RUO

For Professional Use Only

GUIDELINES

to AmpliSens® DNA-*HIV*-FRT PCR kit

for qualitative detection of human immunodeficiency virus type 1 (*HIV*-1) proviral DNA in the biological material by polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection

AmpliSens®

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INTENDED USE

The guidelines describe the procedure of using **AmpliSens® DNA-*HIV*-FRT** PCR kit for qualitative detection of human immunodeficiency virus type 1 (*HIV*-1) proviral DNA in the biological material (whole blood) by polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection using the following instruments:

- Rotor-Gene 3000, Rotor-Gene 6000 (Corbett Research, Australia);
- iCycler iQ, iCycler iQ5 (Bio-Rad, USA).

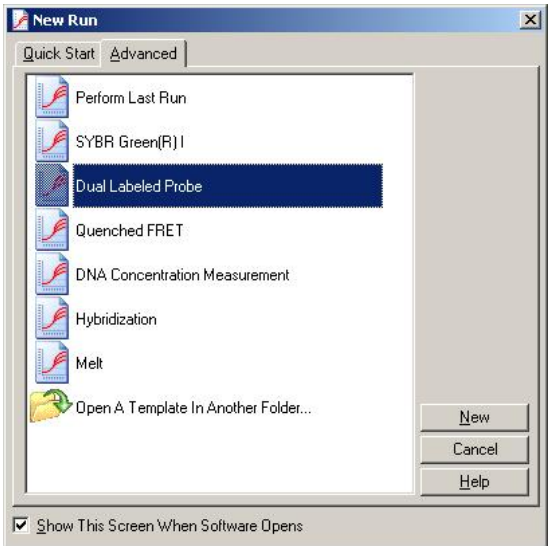
AMPLIFICATION AND DATA ANALYSIS USING Rotor-Gene 3000/6000 (Corbett Research, Australia) INSTRUMENTS

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 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.

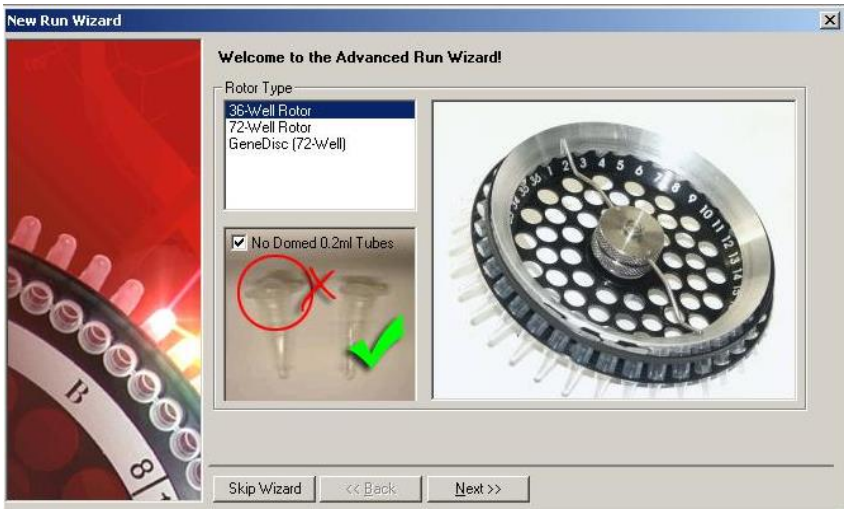
Programming the thermocycler

1. Insert the tubes or strips into the rotor of the Rotor-Gene 3000/6000 instrument (the rotor wells are numbered, the numbers are used for the further programming of the samples' order in the thermocycler).
2. Click the ***New*** button in the software main menu.
3. To create the template select the ***Advanced*** tab in the opened window. Select ***Dual Labeled Probe*/ *Hydrolysis probes***. Activate the ***New*** button.



4. In the opened window select the ***36-Well Rotor*** and tick the ***No Domed 0,2ml Tubes*** option. Click the ***Next*** button.
5. In the opened window enter the operator name, select the reaction volume – 50 µl.

Click the ***Next*** button.

