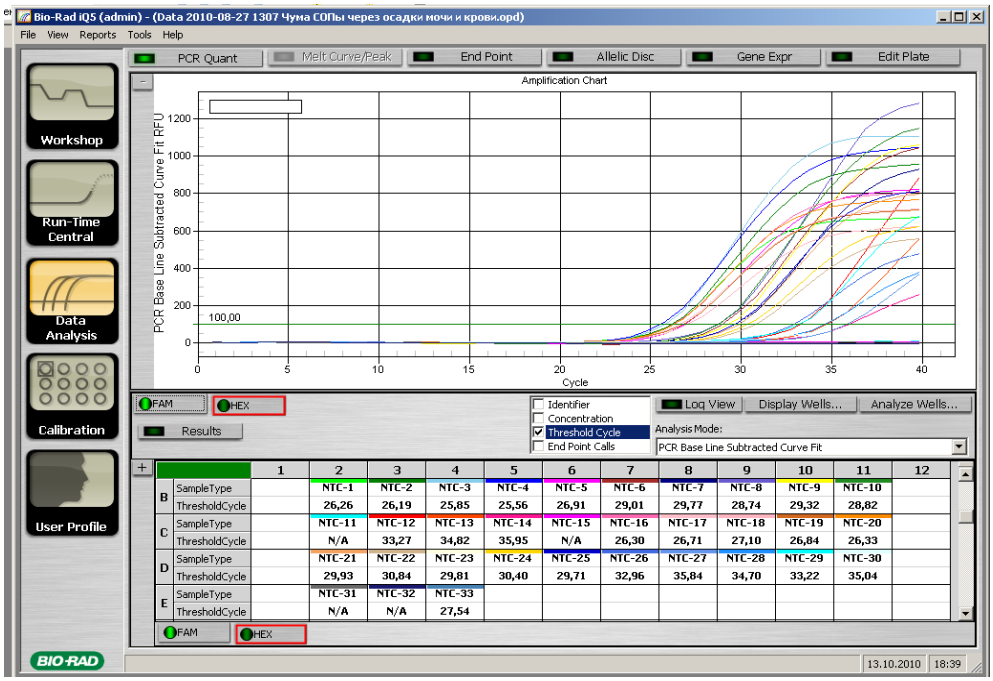
The result of the PCR analysis is considered reliable only if the results for the controls of the amplification and the extraction are correct in accordance with the table of assessment of results for controls (see the Instruction manual) and boundary values specified in the *Important Product Information Bulletin* enclosed to the PCR kit.

The interpretation of the test samples is to be carried out in accordance with the Instruction Manual and the *Important Product Information Bulletin* enclosed to the PCR kit.

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 Guidelines

VER	Location of changes	Essence of changes
05.12.24 HM	Through the text	Corrections in accordance to the template