

GUIDELINES

to AmpliSens[®] *Parvovirus* B19-FRT PCR kit

for qualitative and quantitative detection of *Parvovirus* B19 DNA in the biological material by the polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection

AmpliSens[®]



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TABLE OF CONTENTS

INTENDED USE	3
WORK with the NucliSENS easyMAG AUTOMATED NUCLEIC ACID EXTRACTION SYSTEM.....	3
AMPLIFICATION AND DATA ANALYSIS USING Rotor-Gene 3000/6000 (Corbett Research, Australia) AND Rotor-Gene Q (QIAGEN, Germany) INSTRUMENTS	5
AMPLIFICATION AND DATA ANALYSIS USING iCycler iQ and iCycler iQ5 (Bio-Rad, USA).....	8
AMPLIFICATION AND DATA ANALYSIS USING CFX96 (Bio-Rad, USA)	11
AMPLIFICATION AND DATA ANALYSIS USING Mx3000P, Mx3005P (Stratagene, USA)..	14
CALCULATION OF <i>Parvovirus</i> B19 DNA CONCENTRATION.....	17
TROUBLESHOOTING	17

INTENDED USE

The guidelines describe the procedure of using **AmpliSens® Parvovirus B19-FRT** PCR kit for qualitative and quantitative detection of *Parvovirus* B19 DNA in the biological material (peripheral and umbilical blood, plasma or serum of peripheral and umbilical blood, oropharyngeal washes and swabs, saliva, cerebrospinal fluid, bone marrow biopsy samples, amniotic fluid, chorionic villi, placental biopsy samples, transudate (ascitic fluid) in case of non-immune fetal hydrops) 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, iCycler iQ5 (Bio-Rad, USA);
- CFX96 (Bio-Rad, USA);
- Mx3000P, Mx3005P (Stratagene, 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

When NucliSENS easyMAG instrument is used, the reagent kit allows working with sample volumes from 0.1 to 1 ml.

Variant 1

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

This extraction method allows to reduce the consumption of NucliSens lysis buffer. It is preferred for work with test samples which contain clots.

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 DNA extraction (select *Plasma*)
 - **Volume** – 0.1 ml
 - **Eluate** – 55 µl
 - **Type** – Lysed
 - **Priority** – Normal.
3. Create a new protocol of DNA extraction and save it. In 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 the Negative Control of extraction (C-)). Add **10 µl** of **Internal Control STI-87 (IC)** into each tube. Add **550 µl** of **NucliSens lysis buffer** into each tube.

When working with test samples which contain clots, the lysis in 1.5-ml tubes is recommended. After the end of incubation (item 8) the tubes should be

NOTE: centrifuged at 10,000 rpm for 1 min on a microcentrifuge and the supernatant should be transferred into special tubes, intended for DNA extraction in the NucliSENS easyMAG instrument.

6. Add **100 µl** of pretreated samples into each tube with NucliSens lysis buffer and of Internal Control STI-87 (IC) by using filter tips and carefully mix by pipetting. Avoid transferring the mucous clots and big particles into the tubes.
7. Add **100 µl of Negative Control (C-)** to the tube labelled C- (Negative Control of Extraction).
8. Incubate the tubes for 10 min at room temperature.
9. Resuspend the tube with **NucliSens magnetic silica** by vortexing. Add **10 µl** of **NucliSens magnetic silica** into each tube by a separate filter tip and mix thoroughly by pipetting. The magnetic silica should be dispensed homogeneously.
10. Place the 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 the extraction procedure is completed, remove the tubes from the instrument. Move the tubes with the DNA samples in the Amplification zone.

