

GUIDELINES

to **AmpliSens[®] HAV-FRT** PCR kit

for qualitative detection of *hepatitis A virus (HAV)* RNA in biological material and environmental samples by the polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection

AmpliSens[®]



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INTENDED USE

Guidelines describe the procedure of the use of **AmpliSens® HAV-FRT** PCR kit for qualitative detection of *hepatitis A virus (HAV)* RNA in the biological material and environmental samples by means of polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection with the use of:

- Rotor-Gene 3000 (two and more channels) (Corbett Research, Australia),
- Rotor-Gene 6000 (five or six channels) (Corbett Research, Australia),
- iCycler iQ (three and more channels), iQ5 (Bio-Rad, USA),
- Mx3000P (Stratagene, USA).

WORK WITH NucliSENS easyMAG AUTOMATED NUCLEIC ACID EXTRACTION PLATFORM

Variant 1

RNA extraction with off-board sample lysis (off-board mode)

1. Switch on the NucliSENS easyMAG instrument and prepare it for the RNA/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 extraction (select **Other**)
 - **Volume** – 0.1 ml
 - **Eluate** – 55 µl
 - **Type** – Lysed
 - **Priority** – Normal.
3. Create a new protocol of RNA/DNA extraction and save it. Select **On-board Lysis Buffer Dispensing - No, On-board Lysis Incubation - No** in the protocol.
4. Relocate the sample table into the created protocol.
5. Take the required number of special disposable tubes intended for RNA/DNA extraction in the NucliSENS easyMAG instrument (include positive and negative controls of extraction). Add **10 µl of Internal Control STI-248-rec** to each tube and then add **450 µl of NucliSens lysis buffer**.
6. Add **100 µl** of the prepared samples into each tube with lysis solution and internal control by using tips with aerosol barriers and carefully mix by pipetting (avoid adding mucus clots and large particles to the tube).
7. Add **90 µl of Negative Control** and **10 µl of Positive Control HAV-FL-rec** to the tube intended for the **Positive Control of Extraction (PCE)** and carefully mix by pipetting.

8. Add **100 µl** of **Negative Control** into the tube intended for the **Negative Control of Extraction (C–)** and carefully mix by pipetting.
9. Incubate the tubes at room temperature for 10 min to ensure lysis.
10. Thoroughly vortex the tube with **magnetic silica**. Transfer **20 µl of magnetic silica** into each tube using a tip with aerosol barrier and mix by pipetting. Make sure that magnetic silica is evenly distributed throughout the tube.
11. Place the tubes with samples into the instrument, insert tips, and start the RNA extraction program with lysis of samples by selecting the *off board* mode.
12. After the extraction is completed, take the tubes out of the instrument and carry out the RT-PCR not later than 30 min after RNA extraction.

The purified RNA can be stored at 2–8 °C for up to 8 h or at or below –68 °C for a longer period of time.

Variant 2

RNA extraction with on-board automated sample lysis (on-board mode)

1. Switch on the NucliSENS easyMAG instrument and prepare it for the RNA/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 extraction (select **Other**)
 - **Volume** – 0.1 ml
 - **Eluate** – 55 µl
 - **Type** – Primary
 - **Priority** – Normal.
3. Create a new protocol of RNA/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 RNA/DNA extraction in the NucliSENS easyMAG instrument (include positive and negative controls of extraction). Add **10 µl of Internal Control STI-248-rec** to each tube.
6. Add **100 µl** of the prepared samples into each tube with internal control by using disposable tips with aerosol barriers and carefully mix by pipetting (avoid adding mucus clots and large particles to the tube).
7. Add **90 µl** of **Negative Control** and **10 µl** of **Positive Control HAV-FL-rec** to the tube intended for the **Positive Control of Extraction (PCE)** and carefully mix by pipetting.
8. Add **100 µl** of **Negative Control** into the tube intended for the **Negative Control of**

Extraction (C–) and carefully mix by pipetting.

