GUIDELINES

to AmpliSens® *Bordetella* multi-FRT PCR kit

for qualitative detection and differentiation of genome specific fragments of pathogens causing pertussis (*Bordetella pertussis*), parapertussis (*Bordetella parapertussis*), and *Bordetella bronchiseptica* infection (*Bordetella bronchiseptica*) in the clinical material by the polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection

AmpliSens®

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INTENDED USE

The guidelines describe the procedure of using **AmpliSens®** *Bordetella* **multi-FRT** PCR kit for qualitative detection and differentiation of genome specific fragments of pathogens causing pertussis (*Bordetella pertussis*), parapertussis (*Bordetella parapertussis*), and *Bordetella bronchiseptica* infection (*Bordetella bronchiseptica*) in the clinical material (swabs taken from the mucous membrane of inferior nasal meatus and posterior pharyngeal wall, cultures of microorganisms) 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);

− Rotor-Gene Q (QIAGEN, Germany);

− iCycler iQ, iQ5 (Bio-Rad, USA);

− CFX96 (Bio-Rad, USA)

and also in combination with the automatic station for the nucleic acids extraction NucliSENS easyMAG (bioMérieux, France).

WORK with the NucliSENS easyMAG AUTOMATED NUCLEIC ACID EXTRACTION SYSTEM

The volume of clinical material for DNA extraction – **100 µl**.

Variant 1

DNA extraction with lysis of sample outside of the instrument (off-board mode)

This method of extraction allows reducing the consumption of NucliSens lysis buffer. It is preferred for working with clinical samples which contain clots (sputum, aspirates).

1. Switch on the NucliSENS easyMAG instrument and prepare it to the DNA extraction according to the instruction manual.

2. In the window for input of test samples enter the following parameters:

   - Sample name

   - ***Matrix*** for RNA/DNA extraction (select ***Other***)

   - ***Volume*** – 0.1 ml

   - ***Eluate*** – 25 µl

   - ***Type*** – Lysed

   - ***Priority*** – Normal.

3. Create a new protocol of DNA extraction and save it. In the protocol select ***On-board Lysis Buffer Dispensing – no, On-board Lysis Incubation – no.***

4. Relocate sample table into the created protocol.

5. Take the required number of special disposable tubes intended for DNA extraction in

the NucliSENS easyMAG instrument (including negative control of extraction). Add **10 µl of Internal Control STI-87** to inner walls of each tube and then add **550 µl** of **NucliSens lysis buffer.**

NOTE:	When working with material which contains clots, lysis should be carried out in 1.5-ml tubes. After finishing the incubation (see item 8), tubes should be centrifuged at 10,000 rpm for 1 min. Then transfer the supernatant into special tubes intended for RNA/DNA extraction in the NucliSENS easyMAG instrument.

6. Add **100 µl** of the prepared samples into each tube with **Lysis buffer** and **Internal Control STI-87** using tips with aerosol barriers and carefully mix by pipetting (avoid adding mucus clots and large particles to the tube).

7. Add **100 µl** of **Negative Control** into the tube with Negative Control of Extraction (C-).

8. Incubate tubes for 10 min at room temperature.

9. Resuspend the tube with **magnetic silica NucliSens** by intensive vortexing. Add **25 µl** of **magnetic silica** using single tips with aerosol barriers and carefully mix by pipetting. Magnetic silica should be distributed evenly throughout the tube volume.

10. Place tubes with samples into the instrument, insert the tips, and start the DNA extraction program with lysis of samples by selecting the ***off board*** mode.

11. After finishing DNA extraction take the tubes out of the instrument. The supernatant contains purified DNA.

If necessary to store, transfer purified DNA into sterile tubes within 30 min after DNA extraction.

The purified DNA can be stored at 2-8 °C for 1 week, at the temperature ≤ −16 °C for 1 year, and at the temperature ≤ − 68 °C for a longer period of time.

Variant 2

DNA extraction with automated lysis of sample in the instrument (on-board mode)

1. Switch on the NucliSENS easyMAG instrument and prepare it to the DNA extraction according to the instruction manual.

2. In the window for input of test samples enter the following parameters:

Sample name

Matrix for RNA/DNA extraction (select ***Other***)

Volume – 0.1 ml

Eluate – 25 µl

Type – Primary

Priority – Normal.

3. Create a new protocol of DNA extraction and save it. Select ***On-board Lysis Buffer***

Dispensing – Yes, *On-board Lysis Incubation – Yes* in the protocol.

4. Relocate the sample table into the created protocol.

5. Take the required number of special disposable tubes intended for DNA extraction in the NucliSENS easyMAG instrument (including negative control of extraction). Add **10 µl of Internal Control STI-87** to inner walls of each tube.