Variant 2

DNA extraction with 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 DNA extraction (select *Plasma*)
 - **Volume** – 0,1-1 ml
 - **Eluate** – 55 µl
 - **Type** – Primary
 - **Priority** – Normal.
3. Create a new protocol of DNA extraction and save it. In protocol select **On-board Lysis Buffer Dispensing – Yes, On-board Lysis Incubation – Yes.**

4. Relocate sample table into the created protocol.
5. Add **100 µl** of pretreated samples into each tube for DNA extraction in the NucliSENS easyMAG instrument using a separate filter tip.
6. Add **100 µl of Negative Control (C–)** to the tube labelled C– (Negative Control of Extraction).
7. Mix in a separate 2-ml tube the **NucliSens magnetic silica** and **Internal Control STI-87 (IC)** using sterile filter tips in the following ratio:

Number of samples for DNA extraction	NucliSens magnetic silica, µl	Internal Control STI-87 (IC), µl
1	10	10
24 (complete load instrument)	250 (extra for 25 samples)	250 (from two tubes)

8. Thoroughly mix the content of the tube. The mixture of NucliSens magnetic silica and Internal Control STI-87 (IC) can be stored no more than 30 min.
9. Place the 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.
10. Wait for the NucliSENS easyMAG instrument stop working in **Instrument State-Idle** position (near 15 min).
11. Thoroughly mix the tube with the prepared mixture of NucliSens magnetic silica and Internal Control STI-87 (IC) on vortex. Open the lid of the instrument and add **20 µl of prepared mixture** into each tube. Carefully mix the contents of each tube using a multichannel pipette with 200 µl disposable filter tips.
12. Continue the DNA extraction program.
13. After the DNA extraction procedure is completed, remove the tubes from the instrument.

Move the tubes with the DNA samples in the Amplification zone.

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

If the instrument rotor is not completed, it should be balanced. To do this fill the empty wells by the tubes (do not use the tubes from the previous runs). 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. Click the **New** button in the main menu.
2. In the open window, select **Advanced** and **Dual Labeled Probe/Hydrolysis probes**. Click the **New** button.
3. In the opened window select the **36-Well Rotor** (or **72-Well Rotor**) and tick the **No Domed 0.2 ml Tubes/Locking ring attached** option. 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** option. Click the **Next** button.
5. In the opened 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
1 Cycling	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
2 Cycling	95	5 s	–	40
	60	20 s	FAM/Green, JOE/Yellow	
	72	15 s	–	

This program allows carrying out several AmpliSens® tests within one run if they apply the same amplification program (for example PCR kits for detection of STIs)

Note – The **ROX/Orange, Cy5/Red, Cy5.5/Crimson** channels are enabled when required if the “multiprime” format tests are performed.

6. After setting up the temperature profile click the **OK** button.
7. Click the **Calibrate/Gain Optimisation...** button in the **New Run Wizard** window:
 - a) perform the calibration in the FAM/Green, JOE/Yellow channels (click the **Calibrate Acquiring/Optimise Acquiring** button);
 - b) perform the calibration before the first detection (tick the **Perform Calibration Before 1st Acquisition/Perform Optimisation Before 1st Acquisition** option).
 - c) set calibration for all channels. Set **Min Reading** as 5, **Max Reading** as 10 for all channels. Tick the **Perform Calibration Before 1st Acquisition/Perform Optimisation Before 1st Acquisition** option. Click the **Close** button.
8. Click the **Next** button. Start the amplification by the **Start run** button.
9. Name the experiment and save it on the disk (the results 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). 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 calibrators 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.

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

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. The calibration curve plotting and calculation of the *Parvovirus B19* DNA concentration are performed automatically in accordance with the *Ct* values.

Amplification data analysis of *Parvovirus B19* DNA (JOE/Yellow channel):

1. Make sure that calibrators and their concentrations are specified in the table of samples.
2. Activate the button **Analysis** in the menu, select the mode of the analysis **Quantitation**, activate the buttons **Cycling A. JOE/Cycling A. Yellow, Show**.
3. Cancel the automatic choice of the threshold line level **Threshold**.

4. Activate the ***Dynamic tube*** and ***Slope Correct*** buttons in the menu of main window (***Quantitation analysis***).
5. In the ***CT Calculation*** menu (in the right part of the window) indicate the threshold line level **0.03** in the ***Threshold*** box.
6. Choose the parameter ***More settings/Outlier Removal*** and set **10 %** for the value of negative samples threshold (***NTC/Threshold***).
7. In the results grid (the ***Quantitation Results*** window) one will be able to see the *Ct* values and DNA concentration values (***Calc Conc (copies/reaction)***).