9. Place tubes with samples into the instrument, insert the tips, and start the RNA extraction program with lysis of samples by selecting the **on board** mode.
10. Wait until NucliSENS easyMAG proceeds to the **Instrument State - Idle** option and pauses (for about 15 min).
11. Thoroughly vortex the tube with **magnetic silica**. Open the lid of the instrument and transfer **20 µl of magnetic silica** into each tube using a tip with aerosol barrier (or using a multichannel pipettor with disposable tips with aerosol barriers for 200 µl) and mix by pipetting. Make sure that magnetic silica is evenly distributed throughout the tube.
12. Close the lid and continue the RNA extraction program.
13. After the extraction is completed, take the tubes out of the instrument and carry out the RT-PCR not later than 30 min after RNA extraction.

The purified RNA can be stored at 28 °C for up to 8 h or at or below minus 68 °C for a longer period of time.

REAL-TIME PCR AND DATA ANALYSIS WITH THE USE OF Rotor-Gene 3000/6000 (Corbett Research, Australia) INSTRUMENTS

Pretreat the sample and prepare the reaction mixture according to the instruction manual. Transparent no-domed 0.2-ml PCR tubes and 0.1-ml tubes are recommended for use. Place the tubes in the rotor so that the first tube is in No. 1 well, insert the rotor in the reaction module, and secure the lid (the rotor cells are numbered and these numbers are used for programming the sample order).

The first rotor well should be filled with a test tube from the current experiment. If the rotor is loaded with tubes from different PCR kits, the No. 1 well should

NOTE: contain the tube with the largest number of fluorophores. For example, for simultaneous *HAV* detection and *HCV*-genotyping analysis, the tubes for *HCV*-genotyping should be placed in the rotor first.

Hereinafter, the terms specific for different instruments are listed in the following order: for the Rotor-Gene 3000 instrument / for the Rotor-Gene 6000. If terms for different instruments coincide, only one term is shown.

Programming the Rotor-Gene 3000/6000 instrument

1. Click the **New** button in the main program menu.
2. In the opened window, select **Advanced** menu and **Dual Labeled Probe/Hydrolysis probes**. 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

AmpliSens-3 RG program

Step	Temperature, °C	Time	Fluorescence detection	Cycles
Hold	50	30 min	–	1
Hold	95	15 min	–	1
Cycling 1	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
Cycling 2	95	5 s	–	40
	60	20 s	FAM/Green, JOE/Yellow	
	72	15 s	–	

NOTE: It is possible to carry out any combination of tests (for example *HAV*, *HCV*, etc.) that use AmpliSens-3 RG program within the same run.

6. Click **OK**.
7. Select the **Calibrate/Gain Optimisation** button in the **New Run Wizard** window. Select FAM/Green and JOE/Yellow channels in the **Channel Settings** line of the opened window. In **Tube Position** set 1, in **Min Reading** set 5, in **Max Reading** set 10. Mark **Perform Calibration Before 1st Acquisition/Perform Optimisation Before 1st Acquisition**. Press the **Close** button.
8. Click **Next** then 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. Set the data in the table of samples (opens automatically after the thermocycling process starts). Indicate the names/numbers of test samples in the **Name** column. Set the **Unknown** type for all test samples, set the **Positive Control** type for the positive control of PCR, set the **Negative Control** type for the negative control of PCR, and set the **None** type for empty wells.

NOTE: Samples defined as **None** will not be analyzed.

Data analysis

IC cDNA is detected in the FAM/Green fluorescence channel, *HAV* cDNA is detected in the JOE/Yellow fluorescence channel.

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 presence (absence) of the Ct (cycle threshold) value in the results grid.

Data analysis of the HAV cDNA (JOE/Yellow channel)

1. Activate the **Analysis** button, then select **Quantitation** button and activate the **Cycling A. JOE** or the **Cycling A. Yellow** button. Click **Show**.
2. Cancel the **Threshold** automatic choice.
3. Select the **Dynamic tube** and **Slope Correct** buttons in the main window menu (set by default).
4. In **CT Calculation** menu, set **Threshold = 0.03**.
5. In main window menu **More Settings/Outlier Removal**, set the NTC threshold value **10 %**.
6. In the results grid (**Quant. Results** window), the *Ct* (threshold cycle) values for each test sample will appear.