6. Add **100 µl** of the **prepared samples** into each tube with **Internal Control STI-87** using tips with aerosol barriers (avoid adding mucus clots and large particles to the tube).

7. Add **100 µl** of **Negative Control** into the tube intended for the **Negative Control of Extraction (C–).**

8. Place tubes with samples into the instrument, insert the tips, and start the DNA extraction program with lysis of samples by selecting the *on board* mode.

9. Wait until NucliSENS easyMAG instrument proceeds to the *Instrument State - Idle* option and pauses.

10. Thoroughly vortex the tube with **magnetic silica NucliSens**. Open the lid of the instrument and transfer **25 µl of magnetic silica** into each tube using single tip with aerosol barrier and mix thoroughly by pipetting. Make sure that magnetic silica is evenly distributed throughout the tube.

11. Close the lid and continue the DNA extraction program.

12. After the extraction is completed, take the tubes out of the instrument. The supernatant contains purified DNA.

If necessary to store, transfer purified DNA into sterile tubes within 30 min after DNA extraction.

The purified DNA can be stored at 2-8 °C for 1 week, at the temperature ≤ –16 °C for 1 year, and at the temperature ≤ – 68 °C for a longer period of time.

AMPLIFICATION AND DATA ANALYSIS USING Rotor-Gene 3000, Rotor-Gene 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. **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 working with Rotor-Gene 3000, Rotor-Gene 6000 and Rotor-Gene Q instruments, it is recommended to use 0.2-ml clear tubes with flat caps (detection through the bottom of the tube) or 0.1 ml tubes.

Insert the tubes into the rotor so that the first tube is in No. 1 well (the rotor cells are numbered and these numbers are used for programming the sample order), insert the rotor in the reaction module, and secure the lid.

<u>Programming the thermocycler</u>

1. Click the **New** button in the main program menu.
2. In the opened window, select **Advanced** menu and **Dual Labeled Probe/Hydrolysis probes (TaqMan)**. Click the **New** button.
3. Select **36-Well Rotor** (or **72-Well Rotor**) and **No Domed 0.2 ml Tubes/Locking ring attached**. Click **Next**.
4. Select the operator and set the **Reaction volume** as **25 µl**. Tick the **15 µl oil layer volume** for Rotor-Gene 6000. Click **Next**.
5. Select the **Edit profile** button and set the temperature profile of the experiment (see Table 1).

Table 1

Amplification program for *Bordetella* multi-FRT

Step	Temperature, °C	Time	Fluorescence detection	Cycles
1	95	15 min	–	1
2	95	10 s	–	10
	60	20 s	–	
	72	10 s	–	
3	95	10 s	–	35
	60	20 s	FAM/Green, JOE/Yellow, ROX/Orange, Cy5/Red	
	72	10 s	-	

6. Click **OK**.
7. Select the **Calibrate/Gain Optimisation** button in the **New Run Wizard** window:

– perform the fluorescence detection in FAM/Green, JOE/Yellow, ROX/Orange and Cy5/Red channels (activate the **Calibrate Acquiring/Optimise Acquiring** button);

– perform the calibration before the first detection (activate the **Perform Calibration Before 1st Acquisition/ Perform Optimisation Before 1st Acquisition** button);

– set channel calibration from **5FI** to **10FI** for all dyes (the **Edit** button of the **Auto gain calibration channel settings**). Click **Close**.

8. Click **Next,** then start the amplification program by activating the **Start Run** button.

9. Name the experiment and save it to the disk (results of the run will be automatically saved in this file).

10. Enter the data into the grid of the samples (it opens automatically after the amplification has been started). Indicate the names/numbers of clinical and control samples in the **Name** column. Set the **None** type for empty wells.

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

Data analysis

Data analysis in the FAM/Green channel (Internal Control)

1. Activate the **Analysis** button, then select **Quantitation** button and activate the **Cycling A. FAM**/**Cycling A. Green** button. Click **Show**.

2. Cancel the **Threshold** automatic choice.

3. Activate the **Dynamic tube** button in the main window menu (**Quantitation analysis**).

4. In **CT Calculation** menu (in the right part of the window) set **Threshold = 0.1.**

5. Choose the parameter **More settings/Outlier Removal** and set **0 %** for the value of negative samples threshold (**NTC/Threshold**).

6. In the results grid (the **Quant. Results** window) one will be able to see the *Ct* values.

Data analysis in the JOE/Yellow channel (*Bordetella*)

1. Activate the **Analysis** button, then select the **Quantitation** button and activate the **Cycling A. JOE/Cycling A. Yellow** button. Click **Show**.

2. Cancel the **Threshold** automatic choice.

3. Activate the **Dynamic tube** button in the main window menu (**Quantitation analysis**).

4. In **CT Calculation** menu (in the right part of the window) set **Threshold = 0.1.**

5. Choose the parameter **More settings/Outlier Removal** and set **5 %** for the value of negative samples threshold (**NTC/Threshold**).

