GUIDELINES

to AmpliSens® *Yersinia enterocolitica /*

Y.pseudotuberculosis-FRT

PCR kit

for qualitative detection and differentiation of DNA of virulent and avirulent strains of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* strains in environmental samples and clinical material by the polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection

AmpliSens®

Not for use in the Russian Federation

TABLE OF CONTENTS

INTENDED USE

The guidelines describe the procedure of using **AmpliSens® *Yersinia enterocolitica / Y.pseudotuberculosis*-FRT** PCR kit for qualitative detection and differentiation of DNA of virulent and avirulent *Yersinia enterocolitica* strains (virulence is assessed by genes encoding enterotoxin (*Yst*), attachment invasion locus (*ail*), and plasmid pYV adhesion (*yadA*)) and *Yersinia pseudotuberculosis* strains in environmental samples and clinical material 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),

− CFX96 (Bio-Rad, USA);

− iCycler iQ5 (Bio-Rad, USA),

− Mx3000P (Stratagene, USA).

Correspondence of fluorophores' and detection channels' names

Channel for the fluorophore	Detection channel name for different instrument models[1]
FAM	FAM/Green
JOE	JOE/HEX/R6G/Yellow/Cy3
ROX	ROX/Orange/TxR

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

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 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 transparent 0.2-ml PCR tubes with flat caps (detection through the bottom of the tube) or 0.1-ml tubes.

Programming the thermocycler

1. Turn on the instrument.

2. Insert the tubes or strips into the rotor of the Rotor-Gene 3000/6000/Q instrument

---

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

NOTE: Well No 1 must be loaded with a test tube from the current experiment. If the tubes containing different PCR-mixes are placed in the rotor simultaneously, the first two wells should be loaded with the tubes containing **PCR-mix-1-FEP/FRT** *Yersinia enterocolitica* **type***.*

3. Click the **New** button in the main software menu.

4. Select the reaction mixture volume and the rotor type:

   Reaction volume – 25 µl

   Rotor type – 36-Well Rotor or **72-Well Rotor** according to the rotor type.

5. Set the amplification program. To do this, press the **Edit profile** button.

Table 1

Amplification program for rotor-type instruments

Step	Temperature, °C	Time	Number of cycles
1	95	15 min	1
2	95	10 s	45
	60	25 s *fluorescent signal detection*	
	72	10 s	

Detection of fluorescent signal is enabled in the channels for the FAM, JOE, and ROX fluorophores (if different tests are carried out simultaneously in the same run, other channels can be used as well).

6. Set calibration parameters (activate **Calibrate/Gain Optimisation…** in a new experiment module**)**:

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

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

   – set the channels calibrations from 5 Fl to 10Fl for all fluorophores (activate **Edit…, Auto gain calibration channel settings** window). Press the **Close** button.

NOTE: If tubes with several types of reaction mixtures are simultaneously loaded in the rotor, tubes with **PCR-mix-1-FEP/FRT** *Yersinia enterocolitica* **type** should be placed in the first two wells.

7. Start the amplification program by activating the **Start Run** button**,** and name the experiment.

8. During or after amplification, it is necessary to program the order of test samples, negative control of extraction, positive and negative controls of amplification. Enter the

data in the grid of samples (it opens automatically after the amplification has been started). Indicate the names/numbers of tested samples in the **Name** column. Indicate Positive Control of amplification as **C+;** Negative Control of Amplification as **NCA**. Set the **Unknown** type next to all test samples, the **Positive Control** type next to the Positive Controls, the **Negative Control** type next to the Negative Control of Extraction, and the **NTC** type next to the Negative Control of Amplification. For empty wells, indicate **None**.

Data analysis

NOTE: Data for each type of reaction mixtures simultaneously loaded in the rotor are analyzed separately.

1. Activate the **Analysis** button in the menu, then select the **Quantitation** analysis mode and press the **Cycling A. FAM / Cycling A. Green, Show, Cycling A. JOE / Cycling A. Yellow**, **Show** or **Cycling A. ROX / Cycling A. Orange, Show** buttons.

2. Cancel the automatic choice of the threshold line level **Threshold**.

3. Activate the **Dynamic tube** and **Slope Correct** buttons in the menu of main window (**Quantitation analysis**) and set **More settings** as **10 %**.

4. In the **CT Calculation** menu (in the right part of the window), indicate the threshold line level **Threshold** as **0.05**.

5. In the results grid (the **Quant. Results** window), *Ct* values for each tested sample will appear. *Ct* values for test samples are taken into account only if the results obtained for control samples C−, C+, and NCA are correct according to boundary *Ct* values specified in the *Important Product Information Bulletin* for the PCR kit.