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

1. Activate the **Analysis** button, then select the **Quantitation** button and activate the **Cycling A. FAM** or **Cycling A. Green** button. Click **Show**.
2. Cancel the **Threshold** automatic choice.
3. Select the **Dynamic tube** and **Slope Correct** buttons in the main window menu (set by default).
4. In **CT Calculation** menu, set **Threshold = 0.03**.
5. In main window menu **More Settings/Outlier Removal**, the set NTC threshold value **10 %**.
6. In the results grid (**Quant. Results** window), the *Ct* (Threshold cycle) values for each test sample will appear.

Analysis of results for control samples

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 positive and negative controls of extraction are correct. These results should not be greater than the boundary *Ct* values specified for Rotor-Gene instruments in the *Important Product Information Bulletin*.

Table 2

Results for controls

Control	Stage for control	Ct value in channel	
		JOE/Yellow	FAM/Green
C-	RNA extraction	Negative	≤ B1 boundary value* (positive)
PCE	RNA extraction	≤ K1 boundary value* (positive)	≤ B1 boundary value* (positive)
NCA	RT-PCR	Negative	Negative
C+ _{HAV/IC}	RT-PCR	≤ K2 boundary value* (positive)	≤ B2 boundary value* (positive)

* For K1, K2, B1, B2 boundary *Ct* values, see the *Important Product Information Bulletin*.

Analysis of results for test samples

1. HAV RNA is **detected** in a sample if the *Ct* value of a sample in the JOE/Yellow channel does not exceed **35**.
2. HAV RNA is **not detected** in a sample if its *Ct* value is not detected in the results grid in the JOE/Yellow channel (the fluorescence curve does not cross the threshold line) while the *Ct* value in the FAM/Green channel does not exceed the boundary *Ct* value for IC specified for Rotor-Gene instruments in the *Important Product Information Bulletin*.
3. The result is considered to be **equivocal** if the *Ct* value of a sample in the JOE/Yellow channel exceeds **35**. Repeat RNA extraction for such a sample. If the result repeats as positive, the sample is considered to be positive. If the result repeats as negative, the sample is considered to be equivocal.

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

1. If the *Ct* value for PCE is absent or exceeds the boundary *Ct* value, repeat RNA extraction stage.
2. If no signal is detected for the positive control of amplification (C+) in the **JOE/Yellow** channel, it may suggest that the programming of the temperature profile of the used Instrument was incorrect, or that the configuration of the PCR reaction was incorrect, or that the storage conditions for kit components did not comply with the manufacturer's instruction, or that the reagent kit expired. Programming of the used instrument, storage conditions, and the expiration date of the reagents should be checked, and then PCR should be repeated.
3. If the *Ct* value is absent in both JOE/Yellow and FAM/Green channels or the *Ct* value in the JOE/Yellow channel is higher than the specified boundary *Ct* value, PCR should be repeated starting from the RNA extraction stage.
4. If the *Ct* value is present for C– in the JOE/Yellow channel and/or for NCA in both channels in the results grid, it indicates contamination of reagents or samples. In such cases, the results of analysis must be considered as invalid. Test analysis must be repeated and measures to detect and eliminate the source of contamination must be taken.

REAL-TIME PCR AND DATA ANALYSIS WITH THE USE OF iCycler iQ and iQ5 (Bio-Rad, USA) INSTRUMENTS

Pretreat the sample and prepare the reaction mixture according to the instruction manual. Transparent domed-cap 0.2-ml tubes (detection through the tube cap) are recommended for use.

Prior to amplification run, make sure that the iQ5 instrument was adjusted for 25 µl reaction volume and calibrated using the same type of plastic consumables that will be used for the run.

1. Switch on the instrument. Start the iQ5 program.

NOTE: The lamp should be warmed up for at least 15 min before the experiment starts.

2. Place the tubes/strips into the reaction chamber and program the instrument.

Prior to analysis, remove drops from tube walls, because drops falling during

NOTE: amplification cause signal errors and complicate data analysis. Do not turn strips/plate when placing it into the PCR instrument.

3. Select **Create new** in the **Workshop** module.
4. Set the parameters of amplification.

Table 3

AmpliSens-3 iQ

Step	Temperature, °C	Time	Fluorescence detection	Cycles
Hold	50	30 min	–	1
Hold	95	15 min	–	1
Cycling 1	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
Cycling 2	95	5 s	–	40
	60	30 s	FAM, HEX	
	72	15 s	–	

NOTE: It is possible to carry out any combination of tests (for example *HAV*, *HCV* etc.) that use AmpliSens-3 RG program within the same run.