6. In the results grid (the **Quant. Results** window) one will be able to see the *Ct* values.

Data analysis in the ROX/Orange channel (*Bordetella pertussis*)

1. Activate the *Analysis* button, then select *Quantitation* button and activate the *Cycling A. ROX* / *Cycling A. Orange* button. Click *Show*.
2. Cancel the *Threshold* automatic choice.
3. Activate the *Dynamic tube* button in the main window menu (*Quantitation analysis*).
4. In *CT Calculation* menu (in the right part of the window) set *Threshold = 0.1.*
5. Choose the parameter *More settings/Outlier Removal* and set **5 %** for the value of negative samples threshold (*NTC/Threshold*).
6. In the results grid (the *Quant. Results* window), one will be able to see the *Ct* values.

Data analysis in the Cy5/Red (*Bordetella bronchiseptica*)

1. Activate the *Analysis* button, then select *Quantitation* button and activate the *Cycling A. Cy5*/*Cycling A. Red* button. Click *Show*.
2. Cancel the *Threshold* automatic choice.
3. Activate the *Dynamic tube* button in the main window menu (*Quantitation analysis*).
4. In *CT Calculation* menu (in the right part of the window) set *Threshold = 0.1.*
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.

Results interpretation

The results are interpreted by the software of the instrument by the crossing (or not crossing) of the fluorescence curve in each channel with the threshold line set at the specific level that corresponds to presence (or absence) of the *Ct* (threshold cycle) value in the results grid.

The principle of interpretation is the following:

The curves of fluorescence signal accumulation are analyzed in four channels (FAM/Green, JOE/Yellow, ROX/Orange and Cy5/Red):

Detection channel	FAM/Green	JOE/Yellow	ROX/Orange	Cy5/Red
Reaction	IC detection	Detection of pertussis toxin gene, *ptxA*	Identification of *Bordetella pertussis*	Identification of *Bordetella bronchiseptica*

The results of the analysis are considered reliable only if the results obtained for both positive and negative controls of amplification as well as for the negative control of extraction are correct (see Table 2).

Table 2

Results for controls

Control	Stage for control	Ct value			
		FAM/Green	JOE/Yellow	ROX/Orange	Cy5/Red
		IC detection	Detection of Pertussis toxin gene, *ptxA*	Identification of *Bordetella pertussis*	Identification of *Bordetella bronchiseptica*
C–	DNA extraction	<28	absent	absent	absent
NCA	PCR	absent	absent	absent	absent
CS+	PCR	<26	absent	absent	absent
C+	PCR	absent	<25	<25	<26

Interpretation of results of PCR analysis for detection and differentiation of pathogens that cause pertussis (*Bordetella pertussis*), parapertussis (*Bordetella parapertussis*), and Bordetella bronchiseptica infection (*Bordetella bronchiseptica*) is based on amplification results in accordance with Table 3.

Table 3

Interpretation of results of PCR-analysis for clinical samples

Vari-ants	FAM/Green	JOE/Yellow	ROX/Orange	Cy5/Red	Result
		Ct value			
1	absent or >27	absent or >25	absent	absent	**Invalid**
2	<27	absent	absent	absent	*B.pertussis* *B.parapertussis* *B.bronchiseptica* **are NOT detected**
3	≤35 or absent	≤35	≤35	absent	*B.pertussis* DNA **is detected**
4	≤35 or absent	≤35	absent	≤35	*B.bronchiseptica* DNA **is detected**
5	≤35 or absent	<25	absent	absent	*B.parapertussis* DNA **is detected**
6	<27	>25	absent	absent	*Bordetella* spp. DNA **is detected**: *B.pertussis,* or *B.parapertussis,* or *B.bronchiseptica.* For species differentiation, repeat sampling.
7	<27	absent	present	absent	If the result is repeated in PCR, interpret as **equivocal**
8	<27	absent	absent	present	If the result is repeated in PCR, interpret as **equivocal**

− *Bordetella pertussis*, *Bordetella parapertussis* and *Bordetella bronchiseptica* are **not detected** in a sample if the *Ct* value in the **JOE/Yellow, ROX/Orange**, and **Cy5/Red**

channel is not determined (fluorescence curve does not cross the threshold line), whereas *Ct* value determined in the **FAM/Green** channel (detection of the Internal Control) does not exceed 27.

- *Bordetella pertussis* DNA is **detected** in a sample if the *Ct* value of a sample determined in the results grid in the **JOE/Yellow** and **ROX/Orange** channels does not exceed 35, and the PCR fluorescent curve should have typical exponential increase of fluorescent signal. In such samples, *Ct* determined in the results grid in the channel for detection of internal control (IC) (**FAM/Green**) can have any value or be absent in case of high pathogen load in a sample.