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

1. Make sure that calibrators and their concentrations are specified in the table of samples.
2. Activate the button ***Analysis*** in the menu, select the mode of the analysis ***Quantitation***, activate the buttons ***Cycling A. FAM/Cycling A. Green, Show***.
3. Cancel the automatic choice of the threshold line level ***Threshold***.
4. Activate the ***Dynamic tube*** and ***Slope Correct*** buttons in the menu of main window (***Quantitation analysis***).
5. In the ***CT Calculation*** menu (in the right part of the window) indicate the threshold line level **0.03** in the ***Threshold*** box.
6. Choose the parameter ***More settings/Outlier Removal*** and set **10 %** for the value of negative samples threshold (***NTC/Threshold***).
7. In the results grid (the ***Quantitation Results*** window) one will be able to see the *Ct* values and DNA concentration values (***Calc Conc (copies/reaction)***).

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 (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 iQ5.

3. Insert the tubes or strips into the reaction of the 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 tubes (strips) upside down while inserting them into the instrument.

Program the instrument in accordance with the manufacturer's manual:

1. Select the **Create new** button in the **Workshop** module.

2. In the opened window set the amplification program

Amplification program

Step	Temperature, °C	Time	Fluorescence detection	Number of cycles
Hold	95	15 min	–	1
1 Cycling	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
2 Cycling	95	5 s	–	40
	60	30 s	FAM, JOE/HEX	
	72	15 s	–	

NOTE: This program allows carrying out several AmpliSens® tests within one run if they apply the same amplification program (for example PCR kits for detection of STIs)

Note – The **ROX**, **Cy5** channels are enabled when required if the “multiprime” format tests are performed.

3. Name a new protocol and save it.

4. Create a new **Plate Setup**. Set the order of the tubes in the reaction chamber.

5. In the opened window define all test samples as **Unknown**, positive controls as “+”, negative controls as “–”. Define calibrators as **Standard** in the **HEX** and **FAM** channels and enter their concentration value specified in the *Important Product Information Bulletin* enclosed to the PCR kit (make sure that the **Whole Plate Loading** button is inactivated). Set fluorescence detection for all samples and calibrators in **HEX-530** and **FAM-490** channels.

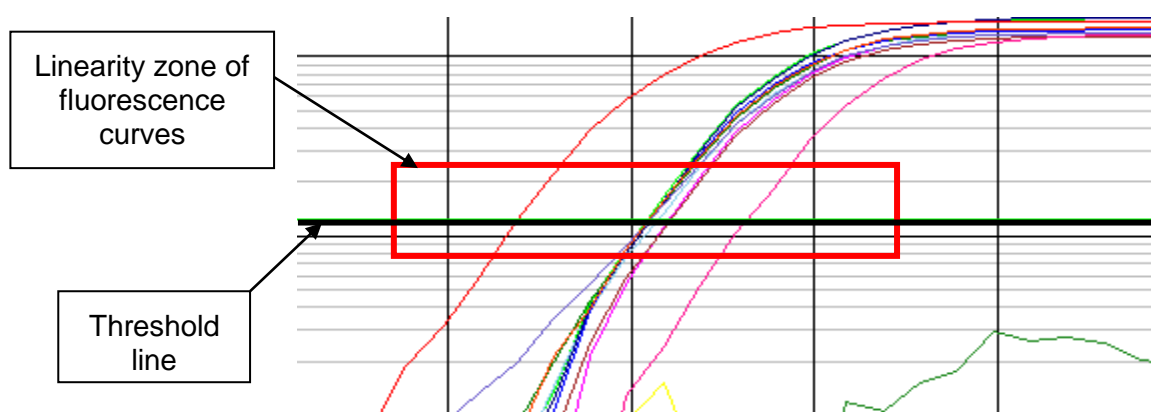
6. Name the **Plate Setup** and save it.

7. Click the **Run** (for iCycler iQ5) or **Run with selected Protocol** (for iCycler iQ) button for instrument running. In the opened window set the sample volume as **25 µl**. Select **Experimental Plate** for well factor detection for iCycler iQ instrument. For iCycler iQ5 **Use Persistent Well Factor** is recommended. Click the **Begin Run** button and save the experiment.

Data analysis

The obtained results are analyzed by the iCycler iQ5 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. The calibration curve plotting and calculation of the *Parvovirus B19* DNA concentration are performed automatically in accordance with the *Ct* values.