5. Name a new protocol and save it.
6. Set the **Plate Setup** (set the order of the tubes in the reaction chamber).
7. In the opened window, define all test samples as **Unknown**, positive controls as “+”, and negative controls as “–”. Set fluorescence detection in **FAM-490** and **HEX-530** channels.
8. Name the plate setup and save it.
9. Click **Run** (for iCycler iQ5) or **Run with selected protocol** (for iCycler iQ). Set the **Sample Volume** as **25 µl** in the opened window. Select the **Experimental Plate** option for iCycler iQ for determination of the well factor. For iCycler iQ5, the well factor can be determined by either experimental plate or persistent plate (recommended).
10. Click the **Begin Run** button and save the experiment.

Data analysis

IC cDNA is detected in the FAM fluorescence channel, *HAV* cDNA is detected in the HEX fluorescence channel.

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.

Data processing

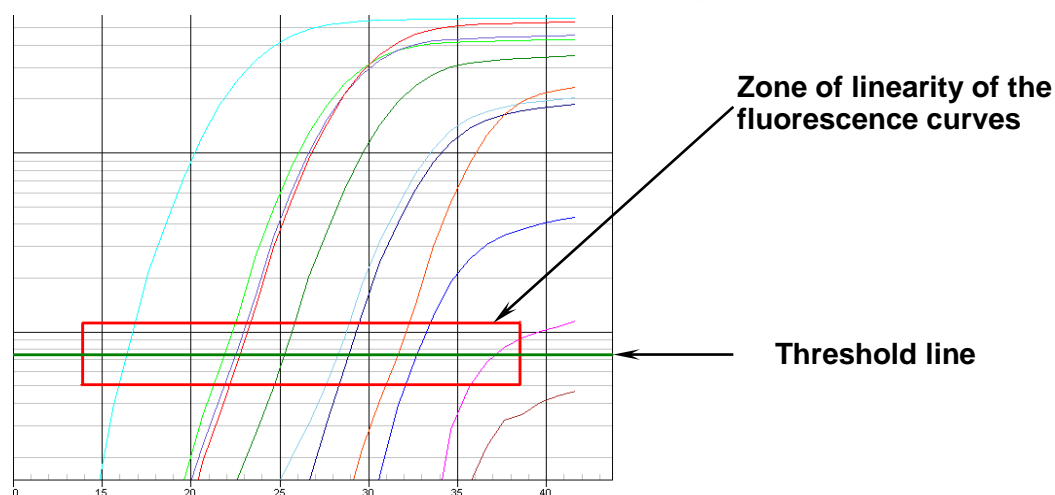
1. Start the program and open the saved file. To do this, click **Data file** in the **Workshop** module and select the required data file. Proceed to the **Data Analysis** mode.
2. View data in each channel separately.

Data analysis of the Internal Control amplification (FAM channel)

Click the **Log View** button. Set the level of the threshold line (with the mouse left button) at the level where fluorescence curves are linear (see Fig. 1).

Data analysis of HAV RNA amplification (HEX channel)

Click the **Log View** button. Set the level of the threshold line (with the mouse left button) at the level where fluorescence curves are linear (see Fig. 1).



3. Click the **PCR Quant** button (for iCycler iQ) or **Results** (for iQ5, the button is located below the buttons with the names of the fluorophores).

Analysis of result for control samples

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 positive and negative controls of extraction are correct (see Table 3). These results should not exceed the boundary *Ct* values specified for iCycler iQ and iQ5 instruments in the *Important Product Information Bulletin*.

Results for controls

Control	Stage for control	Ct value in channel	
		JOE/HEX/JOE-530	FAM/FAM-490
C-	RNA extraction	Negative	≤ B1 boundary value* (positive)
PCE	RNA extraction	≤ K1 boundary value* (positive)	≤ B1 boundary value* (positive)
NCA	RT-PCR	Negative	Negative
C+_{HAV/IC}	RT-PCR	≤ K2 boundary value* (positive)	≤ B2 boundary value* (positive)

* For K1, K2, B1, B2 boundary Ct values, see the **Important Product Information Bulletin**.