- *B.bronchiseptica* DNA is **detected** in a sample if the *Ct* value of a sample determined in the results grid in the **JOE/Yellow** and **Cy5/Red** channels does not exceed 35, and the PCR fluorescent curve should have typical exponential increase of fluorescent signal. In such samples, *Ct* determined in the results grid in the channel for detection of internal control (IC) (**FAM/Green**) can have any value or be absent in case of high pathogen load in a sample.

- *B.parapertussis* DNA is **detected** in a sample if the *Ct* value of a sample determined in the results grid in the **JOE/Yellow** channel does not exceed 25, and *Ct* values in the **ROX/Orange** and **Cy5/Red** channels are absent. In such samples, *Ct* determined in the results grid in the channel for detection of internal control (IC) (**FAM/Green**) can have any value or be absent in case of high pathogen load in a sample.

- *Bordetella* spp. (*B.pertussis,* or *B.parapertussis,* or *B.bronchiseptica*) DNA is **detected** if *Ct* value of a sample determined in the results grid in the **JOE/Yellow** channel exceed 25, *Ct* values in the **ROX/Orange** and **Cy5/Red** channels are absent, *Ct* in the channel for detection of internal control (IC) (**FAM/Green**) does not exceed 27. That can take place if the quantity of extracted DNA is insufficient for species differentiation. If the species differentiation is necessary, repeat sampling.

- If *Ct* value of a sample is absent in the result grid in the **JOE/Yellow** channel, whereas *Ct* values are determined in the **ROX/Orange** and **Cy5/Red** channels and the *Ct* value in the **FAM/Green** channel (detection of IC) is less than 27, then repeat the PCR analysis for this sample. If the same result is repeated, the result is considered **equivocal** and sampling of clinical material should be repeated.

- Results should be interpreted as **invalid** if the *Ct* value in the **ROX/Orange** and **Cy5/Red** channels is absent, in the **JOE/Yellow** channel – is absent or exceed 25, and in the channel for detection of internal control (IC) (**FAM/Green**) – is either absent or exceed 27. In this case, repeat nucleic acid extraction step for the required clinical

sample.

- If *Ct* value for the Positive control of amplification (C+) in the respective channel is absent or exceeds the boundary value (see Table 2), repeat amplification for all negative clinical samples.

- If the *Ct* value is present for the Negative Control of extraction (C–) and/or Negative Control of amplification (NCA) in the **JOE/Yellow, ROX/Orange** and **Cy5/Red** channels, eliminate a source of possible contamination and repeat the analysis for all positive samples to rule out the consequence of possible contamination.

AMPLIFICATION AND DATA ANALYSIS USING iCycler iQ and iQ5 (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).

1. Switch on the instrument and the power supply unit of the optical part of the instrument. The lamp is to be warmed up during 30 min before starting the experiment.
2. Start the iCycler iQ or iQ5 program.
3. Set the plate setup (set the order of the tubes in the reaction chamber and the detection of fluorescent signal in the **FAM, JOE/HEX, ROX** and **Cy5** channels for all tubes).
    − **iCycler iQ5**. Click the *Create New* or *Edit* button in the *Selected Plate Setup* window of the *Workshop* module. Edit the plate setup in the *Whole Plate loading* mode. Set the *Sample Volume* as *25 µl*, *Seal Type* as *Domed Cap*, and *Vessel Type* as *Tubes*. Click *Save &Exit Plate Editing*.
    − **iCycler iQ**. Edit the plate setup in the *Edit Plate Setup* window of the *Workshop* module. To do this, set the order of the tubes in the reaction chamber in the *Samples: Whole Plate Loading* option and indicate the name of each sample in the *Sample Identifier* window. Set fluorescent signal detection in **FAM, JOE**, **ROX** and **Cy5** channels for all tubes in the *Select and Load Fluorophores* option. Save the plate setup. To do this, name the file in the *Plate Setup Filename* window (use the .pts extension) and activate the *Save This Plate Setup* button (in the upper part of the screen). It is possible to edit the *Plate Setup* used earlier. To do this, open the *View Plate Setup* in the *Library* window, select the appropriate *Plate Setup* (a file with .pts extension), and activate the *Edit* button. Prior to using, save the edited file. Select *Run with selected protocol* to use the plate setup.
4. Set the amplification program for plate-type instruments (see Table 4).

Table 4

Amplification program for *Bordetella* multi-FRT

Step	Temperature, °C	Time	Fluorescence detection	Cycles
1	95	15 min	–	1
2	95	10 s	–	10
	60	25 s	–	
	72	25 s	–	
3	95	10 s	–	35
	60	25 s	FAM, JOE/HEX, ROX, Cy5	
	72	25 s	–	

- **iCycler iQ5**. To enter an amplification program, select the *Create New* or *Edit* button in the *Selected Protocol* window of the *Workshop* module. Enter parameters of amplification and save the protocol by clicking the *Save&Exit Protocol Editing* button. This file can be used for further runs if selected from the *Protocol* block (by default protocol files are saved in the *Users* folder).

