

GUIDELINES

to **AmpliSens[®] VZV-FRT** PCR kit

for qualitative detection of *Varicella-Zoster virus* DNA in the biological material (peripheral blood plasma, umbilical blood plasma, amniotic fluid, cerebrospinal fluid (CSF), blister content, saliva, oropharyngeal washes and swabs) using 'real-time' fluorescence-hybridization detection

AmpliSens[®]



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INTENDED USE

Guidelines describe the procedure of the use of **AmpliSens® VZV-FRT** PCR kit for qualitative detection of *Varicella-Zoster virus* DNA in the biological material (peripheral blood plasma, umbilical blood plasma, amniotic fluid, cerebrospinal fluid (CSF), blister content, saliva, oropharyngeal washes and swabs) by the polymerase chain reaction (PCR) with 'real-time' fluorescence-hybridization detection using the following instruments:

- Rotor-Gene 3000, Rotor-Gene 6000 (Corbett Research, Australia);
- Rotor-Gene Q (QIAGEN, Germany)

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

Variant 1

DNA extraction from 100 µl - sample with lysis of sample outside of the instrument (off-board mode)

1. Switch on the NucliSENS easyMAG instrument and prepare it for DNA extraction as recommended in the instructions provided by the manufacturer.
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 for 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. Prepare the required number (including the negative control of extraction) of the tubes intended for DNA extraction with NucliSENS easyMAG automated platform. Add **10 µl of Internal Control STI-87** into each tube then add **550 µl of NucliSens lysis buffer**.

NOTE: If biological material contains clots, use 1.5-ml tubes for lysis procedure. When incubation is completed (step 8), centrifuge the tubes at 10,000 rpm for 1 min and transfer the supernatant to the tubes intended for DNA extraction with NucliSENS easyMAG system.

6. Add **100 µl of prepared samples** to each tube containing the **Internal Control STI-87**

and **NucliSens lysis buffer** with a new tip with aerosol filter and thoroughly mix by pipetting (avoid adding clots and large particles to the test tubes).

7. To the tube for Negative Control of Extraction (C–) add **100 µl** of the **Negative Control** sample.
8. Incubate the tubes at room temperature for 10 min to allow lysis to occur.
9. Thoroughly mix the tube with the **NucliSens magnetic silica** on vortex. Add **10 µl** of **NucliSens magnetic silica** to each tube with a new tip with aerosol barrier and thoroughly mix by pipetting. Make sure that the silica is evenly dispensed throughout the tube.
10. Load tubes into the instrument, insert aspiration tip sets, and start the DNA extraction program with **off-board** lysis of samples.
11. When the DNA extraction procedure is completed, remove the tubes from the instrument.
12. Transfer the tubes with DNA samples to the PCR and detection zone.

Variant 2

RNA/DNA extraction from 0.1 – 1 ml sample with automated lysis of sample in the instrument (on-board mode)

1. Switch on the NucliSENS easyMAG instrument and prepare it for 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 *Plasma*)
 - **Volume** – from 100 µl to 1 ml
 - **Eluate** – 55 µl
 - **Type** – Primary
 - **Priority** – Normal.
3. Create a new protocol for DNA extraction and save it. In the protocol, select **On-board Lysis Buffer Dispensing - yes, On-board Lysis Incubation - yes**.
4. Relocate programmed sample to created protocol.
5. Add **100 µl** of **prepared samples** to each tube intended for DNA extraction with the NucliSENS easyMAG system using a new tip with aerosol filter.
6. To the tube for Negative Control of Extraction (C–) add **100 µl** of the **Negative Control** sample.
7. Load tubes into the instrument, insert aspiration tip sets, and start the DNA extraction program with **on-board** lysis of samples.

8. Wait for the NucliSENS easyMAG instrument stop working in **Instrument State-Idle** position (near 15 min).
9. Mix in a new sterile 2-ml tube NucliSens magnetic silica and appropriate Internal Control using disposable tips with filters (see Table 1).

Table 1

Number of samples for DNA extraction	Volume of NucliSens magnetic silica (µl)	Volume of Internal Control STI-87 (µl)
1	10	10
24 (instrument complete load)	250 (1 extra sample is included)	250

10. Mix the prepared mixture thoroughly. The mixture of NucliSens magnetic silica and Internal Control STI-87 can be stored for no longer than 30 min. Thoroughly vortex the tube with the mixture of NucliSens magnetic silica and Internal Control STI-87 until homogenous.
11. Open the lid of the instrument and add **20 µl of prepared mixture of NucliSens magnetic silica and Internal Control** to each well of the reagent cartridge. Carefully mix the contents of each well by using a pipette with 1000 µl disposable tips with filters.
12. Click the program to continue the DNA extraction procedure.
13. When the DNA extraction procedure is completed, remove the tubes from the instrument and transfer to the PCR and detection zone.