Analysis of results for test samples

1. *HAV* RNA is **detected** in a sample if the Ct value of a sample in the HEX channel does not exceed **35**.
2. *HAV* RNA is **not detected** in a sample if its Ct value is not detected in the results grid in the HEX channel (the fluorescence curve does not cross the threshold line) while the Ct value in the FAM channel does not exceed the boundary Ct value for IC specified for iCycler iQ and iQ5 instruments in the Important Product Information Bulletin.
3. The result is considered to be **equivocal** if the Ct value of a sample in the HEX channel exceeds **35**. Repeat RNA extraction for such a sample. If the result repeats as positive, the sample is considered to be positive. If the result repeats as negative, the sample is considered to be equivocal.

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

1. If the Ct value for PCE is absent it indicates mistakes in the RNA extraction. The analysis should be repeated starting from RNA extraction stage.
2. If no signal is detected for the positive control of amplification (C+) in the **HEX** channel, it may suggest that the programming of the temperature profile of the used Instrument was incorrect, or that the configuration of the PCR reaction was incorrect, or that the storage conditions for kit components did not comply with the manufacturer's instruction, or that the reagent kit expired. Programming of the used instrument, storage conditions, and the expiration date of the reagents should be checked, and then PCR should be repeated.
3. If the Ct value is absent in both HEX and FAM channels or the Ct value in the HEX channel is greater than the boundary Ct value for IC specified for iCycler instruments in the *Important Product Information Bulletin*, PCR should be repeated.
4. If the Ct value is present for C- in the HEX channel and/or for NCA in any channels in

the results grid, it indicates contamination of reagents or samples. In such cases, the results of analysis must be considered as invalid. Test analysis must be repeated and measures to detect and eliminate the source of contamination must be taken.

REAL-TIME PCR AND DATA ANALYSIS WITH THE USE OF Mx3000P (Stratagene, USA) INSTRUMENT

Carry out sample pretreatment and reaction mixture preparation stages according to the instruction manual. Program the instrument according to the user manual provided by the manufacturer. Transparent domed-cap 0.2-ml tubes (detection through the tube cap) are recommended for use.

1. Switch on the instrument. Start the **Stratagene Mx3000P** program.
2. Select **Quantitative PCR (Multiple Standards)** and check **Turn lamp on for warm-up** in the **New Experiment Options** window.

NOTE: The lamp should be warmed up for at least 15 min before the experiment starts.

3. Place experimental tubes into the module and lock it.

NOTE: Prior to analysis, remove drops from tube walls, because drops falling during amplification cause signal errors and complicate data analysis. Do not turn strips/plate when placing it into the PCR instrument.

4. Select **Optics Configuration** in the **Options** menu. In the **Dye Assignment** window, set **FAM** next to the **FAM filter set** line, **JOE** next to the **HEX/JOE filter set** line.
5. Select **Quantitative PCR (Multiple Standards)** and check **Turn lamp on for warm-up** in the **New Experiment Options** window.
6. Set fluorescence detection parameters in the **Plate Setup** menu. To do this,
 - select all cells with test tubes using the mouse and holding the **Ctrl** button down,
 - define them as **Unknown** in the **Well type** drop-down window. Select **FAM** and **JOE** fluorophores in the **Collect fluorescence data** option. Then, double click each cell and enter its name (**Well information** window). Define the positive control as “+” and the negative control as “-”.
7. Set fluorescence detection in the **Plate Setup** screen. Select all cells with test tubes using the mouse and holding **Ctrl** button. In the **Well type** drop-down window select **Unknown** type and tick **FAM** and **JOE** in the **Collect fluorescence data** field. In the **Well Information** window double click each cell and enter tube names. Define Positive Control as “+” and Negative Control as “-”.
8. Set the amplification program (see the table below) in **Thermal Profile Setup**.