- **iCycler iQ**. To enter an amplification program, select the *Edit* option of the *Workshop* module. Set the parameters of amplification (cycles numbers, temperature, and time) in the bottom window and specify *Cycle 3 - Step 2* in the right window. To save the protocol, name the file in the *Protocol Filename* window and click the *Save this protocol* button (at the top of the screen). This file can be used in further runs if selected from the *View Protocol* bookmark of the *Library* module. Click the *Run with selected plate setup* button to assign the program.

5. Insert the tubes into the reaction module of the amplifier in accordance with plate setup specified.

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.

- **iCycler iQ5**. Ensure the correctness of the *Selected Protocol* and *Selected Plate Setup* before starting the program. To start the program, click *Run.* For detection of the well factor, select *Collect Well Factors from Experimental Plate*. Click the *Begin Run* button. Name the experiment (the results of the experiment will be automatically saved in this file) and click *OK*.

- **iCycler iQ**. Ensure the correctness of the selected protocol and plate setup in the *Run Prep* window. For the detection of the well factor select *Experimental Plate* under the *Select well factor source* line. Set the reaction mix volume as **25 µl**. Press *Begin Run* to start. Name the experiment (the results of the experiment will be automatically saved to this file) and click *OK* button

6. Proceed to the results analysis at the end of the program.

Data analysis

The results are interpreted by the software of the instrument by the crossing (or not crossing) of the fluorescence curve with the threshold line set at the specific level that corresponds to the presence (absence) of the *Ct* (cycle threshold) value in the results grid.

- 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 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 of the threshold line and automatic baseline calculation. Select the **Auto Calculated** in the **Baseline Cycles** submenu, select **User Defined** in the **Threshold Position** submenu. Click on it and then drag it by holding the left mouse button down.

− For the **iCycler iQ5** instrument, select the required file with data for 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). To set the threshold line level, click on it and then drag it by holding the left mouse button down. Press the **Results** button to display the results grid.

− Set the threshold line for the **FAM** channel (by pressing the left mouse button down and dragging) at a level corresponding to **20 %** of the maximum fluorescence level recorded for the positive control samples, C+, during the last amplification cycle. The fluorescent curve of C+ should cross the threshold line on the interval of exponential growth of fluorescence coming into the linear growth phase.

− Set the threshold line sequentially for the **JOE/HEX**, **ROX** and **Cy5** channels (by pressing the left mouse button down and dragging) at a level corresponding to **10 %** of the maximum fluorescence level obtained for the positive control samples, C+, during the last amplification cycle. The fluorescent curve of C+ should cross threshold line on the interval of exponential growth of fluorescence coming into the linear growth phase.

− Press the **Recalculate Threshold Cycles** button. *Ct* values will appear in the results grid.

Results interpretation

The principle of interpretation is the following:

The curves of fluorescence signal accumulation are analyzed in four channels (FAM, JOE/HEX, ROX and Cy5):

Detection channel	FAM	JOE/HEX	ROX	Cy5
Reaction	IC detection	Detection of pertussis toxin gene, *ptxA*	Identification of *Bordetella pertussis*	Identification of *Bordetella bronchiseptica*

The result of the analysis is considered reliable only if the results obtained for both positive and negative controls of amplification as well as for the negative control of

extraction are correct (see Table 5).

<div align="right">Table 5</div>

Results for controls

Control	Stage for control	Ct value			
		FAM	**JOE/HEX**	**ROX**	**Cy5**
		IC	**Pertussis toxin gene, *ptxA***	***Bordetella pertussis***	***Bordetella bronchiseptica***
C–	DNA extraction	<30	absent	absent	absent
NCA	Amplification	absent	absent	absent	absent
CS+	Amplification	<27	absent	absent	absent
C+	Amplification	absent	<28	<26	<26

Interpretation of results of PCR analysis for detection and differentiation of pathogens that cause pertussis (*Bordetella pertussis*), parapertussis (*Bordetella parapertussis*), and Bordetella bronchiseptica infection (*Bordetella bronchiseptica*) is based on amplification results in accordance with Table 6.