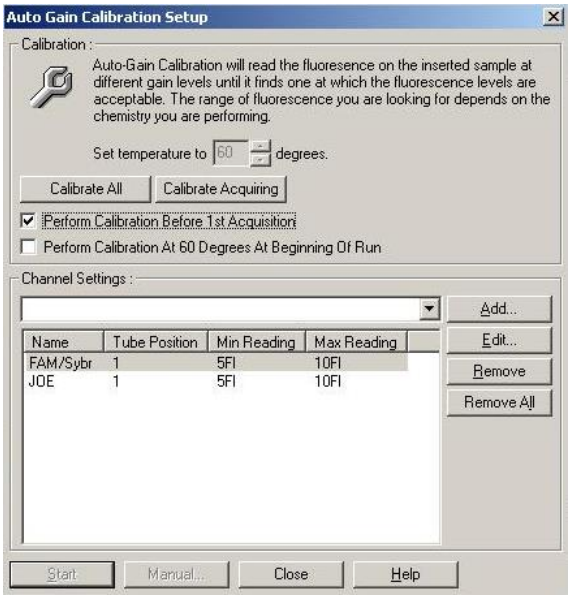


6. In the opened window set the temperature profile of the experiment. To do this click the ***Edit profile*** button and set the amplification program:

Amplification program

Step	***Temperature, °C***	***Time***	***Fluorescence detection***	***Number of cycles***
Hold	95	15 min	–	1
Cycling	95	20 s	–	5
	52	30 s	–	
	72	30 s	–	
Cycling 2	95	20 s	–	40
	55	30 s	FAM/Green, JOE/Yellow	
	72	30 s	–	

7. After setting up the temperature profile click the ***OK*** button.
8. Click the ***Calibrate/Gain Optimisation…*** button in the ***New Run Wizard*** window. In the opened window in the ***Channel Setting*** field select the FAM/Green, JOE/Yellow cannels. Set ***Tube Position*** – 1, ***Min Reading*** – 5***, Max Reading*** – 10. Tick the ***Perform Calibration Before 1st Acquisition/ Perform Optimisation Before 1st Acquisition*** option. Click ***Close***.



9. Click the ***Next*** button. Run the amplification by the ***Start run*** button.
10. Name the experiment and save it to the disc (the results of this experiment will be automatically saved in this file).

Set the tubes positions in the rotor during the thermocycler work or at the end of its work. Mark all clinical samples and controls as ***Unknown*** in the ***Samples*** menu.

Data analysis:

Amplification results are analyzed for *HIV* DNA and IC regions. Accumulation of the amplified product of *HIV* DNA region is detected in the JOE/Yellow channel, and accumulation of the amplified product of IC is detected in the FAM/Green channel.

Amplification data analysis in the FAM/Green channel for IC (endogenous internal control):

1. Activate the button ***Analysis*** in the menu, select the mode of the analysis ***Quantitation***, activate the ***Cycling A. FAM/Cycling A. Green***, ***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***).
4. Choose the parameter ***More settings/Outlier Removal*** and set **10 %** for the value of negative samples threshold (***NTC/Threshold***).
5. In the ***Calculation*** menu (in the right part of the window) indicate the threshold line level **0.03** in the ***Threshold*** box.
6. In the results grid (the ***Quantitation Results*** window) one will be able to see the *Ct* values.

Amplification data analysis in the JOE/Yellow channel for *HIV* DNA:

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***.

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***).
4. Choose the parameter ***More settings/Outlier Removal*** and set **5 %** for the value of negative samples threshold (***NTC/Threshold***).
5. In the ***Calculation*** menu (in the right part of the window) indicate the threshold line level **0.03** in the ***Threshold*** box.
6. In the results grid (the ***Quantitation Results*** window) one will be able to see the *Ct* values.

Results interpretation

1. 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 below.

Results for controls

Control	Stage for control	*Ct* value in the channel for fluorophore	
		JOE	FAM
C–	DNA extraction	Absent	Absent
PCE	DNA extraction	**<**40 (positive)	Absent
NCA	PCR	Absent	Absent
C+$_{HIV\text{-}1}$	PCR	**<**40 (positive)	not evaluated
C+$_{\text{cellular DNA}}$	PCR	not evaluated	**<**20 (positive)

2. The sample is considered **positive**, if *Ct* value in the JOE/Yellow channel is less than 40.
3. The sample is considered **negative**, if *Ct* value in the JOE/Yellow channel is absent, and *Ct* value in FAM/Green channel is less than 20.