AmpliSens-3 Mx

Step	Temperature, °C	Time	Fluorescence detection	Cycles
1	50	30 min	–	1
2	95	15 min	–	1
3	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
4	95	5 s	–	40
	60	30 s	FAM, JOE	
	72	15 s	–	

NOTE: It is possible to carry out any combination of tests (for example *HAV*, *HCV* etc.) that use AmpliSens-3 Mx program within the same run.

9. To start the amplification program click **Run, Start**, and then enter the file name.

Analysis of results

IC cDNA is detected in the FAM fluorescence channel, *HAV* cDNA is detected in the JOE fluorescence channel.

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.

Data processing

1. Shift to the **Analysis** mode by clicking the corresponding button on the tool bar.
2. Make sure that all test samples are activated (the cells have different shadow) in the opened **Analysis Selection/Setup** tab. Otherwise, activate all analyzed samples by selecting them with the left mouse button while holding the **Ctrl** button down.
3. Proceed to the **Results** window in the menu.
4. Make sure that two fluorescence channel are activated (**JOE** and **FAM** buttons are ticked in the **Dyes Shown** field at the bottom of the program window).
5. Make sure that JOE and FAM channels are ticked in the **Threshold fluorescence** field. Make sure that the automatic selection of the threshold level for each channel is correct. Normally, the threshold line should cross only the sigmoid curves¹ of signal accumulation of positive samples and controls and should not intersect the baseline. Otherwise, the threshold level should be raised.

Analysis of result for control samples

The result of the analysis is considered reliable only if the results obtained for both positive

¹ By default displayed signal accumulation curves are linear. To switch from linear to logarithmic scale, double click one of the axis (X or Y) and tick the **Log** option for Y axis in the **Scale** field of the opened **Graph properties** window.

and negative controls of amplification as well as for the positive and negative controls of extraction are correct (see Table 3). These results should not exceed the boundary Ct values specified for the Mx3000 instrument in the Important Product Information Bulletin.

Table 6

Results for controls

Control	Stage for control	Ct value in channel	
		JOE/HEX	FAM
C-	RNA extraction	Negative	≤ B1 boundary value* (positive)
PCE	RNA extraction	≤ K1 boundary value* (positive)	≤ B1 boundary value* (positive)
NCA	RT-PCR	Negative	Negative
C+_{HAV/IC}	RT-PCR	≤ K2 boundary value* (positive)	≤ B2 boundary value* (positive)

* For K1, K2, B1, B2 boundary Ct values, see the **Important Product Information Bulletin**.

Analysis of results for test samples

1. *HAV* RNA is detected in a sample if the Ct value of a sample in the JOE channel does not exceed **35**.
2. *HAV* RNA is **not detected** in a sample if its Ct value is not detected in the results grid in the JOE channel (the fluorescence curve does not cross the threshold line) while the Ct value in the FAM channel does not exceed the boundary Ct value of IC specified for the Mx3000 instrument in the Important Product Information Bulletin.
3. The result is considered to be **equivocal** if the Ct value of a sample in the JOE channel exceeds **35**. Repeat RNA extraction for such a sample. If the result repeats as positive, the sample is considered to be positive. If the result repeats as negative, the sample is considered to be equivocal.

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

1. If the Ct value for PCE is absent, it indicates errors in RNA extraction. The analysis should be repeated starting from the RNA extraction stage.
2. If no signal is detected for the positive control of amplification (C+) in the **JOE** channel, it may suggest that the programming of the temperature profile of the used Instrument was incorrect, or that the configuration of the PCR reaction was incorrect, or that the storage conditions for kit components did not comply with the manufacturer's instruction, or that the reagent kit expired. Programming of the used instrument, storage conditions, and the expiration date of the reagents should be checked, and then PCR should be repeated.

3. If the Ct value is absent in both JOE and FAM channels or the Ct value in the JOE channel is greater than the boundary Ct value for IC specified for the Mx3000 instrument in the *Important Product Information Bulletin*, PCR should be repeated. It can be caused by errors in processing that entailed the loss of DNA or inhibition of PCR. The analysis should be repeated starting from the RNA extraction stage.
4. If the Ct value appeared for C– in the JOE channel and/or for NCA in any channels in the results grid, it indicates contamination of reagents or samples. In such cases, the results of analysis must be considered as invalid. Test analysis must be repeated and measures to detect and eliminate the source of contamination must be taken.