<div align="right">Table 6</div>

Interpretation of results of PCR-analysis for clinical samples

Variants	FAM	JOE/HEX	ROX	Cy5	Result
	Ct value				
1	absent or >29	absent or >25	absent	absent	**Invalid**
2	<29	absent	absent	absent	*B.pertussis* *B.parapertussis* *B.bronchiseptica* **are NOT detected**
3	≤35 or absent	≤35	≤35	absent	*B.pertussis* DNA **is detected**
4	≤35 or absent	≤35	absent	≤35	*B.bronchiseptica* DNA **is detected**
5	≤35 or absent	<25	absent	absent	*B.parapertussis* DNA **is detected**
6	<29	>25	absent	absent	*Bordetella* spp. DNA **is detected**: *B.pertussis,* or *B.parapertussis,* or *B.bronchiseptica.* For species differentiation, repeat sampling and extraction are needed.
7	<29	absent	present	absent	If the result is repeated in PCR, interpret as **equivocal**
8	<29	absent	absent	present	If the result is repeated in PCR, interpret as **equivocal**

– *Bordetella pertussis*, *Bordetella parapertussis* and *Bordetella bronchiseptica* are **not**

detected in a sample if the *Ct* value in the **JOE/HEX, ROX**, and **Cy5** channel is not determined (fluorescence curve does not cross the threshold line), whereas *Ct* value determined in the **FAM** channel (detection of the Internal Control) does not exceed 29.

- *Bordetella pertussis* DNA is **detected** in a sample if the *Ct* value of a sample determined in the results grid in the **JOE/HEX** and **ROX** channels does not exceed 35, and the PCR fluorescent curve should have typical exponential increase of fluorescent signal. In such samples, *Ct* determined in the results grid in the channel for detection of internal control (IC) (**FAM**) can have any value or be absent in case of high pathogen load in a sample.

- *B.bronchiseptica* DNA is **detected** in a sample if the *Ct* value of a sample determined in the results grid in the **JOE/HEX** and **Cy5** channels does not exceed 35, and the PCR fluorescent curve should have typical exponential increase of fluorescent signal. In such samples, *Ct* determined in the results grid in the channel for detection of internal control (IC) (**FAM**) can have any value or be absent in case of high pathogen load in a sample.

- *B.parapertussis* DNA is **detected** in a sample if the *Ct* value of a sample determined in the results grid in the **JOE/HEX** channel does not exceed 25, and *Ct* values in the **ROX** and **Cy5** channels are absent. In such samples, *Ct* determined in the results grid in the channel for detection of internal control (IC) (**FAM**) can have any value or be absent in case of high pathogen load in a sample.

- *Bordetella* spp. (*B.pertussis,* or *B.parapertussis,* or *B.bronchiseptica*) DNA is **detected** if *Ct* value of a sample determined in the results grid in the **JOE/HEX** channel exceed 25, *Ct* values in the **ROX** and **Cy5** channels are absent, *Ct* in the channel for detection of internal control (IC) (**FAM**) does not exceed 29. That can take place if the quantity of extracted DNA is insufficient for species differentiation. If the species differentiation is necessary, repeat sampling.

- If *Ct* value of a sample is absent in the result grid in the **JOE/HEX** channel, whereas *Ct* values are determined in the **ROX** and **Cy5** channels and the *Ct* value in the **FAM** channel (detection of IC) is less than 29, then repeat the PCR analysis for this sample. If the same result is repeated, the result is considered **equivocal** and sampling of clinical material should be repeated.

- Results should be interpreted as **invalid** if the *Ct* value in the **ROX** and **Cy5** channels is absent, in the **JOE/HEX** channel – is absent or exceed 25, and in the channel for detection of internal control (IC) (**FAM**) - is either absent or exceed 29. In this case, repeat nucleic acid extraction step for the required clinical sample.

- If *Ct* value for the Positive control of amplification (C+) in the respective channel is absent or exceeds the boundary value (see Table 5), repeat amplification for all negative clinical samples.
- If the *Ct* value is present for the Negative Control of extraction (C–) and/or Negative Control of amplification (NCA) in the **JOE/HEX, ROX** and **Cy5** channels, eliminate a source of possible contamination and repeat the analysis for all positive samples to rule out the consequence of possible contamination.

AMPLIFICATION AND DATA ANALYSIS USING CFX96 (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 (detection through the cap of the tube).

Programming the instrument:

1. Turn on the instrument and start the **Bio-Rad CFX Manager** program.
2. Program the instrument according to the *Instruction Manual* provided by the manufacturer.

Creating the template for a run

1. Select **Create a new Run/Experiment** in the **Startup Wizard** start window (or select **New** and then **Run…/Experiment…** in the **File** menu).
2. In the **Run Setup** window, select **Protocol** and click the **Create new…** button. Set amplification parameters (time, temperature, cycles, and fluorescence acquiring cycle) in the opened **Protocol Editor – New** window (see Table 7). Set **Sample Volume – 25 µl.**

Table 7

Amplification program for plate-type instruments

Step	Temperature, °C	Time	Fluorescence detection	Cycles
1	95	15 min	–	1
2	95	10 s	–	10
	60	25 s	–	
	72	25 s	–	
3	95	10 s	–	35
	60	25 s	FAM, HEX, ROX, Cy5	
	72	25 s	–	

NOTE! Set **Ramp Rate 2,5 °C/s** by clicking the **Step Options** button for each step of cycling.