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

When working with Rotor-Gene 3000 one should use the Rotor-Gene Version 6 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.

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 Rotor-Gene 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).

Insert the tubes in the rotor so that the first tube is in well No. 1, 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).

NOTE: Balance the rotor of the instrument if it is not loaded entirely. Fill the spare wells with empty tubes (do not use the tubes left after previous experiments). Well 1 must be filled with any studied tube except for an empty one.

Programming the thermocycler

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 2). Click **OK**.

Table 2

AmpliSens-1 amplification program for rotor-type instruments¹

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

6. Select the **Calibrate/Gain Optimisation** button in the **New Run Wizard** window:
 - perform the fluorescence detection in FAM/Green and JOE/Yellow channels (activate the **Calibrate Acquiring/Optimise Acquiring** button);
 - to set channels calibration, indicate **5** in the **Min Reading** box and **10** in the **Max Reading** box (activate **Edit...**, the window **Auto gain calibration channel settings**). Tick the **Perform Calibration Before 1st Acquisition/ Perform Optimisation Before 1st Acquisition** box.
7. Click **Next** then the **Start run** button.
8. Name the experiment and save it to the disk (results of the run will be automatically saved in this file).
9. Enter 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. Indicate Negative Control of amplification as **C-** and Positive Control of Amplification as **C+**. Set the **Unknown** type next to all tested samples, the **Positive Control** type next

¹ For example, Rotor-Gene 3000, Rotor-Gene 6000, Rotor-Gene Q, or equivalent.

to the Positive Control of Amplification, and the **NTC** type next to the Negative Control of Amplification. Set the **None** type for the wells loaded with empty tubes.

NOTE: Samples indicated as **None** will not be analysed.

Data analysis

Analysis of results is performed by the software of the real-time PCR instrument used by measuring fluorescence signal accumulation in two channels:

- The signal of the IC DNA amplification product is detected in the channel for the FAM fluorophore.
- The signal of the **Varicella-Zoster virus DNA** amplification product is detected in the channel for the JOE fluorophore.

The results are interpreted by the presence (or absence) of an intercept between the fluorescence curve and the threshold line set at the specific level that corresponds to the presence (or absence) of a *Ct* value of the DNA sample in the corresponding column of the results grid.

Data analysis of the VZV DNA (JOE/Yellow channel)

1. Activate the button **Analysis** in the menu, select the mode of the analysis **Quantitation**, activate the buttons **Cycling A. JOE/Cycling A. Yellow, Show, Cycling A. FAM/Cycling A. Green, Show**.
2. Cancel the automatic choice of the threshold line level for each of the main open windows (FAM/Green, JOE/Yellow) by activating the **Threshold** button.
3. Activate the **Dynamic tube** and **Slope Correct** buttons in the menu of each main window (**Quantitation analysis**).
4. In the **Calculation** menu (in the right part of the window) indicate the threshold line level **0.03** in the **Threshold** box.
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 (the **Quant. Results** window) one will be able to see the *Ct* values.
7. *Ct* value should appear for the Negative Control of extraction, C– (**Negative Control**).
8. *Ct* value should be absent for the Negative Control of amplification, NCA (**TE-buffer**).
9. *Ct* value less than the specified boundary value should appear for the Positive Control of amplification, C+ (**Positive Control DNA VZV-FL (C+vzv)**).

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. Activate the **Dynamic tube** and **Slope Correct** buttons in the main window menu (**Quantitation analysis**).
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* (Cycle threshold) values of **Internal Control STI-87** should appear for Negative Control of extraction (C–) as well as for each test sample. Moreover, the *Ct* value should not exceed the specified boundary value.
7. *Ct* value should be absent for the Negative Control of amplification, NCA (**TE-buffer**).
8. *Ct* value should be absent for the Positive Control of amplification, C+ (**Positive Control DNA VZV-FL (C+vzv)**).

TROUBLESHOOTING

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

- If the *Ct* value is detected for C– in the JOE/Yellow channel and/or for NCA in the FAM/Green and JOE/Yellow channels in the results grid, this 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.
- If the *Ct* for the Positive Control of amplification (C+) in the JOE/Yellow channel is absent or greater than the boundary *Ct* value, PCR and detection should be repeated for all samples in which *Varicella-Zoster virus* DNA was not detected.
- If test samples do not show *Ct* value in the FAM/Green channel (internal control), this indicates DNA extraction failure. Repeat the analysis for such samples starting from DNA extraction.
- If the *Ct* value in the FAM/Green channel (IC) is greater than the specified boundary *Ct* value and the *Ct* value in the JOE/Yellow channel (VZV) is greater than 35, the sample should be analysed once again starting from the DNA extraction stage. High *Ct* values can be caused by DNA loss during extraction or by the presence of inhibitors.

List of Changes Made in the Guidelines

VER	Location of changes	Essence of changes
13.07.23 EM	Footer	REF R-V61-50-F(RG)-CE was added