6. Data for the test samples are analyzed according to the *Instruction manual* and boundary *Ct* values specified in the *Important Product Information Bulletin* for the PCR kit.

AMPLIFICATION AND DATA ANALYSIS USING iCycler 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 0.2-ml PCR tubes with domed caps (for example, Axygen, USA).

Programming the thermocycler

1. Turn on the instrument and optical module at least 20–30 min before measurement.
2. Click the **Create new** button in the **Workshop** module.
3. Set the amplification program (**Protocol**):

Table 2

Amplification program for plate-type instruments

Step	Temperature, °C	Time	Number of cycles
1	95	15 min	1
2	95	10 s	45
	60	25 s *fluorescent signal detection*	
	72	10 s	

Name the new protocol and save it.

4. Set the order of samples in the plate (**Plate**), select channels for fluorophores FAM and JOE/HEX (**Select/add fluorophores)** and click the **Fluorophore loading in Whole Plate mode** button to activate fluorophores for the samples in the created protocol. Set **Sample Volume - 25 µl, Seal Type – Domed Cap**, **Vessel Type – Tubes.** Save the created protocol by pressing the **Save/Exit Plate Editing** button.
5. Add reagents for amplification and DNA samples into the tubes and place the latter into the instrument.
6. Press the **Run** button. In the opened window, set **Use Persistent Well Factors,** press the **Begin Run** button, and save the experiment.

Data analysis

1. Data are analyzed in the FAM, HEX, and ROX channels.
2. Activate the **Data Analysis** button in the menu.
3. Select the **Base Line** in the range of **20-25** in the **Crossing Threshold User Defined** menu. Normally, the threshold line should cross only sigmoid curves of signal accumulation of positive samples and controls and should not cross the curves of different shapes. Otherwise, the threshold level should be raised.
4. *Ct* values for test samples are taken into account only if the results obtained for control samples C–, C+, and NCA are correct according to the *Instruction manual* and *Important Product Information Bulletin* for the PCR kit.
5. *Ct* values should be absent for the Negative Control of extraction (C–) (except for the

result in the FAM channel when using PCR-mix-1-FEP/FRT *Y. enterocolitica / Y.pseudotuberculosis*).

6. The results for test samples are analyzed according to the *Instruction manual* and boundary *Ct* values specified in the *Important Product Information Bulletin* for the PCR kit. **Sample is considered positive** if the *Ct* value does not exceed the boundary *Ct* value specified in the *Important Product Information Bulletin* for the PCR kit.

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 (e.g. Axygen, USA) (detection through the cap of the tube).

Programming the thermocycler

1. Turn on the instrument and start the Bio-Rad CFX Manager software.

2. Program the instrument according to the *Instruction Manual* provided by the manufacturer.

Creating the template for the run

1. In the **Startup Wizard** window it is necessary to select the **Create a new Run/Experiment** (or select **New** in the **File** menu and then select **Run…/Experiment**…). Click **OK**.

2. In the **Run Setup** window, select **Protocol** tab and click the **Create new…**. Set the amplification parameters in the opened **Protocol Editor – New** window. Set **Sample Volume – 25 μl.**

Amplification program for plate-type instruments

Step	Temperature, °C	Time	Number of cycles
1	95	15 min	1
2	95	10 s	45
	60	25 s *fluorescent signal detection*	
	72	10 s	

NOTE: Set **Ramp Rate 2,5 °C/s** by clicking the **Step Options** button for each step of cycling (see the figure below). Click **OK**.

```
    1    95,0  C  for  15:00
 →  2    95,0  C  for  0:10
         Slow Ramp Rate to   2,5  C per second
    3    60,0  C  for  0:25
         + Plate Read
         Slow Ramp Rate to   2,5  C per second
    4    72,0  C  for  0:10
         Slow Ramp Rate to   2,5  C per second
    5    GOTO   2 ,   44  more times
         END
```

3. Save the protocol: in the **Protocol Editor New** window select **File**, then **Save As**, name the file and click **Save**.

4. Set the plate setup: 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 click the **Selected** checkbox next to the **FAM, HEX, ROX, Cy5, Quasar 705** fluorophores and click **OK**. In the **Sample type** menu select **Unknown** for all the samples. Then in the **Load** column (in the right part of the window) tick the fluorescence signal acquiring for all the samples in the required channels. Define sample names in the **Sample name** window, moreover the **Load** function is to be ticked.

5. Save the plate setup: select **File** and then **Save as** in the **Plate Editor New** window. Enter the file name, click **Save**.