3. In the **Protocol Editor New** window select **File**, then **Save As**, and name the protocol. This protocol can be used for further runs by clicking the **Select Existing…** button in the **Protocol** tab.

   When the required program is entered or edited, click **OK** at the bottom of the window.

4. Set the plate scheme. In the **Plate** tab click the **Create new…** button. Set the tube order in the opened **Plate Editor – New** window. Click the **Select Fluorophores…** button and tick the **FAM, HEX, ROX** fluorophores in the **Selected** column. Select

Unknown for all samples in the **Sample type** menu. Then indicate with a checkmark the fluorescence signal acquiring for all samples in the required channels in the **Load** column (in the right part of the window). Define sample names in the **Sample name** window and confirm each name by the **Load** button.

5. Save the plate scheme. In the **Plate Editor New** window select **File**, then **Save As**, and name the plate, click **Save**.

6. Select the **Start Run** tab. Open the instrument lid by clicking **Open Lid.** Place the reaction tubes in the wells of the instrument in accordance with the entered plate setup. Close the lid by the **Close Lid** button.

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.

7. Run the amplification program with the specified plate scheme by the **Start Run** button. Proceed to the analysis of results after the end of the run. Select the directory for saving the file of the run. Name the file. Click **Save.**

Using the existing template for a run

For the further runs one can use the parameters and plate scheme set earlier. To do this:

– Click the **Select Existing…** button in the **Run setup** window of the **Protocol** tab. In the **Select Protocol** window select the needed file with amplification program. Click **Open**;

– In the **Run Setup** window go to the **Plate** tab and click **Select Existing…** In the **Select Plate** window select the needed file with plate scheme, click Open. One can edit the scheme by clicking the **Edit selected** button.

<u>**Data analysis**</u>

The results are interpreted by the real-time PCR instrument software according to the crossing (or not-crossing) of the S-shaped (sigmoid) fluorescence curve with the threshold line set at the specific level and shown as the presence (or absence) of the *Ct* (threshold cycle) value in the results grid.

1. Run the program, open the saved file with analysis data. To do this select **File** in the menu, then **Open** and **Data file** and select the needed file.

2. The fluorescence curves, plate setup, and results grid with *Ct* values are displayed in the **Quantification** tab. Make sure that the threshold line was set correctly for each channel. Use one of the following variants:

For each channel at a time set the threshold line (drag it with a cursor while pressing the left mouse button) at the level of 10-20 % of maximum fluorescence obtained for the

Positive Controls in the last amplification cycle. Make sure that fluorescence curve of the Positive Control crosses the threshold line at the zone of exponential growth of fluorescence passing onto linear growth.

3. Click **Tools** on the toolbar, then **Reports…**, and then save the generated report.

The result of the analysis is considered reliable only if the results obtained for both positive and negative controls of amplification as well as for the negative control of extraction are correct (see Table 8).

Table 8

Results for controls

Control	Stage for control	Ct value			
		FAM	HEX	ROX	Cy5
		IC	Pertussis toxin gene, *ptxA*	*Bordetella pertussis*	*Bordetella bronchiseptica*
C−	DNA extraction	<30	absent	absent	absent
NCA	Amplification	absent	absent	absent	absent
CS+	Amplification	<27	absent	absent	absent
C+	Amplification	absent	<28	<26	<26

Interpretation of results of PCR analysis for detection and differentiation of pathogens that cause pertussis (*Bordetella pertussis*), parapertussis (*Bordetella parapertussis*), and Bordetella bronchiseptica infection (*Bordetella bronchiseptica*) is based on amplification results in accordance with Table 9.

Table 9

Interpretation of results of PCR-analysis for clinical samples

Variants	FAM	HEX	ROX	Cy5	Result
	Ct value				
1	absent or >29	absent or >25	absent	absent	**Invalid**
2	<29	absent	absent	absent	*B.pertussis B.parapertussis B.bronchiseptica* **are NOT detected**
3	≤35 or absent	≤35	≤35	absent	*B.pertussis* DNA **is detected**
4	≤35 or absent	≤35	absent	≤35	*B.bronchiseptica* DNA **is detected**
5	≤35 or absent	<25	absent	absent	*B.parapertussis* DNA **is detected**
6	<29	>25	absent	absent	*Bordetella* spp. DNA **is detected**: *B.pertussis,* or *B.parapertussis,* or *B.bronchiseptica*. For species differentiation, repeat sampling and extraction are needed.
7	<29	absent	present	absent	If the result is repeated in PCR, interpret as **equivocal**
8	<29	absent	absent	present	If the result is repeated in PCR, interpret as **equivocal**

− *Bordetella pertussis*, *Bordetella parapertussis* and *Bordetella bronchiseptica* are **not detected** in a sample if the *Ct* value in the **HEX, ROX**, and **Cy5** channel is not determined (fluorescence curve does not cross the threshold line), whereas *Ct* value determined in the **FAM** channel (detection of the Internal Control) does not exceed 29.