1. Start the software and open the needed file with data of the analysis in the **Data File** window of the **Workshop** module. Proceed to the **Data Analysis** mode.
2. Data obtained in each channel should be analyzed separately.
3. 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 raise the threshold level. To do this, click the **Log View** and set (with the left mouse button) the threshold line at the level where the fluorescence curves have a linear character and do not cross with the curves of the negative samples (see below).



Ct values and DNA concentration values (**copies/reaction**) should appear in the results grid (**Quant. Results** window) for the analyzed channel.

4. For the data analysis press the **PCR Quant** button (iCycler iQ) or activate the **Results** button (iCycler iQ5).

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 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 (time, temperature, cycles, and fluorescence acquiring cycle) in the opened **Protocol Editor – New** window. Set **Sample Volume – 25 µl**.

Amplification program

Step	Temperature, °C	Time	Fluorescence detection	Number of cycles
Hold	95	15 min	—	1
1 Cyclinge	95	5 s	—	5
	60	20 s	—	
	72	15 s	—	
2 Cycling	95	5 s	—	40
	60	30 s	FAM, HEX	
	72	15 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 0:05
	Slow Ramp Rate to 2,5 C per second
3	60,0 C for 0:20
	Slow Ramp Rate to 2,5 C per second
4	72,0 C for 0:15
	Slow Ramp Rate to 2,5 C per second
5	GOTO 2 , 4 more times
→ 6	95,0 C for 0:05
	Slow Ramp Rate to 2,5 C per second
7	60,0 C for 0:30
	+ Plate Read
	Slow Ramp Rate to 2,5 C per second
8	72,0 C for 0:15
	Slow Ramp Rate to 2,5 C per second
← 9	GOTO 6 , 39 more times
	END

NOTE: This program allows carrying out several AmpliSens® tests within one run if they apply the same amplification program (for example PCR kits for detection of STIs)

Note – The **ROX, Cy5, Quasar 705** channels are enabled when required if the “multiprime” format tests are performed.

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** fluorophores and click **OK**. In the **Sample type** menu select **Unknown** for all the samples, except for the DNA-calibrators. 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.

Set the calibrators C1 and C2 for all the channels as **Sample type – Standard**. Enter a concentration in the **Concentration** box according to the *Important Product Information Bulletin* enclosed to the PCR kit, 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. The calibration curve plotting and calculation of the *Parvovirus* B19 DNA concentration are performed automatically in accordance with the *Ct* values.

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.

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.

Possible problems and special aspects of results analysis with the use of CFX96 for research software

Possible problems	Characters	Ways to eliminate
The threshold level is set incorrectly	The threshold line passes with the negative samples or above some or all positive curves (herewith, curves are S-shaped in a linear scale)	Set the threshold line at the level of 10-20 % of maximum fluorescence obtained for the Positive control in the last amplification cycle (in logarithmic scale)
The drops are not sedimented from the walls of the tubes before the run	The appearance of negative or positive “steps” in the curves of the fluorescence accumulation (fig.)	Repeat the amplification for the sample

AMPLIFICATION AND DATA ANALYSIS USING Mx3000P, Mx3005P (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 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 Stratagene Mx3000P program.
2. In the **New Experiment Options** window, select **Quantitative PCR (Multiple Standards)** and check **Turn lamp on for warm-up**.

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

3. Insert the tubes in the reaction module, close the lid.
4. In the **Options** menu select **Optics Configuration**. In **Dye Assignment** insert **JOE** parameter next to **HEX/JOE filter set** option, and insert **FAM** parameter next to **FAM filter set** option.

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/plate upside down while inserting them into the instrument.