6. Select the **Start Run** tab. Open the lid of the instrument by the **Open Lid** button. Insert 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 tubes (strips) upside down while inserting them into the instrument.

7. Click the **Start Run** button and start the program with the selected plate setup. Select the directory for the file saving, name the file, click **Save**.

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:

− click the **Select Existing…** button in the **Run Setup** window of the **Protocol** tab. Select the needed file with the amplification program in the **Select Protocol** window. Click **Open**.

− go to the **Plate** tab in the **Run Setup** window. Click the **Select Existing…** button. Select the needed file with the plate setup in the **Select Plate** window. Click **Open**. Click the **Edit selected** button to edit the plate setup.

Data analysis

The obtained results are analyzed by the software of the CFX96 instrument. 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.

1. Start the software and open the saved file with data of the analysis. To do this, select **File** in the menu, then **Open** and **Data file** and select the needed file.
2. The fluorescence curves, the tube order in the plate and the table with the *Ct* values are represented in the **Data Analysis** window of the **Quantification** tab.

    Check the correctness of threshold line automatic choice for each channel. The threshold line is to cross only with S-shaped (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, it is necessary to set the threshold line level for each channel manually. To do this, tick the **Log Scale** item (logarithmic scale selection) 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. As a rule, the threshold line is set at the level of 10-20 % of maximum fluorescence obtained for the Positive control in the last amplification cycle. Make sure that the fluorescence curve of the Positive control has the typical exponential growth of fluorescence. To select the curve of C+ sample (or another one appropriate sample), set the cursor to the plate setup or to the results grid.
3. Click the **View/Edit Plate…** button on the toolbar and set the samples names in the opened window.

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 Mx3000P (Stratagene, 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 PCR tubes (0.2 ml) with domed caps (for example, Axygen, USA).

Programming the thermocycler

1. Switch the instrument on and start the **Stratagene Mx3000P** program.

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 for at least 15 min before starting experiment.

3. Insert the tubes into the instrument, lock the fixing arm and the door of the instrument.

4. Select **Optics Configuration** in **Options** menu and, in the **Dye Assignment** tab, set parameter **FAM** next to the **FAM filter set** item, parameter **JOE** next to the **HEX/JOE filter set** and parameter **ROX**, next to the **ROX filter set**.

5. Set fluorescence detection parameters in the **Plate Setup** menu. To do this, select all cells with the test tubes or strips; and mark them as **Unknown** in the **Well type** window. Mark **FAM**, **JOE** and **ROX** fluorophores for the **Collect fluorescence data** option.

6. Name each sample in the **Well Information** window (appears after double click on each cell).

7. Set amplification program in **Thermal Profile Setup** tab for the plate-type instrument (see the *Instruction manual* for the PCR kit). To do this use one of the following methods:

   – Click the **Import…** button. Navigate to the folder containing the previous experimental file and open it. The needed thermocycling profile will appear in the **Thermal Profile** window;

   – Set the appropriate amplification program in the **Thermal Profile Setup** menu.

Table 3

Amplification program for plate-type instruments

Step	Temperature, °C	Time	Number of cycles
1	95	15 min	1
2	95	10 s	45
	60	25 s *fluorescent signal detection*	
	72	10 s	

To set a parameter for measuring the fluorescent signal at the given temperature, select **All points** for the **Data collection marker by dragging** parameter and drag it from the right part of the box to the field with the necessary temperature.

8. Start the amplification by clicking the **Run** and **Start** buttons, then name the experiment.

Data analysis

1. Proceed to the **Analysis** mode by pressing 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).
3. Select to the **Results** tab.
4. Make sure that the automatic selection of the threshold level for each channel is correct. Normally, the threshold line should cross only the sigmoid curves of signal accumulation of positive samples and controls and should not cross the baseline. Otherwise, the threshold level should be raised. To do this, activate each channel image separately in the lower **Dyes shown** panel. Check the threshold line location and change it, if necessary.
5. *Ct* values for test samples are taken into account only if the results obtained for control samples C–, C+, and NCA are correct according to the *Instruction manual* and the *Important Product Information Bulletin* for the PCR kit.
6. The results for test samples are analyzed according to the *Instruction manual* and boundary *Ct* values specified in the *Important Product Information Bulletin* for the PCR kit.

List of Changes Made in the Guidelines

VER	Location of changes	Essence of changes
09.09.11 RT	Tables 1, 2, 3 (amplification program)	Cycles 2 and 3 are combined into one cycle (cycle 2)
02.04.14 SA	Cover page	Address of European representative was added
29.10.15 PM	Through the text	Corrections according to the template
24.05.16 PM	Amplification and data analysis using CFX96 (Bio-Rad, USA)	The section was added
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