− *Bordetella pertussis* DNA is **detected** in a sample if the *Ct* value of a sample determined in the results grid in the **HEX** and **ROX** channels does not exceed 35, and the PCR fluorescent curve should have typical exponential increase of fluorescent signal. In such samples, *Ct* determined in the results grid in the channel for detection of internal control (IC) (**FAM**) can have any value or be absent in case of high pathogen load in a sample.

− *B.bronchiseptica* DNA is **detected** in a sample if the *Ct* value of a sample determined in the results grid in the **HEX** and **Cy5** channels does not exceed 35, and the PCR fluorescent curve should have typical exponential increase of fluorescent signal. In such samples, *Ct* determined in the results grid in the channel for detection of internal control (IC) (**FAM**) can have any value or be absent in case of high pathogen load in a

sample.

- *B.parapertussis* DNA is **detected** in a sample if the *Ct* value of a sample determined in the results grid in the **HEX** channel does not exceed 25, and *Ct* values in the **ROX** and **Cy5** channels are absent. In such samples, *Ct* determined in the results grid in the channel for detection of internal control (IC) (**FAM**) can have any value or be absent in case of high pathogen load in a sample.

- *Bordetella* spp. (*B.pertussis,* or *B.parapertussis,* or *B.bronchiseptica*) DNA is **detected** if *Ct* value of a sample determined in the results grid in the **HEX** channel exceed 25, *Ct* values in the **ROX** and **Cy5** channels are absent, *Ct* in the channel for detection of internal control (IC) (**FAM**) does not exceed 29. That can take place if the quantity of extracted DNA is insufficient for species differentiation. If the species differentiation is necessary, repeat sampling.

- If *Ct* value of a sample is absent in the result grid in the **HEX** channel, whereas *Ct* values are determined in the **ROX** and **Cy5** channels and the *Ct* value in the **FAM** channel (detection of IC) is less than 29, then repeat the PCR analysis for this sample. If the same result is repeated, the result is considered **equivocal** and sampling of clinical material should be repeated.

- Results should be interpreted as **invalid** if the *Ct* value in the **ROX** and **Cy5** channels is absent, in the **HEX** channel – is absent or exceed 25, and in the channel for detection of internal control (IC) (**FAM**) - is either absent or exceed 29. In this case, repeat nucleic acid extraction step for the required clinical sample.

- If *Ct* value for the Positive control of amplification (C+) in the respective channel is absent or exceeds the boundary value (see Table 8), repeat amplification for all negative clinical samples.

- If the *Ct* value is present for the Negative Control of extraction (C–) and/or Negative Control of amplification (NCA) in the **HEX, ROX** and **Cy5** channels, eliminate a source of possible contamination and repeat the analysis for all positive samples to rule out the consequence of possible contamination.

List of Changes Made in the Guidelines

VER	Location of changes	Essence of changes
19.12.14 ME	Intended use	CFX96 instrument was added
	Amplification and data analysis using Rotor-Gene 3000, Rotor-Gene 6000 (Corbett Research, Australia) and Rotor-Gene Q (QIAGEN, Germany); Amplification and data analysis using iCycler iQ and iQ5 (Bio-Rad, USA)	Corrections in accordance with the template. The description of results interpretation was corrected in accordance with table "Interpretation of results of PCR-analysis for clinical samples"
	Amplification and data analysis using CFX96 (Bio-Rad, USA)	The section was added
29.04.15 PM	Work with the NucliSENS easyMAG automated nucleic acid extraction system	The chapter was added
18.02.16 ME	Intended use	The type of clinical material was indicated
	Amplification and data analysis using Rotor-Gene 3000, Rotor-Gene 6000 (Corbett Research, Australia) and Rotor-Gene Q (QIAGEN, Germany)	The boundary Ct values in the ROX/Orange and Cy5/Red channels was deleted from the description of equivocal sample interpretation
	Amplification and data analysis using iCycler iQ and iQ5 (Bio-Rad, USA)	
	Amplification and data analysis using CFX96 (Bio-Rad, USA)	
29.12.20 KK	Through the text	The symbol ⚠ was changed to NOTE:
	Cover page	The phrase "Not for use in the Russian Federation" was added
17.03.21 VA	Front page	The name, address and contact information for Authorized representative in the European Community was changed