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

1. If the *Ct* value in the FAM/Green channel is greater than 20, PCR should be repeated from the DNA extraction stage.
2. If the *Ct* value is present for the Negative Control of Extraction (C–) in the JOE/Yellow channel and for the Negative Control of Amplification (NCA) in any channel in the results grid, it indicates contamination of reagents or samples. In such cases, the results of analysis for all samples must be considered as invalid. The analysis of all samples must be repeated and measures to detect and eliminate the source of contamination must be taken.

AMPLIFICATION AND DATA ANALYSIS USING iCycler iQ5 and iCycler iQ (Bio-Rad, USA) INSTRUMENTS

Make sure that the iCycler iQ5 instrument is calibrated for work with 50 µl reaction mixture. Perform calibration for the type of plastic that is used for amplification.

Make sure that the following mode for dynamicwf is set:

1 cycle 95 °C – 30 s

2 cycles 95 °C – 30 s*

* Fluorescent signal is detected at the 2nd step.

Program the instrument according to the instructions given by the manufacturer:

1. Set the following parameters of amplification:

Step	***Temperature, °C***	***Time***	***Fluorescence detection***	***Number of cycles***
1	95	15 min	–	1
2	95	20 s	–	5
	52	30 s	–	
	72	30 s	–	
3	95	20 s	–	42
	55	40 s	FAM, JOE/HEX	
	72	30 s	–	

2. Set the plate setup. Mark all samples as ***Unknown*****.**
3. For all samples set the fluorophores ***FAM*** and ***HEX***.
4. Set ***Sample volume*** – **50 µL**, select ***Seal type*** and ***Vessel type***. Use the same type of plastic for amplification as for calibration. Save the plate setup.
5. Start the amplification.

   iCycler iQ. Select the ***Run with selected plate*** button. In the opened window enter **50 µl** reaction volume. Select the ***PCR Quantification Melt Curve*** and ***Experimental Plate*** options. Click the ***Begin Run*** button and save the experiment.

   iCycler iQ5. Select the ***Run*** button. In the opened window select the ***Collect Well Factors from Experimental Plate*** and click the ***Begin Run*** button. Save the experiment.

Data analysis

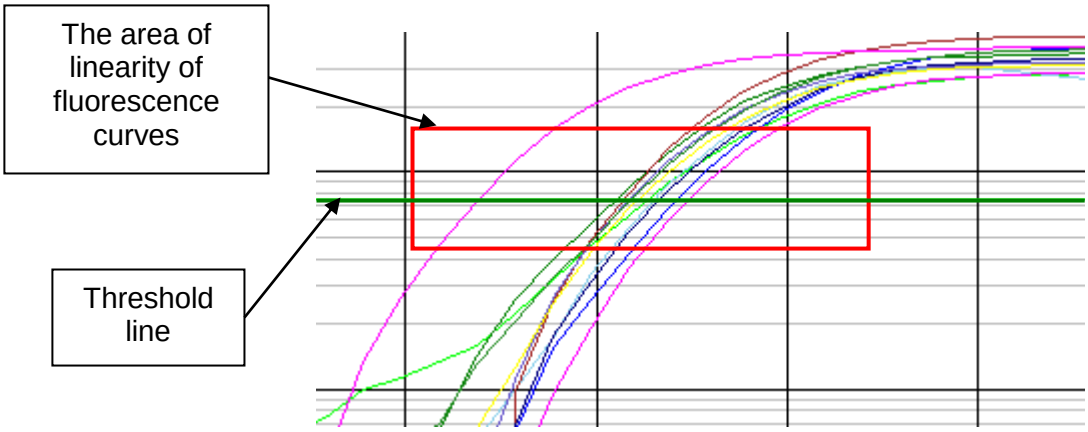
Data are analyzed automatically by the real-time PCR instrument software. Interpretation of results is based on the crossing or not crossing of the threshold line by the fluorescence curve, which corresponds to the presence or absence of the *Ct* value in the corresponding column of the results grid. Amplification results are analyzed for *HIV* DNA and IC regions. Accumulation of the amplified product of *HIV* DNA region is detected in the JOE/HEX channel, and accumulation of the amplified product of IC is detected in the FAM channel. Results obtained with iCycler iQ instrument should be analyzed with the use of iCycler iQ5 instrument software.