5. Lock the fixing arm and the door of the instrument.
6. Select **Quantitative PCR (Multiple Standards)** in the **New Experiment Options** window and check **Turn lamp on for warm-up** with a checkmark.
7. In the **Plate Setup** menu set parameters of fluorescence detection. To do this:
 - select all wells with analyzed samples (hold **Ctrl** while moving the mouse over the required field);

- in the **Well type** drop-down menu select the **Unknown** type and in **Collect fluorescence data** field tick **FAM** and **JOE**; enter sample names by double-clicking on each well (the **Well Information** window). It is possible to enter sample names both during and after amplification run in the **Plate Setup** menu.
8. In the **Plate Setup** menu set parameters of fluorescence detection. To do this:
 - select all wells with analyzed samples (hold **Ctrl** while moving the mouse over the required field);
 - in the **Well type** drop-down menu select **Unknown** and in **Collect fluorescence data** field check **FAM** and **JOE**; double-click on each well and enter tube name (**Well Information** window), Positive Control specify as “+”, Negative Control as “-”.
 9. Define calibrators as **Standard** in **HEX** and **FAM** channels and enter their concentrations specified in the *Important Product Information Bulletin* enclosed to the PCR kit.
 10. Proceed to the **Thermal Profile Setup** menu. Set amplification program (see below).
Use on the following methods:

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

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

Individual programming

1. When all required parameters are set, select all wells where the test tubes are set.
Select the **Thermal Profile Setup** tab and set amplification program.

Amplification program

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

NOTE: This program allows carrying out several AmpliSens® tests within one run if they apply the same amplification program (for example PCR kits for detection of STIs)

Note – The **ROX**, **Cy5** channels are enabled when required if the “multiprime” format tests are performed.

2. Set fluorescent signal detection at the specific temperature. Select the **All points** option for **Data collection marker by dragging** parameter and move it to the field with required temperature.

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

Data analysis

The obtained results are analyzed by the software of the Mx3000P/Mx3005P 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 *C_t* (threshold cycle) value in the corresponding column of the results table. The calibration curve plotting and calculation of the *Parvovirus* B19 DNA concentration are performed automatically in accordance with the *C_t* values.

1. Make sure that calibrators and their concentrations are specified in the table of samples.
2. Proceed to **Analysis** by clicking the button on the toolbar.
3. Ensure that all analyzed samples are activated in the **Analysis Selection/Setup** window. Otherwise, select the samples to be studied (hold **Ctrl** while moving the mouse over required field);
4. Proceed to the **Results** window.
5. Ensure that both fluorescent channels are activated (buttons **HEX** and **FAM** are pressed in **Dyes Shown** field at the bottom of the program window).
6. Ensure that JOE/HEX and FAM channels are checked in **Threshold fluorescence** field. Ensure that automatic selection of threshold level is correct. Normally, threshold line should cross only sigmoid curves of signal accumulation of positive samples and controls and not to intersect base lane. If any, threshold level should be raised. By default shown signal accumulation curves are linear. To switch to log view, double click on one of the axis (Y or X) and in the **Graph properties** window check off **Log** option in **Scale** field for **Y axis**.
7. *C_t* values and concentration values (**copies/reaction**) should appear in the result grid.

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.

CALCULATION OF *Parvovirus* B19 DNA CONCENTRATION

Calculation of *Parvovirus* B19 DNA concentration in test and control samples is performed in accordance with the following formula:

$$\frac{\text{Quantity of } \textit{Parvovirus} \text{ B19 DNA (IU/reaction)}}{\text{Quantity of IC STI-87 DNA (copies/reaction)}} \times \text{coefficient A} \times \text{coefficient B (copies/ml)} = \text{IU/ml}$$

$$\text{Coefficient A} = \frac{100}{\text{extraction volume, } \mu\text{l}}$$

TROUBLESHOOTING

1. If any *Ct* value is determined for the Negative Control of Extraction (C–) in the channel for JOE fluorophore and for the Negative Control of Amplification (NCA) in the channels for FAM and/or JOE fluorophores it indicates contamination of reagents or samples. Results of analysis are considered as invalid for all samples. The analysis should be repeated for all samples. Measures for detecting and elimination of contamination source must be taken.
2. If *Ct* value is absent for the Positive Control of amplification (C+) – Positive Control DNA *Parvovirus* B19 / STI (C+B19/STI), results of analysis are considered as invalid for all samples. Repeat PCR for all samples once again.
3. If obtained calibrator values (IU/reaction) differ from the specified values in more than 30 % check the tube order in the instrument.
4. If correlation coefficient in the **Standard Curve** window is less than 0.9 it indicates calibration failure. Check correctness of parameters of calibrators. If it doesn't help repeat PCR for all samples and calibrators.