Start the program and open the saved file.

Select the ***PCR Quant*** button for data analysis.

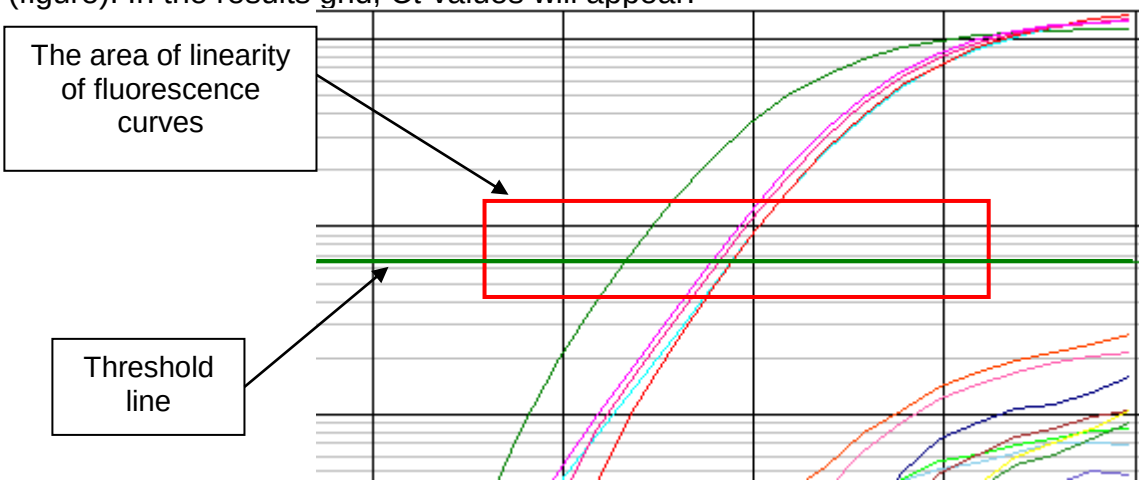
Amplification data analysis in the FAM channel for IC:

Set the values of ***Baseline Cycles*** from 2 to 10. Click the ***Log View*** button. Set the threshold line (by the left mouse button) at a level where fluorescence curves are linear (see the figure). In the results grid, *Ct* values will appear.



Amplification data analysis in the JOE/HEX channel for *HIV* DNA:

Set the values of ***Baseline Cycles*** from 2 to 10. Press the ***Log View*** button. Set the threshold line (with the left mouse button) at a level where fluorescence curves are linear (figure). In the results grid, *Ct* values will appear.



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Results for controls

Control	Stage for control	*Ct* value in the channel for fluorophore	
		JOE	FAM
C–	DNA extraction	Absent	Absent
PCE	DNA extraction	<40 (positive)	Absent
NCA	PCR	Absent	Absent
C+$_{HIV\text{-}1}$	PCR	<40 (positive)	not evaluated
C+$_{\text{cellular DNA}}$	PCR	not evaluated	<24 (positive)

2. The sample is considered **positive**, if *Ct* value in the JOE/HEX channel is less than 40.
3. The sample is considered **negative**, if *Ct* value in the JOE/HEX channel is absent, and *Ct* value in FAM channel is less than 24.

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

1. If the *Ct* value in the FAM channel is greater than 24, PCR should be repeated from the DNA extraction stage.
2. If the *Ct* value is present for the Negative Control of Extraction (C–) in the JOE/HEX channel and for the Negative Control of Amplification (NCA) in any channel in the results grid, it indicates contamination of reagents or samples. In such cases, the results of analysis for all samples must be considered as invalid. The analysis of all samples must be repeated and measures to detect and eliminate the source of contamination must be taken.

List of Changes Made in the Guidelines

VER	Location of changes	Essence of changes
14.05.15 PM	Through the text	Clinical material was changed to biological
16.11.20 MM	Footer	**REF** TR-V0-G(RG,iQ)-CE-B was deleted
02.06.21 KK	Cover page	The phrase “For research use only. Not for diagnostic procedures” was added
04.08.23 BA	Footer	The **REF** TR-V0-G(RG,iQ)-CE-B was added